Pulmonary surfactants and respiratory distress syndrome.

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PULMONARY SURFACTANTS
AND
RESPIRATORY-DISTRESS SYNDROME

BY
Patricia K. (Doherty) Kangas

A Major Clinical Chemistry Critique
Submitted to the Faculty of Graduate Studies Through the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
1985
ABSTRACT

PULMONARY SURFACTANTS
AND
RESPIRATORY DISTRESS SYNDROME

by

PATRICIA K. (DOHERTY) KANGAS

This critique reviews the current available knowledge concerning respiratory distress syndrome (RDS), pulmonary surfactants, and the methods utilized in assessing fetal pulmonary maturity. Respiratory distress syndrome, the major cause of mortality and morbidity among prematurely born infants, is due to inadequate surfactant concentration in the neonatal lung. A description of the basic aspects of fetal pulmonary anatomy and physiology, pulmonary surfactant biochemistry, and metabolic activities, respiratory distress syndrome and all its facets, and the various biochemical and biophysical methods of fetal pulmonary assessment are discussed.

The section dealing with the process of fetal pulmonary development discusses anatomy, the phases of prenatal lung development, birth and the onset of respiration, and the vascular changes during development. The ultimate product of these changes in structure, function, and metabolism is an organ with alveoli having adequate surface area and capable of sustained ventilatory excursions for efficient gas exchange.

The next section deals exclusively with pulmonary surfactant physiology, metabolic activities, composition, functional role, and
biosynthesis. Pulmonary surfactant is a group of phospholipids that lowers surface tension in the alveoli and thus prevents complete collapse of the alveoli with expiration. Surfactant is synthesized and stored in the lamellar inclusion bodies of type II alveolar cells. Much has been learned about pulmonary surfactants, however, clearly much remains to be determined concerning the composition and mechanism of the various components of alveolar froth. The major phospholipids of surfactant have characteristic gestational appearances during prenatal development which constitutes the basis for amniotic fluid surfactant testing.

The next part of the paper deals with respiratory distress syndrome, its etiology, incidence, mortality, diagnostic criteria, differential diagnosis, treatment, complications, pathology, and prevention. In the last ten years there have been major advances in RDS treatment and prevention.

The methods utilized in assessing fetal pulmonary maturity occupy a large portion of this critique, for there have been a bewildering number, suggesting many shortcomings. The following methods are discussed, biochemical include—lecithin/sphingomyelin ratio (L/S ratio), lung profile, optical density, cortisol, palmitic acid, lamellar body analysis, lecithin, enzymatic, and fast atom bombardment mass spectrometry; biophysical include—surface tension, foam, and fluorescence polarization. The principle, procedure, results, contamination effects, and advantages and disadvantages for each method are discussed in detail. During this extensive literature search of these various methods numerous variations in the procedures utilized and results obtained were
encountered. These variations included, the reliability of specific
tests, predictive values of results obtained, and lack of standardization
of procedures utilized. Also many investigators correlated their
procedure's results with the universally accepted L/S ratio standard test,
even though their correlation was good, their sample size was inadequate
and population tested not inclusive (complicated pregnancies and RDS
cases omitted). Therefore a clinical survey of the Metro Detroit area
hospitals was conducted to determine which procedures were being utilized
by physicians to monitor and diagnose fetal pulmonary maturity. This is
followed by a discussion on the choice of methods currently being
utilized.

The paper concludes by discussing the diagnostic usefulness of
assessing fetal pulmonary maturity in normal and complicated pregnancies.
Also a discussion of which recent methods are best suited for clinical
laboratory testing is included.
DEDICATION

To Darrell, my dear husband;
To my mother, Mary;
To my mother-in-law, Virginia,
and my two precious children,
Stephanie and Mark.
ACKNOWLEDGEMENTS

I would like to especially thank Dr. R. J. Thibert for his continuous support and interest in completing this work, as well as his ever present guidance over the duration of my program.

I would also like to express my appreciation and thanks to the other members of my committee: Dr. T. A. Hyde, Dr. H. B. Fackrell, and Dr. B. Mutus for their suggestions and cooperation given throughout the course of my project.

Also I would like to thank all the people who took the time to participate in my survey.

I also wish to extend my personal thanks to Mrs. Connie Schaap for the expert typing of this manuscript.

Finally, I would like to express my deepest gratitude to my husband for assisting me, and my mother, mother-in-law, and brother, for watching my children so that I could work on my paper. Also for all of their support and understanding during this project.

In retrospect, I believe this critique could be shortened if the lung anatomy and the respiratory distress syndrome (RDS) sections were dealt with separately. Then the numerous methods of evaluating fetal pulmonary maturity could be a critique with its own identity. This critique is quite lengthy due to its encompassing all the facets of the disease, RDS.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xxx</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xxxv</td>
</tr>
<tr>
<td>ABBREVIATIONS AND DEFINITIONS</td>
<td>xxxvi</td>
</tr>
</tbody>
</table>

## CHAPTER

I. INTRODUCTION

II. THE PROCESS OF FETAL LUNG DEVELOPMENT

A. ANATOMY

   1. Nonrespiratory Structures
   2. Respiratory Structures

B. PRENATAL LUNG DEVELOPMENT

   1. Embryonic Phase
   2. Pseudoglandular Phase
   3. Canalicular Phase
   4. Alveolar Sac Phase

C. GAS EXCHANGE UNIT

   1. 17 Weeks of Gestation
   2. 20 Weeks of Gestation
   3. 22 Weeks of Gestation
   4. 24 Weeks of Gestation
5. 26 Weeks of Gestation ........................................... 33
6. 28 Weeks of Gestation ........................................... 33
7. 30 Weeks of Gestation ........................................... 33
8. 34 Weeks of Gestation ........................................... 36
9. 38-40 Weeks of Gestation ...................................... 36
10. Birth ............................................................. 36
11. Postnatal .......................................................... 37

D. BIRTH AND THE ONSET OF RESPIRATION .................... 38

E. VASCULAR CHANGES DURING PULMONARY DEVELOPMENT .... 40

F. SUMMARY LUNG DEVELOPMENT ................................ 43

III. PULMONARY SURFACTANTS ................................... 44

A. PHYSIOLOGY OF SURFACTANTS ................................ 44

1. Historical Review of Pulmonary Surfactant .................. 45
2. Surface Tension .................................................. 47
3. Metabolic Activities of Surfactants: Synthesis, Storage, Secretion, and Degradation ......................... 51
4. Composition of Pulmonary Surfactants ....................... 59
5. Phospholipid Chemistry ........................................ 68

6. Gestational Appearance of Surfactant Phospholipids and Their Functional Role ............................... 69
   (a). Phosphatidylcholine ........................................ 73
   (b). Phosphatidylglycerol ...................................... 74
   (c). Phosphatidylinositol ..................................... 77
   (d). Sphingomyelin ............................................. 78
   (e). Phosphatidylethanolamine and Phosphatidylserine .... 78

B. BIOSYNTHESIS OF PULMONARY PHOSPHOLIPIDS ............... 78
1. Lecithin (PC) Biosynthesis .................. 79
   (a). The Choline (CDP) Pathway ............. 79
   (b). Methylation Pathway .................. 83
   (c). Auxiliary Mechanisms .................. 87
2. Biosynthesis of the Other Pulmonary Phospholipids .... 93
   (a). Phosphatidylglycerol (PG) Biosynthesis .... 94
   (b). Phosphatidylinositol (PI) Biosynthesis ... 95
   (c). Phosphatidylethanolamine (PE) Biosynthesis ... 95
   (d). Sphingomyelin Biosynthesis ............... 96
   (e). Phosphatidylserine (PS) Biosynthesis .... 96
3. Enzymatic Regulation of Synthesis ................. 96
   (a). Palmitic Acid Synthesis ................ 97
   (b). Formation of 1,2-Diglyceride as a Common Precursor .... 97
   (c). Choline (CDP) Pathway .................. 97
       (i). CK activity ...................... 97
       (ii). CYT activity .................... 98
       (iii). CPT activity .................... 98
   (d). Methylation Pathway ................... 98
   (e). Transacylation of PC Fatty Acids into Dipalmitic Lecithin .... 99
   (g). Phosphatidylinositol (PI) Synthesis .... 99
4. Metabolic Interrelationships in Phospholipid Metabolism .......... 100
   (a). Carbohydrate and Fatty Acid Metabolism ...... 100
   (b). Protein Metabolism .................... 100
5. Summary .................................................. 102

IV. RESPIRATORY DISTRESS SYNDROME ................. 103
   A. ETIOLOGY .............................................. 103
   B. TERMINOLOGY OF THE DISEASE ..................... 109
   C. FACTORS AFFECTING RDS .............................. 109
      1. Predisposing Factors ............................... 109
      2. Factors That Reduce The Risk of RDS ............ 111
   D. INCIDENCE AND MORTALITY .......................... 111
   E. DIAGNOSTIC CRITERIA ............................... 113
      1. Physical Findings .................................. 113
         (a). Tachypnea .................................... 116
         (b). Chest Wall Retraction ......................... 116
         (c). Flaring of the Nares .......................... 116
         (d). Cyanosis ..................................... 119
         (e). Other Physical Findings ....................... 119
      2. Blood Gases ........................................ 120
      3. Radiographic Findings .............................. 120
   F. DIFFERENTIAL DIAGNOSIS ............................. 121
      1. Pneumonia .......................................... 126
      2. Pulmonary Hemorrhage ............................. 132
      3. Transient Tachypnea ................................ 138
      4. Meconium Aspiration ................................ 141
         5. Congenital Heart Disease ......................... 144
      6. Other Diseases ..................................... 147
      7. Summary ............................................ 147
G. TREATMENT ........................................ 147

1. Ventilation Support and Oxygen Therapy .. 151
2. Acid-Base Balance .......................... 156
3. Body Temperature .......................... 157
5. Nutrition .......................... 158
6. Antibiotic Administration .................. 158
7. The Team Approach and Intensive Care Unit .. 159
8. Summary .................................. 160

H. COMPLICATIONS .................................. 160

1. Disease and Prematurity Associated Complications .. 160
   (a). Pneumothorax .......................... 160
   (b). Pneumomediastinum .................. 184
   (c). Metabolic Disturbances ............... 187
   (d). Patent Ductus Arteriosus (PDA) .... 187

2. Intensive Care Associated Complications ........ 173
   (a). Complications of Endotracheal Intubation .. 173
   (b). Complications of Umbilical Vessel Catherization .. 173
   (c). Complications of Infection .......... 173
   (d). Fluid Administration Complications .... 174
   (e). Intracranial Hemorrhage .............. 174
   (f). Oxygen Therapy Complications ........ 174

I. PATHOLOGY .................................. 180

J. PREVENTION OF RDS ........................ 183

1. Prematurity .......................... 186
2. Usage of Glucocorticoids ........................................ 186
   (a). Mechanism of Action ........................................ 187
   (b). Morphologic Changes ....................................... 191
   (c). Physiologic Changes ........................................ 191
   (d). Biochemical Changes ....................................... 192
   (e). Therapeutic Trials of Glucocorticoids ..................... 193
       (i). Time of Treatment ...................................... 198
       (ii). Duration and Frequency of Treatment ............... 199
       (iii). Mode of Glucocorticoid Administration .......... 200
       (iv). Choice of Glucocorticoid ............................. 200
       (v). Usage of Betamethasone and Dexamethasone ....... 201
       (vi). Fetal Side Effects .................................... 203
       (vii). Maternal Side Effects ................................ 205
       (viii). Medical-Legal Aspects of Glucocorticoid
               Administration ........................................ 208
       (ix). Summary .................................................. 208

3. Effects of Other Hormones on Lung Maturation ................ 210
   (a). Thyroid Hormones .......................................... 210
   (b). Insulin ..................................................... 211
   (c). Prolactin ................................................... 212

4. Surfactant Substitution By Exogenously Administered
   Surfactant ................................................................... 212
   (a). Animal Studies ................................................ 212
   (b). Human Studies ................................................ 213
   (c). Current Available Knowledge ................................ 216
       (i). Preparation .................................................. 216
(ii). Side Effects .................................. 217

(iii). Clinical Application of Surfactant Replacement in Managing RDS ............... 217

(d). Summary ...................................... 219

K. INFANTS SURVIVING RDS ........................................ 219

V. METHODS OF EVALUATING FETAL LUNG MATURITY ............................................ 222

A. BIOCHEMICAL PROCEDURES ..................................................... 223

1. Lecithin/Sphingomyelin Ratio (L/S Ratio) ........................................ 223

(a). Principle ............................................. 223

(b). Procedure (Gluck's Original L/S Ratio) ........................................ 226

(c). L/S Ratio Procedural Steps and Their Effects. ........................................ 229

(i). Centrifugation Effect ........................................ 229

(ii). Lipid Extraction Effect ........................................ 239

(iii). Acetone-Precipitation Effect ........................................ 239

1. Utilization of the Acetone-Precipitation Step ........................................ 241

2. Omit Acetone-Precipitation Step ........................................ 247

(iv). Thin-layer Chromatography (TLC) ........................................ 251

(v). Direct Quantitation of Lecithin and Sphingomyelin .................................. 252

1. Step One: Staining ........................................ 253

2. Step One: Charring ........................................ 254

a. Charring with Sulfuric Acid ........................................ 255

b. Charring with Potassium Dichromate in Sulfuric Acid .................................. 255

c. Charring with Ammonium Sulfate ........................................ 258

3. Methods to Quantitate Staining and Charring ........................................ 259
a. Densitometry .................................. 270
b. Planimetry .................................... 271
c. Gravimetry .................................... 272
d. Visual Assessment .............................. 272

(d). Variations in the L/S Ratio Procedure .... 273

(e). The L/S Ratio Clinical Interpretation and
Usefulness ......................................... 273

(i). High Incidence of False Negative Results. 284

(ii). L/S Ratio's Value in Complicated
Pregnancies ....................................... 287

(f). Sources of Contamination ................. 290

(i). Blood ......................................... 290

(ii). Meconium ................................... 291

(iii). Miscellaneous Sources of Contamination

1. Bilirubin ....................................... 292
2. Para-Aminohippuric Acid...................... 292
3. Lack of Refrigeration ......................... 293
4. Fetal and Maternal Urine ...................... 293
5. Fetal and Maternal Ascitic Fluid .......... 293
6. Vaginal Samples .............................. 293
7. Serum ......................................... 293

(g). Summary ..................................... 293

2. Lung Profile ................................... 294

(a). Principle ................................... 294

(b). Procedure ................................... 295

(i). Two-dimensional Thin-layer Chromatography
(TLC) Procedures ............................... 295
1. Results of Two-dimensional Thin-layer Chromatography Procedure .... 299
   a. L/S Ratios ........................................ 299
   b. Percentage of PC .................................. 299
   c. Percentage of PI .................................. 299
   d. Disaturated Lecithin Fraction .................. 299

2. Summary of Lung Profile Compared to the L/S Ratio .................. 304

(ii). One-dimensional Thin-layer Chromatography .................. 309

   1. Stepwise Development Procedure .......................... 309
      a. Results ........................................... 310

   2. Continuous Development Procedure ..................... 310
      a. Results ........................................... 315

   3. Simultaneously Determined Procedure .................... 318
      a. Results ........................................... 319

   4. Improved One-dimensional Thin-layer Chromatography Procedure .. 322
      a. Results ........................................... 323

(iii). Comparison of Two and One-dimensional Thin-layer Chromatography Procedure for the Phospholipids of Amniotic Fluid ........ 329

(c). The Clinical Usefulness and Interpretation of the Lung Profile .... 330

(d). Problems Associated with Phosphatidylglycerol and Phosphatidylinositol Levels in Amniotic Fluid as Indices of Fetal Lung Maturation 337

(e). Complicated Pregnancies in Relation to the Lung Profile .......... 335

(i). Diabetes Mellitus .................................... 340
   1. L/S Ratio ........................................... 345
2. Disaturated Lecithin 340
3. Phosphatidylinositol 340
4. Phosphatidylglycerol 345
5. Disease Relationship to Fetal Lung Maturation 346

(ii). Premature Rupture of Membrane (PROM): 354
1. L/S Ratio 354
2. Disaturated Lecithin 354
3. Phosphatidylinositol 354
4. Phosphatidylglycerol 354
5. Disease Relationship to Fetal Lung Maturation 359

(iii). Hypertension 359
1. L/S Ratio, Disaturated Lecithin, PI, and PG 360
2. Disease Relationship to Fetal Lung Maturation 360

(f). Sources of Contamination 361

(g). Summary 361

3. Optical Density 362
(a). Principle 362
(b). Procedure 363
(c). Results 364
(i). Sbarra et al. 364
(ii). Copeland et al. 367
(iii). Moodley et al. 367
(iv). Arias et al. 367
(v). Spellacy et al. ........................................... 367
(vi). Cetrulo et al. ........................................... 370
(vii). Sbarra et al. ........................................... 370
(d). Factors Which Affect the Optical Density Test .................................................... 374
   (i). Refrigeration ............................................. 374
   (ii). Centrifugation, Blood, and Meconium ......................................................... 374
   (iii). Gestational Age and Polyhydramnios ......................................................... 377
(e). Contamination ............................................. 377
(f). Summary ...................................................... 377

4. Cortisol ....................................................... 379
   (a). Principle .................................................. 379
   (b). Different Procedures and Their Corresponding Results ..................................... 380
       (i). Fenc1 and Tulchinsky—Total Cortisol ...................................................... 380
       (ii). Tan et al.—Unconjugated RIA Cortisol .................................................. 384
       (iii). McCann et al.—RIA Cortisol ............................................................ 388
       (iv). Doran et al.—Total Cortisol ............................................................. 392
(c). Pregnancy Disease Conditions Which Affect the Amniotic Fluid Cortisol Concentrations ......................................................... 395
(d). Contamination ............................................. 395
(e). Summary ...................................................... 396

5. Palmitic Acid .................................................. 397
   (a). Principle .................................................. 397
   (b). Different Procedures and Their Corresponding Results ..................................... 397
       (i). Gás—Liquid Chromatography (GLC) ....................................................... 397
       (ii). Thin-Layer Chromatography (TLC) ...................................................... 398
(iii). Ip et al. Modified Extraction Gas-Liquid Chromatography Procedure .................. 400 a

(c). Contamination ........................................ 409
(d). Summary ............................................... 409

6. Lamellar Body Analysis. ................................. 411

(a). Principle .............................................. 411
(b). Procedures ............................................ 412
(i). 10,000 x g Pellet Method ......................... 412
  1. Procedure ............................................ 412
  2. Results ............................................. 413
  3. Comments ........................................... 418
  4. Contamination Effects .............................. 420

(ii). The Lamellar Body Phospholipid (LB-PL) Method ................... 420
  1. Procedure ............................................ 421
  2. Results ............................................. 422
  3. Comments ........................................... 426
  4. Contamination Effects .............................. 431

(iii). Fluorometric Procedure ........................... 431
  1. Procedure ............................................ 432
  2. Results ............................................. 435
  3. Comments ........................................... 435
  4. Contamination Effects .............................. 438

(c). Summary ............................................... 438

7. Lecithin ................................................. 440
(a). Principle ............................................. 440
(b). Procedure ........................................ 441
(c). Results ............................................ 442
(d). Contamination ..................................... 446
(e). Summary ........................................... 446

8. Enzymatic Procedures. ................................ 447
(a). Principle .......................................... 447
(b). Procedures ......................................... 447

(i). McDonald et al.-L/S Enzymatic Radiochemical Method ........... 447
1. Procedure .......................................... 447
2. Results ............................................. 448

(ii). Siegel et al.-Phosphatidylglycerol (PG) Enzymatic Radiochemical Method ................................. 453
1. Procedure .......................................... 453
2. Results ............................................. 454

(iii). McGowan et al.-L/S Enzymatic Colorimetric Method .............. 454
1. Procedure .......................................... 454
2. Results ............................................. 457

1. Procedure .......................................... 457
2. Results ............................................. 461

(c). Contamination ...................................... 469
(d). Summary ........................................... 469

(a). Principle .......................................... 470
(b). Procedure ............................................. 471
(c). Results ................................................. 472
(d). Summary ................................................. 479

B. BIOPHYSICAL METHODS ........................................ 481

1. Surface Tension .............................................. 483
   (a). Principle ............................................... 485
   (b). Different Procedures and Their Corresponding
        Results ................................................ 483
        (i). Shelley et al. Method ............................... 483
        (ii). Muller-Tyl et al. Method ......................... 489
        (iii). Goldkrand et al. Method ......................... 494
        (iv). Goldkrand and Slattery Method ................... 502
   (c). Contamination ........................................... 511
   (d). Summary ................................................. 511

2. Shake Test .................................................... 514
   (a). Principle ............................................... 514
   (b). Different Procedures and Their Results ............. 517
       (i). Clements et al.-Original Shake Test .......... 517
       (ii). Edwards and Baillie-Modified Shake Test .... 518
       (iii). Sher et al.-Foam Stability Index
               (FSI) Test .................................... 521
       (iv). Schlueter et al.-Improved Shake Test ....... 523
   (c). Sources of Error and Interference .................. 529
   (d). Contamination ........................................... 530
   (e). Summary ................................................. 530

3. Fluorescence Polarization ................................... 532
(a). Principle ........................................... 532
(b). Procedure ......................................... 533
(c). Modifications of Original Procedure .......... 539
   (i). Centrifugation ................................. 539
   (ii). Incubation Conditions ...................... 543
   (iii). Cut-Off Value and Its Relationship to
          Neonatal RDS ................................ 545
(d). Contamination .................................... 550
(e). Summary .......................................... 551
(f). Items for Future Investigation ............... 552

C. COLLECTION AND HANDLING OF SPECIMENS FOR FETAL PULMONARY
   MATURITY ASSESSMENT ............................. 554
   1. Collection ....................................... 554
   2. Handling ......................................... 555

VI. SURVEYS OF CURRENT CLINICAL METHODS AND PHILOSOPHIES ON FETAL
    LUNG MATURITY ASSESSMENT ....................... 557
   A. FETAL LUNG MATURITY CLINICAL TESTING SURVEY .... 557
      1. Procedure Utilized ............................. 558
         (a). Test Time .................................. 570
         (b). Quantity of Tests Performed ............. 570
      2. Handling of the Sample and Contamination .... 571
      3. Centrifugation ................................ 571
      4. Lipid Extraction ............................... 572
      5. Acetone-Precipitation ......................... 572
      6. Thin-layer Chromatography .................... 572
      7. Staining/Charring ............................. 573
      8. Quantitation .................................. 573
9. Cut-off Values/Reproducibility .................................................. 573
10. Comments .................................................................................. 574
11. New Procedures Being Investigated .......................................... 574
   (a). Amniostat-FLM ....................................................................... 574
   (b). Lumadex-FSI ........................................................................... 575
B. RESPIRATORY DISTRESS SYNDROME: NEONATOLOGIST SURVEY .......................... 577
   1. Predisposing Factors ................................................................. 577
   2. Terminology .............................................................................. 577
   3. Physical Findings ....................................................................... 584
   4. Radiographic Findings .............................................................. 584
   5. Differential Diagnosis .............................................................. 585
   6. Treatment ................................................................................. 585
   7. Complications .......................................................................... 585
   8. RDS Survivor Follow-Up .......................................................... 586
   9. Comments ................................................................................. 587
C. FETAL LUNG MATURITY ASSESSMENT: OF HIGH RISK OBSTETRICIAN SURVEY .......... 587
   1. Prematurity Prevention ............................................................. 591
   2. Fetal Lung Maturity Assessment in Complicated Pregnancies .......... 591
   3. Glucocorticoid (Steroid) Treatment to Enhance Fetal Lung Maturation .... 591

VII. SUMMARY AND CONCLUSIONS .................................................. 593
APPENDICES ..................................................................................... 527
BIBLIOGRAPHY ............................................................................... 644
VITA AUCTORIS ............................................................................... 662
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nonrespiratory Conducting Airways</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>The Acinus (Respiratory Structures) and Stages of Intra-uterine Development</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Embryonic and Pseudoglandular Phases of Intrauterine Lung Development</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Pulmonary Epithelium Transformation Through Three Phases of Development</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Electron Micrograph Depicting Pseudoglandular and Cánalicular Phases</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Electron Micrograph Depicting Alveolar Sac Phase</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Electron Micrograph Depicting Type I and Type II Cells of the Alveolar Sac Phase</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Development of the Acinus</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>24 and 30 Weeks Gestation</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>Fetal, Transitional, and Adult-Type Circulations</td>
<td>42</td>
</tr>
<tr>
<td>11</td>
<td>Surface Area Diagrams, Surface Tension, and Molecular Orientation of Phosphatidylcholine at the Air Liquid Interface</td>
<td>49</td>
</tr>
<tr>
<td>12</td>
<td>Type II Alveolar Pneumocyte</td>
<td>54</td>
</tr>
<tr>
<td>13</td>
<td>Cellular and Extracellular Aspects of Pulmonary Surfactant</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>Stereomodels of the Alveolar Pool Constituents</td>
<td>63</td>
</tr>
<tr>
<td>15</td>
<td>L-α-Glycerophosphate and Sphingomyelin</td>
<td>71</td>
</tr>
</tbody>
</table>
38. Bronchopulmonary Dysplasia
39. Bronchopulmonary Dysplasia
40. Hyaline Membranes
41. Hyaline Membranes
42. Proposal Mechanism for Accelerated Maturation of Fetal
    Alveolar Lining Cells by Steroids
43. Gestational Concentrations of Lecithin and Sphingomyelin
44. L/S Ratio Variation Related to g-Force and Weeks of Gestation
45. Lecithin and Sphingomyelin Migration on the Silica Gel
    H Plate and the L/S Ratio Related to g-Force
46. Standard Curves for Lecithin and Sphingomyelin Spots with
    Sulfuric Acid Charring, Potassium Dichromate - Sulfuric Acid
    Charring, and Ammonia Sulfate Charring
47. Two-Dimensional TLC Showing Separation of Phospholipids
48. Two-Dimensional TLC Procedure Results for Normal Pregnancy
    L/S Ratio and Percentage of Phosphatidyglycerol
49. Two-Dimensional TLC Procedure Results for Percentage of
    Phosphatidylinositol and Disaturated Lecithin
50. Lung Profile Form
51. Stepwise Development One-Dimensional Thin-Layer
    Chromatography and Phosphatidylinositol Results
52. Stepwise Development One-Dimensional Thin-Layer Chromatography
    Phosphatidyglycerol Results and the Phosphatidylinositol/
    Phosphatidyglycerol Results in Mild Diabetics
54. Simultaneous Determination of One-Dimensional Thin-Layer Chromatography, the L/S Ratio Change with Gestational Age and the Change in PI/S and PC/S Ratios Versus the L/S Ratio ... 321
55. Typical Thin-Layer Chromatography Plate from Improved One-Dimensional Thin-Layer Chromatography Method ... 325
56. L/S Ratio and Percentage of Disaturated Acetone Precipitated Lecithin by Class of Diabetes During Gestation ... 342
57. Percentage of Phosphatidylinositol and Phosphatidylglycerol During Gestation by Class of Diabetes ... 344
58. Possible Mechanism of the Impairment of Fetal Lung Functional Maturation in the Diabetic Pregnancy ... 350
59. Possible Effects of Hyperinsulinism on the Production of Surfactant ... 353
60. L/S Ratio and Percentage of Disaturated Lecithin Curves in Prolonged Rupture of Membrane and Hypertension Conditions During Gestation ... 356
61. Percentage of Phosphatidylinositol and Phosphatidylglycerol in Prolonged Rupture of Membranes and Hypertension Conditions During Gestation ... 358
62. Correlation Between Amniotic Fluid Results of Optical Density (OD₅₀) and the L/S Ratio ... 369
63. Effect of Added Phospholipids on Amniotic Fluid Optical Density at 650 nm and of Different Absolute Concentrations of Lecithin and Sphingomyelin with a Constant L/S Ratio of OD$_{650}$nm .................................................. 373

64. The Effect Refrigeration has on Amniotic Fluid Optical Density ............................................................... 376

65. Total Cortisol Concentration in Amniotic Fluid at Various Stages of Pregnancy ........................................... 383

66. Amniotic Fluid Cortisol Values from Competitive Protein Binding Radioassay Procedure and the Correlation of Amniotic Fluid Cortisol and L/S Ratio Values ........................................................................ 387

67. Amniotic Fluid Cortisol Concentration Versus L/S Ratio Values ...................................................................... 391

68. Gas-Liquid Chromatogram of the Amniotic Fluid Phospholipid Fatty Acid Esters ............................................. 403

69. Palmitic Acid Levels Versus Gestational Periods ............................................................................................ 408

70. Stage I, and V of Developmental Profile and Characteristic Chromatograms for the Developmental Changes in Stages I, III, and V .................................................................................. 416

71. Relationship Between Respiratory Performance of the Newborn and the LB-PL Concentration ....................... 428

72. Fluorometric Automated Procedure for the Lamellar Body Fraction and the Relationship Between the Fluorometric and Colorimetric Results .................................................................... 434
73. Effect of Reacting Osmium Tetroxide with Various Phospholipids .............................................. 444
74. Enzymatic Radiochemical Method Flow Chart ................................................................. 450
75. Standard Chromatographic L/S Ratio Compared to Three Indices and the Percentage Total Lecithin ............... 452
76. Enzymatic Radiochemical Phosphatidylglycerol Determination Compared to the Chromatographic L/S Ratio and the Concentration Corresponding to Weeks of Phosphatidylglycerol Gestation ................................................................. 455
77. Reaction Sequence for McGowan et al. Enzymatic Determination of Lecithin and Sphingomyelin ........................................ 456
78. Reaction Sequence for Enzymic Phosphatidylglycerol Determination ......................................................... 463
79. Optimum Conditions for Phospholipase D (PL-D) Assay for Phosphatidylglycerol and the Comparison of Colorimetric Enzymic to the Thin-layer Chromatographic Results for Phosphatidylglycerol ................................................................. 465
80. Comparison of Phosphatidylglycerol Concentrations Versus Gestational Age by the Thin-layer Chromatography and Enzymic Procedures ................................................................. 468
81. Partial Positive-Ion Fast Atom Bombardment Mass Spectrum ..................................................... 474
82. Log-Log Plot of the Ratio Intensities of Peaks Versus Relative Amounts of $D_0$ and $D_9$ Dipalmitoylphosphatidylcholine ......................................................... 478
83. Surface Tension Area Diagrams ................................................................. 486
84. Surface Balance and Surface Tension Area Diagrams ................................................................. 491
85. Surface Tension Parameters of Amniotic Fluid .................. 493
86. Comparison Between Surface Tension \( \gamma_{\text{min}} \), the L/S Ratio, the
    Lecithin Content and Bubble Stability Test ..................... 496
87. Mean Surface Tension Values ................................. 499
88. Formation of the Subsurface Globule and the Comparison of
    the Surface Tension Values and Volume Required for
    Globule Formation ............................................. 504
89. Pattern of Pulmonary Maturation in Normal Pregnancy and in
    Diabetes Mellitus .............................................. 507
90. Pattern of Pulmonary Maturation in Rh-Sensitization
    and Pre-eclampsia ............................................. 510
91. Decrease in Surface Tension at an Air-Liquid Interface,
    with Increasing Volume Fraction of Ethanol ...................... 516
92. Foam as a Function of Ethanol Volume Fraction and
    Concentration of Lecithin and the Comparison of Foam
    Stability Index Values with Neonatal Outcome .................. 520
93. Eight Possible Results of the Foam Test ....................... 525
94. Measurement of Microviscosity by Fluorescence Depolarization
    and Amniotic Fluid Microviscosity Changes During Gestation .... 535
95. Elescint Fetal Lung Maturity Analyzer (FELMA) .................. 538
96. Effect of Centrifugation on Fluorescence Polarization of
    Amniotic Fluid ............................................... 542
97. Microviscosity of Phospholipid Mixtures Representing Three
    Phases of Gestation ......................................... 549
LIST OF TABLES

Table | TITLE | Page
-----|-------|------
I | Surfactant Fractions Obtained from Isolating Dog Lung Homogenates | 60
II | Approximate Molar Ratios in Surface Active Material | 65
III | Comparison of the Phospholipid Composition of Surfactant and Whole Lung Tissue | 67
IV | Major Classes of Phosphoglycerides | 72
V | Categorization of Observations in Respiratory Distress Syndrome | 106
VI | The most Frequent Diseases which must be Differentiated from RDS | 127
VII | Therapeutic Trials of Glucocorticoids | 194
VIII | Biologic Activity of the most used Glucocorticoids in Clinical Studies | 202
IX | Human Trials of Surfactant Replacement Therapy | 214
X | Comparison of L/S Ratio Values where Acetone-Precipitation was done or Omitted on Aliquots of the same Fluid | 242
XI | L/S Ratios of Total Lipid and Acetone Insoluble Lipid Extracts of Fourteen Samples of Amniotic Fluid | 243
XII | Percent Fatty Acid Composition of Lecithin from Term Amniotic Fluid and from Egg Yolk | 246
XIII | Comparison of the Extraction Procedure with and without Inclusion of Cold-Acetone Precipitation | 248
XIV  Staining and Charring Methods Utilized in the L/S Ratio Procedure ........................................ 254
XV  Modifications of the L/S Ratio Procedure .............................................................. 274
XVI  Comparison Between L/S Ratio Alone and the Lung Profile in Predicting Fetal Lung Maturity ......................................................... 307
XVII Comparison of Two- and One-Dimensional Thin-Layer Chromatography Procedure for the Phospholipids of Amniotic Fluid ......................................................... 327
XVIII Comparison of the Surface Properties of Phosphatidylglycerol and Phosphatidylinositol Surfactants ................................................................. 336
XIX  Maternal and Fetal Complications Affecting Maturation of the Fetal Lung ................................................................. 339
XX  Optical Density₆₅₀ Measurements for Predicting Fetal Lung Maturity and Correlation to the L/S Ratio ................................................................. 365
XXI  Critical Values and Differential Percentages Indicating Accuracy of Assessment of Fetal Maturity ................................................................. 394
XXII Mean Palmitic/Stearic Acid Ratios in Lecithin ................................................................. 399
XXIII Fatty Acid Variation in Concentration with Gestation ................................................................. 400
XXIV Amount of Selected Lipid Components in Human Amniotic Fluid at 40 Weeks of Normal Pregnancy ................................................................. 404
XXV Amniotic Fluid Phospholipid Palmitic Acid as Determined By Two Gas-Chromatographic Methods ................................................................. 405
XXVI Effect of Presence of Blood Components on Results of Phospholipid Palmitic Acid in Amniotic Fluid ................................................................. 410
XXVII Concentration and Composition of Structures Present in the 10,000 x g Pellet at Various Developmental Stages

XXVIII Respiratory Status of Infants Delivered at Various Stages of Development

XXIX The Lamellar Body Phospholipid (LB-PL) Content of Amniotic Fluid from all Pregnancies Resulting in Serious Respiratory Problems

XXX Lamellar Body Phospholipid (LB-PL) Content of Amniotic Fluid from all Pregnancies Resulting in no Respiratory Problems or Minimal Respiratory Distress

XXXI Lamellar Body Phospholipid (LB-PL) Content of Amniotic Fluid from all Pregnancies Resulting in Transient Tachypnea

XXXII The Lamellar Body Phospholipid (LB-PL) Content of Amniotic Fluid from Pregnancies Complicated by Maternal Diabetes

XXXIII Correspondence Between L/S Ratios and Fluorometric Phospholipid Values for Amniotic Fluids Sampled from 30-37 Weeks of Gestation

XXXIV L/S Ratio in 10 Specimens of Amniotic Fluid as Determined by the Proposed Enzymatic Method and by Thin-layer Chromatography

XXXV Dipalmitoyl Phosphatidylcholine (DPC) Content of Amniotic Fluids
XXXVI  Effect of Centrifugal Force on the Concentration of Phosphatidylcholine (PC) and Dipalmitoylphosphatidylcholine (DPC) in Amniotic Fluid .................. 480

XXXVII Surface Tension Measurements and Phospholipid Values of Amniotic Fluid Samples Collected at Delivery of Infants who Developed Respiratory Distress .......... 487

XXXVIII Surface Tension Measurements and Phospholipid Values of Amniotic Fluid Specimens Collected at Delivery of Five Healthy Premature Infants and Two Infants of Diabetic Mothers .................. 488

XXXIX  Comparison of the Amniotic Fluid Lipid Extract Globule Formation and the L/S Ratio .................. 501

XL    Relationship of Ethanol Volume Fraction in Final Assay Mixture to Volume of 95% Ethanol Added to 0.05 mL of Amniotic Fluid .................. 522

XLI    Risk for Developing HMD if Delivery is Within 24 Hours of Amniotic Fluid-Foam Test .................. 526

XLII   Independent Associations of Foam Test and Gestational Age with the Risk of HMD .................. 528

XLIII  Sensitivity and Specificity of Fluorescence Polarization Values Measured at 25 and 37°C .................. 544

XLIV   Sensitivity, Specificity, and Predictive Value of Fluorescence Polarization Values .................. 549

XLV    Metro Detroit Area Survey of the Clinical Testing for Fetal Lung Maturity .................. 559
XLVI  Metro Detroit Area Survey of Neonatologists Concerning Respiratory Distress Syndrome ............... 578
XLVII Metro Detroit Area Survey of High Risk Obstetricians Concerning High Risk Pregnancies' Fetal Lung Maturity Assessment ............................................. 588
XLVIII Fetal Lung Maturity Evaluation Methods Summary ...................................................... 601
<table>
<thead>
<tr>
<th>Appendix</th>
<th>TITLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Clinical Laboratory Survey Questionnaire</td>
<td>627</td>
</tr>
<tr>
<td>B.</td>
<td>Amniostat-FLM Procedure and Literature</td>
<td>631</td>
</tr>
<tr>
<td>C.</td>
<td>Lumadex-FSI Procedure and Literature</td>
<td>636</td>
</tr>
</tbody>
</table>
ABBREVIATIONS AND DEFINITIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>Amniotic fluid</td>
</tr>
<tr>
<td>BPD</td>
<td>Bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>CDP</td>
<td>Cytidine diphosphate choline</td>
</tr>
<tr>
<td>CK</td>
<td>Choline kinase</td>
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<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
</tr>
<tr>
<td>CPT</td>
<td>Choline phosphotransferase</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYT</td>
<td>Cytidyl transferase</td>
</tr>
<tr>
<td>DPL</td>
<td>Dipalmitoyl lecithin</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FLM</td>
<td>Fetal lung maturity (maturation)</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence polarization</td>
</tr>
<tr>
<td>FSI</td>
<td>Foam stability index</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatography</td>
</tr>
<tr>
<td>HMD</td>
<td>Hyaline membrane disease</td>
</tr>
<tr>
<td>HP</td>
<td>High performance</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth retardation</td>
</tr>
<tr>
<td>L</td>
<td>Lecithin</td>
</tr>
<tr>
<td>LB</td>
<td>Lamellar bodies</td>
</tr>
<tr>
<td>LB-PL</td>
<td>Lamellar body phospholipid</td>
</tr>
<tr>
<td>MAE</td>
<td>Methionine-activating enzyme</td>
</tr>
<tr>
<td>MT</td>
<td>Phosphatidyl methyltransferase</td>
</tr>
</tbody>
</table>
OD  Optical density
OLB  Osmiophilic lamellar bodies
P   Detected degree of fluorescence polarization
PA  Palmitic acid
PC  Phosphatidylcholine
PC  Value of dipalmitoyl lecithin
PDA Patent ductus arteriosus
PE  Phosphatidylethanolamine
PG  Phosphatidylylglycerol
PI  Phosphatidylinositol
PDME Phosphatidyl dimethylethanolamine
PL-D Phospholipase D
PROM Prolonged rupture of membranes
PS  Phosphatidylserine
RCF Relative centrifugal force
RDS Respiratory distress syndrome
RLF Retrolental fibroplasia
S   Sphingomyelin
SEM Standard error of the mean
SPC Saturated
TLC Thin-layer chromatography
TPL Total phospholipid
Predictive value – The frequency of diseased patients among all patients with positive test results for a particular disease.

Sensitivity – The frequency of positive test results in patients with a particular disease.

Specificity – The frequency of negative test results in patients without the particular disease.
CHAPTER I

INTRODUCTION

This critique is an extensive review of the currently available knowledge on respiratory distress syndrome, pulmonary surfactants, and methods of assessing fetal pulmonary maturity.

Prematurity is a significant cause of perinatal mortality, for the preterm infant is subject to many problems, the most important of which is the risk of respiratory distress syndrome (RDS), causing respiratory distress. The lungs of these neonates are deficient in surfactant, which because of its unique variable surface tension effect when compressed prevents atelectasis and collapse of the alveoli at the end of expiration, thereby maintaining a residual capacity and facilitating expansion of the alveoli on inspiration.

The last thirty-five years, since the first description of infants with RDS, have been marked by extensive research concerning this syndrome. Currently the pathophysiology is carefully documented, and there have been major advances in neonatal lung development, surfactant biochemistry and metabolic activities, RDS therapy and prevention, and in neonatal pulmonary maturity assessment. The chapter's dealing with these above mentioned elements of RDS and pulmonary surfactants will now be briefly discussed.
Chapter II details fetal lung development which requires coordination of the anatomic, physiologic and biochemical processes. The amniotic development involves the appearance of lamellar bodies, in the type II alveolar cells, where pulmonary surfactant is synthesized, stored, and secreted. These lamellar bodies proliferate with gestation, come to the cell surface and are extruded into the alveolar fluid from about 24 to 26 weeks and are carried up into the amniotic fluid.

Chapter III describes surfactant physiology and biochemistry. Surfactant is the generic name for the biological detergent that adsorbs to the water layer lining the alveoli, after air displaces fetal alveolar fluid when breathing is begun. Surfactant lowers the surface tension of this water layer at its interface with air; otherwise the water would tend to make itself as small a surface as possible by the strong attraction of the water molecules in the surface and, in doing, would collapse the alveoli.


Surfactant is composed of about 90% lipid and 10% protein\(^4\). In turn about 85% of the lipid is phospholipid, the most abundant of which is lecithin or phosphatidylcholine (PC). Most of the studies on surfactant metabolism and physiology have been done with PC, although now it is recognized that the maturation of surfactant and the stabilization of lecithin in surfactant are dependent upon two acidic phospholipids: phosphatidylglycerol (PG) and phosphatidylinositol (PI).

Chapter IV details RDS etiology, diagnosis, treatment, complications and prevention. Deficient stores of pulmonary surfactant are a major etiologic factor in RDS of the newborn. Respiratory distress syndrome of the newborn infant consists of respiratory grunting, thoracic retractions and tachypnea of over 60 respirations per minute\(^5\). Confirmation of the diagnosis of RDS requires physical, blood gas, and radiographic findings. Over the last ten years there has been much improvement in the treatment of RDS infants, because of the advent of the neonatal intensive care unit

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and team approach theory. Also, the recent research on surfactant replacement therapy represents a potentially useful treatment of RDS infants, because it directly attacks the primary cause of RDS, which is surfactant deficiency. Another aspect of RDS which also requires additional research is prevention. It has been demonstrated that administration of glucocorticoids to pregnant mothers accelerates fetal pulmonary maturation. However, in the last ten years, there has been much controversy concerning glucocorticoids administration and its potential maternal and fetal side effects. Only the current long-term follow-up studies will answer some of these controversial questions.

Chapter V describes the methods utilized in assessing fetal pulmonary maturity. Fetal pulmonary maturity is evaluated by measuring the surfactant phospholipids, which change qualitatively as well as quantitatively during gestation. Therefore, the most accurate assessment of fetal pulmonary maturity is to measure several surfactant phospholipids. The standard and most widely utilized test for fetal pulmonary maturity is the lecithin/sphingomyelin ratio (L/S ratio).

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Also recently the rise and fall in concentration of PI and the appearance of PG can be measured and are being routinely assessed with the L/S ratio as the lung profile. Many variations of these two procedures have arisen because the tests are complex and long, the procedural steps are controversial and not standardized, and some false immature results have been obtained. Therefore other tests have been developed to overcome these shortcomings which include: optical density, cortisol, palmitic acid, lamellar body analysis, lecithin, enzymatic, fast-atom bombardment mass spectrometry, surface tension, shake and fluorescence polarization.

For each of the above mentioned methods the principle, procedure, results, contamination effects, and advantages and disadvantages of the procedure are discussed in detail. Because of these numerous amniotic fluid parameters in the literature, a survey of the Metro Detroit area hospitals was conducted to determine which procedures were being utilized by physicians to monitor and diagnose fetal pulmonary maturity. Currently the majority of laboratories utilize a one-dimensional thin-layer chromatographic procedure for the L/S ratio and PG determination. Therefore, in spite of its methodological problems, the revised and improved determination of the L/S ratio with PG determination has proven useful due to its many years of clinical correlation. Also the

enzymatic method is discussed as a future replacement for the LS ratio due to its numerous advantages.

In conclusion, whichever method is utilized in assessing fetal lung maturity there must be standardization of the procedural steps so that appropriate clinical cut-off values are determined.
CHAPTER II
THE PROCESS OF FETAL LUNG DEVELOPMENT

The transition from fetus to neonate imposes critical demands upon many organ systems, but in none are the demands so urgent or so abruptly imposed as in the case of the pulmonary system. For example, the cardiovascular system has been functioning for months in the fetus and has had ample exposure to circulatory requirements. The gastrointestinal system, although relatively inactive in the fetus, has at least several days to adjust to neonatal needs. However the lungs, which fulfill no role in fetal respiratory demands, must suddenly fulfill all neonatal respiratory demands from the moment of birth, or else the infant succumbs. The fact that most infants survive this amazing transition from placental to pulmonary respiration is indicative of the extensive and elaborate preparation that precedes birth. This preparation includes the development of not only an anatomic structure and circulatory system that will afford adequate gas exchange, but also the important ventilatory properties and neural control that will facilitate such exchange. The anatomic and morphologic development of the fetal lung will now be discussed.

A. ANATOMY

The lung is composed of nonrespiratory and respiratory structures.

1. Nonrespiratory Structures

The nonrespiratory structures, otherwise known as the conducting airways, consist of the trachea, right and left main-stem bronchi, segmental and subsegmental bronchi, varying generations of smaller
bronchi and bronchioles and a generation of terminal bronchioles. Figure 1 illustrates the above mentioned conducting airways (1). Also Fig. 1A illustrates; two systems of numbering the conducting airways: A (1), the traditional way, where counting starts with the upper airways and progresses downward; and B (1), the newer precise way, where counting begins with the last fully epithelialized generation and progresses upward. The numbers given represent mean counts.

All the axial generations of the bronchial tree (the nonrespiratory portions) have been formed by the 16th week of gestation. Until the 16th week, division and growth of the conducting airways continues in a dichotomous, asymmetric branching pattern. Between the 10th and 14th weeks a surge of activity, which develops 65-75% of the total number of branchings occurs. The branching process gives rise to differing path lengths, resulting in 8-32 divisions of pathways from the carina to the terminal bronchioles of various segments. The lingula, the middle lobe and the anterior and posterior basilar segments of the lower lobes contain the longest conducting airways. Figure 1B, shows that in the developed lung a characteristic pattern of asymmetric dichotomous branching can be found in each lobe. In each lobe there is variation not only in the number of airways but also in the path lengths of the different segments.

The functional significance of airway branching is to increase the number of terminal bronchioles from which the respiratory portions of the lung grow.
FIGURE 1
NONRESPIRATORY CONDUCTING AIRWAYS

Legend

A. Mean numbers of nonrespiratory conducting pathways. There are two systems of numbering the conducting airways: A, counting starts with the upper airways and progresses downward; and B, counting begins with the last fully epithelialized generation and progress upward. The numbers given represent mean counts. The respiratory bronchioles, alveolar ducts and alveolar sacs comprise the acinus.

B. This figure shows that in the developed lung there is a characteristic pattern of asymmetric dichotomous branching in each lobe.


2. Respiratory Structures

The respiratory structures, otherwise known as the acinus-portion, consist of the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli, which are shown in Fig. 2A. The acinus is the gas exchanging unit of the mature lung, consisting of structures peripheral to the terminal bronchiole (the most distal structure with a fibrous wall and ciliated epithelium). It contains three or four generations of respiratory (alveolar bearing) bronchioles and each of these gives rise to two or three generations of alveolar ducts (entire wall made up of alveoli) terminating in alveolar sacs (2).

The first appearance of the respiratory system is at 24 days gestation, at which time, the developing embryo is forming three primary germ layers: ectoderm, mesoderm (mesenchyme), and endoderm, by a process of cellular differentiation. However, only the mesoderm and endoderm are involved in the formation of the respiratory structures.

The 24th day of gestation marks the earliest evidence of embryonic respiratory structures with the appearance of a ventral groove in the cervical region of the endodermal tube. This groove develops into a ventral pouch to form the primitive lung bud, which grows into and develops within the median mass of mesoderm (Fig. 2B 1). Between the endoderm and mesoderm an interdependence exists, and it is likely that the mesoderm directs the development of the endodermal structures. At days 26-28, development of the bronchial tree is initiated by a series of asymmetric dichotomous branchings of the primitive lung bud (Fig. 2B 2). By 12 weeks of gestation the dichotomous branching of the lung has
FIGURE 2
THE ACINUS (RESPIRATORY STRUCTURES) AND STAGES
OF INTRAUTERINE DEVELOPMENT

Legend

A. The respiratory structures, otherwise known as the acinus portion consists of the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveolus.

B. Stages of intrauterine lung development begin (1) on the 24th day of gestation with the appearance of the ventral groove which develops into a ventral pouch to form the primitive lung bud. At days 26-28 (2), development of the bronchial tree is initiated by a series of asymmetric dichotomous branchings of the primitive lung bud. By 12 weeks of gestation (3), the dichotomous branching of the lung has continued and is characterized by the appearance of ciliated columnar epithelium which line the airways. As development continues (4) the lobes of the lung take form and the cellular mesenchyma surrounding the bronchial tree ultimately differentiates into the muscle, connective tissue and cartilaginous plates of the airways and the supporting tissues of the alveolar walls.

Abbreviations: TB = terminal bronchiole, RB, = respiratory bronchiole generation, AD = alveolar ducts, AS = alveolar sacs.


FIGURE 2

1. Trachea
   Lung Bud
   Esophagus

26-28 days
First division branching
Protrudes from gut

24 days
Early lung bud

3. 4-12 weeks
   Dichotomous branching,
   Columnar epithelium
   Lined airway

4. Later intrauterine development—
   Lobes of the lung
   Take form
continued and is characterized by the appearance of ciliated columnar epithelium which line the airways (Fig. 2B 3). Also the right and left lung buds branch out laterally into their respective pleural cavities, carrying with them the mesenchymal tissue, which continues to adapt itself to the shape of the developing bronchial tree. As intrauterine development continues the lobes of the lungs gradually take form and the cellular mesenchyma surrounding the bronchial tree ultimately differentiates into the muscle, connective tissue and cartilaginous plates of the airways and the supporting tissues of the alveolar walls (Fig. 2B 4). The elastic and collagen tissues of the airways, pleura, septa, and lung are formed from noncellular mesenchyma (1).

During the 14th to 16th weeks of gestation the respiratory bronchioles begin to develop, and relatively primitive alveolar structures are present at birth, the major development of the respiratory portion of the lung will occur after birth. Only 10% of the adult gas exchange unit is present in full-term delivered infant, for major changes occur as early as the first two months of life.

B. PRENATAL LUNG DEVELOPMENT

Currently four phases of prenatal lung development are recognized in humans, consisting of embryonic, pseudoglandular, canalicular, and alveolar sac phases. Each of these phases will now be discussed in detail.

1. Embryonic Phase

This is when the lung primordium is formed from the 3rd to 6th week. Morphogenesis of the lung depends on interactions between the epithelial
primordium and its underlying mesoderm. The isolated epithelium of a lung bud, separated from its mesoderm, continues to grow but branches fail to form; reunification of the epithelium and mesoderm makes branching begin. Electron microscopy of the mesoderm overlying the trachea and other non-budding areas of epithelium shows a highly ordered structure consisting of: rectangular shaped cells which are regularly arranged in rows with their long axis perpendicular to the epithelial tube and an array of collagen fibers running parallel to the axis of the airway between the mesodermal cells and the epithelium. The collagen fibers and the mesodermal cells are not arranged in this regular way at the tips of the lung bud. Wessels (3), and Alescia (4), found that, growth of the epithelium is controlled and stabilized by the regular arrangement of the collagen fibers, which create a barrier to the formation of branches, except at special points where the collagen bundles are interrupted, allowing epithelial cells to grow out through the gap. Therefore, epithelial cell subdivision is probably determined by general epithelium growth, and the sites of budding by the pattern of collagen fiber formation. During this phase, on the 26th day of gestation the lung bud appears and soon divides and elongates into two sacs (Fig. 3A and B). The sacs grow dorsad on either side of the esophagus (Fig. 3C). Then upper, middle, and lower lobar buds develop and upon these, appear the primary buds of the ten bronchopulmonary segments (Fig. 3D), and from these arise the future airways (Fig. 3E).

2. Pseudoglandular Phase (bronchial divisions are established (Fig. 3F), but in which respiration is not yet possible).
FIGURE 3

EMBRYONIC AND PSEUDOGLANDULAR PHASES OF INTRAUTERINE LUNG DEVELOPMENT

Legend

Diagrammatic representation of the development of the human lung in the embryonic (A-E), and pseudoglandular (F), phases (A is depicted from the front and the side, all others from the front only). During the embryonic phase on the 26th day of gestation the lung bud appears (A), and soon divides and elongates into two sacs (B). These sacs grow dorsad on either side of the esophagus (C). Then upper (U), middle (M) and lower (L) lobar buds develop and upon these appear the primary buds of the ten bronchopulmonary segments (D), and from these arises the future airways (E). All subsegmental bronchi are represented, with many undergoing further subdivision by days 38 to 40 (F).

The pseudoglandular phase obtained its name from the fact that between the 6th and 17th weeks of gestation the lung resembles the acinar portion of an exocrine gland. Tall columnar cells are present which have elongated basal nuclei, prominent, rough endoplasmic reticulum, an abundant store of glycogen found at the growing tips of the lung rudiment and are surrounded by small liquid filled spaces (Fig. 4A). Also, the columnar cells have large amounts of mesoderm present and the capillaries are some distance from the epithelium surface (Fig. 4A) (5).

Figure 5A illustrates this pseudoglandular phase, with an electron micrograph.

There are currently two theories which postulate why there are such massive quantities of glycogen present in the epithelial cells during this pseudoglandular phase. The first theory is that since the epithelial cells are some 5-10 μm from the blood capillaries they store the glycogen to be utilized at the time of cell division for the cells' energy demands are such that only oxidation of glucose can provide a sufficient supply (5). The second theory is that the pseudoglandular phase is followed by a period of cell differentiation, during which the rate of cell proliferation and amount of glycogen in the epithelial cells decreases, and surfactant synthesis increases, and type II cells become recognizable by the presence of lamellar bodies. It is postulated that a reciprocal relationship exists between glycogen accumulation, and lamellar body formation, therefore fetal lung glycogen is utilized for the initial synthesis of surfactant (5).

During this phase the lung is seen as loose mesenchymal tissue
FIGURE 4

PULMONARY EPITHELium TRANSFORMATION THROUGH THREE PHASES OF DEVELOPMENT

Legend

Pulmonary epithelium transformation through: A. pseudoglandular; B, canalicular; and C, alveolar sac phases. Left-hand diagram showing cell and lining; while right-hand diagram based on electron microscopic appearance of cellular contents.

A. The pseudoglandular phase has tall columnar epithelium containing abundant glycogen (shaded areas) and glycogen-free spaces (GFS); note the capillaries (C) are some distance from epithelium.

B. The canalicular phase with cuboidal epithelium consisting of secretory cells and prospective lining cells. Secretory cells contain osmiophilic lamellar bodies whereas the future lining cells are labeled by the low position of the junctional complex with neighboring cells. These cuboidal cells also contain less glycogen and the capillaries are closer to the epithelium.

C. The alveolar sac phase is where the lining cells have formed broad and thin cytoplasmic extensions allowing for a thin air-blood barrier while the secretory cells remain unchanged. Also the epithelium have differentiated into type I and type II cells and the capillaries are very close to the epithelium.

Abbreviations: L = lumen, C = capillary, L₁ = lamellar body, Mes = mesodermal cell.


FIGURE 4

A. Pseudoglandular Phase

B. Canalicular Phase

C. Alveolar Sac Phase
FIGURE 5.
ELECTRON MICROGRAPH DEPICTING PSEUDOGLANDULAR
AND CANALICULAR PHASES

Legend
A. Electron micrograph depicting high columnar cells of epithelial tube
of later pseudoglandular stage (day 15 of gestation). The cytoplasm is
packed with glycogen (gly); it also contains small mitochondria and some
scare cisternae of rough endoplasmic reticulum. Arrow is pointing
towards tight junctions at the luminal surface of epithelial cells. (x
8,800).

B. Electron micrograph depicting canalicular phase at the 25th day of
gestation in fetal rabbit, where the thinned epithelium are present in
the future alveolar region. The interstitium appears thinner than during
the pseudoglandular phase, probably due to loss of interstitial fluid.
(x 3,200)

Abbreviations: sac = sacculus, cap = capillary.

Reproduced without permission from: A. Burri, P. H., Weibel, E. R.
(1977) in Development of the Lung: Lung Ultrastructure and Morphometry
(Lenfant C., Hodson, W. A., eds.), p. 220, Marcel Dekker, Inc.

B. Meyrick, B., Reid, L. (1977) in Development of the Lung:
160, Marcel Dekker, Inc.
surrounding buds of endodermal cells and vascularization of the mesenchymal tissue is not apparent. Also, the preacinar artery and airway branching pattern are established and elastic fibers are found around main bronchi, arteries and the pleura.

3. Canalicular Phase (Viable stage in which the respiratory portion becomes delineated and vascularized (Fig. 4B)).

The canalicular phase is from the 17th - 24th week of gestation and is characterized by subdivision of the branches which elongate and the epithelial cells change from columnar to cuboidal. The cell nuclei and glycogen condense and the mesoderm begins to thin out and its plexus of blood capillaries becomes more abundant (6). Towards the end of this phase, parts of the epithelium in the peripheral air passages become thinner, and great proliferation of the microcirculation takes place, bringing blood capillaries into close relation to potential air spaces for the first time (Fig. 4B). Figure 5B illustrates that the epithelial lining the cells is much flatter and that the cell organelles are at the cell edges.

At the beginning of this canalicular phase the respiratory portion of the lung is recognized and during it, increasing vascularization of the mesenchyme occurs. The epithelium is thinning due to the capillaries migrating towards the terminal portions of the endodermal bud and pushing into the epithelium. The terminal end of the buds also branch and grow to form the future terminal air sacs, while transformation of the last airways occurs to form the respiratory bronchiolus. Also elastic fibers are found close to the saccules.
4. Alveolar Sac Phase (Definitive alveoli are differentiated (Fig. 4C)).

The alveolar (or terminal) sac phase is from the 24th week of gestation to term and is characterized by two features: 1) close approximation of blood capillaries to epithelium throughout the lung, and 2) differentiation of epithelial cells into their mature forms (type I and type II cells), as shown in Fig. 4C. Also Fig. 6 depicts this phase and the type I and II cells.

By 30-32 weeks, the terminal bronchioles give rise to three or four generations of thin-walled structures, the forerunners of respiratory bronchioles, the most peripheral of which bear two clusters of elongated saccules (7). At term, the saccules have undergone subdivision to the extent that each terminal bronchiole may carry 176 saccules.

During this phase further differentiation occurs of the respiratory region with the appearance of more saccules (later to become alveolar ducts) and considerable thinning of the walls of those already present. This represents a considerable increase in surface area for the lung.

In 1963, Campiche et al. first identified type I and type II alveolar cells at 24 weeks gestation (6). Type I cells cover about 96% of the mature alveolar surface by virtue of their long cytoplasmic extensions and are flattened structures with a diameter of 50-60 \( \mu \text{m} \) and an average thickness of 0.1\( \mu \text{m} \). They interdigitate by tight junctions with other type I cells and with type II cells. As seen in Fig. 7A, type I cells contain few intracellular organelles. The nucleus is centrally placed in the cell giving the appearance of a fried egg (if the nucleus is
FIGURE 6
ELECTRON MICROGRAPH DEPICTING ALVEOLAR SAC PHASE

Legend
Electron micrograph depicting alveolar sac phase in fetal rabbit at 27 days gestation, where both the type I (I) and type II (II) pneumonocytes can be recognized. (× 9,000)

Abbreviations: sac = saccule, cap = capillary, lb = lamellated bodies. I = type I pneumonocyte, II = type II pneumonocyte.

FIGURE 7

ELECTRON MICROGRAPH DEPICTING TYPE I AND TYPE II CELLS OF THE ALVEOLAR SAC PHASE

Legend

A. Electron micrograph depicting type I cell of the human alveolar wall. A red blood cell is in the capillary lumen which is lined by endothelium. The type I pneumocyte has long cytoplasmic extensions, which line most of the alveolus and form part of the blood-gas barrier. The varying thickness of this barrier should be noted. To the left the barrier also contains elastin collagen, and part of a fibroblast. Cell junction (♂) of type I pneumocytes. (x 4,800)

B. Electron micrograph depicting type II cell from a 6 year-old child. This cell is characterized by the lamellated bodies (.lb) and the cytoplasmic projections at the alveolar surface (pr). This cell is associated with the production and storage of pulmonary surfactant. (x 10,400)

Abbreviations: cap = capillary lumen, end = endothelium, I = type I pneumocyte, al = alveolus, el = elastin, lb = lamellated bodies, pr = cytoplasmic projections.


considered the yolk). The nucleus is covered by a thin layer of cytoplasm in which Golgi apparatus, ribosomes, odd cisternae, and rough endoplasmic reticulum, and occasionally, small mitochondria may be found. Theory assumes that the blood-gas exchange takes place mainly in the thinnest part of the alveolar wall. In this region the blood-gas barrier is comprised of a part of a type I cell and endothelial cell and the basement membrane or fused membrane of both cells (7).

Type II cells are rounded structures about 10 μm in diameter bearing microvilli and have cytoplasmic projections along their free alveolar edge. The type II cells (another name, granular pneumocytes) occupy less than a tenth of the lung surface, and about half of the epithelial cells of the lung periphery are of this type. Figure 7B depicts the type II cell, which contains numerous mitochondria with tightly packed cristae, abundant rough endoplasmic reticulum, well developed golgi zones and high concentration of oxidative enzymes (7). The most characteristic feature of mature type II cells is the osmiophilic lamellar bodies which are intracellular structures measuring 0.2-0.5 μm in diameter and consisting of stacks of membranous material (Fig. 7B). The lamellar bodies first appear in the lungs at the same gestational age (human, 24 weeks gestation) as the surface active surfactant can be extracted from lung tissue, and are believed to represent the intracellular store of surfactant (7). Another component of type II cells to show definite changes in late fetal life, is the peroxisome, which is a dense body (0.2-1.0 μm in diameter) containing H₂O₂ producing enzymes and catalase. As mentioned above, the type II cells' function is considered to be the
site of production and secretion of pulmonary surfactant.

C. GAS EXCHANGE UNIT

Gas exchange is composed of three interrelated functions: 1) ventilation of the respiratory tissues, 2) diffusion of gas, and 3) adequate perfusion of the pulmonary capillaries. The maintenance of extraterine life is totally dependent on the interrelated functioning of these three components. The gas exchange unit is composed of respiratory bronchioles, alveolar ducts, alveolar saccules, and alveoli together with the tissues that form their boundaries and the pulmonary capillaries.

Following is a summary of the chronological development of the gas exchange unit, which includes both the pre- and postnatal periods (8). Figure 8A diagrammatically illustrates the acinus at six stages of development.

1. 17 Weeks of Gestation

This period is the timing of the birth of the acinus, characterized by the delineation of the pulmonary acini and the initial invasion of their peripheral branches by capillaries. The essential components of the acinus that can be identified at this time are: terminal bronchioles, a series of two to four prospective respiratory bronchioles and a peripheral cluster of six to seven generations of closely branched buds destined to become the saccules of the terminal sac period.

2. 20 Weeks of Gestation

There is a thinning of the epithelium at the distal ends of the conducting airways and a minimal amount of capillaries are in contact with potential air spaces.
FIGURE 8
DEVELOPMENT OF THE ACINUS

Legend

A. Figure illustrates diagrammatic representation of the acinus at six stages of development. At all ages airway generations are drawn the same length so that increase in length represents an increase in generations. A given generation may be traced down the same vertical line, permitting remodeling in its structure to be followed. Actual increase in size is shown by the length from the terminal bronchiole to the pleura.

B. Drawing of a section through a respiratory bronchiole and two alveolar ducts of adult human lung. This drawing shows the gas exchanging acinus portion and the components it consists of: respiratory bronchioles (three orders), a generation of alveolar ducts, and terminal clusters of alveolar sacs.

Abbreviations include: TB = terminal bronchiole, RB = respiratory bronchiole, TD = transitional duct, S = saccule, TS = terminal saccule, AD = alveolar duct. At = atrium, AS = alveolar sac, TB = terminal bronchiole.


3. 22 Weeks of Gestation

Evagination of the thinner areas of epithelium is noted and the capillary buds found are in more intimate contact with the epithelium.

4. 24 Weeks of Gestation (Fig. 9A)

Appearance of terminal portions of the acinus are present, which begin to resemble those of the succeeding terminal sac period (Fig. 9A). Cellular differentiation into type I and type II alveolar cells occurs and the capillary invasion is very advanced. However, the potential air-blood interface is not well defined and the air spaces are poorly developed. Figure 9A shows the appearance of the peripheral airways after being invaded by capillaries.

5. 26 Weeks of Gestation

Onset of the terminal saccule phase is characterized by further development of the alveolar saccules, development of the intersaccular septa and marked proliferation of the capillary bed. The developing pulmonary arterioles and the capillary bed gradually become contiguous.

6. 28 Weeks of Gestation

The alveolar saccules are lined by cuboidal epithelium and the capillaries are numerous. Also the respiratory bronchioles and alveolar ducts are distinguishable. Lamellar bodies proliferation in the type II alveolar cells occurs.

7. 30 Weeks of Gestation (Fig. 9B)

The intersaccular septa with prominent elastic tissue fibers are found and partial alveolarization has occurred with thinning of the saccular epithelium. Figure 9B shows that the capillaries have invaded
FIGURE 9

24 and 30 WEEKS GESTATION

Legend

A. Photomicrograph (x 800) of a 5 μm section of the terminal airspaces in a stillborn infant of 24 weeks gestation (after ovulation measuring 247 mm and weighing 870 g). This micrograph shows the appearance of peripheral airways after being invaded by capillaries. Capillaries seem to line air channels but are actually covered by thin membrane of entodermal origin.

B. Photomicrograph (x 70) of a 5 μm section of the left superior segment of a premature infant of 30 to 32 weeks gestation (weighed 1300 g and lived 19 hr and 15 min). Note the large subterminal bronchiole at top of figure which is dividing into two terminal bronchioles. The one on the right can be followed through three series of respiratory bronchioles. Asterisk, * = indicates a 'third respiratory bronchiole dividing into two transitional ducts; 5a = preceded by a transitional duct passing into a cluster of sacs. Capillaries have invaded the thin layers of the respiratory bronchioles (R'a and b). Its thick layer consists of dense ciliated columnar epithelium. The distance from the big subterminal bronchiole to the pleura is 1.6 mm.

Abbreviation: cap = capillaries, ch = channels.

the thin layers of the respiratory bronchioles. At this time the three orders of respiratory bronchioles are tubes lined on one side by dense ciliated cuboidal epithelium, and on the other side by flat capillarized epithelium.

8. 34 Weeks of Gestation

At this time the gas exchange unit is well developed with respiratory bronchioles, alveolar ducts, and primitive saccular structures. Also capillary invasion is prominent and the type I alveolar cells are now thin lining cells which facilitate diffusion of gas from the alveoli to the capillary spaces. In the septa and at the mouths of the saccular structures are the metabolically active type II cells.

9. 38-40 Weeks of Gestation

The intersaccular septa have numerous shallow, saucer-shaped depressions known as the alveoli of the newborn lung. As intrauterine lung maturation progresses, thinning of the intersaccular septa occurs, but these septa are obviously broader in infants of term than the typical interalveolar septa present in the mature lung.

Also many type II alveolar cells which contain the lamellar inclusion structures (inclusions are comparable in fetal neonatal and adult lungs) are present, and a few type I cells are found in the alveolar section of the fetal lung. The respiratory bronchioles and alveolar ducts contain numerous type I cells.

10. Birth

The number of alveolus-like structures is 340 alveoli per terminal lung unit. The gas-exchanging-acinus portion consists of three orders of
respiratory bronchioles, a generation of transitional (alveolar) ducts, and terminal clusters of alveolar sacs (Fig. 8B) with relatively few narrow and shallow alveoli.

11. Postnatal

A great deal of lung development occurs after birth. For the changes in pulmonary maturation are immense and must occur by various mechanisms. The respiratory system of the newborn infant, although sufficiently developed to support extrauterine respiration, would soon become inadequate if the following rapid postnatal growth and development did not occur (8).

Postnatal development of the respiratory portion of the lung is evident in infants as early as the first two months of life. At birth, the respiratory portion of the fetal lung consists of three generations of respiratory bronchioles and one order of alveolar ducts with alveolar sacs. Approximately 24 million rudimentary alveoli are present, but they are shallow and have an average diameter of only 50 μm. However, by two months there are at least four orders of respiratory bronchioles and three orders of alveolar ducts. The alveolar sacs and alveoli have a diameter in the range of 50-120 μm. The saccular structure of the newborn lung develops into the alveolar structures of the mature lung. The number of alveoli increases approximately tenfold and the air-tissue interface increases to a magnitude twenty-one times that which exists in the newborn. The interalveolar septa and alveolar walls undergo changes in morphology and relative size along with an increase in the lateral chest wall dimensions. An integration of these processes results in the
mature respiratory system (8).

D. BIRTH AND THE ONSET OF RESPIRATION

At birth the newborn infant enters an uncontrolled environment and must initiate respiration, readjust the circulation, maintain body temperature and adjust the acid-base balance. All of these interrelated functions must be performed rapidly, for failure of any one of these functions will have a devastating effect on the others. The perinatal change which consumes enormous amounts of energy and must precede all of the other functions is the initiation of respiration, for initial respiratory forces are directed toward removing lung fluid, overcoming the surface tension encountered in the respiratory portion of the lung, and reshaping the nonrespiratory pulmonary tissues.

The major rôle of gas exchange is facilitated by the lungs and pulmonary circulation which, both develop biochemically and morphologically in utero in preparation for birth. A rapid transformation occurs in the lung from a fluid-filled to an air-filled lung with an end-expiratory stability at birth. The four processes which facilitate this transformation include: 1) the type of delivery; 2) the establishment of an effective pulmonary circulation; 3) the initiation and maintenance of the mechanism of breathing; and 4) pulmonary gas exchange (8).

In a normal vertex presentation through the vagina, the fetal chest is compressed by the cervix during passage through the birth canal and a quantity of the pulmonary fluid is forced out through the fetal mouth. The elastic chest wall of the fetus regains its expanded shape and a
relative intrapulmonary vacuum develops after the chest passes through the cervix. Also at this time, air is drawn into the lungs and the beginning of the pulmonary gas volume is rapidly established. However, if the fetus is delivered breech or by a cesarean section, the expulsion of fluid caused by cervical chest compression does not occur and therefore more fluid must be removed via the pulmonary lymphatics and circulation. Cervical chest compression removes an estimated 10–15% of the pulmonary fluid and the remainder is removed from the lung by the pulmonary lymphatic circulation during the first few days of life. The Valsalva effect and relatively low oncotic pressure of the lung fluid facilitate in this clearance of lung fluid. Norman noted that there is a definite relationship between the onset of ventilation, the decrease in alveolar fluid and the steady increase in lymphatic flow (9).

A combination of the negative intrathoracic pressure resulting from passage through the birth canal and diaphragmatic contraction result in the fetus' first extrauterine breath.

The following conditions occur with the first breath:

- The volume of the first breath ranges between 15 and 80 mL in a full-term infant, this is a volume between tidal volume and crying vital capacity.

- The negative intrathoracic pressure ranges between −40 and −80 cm H₂O pressure to overcome all of the opposing forces and to establish a gas-liquid interface.

- Inflation of the lung is probably complete with the first few breaths, but increases in compliance and changes in airway
resistance, residual volume, and functional residual capacity continue over a period of days.

- Expiratory tidal volume is less than inspiratory volume during the first few breaths, while the residual volume is being established.
- Although surfactant is present prenatally, it apparently does not lower surface tension until the air-liquid interface is established (10).

E. VASCULAR CHANGES DURING PULMONARY DEVELOPMENT

As mentioned previously, increases in pulmonary blood flow along with the establishment of effective ventilation permit the lung to function as an extrauterine gas exchange organ. For with the filling of the lungs with air, the newborn PaO₂ increases dramatically and there is a rapid, marked decrease in pulmonary vascular resistance and a rapid increase in pulmonary blood flow.

Gas exchange is facilitated between the fetus and mother across the placenta in intrauterine life (Fig. 10A). Therefore, the circulation of the fetus is adapted for supplying a large blood flow to the placenta and only a small one to the lungs. However, at birth, there are three major changes: 1) the placental circulation is eliminated, 2) blood flow through the lungs increases (systemic vascular resistance rises); and 3) a reduction of pulmonary vascular resistance occurs (10). These last two factors cause a reversal of the ratio of systemic to pulmonary vascular resistance that reverses the direction of ductal blood flow (another factor which serves to increase the pulmonary blood flow). The increase in PaO₂ also stimulates the ductus arteriosus to constrict and it
FIGURE 10

FETAL, TRANSITIONAL, AND ADULT-TYPE CIRCULATION'S

Legend

A. Gas exchange in facilitated between the fetus and mother across the placenta in intrauterine life. This schematic diagram is showing the fetal circulation and the foramen ovale is between the right and left atrium.

B. The transitional circulation occurs shortly after birth and is noted by an increase in the left atrial pressure acting to close to the foramen ovale. Also by twelve hours of life in the full-term neonate, the ductus arteriosus, normally closes and there is no longer a left-to-right shunt at the ductal level.

C. After seventy-two hours of extrauterine life, the ductus arteriosus is permanently closed. The foramen ovale is also functionally closed and an adult-type circulatory pattern is established.

Abbreviations: RV = right ventricle, LV = left ventricle, RA = right atrium, LA = left atrium.

normally becomes progressively restricted with an increased intrinsic resistance. With the higher pressure and resistance in the aorta as compared to those in the pulmonary artery, the left atrial pressure becomes greater than the right atrial pressure and serves to functionally close the foramen ovale. These changes in circulation which occur shortly after birth are termed transitional circulation and are illustrated in Fig. 10B. By twelve hours of life in the full-term neonate, the ductus arteriosus normally closes and there is no longer a left-to-right shunt at the ductal level. After seventy-two hours of extraterine life, the ductus arteriosus is permanently closed. The foramen ovale is also functionally closed and an adult-type circulatory pattern is established, as shown in Fig. 10C (10).

F. SUMMARY LUNG DEVELOPMENT

Differentiation of the lung must bring about complete structural remodeling, ensure the presence of pulmonary surfactant (surface tension-lowering material), and provide the capacity for rapid resynthesis and renewal of this phospholipid-rich substance lining the alveoli. Therefore, fetal lung development requires coordination of anatomic, physiologic, and biochemical processes, the timing of which must be carefully regulated. The ultimate product of these changes in structure, function and metabolism is an organ with alveoli having adequate surface area and capable of sustained ventilatory excursions for efficient gas exchange. The surfactant system plays a major role in development of the fetal lung and in postnatal respiratory function and will now be discussed.
CHAPTER III
PULMONARY SURFACTANT

A. PHYSIOLOGY OF SURFACTANT

The definition for pulmonary surfactant is that it is a unique lipoprotein complex, particularly rich in highly saturated lecithins, that is produced in the alveolar type II pneumocytes and secreted onto the alveolar surfaces; by providing a low and variable interfacial surface tension, it serves to facilitate maintenance of alveolar stability.

The surfactant system plays a major role in the development of the fetal lung and in postnatal respiratory function. The structural integrity of the alveoli are highly dependent on the presence of the pulmonary surfactant system in the alveolar lining layer. The alveolar surface of the lung is normally covered with an acellular lining layer. This layer is known as pulmonary surfactant, because it serves to lower surface tension at the air-water interface of the lung, thus maintaining alveolar stability. Therefore, surfactant is often called the anti-atelectasis factor, located in the alveolar lining layer.

In the newborn infant, surfactant has two basic functions: first it must maintain alveolar stability or prevent diffuse collapse of the terminal respiratory units; and secondly, it must reduce the pressure needed to distend the lung initially. These two functions are attributable to surfactant's capacity for lowering surface tension. For more specifically, the presence of surfactant along the air-water interface of the alveolar surfaces ensures a low and variable surface
tension, such that the hysteresis requirement for stable alveolar excursions is met.

The remainder of this chapter will be a review and discussion of the presently known information concerning the surfactant system.

1. Historical Review of Pulmonary Surfactant

For all practical clinical purposes the biochemical development of the lung is synonymous with that of pulmonary surfactant, for if production of these phospholipids is inadequate, the alveoli do not stabilize and the neonate will not survive.

Following is an account of the historical timetable of surfactant, starting with the original concept of surfactant being the anti-atelectasis factor in the lung, to its association with fetal pulmonary maturity.

- Gluck (11) found that in 1929, Von Neergaard described the relation between pulmonary pressure-volume characteristics and surface tension of alveolar fluid.
- In 1958, Clements et al. demonstrated the presence of an insoluble film lining the alveoli at the air-liquid interface (12). This film proved to be a surfactant complex, whose prime function was to lower the alveolar surface tension and to prevent alveolar collapse and lung retraction during expiration. The surfactant was isolated by saline lung lavage and tracheal aspiration and was found to display a high surface activity.
- In 1961, Klaus et al. isolated pulmonary surfactant from bovine lung, extracting a phospholipid fraction with compression and
expansion characteristics on the modified Wilhelmy balance,
similar to those of the extract of total normal lung (13). The
similarity of these findings to those produced by synthetic
dipalmitoyl lecithin led to the postulation that dipalmitoyl
lecithin was the lung surfactant. However, more recent work shows
that other lecithins and phospholipids found in the lung also are
surface active.

- In 1959, Avéry and Mead were the first, to clinically correlate
development of RDS with surfactant properties (consequence of
alveolar collapse and fluid accumulation) (14).

- In 1967, Scarpelli suggested that fetal respiratory movements
result in extrusion of endotracheal fluid containing pulmonary
surfactant into amniotic fluid (15). This observation resulted in
the study of the composition of amniotic fluid lipids.

- In 1968, Biezinski et al. reported the first detailed study of the
composition of human amniotic fluid lipids (16).

- In 1969, Nelson made the initial observation of the relationship
between low amniotic fluid phospholipid concentration and
subsequent development of RDS (17). However, the most significant
clinical breakthrough came in 1971, with the Gluck publication on
RDS being caused by a surfactant deficiency (discussed in detail
CHAPTER V 1.b). From these findings followed the assessment of
amniotic fluid phospholipids to define the formation of pulmonary
surfactant and pulmonary maturity and in turn reflect fetal
maturity.
2. Surface Tension

Surface tension represents a phenomenon observed at an air-liquid interface. The fluid lining of alveoli qualifies the alveolar sac as an air-liquid interface. The surface tension observed is related to the intermolecular attraction of liquid molecules on the surface which bind each other more readily than do molecules of air above the liquid layers. The observed resultant phenomenon is net work (force measured in dynlem), in which the strong mutual attraction of the water molecules on the surface decreases the surface to as small an area as possible.

The majority of in vitro studies of surfactant film (washed or extracted from mammalian lungs) surface properties, have been conducted by spreading the surfactant in a Wilhelmy trough and determining the surface tension, while compressing and expanding the surface film. All lung films of this type exhibit two characteristics. First these films exhibit a large, reproducible hysteresis (a difference in film behavior on expansion from that observed on compression) (Fig. 11 A and B) when they are cycled on a electrolytic subphase in a surface balance, and secondly, the surface tension lowering attained at maximum compression is extremely high (60-70 dyn/cm based on the σ of water) (10). These two characteristics have caused researchers to conceive of the pulmonary surfactant system in vivo as a monomolecular film at the liquid-air alveolar interface, which acts to cause large or inflated alveoli to have
FIGURE 11

SURFACE AREA DIAGRAMS, SURFACE TENSION,
AND MOLECULAR ORIENTATION OF PHOSPHATIDYLCHOLINE
AT THE AIR LIQUID INTERFACE

Legend

A and B. Surface tension/surface area diagrams of extracts of lung from an infant with respiratory distress syndrome (right) and a lung from an infant with normal lungs (left). These were obtained from a modified Wilhelmy surface tension balance in which surface tension is continuously recorded as the surface area of the lung extract is reduced and increased. When the surface area is reduced to 20% (equivalent to a lung changing from total lung capacity to residual volume) the surface tension of the extract from the lung with respiratory distress syndrome is 20 mN/m, a high tension, which if present at the surface of the alveolus, would favor atelectasis. The surface tension at 20% surface area for extracts of normal lungs is 6 mN/m.

C. Figure is a diagrammatic representation of molecular explanation of surface tension. A molecule of water within the bulk phase is attracted by all molecules surrounding it, however, molecules of water at the surface experience a net attraction into the interior.

D. Figure illustrates orientation of various molecular species of PC at the air-liquid interface. The polar PC group binds water by means of its positive and negative charges. Owing to the kinks at the double bonds, PC’s with unsaturated fatty acids cannot be compressed to the same extent as DPPC and, therefore, are not as effective in reducing surface tension. Numerals refer to the number of carbons and the number of double bonds on the fatty acids: 16:0, palmitate; 18:1, oleate; 18:2, linoleate; 18:3, linolenate.


FIGURE 11

Molecular Explanation of Surface Tension

AVERAGE MOLECULAR CROSS SECTION OCCUPIED AT THE AIR-WATER INTERFACE BY DIFFERENT PHOSPHATIDYLCHOLINES
a high surface tension ($\sim 50 \text{ dyn/cm}^*$), while causing small or deflated alveoli to have a very low surface tension ($\sim 1 - 10 \text{ dyn/cm}$). Therefore, the pulmonary surfactant system acts to help stabilize the alveolar structure of the lung (10).

Practical implications to the clinician reside in a phenomenon described by the Law of Laplace: $\Delta P = \frac{2\sigma}{R}$, where $\Delta P$ = change in pressure across the surface ($\text{ dyn/cm}^2$), $\sigma$ = surface tension ($\text{ dyn/cm}^{-1}$), and $R$ = radius (cm), which states that wall tensions of expandable and compressible spheres are inversely proportional to their radii.

The smaller the radius of a sphere, such as a bubble of air or an alveolus (which functions as a bubble) the greater the wall tension. According to Pattle's postulation, as it becomes smaller, should the alveolus not be stabilized, two forces pertain. First, the alveolus would collapse due to the extremely high wall tensions, which are too high to permit alveoli to remain patent on expiration, and the second force, is the high pressures required to reexpand the alveolus which results in transudation of fluid from the blood vessels into the alveoli. Therefore to summarize the concept of tension and comprehend the manner in which pulmonary surfactant stabilizes the lung, a molecule of water in a beaker shall be utilized as an illustration. As shown in Fig. 11C,$^*$

*The maximum surface tension of 50 dyn/cm is due to the fact that lung extract films contain soluble as well as insoluble surfactants. It is these soluble or partially soluble surfactants which give the system a maximum of about 50 dyn/cm rather than the 72 dyn/cm of a pure water system.
a molecule of water in a beaker is attracted by the molecules of water around it, therefore, resulting in zero net force. On the other hand, molecules of water at the surface experience a net attraction into the bulk phase; therefore, it requires 70 ergs (work) (1 erg = 0.1 J) at 37° C to move sufficient water and expand the surface by 1 cm (18). The above-mentioned Laplace Law pressure difference is the result of the net attraction of the water molecules into the bulk phase and the tendency to assume a minimum surface area (18).

Figure 11D illustrates the orientation of various molecular species of phosphatidylcholine (PC) at the air-liquid interface. Dipalmitoyl phosphatidylcholine (DPPC), or unsaturated phosphatidylcholine (PC) can form monolayers at the air-liquid interface with the polar head groups in the water and the hydrophobic fatty acids extending outwards (18). The polar PC group binds by means of its positive and negative charges. Since surface tension arises from the attraction of water into the bulk phase, therefore binding water at the surface which counteracts this attraction, will reduce surface tension (18). Pulmonary surfactant films are very stable and can be compressed until the surface tension falls to 0.7 dyn/cm (18). Owing to the kinks at the double bonds, PC's with unsaturated fatty acids cannot be compressed to the same extent as DPPC and therefore, are not as effective in reducing surface tension. (Unsaturated PC's can achieve low surface tensions but only below the gel-liquid crystalline phase transition temperature) (18).

3. Metabolic Activities of Surfactants: Synthesis, Storage, Secretion and Degradation
The major site of all surfactants' metabolic activities is the large alveolar epithelial cell known as the type II pneumocyte. The type II cell comprises approximately 10% of the lung's cells and contains osmiophilic lamellar bodies (OLB's), the presumed storage granules of the surfactant complex (Fig. 12). These cells' perform a triad of functions, for they synthesize, store, and secrete pulmonary surfactant (20).

Meyrick and Reid have conducted an extensive literature search and found that the following evidence has accumulated supporting the theory that the osmiophilic lamellar bodies (OLB's) of the type II pneumocyte represent the intracellular storage and sites of synthesis of pulmonary surfactant (20):

- Investigators have found a rise in surface tension accompanied by a decrease in osmiophilia of lamellated bodies, but not a decrease in number.
- It has been demonstrated from the lungs of infants dying of RDS, that there was a lack or reduction in the number of lamellated bodies.
- A secondary osmium tetroxide-ethanol fixation method was utilized to demonstrate synthetic dipalmitoyl lecithin. This fixation method was also found to increase osmophilia within the lamellated body of the type II pneumocyte, indicating this as the source of pulmonary surfactant.
- OLB's tend to increase in numbers during the course of fetal lung development and at term appear to discharge their contents (Fig.,
FIGURE 12

TYPE II ALVEOLAR PNEUMOCYTE

Legend

Photomicrograph showing a type II pneumocyte (II) with a lamellated body discharging into the alveolus. The lamellar bodies are the presumed storage granules of the surfactant complex. The type II pneumocytes perform a triad of functions, for they synthesize, store and secrete pulmonary surfactant. (x 22,500)

Abbreviations: II = type II pneumocyte, lb = lamellated body, al = alveolus.

12) into the alveolar spaces, for temporally their increase in numbers and size correlates with adequacy of pulmonary function and with the ability to recover pulmonary surfactant.

- Finally, OLB's have the following characteristics: they contain phosphoglycerides of the type important in surfactant; they sediment in subcellular fractions containing concentrated surface activity; and finally they have a membrane-like structure, resembling the stacked-up lamellae to be seen in liposomes in phosphoglyceride water suspensions.

- Chevalier and Collet investigated the OLB's mechanism of secretion by following the fate in type II cells of labelled choline (a specific precursor of phosphatidylcholine) (21). The labelled choline was initially localized in endoplasmic reticulum, then rapidly transferred through the Golgi complex stored in OLB's and later discharged to the alveolar surface (Fig. 13A). Also tritiated galactose and leucine, carbohydrate and protein precursors, respectively, were found to be incorporated into the type II pneumonocytes and finally localized in the lamellated bodies by slightly differing mechanism (Fig. 13A). This study demonstrated that the secretory product formed in the type II pneumonocyte is comprised of phospholipid, protein and carbohydrate, three components of surfactant (21).

Additionally, other studies conducted agree with the above findings of pulmonary surfactants' synthesis in the type II cells and have determined the extracellular mechanism of lamellar bodies (Fig. 13B) (18), for
FIGURE 13

CELLULAR AND EXTRACELLULAR ASPECTS OF PULMONARY SURFACTANT

Legend

A. Utilizing electron microscopic autoradiography investigated the in vivo incorporation of [3H] leucine and [3H] galactose by the type II pneumonocyte. The tritiated choline, a specific precursor of phosphatidylcholine, was found to incorporate selectively into the type II pneumonocyte by the following mechanism: initially localized in the endoplasmic reticulum then rapidly transferred through the Golgi complex, stored in the lamellar bodies and later discharged to the alveolar surface. Also the tritiated galactose and leucine, carbohydrate and protein precursors, respectively, were found to be incorporated into the type II pneumonocytes and finally localized in the lamellated bodies by slightly differing mechanisms. This study demonstrates that the secretory products formed in the type II pneumonocyte is comprised of phospholipid, protein, and carbohydrate, three components of surfactant.

B. Figure is a diagrammatic representation of the formation of and excretion of pulmonary surfactant. After exocytosis, the lamellar bodies form tubular myelin which is thought to be the source of the monolayer. Only the monolayer can act in the reduction of surface tension. Also the surfactant lipids, presumably derived from the surface layer during repeated contractions, can be taken up by the type II cells and reutilized for surfactant production.

Abbreviations: er = endoplasmic reticulum, g = golgi complex, tb = lamellar bodies, M = monolayer, TM = tubular myelin, cho = choline, gal = galactose, leu = leucine, I = type I cell, II = type II cell

after release into the extracellular spaces, the lamellar bodies become hydrated and form tubular myelin. Tubular myelin consists of elongated rectangular tubes, stacked together like a box of square cigars (precise molecular configuration unknown). Accumulated evidence indicates that tubular myelin is directly responsible for the formation of the surface active monolayer in the lung (18). Also recent evidence has shown that surfactant lipids, presumably derived from the surface layer during repeated contractions, can be taken up by the type II cells and reutilized for surfactant production (18). However, surfactant apoproteins are probably not reutilized to any great extent (18).

In summary, surfactant is produced by mature squamous alveolar lining cells (type II pneumocytes) which contain membrane-bound vesicles. Electron microscopy reveals that the vesicles contain lamellar bodies which are secreted as precursors to surfactant. Secretion involves fusion of each vesicle membrane with the cells' plasma membrane and extrusion of lamellar bodies. The secreted material forms a lipoprotein tubular myelin layer. The protein moiety possibly serves as a subsurface layer that organizes the secretory material and adsorbs it to the alveolus. When the tubular myelin layer is compressed during pulmonary expiration, phospholipid is physically forced out of a subphase into an active surface film (19).

There is not much information available regarding the degradation of pulmonary surfactant from the alveoli. Pulmonary surfactants' half-life is about 10 hours. The following possibilities have been proposed for the degradation of pulmonary surfactant:
• Removal by alveolar macrophages, which itself is removed by way of lymphatics after the cells have reentered the alveolar wall (manner unknown).

• Passage up the respiratory tree and clearance up the airways, for surfactant phospholipids were detected in pharyngeal aspirates of newborn infants.

• Reabsorption into the alveolar epithelium and in situ enzymatic degradation.

4. Composition of Pulmonary Surfactants

Pulmonary surfactant is a unique lipoprotein, particularly rich in highly saturated (anenoic and monoenoic) lecithins and containing lesser amounts of cholesterol, neutral lipid, and other phospholipids (22).

In 1972, King and Clements isolated surfactant from dog lung homogenates and lavage fluid, and obtained four surface active fractions which have similar chemical and physical properties (23). As listed in Table I, lipids, especially lecithins, were found in greatest abundance. Of the esterified fatty acids, saturated species predominated, palmitate representing nearly 90% of the anenoic components. Additionally, an appreciable content (15%) of monounsaturated species was identified. Assuming that these unsaturated esters are linked at their usual C-2 position, it is possible from further analytic data of King and Clements to provide a reasonable estimate of the contribution of various molecular species to the lecithin pool of pulmonary surface active material (23). As shown in Table I the expected principle component is dipalmitoyl lecithin; however, in agreement with the observation of Shah, a
TABLE I
SURFACTANT FRACTIONS OBTAINED FROM
ISOLATING DOG LUNG HOMOGENATES

<table>
<thead>
<tr>
<th>Chemical components</th>
<th>Lipid fractions</th>
<th>Phosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>85</td>
<td>Phosphatidyl- 75.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Palmitate (16:0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71.0</td>
</tr>
<tr>
<td>Protein</td>
<td>13</td>
<td>Neutral lipid 9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myristate (14:0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.1</td>
</tr>
<tr>
<td>Hexose</td>
<td>1.7</td>
<td>Cholesterol 6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stearate (18:0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>0.7</td>
<td>Phosphatidyl-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Palmitolo-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ethanolamine 6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oleate (16:1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.0</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.5</td>
<td>Lysolecithin 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unidentified 3.6</td>
</tr>
</tbody>
</table>

*It has been proposed that this fraction contains a protein unique to lung, the surfactant apoprotein (based on a immunoreactivity study).

surprisingly large amount of monoenic lecithins (a minimum of 30%) is also present (24). Vandenhauvel, on the basis of stereochemistry, has emphasized that monoenic phosphatidylcholine can contribute to the alveolar pool and can be physically accommodated in compressible surface films (Fig. 14A) in a sterically feasible arrangement (25). Thus monoenic lecithins are not functionally precluded from the surfactant system. King and Clements have proposed that the role of monounsaturated lecithin, as well as neutral lipid and protein, might be to accelerate adsorption onto the surface (23).

Clements has developed a simplistic scheme of the lamellar structure in the lamellar body (Fig. 14B) which consists of a repeating bimolecular array of lipids with protein which are partially embedded in the surface resulting in a cobbled appearance on fracturing (26). Also Clements developed a simplistic scheme of the surfactant structure of the alveolar surface (Fig. 14C) performing its mechanical function (26). Figure 14C illustrates a monolayer of lipid and the antigenic protein, which Clements termed apoprotein (26). The properties thus far determined for this antigenic protein are (22, 23, 26):

- Of the many tissues (i.e., kidney, spleen, heart, brain, etc.) immunoassayed and/or immunofluoresced (localized at the alveolar interface) this surfactant antigen is unique to the lung.
- On polyacrylamide gel electrophoresis this fraction reveals mainly one protein band.
- Testing on an Ouchterlony double diffusion plate reveals that
FIGURE 14
STEREOMODELS OF THE AVEOLAR POOL CONSTITUENTS

Legend

A. Figure illustrates contribution of monoenoic phosphatidylcholine to the aveolar pool. Presumed interfacial...S-PC.

Presumed interfacial arrangement of saturated (S) and unsaturated (U) phosphatidylcholine (PC) and cholesterol (C) molecules in the pulmonary surfactant. Molecules sequentially depicted are: S:PC, S:PC, S:PC, S:PC, U:PC, C, S:PC, U:PC, C, and S-PC.

B. Figure illustrates model of lamellar structure in the lamellar body. Structure is a repeating bimolecular array of lipids with particles, presumably proteins, partially embedded in the surface giving rise to the cobbled appearance on fracturing (cobbles are about 100\textsuperscript{Angstrom} units wide).

C. Figure illustrates model of surfactant structure at alveolar surface performing its mechanical function. Diagram illustrates a monolayer of lipid and the antigenic protein (apoprotein) associated with it.

there is a considerable measure of antigenic identity between the entire surface active material and this single protein.

- At a physiologic pH this is an anionic protein with approximately two acidic amino acids for each basic amino acid and approximately 60% of the amino acids are nonpolar.

- Table II gives the approximate molar ratio of the canine pulmonary surfactant repeating unit, which is approximately 12 amino acids long if the chain is extended.

- Utilizing procedures designated to separate away soluble proteins, the apoprotein is purified in preparations of pulmonary surfactants.

- Clements and King have found the apoprotein in human amniotic fluid coinciding with the biochemical and clinical appearance of pulmonary surfactant (23). Also apoprotein concentration parallels the change of surface active phospholipid concentration with increasing gestational age. Clements and King have found that pulmonary surfactant containing apoprotein adsorbs from the saline subphase to its air liquid interface more rapidly than do sonicated lipids extracted from the same material (23).

Clements' analysis of canine pulmonary surfactants' composition can be summarized as follows: the predominant molecular species is dipalmitoyllecithin, with smaller quantities of other lipids; it contains a unique protein (apoprotein) which is especially suited for phospholipid association, whose molecular weight is about 10,700 (26).

Clements' overall view of pulmonary surfactants' mechanical function is
TABLE II
APPROXIMATE MOLAR RATIOS IN SURFACE ACTIVE MATERIAL

1 Sialic acid/protein

7 Hexose/protein (i.e., 1 per 12 amino acids)

- Amino Acids: 1/3 polar, 1/3 nonpolar, 1/3 "spacer"

2 Anionic amino acids/cationic amino acid

1 Cationic amino acid/anionic lipid

1 Cholesterol/unsaturated phospholipid

4 Lipid molecules/amino acid side chain, including "electrostatically-bound" phospholipid and fatty acid

that the dipalmitoyl lecithin functions as the primary molecular component of the surface active material in the pulmonary alveoli and that it sets up tightly-packed stable surface films which can withstand high compression and thereby give low values for surface tension in the alveoli (26). The surfactant is complexed with other components such as unsaturated phospholipid, apoprotein and neutral lipids which work to form a film rapidly, because in pure form dipalmitoyl lecithin has unsuitable physical properties for adsorption (26). Clements has speculated on the protein moiety function saying that it may contain subtle information which orders the surfactant complex for its adsorption and possibly may even modify the films stability when it is in its working position (26). Therefore, the apoprotein may accelerate the extracellular transport of pulmonary surfactant to the alveolar interface, and in this manner insure that adequate amounts are available to the surface in response to physiologic requirements. Many questions concerning the apoproteins' role in the regulation of the composition and metabolism of surface active material remained to be answered.

Table III gives a comparison of the phospholipid composition of surfactant and whole lung tissue, expressed as a percentage of the total lipid weight. Table III also shows that about 85% of surfactant is phospholipid, which unlike other body phospholipids, contains high saturated fatty acids. The most abundant component of surfactant is a disaturated phosphatidylcholine (PC), dipalmitoyl phosphatidylcholine or
### TABLE III.

**COMPARISON OF THE PHOSPHOLIPID COMPOSITION OF SURFACTANT AND WHOLE LUNG TISSUE**

<table>
<thead>
<tr>
<th>Phospholipid composition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Surfactant</th>
<th>Whole lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral lipids, %</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>Phospholipids, %</td>
<td>85</td>
<td>40</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>--</td>
<td>0.6</td>
</tr>
<tr>
<td>Lyso-bis-phosphatidic acid</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphatidylglycerol (PG)</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>7.1</td>
<td>14.4</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysophosphatidylethanolamine</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>67.1</td>
<td>19.6</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.5</td>
<td>Trace</td>
</tr>
<tr>
<td>Plasmalogen, % of total lipid extract</td>
<td>1.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as a percentage of the total lipid weight

dipalmitoyl lecithin*, which accounts for about 67% of lung surface active material. The other minor phospholipid components include phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), sphingomyelin, lysolecithin, free fatty acids, mono-, di-, and triglycerides, cholesterol and cholesterol esters.

Table III also displays the fact that the lipid composition of surfactant differs considerably from that of whole lung tissue. Whereas, lung tissue phospholipids comprise approximately 40% of the total lipids, in surfactant they represent as much as 85% of the total lipid pool. Also the distribution among the various phospholipids classes appears to be different. Finally it should be noted that the molecular composition of surfactant PC differs from whole lung tissue PC, in that surfactant PC contains much higher concentrations of disaturated molecules (41 to 58% of the total surfactant lipids) (27).

5. Phospholipids Chemistry

Phospholipids may be divided into two general groups: derivatives of L-glycerol-3-phosphate, or phosphoglycerides which they are usually called; and derivatives of sphingosine.

---

*Lecithin is the trivial name for choline phosphoglyceride known systematically as 1,2-diacyl-sn-glycero-3-phosphorylcholine; the recommended generic term is 3-sn-phosphatidylcholine or, as generally used, phosphatidylcholine (PC). Lecithin enjoys widespread clinical usage and is permitted by the combined commission on nomenclature (IUPAC - IUB). It is used herein to designate the compound, along with dipalmitoyl lecithin (DPL) for 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine.
Phospholipids are important constituents of cell membranes and membranes of cytoplasmic organelles such as mitochondria. The lung phospholipids possess the unique quality of surface tension lowering properties. L-glycerol-3-phosphate is the parent compound of the phosphoglycerides (Fig. 15A). The various phosphoglycerides are formed by esterifying fatty acids to the hydroxyl groups of carbons one and two. In addition, an alcoholic component, X-OH, is esterified to phosphoric acid, forming a phosphodiester. Figure 15A shows the general structure of a phosphoglyceride. Table IV lists the alcoholic X groups of the major phosphoglycerides. The compounds are named according to the alcoholic component (i.e., phosphatidylcholine (lecithin)). Each phosphoglyceride class can have many species, depending on the fatty acids esterified to the glycerol backbone.

Sphingomyelin is composed of sphingosine, a long chain fatty acid, phosphoric acid and choline (Fig. 15B). In contrast to the phosphoglycerides with acyl esters, the fatty acid is joined to the sphingosine backbone by an amide linkage. The most common fatty acid in lung sphingomyelin is palmitate. Lung sphingomyelin has a structure similar to that of dipalmitoyl lecithin and is surface active.

6. Gestational Appearance of Surfactant Phospholipids and Their Functional Role

Surfactant has a period of tissue storage, followed by a period when it is delivered to the lung surface. Biosynthesis of pulmonary phospholipids begins at the time that fetal alveolar epithelium begins to change from simple cuboidal to squamous (type II cells at 21-24 weeks
FIGURE 15'

L-\(\alpha\)-GLYCEROPHOSPHATE AND SPHINGOMYELIN

Legend

A. L-\(\alpha\)-glycerophosphate is the parent compound of phosphoglycerides. Various phosphoglycerides are formed by esterifying fatty acids to hydroxyl groups of carbon one and two of L-\(\alpha\)-glycerophosphate. In addition, alcoholic amines are frequently esterified to phosphoric acid forming a phosphodiester.

B. Surfactant contains significant amounts of sphingomyelin. This surface active phospholipid is derived from sphingosine, the parent molecule of sphingolipids.
FIGURE 15

L-γ - Glycerophosphate / Phosphoglyceride

SPHINGOSINE  SPHINGOMYELIN
### TABLE IV

**MAJOR CLASSES OF PHOSPHOGLYCERIDES**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Phosphoglyceride</th>
<th>Head alcohols</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>Non-acidic</td>
<td>HOCH₂CH₂NH₂</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylethanolamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphatidylmonomethylethanolamine</td>
<td>HOCH₂CH₂NH₂CH₃</td>
</tr>
<tr>
<td></td>
<td>Phosphatidildimethylethanolamine</td>
<td>HOCH₂CH₂NH(CH₃)₂</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine (lecithin)</td>
<td>HOCH₂CH₂N(CH₃)₃</td>
</tr>
<tr>
<td></td>
<td>Acidic. Phosphatidylserine</td>
<td>HOCH₂CHNH₃COOH</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylinositol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphatidylglycerol</td>
<td>HOCH₂CHOHCH₂OH</td>
</tr>
</tbody>
</table>
gestation). Two metabolic pathways are known to produce lecithins in human tissues: The first metabolic pathway (minor), methylation, is observed in fetal lungs after 22 weeks gestation and produces a palmitoylmyristoyl lecithin. The second pathway (major), choline incorporation, is augmented at about 33 weeks and markedly increases dipalmitoyl lecithin production. The dipalmitoyl lecithin (phosphatidylcholine) is the most important surfactant phospholipid and is often simply referred to as lecithin. There is normally a marked increase in amniotic fluid lecithin concentration, at some time between 35-37 weeks. The increase in surfactant develops over a three- or four-day interval so that deficiency and abundance may be observed in two amniotic fluid samples that are obtained only a few days apart.

As mentioned above, the major phospholipid is anenoic (saturated), dipalmitoyl phosphatidylcholine. This particular phospholipid is the most surface active phospholipid of the several present in the normally complex surfactant of human lungs. Other phospholipids, although they are surface active, may be more important in adsorbing the film onto the alveolar surface when it is first secreted or in respreading of the film during respiratory changes of alveolar surface area. These other helper phospholipids include phosphatidglycerol, (PC), phosphatidylinosital (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin.

Each of the surfactant phospholipids has a characteristic gestational appearance and functional role, which will now be discussed.

(a). Phosphatidylcholine (PC)/Lecithin

Lecithin is the major phospholipid of pulmonary surfactant. The
lecithin concentration rises gradually until 34-35 weeks after which it rises rapidly (Fig. 16). At term, lecithin accounts for 75% of amniotic fluid phospholipid.

Lecithin is the most surface active phospholipid in surfactant of the several phospholipids present. Lecithin's function in surfactant seems to be dependent on PI and PG, for without them, Gluck speculates that lecithin may not be stable (28). Gluck has also shown that surfactant containing PI and PG gives better alveolar stability than does surfactant containing only PI (28).

Rapid turnover is a characteristic feature of phosphatidylcholines (PC) in the lung. Tierney et al. followed labelled PC and estimated a mean half-life of 14 hours for PC bearing saturated or mono-unsaturated hydrocarbon chains (29). Other studies conducted showed how much shorter lung PC half-life (14 h) is compared to 1-3 years half-life of phospholipids in brain myelin, or 20 days for PC in the whole body of the rat. Therefore, surfactant deficiency may probably arise quite quickly as a result of depressed synthesis or increased destruction.

(b). Phosphatidylglycerol (PG)

At approximately 36 weeks gestation the concentration of PG which was previously extremely low or not detectable at all, begins to increase rather sharply. By term, the concentration of PG exceeds that of PI and PG becomes the most abundant phospholipid in surfactant, second only to lecithin (Fig. 16). The present evidence thus far suggests that PG improves the quality of surfactant that may be critical in stabilizing the alveoli (28). Measurements on a surface balance suggest that
FIGURE 16
SURFACTANT PHOSPHOLIPIDS CHARACTERISTIC GESTATIONAL APPEARANCE

Legend

A. Figure illustrates mean concentrations in amniotic fluid of lecithin, and sphingomyelin. During gestation, total lipids in amniotic fluid increase in concentration, highlighted by a sudden increase at 35 weeks. For clinical evaluation, the nonacidic phospholipid fraction, consisting principally of lecithin and sphingomyelin, was the most important and showed a sharp spurt in concentration at 35 weeks. The spurt is in lecithin not in sphingomyelin. The concentrations of the two phospholipids were nearly equal prior to 35 weeks when the lecithin concentration rose to 4 times that of sphingomyelin. In subsequent weeks lecithin concentration continued to rise while sphingomyelin declined.

*PG— At approximately 36 weeks gestation the concentration of PG which was previously extremely low or not detectable at all, begins to increase rather sharply. By term the concentration of PG exceeds that of PI and PG becomes the most abundant phospholipid in surfactant, second only to lecithin.

*PI— Increase in the concentration of PI parallels that of lecithin achieving a peak at 35-36 weeks, and thereafter it then decreases inversely, to the increase in PG. PI accounts for 15% phospholipid at term.

*PE and PS— These two phospholipids are present in nearly equal concentrations in the early trimester, at which time the concentration of each is roughly equal to lecithin. However, after 30-32 weeks, the concentrations of PE and PS level off and then fall, while that of lecithin continues to rise.

* Denotes factual concentration of these phospholipids at specific gestational weeks, but is not actual measured concentration.

B. Figure illustrates regression curve for percentage of PG during gestation in normal pregnancy.

C. Figure illustrates the rise and fall in percentage concentration of PI in normal pregnancy during gestation.

surfactant with PG as compared to that without PG has better physicochemical characteristics to guarantee alveolar stability. Therefore, absence of PG in amniotic fluid indicates that surfactant may not be quite mature. Clinically it is generally accepted that PG, as a component of the surfactant complex, serves as a biochemical marker, for in its absence, there is a significant risk for development of respiratory distress syndrome (RDS), while the presence of PG suggests biochemical maturity of the surfactant. However, PG's absence does not necessarily mean that RDS is inevitable. Hallman et al. have documented cases where PG first appeared within two hours after birth with no symptoms of RDS, but with a mature L/S ratio (30). On the other hand, Hallman et al. also found that surfactant with prominent PI sometimes was recovered from lung effluent at birth, but PG did not appear and RDS developed (30).

Phosphatidylglycerol needs to be further defined as an index of maturity, and its function and the factors that regulate and interfere with its development need to be clarified.

(c). Phosphatidylinositol (PI)

Increase in the concentration of PI parallels that of lecithin achieving a peak at 35-36 weeks, and thereafter it then decreases inversely, to the increase in PG (Fig. 16). PI accounts for 15% phospholipids at term. The following factors have been determined for PI:

- High PI values suggest that the lungs are about to be mature, whereas low PI values indicate immature lungs (before 38 weeks).
- PI appears to be more informative the younger the fetus is, since
the alveolar system of a tiny premature baby may remain stable even without PG.

Phosphatidylinositols' function and the factors that regulate and interfere with its development need to be clarified.

(d). Sphingomyelin

Sphingomyelin is present in nearly equal concentrations in the early third trimester, at which time the concentration is roughly equal to that of lecithin. However, at 30-32 weeks, the concentrations of sphingomyelin levels off and then falls, while that of lecithin continues to rise (Fig. 16). (similar to PE and PS concentrations). Sphingomyelin is present in low concentrations (2%) and its exact function as a phospholipid is undetermined.

(e.) Phosphatidylethanolamine (PE) and Phosphatidylserine (PS)

PE and PS are present in nearly equal concentrations in the early trimester, at which time the concentration of each is roughly equal to lecithin. However, after 30-32 weeks, the concentrations of PE and PS level off and then fall, while that of lecithin continues to rise (Fig. 16. It has been found that PE and PS have little surface activity, however, their function needs determination.

B. BIOSYNTHESIS OF PULMONARY PHOSPHOLIPIDS

Following is an account of the principal pulmonary phospholipid components, their pathways for de novo synthesis, and the enzymes involved in the pathways which play a role in regulating surfactant component production.
1. Lecithin (PC) Biosynthesis

Most of the studies on the biosynthesis and renewal of surfactant have been focused on its principal phospholipid component, lecithin (PC).

The importance of lecithin in the formation of pulmonary surfactant and its relation to the development of RDS has spurred investigations of lung lecithin biosynthesis. There are two known primary de novo pathways of lecithin synthesis in the mammalian body: 1) cytidine diphosphate choline (CDP) pathway; and 2) the conversion of PE, synthesized de novo via CDP ethanolamine into PC by three sequential N-methylation steps. Also, two auxiliary mechanisms may function in the lung to retalior the fatty acyl moieties of PC molecules that have synthesized de novo via either pathway 1 or 2, which are, a deacylation-reacylation cycle, and a deacylation-transacylation process. However, these two auxiliary mechanisms cannot lead to a net increase of the total PC pool, but can only accomplish a shift in the molecular composition of the total PC fraction (31).

It should be noted that the two primary pathways for the de novo synthesis of lecithin have been found both in the mature lung of most mammals and in the fetal lung. However, fetal lungs differ markedly from one species to another, in timetables for the synthesis of lecithin when correlated with development of the fetus (32).

(a). The Choline (CDP) Pathway

Kennedy and Weiss showed that the CDP pathway was the major pathway of all phosphoglyceride synthesis (33). The compound formed depends on the specific phospholipid base, such as choline or
ethanolamine, that combines with the specific coenzyme. This pathway comprises three steps (Fig. 17), each requiring a specific enzyme and each may be rate limiting.

By this pathway (Fig. 17), choline is phosphorylated, then activated by conversion to cytidine diphosphate (CDP) choline; which is finally transferred to a diglyceride to form phosphatidylcholine. The three enzymes of this pathway function in the following manner (Fig. 17): choline kinase phosphorylates choline, cytidyl transferase forms the CDP-choline complex, and glyceride transferase catalyzes the addition of phosphoryl choline to \( \alpha, \beta \)-diglyceride (34). The kinase enzyme is more active than the transferases. All three of these enzymes were found to be more abundant in lungs of premature infants than in adult lungs. This agrees with the observation that under stressful conditions the whole pathway might be operative in early pregnancy (35).

The diglyceride that enters into the formation of lecithin is an intermediate in the synthesis, but its fatty acid composition apparently varies with the stage of gestation and the glycerol carbon. In most mammalian phospholipids, saturated fatty acids are found on the alpha carbon and unsaturated on the beta carbon. Surfactant lecithin is an exception, for a high percentage of saturated acids are on both carbons. It has been observed that abundant palmitic acid is present in the lung tissue before the CDP choline pathway is operative, and therefore, palmitic deficiency is not likely to be rate-limiting in the formation of DPPC.

The following evidence has been reported for the choline pathway:
FIGURE 17

CYTIDINE DIPHOSPHATE CHOLINE PATHWAY
IN THE SYNTHESIS OF PHOSPHATIDYLCHOLINE

Legend

By this cytidine diphosphate choline pathway, choline is phosphorylated, then activated by conversion to cytidine diphosphate (CDP) choline, which is finally transferred to a diglyceride to form phosphatidylcholine.
FIGURE 17

1) Choline + ATP \( \text{CHOLINE KINASE} \)
\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{N-CH}_2\text{-CH}_2\text{OH} + \text{ATP} & \rightarrow \text{Phosphorylcholine} + \text{ADP} \\
& \quad \text{Mg}^{2+}
\end{align*}
\]

2) Phosphorylcholine + CTP \( \text{CYTIDYLTRANSFERASE} \)
\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{N-CH}_2\text{CH}_2\text{-O-P-OH} & \rightarrow \text{CDP-Choline} + \text{PPi} \\
& \quad \text{Mg}^{2+}
\end{align*}
\]

3) CDP-Choline + \( \triangle 1,2\)-Diglyceride \( \text{GLYCERIDE TRANSFERASE} \)
\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{N-CH}_2\text{CH}_2\text{-O-P-O-P-D-Cytidine} + \text{CH}_2\text{-O-C-R}_1 & \rightarrow \text{Phosphatidylcholine} \\
& \quad \text{R}_2\text{-C-O-CH} \quad \text{Mg}^{2+}
\end{align*}
\]

\( \text{Phosphatidylcholine} \) + CMP

\( \text{(LECITHIN)} \)
- Farrell reported on the predominance of the choline pathway in fetal rats, rabbits, monkeys and humans (31).
- Gluck et al. (32) initially reported of this pathway in the human lung, and others found the enzymes of this pathway in human fetal lung tissue (36).
- The choline pathway is active in the lungs of human fetuses of 18 to 20 weeks gestation and the pathway surges markedly at 35-36 weeks (90%) of gestation. From this time until term, dipalmitoyl phosphatidylcholine synthesis was found to be predominant (37). The surge in the choline pathway correlates closely with the increase in the lecithin/sphingomyelin ratio.

The presence of high myristic acid content in pulmonary surfactant lecithin is probably associated with a different process of lecithin formation, namely the methylation pathway, which will now be discussed.

(b) The Methylation Pathway

Bremer and Greenburg initially described lecithin synthesis by the methylation pathway in the rat liver (38). This synthesis involves (Fig. 18) phosphorylation, activation, and diglyceride linkage of ethanolamine to form phosphatidylethanolamine, which then undergoes three successive methylations from the methyl donor, S-adenosyl-L-methionine, forming mono- and dimethyl phosphatidyl ethanolamine (PDME) intermediates of phosphatidylcholine.

Studies of the kinetics of this methylation pathway revealed that reaction rates were highest in the presence of saturated (specifically dipalmitoyl) acyl substrates, thus suggesting possible importance of this
FIGURE 18

THE METHYLATION PATHWAY

Legend

This figure depicts the methylation pathway in the synthesis of phosphatidylcholine (lecithin). This synthesis involves phosphorylation, activation and diglyceride linkage of ethanolamine to form phosphatidylethanolamine, which then undergoes three successive methylations from the methyl donor, S-adenosyl-L-methionine, forming mono- and dimethylethanolamine (PDME) intermediates of phosphatidylcholine.
FIGURE 16

\[
\begin{align*}
\text{Phosphatidyl Ethanolamine} & \rightarrow \text{Phosphatidyl Ethanolamine Methyl Transferase} \\
\text{Monomethyl Phosphatidyl Ethanolamine} & \rightarrow \text{Phosphatidyl Ethanolamine Methyl Transferase} \\
\text{Dimethyl Phosphatidyl Ethanolamine} & \rightarrow \text{Phosphatidyl Ethanolamine Methyl Transferase} \\
\end{align*}
\]

\[+ S\text{-Adenosyl Methionine}\]

\[+ S\text{-Adenosyl Methionine}\]

\[+ S\text{-Adenosyl Homocysteine}\]

Phosphatidyl Choline (Lecithin)
pathway in the synthesis of highly surface active material. However, there have been conflicting studies which stated that the methylation pathway plays a minor role in PC synthesis (39, 40). Also it has been suggested that PC in the lung is a heterogeneous mixture of unsaturated PC, which is synthesized via the methylation pathway, while surface active, highly saturated, fatty acids are synthesized selectively by the CDP-choline pathway.

From all the conflicting reports and studies conducted to determine the methylation pathways' role in PC synthesis of the lung, the following conclusions can be derived:

• There is general agreement that the methylation pathway is minor (only 5% compared to 95% for choline pathway) for PC synthesis in the human lung.

• Previous observations relevant to this pathway were made using a presumed pathway intermediate, PDME, since identified as phosphatidylglycerol (PG).

• This pathway may remain of some physiologic significance in the prematurely born human neonate prior to the onset of the CDP-choline incorporation surge for PC production. For the methylation pathway was found to be active by 22-24 weeks of gestation. A study conducted on premature infants concluded the following results: RDS infants demonstrated a decrease in myristic acid on the second carbon in the PC structure, while, non-RDS infants demonstrated presumed methylation pathway intermediate by TLC and gas chromatographic techniques and second
carbon acyl group determinations. Also acidosis and perinatal asphyxia were found to selectively inhibit the appearance of the methylation pathway intermediate in human infants. Therefore, it was concluded that from 22 to 24 weeks' gestation, the methylation pathway provided initial surface active PC synthesis, which could be altered markedly by adverse conditions (acidosis and perinatal asphyxia), while the major pathway for PC synthesis is the choline pathway, which becomes active at about 35 weeks gestation (31).

Figure 19 illustrates the scheme of the timing for these two primary pathways of lecithin synthesis, and the stress effects and possible modes of inhibition on each.

(c). Auxiliary Mechanisms (Formation of Lecithin from Lysolecithin)

Two auxiliary mechanisms exist for the transformation of unsaturated PC (product from the choline) into dipalmitoyl PC (surfactant PC), which are: the deacylation-reacylation process and the deacylation-transacylation process. These two mechanisms can only produce net formation of dipalmitoyl PC if they operate in conjunction with the CDP-choline pathway. These pathways have been described for the remodeling of lecithin by synthesis from lysolecithin. A prerequisite for either of these auxiliary mechanisms is the formation of dipalmitoyl PC (or the introduction of palmitic acid at the 2-position of dipalmitoyl PC) and the availability of 1-palmitoyl-sn-glycero-3-phosphorylcholine (1-palmitoyl-lyso PC). In view of the obligated concerted action of the auxiliary mechanisms with the de novo synthesis, this 1-palmitoyl-lyso PC should be produced in the lung from unsaturated phosphatidylcholine.
FIGURE 19
TWO PRIMARY PATHWAYS FOR LECITHIN SYNTHESIS
AND POSSIBLE MODES OF INHIBITION

Legend

Scheme of timing of two primary pathways (left-side CDP-choline pathway, right-side methylation pathway) for lecithin synthesis. Indicated are the stresses, effects, and possible modes of inhibition on each pathway. Initially the CDP-choline pathway is not affected by conditions such as hypothermia and acidosis.
Alveolar stability depends upon adequate

\[ \text{LECHEHIN SYNTHESIS} \]

\[
\begin{align*}
\text{CDP-choline} & \quad \text{Phosphatidyl ethanolamine} \\
+ \quad \text{D-} & \quad + 3 \text{CH}_3 \\
\text{X} & \quad \text{Major pathway possibly as early as} \\
& \quad \text{22 wks, definitely by 24 wks gestation.} \\
\text{Y} & \quad \text{Can sustain alveolar stability alone} \\
& \quad \text{until other pathway matures unless} \\
\text{Z} & \quad \text{stressed. Increases after birth.} \\
\text{X-palmitic/} & \quad \text{X-palmitic/} \\
\text{Y-palmitic lecithin} & \quad \text{Y-myristic lecithin} \\
\end{align*}
\]

\[
\begin{align*}
\text{Hypothermia} & \quad \text{Effects thought due to catecholeamine} \\
\text{Hypoxia-Acidosis} & \quad \text{response producing alveolar} \\
\text{Hypercapnea} & \quad \text{vasoconstriction. (Possibly also} \\
& \quad \text{catecholeamine metabolism competes} \\
& \quad \text{for CH}_3 \text{ groups).} \\
\end{align*}
\]

After onset breathing in full term is active in few hours; in premature may require up to 5 days.
- Deacylation-Reacylation Mechanism

Figure 20 (reaction A) shows lyssolecithin formation by deacylation by phospholipases at either the 1- or 2- position (although 1- acyl lyssolecithin more commonly formed). Lands and Merkl demonstrated in 1963, the acylation of 2-acyl lyssolecithin compounds (41). Since then, the deacylation-reacylation mechanisms Fig. 20, reactions A and B have been shown to occur in the lung and some very complex consistencies (Fig. 20) to this oversimplified scheme found in Fig. 20, have been found.

The reverse of reaction A, is the Lands mechanism, reaction B which includes remodelling of 1-saturated, 2-unsaturated lecithin in the lung to 1,2 disaturated lecithin and favors the formation of more unsaturated lecithin (42). In 1971, Fronolono et al. indirectly supported the role of the Lands mechanism in the formation of disaturated lung lecithin, however, they did not dismiss the deacylation transacylation mechanism (43). Also Kyei-Aboagye et al. were able to incorporate palmitic acid directly by acylation into a lyssolecithin molecule of a rabbit lung homogenate by addition of CoA and ATP to the reaction mixture (44). These same investigators showed the exchange of a molecule of palmitic acid, for a molecule of another acid, i.e., oleic, within the lecithin molecule without disturbing the remaining molecular structure.

- Deacylation - Transacylation Mechanism

The transacylation reaction involves direct addition of the molecule
FIGURE 20

AUXILIARY MECHANISMS

FORMATION OF LECITHIN FROM LYSOLECITHIN

Legend

Reaction A shows the deacylation of lecithin to lysolecithin catalyzed by phospholipases at either the 1- or 2- position.

Reaction B is the so-called Lands mechanism and is the reverse of reaction A. This reaction involves the remodelling of 1-saturated, 2-unsaturated lecithin in lung to 1,2 disaturated lecithin and favors the formation of more unsaturated lecithin.

Reactions A and B have been shown to occur in the lung and some very complex consistencies to this oversimplified scheme include: isolation of the acyl-CoA: monoacyl phosphoglycerides acyltransferases; discovery that microsomes appear to be the loci of this enzymes' activity, however, mitochondria and plasma membrane may also have some activity, and finally they have found the substrates of the enzymes to be various monoacyl phospholipida and acyl thioesters (not fatty acids) of varying chain length and unsaturation.

Reaction C is the Marinetti mechanism for the formation of lecithin from two molecules of lysolecithin. This transacylation reaction involves direct addition of the molecule of fatty acid to the lysolecithin molecule. Therefore a monopalmitoyl lysophosphatidyl choline is acylated with palmitic acid to DPPC.

Abbreviations: SFA = saturated fatty acid, UFA = unsaturated fatty acid.

FIGURE 20

Reaction A and B deacetylation-reacylation mechanisms

Reaction C deacetylation-transacylation mechanisms
of fatty acid to the lyssolecithin molecule. Thus, a monopalmitoyl lysophosphatidyl choline is acylated with palmitic acid to DPPC (Fig. 20C). The pathway was first described by Erbland and Marinetti (45) who found that by the action of lyssolecithin transferase, the fatty acid from one lyssolecithin molecule was transferred to another lyssolecithin molecule as follows:

\[
\begin{align*}
2 \text{lyssolecithin} & \quad \text{lyssolecithin} \to \text{lecithin + glycerylphosphoryl-choline} \\
\text{transferase}
\end{align*}
\]

It was found by Abe et al. that fetal rabbit lung slices displayed the pathway after 20 weeks gestation, but the total amount of lecithin produced was small (46).

In conclusion, these two auxiliary mechanisms of deacylation-reacylation and deacylation-transacylation are involved in the transformation of de novo synthesized, unsaturated PC molecules into dipalmitoyl PC. Increasing evidence suggests that both mechanisms may function with sufficient specificity to account for the introduction of palmitate at the 2-position of dipalmitoyl-PC. The relative importance, and quantitative significance of these two mechanisms, however, is still unknown. Only future research with the type II cells and pulmonary surfactant biochemical system will answer these questions.

2. Biosynthesis of the Other Pulmonary Phospholipids

Most of the biosynthesis research has been conducted on dipalmitoyl PC because of its prominent role in pulmonary surfactant. However, the other phospholipids of: sphingomyelin, PE, PG, PI, and PS occur in
conjunction with PC in pulmonary surfactant. These other phospholipids specific roles are uncertain, however some research has been conducted concerning their biosynthesis and will now be discussed.

(a). Phosphatidylglycerol (PG) Biosynthesis

In the lung and lung surfactant, PG consists of as much as 10% of the total phospholipids.

Sanders and Longmore conducted studies on perfused rat lungs for 2 hours with labeled glucose, palmitate, and acetate and determined the incorporation of these substrates into PC and PG of surfactant (48). From their study Sanders and Longmore concluded the following facts (48):

- Results suggested that the esterification of palmitate at the 1- and 2- positions of PC and PG occurred at different rates and is dependent on the precursor of palmitate.
- De novo palmitate synthesis from glucose, acetate or lactate labeled PG more efficiently than PC.
- Perfusion with exogenous palmitate on the other hand gave higher specific activities for PC than for PG.

They suggested that a precursor-product relationship exists between PG and PC, but much more concentrated studies must be conducted.

Hallman and Gluck also conducted studies and showed the in vivo two step formation of PG from CDP diacylglycerol and sn-glycerol-3-P, as follows (49):

1) CDP-diacylglycerol + sn-glycerol-3-P → phosphatidylglycerol-3'-phosphate + cytidine monophosphate
2) phosphatidylglycerol-3’'-phosphate →
phosphatidylglycerol + phosphate

Hallman and Gluck also found that in the lung, microsomes were more active than mitochondria (in other tissues main locus of PG synthesis) in catalyzing PG synthesis (49). Finally, Hallman and Gluck have suggested that PG synthesis in the microsomes produces PG destined for storage in the lamellar bodies and excretion into the surfactant (49).

(b). Phosphatidylinositol (PI) Biosynthesis

Very little has been published on the synthesis of the phospholipid PI. The current information available consists of the following:

- Hallman and Gluck showed the capacity of lung microsomes to catalyze the synthesis of PI from CDP diacylglycerol and myoinositol (50).
- Also, Abe and Akiho showed that the de novo synthesis of PI operates primarily in the formation of polyunsaturated species of PI (51).

(c). Phosphatidylethanolamine (PE) Biosynthesis

Scarpelli demonstrated the active metabolite behavior of this phospholipid, by showing that palmitate was incorporated very rapidly into PE of the fetal lung and pulmonary fluid (52).

Tombropoulos studied the simultaneous incorporation of sn-glycerol-3-P and palmitic acid into various phospholipid classes and arrived at the following conclusions (53):

- A large proportion of the palmitate was incorporated into PC via a route other than CDP choline pathway.
- Observed a higher contribution of the de novo synthesis to the
Introduction of palmitate into PE (PE does not contain significant amounts of disaturated molecules like PC).

And finally, Abe and Akino studied the incorporation of labeled glycerol into various molecular species of liver and lung PE and concluded that the de novo synthesis via CDP ethanolamine contributed mainly to the formation of hexaenoic, monoenoiic, and dienoic PE in both the lung and liver (51). Also presumably, the tetraenoic species of PE are formed largely by a deacylation-reacylation mechanism in the lung.

(d). Sphingomyelin Biosynthesis

Sphingomyelin is present in high concentrations in the lung, however not much is known about its metabolism. Details of sphingomyelin synthesis have been worked out in other tissues, and are presumed to be the same in the lung.

Sphingosine, the parent compound of sphingolipids, is formed from palmitoyl CoA by a series of enzymatic steps and then N-acylated to form a ceramide (N-acyl-sphingosine). In the lung this acyl group is usually palmitic acid. Ceramide then reacts with CDP-choline in the presence of phosphocholine-ceramide transferase to produce sphingomyelin.

(e). Phosphatidylserine (PS) Biosynthesis

The biosynthesis of this phospholipid has not been determined to date.

3. Enzymatic Regulation of Synthesis

The previous section discussed in detail the major pathways which contribute to synthesis of the pulmonary phospholipids. Now the enzymes which regulate pulmonary phospholipids synthesis
will be discussed.

(a). Palmitic Acid Synthesis

Studies conducted in the fetal rabbit lung demonstrated a constant rate of de novo fatty acid synthesis (primarily palmitic acid) via fatty acid synthetase from 23 days gestation to the adult. Therefore, early maturation of fatty acid synthetase and fatty acid precursors occurs at a time of enhanced lung cell differentiation and surfactant synthesis.

(b). Formation of 1,2-Diglyceride as a Common Precursor

Johnson et al. identified the phosphatidic acid phosphohydrolase (PAPase) reaction, which catalyzes the hydrolytic cleavage of phosphatidic acid to form 1,2-diglyceride (54). This catalyst plays an initial role in substrate regulation in PC synthesis. Isolated lamellar bodies in fetal rabbit lung at 26 days' gestation had a 4 to 8-fold increase in PAPase activity, which occurred one day before the first detectable accumulation of dipalmitylphosphatidyl choline (40).

(c). Choline CDP Pathway

The three enzymes which regulate lecithin synthesis via the choline CDP pathway are: CK (choline kinase), CYT (cytidyl transferase) and CPT (choline phosphotransferase) (Fig. 17) (31). Several investigators (31, 34) have found that specific gestational periods have enzyme-mediated effects on the synthetic pathway of PC by the choline pathway, such as:

(i). CK activity is 2-3 fold higher in immature human neonates of 28 weeks gestation than in those of 33-40 weeks. Also in
neonates of 24 hours of age the CK activity began to fall. The following characteristics have been established for CK activity (in supernatant): temperature optimum of 25-38°C; pH optimum of 10.8; and APT, and magnesium are required as cofactors.

(ii). CYT activity studied in the rat lung reached full activity at birth. Also CYT activity in the rat liver was enhanced by the presence of lysophosphatidylethanolamine and other phospholipids. The following characteristics have been established for CYT activity (in whole lung): temperature optimum of 50°C; pH optimum of 6.0-6.5; magnesium and CTP required as cofactors; stable after freezing; and not inhibited by oxygen (55).

(iii). CPT activity studied in human neonates between 20-30 weeks' gestation was increased 2-fold over the activity in those born at term (55). The following characteristics have been established for CPT activity (in whole lung): temperature optimum of 35-37°C; pH optimum of 7.5; and magnesium is required as a cofactor, while calcium ion inhibits CPT activity (48). Also CPT is found to be the rate limiting enzyme with the lowest specific activity and last catalyst in the choline pathway.

(d). Methylation Pathway

The two enzymes which regulate lecithin synthesis via the methylation pathway are MAE (methionine-activating enzyme), and MT (phosphatidyl methyltransferase). Since the methylation pathway has a minor role in lecithin synthesis, the modulation of these enzymes also appears to be insignificant when compared to those of the choline pathway. Both of these enzymes' activity was found to be higher before
birth than either immediately after birth or in the adult. The following characteristics have been established for MAE activity (in supernatant): temperature optimum of 35-39°C; and magnesium and APT required as cofactors. The following characteristics have been established for MT activity (in supernatant): temperature optimum of 35-39°C; and optimum pH 7.5.

(e). Transacylation of PC Fatty Acids into Dipalmitic Lecithin

As discussed previously, not all PC is in the disaturated surface active form and a transacylation reaction from the position of a 1-acyl-2-lysolecithin molecule to form disaturated lecithin occurs. It appears that the choline pathway combines with a lyso phosphatidylcholine loop forming disaturated PC. It appears that the same enzyme is responsible for both lyso phosphatidylcholine acylhydrolase and acyltransferase activities. Hasegawa and Ohno found that there are two different acyltransferases in rat lung microsomes; the dienoic and monoenoic PC species are provided mainly by the de novo synthetic pathway, while the saturated species and the polyenoic species are produced primarily by other than the de novo synthesis (56).

(f). Phosphatidylglycerol (PG) Synthesis

Microsomal glycerol-3-phosphate: CMP-phosphatidyltransferase activity limits the rate of PG synthesis. For the palmitate esterified to positions 1 and 2 of PG and PC occurs at different rates, and appears to be dependent on the source of the precursor palmitate.

(g). Phosphatidylinositol (PI) Synthesis

The formation of PI from CDP-diglyceride and inositol has
been found in a large number of tissues. Also it has been found that enhanced synthesis may reflect increased tissue levels of cyclic inositol phosphate. Recently, Obladen et al. observed a rise in PI in the early recovery phase of infants with severe RDS and inversely, PC and PC were reduced (57). This increased concentrations of PI can be explained by reduction of glycerol-3-phosphate: phosphatidyl transferase activity.

4. Metabolic Interrelationships in Phospholipid Metabolism

As shown in Fig. 21, carbohydrates and proteins play a important role in the interrelation of phospholipid metabolism. Following is a discussion of how carbohydrate, fat and protein metabolism play a major role in the synthesis of phospholipids by supplying both energy and precursors (58).

(a). Carbohydrate and Fatty Acid Metabolism

Glucose supplies many important precursors such as acetyl CoA and L-\(\alpha\)-glycerophosphate, and is of great importance as it influences almost every reaction in the cell. Also, glucose influences the metabolism of acetate, directing incorporation of C-2 units into fatty acids (rather than oxidation to CO\(_2\)). The lung incorporates the fatty acids into phospholipids. Metabolism of glucose via the hexose monophosphate shunt also generates the NADH needed for the synthesis of these fatty acids from acetyl CoA and malonyl CoA (58). From the above data it can be noted that glucose plays an important part in lung metabolism.

(b). Protein Metabolism

Protein metabolism also plays a significant role in
FIGURE 21
METABOLIC INTERRELATIONSHIPS THAT CARBOHYDRATES
AND PROTEINS PLAY IN PHOSPHOLIPID METABOLISM

Legend
Carbohydrate, fat, and protein metabolism play a major role in
synthesis of phospholipids by supplying both precursors and energy.

Reproduced without permission from: Gluck, L., and Keidel, W. N. (1975)
in Pulmonary Physiology of the Fetus, Newborn and Child (Scarpelli, E.
phospholipid metabolism in that it provides the nitrogenous bases choline and ethanolamine. ATP and CTP are required to convert these bases into their precursors, CDP-choline, CDP-ethanolamine, etc., needed for synthesis of phosphoglycerides and sphingomyelin (58).

5. Summary

Currently, the characterization of components of pulmonary surface active material is far from complete. Generally the nature of the surfactant composition is agreed upon by most investigators, however a precise stoichiometry for its components has not been demonstrated. The key to unlocking all of the surface active materials unknowns is the lamellar inclusions bodies of the type II alveolar cells. Clearly, much remains to be learned about the composition of the various components of alveolar froth.
CHAPTER IV
RESPIRATORY DISTRESS SYNDROME (RDS)

A. ETIOLOGY

RDS is an acute pulmonary disease, predominantly of the prematurely born infant.

Enough is now known about the condition usually specified either as the respiratory distress syndrome (RDS) or as hyaline membrane disease (HMD) to name it according to its principle cause, surfactant deficiency. For surfactant lines the alveoli of the mature lung and lowers the surface tension required to keep the alveolus open during expiration. However, when surfactant is deficient, as in RDS, diffuse atelectasis ensues, resulting in pulmonary insufficiency. The end result of surfactant deficiency is failure of proper lung expansion and absence of alveolar stability (collapse at end-expiration, atelectasis) (Fig. 22).

Essentially and in varying degrees the following happens in RDS (Table V):

- The fetal pattern of cardiopulmonary circulation persists after birth, in that pulmonary vascular resistance is elevated, because of vasoconstriction and failure of capillaries to dilate, thus diverting blood from the lungs.

- Compliance of the lung is significantly diminished and collapse of the alveoli and poor lung perfusion cause diminished gas exchange.

- Some expanded alveoli, which receive air extremely well, are not supplied with sufficient blood, whereas other alveoli in a
FIGURE 22
SURFACTANT EFFECTS

Legend
Surfactant has a detergent property of lowering surface tension in the fluid layer which lines the primitive alveoli once air enters the lungs, and it acts as an antiatelactasis factor to maintain patency of terminal airspaces. Surface tension of a fluid is measured in dynes/cm. A drop of water on a sheet of glass tends to round up into a compact mass because of its surface tension at about 72 dynes/cm at the air-water interface. If household detergent is added to the drop of water, its surface tension is reduced to about 20 dynes/cm and it spreads into a very thin film on the glass. In a similar manner surfactant reduces surface tension of the fluid layer lining the alveolus to about 5 dynes/cm. This ability to form a monomolecular layer at the interface between air and the alveolar lining fluid allows some air to be retained with the alveolus at all times.

Surfactant Effects

Drop of water with surface tension of 72 dynes cm forms a globule.

Drop of water mixed with household detergent, surface tension reduced to 20 dynes cm and thus water spreads out.

Minimum surface tension is 50 dynes cm. As much as 20 cm H2O of negative pressure needed to inflate sac alveolus during 4th and subsequent breaths.

Negative pressure of 40 to 100 cm H2O needed to inflate sac alveolus with air.

Minimum molecular layer of surfactant lining fluid layer on surface of terminal sac alveolus.

Surface tension is 5 dynes cm or less. Negative pressure of only 2 cm H2O needed to inflate sac alveolus to maximum diameter during 4th and subsequent breaths.

Before 1st breath

During 1st breath

After 3rd breath
### TABLE V
CATEGORIZATION OF OBSERVATIONS IN RESPIRATORY DISTRESS SYNDROME

<table>
<thead>
<tr>
<th>Established</th>
<th>Probable</th>
<th>Possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surfactant deficiency during disease.</td>
<td>Primary surfactant deficiency (<em>in utero</em>)</td>
<td>Absent corticoid stimulus (<em>in utero</em>)</td>
</tr>
<tr>
<td>Epidemiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worldwide</td>
<td></td>
<td>Maternal diabetes predisposes.</td>
</tr>
<tr>
<td>Prematurity predisposes</td>
<td></td>
<td>Maternal hemmorhage predisposes.</td>
</tr>
<tr>
<td>C-section w/o labor predisposes</td>
<td></td>
<td>Familial predisposition</td>
</tr>
<tr>
<td>Prenatal asphyxia predisposes Male mortality&gt;female</td>
<td></td>
<td>Prenatal corticoids spares.</td>
</tr>
<tr>
<td>Clinical Signs: Onset near the time of birth</td>
<td>Fine inspiratory rales Hypothermia Peripheral edema</td>
<td>Pulmonary edema PDA murmur</td>
</tr>
<tr>
<td>Retractions and tachypnea Expiratory grunt Cyanosis Systematic hypotension Characteristic chest x-ray</td>
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<td></td>
</tr>
<tr>
<td>Course to death or recovery lasts 3 to 5 days</td>
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### TABLE V-Continued

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<thead>
<tr>
<th>Established</th>
<th>Probable</th>
<th>Possible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pathophysiology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced lung compliance</td>
<td>Poor peripheral perfusion</td>
<td>Myocardial malconduction</td>
</tr>
<tr>
<td>Reduced FRC</td>
<td>Poor renal perfusion</td>
<td></td>
</tr>
<tr>
<td>Poor lung distensibility</td>
<td>Poor alveolar stability</td>
<td></td>
</tr>
<tr>
<td>Poor alveolar stability</td>
<td>Right-to-left shunts</td>
<td></td>
</tr>
<tr>
<td>Right-to-left shunts</td>
<td>Reduced effective pulmonary blood flow.</td>
<td></td>
</tr>
<tr>
<td><strong>Pathobiochemistry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory acidosis</td>
<td>Hyperbilirubinemia</td>
<td>Hyperkalemia</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>Decreased total serum proteins.</td>
<td>Pepsinogen in lung</td>
</tr>
<tr>
<td>Decreased saturated phospholipids</td>
<td>Decreased fibrinolysins</td>
<td></td>
</tr>
<tr>
<td>Preceded by low AF L/S ratio.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preceded by low AF surfactant titer.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atelectasis</td>
<td>Osmiophilic lamellar bodies decreased early, increased later.</td>
<td>Small adrenal glands Intracranial hemorrhage</td>
</tr>
<tr>
<td>Injury to epithelial cells.</td>
<td>Membrane contains fibrin and cellular products.</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PROM = prolonged rupture of membranes (>16 hours), IUGR = intrauterine growth retardation, PDA = patent ductus arteriosus, FRC = functional residual capacity, AF = amniotic fluid, L/S = lecithin/sphingomyelin ratio, DPL = dipalmitoyl lecithin.

collapsed state are poorly ventilated and are of little use, even if blood supply were optimal. The predominant effect is from poor alveolar ventilation due to widespread atelectasis.

- Overall, the balance of ventilation and perfusion is abnormal for oxygen intake is decreased, and retention of carbon dioxide is increased. Continued hypoxia is associated with metabolic acidosis, whereas accumulation of carbon dioxide causes respiratory acidosis.

Avery and Mead were the first to discover the intimate association between lung surfactant and RDS by reporting that surfactant lowering material was absent in lungs removed at autopsy from infants succumbing to RDS (59). Over the years, much evidence has been accumulated supporting the hypothesis that RDS is in most cases an expression of underlying pulmonary surfactant deficiency. The observations which support this proposal include: 1) the abnormal mechanical properties of isolated lungs; 2) the abnormal surface tension characteristics of lung extracts; 3) the low concentrations of saturated lecithin in lung; 4) the predictability of amniotic fluid lecithin concentration (according to gestation age); and 5) the low lecithin concentration in tracheal effluent. Very little evidence arguing against the primacy of diminished surfactant in RDS has appeared and withstood the test of time. Therefore, it is reasonable to assert that in most cases RDS occurs as a consequence of the relative inability of the lung to synthesize or secrete surfactant in amounts sufficient for normal neonatal respiratory adaption.
B. TERMINOLOGY OF THE DISEASE

The subject of respiratory problems in the newborn period has had some terminologic difficulties, since hyaline membrane disease (HMD) was an illness only diagnosed at autopsy. When it was found that hyaline membranes were often absent from the lungs of infants who died very early in the course of the illness, the name "hyaline membrane disease" (HMD) was replaced with "idiopathic respiratory distress syndrome" of the newborn (60). Since that time, the term has been abbreviated to "respiratory distress syndrome" (RDS). Therefore the term HMD is less suitable because membrane formation is not invariably present and, indeed, may be the result rather than the cause of the condition.

A survey of the areas' neonatologists resulted in both names being utilized. RDS (respiratory distress syndrome) is utilized to classify, in a broad sense, any initial respiratory problem found in the newborn (respiratory frequency over 60/min.). HMD (hyaline membrane disease) is utilized to classify the newborn with the typical symptoms of surfactant deficiency (see Diagnostic Criteria) and is labeled according to the clinical symptoms and severity of disease present (Type I is most severe). In this paper the disease identity of surfactant deficiency is referred to as RDS.

C. FACTORS AFFECTING RDS

1. Predisposing Factors

By far the most important factor predisposing an infant to RDS is immaturity. Gestational age is the single most important factor determining whether the lungs at birth contain sufficient surfactant to
sustain normal respiratory function. For in the human fetus the steady rise in the mean concentration of surfactant in amniotic fluid is between 30 and 36 weeks gestation. Also the birth weight is associated with prematurity, for if the weight is greater than 2500 g RDS is not likely, however if less than 2500 g, the likelihood of RDS increases in relation to the lower weight.

There are also several factors, besides premature birth, that probably affect the synthesis, secretion, or action of surfactant (60). These include sex, mode of delivery, maternal disease and conditions of the infant at birth. For RDS is twice as common in males as in females at every gestational age. The disease frequently follows delivery by Cesarean section, particularly if this is done before labor has begun and the infant is less than 32 weeks gestation. The associated maternal factors which predispose an infant to RDS are: diabetes for infants of diabetic mothers (Class A-C well controlled) are five times more likely to develop RDS than infants of nondiabetic mothers (with the same age, sex, and mode of delivery); and history of recurring premature deliveries. The conditions of the infant at birth which increase the possibility of RDS are: the second-born twin is at greater risk for development of RDS than is the first-born; hydrops fetalis and severe hemolytic disease delay surfactant production; and perinatal asphyxia and neonatal hypotension (for depressed asphyxiated infants may not be able to make sufficiently vigorous initial respiratory efforts to fully inflate their lungs at birth).
2. Factors That Reduce The Risk of RDS

Several factors have been suggested as playing a role in reducing the risk of developing RDS and can be classified as those factors that produce stress in the at-risk infant. The factors which reduce risk of RDS development are: maternal diabetes (Class D-F poorly controlled); prolonged rupture of membranes (24 hours or more before birth); intrauterine stress; intrauterine growth retardation; maternal heroin addiction, thyroxine and steroid therapy to the mother 24 hours prior to delivery (see prevention section for details on the last two factors) (60).

It has been suggested that the protective effect of these factors is possibly exerted through a steroid mechanism. However, in all instances the difficulty in accurately assessing gestational age complicates the evaluation of which factors have protective effect. Intrauterine growth retardation and maternal heroin addiction are closely related, in that the intrauterine growth retardation may be the factor in patients with heroin addiction as well.

D. Incidence and Mortality

RDS is probably the commonest cause of death in live-born premature (short gestation) infants and the incidence of the disease increases with decreasing maturity.

At a birth weight of 1000 to 1500 g (gestation approximately 27 to 31 weeks) the incidence is about 50% of all infants, whereas at a birth weight of 2000 to 2500 g (gestation approximately 34 to 36 weeks) the incidence is about 5% (61).

The incidence and mortality of RDS has changed considerably since the
early 1960's to the current 1980's because of increased understanding of its pathophysiology and improved methods of treatment. For in the early 1960's it was reported reported that fatal RDS occurred in 3.8/1000 births of infants under 2500 g equivalent to 12,000 deaths per year (61). Then, in 1974 it was reported that 10,897 deaths were attributed to RDS (19% of all neonatal deaths in 1968) (61). The incidence of RDS was estimated to be 40,000 cases per year, or 1% of all births.

According to Wegman in 1979, the improvement of treatment (especially the utilization of ventilators and continuous distending airway pressure) greatly decreased neonatal mortality from 15.9/1000 live births in 1968 to 9.4/1000 in 1978 (61). Also in 1979 it was reported, that larger infant death rates from RDS decreased, however more low birth infants lived long enough to develop RDS and its complications (61), and that only 276 infants were born with RDS from 1969 through 1978. The most dramatic change was in infants weighing under 1000 g, for in 1978 there were 50% survivors and from 1969 to 1971 they were no survivors (61).

RDS is more likely to occur in the more immature infant. Mortality is greatest in the most immature infants, with improvement in outcome occurring as gestational age increases. Over 90% of deaths occur by the 4th day of life, decreasing exponentially from a high point in the first 24 hours. For RDS continues to occur in about 10 to 15% of infants under 2500 g (or about 1% of live births), but deaths are rare in infants over 1500 g (61). Current mortality from RDS is approximately 3 to 4 per 100
infants under 2500 g or about 7000 deaths per year (61).

E. DIAGNOSTIC CRITERIA (Table V)

The clinical features of the disease are well defined. For clinical presentation of RDS involves most infants born prematurely and having birth weights less than 2500 g and gestation age less than 37 weeks.

One of the most important diagnostic features is the early onset of symptoms and with increasingly careful observation it has become apparent that the infants are affected by the disease process from birth or at any rate within the first hour or two of life. Some infants with RDS fail to expand their lungs at birth even with vigorous inspiratory efforts and have respiratory distress from the first minutes after birth. Others inflate their lungs initially, but develop progressive atelectasis and increasingly labored breathing in the first few hours. The signs and symptoms of RDS worsen through the first three days of life, after which improvement and recovery are the rule, unless complications of RDS arise.

The criteria for diagnosis of RDS include: immaturity; maternal history, which usually contains one of the predisposing factors; physical findings; blood gas results; and radiographic findings (61, 62).

1. Physical Findings

The main physical findings of RDS include tachypnea, chest wall retractions, expiratory grunting, poor air exchange, and cyanosis (61, 62). These findings will now be discussed in detail and are shown in Fig. 23.
FIGURE 23

CLINICAL MANIFESTATIONS OF RDS

Legend

A. Clinical manifestations of RDS.
   1. Flaring of nostrils
   2. Respiratory rate accelerated
   3. Expiratory grunt or whimper
   4. Retraction of soft tissues on inspiration
   5. Umbilical artery sampling
   6. Excessive peripheral edema
   7. Pooling of circulation
   8. Breath sounds harsh
   9. Cyanosis or ashen pallor

B. Photograph is taken of infant shortly before death. Note the
   intercostal and sternal retractions, flared alae nasae, edema, and
   flaccid posture.

C. Pulmonary function studies in RDS.

Reproduced without permission from: A. Netter, F. H. (1979) in the CIBA
Collection of Medical Illustrations, vol. 7: Respiratory System (D()ertie,
B and C: Gregg, R. H., and Berstein, J. (1961) Am. J. Dis. Children 102,
p. 127
FIGURE 23

Clinical Manifestations of Hyaline Membrane Disease

A.

1. Flaring of nostrils

2. Respiratory rate accelerated (interposed apnoic periods of over 10 sec indicate poor prognosis)

3. Expiratory grunt or whimper (may be clearly audible or heard only via stethoscope)

4. Retraction of soft tissues on inspiration (lower sternum may retract and abdomen distend "seesaw" fashion)

5. Umbilical artery streaming

6. Cyanosis or abnormal patellar reflex in left lower limb

7. Pitting edema of extremities especially of dependent areas

8. Breath sounds harsh and low tones may be present

9. Cyanosis or blue tinge to left arm

C. Illustrative Pulmonary Function Studies in the Respiratory Distress Syndrome

<table>
<thead>
<tr>
<th>Normal</th>
<th>Infant with RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>2.5 Kg</td>
</tr>
<tr>
<td></td>
<td>3 Kg</td>
</tr>
</tbody>
</table>

| Tidal volume | 12 ml | 7 ml |
| Rate | 35/min | 30/min |
| Minute volume | 60 ml | 40 ml |
| Functional dead space | 5 ml | 8 ml |
| Alveolar ventilation | 255 ml/min | 150 ml/min |
| Lung compliance | 5 ml/cm | 20 ml/cm |
| Pulmonary work per minute | 1400 gm/cm²/min | 800 gm/cm²/min |
(a). Tachypnea

The classical symptom is tachypnea or a respiratory frequency above 60 per minute by four hours of age and sometimes increasing to 90 per minute or more.

(b). Chest Wall Retractions

One of the first signs, is usually retraction of the chest wall with each inspiration, which means that the lungs are diffusely atelectatic. High pressures are required to inflate the lungs, therefore the infant will have to exert a large negative interpleural pressure with every breath causing chest wall retractions, while the pressure outside the chest wall is atmospheric.

These retractions can vary from mild seesawing of the chest and abdomen with respirations to a tracheal tug and deep substernal retractions. Figure 24A depicts some of these chest wall retractions and is the old fashion grading system (61) of the severity of retractions devised by Silverman and Anderson (1956). Currently, neonatologists are utilizing the therapeutic scoring system in Fig. 24B, on infants with RDS. Another convenient sign of inadequate chest expansion is detection by auscultation with the stethoscope of poor alveolar air entry. Another helpful aid that can be noted clinically if the infant has required resuscitation, is that the lungs in RDS feel stiff when one is bagging by hand to establish respiration.

(c). Flaring of the Nares

Flaring of the nares occurs and as the retractions become increasingly severe an expiratory grunt, which arises in the larynx
FIGURE 24
CHEST WALL RETRACTIONS AND THE THERAPEUTIC SCORING SYSTEM

Legend
A. Silverman scoring of chest wall retractions. This is a suggested system for grading retractions in RDS infants.
B. Currently some neonatologists are utilizing the therapeutic scoring systems on infants with RDS. Respiratory distress syndrome scores are to be recorded with vital signs for any infant under an oxygen hood. When a score of 6 or more is noted, a physician is to be notified for further evaluation (i.e., x-ray, blood gas, etc.)

B. Pontiac General Hospital Neonatal Intensive Care Unit, Dr. Rao.
FIGURE 24

A. Grade 0
- UPPER CHEST: SYNCHRONIZED
- LOWER CHEST: NO RETRACT.
- XIPHOID RETRACT: NONE
- NAresa: NONE
- EXP: GRUNT: NONE

Grade 1
- UPPER CHEST: LAG ON INSP.
- LOWER CHEST: JUST VISIBLE
- XIPHOID RETRACT: JUST VISIBLE
- NAresa: MINIMAL
- EXP: GRUNT: STETHOS ONLY

Grade 2
- UPPER CHEST: SEE-SAW
- LOWER CHEST: MARKED
- XIPHOID RETRACT: MARKED
- NAresa: MARKED
- EXP: GRUNT: NAKED EAR

B. RESPIRATORY DISTRESS SYNDROME SCORE
(RDS SCORE)

<table>
<thead>
<tr>
<th>Grade</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.R.</td>
<td>40-60</td>
<td>61-80</td>
<td>80</td>
</tr>
<tr>
<td>Retraction</td>
<td>no</td>
<td>Minimal</td>
<td>Severe</td>
</tr>
<tr>
<td>Grunting</td>
<td>no</td>
<td>With Stethoscope</td>
<td>Without Stethoscope</td>
</tr>
<tr>
<td>Color</td>
<td>Pink With room air</td>
<td>Pink With 21-40% O2</td>
<td>Pink With 02 40%</td>
</tr>
<tr>
<td>Airway Entry</td>
<td>Good</td>
<td>Fair</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Respiratory Distress Syndrome Scores are to be recorded with vital signs for any infant under an oxygen hood. When a score of 6 or more is noted, a physician is to be notified for further evaluation. (i.e., x-ray, etc.)
become’s audible. This grunting is produced by partial closure of the larynx during expiration; it is possible that the interposition of an expiratory resistance at the larynx helps to maintain inflation of the air sacs for a longer proportion of each breath.

(d). Cyanosis

Except in the mildest cases cyanosis is present when breathing room air and as the disease worsens cyanosis persists even during oxygen breathing (62). Progressive cyanosis is one of the gravest prognostic signs of the disease and is succeeded by apneic attacks and peripheral circulatory collapse. Interference with gas exchange caused by unstable air sacs produces cyanosis, but the situation is worsen because during the newborn period hypoxia, low pH or hypercarbia in the lung causes intense reflex pulmonary vasoconstriction. Not only is pulmonary blood flow reduced, but also large right-to-left shunts develop through the foramen ovale and the ductus arteriosus, which are still patent. These shunts were first noticed when more than 80% of the cardiac output bypassed the lung. There is no doubt that shunting of blood is the most important factor in producing cyanosis during the acute stages of RDS (61). After recovery has begun, the right-to-left shunt becomes smaller and by about the fifth day, maldistribution of inspired gas and pulmonary blood flow is probably the most important cause of impaired gas exchange.

(e). Other Physical Findings

Other physical findings include: diminished air entry, and fine rales (abnormal sounds) in both lungs are present, especially at the
end of a deep inspiration that precedes a cry. Infants often appear flaccid and inactive. Peripheral edema is usually present, although there is no good evidence that the infants are in heart failure. The systemic blood pressure is low. Periods of very rapid breathing interposed with apneic periods of more than 10 seconds are a poor diagnostic sign in RDS.

2. Blood Gases

When RDS progresses, the hypoxemia increases (decreased arterial oxygen saturation $p_{O_2}$) and results in organic acid appearance, and potassium leaves the cellular interior, and therefore serum potassium concentration rises. Also the partial pressure of carbon dioxide increases ($p_{CO_2}$) because of reduced lung compliance and ineffective ventilation. The end result of these changes is a mixed metabolic and respiratory acidosis (low pH). Correction of the acidosis plays a key role in the overall management of RDS. However, sustained and continued severe acidosis results in hypovolemia and arterial hypotension with poor peripheral perfusion (62).

Respiratory acidosis results from the failure of ventilation, and metabolic acidosis results from the defect in $O_2$ supply causing lactic and pyruvic acid accumulation. Without treatment the pH of arterial blood frequently drops below 7.0. There is also evidence of increased cellular breakdown; the renal excretion of $Na^+$, $K^+$, and $N^+$ is increased and plasma levels of $K^+$, urea, and phosphates rise.

3. Radiographic Findings

Diagnosis of RDS can usually be confirmed by the characteristic
radiograph, however, variations of the classical pattern occur frequently (63). The initial radiograph taken within the first two hours of life may show a bell-shaped chest, with curved ribs, and hypoinflation (seven to eight rib expansion during inspiration on a frontal projection). A radiograph taken six to eight hours after birth is the most diagnostic and characteristic for RDS. Figures 25 and 26 depict the classical radiograph found in RDS with a reticulogranular pattern and distinct air-bronchograms extending throughout the lung fields (63). In moderately severe RDS infants a ground-glass appearance is characteristic and air-bronchograms and reduced lung volume are found on the radiograph. The very severely afflicted RDS infant’s radiograph shows total opacity of the lungs and air-bronchograms (Fig. 26 A and B).

There is good correlation between the radiographic findings and clinical symptomatology, however, variations of the classical radiographic pattern do occur.

Sequential radiographs are of help in managing treatment and in distinguishing RDS from diseases that occasionally mimic RDS (explanation in next section on DIFFERENTIAL DIAGNOSIS).

F. DIFFERENTIAL DIAGNOSIS

The likelihood of RDS is relatively high in a premature infant with early respiratory difficulty; however, other diseases can lead to respiratory distress in the first few hours of life (62). The presiding physician must carefully differentiate these diseases to arrive at optimum therapeutic decisions. The tools the physicians works with are the physical examination, with clinical findings, and the radiographic.
FIGURE 25
RESPIRATORY DISTRESS SYNDROME

Legend

A. Figure illustrates chest radiograph with characteristic granular pattern of hyaline membrane disease.

B. Figure illustrates repeat chest radiograph of infant above, at the end of a week which shows no abnormality.

FIGURE 26

RESPIRATORY DISTRESS SYNDROME

Legend

A and B. Frontal (A) and lateral (B) radiographs show nearly complete opacification of both lungs in an infant with RDS whose previous chest radiograph showed characteristic lung changes.

C. Figure illustrates RDS in a 1500 g premature infant. The granular densities are poorly seen, but the distinct air bronchogram and the high position of the diaphragm suggest RDS. Skin folds are seen on the right lower hemithorax.

picture. The findings may lead the physician to suspect certain disorders and offers the opportunity to rule out others. Two conditions in particular are difficult to differentiate from RDS i.e., pneumonia, and massive pulmonary hemorrhage. In both of these conditions the physical and radiographic findings may be closely similar to those of RDS.

The most frequent disease which must be differentiated from RDS are listed in Table VI, which lists the frequency, symptoms, and radiographic findings for each disease. These diseases will now be discussed in detail (62, 64).

1. Pneumonia (Table VI)

There are two main types of pneumonia found in the neonatal period, which are aspiration pneumonia and infectious pneumonia (62, 64).

Aspiration pneumonia occurs during the first few days of life and its predisposing factors are: premature rupture of membranes; prolonged labor; premature labor; maternal fever or fetal distress. The above conditions often result in aspiration of amniotic fluid which contains debris such as vernix caseosa, epithelial cells, meconium or material from the birth canal such as vaginal secretions. The debris blocks the smallest airways and interferes with alveolar exchange of oxygen and carbon dioxide. The aspirated material is frequently accompanied by pathogenic bacteria and pneumonia develops. However, even in the noninfected cases there are respiratory distress symptoms, and usually radiographic evidence of aspiration. Other situations in which pulmonary aspiration of foreign material may contribute to serious consequences in
### TABLE VI

**The Most Frightening Diseases Which Must Be Differentiated from RIS**

<table>
<thead>
<tr>
<th>Disease/cause</th>
<th>Frequency of disease</th>
<th>Symptoms: similar to RIS</th>
<th>Symptoms aiding in differentiation from RIS</th>
<th>Radiographic findings</th>
<th>Figure/page</th>
<th>Radiograph found</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNEUMONIA</td>
<td></td>
<td></td>
<td>Occurs during the first few days of life.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pathogenic organism may be grown from blood cultures or for tracheal aspirate.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Characteristic paravertebral abnormality involving right upper and lower lobes and perihilar areas.</td>
<td></td>
<td>Fig. 21A and B</td>
<td>Page 135</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In both types of pneumonia: usual signs of respiratory distress:
- cyanosis
- tachypnea
- grunting
- retraction
- possible peripheral asphyxia

<table>
<thead>
<tr>
<th>Disease/cause</th>
<th>Frequency of disease</th>
<th>Symptoms: similar to RIS</th>
<th>Symptoms aiding in differentiation from RIS</th>
<th>Radiographic findings</th>
<th>Figure/page</th>
<th>Radiograph found</th>
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</tbody>
</table>

Begin after the first few days of life. Pathogenic organism may be grown from blood cultures or tracheal aspirate.

Reuse: linear areas of density which radiate from the hilar areas, segmental areas of consolidation, atelectasis and air trapping.

Flattening of the diaphragm at a lower position and an increase in anteroposterior diameter of the chest. | Fig. 221 and B | Page 137 |

127
<table>
<thead>
<tr>
<th>Disease/cause</th>
<th>Frequency of disease</th>
<th>Similar to RDS</th>
<th>Symptom aid in differentiation from RDS</th>
<th>Radiographic findings</th>
<th>Figure/Reference</th>
<th>Radiograph Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>PULMONARY EDMASSAGE</td>
<td>Unknown</td>
<td>Paroxysmal symptoms as seen in RDS</td>
<td>Obvious blood-stained liquid exsanguination from trachea</td>
<td>Total opacification of lung fields, ill-defined areas of density in one or more pulmonary segments and occasionally, a reticulo-granular appearance and air bronchogram (These last two findings are characteristic for RDS).</td>
<td>Fig. 29</td>
<td>Page 140</td>
</tr>
<tr>
<td>TRANSIENT TACHYPNEA</td>
<td>Respiratory rate range from 80 to 140/minute (but become normal between 5 to 10 days)</td>
<td>Mild cyanosis (may require 35-40% oxygen to remain pink)</td>
<td>Seen in term infants or large preterm infants. Usually normal at birth but during first day of life show gradual onset of tachypnea.</td>
<td>Peri-hilar Streaking (which are prominent linear vascular markings radiating from the hilar areas) and cardiac silhouette is slightly enlarged.</td>
<td>Fig. 30</td>
<td>Page 143</td>
</tr>
</tbody>
</table>

128
<table>
<thead>
<tr>
<th>Disease/cause</th>
<th>Frequency of disease</th>
<th>Symptoms similar to RDS</th>
<th>Symptoms aid in differentiation from RDS</th>
<th>Radiographic Findings</th>
<th>Figure/page found</th>
</tr>
</thead>
</table>
| MECONIUM ASPIRATION | 60% | Infants have varying degrees of:  
- tachypnea  
- chest retractions  
- cyanosis  
- rales may be heard  
Encountered in infants with intrauterine asphyxia. | Marked recovery usually occurs within 48 hours of life. | Paramount feature is air-trapping (in more severe cases) and is manifested by an increased anteroposterior diameter of the chest evident by chest x-ray and inspection. | Fig. 31A and B Page 146 |
| CONGENITAL HEART DISEASE | 2% | Cyanotic | Cyanosis exists and is not improved significantly by oxygen administration.  
Presence of heart murmurs.  
Abnormal electrocardiogram. | Abnormal cardiac configuration with enlarged heart and usually both the arterial and venous pulmonary vascular markings are more prominent. |
### TABLE VI Continued

<table>
<thead>
<tr>
<th>Disease/cause</th>
<th>Frequency of disease</th>
<th>Symptoms similar to RDS</th>
<th>Symptoms aiding in differentiation from RDS</th>
<th>Radiographic findings</th>
<th>Figure/page Radiograph found</th>
</tr>
</thead>
</table>
| **OTHER DISEASES**<br>Such as:  
Diaphragmatic hernia | 7%                    | All these diseases cause respiratory insufficiency in the neonate period. Heart tones are shifted significantly to one side or the other and hernia has forced the mediastinum laterally. | Each disease has distinctive features present on routine chest radiograph. |                       |                             |
| Diaphragmatic hernia |                       |                         |                                             |                       |                             |
| Parotic nerve paralysis |                       |                         |                                             |                       |                             |
| Oesophageal atresia with tracheo-oesophageal fistula |                       |                         |                                             |                       |                             |

### RESPIRATORY DISTRESS SYNDROME

<table>
<thead>
<tr>
<th></th>
<th>CLINICAL FINDINGS</th>
<th>RADIOGRAPHIC FINDINGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDS</td>
<td>Diagnostic criteria include: prematurity (&lt;37 wks gestation) and birth weight &lt; 2500 g, Tachypnoeic respiratory frequency &gt; 60/mi, Chest wall retraction, Respiratory grunting and flaring of</td>
<td>Radiograph taken 1 to 6 h after birth is most diagnostic and characteristic for RDS. Reticulogranular pattern and distinct air-branchograms extending throughout the air fields. Moderately severe RDS.</td>
</tr>
<tr>
<td>Disease/cause</td>
<td>Frequency of disease</td>
<td>Symptoms similar to RDS</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>The aores</td>
<td></td>
<td>Cough</td>
</tr>
<tr>
<td>Cyanosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory acidosis</td>
<td></td>
<td>Infants often appear flacid and inactive.</td>
</tr>
<tr>
<td>Very severely afflicted RDS infants</td>
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the newborn include: esophageal and duodenal obstructions; esophageal atresia, tracheoesophageal fistula; and improper feeding practices. Any situation resulting in the inability of the infant to swallow properly or which leads to vomiting may result in aspiration pneumonia.

The second type of pneumonia found in infants is infectious pneumonia, which begins after the first few days of life and is usually acquired through contact with adults or children with respiratory infections, or through bacteremia.

Neonatal pneumonia may be a primary cause of respiratory distress, or it may occur in association with RDS. In both of these instances it frequently occurs as part of a generalized sepsis. Pneumonia may also occur in sepsis that is a complication of indwelling catheters and other manipulations of intensive care.

The most frequent causative organisms for either type of pneumonia include beta-hemolytic *Streptococcus* group B, *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas*, *Proteus*, and *Salmonella*.

RDS in newborns is generally obvious soon after birth. Also depending on the severity, pneumonia may be present soon after birth, however, sometimes it may not produce symptoms for several days. Infants with pneumonia display the usual signs of respiratory distress of which cyanosis, tachypnea, grunting and retractions are the most common. Also with pneumonia perinatal asphyxia may be present and the infant can be a resuscitation problem at birth. Fever may be present, put hypothermia and difficulty in maintaining temperature are more common. Also it must be remembered that a pathogenic organism may be grown from blood cultures.
or tracheal aspirate.

The radiographic findings with aspiration pneumonia are the following: with duodenal obstruction, atelectasis of the right upper lobe secondary to aspiration and following vomiting in an infant is shown in Fig. 27A. Figure 27B shows the characteristic parenchymal abnormality involving the right upper and lower lobes, and perihilar areas (these being the most dependent areas of the lungs) with aspiration pneumonia (63).

Infectious pneumonia displays the following radiographic characteristics: coarse linear areas of density which radiate from the hilar areas; segmental areas of consolidation; atelectasis; and air-trapping are usually present, as shown in Fig. 28A. Also Fig. 28B is a radiograph displaying the flattening of the diaphragm at a lower position and an increase in the anteroposterior diameter of the chest (overinflated lungs, bacteriologic evidence of streptococcal pneumonia) (63).

Correlation of the physical, laboratory, and radiographic findings is very important in establishing a correct diagnosis of pneumonia.

2. Pulmonary Hemorrhage (Table VI)

It is not clear whether pulmonary hemorrhage is a disease entity or just secondary to a systemic process, possibly anoxia or stress (62, 64). With pulmonary hemorrhage there is generalized hemorrhage throughout the lungs (capillary permeability affected causing gross hemorrhage into the alveolar bed). Pulmonary hemorrhage is suspected clinically when obvious blood-stained liquid is seen arising from the trachea. Chest radiographs
FIGURE 27
ASPIRATION PNEUMONITIS

Legend

A. Chest radiograph illustrates atelectasis of the right upper lobe secondary to aspiration following vomiting in an infant with duodenal obstruction. Pneumonia or atelectasis in this area is characteristic of aspiration pneumonia.

B. Chest radiograph made after episode of vomiting and strangling shows parenchymal abnormality involving the right upper and lower lobes.

FIGURE 28

INTRAUTERINE INFECTIOUS PNEUMONIA

Legend

A. Chest radiograph shows segmental areas of atelectasis and air-trapping as well as coarse linear densities occupying both lungs.

B. Lateral view shows flattening of the diaphragm and increase in anteroposterior diameter of the chest (bacteriologic evidence of streptococcal pneumonia).

(Fig. 29A and B), most frequently show total opacity of the lung fields, ill-defined areas of density in one or more pulmonary segments, on occasion a reticulogranular appearance, and air bronchograms are noted (these last two features are characteristic of RDS) (63). The radiographic findings (except for the two characteristic of RDS) with the clinical features should result in a pulmonary hemorrhage diagnosis. Treatment is supportive and if a coagulation defect is present it should be treated. Problems are similar to those of RDS, and therefore monitoring procedures are required, including frequent determination of blood gases. Resolution of pulmonary hemorrhage occurs after two to three days, and the chest has a normal appearance. However, frequently, any underlying cerebral stress or anoxic phenomenon which is severe enough to produce pulmonary hemorrhage may be fatal to the infant (61).

3. Transient Tachypnea (Table VI)

The most common cause of abnormal respiratory function following birth, is a condition labeled transitory tachypnea (61, 62). The condition is usually seen in term infants or large premature infants who are usually normal at birth, but during the first day of life show a gradual onset of tachypnea. The respiratory rates usually range from 80 to 140 per min, but become normal between two to five days. The tachypnea is occasionally accompanied by minimal retraction, and mild cyanosis (some infants may require 35-40% oxygen to remain pink). A prominent feature of the physical examination of infants with this condition is a barrel-chested appearance, and as the infant improves the
FIGURE 29
PULMONARY HEMORRHAGE

Legend
A. Abnormal density occupies most of the right lung. Clinically the infant was thought to have pulmonary hemorrhage.
B. Repeat radiograph three days later shows complete resolution of the hemorrhage.

chest returns to a more normal size. The radiographic findings show a perihilar streaking (which are prominent linear vascular markings radiating from the hilar areas) and often the cardiac silhouette is slightly enlarged (Fig. 30A and B) (63).

Clinically, this condition can resemble RDS, meconium aspiration, or congenital heart disease. However, RDS is ruled out because of the radiographic findings, mild symptoms, and unusually short course of the disease. Meconium aspiration is ruled out because the infants are not meconium stained and their amniotic fluid is clear. Congenital heart disease is ruled out when a normal electrocardiogram is present and follow-up radiographs show regression in heart size, and in the prominence of the pulmonary vascular channels.

The pathogenesis is still presumptive; however, it has been suggested that this condition may be secondary to slow absorption of lung fluid. Fluid remaining in the periarterial tissue would explain the radiographic findings, and lung compliance would be decreased because of the additional fluid. The infant's increased respiratory rate then would minimize respiratory work. This condition appears to be self-limited, and the course of this condition is quite benign (63).

4. Meconium Aspiration (Table VI)

Meconium aspiration is encountered in the newly born with intrauterine asphyxia (62). With this condition, amounts of meconium invade the lower respiratory tract and frequently these infants require resuscitation at birth. While meconium can be aspirated from the tracheas of 60% of all infants who are born covered with meconium, only
FIGURE 30
TRANSIENT TACHYPNEA OF THE NEWBORN

Legend
A. Chest radiograph made during the first day of life shows linear areas of density extending from the hilus into each lung with associated cardiac enlargement.
B. Repeat chest radiograph one week later shows no abnormality.

20-25% of these infants will develop symptoms of respiratory distress or pulmonary radiographic changes.

These infants have varying degrees of tachypnea, chest retractions, cyanosis, and rales may be heard (61). The chest radiograph shows air trapping which is a paramount feature of the more severe cases and is manifested by an increased anteroposterior diameter of the chest evident by both inspection and chest radiograph (Fig. 31A). Additional radiographic findings show areas of increased density and areas of overexpansion irregularly distributed throughout the lung (Fig. 31B) (63).

Marked recovery usually occurs after 48 hours of life because the lung can clear meconium rapidly (suggesting that meconium quickly moves to the periphery of the lung). However, a small percentage of infants with meconium aspiration recover over a prolonged period of time. One complication which occurs in 10% of meconium-covered infants is that the partially blocked, overexpanded areas of the lungs rupture with pneumothorax (explained in COMPLICATIONS section).

The clinical features are usually sufficiently distinctive to allow for clear-cut differentiation from RDS.

5. Congenital Heart Disease (Table VI)

Congenital heart disease is highly suspected when cyanosis exists and is not improved significantly by oxygen administration (62). An abnormal cardiac configuration by radiograph, the presence of murmurs, and an abnormal electrocardiogram are helpful clues in differentiating congenital heart disease. The radiographic findings show an enlarged
FIGURE 31
ASPIRATION SYNDROME

Legend
A. Frontal projection shows hyperaeration of the lungs with coarse linear peribronchial infiltrations radiating from the hilar areas into the lungs.
B. Chest radiograph of meconium-stained baby shows extensive pulmonary abnormality.

heart and, usually both the arterial and venous pulmonary vascular
markings are more prominent.

6. Other Diseases (Table VI)

This is a group of rarely occurring diseases which are important
causes of respiratory insufficiency in the newborn period, which include
diaphragmatic hernia, phrenic nerve paralysis, oesophageal atresia with
trachea-oesophageal fistula, spontaneous or induced pneumothorax, and
pneumomediastinum (these last two conditions will be discussed in detail
under COMPLICATIONS) (62). Fortunately, however, each of these
conditions can be readily differentiated on the basis of distinctive
features present on a chest radiograph (61).

7. Summary

After careful examination of physical, clinical, and radiographic
findings other causes of respiratory distress can be excluded and RDS can
be diagnosed with about 90% certainty.

G. TREATMENT

Therapeutic treatment of RDS is complex and multifaceted. Successful
treatment of infants with RDS depends on their early identification and
the understanding of the physiopathology of the disease in concert with
the biological needs of prematurity. Proper management of newborns in
the delivery room is the initial step. As mentioned in the previous
section, the clinical presentation, chest radiograph, and blood gases are
the criteria for diagnosis.

The degree of respiratory difficulty in RDS varies over a wide range.
Management of RDS is dependent on the severity of respiratory
insufficiency and involves maximum supportive measures for the infant. A critical balance of essential interacting components is necessary to maintain normal pulmonary metabolism, ventilation, and perfusion.

Adequate surfactant production depends on active metabolic processes in type II alveolar cells. Surfactant synthesis and secretion allow for lung expansion, thereby facilitating ventilation. In turn, satisfactory ventilation and continued lung expansion allow for normal oxygenation, which helps to ensure good blood flow through the pulmonary arteries. Adequate lung perfusion ensures delivery of oxygen, glucose, fatty acids, and other nutrients to the alveolar cells, thus completing the cycle and permitting synthesis of abundant surfactant. However, with RDS there is a notable lack of alveolar surfactant (anti-atelectasis factor) in the lungs of these infants and a significant alteration of the ventilation/perfusion ratio of the alveoli (64). The surfactant deficiency results in a decrease of lung compliance ("stiff lung") and an increase in the work of breathing. This leads to constriction of the pulmonary arteries and increased right-to-left shunting both on a intrapulmonary level and through the foramen ovale and ductus arteriosus. The constriction of the pulmonary arteries may lead to reduced lung perfusion, poor alveolar cell metabolism, and diminished surfactant synthesis. These conditions aggravate the atelectasis and complete the vicious cycle of neonatal RDS, (shown in Fig. 32) leading to hypoventilation, alveolar hypoperfusion, right-to-left shunts, hypoxia, acidosis, and ultimate asphyxia. If the cycle is not interrupted, the outcome can be fatal (64).
FIGURE 32
VICIOUS CYCLE OF HYPOXEMIA

Legend

With RDS there is a notable lack of alveolar surfactant in the lungs of these infants and a significant alteration of the ventilation/perfusion ratio of the alveoli. The surfactant deficiency results in a decrease of lung compliance (stiff lung) and an increase in the work of breathing. This leads to the constriction of the pulmonary arteries and increased right-to-left shunting both on a intrapulmonary level, and through the foramen ovale, and ductus arteriosus. The constriction of the pulmonary arteries may lead to reduced lung perfusion, poor alveolar cell metabolism, and diminished surfactant synthesis. These conditions aggravate the atelectasis and complete the vicious cycle of neonatal RDS, leading to hypoventilation, alveolar hypoperfusion, right-to-left shunts, hypoxia, acidosis and ultimate asphyxia. If the cycle is not interrupted, the outcome can be fatal.
FIGURE 32

VICIOUS CYCLE OF HYPOXEMIA.

Hypotension → Sudden
Hypovolemia → Intrauterine
Asphyxia → Hypoxemia
↓ pH, ↓ pO₂
Pulmonary Vasoconstriction

Hypoxemia → Alveolar Hypoperfusion

Alveolar Cell Dysfunction

Surfactant Deficiency

Intrapartum Asphyxia
C-section
Familial Predisposition
Prematurity

Acidosis

Transient Tachypnea
Neonatal Asphyxia
Hypothermia
Apnea

Hypoventilation Atelectasis

Hyaline Membrane Formation
Increased Capillary Permeability
The design of immediate and long-term supportive therapy for RDS follows from consideration of this metabolic-ventilation-perfusion cycle. Therefore management of RDS is concerned with provision of optimal ventilation (with or without support), maintenance of acid-base balance, normal body temperature, blood volume, and nutrition (65, 66). Each of these treatment factors will now be discussed in detail.

1. Ventilation Support and Oxygen Therapy

In RDS the main clinical expression is an immature lung whose small respiratory units inflate with difficulty and do not remain filled with gas between respiratory efforts. Some infants with this disease cannot completely expand their lungs at birth, even with vigorous inspiratory efforts, and they have respiratory distress beginning in the first minutes of life. However, other infants inflate their lungs initially, but develop progressive atelectasis and increasingly labored breathing during the first hours after birth.

Provision of optimal ventilation may simply require an increment in FIO$_2$ (fraction of inspired oxygen) in a plastic hood, or it may be complex in that some form of ventilatory support is required (61). In either event, assiduous monitoring of arterial blood gases and pH is the only reliable method for evaluation of the effectiveness of therapy. These determinations are essential after any change in respiratory therapy, no matter how frequent or, in the absence of change in therapy, at least every four hours. Also to meet the immediate and changing oxygen, metabolic and ventilatory requirements of the infant, color activity, and skin or rectal temperatures are monitored every hour, and respiration and
heart rates are monitored continuously to prevent a long apneic period.

In general, the following blood gas parameters should be maintained: PaO₂ between 50 and 100 mm Hg, pH above 7.25, and PaCO₂ between 35 to 45 mm Hg. The most useful way of obtaining blood for blood gas and pH determinations is by the insertion of an umbilical arterial catheter. Some institutions have also routinely used blood samples from the temporal and radial arteries for blood gas determinations.

In general, if an infant is unable to attain a PaO₂ of 50 mm Hg in 100% O₂, assisted ventilation is indicated. Such an approach helps maintain the functional residual capacity, prevents further atelectasis, and facilitates oxygen uptake. When oxygen alone is insufficient, continuous distending pressure may aid in preventing alveolar collapse and thus improve respiratory function. Distending pressure during spontaneous breathing may be delivered as continuous positive airway pressure (CPAP) by means of an endotracheal tube, nasal prongs, or face mask, or as negative pressure by enclosing the chest in a partial vacuum chamber while the head remains exposed to atmospheric pressure.

In 1971 Gregory et al. introduced CPAP for treatment of RDS (67). CPAP significantly improves gas exchange, which in essence maintains varying degrees of lung expansion and end-expiration, depending on the amount of applied positive pressure (Fig. 33). The distention of collapsed, but perfused alveoli provides a greater surface for effective gas exchange. The maintenance of positive-pressure throughout the respiratory cycle increases functional residual capacity. This procedure's benefits are reflected in an increased PaO₂ where hypoxic
FIGURE 33

CONTINUOUS POSITIVE AIRWAY PRESSURE

(CPAP)

Legend

Figure compares respiratory units in normal, HMD, and CPAP conditions.

A. At end expiration in the lung, surfactant (the stippled zone at the surface of the alveolus) is tightly packed and surface tension at the interface between alveolar gas and the alveolar wall is low, so that pulmonary units remain inflated. With inspiration, surfactant is spread over a larger area, which allows some nonsurface active material to reach the surface and surface tension rises. With repeated respiratory cycles respiratory units do not change size.

B. With HMD where surfactant is absent or in low quantities the surface tension at end-expiration is high. In these infants respiratory units tend to become smaller with each successive expiration, until they are airless.

C. Continuous positive airway pressure (CPAP) applied through an endotracheal tube opposes the high surface tension and, with successive respirations, the respiratory units become larger until they reach a stable volume.

levels had existed previously. CPAP has reduced the mortality of RDS significantly and its effectiveness depends on the infants' ability to ventilate spontaneously (68). If the infant cannot maintain an adequate minute volume, \( \text{PaCO}_2 \) will rise even if \( \text{PaO}_2 \) remains within satisfactory limits. When carbon dioxide retention elevates the \( \text{PaO}_2 \) above 70 to 75 mm Hg, or with CPAP the arterial oxygen tension is less than 30-40 mm Hg with the infant breathing 100% oxygen this is an indication that a mechanical ventilator must be utilized (68). At this oxygen tension the chance of the infant surviving without ventilatory assistance is less than 10%.

Mechanical ventilation is used as part of the supportive management of newborn infants with respiratory failure. The primary objective of mechanical ventilation is to undertake gas exchange for the infant until there is recovery from the potentially reversible pathologic process that has caused respiratory failure. Respiratory failure is present when the \( \text{PaCO}_2 \) is increased and is associated with hypoxemia and acidosis. The clinical manifestations of respiratory failure in the newborn are a decrease in respiratory rate, periods of apnea, cyanosis unrelieved by high oxygen concentrations, and ultimately, a fall in blood pressure, tachycardia, pallor, evidence of peripheral circulatory failure, and bradycardia (65, 66).

Monitoring of the patient is vital, and requires continuous observation, and frequent blood gas determinations. Frequency of respiratory rate is closely associated with inspiratory/expiratory (I/E) ratios and involves inspiratory flow rate. Depending on the condition of
the patient's lungs, the optimal I/E ratio varies. However, in 1971 Reynolds, found that progressive increases in the inspiratory phase to an I/E ratio of 4:1 results in progressive rises in PaO₂ in infants with RDS (this technique should be used only for RDS) (69). This observation is very significant, since these high ratios and low frequencies permit ventilation of infants with RDS at much lower pressures, thereby decreasing the risk of pneumothorax. The concentration of oxygen used should initially correspond to that necessary to maintain an adequate PaO₂ during spontaneous ventilation. Effective mechanical ventilation sometimes may result in very striking increases in oxygen tension; therefore, these infants are monitored closely to prevent the development of retrolental fibroplasia and pulmonary oxygen toxicity. The complications of mechanical ventilation are infection, retrolental fibroplasia, and bronchopulmonary dysplasia. Also a pulmonary air leak can result in pneumothorax, pneumomediastinum or interstitial emphysema (61). Precautions should be taken to prevent these complications from occurring.

2. Acid-base Balance

Mixed respiratory and metabolic acidosis are found in RDS (65, 66). The respiratory acidosis results from retention of carbon dioxide. The metabolic acidosis results from the increased production of the organic acid end-products of anaerobic glycolysis (mainly lactic acid), and this is the most important contributing factor to the low pH values in RDS.

Judicious administration of intravenous sodium bicarbonate (NaHCO₃) infusion is utilized to elevate the pH and this directly affects the
metabolic acidosis and has no direct neutralizing influence on the respiratory component. Sodium bicarbonate actually raises PaCO₂, which except in the most severely affected infants, is usually excreted through the lungs, at least partially. The dosage of intravenous NaHCO₃ depends on assessment of the infants' acid-base status (65, 66).

The only effective therapy for the hypercapnea of respiratory acidosis is mechanical ventilation.

3. Body Temperature

Infants with RDS should be kept in a neutral thermal environment to conserve heat and reduce the need for oxygen (66). The inspired oxygen concentration must be raised sufficiently to support tissue oxygenation. The best way to insure a correct environmental temperature is to keep the skin temperature between 36 and 37°C (97 and 98.5°F) which correlates fairly well with minimal oxygen consumption. Maintenance of this level is essential (66).

4. Blood Volume

Circulatory disturbances in infants with RDS such as hypovolemia, poor peripheral perfusion, hypotension, and anemia need immediate attention to decrease morbidity and mortality (66). In such instances blood pressure is low, hematocrit is subnormal (less than 45 to 50 vol%), and tachycardia is present (rate over 160 beats/min). More often soon after birth the blood pressure and hematocrit are normal, but tachycardia is evident. If blood is not infused, most of such infants will soon become hypotensive, while the hematocrit drops to levels below 40 vol% within 2 to 3 hours. Whole blood is administered to infants who have
only tachycardia during the first day of life and usually the heart rate will drop to normal (120 to 160 beats/min). The hematocrit is maintained at 45 to 50 vol% throughout the course of the disease.

Also hypovolemia and hypotension are corrected by blood, or blood products if blood is not available.

5. Nutrition

Almost all babies born prematurely with RDS have low caloric stores, including diminished body fat and glycogen; thus nutritional support plays a major role in the overall management of the infant with RDS. To minimize nutritional deprivation parenteral glucose is generously used and nasoduodenal or nasojujenal feedings begin by the third day of life or as soon as possible after diminution of dyspnea, passage of meconium, and voiding of urine. Gastric emptying time is prolonged in infants with RDS. Therefore the interim between feedings should initially be no less than 3 hours. Renal function is compromised in infants with RDS, and fluid administration must be adjusted accordingly. This is especially true because patent ductus arteriosus (PDA) is also a complication of RDS, and signs and symptoms of heart failure may be exacerbated by excessive fluid administration (66).

6. Antibiotic Administration

Immaturity of the immunity system and instrumentation are the two major factors predisposing RDS infants to infection (65, 66). Preventive measures take precedence in the control of infection, and infection complications of RDS require immediate attention; for the quality of care and the progress of the infant should be evaluated several times
daily. Antibiotics are given to RDS infants with the following conditions to circumvent infection: male babies (more prone to infection), increased white blood cell count of infant; maternal fever; maternal prolonged rupture of membranes; and amnionitis (infection of amniotic fluid) (66).

7. The Team Approach and Intensive Care Unit

Management of severely affected infants, who inevitably develop complications of the disease and of the therapy itself, is the most demanding of all commitments of a neonatal intensive care unit. The physician and nurses must be experienced, knowledgeable, and circumspect in making decisions, yet quick to change the plan when the situation requires it. Skilled and gentle handling by both physicians and nurses is crucial to a successful outcome. The importance of a fully trained nursing staff who understands the basic pathophysiology of the disease and can detect abnormalities cannot be overestimated. The care provided by the nurses is the most important factor in reducing the mortality and morbidity of the disease. The nurse must anticipate deterioration and certainly recognize it when it occurs, for the nurses' effectiveness is directly proportional to their knowledge of the disease and their alertness to the many details involved in its adequate therapy.

Because of their unstable condition these infants require frequent monitoring of blood gases, blood pH, respiratory, cardiac, and arterial pressure, and hydration status. Therefore a laboratory capable of determining blood gases and pH within minutes of sample collection is indispensable, and must be manned by competent personnel around the clock
daily. The involvement of several groups of people in such a program of therapy is important and is termed the team approach.

Also in recent years, the concept of regionalization of perinatal health care has become increasingly popular as an approach aimed at reducing neonatal mortality and morbidity. These newborn intensive care units and perinatal care centers are equipped with personnel, and modernized equipment to deal with support required in the treatment and associated complications of RDS.

8. Summary

In summary, optimum treatment of RDS demands a perinatal approach. For anticipation of the risk of developing RDS by amniotic fluid surfactant determination, expert neonatal resuscitation, early recognition of the afflicted neonate, and transfer to a facility equipped to deal with the disease are all of great importance in decreasing the mortality and morbidity associated with RDS.

As mentioned in this section the intensive therapy for RDS has lead to increased survival, but also to new complications in treated infants, as will now be discussed.

H. COMPLICATIONS

The many complications of RDS, basically have three origins: the disease; the associated prematurity; and the intensive treatment itself. Examples of these complications are discussed below (65, 66).

1. Disease and Prematurity Associated Complications

   (a). Pneumothorax

   Pneumothorax is a condition which may occur spontaneously in RDS
or as a complication of respiratory therapy. The pneumothorax which may occur spontaneously in RDS, is the result of an elevated negative intrapleural pressure triggered by noncompliant lungs and its basis is alveolar rupture due to a result of gas trapping from overdistention due to occlusion of outflow of gas. Also, continuous distending pressure and positive pressure ventilation produce pneumothorax in approximately 5 to 20% of cases, respectively; this complication usually causes sudden deterioration in respiratory function. In addition mechanical ventilation, in which positive pressure is applied, poses a constant threat to pleural rupture, and development of tension pneumothorax.

Physical findings of this condition include cyanosis, tachypnea, grunting, and flaring of the alae nasi. Percussion is sometimes helpful, but a shift of the apical impulse is usually more easily noted. Auscultation may be misleading because of the wide referral of breath sounds. The sudden onset of a tense, distended abdomen is often a useful clinical feature signifying a pneumothorax (61, 66).

A characteristic feature of pneumothorax in the sluggish neonate is the gas in the pleural space which tends to collect anteromedial to the lung. Also the chest radiograph taken in the anteroposterior and lateral projection is very diagnostic and allows assessment of the size of the lesion. Figure 34 shows the air lateral, inferior, and medial to the right lung. Also Fig. 34 shows the characteristic radiograph of a pneumothorax in RDS with the absence of larger air space and more complete collapse of the right lung (63). Also a high-intensity transilluminating light using a fiberoptic probe is especially helpful in
FIGURE 34
PNEUMOTHORAX

Legend
Radiographs illustrate RDS with complicating pneumothorax.
A. Air is identified lateral, inferior, and medial to the right lung.
Absence of larger air space and more complete collapse of the right lung
is characteristic of pneumothorax in RDS.
B. Pneumothorax on the left complicating RDS.

Reproduced without permission from: Singleton, E. B., and Wagner, M. L.
(1971) in Radiologic Atlas of Pulmonary Abnormalities in Children, p. 35,
W. B. Saunders Co., Philadelphia.
quickly diagnosing a pneumothorax (61). Figure 35 shows the increased transmission of light from a high-intensity fiberoptic probe by a large pneumothorax (61). The theory of the light, is that abnormal air collections result in increased transmission of light on the involved side and larger air accumulations are more readily detected. The advantages of this method are that infant's predisposed for development of pneumothorax can be checked periodically, and secondly, the rapidity of diagnosis with the light can result in speedier treatment intervention (61).

Treatment is necessary only in instances where the magnitude of pleural air is such as to cause significant respiratory embarrassment. The air can be removed quickly by needle aspiration followed by the insertion of a catheter into the pleural space and connected to a negative pressure drainage system. The pleural leak seals off quickly and the catheter can usually be removed within 48 hours (61). Massive pulmonary hemorrhage may occur at any time during the acute phase of the disease and its exact pathogenesis remains obscure.

(b). Pneumomediastinum

Pneumomediastinum is a complication of RDS and meconium aspiration (65, 66). The basis of this disease is alveolar rupture, presumably as a result of gas trapping from overdistention due to occlusion of outflow gas. When air enters the interstitial tissue along bronchioles and blood vessels, it tracks into the mediastinum. Pneumothorax and pneumomediastinum occur simultaneously; however, pneumomediastinum is invariably benign, and should not be confused with a pneumothorax on
FIGURE 35

FIBEROPTIC PROBE

Legend

Figure illustrates increased transmission of light from a high-intensity fiberoptic probe by a large pneumothorax. Abnormal air collection results in increased transmission of light on the involved side or area. There may be a few false-negative and false-positive interpretations in the presence of small collections of air, however larger accumulations are readily detected. The advantage of this method is the rapidity with which clinicians can intervene with needle aspiration or chest tube replacement.

FIGURE 35
radiographic evaluation. Because of its benign course, pneumomediastinum may go unrecognized, however, it has one characteristically diagnostic feature, distant heart sounds on auscultation (61). The characteristic diagnostic features of a pneumomediastinum radiograph are a halo of air, often with indistinct margins present adjacent to the borders of the heart on an anteroposterior radiograph (Fig. 36A). A lateral view of a pneumomediastinum (Fig. 36B) shows marked retrosternal hyperlucency, a collection of air in the mediastinum anterior to the heart and displacement of the thymus (arrow) away from the anterior border of the heart (61).

No treatment is indicated for pneumomediastinum.

(c). Metabolic Disturbances

Metabolic disturbances include anemia, acidosis, hypocalcemia, hypoglycemia, and hyperbilirubinemia, which are common and may seriously worsen respiratory function if left untreated (66). Hyperbilirubinemia is most frequent; furthermore kernicterus may ensue at bilirubin levels that are generally lower than those associated with kernicterus in non-RDS infants, for a low pH predisposes detachment of bilirubin from albumin-binding sites to permeation of the brain. Therefore hyperbilirubinemia is often more severe in infants with RDS and is more likely to cause kernicterus when acidosis or hypoxemia are present.

(d). Patent Ductus Arteriosus (PDA)

The presence of PDA in premature infants is not understood, however the following theories exist: primarily the ductus is known to
FIGURE 36
PNEUMOMEDIASTINUM

Legend
A. Figure illustrates on this anteroposterior view, that the pneumomediastinum is very poorly defined. The thymic lobes, however, are elevated (arrows), indicating air in the mediastinum.

B. Figure illustrates same patient as A however, this is the horizontal beam lateral view which clearly shows a collection of air in the mediastinum anterior to the heart. The displacement of the thymus (arrow) away from the anterior border of the heart is well demonstrated.

respond to oxygen and therefore, hypoxemia of an RDS infant can open a physiologically closed ductus arteriosus; also hormonal factors and autonomic control may be involved (61). The relationship exists that the impact of the shunt is inversely related to gestational age and the birth weight of the infant; for in very immature infants, the physical (musculature) and chemical (prostaglandin cause constriction) changes of the ductus and pulmonary arteries are incomplete, so that PDA is more likely to occur and result in early congestive heart failure (61). In infants recovering from RDS there are usually both pulmonary and cardiovascular lesions present and also left-to-right shunting occurring, because of increased pulmonary vascular resistance. However, as systemic oxygenation and pulmonary disease improves, the pulmonary vascular resistance falls and blood flows across the ductus, from the aorta to the pulmonary artery.

The clinical presentation of patent ductus arteriosus varies according to the state of respiratory disease, maturity of the infant, and size of the ductus. The presence of the shunt is usually identified in an infant recovering from RDS by a sudden deterioration, such as apneic spells or increased ventilation or inspired oxygen. Also a very diagnostic characteristic of PDA, is the discovery of a systolic cardiac murmur over the left sternal border or pulmonic area. Often positive radiographic and echocardiographic findings indicate the presence of a significant shunt in many premature infants before a cardiac murmur is detected (61). Figure 37 is a radiographic of a patent ductus arteriosus in an immature infant with left-to-right shunting, displaying a
FIGURE 37
PATENT DUCTUS ARTERIOSUS
(PDA)

Legend

Figure illustrates radiograph of a preterm infant with left-to-right shunting via a patent ductus arteriosus (PDA).

Also shown are a cardiomegaly and increased pulmonary vascular markings.

cardiomegaly, and increased pulmonary vascular markings.

2. Intensive Care Associated Complications

(a). Complications of Endotracheal Intubation

Complications of endotracheal intubation include colonization by pathogenic organisms, edema of the larynx, and subglottic stenosis.

Also an extremely common and serious complication of therapy is obstruction of the airway by a misplaced endotracheal tube. A radiograph (PA film) will suffice to identify the location of a radiopaque endotracheal tube, and it is a relatively simple matter to withdraw the tube to an appropriate level. This manoeuvre is invariably followed by rapid improvement both clinically and radiographically (61).

(b). Complications of Umbilical Vessel Catherization

Complication of umbilical vessel catherization include vascular embolization, thrombosis, renal hypertension, and infection.

(c). Complications of Infection

Another major complication associated with treatment and which can be attributed to sudden deterioration (and can not be ascertained by physical examination or chest radiograph) is that of sepsis. If sepsis is suspected, antibiotics should be started immediately, following collection of blood, urine and cerebral spinal fluid for culture (65).

The selection of antibiotics depends largely upon the experience of a given nursery with the prevailing bacterial flora. Also the utilization of ventilatory equipment should always alert the possibility of a Gram negative rod infection, such as Pseudomonas, Serratia marcescans and others.
The risk of infection should be monitored carefully in each RDS infant.

(d). Fluid Administration Complications

Feeding a severely distressed infant necessitates special feeding techniques, such as nasogastric lavage or intravenous alimentation. The fluid administration must be monitored carefully because renal function can be compromised, and signs and symptoms of heart failure may be exacerbated by excessive fluid administration (65).

(e). Intracranial Hemorrhage

The premature infant is at great risk to develop intracranial hemorrhage, most particularly intraventricular hemorrhage. Intraventricular hemorrhage is believed to be related to hypoxic insult of the rich vascular plexuses in the paraventricular region. The susceptibility to bleeding may be accentuated by therapeutic fluid administration (sodium bicarbonate) and hemodynamic factors associated with assisted ventilation.

(f). Oxygen Therapy Complications

Retrolental fibroplasia (retinal damage which may lead to partial or total blindness) and bronchopulmonary dysplasia (oxygen toxicity), are the two known significant complications of oxygen overdosage. Retrolental fibroplasia (RLF) is caused by hyperoxemia in premature babies and rarely occurs in infants whose gestational age is over 36 weeks. The development of RLF is directly related to the PaO₂, (maintain below 90 to 100 mm Hg) not to the percent concentration of inspired oxygen. However, infants with RDS are actually less likely to develop
RLF, in spite of the frequency with which a high FIO₂ must be provided, because their disorder militates against the achievement of high arterial oxygen tensions.

On the other hand, bronchopulmonary dysplasia (oxygen-lung toxicity) appears to be directly related to an FIO₂ over 0.60 when breathed for several days rather than to arterial oxygen tensions. The usage of CPAP has diminished the incidence of oxygen lung toxicity because use of a lower FIO₂ is possible.

With bronchopulmonary dysplasia (BPD) there appears to be prolongation of the healing phase of RDS combined with pulmonary oxygen toxicity and the subsequent development after one month of chronic lung disease in the surviving infants. Coincidental with the greater use of oxygen and ventilators has been the appearance of chronic pulmonary lesions, which compromise gas exchange and may dictate even more prolonged ventilatory assistance (61, 66).

The BPD’s clinical course changes parallel the four distinct changes found in radiographic findings, described by Northway and Rosan in 1968 (61). These four radiographic progressive stages are: 1) Identical radiographically to moderately severe RDS (reticulogranular appearance), and is found from the first and third days of life (Fig. 38A; a 2-day old infant with BPD); 2) the lungs appear consolidated, with more homogenous opacification than the reticulogranularity found in stage one (Fig. 38B; same infant at 2 weeks), and is present from four to ten days of life; 3) many small cystic areas appear as a result of focal alveolar emphysema, from ten to twenty days of life (Fig. 39A; same infant at 8 weeks) this
FIGURE 38
BRONCHOPULMONARY DYSPLASIA
(BPD)

Legend

Figures illustrate the first two stages of bronchopulmonary dysplasia.

A. Stage 1, present in the first 1 to 3 days of life (this radiograph is of a 2 day old infant with BPD) is indistinguishable radiologically from moderately severe respiratory distress syndrome.

B. Stage 2, is present from 4 to 10 days of age (same infant as above at 2 weeks). The lungs become consolidated, with a more homogenous opacification than the reticulogranularity seen in stage 1.

Figure 39
BRONCHOPULMONARY DYSPLASIA
(BPD)

Legend

Figure illustrates the second two stages of BPD.
A. Stage 3 is from 10 to 20 days (same infant as in Fig. 38 at 8 weeks) and is characterized by the appearance of numerous small cystic areas as a result of focal alveolar emphysema, somewhat similar to the bubbly appearance of the lungs in the Wilson-Mikity syndrome, and this feature must also be distinguished from pulmonary interstitial emphysema. The clinical presentation of both of these disorders differ from that of bronchopulmonary dysplasia.

B. Stage 4, the cystic lucencies gradually become replaced by generalized emphysema, and strandlike pulmonary densities appear, particularly in the upper lobes. These may either clear over a period of months or lead to cor pulmonale and death (same infant as above at 10 weeks).

stage radiographically is somewhat similar to the "bubbly" appearance of
Wilson-Mikity syndrome and pulmonary interstitial emphysema, however,
clinically the three diseases differ and: 4) the cystic lucencies
gradually become replaced by generalized emphysema, and strandlike
pulmonary densities appear, especially in the upper lobes (Fig. 39B; same
infant at 10 weeks) (61). This disease's process appears to be one of
continuous injury and repair, causing a delay in both lung and body
growth. However, complete recovery is possible in these infants.

I. PATHOLOGY

Infants who die during acute stages of respiratory insufficiency have
profound generalized atelectasis on autopsy. At postmortem examination,
the lungs from infants with RDS are firm and airless. Atelectasis is
striking on gross inspection, even when the lungs are inflated before
fixation and only the airways and a few alveolar ducts are air filled.
Also the small pulmonary arterioles appear constricted and there is
congestion of the pulmonary capillaries and veins. The intrapulmonary
lymphatics are dilated, particularly those in septa and around small
airways and vessels, suggesting an increase in fluid flux in the lung or
a disruption of the usual mechanism for the clearance of lung water.

Histologically the terminal air spaces are collapsed and the alveolar
ducts and terminal bronchioles are dilated (Fig. 40A and B) (70). If an
attempt is made to inflate the lungs with air, alveolar opening is almost
impossible to achieve, but the distal airways become enormously
distended. However, if expansion is carried out with liquid, the
terminal air sacs open and the structure of the lung is seen to be
FIGURE 40
HYALINE MEMBRANES

Legend

A. This photomicrograph illustrates bronchiolar distention and almost complete alveolar collapse. The dilated alveolar ducts are lined by thin eosinophilic membranes. Hematoxylin and eosin stain; reduced 27% from approximate mag. x 130.

B. Longitudinal section of the left lung of a 1500 g infant born after a 30 week gestation, who died at 60 h of age with RDS. The airways are distended, and a few of the respiratory bronchioles are overinflated. Most of the alveolar ducts and alveoli are airless.

grossly normal (70). Gruenwald concluded that atelectasis and dilation of the terminal airways are important pathologic findings and that the terminal airways are usually lined with a homogenous eosinophilic membrane otherwise named the hyaline membrane (71). The membranes are composed largely of fibrin and other constituents of the blood (hemoglobin products) and the cells underlying them are disrupted. These hemoglobin products and cellular debris coat the terminal bronchioles, and this is the classically described hyaline membrane (Fig. 41) (61). However, hyaline membranes are not present in all cases, nor is it specific for RDS, for hyaline membranes are absent in the lungs of infants who die in the first few hours of life, whereas atelectasis is extensive. In differential diagnosis of the pulmonary lesions of RDS it is important to realize that hyaline membranes may also form in the air spaces in conditions where there has been massive extravasation into the air spaces, especially in massive pulmonary hemorrhage and hydrops fetalis (61). Therefore, a diagnosis of RDS should not be made solely on the presence of hyaline membranes.

J. PREVENTION OF RDS

The occurrence of RDS has gradually decreased during the last decade. Advances in obstetric management of preterm labor and high-risk pregnancies have influenced the decrease of RDS. Recently, there has been an increase in the population of surviving tiny premature newborns who barely a decade ago would have been lost earlier than the diagnosis of RDS could have been made. However, these infants are now chronic occupants of intensive care units, with a long list of problems, mainly
FIGURE 41
HYALINE MEMBRANES

Legend

A. Figure illustrates section from lung with atelectasis and hyaline membranes. The membrane lines some of the aerated spaces. (x 100)

B. Figure illustrates highpower view of the homogenous, eosinophilic membrane. (x 400)

developmental, including RDS and its complications.

1. Prematurity

There are only small trends showing a decrease in prematurity, and the prematurity rate is the most important factor that is responsible for RDS remaining a major neonatal problem. Therefore, any steps taken to avoid delivery before term will reduce the frequency of RDS. Currently, pregnancy is prolonged with bed rest and/or the utilization of tocolytic drug agents, such as ritodrine, to inhibit premature labor (usually before 36 weeks of gestation). Also, concurrently some physicians administer steroids to pregnant mothers in premature labor (between 28-32 weeks) to enhance fetal lung maturation and reduce the incidence of RDS.

2. Usage of Glucocorticoids

The administration of steroids to pregnant mothers enhances fetal lung maturation and therefore aids to prevent or diminish the severity of the RDS. This topic has been very controversial since its initial conception in 1968, for many investigators have cautioned that until the side effects of the use of steroid hormones in the developing fetus are fully understood, this therapy must be considered experimental and used advisedly. However, others counter that it is not right to withhold a therapy that might be lifesaving, regardless of the consequences. This entire controversy will now be discussed in detail.

The occurrence of neonatal RDS is directly correlated with premature delivery. Because of limitations of prenatal risk scoring for premature delivery (often combined with late entry to the labor area for tocolytic therapy) or other obstetric medical complications, approximately 40% of
The mothers at risk for premature delivery are not candidates for corticosteroid therapy. In the remaining 60%, the physician often must still deal with two major clinical considerations: assessment of gestational age (fetal maturity), and diagnosis of true or false premature labor.

In 1969, Liggins was studying the effect of steroid hormones on labor in pregnant ewes (72). Liggins deduced from his results that he was witnessing the accelerating effects of steroids on pulmonary maturation of the sheep fetus (72). This initial observation has triggered many investigations into morphological, physiological, and biochemical mechanisms, confirmatory studies, and into therapeutic trials of glucocorticoids. These factors will all be discussed.

(a). Mechanism of Action

Antepartum administration of both natural and synthetic glucocorticoids via the maternal circulation decreases the incidence of RDS in the prematurely born infant. This is thought to be due to an acceleration of fetal lung development, for in the type II alveolar epithelial cells, the presence of cytoplasmic and nuclear receptors that specifically bind to glucocorticoids indicates that the fetal lung is a target organ for these steroids and that these steroids play an important role in the differentiation and maturation of the lung (73). The following factors are evidence for the lungs cytoplasmic glucocorticoid binding activity: The lung's binding activity has been detected as early as 9 weeks gestation (74). These receptors appear to be detectable throughout most gestation and in the lungs of premature and
term neonates (74). The concentration of these glucocorticoid receptors in the human lung is reportedly constant between 12 and 20 weeks gestation (74). The fetal cytosol contains two to five times more receptor sites per milligram of protein than do other fetal organs (i.e., liver, heart, kidney, brain, muscle, jejunum, and thymus). This high level of binding activity in lung tissue may reflect a greater hormonal responsiveness of this tissue compared to the others (75). Therefore, these glucocorticoid receptors are present in the fetal lung sufficiently early in gestation to permit the lung to respond to glucocorticoids administered between 27 and 36 weeks' gestation.

Although many details of the intracellular mechanisms of glucocorticoid action in the fetal lung are unknown, available evidence supports the following two-step mechanism (Fig. 42) similar to that described for a large number of steroid receptor systems in a variety of target cells (76). The first event involves binding of the steroid with the high affinity glucocorticoid specific receptor protein in the cell cytoplasm. The complex formed is inactive in the sense that it cannot bind efficiently to the nuclei. This first step involves activation of the complex to a form which has an enhanced affinity for the nuclei. This step is highly dependent on temperature and ionic strength (and poorly understood). The second step involves translocation of the activated complex into the nucleus where it is retained by binding to chromatin acceptor sites. It is believed that this nuclear interaction influences the rate of synthesis of specific mRNA's which are eventually transported to the cytoplasm and code for the synthesis of specific
FIGURE 42
PROPOSED MECHANISM FOR ACCELERATED MATURATION OF FETAL ALVEOLAR LINING CELLS BY GLUCOCORTICOID STERIODS

Legend

Figure illustrates two-step mechanism which supports intracellular glucocorticoid action in the fetal lung. The first event involves binding of the steroid with the high-affinity glucocorticoid specific receptor protein in the cell cytoplasm. The complex formed is inactive in the sense that it cannot bind efficiently to the nuclei. This first step involves activation of the complex to a form which has an enhanced affinity for the nuclei. This step is highly dependent on temperature and ionic strength (and poorly understood). The second step involves translocation of the activated complex into the nucleus where it is retained by binding to chromatin acceptor sites. It is believed that this nuclear interaction influences the rate of synthesis of specific mRNAs, which are eventually transported to the cytoplasm and code for the synthesis of specific proteins. Among the newly synthesized proteins are enzymes involved in the synthesis or secretion or both of surface-active phospholipids.

proteins (77). Among the newly synthesized proteins are enzymes involved in the synthesis or secretion or both, of surface-active phospholipids.

Following is an account of the morphologic, physiologic and biochemical changes, which occur in the fetal lung following maternal administration of glucocorticoids.

(b). Morphologic Changes

Kikkawa et al. found the following morphologic changes when studying cortisol administration to the fetal rabbit lung (78):

- Accelerated maturation of the epithelial cells, as evidenced by an accelerated depletion of glycogen and an increase in the number of lamellar inclusions.
- Increased alveolarization.
- Histologic maturation as shown by the faster transition from the glandular to canalicular and alveolar forms of lung structure.
- Increased branching and enlargement of glands during glandular stage.
- Acceleration of canal formation and increased potential air space.
- Increased rate of cell proliferation in early gestation and decreased rate of cell proliferation in late gestation.

(c). Physiologic Changes

The following physiological data support the above morphologic observations:

- Alveolar stability is increased.
- Greater deflation stability and increased maximal lung volumes.
- Increased distensibility.
• Analysis of deflation pressure-volume curves revealed increased lung maturity which correlated with increased survival times.

• Earlier appearance of lamellar bodies in epithelial lung cells of steroid treated fetus (79).

(d). Biochemical Changes

Biochemical studies also provide the following evidence that glucocorticoids accelerate the maturation of the surfactant system.

• The net biochemical glucocorticoid effect is an enhanced capacity of the lung to produce surface active phospholipids at an earlier time in gestation than would normally occur.

• As mentioned, the earlier appearance of lamellar bodies in the lung cells stimulates the synthesis of phospholipids, particularly surface active lecithin. This has been repeatedly demonstrated by the findings of increased amounts of lecithin in lung parenchyma and in lung fluid obtained by saline lung lavage (80).

• Also increased incorporation of choline into lecithin and increased activity of enzymes involved in lecithin synthesis (81).

Although conflicting data from different laboratories, as well as species differences, have been reported, it appears that glucocorticoids may enhance the activity of two groups of enzymes. The first group of enzymes is involved either directly in the biosynthesis of lecithin by the choline incorporation pathway (choline phosphate cytidyltransferase (82), choline phosphotransferase (83)) or in the restructuring of unsaturated into disaturated lecithin by the deacylation-reacylation
cycle (lysolecithin acyltransferase) (82, 83). The second group of enzymes, the activity of which may be affected by glucocorticoids is concerned with the production of precursors for lecithin synthesis, such as the supply of diacylglycerols from phosphatidic acid (phosphatidic acid phosphatase) (84) or the supply of fatty acids from triglycerides (lipoprotein lipase) (84). Further studies are necessary to define which of these enzymes are rate limiting for the synthesis of surface active lecithin and the role of glucocorticoids in modulating their activity. Also, little is known about the mechanism by which glucocorticoids enhance enzyme activity in the fetal lung with the exception of the report of Farrell and Zachman, which showed that cycloheximide but not actinomycin D blocked the glucocorticoid-induced increase in choline phosphotransferase activity, suggesting that the steroid effect may be mediated at the translational level (81).

(e). Therapeutic Trials of Glucocorticoids

Table VII lists the large number of human studies conducted to evaluate the administration of glucocorticoids to pregnant women in which there was a reduction in the incidence of RDS. However, there has been much difficulty in comparing the studies because of the following variables: steroid type, dose, route of administration; course of treatment; patient selection; method of study analysis; concomitant maternal-fetal disease states; and follow-up (Table VII). Some important conclusions, however, may be drawn and will now be summarized.
<table>
<thead>
<tr>
<th>Author, Reference (Date)</th>
<th>Glucocorticoid dose, route, and timing of administration</th>
<th>Gestational age range (wk)</th>
<th>Frequency of RIS (controls/treated)</th>
<th>Perinatal death rate</th>
<th>Maternal side effects</th>
<th>Fetal material side effects</th>
<th>Effect on the L/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucini et al. (1978)</td>
<td>Betamethasone 12mg, IM q 24h for 2 days</td>
<td>&lt;36</td>
<td>33/36</td>
<td>20/36</td>
<td>20/36</td>
<td>20/36</td>
<td>increased</td>
</tr>
<tr>
<td>Presser et al. (1979)</td>
<td>Betamethasone 12mg, IM q 24h for 2 days</td>
<td>31 ± 1.2</td>
<td>7/13</td>
<td>54/36</td>
<td>0/13</td>
<td>0/13</td>
<td></td>
</tr>
<tr>
<td>Kenny (1978)</td>
<td>Betamethasone 12mg, IM q 24h for 2 days</td>
<td>&lt;36</td>
<td>33 ± 1.2</td>
<td>12/26</td>
<td>66 ± 12</td>
<td>1/8</td>
<td>increased</td>
</tr>
<tr>
<td>Russell et al. (1975)</td>
<td>Betamethasone 12mg, IM q 24h for 2 days</td>
<td>&lt;36</td>
<td>33 ± 1.2</td>
<td>12/26</td>
<td>1/8</td>
<td>1/8</td>
<td>increased</td>
</tr>
<tr>
<td>Kenny, J. L. Jr. (1976)</td>
<td>Betamethasone 12mg, IM q 24h for 2 days</td>
<td>&lt;36</td>
<td>33 ± 1.2</td>
<td>6/12</td>
<td>50 ± 12</td>
<td>1/12</td>
<td></td>
</tr>
<tr>
<td>Author reference/ type of study</td>
<td>Glucocorticoid dose route, and timing of administration</td>
<td>Gestational age range (wks)</td>
<td>Frequency of RDS: controls no. (RDS/total) %</td>
<td>Frequency of RDS: treated no. (RDS/total) %</td>
<td>Perinatal death rate: control steroid</td>
<td>Maternal side effects</td>
<td>Fetal-maternal side effects</td>
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<tr>
<td>LUGGINS, G. E., (1976) lung maturation, p. 97* Trible blind</td>
<td>Betamethasone 12mg, IM 24h for 2 days</td>
<td>&lt;30 - 32</td>
<td>15/25 58</td>
<td>10/36 28</td>
<td>2/23 9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MACFADDY et al. (1976) Arch. Dis. Child. 51, 428</td>
<td>Hydrocortisone 100 mg, IM tid (one dose)</td>
<td>27 - 35</td>
<td>18/60 45</td>
<td>5/31 15 (524 hr)</td>
<td>35 10</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CASPI et al. (1976) Br. J. Obstet. Gynaecol. 83, 187</td>
<td>Betamethasone 4 mg, IM tid for 7 days</td>
<td>28 - 32</td>
<td>19/28 68</td>
<td>8/26 19</td>
<td>38.0</td>
<td>Increased</td>
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<tr>
<td>MACFADDY et al. (1977) Br. J. Obstet. Gynaecol. 84, 20</td>
<td>Betamethasone 12 mg, IM 24h for two days</td>
<td>&lt;32 - All premature</td>
<td>9/19 47</td>
<td>4/13 31</td>
<td>14.4 8.0</td>
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<tr>
<td>ROCK et al. 1977. <em>Triple blind</em></td>
<td>Betamethasone 12 mg, IM 24h for 2 days</td>
<td>9/19 47</td>
<td>8/15 53</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Methylergometrine 12mg IM 24h for 2 days</td>
<td>9/19 47</td>
<td>8/15 53</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Author reference/type of study</td>
<td>Glucocorticoid dose, route, and timing of administration</td>
<td>Gestational age range (wk)</td>
<td>Frequency of RIE</td>
<td>Maternal side effects</td>
<td>Fetal/neonatal effect</td>
<td>Effect on LR ratio</td>
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<td>MORGON et al. (1978) Mg, J.</td>
<td>Hydrocortisone 72mg, IV g 0.12E for 2 days</td>
<td>26 - 31 32 - 35</td>
<td>34 14/50 24</td>
<td>6/62 9 12.0 4.4</td>
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<td>Clesi., General 131, 19A,</td>
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<td>Flexible blind.</td>
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<tr>
<td>THERMOD et al. (1978) Mg, J.</td>
<td>Prednisolone 8mg, 24h for 2 weeks: Ethanol to delivery.</td>
<td>32 - 36 236</td>
<td>32 18/35 75</td>
<td>9/16 35 4 0</td>
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<tr>
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<tr>
<td>Controls subjects untreated or inadequately treated. Matched for all factors</td>
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<tr>
<td>BALJAD et al. (1979) Mg, J.</td>
<td>Prednisolone 12mg, 12h for 4 days (plus azete inhibitor)</td>
<td>26 - 34 30/118 51</td>
<td>41/114 38</td>
<td>23 7</td>
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<td>Pelvis, General 95, 97,</td>
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<tr>
<td>Controls patients untreated.</td>
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<tr>
<td>FERRANDI et al. (1979) Statistics</td>
<td>Prednisolone 12mg, qd x 2 plus qd x 2 plus</td>
<td>27 - 34 17/30 57</td>
<td>17/27 15</td>
<td>0</td>
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Legends:
- RIE: Retinopathy of prematurity
- LR: Likelihood ratio
TABLE VII

<table>
<thead>
<tr>
<th>Letter reference</th>
<th>Type of study</th>
<th>Gestational age range (wk)</th>
<th>Frequency of HIE</th>
<th>Perinatal death rate</th>
<th>Maternal side effects</th>
<th>Total maternal effects</th>
<th>Effect on the IAS ratio</th>
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<td>JSHE et al. (1979)</td>
<td>Newborns, General.</td>
<td>31 days, q.d. x 2</td>
<td>12/24</td>
<td>14</td>
<td>14/84</td>
<td>16</td>
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<td>TASHE et al. (1979)</td>
<td>Newborns, General.</td>
<td>26 - 36 weeks</td>
<td>9/26</td>
<td>35</td>
<td>2/10</td>
<td>15</td>
<td>6</td>
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<td></td>
<td>Newborns, General.</td>
<td>48 mg tid x 6 days, plus oral or B-adrenergic.</td>
<td>9/19</td>
<td>47</td>
<td>1/6</td>
<td>17</td>
<td>6</td>
</tr>
</tbody>
</table>


**Abbreviations:** HIE = intracerebral, q = every, tid = three times daily.
198

(1). Time of Treatment

Although steroid therapy was utilized over a wide gestational age range, positive results were most evident in women delivering between 28 to 34 weeks gestation.

In patients under 28 weeks gestation the development of the fetal lung with reference to interstitial tissue and blood vessel proliferation may be insufficient to support adequate ventilatory exchange, even though steroid administration could possibly induce lecithin formation in the type II alveolar cells (85). Obviously since there are survivors among these tiny infants, many factors must be considered in regard to therapy in those under 28 weeks gestation.

It appears that glucocorticoids are effective only when administered to fetuses of less than 34 weeks gestation, but not to older fetuses. One reason for the ineffectiveness of glucocorticoid administration after the 34th week of gestation may be the low incidence of RDS in these older fetuses. Also, it has been suggested the lungs of fetuses older than 34 weeks' gestation may be unresponsive to glucocorticoids and the already lower incidence can not be reduced any further.

Baden et al. found that postnatal administration of glucocorticoids to newborn premature infants with RDS was not effective, indicating that the mechanism of steroid action involves prevention rather than treatment of the disease (86). The failure of postnatal glucocorticoid therapy is not unexpected, since infants with RDS are known to have elevated cortical levels that may be sufficient to evoke an optimal response of the lung. Moreover, many infants with RDS begin to improve spontaneously
during the third day of life, a time when glucocorticoid effects might first be expected.

(ii). Duration and Frequency of Treatment

Most investigators feel that maximal benefit is obtained when treatment has been given for at least 24 hours prior to delivery. The Liggins and Howie studies demonstrate that the incidence of RDS begins to fall in those delivered 18 to 24 hours after therapy is initiated (87). This above data is in accord with the in vitro studies, demonstrating surfactant synthesis of approximately 12 hours after glucocorticoid administration (81). Liggins and Howie have also found that the reduction in the incidence of RDS is achieved only in infants born within seven days of initial corticoid treatment (87). The effectiveness of a second weekly dose of corticoid has not been established.

Equilibration of the corticoid occurs rapidly between the mother and fetus, therefore the frequency of administration is very important. However, different administration schedules reported in the studies makes it impossible to compare this variable. For example, Caspi continues administration of steroids for seven days (88), while most administer the corticoid for only 48 hours.

The total dosage necessary for lung maturation is extremely questionable, since so many regimens are used in the published studies. Besides, each of the steroid agents has a variable potency, protein binding, bioavailability, placental transfer, and metabolic half-life. Therefore, since fetal lung maturation and other endogenous effects occur
with all dosage levels and administration regimens, it is likely that more medication is being given than is actually needed. The lower limits of effective therapy are not known, however, most investigators follow the schedule originally described by Liggins, 12 mg betamethasone every 24 hours for 2 days (87). This regimen of Liggins was selected on the basis of his early animal work and may have little relation to the lowest possible dose for human lung maturation. Definitely, more work is required in this important area, because dosage may play a role in potential drug toxicity relative to neonatal side effects.

(iii). Mode of Glucocorticoid Administration

Glucocorticoids could be administered directly into the fetus by injection into the amniotic fluid, however, the safest and most convenient route of administration is to the mother either orally or intramuscularly, with transplacental transfer to the fetus.

(iv). Choice of Glucocorticoid

In choosing the type of steroid to be utilized for enhancement of lung maturation the following points must be considered (87).

- Steroid must be a potent glucocorticoid with minimal mineralocorticoid activity.
- Steroid should readily cross from the maternal to the fetal circulation, while undergoing minimal metabolism by the placental.
- Steroid's affinity for binding to albumin and/or transcortin (corticosteroid-binding globulin-CBG) is important in controlling the transfer of the glucocorticoid to the fetus.
- The capacity to bind to specific receptors and to induce enzymes are a very important aspects of glucocorticoid activity.
- The degree of steroid metabolism, and its clearance in the maternal and fetal circulation, for the mother and fetus are capable of reducing levels of glucocorticoids through hepatic metabolism. However, currently no data is available on the relative rates of glucocorticoid metabolism by the maternal or fetal liver, which might possibly result in different rates of disappearance from circulation.
- The plasma concentration of glucocorticoids may be an important regulatory factor in the pathogenesis and prevention of RDS. Firstly, the optimal responsiveness of glucocorticoid administration should occur at levels of steroid required to saturate the cytoplasmic receptors. Secondly, steroid binding by lung cells is a reversible process, and the optimal effect of steroid therapy is largely dependent on the continued presence of appropriate concentrations of glucocorticoid within the lung cells.
- Efficacy for producing the desired physiologic effect and possible side effects.

(v). Usage of Betamethasone and Dexamethasone

In the studies conducted several glucocorticoids were utilized (Table VII), however currently it appears that betamethasone and dexamethasone seem to be the best suited for the role of therapeutic agent for the following reasons (Table VIII) (89):
TABLE VIII
BIOLOGIC ACTIVITY OF THE MOST USED GLUCOCORTICOIDS IN CLINICAL STUDIES

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Biologic half-life (h)</th>
<th>Plasma half-life (min)</th>
<th>Transcorin-isotope assay</th>
<th>Maternal/fetal gradient</th>
<th>Relative affinity for human fetal lung receptors</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betamethasone (9α-fluoro-16α-methyl-prednisolone)</td>
<td>36-54 (long activity)</td>
<td>&lt;300</td>
<td>1</td>
<td>3:1</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (9α-fluoro-16α-methyl-prednisolone)</td>
<td>36-54 (long activity)</td>
<td>&lt;300</td>
<td>1</td>
<td>3:1</td>
<td>710</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone (cortisol)</td>
<td>8-12 (short activity)</td>
<td>90</td>
<td>100</td>
<td>5.8:1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>6α-methyl-prednisolone</td>
<td>8-36 (intermediate-acting)</td>
<td>&lt;200</td>
<td>1</td>
<td>—</td>
<td>1190</td>
<td></td>
</tr>
</tbody>
</table>

* Values determined from dose-response experiments with cytosol prepared from lungs of human fetuses of 16-20 weeks gestation incubated with 13 nM [3H]-dexamethasone for 28-32 h at 2°C.

• On the basis of their biologic half-lives, betamethasone and dexamethasone are termed long-acting glucocorticoids (36-54 hours). (Plasma-half-life 200 min).

• Betamethasone and dexamethasone are bound only to albumin and, therefore, may be more readily transferred across the placenta to the fetus.

• Betamethasone and dexamethasone have a low affinity for transcortin (isotope assay); therefore, they diffuse more completely into the tissues, which explains in part their greater relative potency in humans. However, this may lead to excessive levels of free drug and an enhanced susceptibility to side effects.

• Betamethasone and dexamethasone may be more suitable for treating the fetus, since their maternal:fetal concentration gradients after administration to the mother are low at about 3:1.

• Betamethasone and dexamethasone induce enzymes that are important for surfactant production.

• Finally, betamethasone and dexamethasone bind well to cytoplasmic and nuclear receptors (540 and 710, respectively), a property that appears to be a prerequisite for exerting their biologic effects.

(vi). Fetal Side Effects

The outstanding major concern regarding the prenatal administration of glucocorticoids is the possibility of long-term, harmful side effects. For glucocorticoids are known to influence the growth and development not only of the fetal lung, but also of a variety
of other fetal tissues (cerebellum, small intestine, pancreas, adrenal medulla, liver, and retina) and are known to increase the activity of a large number of enzymes in developing animals.

Following are the multisystemic adverse side effects of glucocorticoid treatment in previous animal studies. Fetal side effects are: increased fetal loss; reduced body weight and cell numbers; diminished immune responses; diminished placental growth and increased senescence of the placenta; impaired maternal-fetal glucose transfer; and increased pancreatic islet maturation and degeneration. Neonatal side effects are: diminished immune responses and antibody responses; decreased brain and body weight; decreased locomotor activity, decreased central nervous system DNA with associated increased central nervous system malfunction; decreased glucose metabolism; increased neonatal loss; and increased pulmonary susceptibility O₂ toxicity.

The most severe side effect problem is the diminution in body growth following steroid administration to the fetus which has been verified in a variety of animal species where essentially the whole animal growth is diminished, presumably as a reflection of the widespread effect of steroids on the division of DNA in growing tissues (90). Growth of specific organs including the lung may also be affected, although in the rabbit lung there is evidence of catch-up growth after cessation of steroids (91).

By far the most serious effects of steroids of the many described on organ growth are those on growth of the brain. Studies on rats have shown that total DNA in the cerebrum and the cerebellum is diminished.
and the animals subsequently develop abnormally, have significant neurologic deficits throughout their lives, and the brain growth never catches up (92). Also in some of the animal studies, the brain weight was decreased, lower cholesterol/DNA ratios were found, and clinical associations were made between these findings and decreased performance of fine-motor control and purposeful activity, which persisted into adulthood (93). Similar studies of steroid administration to fetal monkeys have shown diminution in DNA brain, indicating a lesser number of cells than in normals.

While the dosages of steroids in these studies were high and the duration of their administration long, the studies have provided concern about the safety of these agents for the fetus and the newborn when administered to the mother. Currently, no human studies, controlled or otherwise, have substantiated these animal results. For many of these effects were seen in the animals only after massive doses of steroids were given to the animals, who normally have a short, telescoped gestation and life. Therefore, this means that any drug given is subject to great exaggeration of effect, especially in doses that are much larger than the human dosage would be. However, in two animal studies, one by Loeb (94), in which liver, skeletal muscle, and other tissues showed inhibition of growth in rabbits and one by Epstein et al. (95) in which gliosis in fetal monkey brain was seen in almost 50% of fetuses, doses of steroid comparable to the usual therapeutic range of doses in humans were employed.

Note that in most of the animal studies, very large doses of
glucocorticoids were administered over long periods of time during early stages of gestation. In the human, no serious adverse effects have been reported after short-term (two to seven days) treatment with glucocorticoids, using the doses employed by Liggins and Howie (87), except for hypercholesterolemia in the newborn and perhaps also increased maternal susceptibility to infection. The other complication noted has been the increased risk of fetal death in mothers with severe pre-eclampsia and massive proteinuria (87). Follow-up of infants delivered after betamethasone treatment revealed normal IQ scores at 4.5 years of age (87).

(vii). Maternal Side Effects

A few maternal side effects have been noted in the studies from the prenatal administration of glucocorticoids, which are maternal free cortisol levels were depressed (<5 μg/dL) 12 hours after betamethasone administration, but gradually returned to normal 2 to 4 days after therapy was discontinued (96). This probably reflects decreased transfer of maternal precursors as well as reduced production of fetal steroid. Ballard found the maximal levels of betamethasone (75 mg cortisol derivatives per deciliter) 1 hour after each dose, but was unable to detect the agent 2 days after the last dose (96). Several investigators found no increase in postpartum infection, fever, resistant organisms, or prolonged hospital stay and labor or lactation were not affected (97). In the studies cited, there were no long-term maternal effects noted at the six-week postpartum visit. These findings are not unexpected, for in clinical experience, much more prolonged
administration of these compounds has usually been required before significant maternal side effects were noted. However, it has been noted that there is increased incidence in maternal fever due to amnionitis in cases with rupture of the membranes for more than 48 hours who received steroid treatment.

(viii). Medical-Legal Aspects of Glucocorticoid Administration

The medical-legal aspects regarding the administration of withholding of steroid therapy will now be addressed. First, treatment of the mother with glucocorticoids involves the use of a licensed drug for an indication not approved by the FDA (lack of human side-effect evidence). Therapy should be undertaken only with written informed consent and under a strict protocol. Preferably, it should be given in a study where long-term follow-up is assured. Most important is the mother's understanding of: 1) the potential risks to her infant if the drug is not taken; 2) the potential decreased risks of RDS to the infant, should the mother decide to participate in therapy; and 3) the limited amount of confirmed data on the long-term effects of steroid therapy on the infant. Obviously, another critical issue is the standard of medical practice in the community. For the patient, her family and the health care provider should participate actively in this decision to reduce the risk of legal action.

With the present available knowledge, the physician must weigh the risks and benefits in choosing patient candidates for steroid administration. The following are the prerequisites for steroid therapy in preterm patients:
• Live fetus, with immature lungs with a gestational age of 26 to 34 weeks (or L/S ratio < 2.0).
• Absence of maternal-fetal disease precluding continuation of gestation (absence of amnionitis).
• Cervical dilation 4 cm or less and anticipated delivery more than 12 h and less than 7 days.
• Informed consent from patient and family.
• Adequate personnel and equipment for safe tocolytic drug therapy.
• Access to adequate neonatal intensive care nursery.
• Access to intricate long-term neurologic infant follow-up.

The investigations relating prenatal glucocorticoid therapy to decreased incidence and severity of RDS in premature infants offer exciting promise in reducing the leading cause of mortality and morbidity in preterm infants. However, until certain questions, particularly those related to long-term infant sequelae, are answered, it is highly recommended that the drugs be used under thoroughly controlled conditions. The final line for the physician is the rational decision regarding delivery versus continued gestation and regarding therapy versus no steroid treatment.

(ix) Summary

Although the steroids do seem to have an effect in maturing fetal lungs, it is important to remember that they do not mature the lungs of all fetuses treated, and that a significant proportion of babies still will have RDS despite the administration of steroids. Liggins and Howie reduced the incidence of RDS by 33 percent, for an overall figure
of 10 to 15 percent (87).

This is a classic dilemma of benefit versus harm, and more important, the almost perpetual problem in perinatology benefits from interventions are evident long before their harm becomes obvious, for RDS is a serious disease, particularly among younger and smaller prematures. Today, however, with careful techniques, infants over 1200 g with RDS can be expected to survive with a greater than .95% assurance. Follow-ups on these infants suggests that about 90% are normal, 5% may have minor to moderate neurologic and intellectual problems, and only 5% have significant neurologic and intellectual problems. This is certainly a far cry from 2 decades ago when, prior to modern neonatal ICU care, even normal prematures had a high incidence of neurologic and intellectual problems.

In summary, it must be remembered that the most damaging evidence of adverse effects on the neonate from prenatal maternal corticosteroid administration has been developed from animal experimentation and has not been directly demonstrated in humans. Although these adverse effects are disquieting, the dosage and length of administration of steroid to promote human fetal lung maturity and to control maternal disease are generally significantly less when compared to the experimental animal models. Moreover, acute and chronic drug effects of steroids in animals are not necessarily applicable to human offspring.

In the immediate future the following items must be accomplished: First, the potential serious long-term side effects of brain and neurologic development must be investigated in detail and extensively
followed-up for 5 to 10 years in humans. Secondly, actual minimal-effect dosage schedules for the various steroids must be determined for humans.

3. Effects of Other Hormones on Lung Maturation.

The possibility that thyroid, insulin, and prolactin hormones may play a role in lung development has been documented and will now be summarized. These hormones may well work in concert with glucocorticoids (or perhaps at a different time in gestation); the combination may be necessary for normal lung development and normal type II cell differentiation. A thorough consideration of other possible hormonal factors operative in lung development may lead to some resolution of the numerous questions posed by the results of experimental and clinical studies dealing exclusively with the role of glucocorticoids in either normal or accelerated lung development.

(a). Thyroid Hormones

The initial suggestion that thyroid hormones had a role in the process of lung maturation was derived from studies showing lower cord serum thyroxine and triiodothyronine concentrations in infants with RDS than in normal infants. Smith and Torday found that triiodothyronine stimulates the incorporation of choline into lecithin in fetal lung cells in monolayer culture (98). Also Lindenberg et al. found that fetal lung cells contain specific hormone receptors (99). Because of this evidence, thyroxine has been suggested as an intra-amniotic therapeutic agent to be absorbed by the fetus (because it does not cross placenta) that might accelerate the production of surfactant. Preliminary studies on human
fetuses who had intra-amniotic thyroxine injection showed the infants did not develop RDS when they were delivered prematurely (between the 30th and 36th week of gestation) (100). However, this study was not controlled and the sample size was small and inadequate. Therefore, additional testing is required to determine the effect of large doses of thyroid hormone on the developing human fetus and thyroxines therapeutic value as an agent in accelerating lung maturation.

(b). Insulin

Smith et al. have shown that insulin can significantly inhibit the specific action of steroids on the lung receptors and thus reduce lecithin production (101). This suggests that insulin antagonizes the glucocorticoid-induced stimulation of pulmonary lecithin synthesis in the fetus. Clinically, this has been substantiated in diabetic women who have an increased rate of RDS in their infants, if maternal blood glucose (and fetal insulin) is high (102).

In other studies, insulin has been shown to stimulate glycogen accumulation and to decrease the number of lamellar bodies in explants derived from 19-day fetal rat lung in short-term cultures, indicating that insulin may delay maturation of the fetal lung (103). The exact insulin mechanism is not known, however, the following two theories exist: first, insulin may inhibit glycogenolysis and deprive the lung of substrate for phospholipid synthesis; and second, alternatively insulin may act by stimulating glycogen synthesis from glucose, and thereby divert substrate away from phospholipid synthesis (104).
(c). Prolactin

Prolactin administration to fetal rabbits has shown an increase in concentration of both total and disaturated lecithin in fetal lungs (105). Also prolactin receptors have been identified in fetal lungs. However, the importance of prolactin in enhancing lung maturation is questionable for the following two reasons: first, when Ballard et al. injected prolactin into rabbit fetuses and examined them 2 days later, they found no enhancement of choline incorporation into lecithin, nor increased phospholipid tissue content, as compared with a control group (106), secondly, a rise in prolactin may not be obligatory for production of pulmonary surfactant, since infants of mothers who were treated throughout pregnancy with bromocriptine did not develop RDS despite very low cord blood prolactin.

4. Surfactant Substitution By Exogenously Administered Surfactant

The potential utilization of surfactant replacement therapy was first demonstrated by Enhorning and Robertson, who found that a natural surfactant isolated by alveolar lavage and concentrated by centrifugation improved lung expansion of prematurely delivered rabbits (107).

(a). Animal Studies

Following is a list of facts observed by subsequent animal studies (lambs and rabbits) of surfactant replacement therapy: improved aeration of alveoli, increased lucency of lung fields assessed by radiology; increased survival; better arterial blood gases over a 6-hour period;
pressure volume curves measured on lungs showed improved retention of air; and protection against necrosis and desquamation of peripheral airway lining.

It was concluded from these initial studies, which employed crude natural surfactant preparations, that when a natural surfactant product was administered before breathing, lung function and gas exchange improved in prematurely delivered lambs and rabbits; however, the duration of effect of surfactant was dependent upon the timing of administration (108).

(b). Human Studies

Over the last several years five human trials of surfactant replacement therapy have been tried. The following generalizations and conclusions can be made from these trials (Table IX):

- A variety of surfactant preparations were utilized, including: lipid extract of beef surfactant + DPPC + PG; dry surfactant, 90% DPPC + 30% PG; lipids extracted from beef surfactant; DPPC + unsaturated PC + PG + cholesterol; and surfactant from human amniotic fluid.

- Very sick, predominantly tiny infants with severe RDS at risk for serious complications from standard ventilatory techniques were treated. The number of patients was very small and in only one study were control infants included.

- Infants were treated at times from 4 to 33 hours after birth by endotracheal tube instillation with the various surfactants,
## TABLE IX

HUMAN TRIALS OF SURFACTANT REPLACEMENT THERAPY

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of patients</th>
<th>Age at treatment</th>
<th>Surfactant, composition</th>
<th>Dosage (mg/kg)</th>
<th>Clinical outcome</th>
<th>Complications (number involved)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujihara et al. (1980)</td>
<td>10</td>
<td>4-33 h</td>
<td>Lipid extract of beef surfactant + DPPC + PG; natural synthetic combination with small amounts of protein.</td>
<td>125</td>
<td>Very effective.</td>
<td>FDA (9/10)</td>
</tr>
<tr>
<td>promoting Pao2 values within 1-2 h, therefore rapid reduction for O2 requirement (81% before treatment to 38% within 3 h of treatment).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smythe, et al. (1981)</td>
<td>3</td>
<td>15 h</td>
<td>Lipids extracted from beef surfactant; prepared by repeated extraction of the lipids from lung lavage from cattle (protein free).</td>
<td></td>
<td>Effective</td>
<td>FDA (1/3) IVR (1/3)</td>
</tr>
<tr>
<td>Study</td>
<td>Number of patients</td>
<td>Age at treatment</td>
<td>Surfactant composition</td>
<td>Dosage (mg/kg)</td>
<td>Clinical outcome</td>
<td>Complications (number involved)</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td>------------------------</td>
<td>----------------</td>
<td>------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Morley et al. (1981)</td>
<td>22</td>
<td>At birth</td>
<td>Dry surfactant: 90% DPPC, 10% PC</td>
<td>18</td>
<td>Possibly effective</td>
<td>Fever, infants needed ventilation and 11 days required lower pressures in the first 6 hours of life. None of the treated babies died compared with 8 of the control.</td>
</tr>
<tr>
<td><em>Lancet</em> 1, 66.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hallman et al. (1982)</td>
<td>3</td>
<td>6.7-10 h</td>
<td>Surfactant from human amniotic fluid isolated</td>
<td>70</td>
<td>Effective</td>
<td>2/3 required less oxygen and less mean airway pressure.</td>
</tr>
<tr>
<td>Friedman and Doody (1987)</td>
<td>3</td>
<td>4-14 h</td>
<td>DPPC = unsaturated PC + PG = cholesterol</td>
<td>25</td>
<td>Possibly effective 2/3</td>
<td>Modified course of BPD. Rapid clearing of chest x-ray was seen in 2/3 patients before clinical improvement.</td>
</tr>
</tbody>
</table>

*Surfactant dosage given into the endotracheal tube either suspended in an aqueous medium or dry powder.*

**Abbreviations:** PC = phosphatidylcholine, DPPC = dipalmitoyl phosphatidylcholine, PG = phosphatidylglycerol, PDA = patent ductus arteriosus, IVH = intraventricular hemorrhage.
either suspended in an aqueous medium or dry powder.

- The repeated complication in these trials was PDA.

(c). Current Available Knowledge

Although the first surfactant substitution occurred more than fifteen years ago, there are only a few successful trials and many important questions concerning the dosage, administration timing, suitable preparation, metabolic and physiologic side effects, complications related to preparation, and effectiveness, which remain to be answered.

A summary of the current available knowledge concerning surfactant replacement will now be summarized.

(i). Preparation

The most desirable preparation would be nonantigenic synthetic surfactant that performs effectively in substitution. Of the synthetic preparations tested, the mixtures of PC and PG were most effective. The acidic phospholipids (PC and PI) and surfactant protein induce surface adsorption of PC. Instillation of a suspension in a water-based vehicle, is an effective means of delivering surfactant to alveoli. Dry surfactant blown into the lungs of premature infants during resuscitation at birth seemed to have some beneficial effect, but the results were inconclusive. The method by which a surfactant is administered may be important to its distribution and function within the lung. Both the animal and human studies demonstrate that the more closely the artificial surfactant compound approaches the composition of
a natural surfactant, the more effective it is. The ideal surfactant should be consistently equivalent to natural surfactant in function, easily sterilized, stable and probably protein free.

(ii). Side Effects

The introduction of a deficient essential body constituent into the airways of an immature newborn causes a dramatic improvement in gas exchange, but may also have undesirable consequences. Expectedly, effective surfactant increase the left-to-right circulatory shunt through the patent ductus arteriosus (PDA). However, in three of the five human trials (Table X), PDA became clinically evident and complicated subsequent management of these infants. This PDA complication can be explained by the fact that, with rapid improvement in lung function (due to surfactant replacement) there is a concurrent fall in pulmonary vascular resistance, therefore the left-to-right shunt would increase in the infant with a PDA. This raises the question of how to manage the PDA among these tiny newborns who are recipients of exogenous surfactant. Other possible side effects are related to the preparation itself, such as, immunologic abnormalities and autoantibodies against lung tissue, which are associated with serious chronic lung disease.

(iii). Clinical Application of Surfactant Replacement in Managing RDS

Administration of surfactant replacement at birth or as early as possible following the diagnosis of RDS seems indicated, because its effect in protecting from severe respiratory failure and secondary lung
damage should be at their highest. For in comparing the animal (lamb) and human studies it was found that: the lambs given natural surfactant prior to the onset of breathing were protected from respiratory failure for about eight hours, whereas substitution at thirty minutes of age resulted in more modest effects approximately lasting only three to four hours. The disappearance of the beneficial effects seemed to be due to appearance of (plasma derived) inhibitors rather than to clearance of surfactant from the airways (109). However, in comparing the human trials a less evident transient effect was found (110), for in the infants the substitution first took place at an average of thirteen hours and the infants may have had a mild surfactant deficiency compared with the premature lambs that succumbed without substitution in less than one hour even on mechanical ventilation and oxygen. This raises the question at what time of application is the surfactant replacement most effective. Another point is that the animal and human studies only reported a clinical response to surfactant replacement, and the question of how the replacement surfactant lipids interact with the endogenous surfactants and how the replacement dose might affect endogenous surfactant lipid metabolism must be answered. One animal study so far has shown that natural surfactant given to premature animals does not block the synthesis or secretion of endogenous lipids (111). Also a study of radiolabeled natural surfactant given at birth to premature lambs suggests that clearance of surfactant lipids used for treatments may depend on the severity of RDS and/or the gestational age, as well as the
type and physical composition of the administered lipids (112).

(d). Summary

In summary, surfactant replacement therapy represents a potentially useful treatment for RDS infants because it directly attacks the primary cause of RDS, being surfactant deficiency. The clinical studies are encouraging and the early metabolic studies suggest that surfactant therapy will not be detrimental to the newborn infant, if the potential complications are anticipated. However, only controlled trials with long-term follow-up will show whether surfactant substitution decreases serious complications and facilitates the overall management of RDS, without causing serious side effects. Therefore, the search for a safe, effective and easily available surfactant must continue and all the unanswered questions, concerning preparation, dosage, administration timing, effectiveness metabolism and side effects, must be answered. Also recent research has found that mature human surfactant recovered through pulmonary lavage may be of value in preparing partially artificial surfactant although further research is necessary to define the optimum dose for treatment.

K. INFANTS SURVIVING RDS

There have been several studies conducted to evaluate the development of infants surviving RDS. However, these studies have encountered critical research design problems in investigating RDS such as the selection of appropriate controls, and meaningful measures (form of developmental and intellectual assessment). The overall conclusions of
these variable studies are:

- With few exceptions, these children have developed normally, however, perceptual problems are becoming apparent as the children reach school age.
- Some RDS children exhibit signs of hearing, language and behavioral deficits and some symptoms of minimal brain dysfunction.
- The occurrence of these deficits was dependent on several variables such as gestational age, birth weight, forms of RDS treatment, and other complications.
- The average developmental level of infants of less than 2000 g birth weight is less than that of infants above 2000 g birth weight but both are within a normal range.
- Respirator infants are not distinguishable from the population from which they were drawn, suggesting that severity of the initial disease does not seriously affect eventual intelligence in survivors.
- Genetic and socioeconomic status of parents seems to play a significant role in the child's eventual developmental achievement as judged by sibling's development and parental educational and achievement background.
- There were no serious residual pulmonary symptoms at 6 years of age attributable to chronic RDS or its treatment. There were some minimal pulmonary symptoms seen in childhood, however, all improved
after 3 years of age (i.e., abnormal pulmonary function tests without recognizable exercise limitations).

- It was noted that some children had abnormally slow somatic growth, possibly related to iatrogenic malnutrition.

All investigators agree that since the evidence points to delayed motor and language production skills, they suggest the importance of early intervention programs directed specifically at the sensorimotor and communication skills of infants surviving RDS. Overall, the number and severity of problems are thought to be minimal for the RDS population and encourage physicians to continue follow-up studies as the population of survivors and modes of therapy change. Finally, several investigators suggest that RDS may contribute to the sequelae of prematurity itself.
CHAPTER V

METHODS OF EVALUATING FETAL LUNG MATURITY

The composition of the surfactant phospholipids in amniotic fluid reflects the fetal lung maturation process. Surfactant deficiency is one of the most important factors underlying the development of RDS in the immature lung of the newborn. Phosphatidylcholine (lecithin) is the predominant phospholipid component of the surfactant complex and is mainly responsible for the surface active properties. Unsaturated phosphatidylcholine species; phosphatidylglycerol, cholesterol, relatively small amounts of other phospholipids (i.e., phosphatidylserine, phosphatidylinositol, and phosphatidylethanol), as well as protein make up the remainder of the surfactant complex. The lungs, particularly towards the end of gestation, significantly contribute to the phospholipid content of amniotic fluid. Evidence of this is secretion of surface-active phospholipids from the lung into amniotic fluid, which occurs when the lung has reached a stage of maturity characterized by enhanced synthesis and secretion of pulmonary surfactants. A good correlation exists between evidence of surfactant in amniotic fluid and normal postnatal respiratory function. Therefore, numerous chemical and physical methods have been developed for predicting fetal lung maturity by analysis of amniotic fluid.

Following is an extensive literature search of the biochemical and biophysical methods utilized to assess the amount and/or functionality of surfactants in amniotic fluids. The biochemical methods reviewed include: the lecithin/sphingomyelin ratio (L/S) ratio; lung profile;
optical density; cortisol; palmitic acid; lamellar body analysis; enzymatic analysis; and fast atom bombardment. The biophysical methods reviewed include: shake (foam, bubble) test; surface tension; and fluorescence polarization. The following items concerning each method will be discussed if applicable: principle; procedure; results; interferences; sources of error; sensitivity; specificity; reliability; and predictive values.

A. BIOCHEMICAL PROCEDURES

1. Lechitin/Sphingomyelin Ratio (L/S Ratio)

(a). Principle

In 1968, Graven was the first to report of correlation between amniotic fluid phospholipid concentrations and development of RDS (113). After this, the paper that had the most impact, was published by Gluck et al. in 1971, and it reported a correlation between amniotic fluid lecithin concentration, increasing gestational age, and pulmonary maturation (114). Gluck and associates reported that during gestation, total lipids in amniotic fluid increased in concentration, highlighted by a sudden increase at 35 weeks. The most abundant lipids prior to term were the non-acidic phospholipids, lecithin and sphingomyelin. The concentration of lecithin and sphingomyelin in amniotic fluid was nearly equal prior to the 35th gestational week, when the lecithin concentration sharply rises to four times that of sphingomyelin (Fig. 43). In subsequent weeks the lecithin concentration continues to rise, while the sphingomyelin concentration declines (Fig. 43). Therefore, Gluck and associates took advantage of the fact that amniotic fluid sphingomyelin
FIGURE 43

GESTATIONAL CONCENTRATIONS OF LECITHIN AND SPHINGOMYELIN

Legend

Figure illustrates mean concentrations in amniotic fluid of sphingomyelin and lecithin during gestation. The acute rise in lecithin at 35 weeks marks pulmonary maturity.

remains relatively constant throughout gestation, and reported that RDS could indeed be diagnosed by measurement of the ratio of lecithin to sphingomyelin (L/S) in amniotic fluid.

In the ensuing five years, more than 100 reports appeared in the literature, confirming the clinical predictability of amniotic fluid phospholipids, as well as their ontogeny. The key changes in amniotic fluid that accurately predict fetal lung maturity, and that form the basis of several tests are the increase in the concentration of lecithin, and that the sphingomyelin concentration remains nearly constant as gestation advances and may be used as an internal standard and volume correction factor to permit calculation of the L/S ratio as an indicator of increased lecithin. Therefore, the L/S ratio can be used as an index for the degree of fetal lung maturity.

The Gluck method was quickly adopted, and is still widely used for assessing fetal lung maturity. Gluck's general procedure is described below.

(b). Procedure (Glucks' Original L/S Ratio)

Essentially, Glucks' procedure for determining the L/S ratio involves isolating the surface active phospholipids, applying them to a thin-layer chromatographic plate (TLC), developing the plate in an appropriate solvent, and quantitating the spots.

First, the Gluck et al. original procedure will be reviewed, as it proved to be the pioneer study that others tried to emulate and change (114). Unfortunately the first paper published by Gluck et al. in 1971, did not carry full technical detail, however, references were made to
their earlier publications (115, 116, 117).

The original procedure involved the following steps:

- Fresh amniotic fluid was centrifuged to remove cells and sediment (no details) (272 pregnancies were studied with 302 amniocentesis).

- The supernatant was extracted with an equal volume of absolute methanol and 2 volumes of chloroform which removed the lipids. The chloroform layer was evaporated to dryness producing a total lipid extract, and the non surface-active phospholipids taken up from the precipitate in ice-cold acetone. Precipitation in cold acetone concentrates the surface-active lecithin.

- The acetone-insoluble surface-active phospholipids were then dissolved in chloroform prior to spotting on heat activated TLC plates of silica gel H. The plates were developed in chloroform/methanol/water (95:34:4 by volume).

- Phospholipids were identified by spraying the plates with 50% sulfuric acid and charring for 15 minutes at 280°C. The amount of phospholipid in each individual spot was then determined by reflection densitometry.

In the next three years, Gluck published two subsequent papers (Gluck and Kulovich 1973, and Gluck et al. 1974) which provided procedure modifications and more details, which included:

- Gluck and Kulovich 1973 paper (118)

- The fresh amniotic fluid was centrifuged at 5400 rev/min for 5-10 min (no g value given).
- 30μg chloroform aliquots of the acetone-precipitated phospholipids were placed on thin layers of silica gel \( \text{H} \), now prepared from a slurry of 5% ammonium sulphate.

- The plates were developed using a new solvent (chloroform/methanol/water, 65:25:4 by volume), and again charred at 280°C for 15 minutes.

Gluck et al. 1974 paper detailed the extraction techniques (119).

- Whereas in the first description extraction was described as adding an equal volume of methanol and stirring before adding 2 volumes of chloroform, in this later paper a volume of amniotic fluid was vigorously mixed with a 2:1 chloroform/methanol mixture on a vortex mixer.

- After acetone precipitation of the surface-active phospholipids the precipitate was taken up in 30 μL of chloroform (not 30 μg as first described) and the need for accurate measurement at this stage was emphasized.

- TLC was described as previously reported, except that a 30% slurry with 5% ammonium sulphate on Pyrex glass was specified.

Gluck's overall procedure was relatively simple and lipid extraction and examination by TLC following amniocentesis required about 2 hours and about 1 to 5 mL of amniotic fluid.

To date many modifications to Gluck's original method have been reported, each of which may have an effect on the L/S ratios obtained. Care in performing every step in the procedure is essential to achieve reproducible and clinically meaningful results.
(c). L/S Ratio Procedural Steps and Their Effects

Through the years each of these steps of the L/S ratio procedure has been subjected to innumerable variations to improve or simplify the L/S test procedure. These variations are very controversial and will now be discussed in detail for their analytical or clinical significance.

(i). Centrifugation Effect

Centrifugation is utilized to clear amniotic fluid of cells and debris for surfactant analysis. In 1973, it was suggested by Mathur and Kiyasu, that conditions utilized for the preliminary centrifugation of amniotic fluid might be critical in the determination of the L/S ratio (120). Since then, many and often conflicting reports of the effect of centrifugation on the L/S ratio have appeared. However, these reports all suffered difficulties such as methodological problems of interference, inadequate and incomplete sampling of lung development (i.e., immature transition and mature samples), and individual case variations' response to centrifugation.

Most of the reports included investigating the effect of increasing g-forces on the L/S ratio. In 1974 Wagstaff et al. found that there were considerable losses of acetone-precipitated phospholipid with centrifugation (121). Even at relatively low value of 750 × g, a loss of 71% of lecithin from the supernatant occurred, while with increasing values of g, there was a disproportionate loss of lecithin compared with sphingomyelin resulting in a fall in the L/S ratio from 14.25 in the untreated sample to 9.83 at 15,000 × g (121).

In 1977, Wilkinson et al. demonstrated that the L/S ratio is
significantly affected by the g-force, in that higher g-forces will lower
the L/S ratio of mature fluid (122). This phenomenon is illustrated in
Fig. 44, where the L/S ratio values between 1.8 and 4.0 at the lower g-
force (X) are generally encountered between 34 and 37 weeks gestation and
are most significantly affected by exposure to the higher g-force.
In this analysis, 7 fluids (35-39 weeks gestation) of the 27 interpreted
as mature at the lower g-force were immature at the higher g-force (the 7
immature did not suffer with RDS). This study demonstrated that the L/S
ratio is most susceptible to g-force factors, at the same time it is most
susceptible to gestational timing, in assessing for RDS.

Wilkinson et al. also found a problem associated with very low g-
force for L/S ratio determination, in that TLC did not adequately
separate the lecithin and sphingomyelin spots for good densitometric
readings, as shown in Fig. 45A (122). To study this further, ten amniotic
fluids were divided into eight portions each and spun at increasing g-
force and the L/S ratios determined. Figure 45A demonstrates that in the
range from 350 to 2800 x g there are the most significant variations in
the L/S ratio. This is interesting for most major publications on L/S
ratio determinations disagree in this variation range (350 to 2800 x g)
(122). Wilkinson et al. concluded that (122):

- The L/S ratio of mature fluid falls with increasing g-forces,
  therefore, the L/S ratio is significantly affected by the g-force
  the fluid is exposed to prior to its determination.
- Analysis of Fig. 45B suggests a g-force of approximately 3000 x g
  for 10 minutes to work on the plateau of the curve for maximum
FIGURE 44
L/S RATIO VARIATION RELATED TO G-FORCE AND WEEKS OF GESTATION

Legend

A. Illustrates the L/S ratio variation related to g-force. Forty amniotic fluids that were split into two equal volumes and then run at 3520 x g for 5 minutes and 27,138 x g for 15 minutes, respectively. Not all spots are shown in the figure due to overlap.

B. Illustrates the L/S ratio variation related to g-force and weeks of gestation. The dots represent samples run at 27,138 x g for 15 minutes and X's represent the other half of the samples run at 3520 x g for 5 minutes. The fluids are the same as shown in Figure A above. This figure demonstrates that the L/S ratio is significantly affected by the g-force, in that higher g-forces lower the L/S ratio of a mature fluid. L/S ratio values between 1.8 and 4.0 at the lower g-force (x) are generally encountered between 34 and 37 weeks gestation and are most significantly affected by exposure to the higher g-force.

FIGURE 44

A. L/S ratio

3520 x g
5 min

27,138 x g
15 min

B. Weeks' gestation

L/S ratio

22 24 26 28 30 32 34 36 38 40 42 Over
FIGURE 45
LECITHIN AND SPHINGOMYELIN MIGRATION ON THE SILICA GEL H PLATE
AND THE L/S RATIO RELATED TO g-FORCE

Legend

A. Figure illustrates the lecithin and sphingomyelin migration on the silica gel H plate containing 5% ammonium sulfate. The solvent was a mixture of chloroform, methanol, and water in the ratio 65:25:4 by volume. To the far right, in the last 2 lanes, the control lecithin spot is above the sphingomyelin spot. Point of application is at the bottom of the figure. From left to right, the fluids were spun at 350 x g, 700 x g, 1500 x g, 2800 x g, 5000 x g, 11,000 x g, 22,000 x g, and 44,000 x g, all for 10 minutes. It can be seen from the migration pattern that inadequate separation of the L/S peaks for precise densitometer readings occur at g-force of 1400 x g and below for 10 minutes.

B. This figure illustrates the L/S ratio related to g-force. In fluids with an initial L/S ratio above 2, the L/S ratio decreases with increasing g-force. The steepness of the slope from 350 to 2800 x g is evident. Analysis of this figure suggests a g-force of approximately 3000 x g for 10 minutes to work on the plateau of the curve for maximum precision. A g-force exceeding 13,000 at 4°C will give excessively false negative results.

precision. A g-force exceeding 13,000 at 4°C will give excessively false negative results.

- In most samples a progressive decrease in the L/S ratio was observed until a plateau was reached between 2800 and 5900 g for 10 minutes, and that centrifugation at higher g-forces (up to 44,000 g for 10 min) caused a secondary decrease in the L/S ratios of most samples (Fig. 43B).

In conclusion, Wilkinson et al. commented that amniotic fluid should be centrifuged at 3000 x g for 10 minutes at 4°C until further information was obtained (122). Also, they stressed the effect that centrifugation had on the L/S ratio's clinical interpretation, for lowering of the centrifugation speed to harvest more lecithin in the supernatant would result in an invalid L/S ratio of 2 or above. Lowering of the g-force suggests that not only must the level for a mature L/S ratio be increased, but also that the transitional value range of the L/S ratio may become excessively broad, producing an excessive number of false positives.

In 1979, Oulton updated the understanding of the effect that centrifugation has on the L/S ratio (123). Oulton found that analysis of amniotic fluid fractions obtained by differential centrifugation revealed the presence of intact globular-like structures, known as lamellar bodies, which are approximately 1 to 2 nm dimensions (123). These particles, which are representative of fetal lung surfactant, began precipitating at very low g-forces and the extent of their removal by centrifugation was found to significantly alter L/S ratio measurements.
This effect is particularly significant when moderate levels of these structures are present, which is usually between 34 and 37 weeks gestation. False negative L/S ratios could be attributed to this effect.

Oulton utilized amniotic fluid samples from 14 weeks to postterm gestation and the specimens were centrifuged at various g-forces (140, 750, 1,500, 10,000 and 33,000) (123). The pellets were resuspended in 2 to 3 mL 0.85% NaCl for analysis; the supernatant fluids were examined and light microscopy performed. Lipids were extracted from all fractions with 1 volume of methanol and 2 volumes of chloroform and separated by one-dimensional TLC on silica gel G plates. The individual lecithin and sphingomyelin spots were measured by reflectance densitometry (included acetone precipitation step). The following factors, summarized by Oulton, are representative of the current understanding of the effect centrifugation has on the L/S ratio (123):

- The presence of surfactant, as lamellar bodies in amniotic fluid, causes the increase in the L/S ratio which signals lung maturity. The L/S ratio is dependent on the extent of removal of the lamellated-structures during centrifugation.

- It is very difficult to achieve consistent removal of these structures during centrifugation.

- When amniotic fluid contains very little surfactant (as before 33 weeks gestation), variations in the distribution of this material between pellet and supernatant will still result in a prediction of lung immaturity.

- When amniotic fluid contains large amounts of surfactant (after
38 weeks gestation), the L/S ratio of both pellet and supernatant will indicate lung maturity over a wide range of centrifugal conditions.

- In borderline cases (between 34 and 37 weeks gestation), variations in the degree of sedimentability of surfactant during preliminary centrifugation could lead to clinically significant variations in the L/S ratio.

- Investigation has revealed that false-immature L/S ratios could be attributed to excessive loss of these globular structures by the preparative centrifugation steps. It should be emphasized that even with standardized conditions of centrifugation it is very difficult to achieve consistent removal of these structures with g-forces as low as 750 x g, and that this factor significantly affects the L/S ratio, and no doubt affects any of the other surfactant measurements which employ centrifugation as a preparative step.

In conclusion, Oulton stated that from her study it was evident that the globular-like structures are representative of the pulmonary surfactant in amniotic fluid and care must be taken to avoid loss of these structures when preparing amniotic fluid for surfactant analysis (123).

From her study, Oulton suggests that surfactant measurement is better to be made on the isolated globular fraction, because of the extrapulmonary contribution to the supernatant phospholipid pool, which is in some cases much greater than the pulmonary contribution to the
10,000 x g pellet (123). By employing centrifugation this fraction can be harvested in 30 minutes and the phospholipid content can be measured within an hour (only small volumes of amniotic fluid are required, 3 to 5 mL and, if very turbid, smaller volumes can be used). In the future the globular-containing fraction can be analyzed by a specific simplified procedure for assessing the index of fetal lung development.

Finally, in 1982 Brown et al. investigated the effect centrifugation has on the L/S ratio by studying increasing g-forces (124). They utilized 17 different amniotic fluid samples and determined the L/S ratio before and after centrifuging for 5 minutes at g-forces ranging from 140 to 1000 x g. In 13 out of 17 samples, increasing the centrifugal force up to 250 x g (recommended in routine procedure) increased the L/S ratio. However, beyond 250 x g there was a consistent decrease in the L/S ratio (others also reported these findings), which reflects a preferential loss of lecithin-rich surfactant (124). Brown et al. also found that by centrifuging at 250 x g whole cells were virtually removed from the whole amniotic fluid and corresponding supernate demonstrated by phase-contrast microscopy (124). This is the prime purpose of centrifugation i.e., to remove cells and debris (125).

A review of the literature reveals that relatively harsh centrifugal conditions are utilized. The centrifugal conditions must be standardized so that the specific phospholipids present may be analyzed. One consistent finding in the studies already conducted was that higher g-forces will decrease the L/S ratio by loss of the lecithin-rich surfactant.
(ii). Lipid Extraction Effect

The isolation of surfactant phospholipids begins with lipid extraction. Gluck et al., original extraction step, involved mixing the amniotic fluid with an equal volume of methanol, followed by two volumes of chloroform, resulting in a biphasic mixture (114). After vortexing, the biphasic mixture is centrifuged to separate the lower chloroform layer, which contains both phospholipid and neutral lipids, from the upper methanol-water layer. Insoluble proteins form a layer at the interface. The aqueous layer and residual protein are usually discarded at this stage (125).

Several investigators have shown that this extraction step (just described) does not give complete recovery of phospholipids (125, 126). Therefore, some laboratories have suggested employing repeated extraction (125, 126).

However, this extraction step virtually has been universally accepted for the L/S ratio. For even though such a single extraction of amniotic fluid does not quantitatively extract lecithin and sphingomyelin, it is universally assumed that both are extractable to the same extent and the ratio will be unaffected.

(iii). Acetone-Precipitation Effect

After the lipids have been extracted, the organic solvent is evaporated from the extract. The most controversial step in the L/S ratio procedure is this cold-acetone precipitation treatment of the amniotic fluid lipid extract prior to TLC. This step involves the dropwise addition of ice-cold acetone to the residue, and after a
specified time interval, the tube is centrifuged in a refrigerated centrifuge, and the acetone is drained from the white precipitate that remains.

Earlier studies which led Gluck to the use of acetone-precipitation technique showed that surface-active lecithin could be separated from the total lecithin fraction by acetone-precipitation (127). Therefore Gluck originally included the acetone-precipitation step in his L/S ratio procedure "to concentrate the surface-active lecithin" (114).

Gluck and his associates in their original and subsequent procedures placed a great emphasis on the intermediate acetone-precipitation step of the total lipid extract. They believed that the resultant precipitate contained fully-saturated surfactant lecithins, whereas other lecithins remained in the supernatant. They found that when the L/S ratio was more than 2.0 the precipitable lecithin was not less than 45% of the total lecithin. Also they reported that the cold-acetone precipitated lecithin displayed a higher surface activity than did the soluble lecithin.

The value of cold-acetone precipitation has been questioned by many investigators. Many laboratories omit the acetone-precipitation step and still use the L/S ratio critical mature value of 2.0. Application of basic lipid chemical techniques sheds some light on the problem (127). Use of cold-acetone is a time-honored technique for crude separation of polar from nonpolar lipids. The fatty acids on the phospholipid molecule affect the polarity of the molecule to a limited degree, insufficient to be sensitive to the cold-acetone precipitation step. It is likely, however, that unless a very rigid regimen is observed, the acetone warms
up during centrifugation or in other steps to a degree sufficient to
dissolve some phospholipids including lecithin. The more unsaturated the
phospholipids the more soluble they are. Thus, any lecithin lost in the
supernatant is likely to be more unsaturated. Because of similar
polarities, some sphingomyelin is also lost and there need not be any
difference in the L/S ratio.

A detailed discussion of the advantages and disadvantages of the
acetone precipitation step follows.

1. Utilization of the Acetone-Precipitation Step

Originally Gluck et al. separated the surface-active fraction from
total lecithin semiquantitatively with cold-acetone precipitation (114).
Because the method was semiquantitative, there have been some questions
about the usefulness of cold-acetone precipitation. In some proposed
variations of the procedure for the L/S ratio, total lecithin fractions
have been used and acetone-precipitation was eliminated. Therefore in
1974, in two separate studies Gluck et al. (128) and Wagstaff et al.
(121), evaluated L/S ratios with and without the acetone-precipitation
step. The Gluck et al. results of 123 amniotic fluid samples is shown
in Table X and concludes that the L/S ratio with the total lecithin
extract is always significantly greater than that with acetone
precipitation (128).

Wagstaff et al. results of fourteen amniotic fluid samples are shown
in Table XI and concludes that in the L/S ratio measured by both spot
area measurement (p < 0.02) and densitometry (p < 0.001) there was a
significant decrease occurring after acetone-precipitation (121). This
### TABLE I

**Comparison of L/S Ratio Values Where Acetone Precipitation was Done or Omitted on Aliquots of the Same Fluid**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Acetone ppt.</th>
<th>Total extract</th>
<th>Specimen No.</th>
<th>Acetone ppt.</th>
<th>Total extract</th>
<th>Specimen No.</th>
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<th>Acetone ppt.</th>
<th>Total extract</th>
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<td>0.37</td>
<td>1.35</td>
<td>58</td>
<td>1.90</td>
<td>5.60</td>
<td>114</td>
<td>3.30</td>
<td>7.30</td>
<td>115</td>
<td>5.30</td>
<td>10.3</td>
</tr>
<tr>
<td>28</td>
<td>0.60</td>
<td>1.60</td>
<td>59</td>
<td>1.90</td>
<td>5.60</td>
<td>116</td>
<td>3.30</td>
<td>7.30</td>
<td>117</td>
<td>5.30</td>
<td>10.3</td>
</tr>
<tr>
<td>29</td>
<td>0.60</td>
<td>1.60</td>
<td>60</td>
<td>1.90</td>
<td>5.60</td>
<td>118</td>
<td>3.30</td>
<td>7.30</td>
<td>119</td>
<td>5.30</td>
<td>10.3</td>
</tr>
<tr>
<td>30</td>
<td>0.60</td>
<td>1.60</td>
<td>61</td>
<td>2.0</td>
<td>5.60</td>
<td>120</td>
<td>3.30</td>
<td>7.30</td>
<td>121</td>
<td>5.30</td>
<td>10.3</td>
</tr>
<tr>
<td>31</td>
<td>0.60</td>
<td>1.60</td>
<td>62</td>
<td>2.0</td>
<td>5.60</td>
<td>122</td>
<td>3.30</td>
<td>7.30</td>
<td>123</td>
<td>5.30</td>
<td>10.3</td>
</tr>
</tbody>
</table>

L/S ratios of 121 amniotic fluid specimens measured by reflectance densitometry with acetone precipitation values where acetone precipitation was done or omitted on aliquots of the same fluid.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gestation (weeks-days)</th>
<th>Densitometer ratio</th>
<th>Spot Area ratio</th>
<th>Lecithin peak area (mm²)</th>
<th>Sphingomyelin peak area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10-0</td>
<td>1.0</td>
<td>0.9</td>
<td>492</td>
<td>397</td>
</tr>
<tr>
<td>2</td>
<td>16+2</td>
<td>1.6</td>
<td>1.3</td>
<td>695</td>
<td>507</td>
</tr>
<tr>
<td>3</td>
<td>17+0</td>
<td>1.2</td>
<td>1.1</td>
<td>462</td>
<td>429</td>
</tr>
<tr>
<td>4</td>
<td>21+0</td>
<td>4.3</td>
<td>3.1</td>
<td>1119</td>
<td>646</td>
</tr>
<tr>
<td>5</td>
<td>30+2</td>
<td>5.7</td>
<td>4.3</td>
<td>666</td>
<td>556</td>
</tr>
<tr>
<td>6</td>
<td>34+0</td>
<td>4.8</td>
<td>4.1</td>
<td>547</td>
<td>471</td>
</tr>
<tr>
<td>7</td>
<td>34+0</td>
<td>2.7</td>
<td>2.4</td>
<td>611</td>
<td>667</td>
</tr>
<tr>
<td>8</td>
<td>34+5</td>
<td>1.3</td>
<td>1.1</td>
<td>423</td>
<td>318</td>
</tr>
<tr>
<td>9</td>
<td>35+5</td>
<td>3.0</td>
<td>2.4</td>
<td>674</td>
<td>478</td>
</tr>
<tr>
<td>10</td>
<td>35+2</td>
<td>7.6</td>
<td>6.2</td>
<td>384</td>
<td>317</td>
</tr>
<tr>
<td>11</td>
<td>38+3</td>
<td>5.0</td>
<td>4.2</td>
<td>398</td>
<td>543</td>
</tr>
<tr>
<td>12</td>
<td>39+0</td>
<td>6.0</td>
<td>5.8</td>
<td>856</td>
<td>810</td>
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<tr>
<td>13</td>
<td>40+1</td>
<td>4.6</td>
<td>3.9</td>
<td>543</td>
<td>514</td>
</tr>
<tr>
<td>14</td>
<td>40+1</td>
<td>5.3</td>
<td>5.2</td>
<td>615</td>
<td>595</td>
</tr>
</tbody>
</table>

Paired 't' tests: p < 0.001, p < 0.02, p < 0.001, p > 0.7

decrease in ratio is attributed to the preferential loss of lecithin (p <0.001) without a corresponding reduction in sphingomyelin (p <0.7).

Both Gluck et al. (128), and Wagstaff et al. (121) developed the following conclusions concerning their similar studies and results:

- The L/S ratio with the total lecithin extract is always significantly greater than that with acetone precipitation. Total lecithin in amniotic fluid comes from other sources as well as from the fetal lung. In early pregnancy the fraction contributed from extrapulmonary sources may be relatively greater than the fraction from pulmonary sources. Therefore, the L/S ratio in early gestation will be spuriously high if the total unprecipitated extract is used (128).

- The treatment of the total lipid extract with ice-cold acetone alters the L/S ratio principally by a loss of lecithin in the acetone wash. The amount of lecithin lost at acetone precipitation tended to decrease with advancing gestation (Table XI) confirming the suggestion (114) that the proportion of surface active phospholipid increases with progressive pulmonary maturity (121).

- An L/S ratio of 2.0 which represents the end-point of fetal lung maturity, was established with acetone-precipitation. If acetone precipitation is omitted, then some other value must be established to represent fetal lung maturity. From Table X it appears to be very difficult to select one definite value that would denote pulmonary maturity safely because of extreme variability (128).
Since little is known about the unpredictable variations in extrapulmonary lecithin in amniotic fluid and what determines these variations, the use of the total lecithin fraction would seem to be nonspecific and perhaps uninterpretable, for maturity of the fetal lung is measured by the surfactant (acetone-precipitated) lecithin produced by the fetal lung (128).

Also, as recent as 1983, Gebhardt (129) voiced two arguments against omitting the acetone-precipitation step originally used by Gluck et al. (114). The first argument has already been stated, that the threshold value of 2.0 for the L/S ratio would have to be replaced by a new value. Then it is unknown whether the test would retain its clinical usefulness for determining fetal lung maturity. Gluck et al. specifically stated that: "those variations that omit the acetone precipitation may introduce large errors and are not acceptable" (130).

Secondly, Gebhardt et al. have published the complete fatty-acid analysis of the acetone-soluble and acetone-insoluble lecithin of amniotic fluid collected at term (Table XII) (131). They found that since the fatty acid composition of acetone-insoluble lecithin contains mostly saturated fatty acids (82%), including 76% palmitic acid, therefore this appears to be almost identical to that of the lecithin of the lamellar bodies of lung surfactant, while the acetone-soluble lecithin was rich in unsaturated fatty acids. Therefore, Gebhardt concluded that as long as the L/S ratio is measured in the clinical chemistry laboratory as an aid to the obstetrician, the acetone-
<table>
<thead>
<tr>
<th></th>
<th>Acetone-soluble lecithin from term amniotic fluid</th>
<th>Lecithin from egg yolk (Sigma) batch 83C-8140-1</th>
<th>Acetone-insoluble lecithin from term amniotic fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(14:0)</td>
<td>1.8</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>C(16:0)</td>
<td>45.8</td>
<td>30.0</td>
<td>76.2</td>
</tr>
<tr>
<td>C(16:1)</td>
<td>14.3</td>
<td>7.7</td>
<td>4.2</td>
</tr>
<tr>
<td>C(18:0)</td>
<td>5.0</td>
<td>12.7</td>
<td>2.8</td>
</tr>
<tr>
<td>C(18:1)</td>
<td>22.2</td>
<td>31.8</td>
<td>5.3</td>
</tr>
<tr>
<td>C(18:2)</td>
<td>4.8</td>
<td>16.0</td>
<td>1.5</td>
</tr>
<tr>
<td>C(18:3)</td>
<td></td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>C(20:4)</td>
<td>0.3</td>
<td>2.8</td>
<td>1.1</td>
</tr>
<tr>
<td>C(22:6)</td>
<td>1.2</td>
<td>2.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

precipitation step must remain mandatory (129). Gebhardt feels that a better determination method might be easier to standardize and require less time; however, this is still in the future (125).

In conclusion, those L/S ratio procedures which omit the acetone precipitation may introduce large errors and are not acceptable variations, for acetone precipitates the surface-active highly disaturated lecithin fraction and this is what the L/S ratio procedure is based upon. Omitting acetone precipitation results in higher L/S ratios than are seen with acetone precipitation because the phospholipids, with the exception of unsaturated lecithin, are not soluble in cold acetone, although other neutral lipids (i.e., diglycerides, triglycerides) are soluble. Therefore serious and unpredictable errors may be introduced, especially when evaluating pregnancies prior to 32 weeks gestation, when most of the lecithin in surfactant may be unsaturated.

2. Omit-Acetone-Precipitation Step

In 1973, Coch et al. developed a modified procedure for the evaluation of the L/S ratio in amniotic fluid (132). Concerning the acetone precipitation step, they did not find it necessary to include it in their extraction procedure for separating surface-active lecithin from nonsurface-active lecithin. Coch et al. extracted mixtures of dipalmitoyl L/S standards as well as amniotic fluids, both with and without a cold-acetone precipitation step, and found no significant difference in the results obtained; however, they did note a decreased sensitivity in spot size on the TLC sheets (were only half as large or less (Table XIII) (132).
### TABLE XIII

**Comparison of the Extraction Procedure with and without Inclusion of Cold-Acetone Precipitation**

<table>
<thead>
<tr>
<th>Specimen &amp; extracted</th>
<th>Without acetone precipitation</th>
<th>With acetone precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1 Dipalmitoyl L/S standard</td>
<td>L/S 1 (iodine) 1 (bismuth)</td>
<td>L/S 1 (iodine) (neither detectable by bismuth)</td>
</tr>
<tr>
<td>4:1 Dipalmitoyl L/S standard</td>
<td>L/S 4 (iodine) 4 (bismuth)</td>
<td>L/S 4 (iodine) 3 (bismuth)</td>
</tr>
<tr>
<td>L.S. (26 weeks gestation)</td>
<td>L/S 1 (iodine) (neither detectable by bismuth)</td>
<td>neither L nor S detectable by iodine or bismuth</td>
</tr>
<tr>
<td>P.M. (36-37 weeks gestation)</td>
<td>L/S 6-7 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
<td>L/S 5 (iodine) 5 (bismuth)</td>
</tr>
<tr>
<td>P.H. (35-36 weeks gestation)</td>
<td>L/S 2 (iodine) 2-3 (bismuth)</td>
<td>L/S 1 (iodine) (neither detectable by bismuth)</td>
</tr>
<tr>
<td>L.P. (approximately 36 weeks gestation)</td>
<td>L/S 6-7 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
<td>L/S 5-6 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
</tr>
<tr>
<td>M.R. (38 weeks gestation)</td>
<td>L/S 8-9 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
<td>L/S 8-10 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
</tr>
<tr>
<td>M.M. (36 weeks gestation)</td>
<td>L/S 10 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
<td>L/S &gt; 5&lt;sup&gt;b&lt;/sup&gt; by both iodine and bismuth</td>
</tr>
<tr>
<td>J.C. (unknown EDC)</td>
<td>L/S 5-6 (iodine) &gt; 5 (bismuth)</td>
<td>L/S 7-8 (iodine) &gt; 5&lt;sup&gt;b&lt;/sup&gt; (bismuth)</td>
</tr>
</tbody>
</table>
TABLE XIII Continued

<table>
<thead>
<tr>
<th>Specimen extracted</th>
<th>Without acetone precipitation</th>
<th>With acetone precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.V. (&quot;post-mature&quot;)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>L/S 10 (iodine)&lt;sup&gt;a&lt;/sup&gt; &gt; 5 (bismuth)</td>
<td>L/S &gt; 5&lt;sup&gt;b&lt;/sup&gt; by both iodine and bismuth</td>
</tr>
<tr>
<td>L. V. C. (unknown EDC)</td>
<td>L/S &gt; 5&lt;sup&gt;b&lt;/sup&gt; by both iodine and bismuth</td>
<td>L/S &gt; 5&lt;sup&gt;b&lt;/sup&gt; by both iodine and bismuth</td>
</tr>
<tr>
<td>S. M. (unknown EDC)</td>
<td>L/S 5-6&lt;sup&gt;e&lt;/sup&gt; (iodine) &gt; 5&lt;sup&gt;e&lt;/sup&gt; (bismuth)</td>
<td>L/S 5 (iodine) &gt; 5&lt;sup&gt;e&lt;/sup&gt; (bismuth)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Initials of each patient as indicated.
<sup>b</sup> No S detectable and L spot equal to or greater than L spot from mix 2.
<sup>c</sup> Pertinent diagnosis made by doctor requesting L/S assay.
<sup>d</sup> Loss in sensitivity noted — smaller TLC spots.
<sup>e</sup> (S at 20 mg/L)

Also there was a loss in sensitivity noted, for the smaller TLC spots were from specimens undergoing the acetone-precipitation step. Coch et al. concluded that any advantages gained by including the acetone-precipitation step were minor as compared to the greater than 50% decrease in sensitivity of the TLC analysis and the additional assay time obtained when the step was excluded (132).

Also in 1972, Sarkozi et al. (133) utilized the same extraction and almost the same TLC and visualization techniques as Gluck, reported that precipitation with cold-acetone was unnecessary and did not alter the interpretation. However, they did state that it was critical that great care be exercised to ensure that the relative recoveries of dipalmitoyl lecithin and sphingomyelin are the same, if the step is utilized.

Also currently available information refutes the theory that acetone precipitation of amniotic fluid lipid extracts separates disaturated lecithin from unsaturated lecithin. Studies have shown that when acetone precipitation is applied to lecithin, disaturated lecithin is not quantitatively precipitated, for the percentage of dipalmitoyl lecithin precipitated averaged 78% and varied considerably in samples, between 60 to 90% (134). Additionally, evidence shows that the acetone precipitate contains both saturated and unsaturated lecithins, for utilization of methods specific for disaturated lecithin measurement found that the acetone precipitate of amniotic fluid extracts contained only 50 to 60% disaturated lecithin, and a considerable amount of unsaturated lecithin which reacts with cupric acetate staining reagent (this reagent does not react with dipalmitoyl lecithin) (135).
In conclusion, currently many laboratories utilizing the L/S ratio procedure, omit the acetone-precipitation step, because it does not appear to be consistently selective in precipitating lung-derived disaturated lecithin from amniotic fluid and saves assay time (125).

(iv). Thin-layer Chromatography (TLC)

Thin-layer chromatography is used to analyze the isolated lipids, whether or not cold acetone precipitation is utilized. Prior to TLC, the amniotic fluid lipid extract is evaporated to dryness. Then lipids are solubilized in a small amount of solvent and spotted on a chromatographic plate.

The spotting solvent used to transfer the dried extract to the TLC plate is not critical, however, the following factors should be considered in choosing one. Chloroform is a commonly used spotting solvent, however it tends to take up lecithin more readily than sphingomyelin, thus giving an overestimate of the L/S ratio (136). However, this effect is minimized by vortexing after the addition of solvent. The solvent system most frequently utilized includes chloroform/methanol/water (136) and substitution of 25% (by volume) ammonium hydroxide for water eliminates the problem of cochromatographing PI and PS in the L/S region (137).

Because of the recent rise in quantitating of other phospholipids such as PC, PI, PE and PS, the implementation of solvent systems to separate all the important phospholipids has resulted, such as: chloroform/methanol/ammonium hydroxide (30:1:1.5 by volume) (580 g/L) (137).

After the lipids are solubilized in the solvent, the mixture is
spotted on a chromatographic plate. In Gluck’s original procedure handmade TLC plates were utilized (114); however, most laboratories prefer to purchase commercially prepared plates. These commercial plates are available with and without ammonium sulfate and generally contain binders to fix the silica gel to the backing which may be glass, glass fiber, plastic, or aluminum. Because these binders may or may not affect some aspect of the chromatographic behavior of phospholipids, plates from different sources should not be interchanged and the suitability of a given plate’s behavior with individual standards and mixtures of phospholipids should be checked for proper lipid component separation. Also it has been noted by Gluck et al. (114), that incorporation of ammonium sulfate in the gel as a charring agent alters the mobility of PS and PI and therefore, also would be expected to alter L/S ratios as compared with plates without ammonium sulfate. The plates usually consist of silica gel G or H coated on glass plates. It has been found that better separation is achieved if the sample is applied to the TLC plate as a streak rather than a spot, and that the practice of dividing the TLC plates into narrow channels or cutting them into narrow strips for individual sample runs may give rise to edge effects resulting in distortion of spot shape (138).

(v). Direct Quantitation of Lecithin and Sphingomyelin

Direct quantitation of lecithin and sphingomyelin involves the following two steps: staining or charring of the phospholipids spots on the TLC plate followed by quantitation of the ratio of lecithin to sphingomyelin (in most laboratories, the absolute amount of each
phospholipid is not determined) (125). These two steps of quantitation will now be discussed in detail.

1. Step One: Staining

Various reagents have been utilized for staining the phospholipids in the L/S ratio procedure, as shown in Table XIV (125). The table lists the staining reagent, the conditions for color development (i.e., temperature and time), the linearity response and highest standard response of both lecithin and sphingomyelin, specific usage, and the quantitating procedure utilized (i.e., densitometric, planimetry, or visual).

Table XIV lists the staining and charring methods utilized in the L/S ratio procedure and follows are the general factors taken into consideration when choosing a specific stain/staining procedure (125):

- For any given staining procedure, the rate of color development may not be the same for each phospholipid, for some stains react with only a specific functional group of the phospholipid molecule, while some reagents react with equivalent weights of lecithin and sphingomyelin to the same extent, others stain sphingomyelin more intensely than an equivalent weight of lecithin.

- Some staining reagents do not penetrate well, and therefore tend to stain the outer edges of the spots more intensely.

- The amount of staining reagent utilized is very important, for too much stain may produce a high background color, and too little may
<table>
<thead>
<tr>
<th>Staining/charring reagent</th>
<th>Conditions for color development: time/temperature (°C)</th>
<th>Response linearity</th>
<th>Highest standard</th>
<th>Specific usage/comments</th>
<th>Quantitating procedure utilized/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% SULFURIC ACID</td>
<td>Charring for 15 min at 280°</td>
<td>---</td>
<td>---</td>
<td>Gluck original procedure.</td>
<td>Densitometry/1</td>
</tr>
<tr>
<td>BROMOTHYMOL BLUE - NH₃ VAPOR</td>
<td>Room temperature</td>
<td>No</td>
<td>4 ug S/L</td>
<td>As amount of L on the plate increases, the ratio of density of L to density of S would tend to be lower than the ratio of the actual concentration of L to S. Also phospholipid spots only appear in NH₃ vapor and rapidly fade when plate is removed from vapor, therefore difficult to measuredensities of spots except with rapid scanning densitometer.</td>
<td>Densitometry/2</td>
</tr>
<tr>
<td></td>
<td>3 min at 110° and hot air</td>
<td>No</td>
<td>75 ug S/L</td>
<td>Reacts with equivalent weights of L and S to the same extent. The phospholipid spots are indistinct unless exposed to ammonia and when re-exposed to air for purposes of measurement the spots soon fade. The utilization of an ammoniac chamber and a Polaroid camera overcomes the exposure problem, but requires additional equipment and material. By staining for a longer period of time a more permanent spot was produced to facilitate spot area measurement without the use of ammonia.</td>
<td>Densitometry/3</td>
</tr>
</tbody>
</table>

TABLE IV
STAINING REAGENTS UTILIZED IN THE L/S RATIO PROCEDURE
<table>
<thead>
<tr>
<th>Staining/Charring Reagent</th>
<th>Conditions for Color Development: Time/Temperature (°C)</th>
<th>Response Linearity</th>
<th>Highest Standard</th>
<th>Specific Usage/Comments</th>
<th>Quantitating Procedure Utilized/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromothymol Blue NH₃ Vapor (continued)</td>
<td>Air drying NH₃ vapor for 10-15 min. Phospholipids appear as dark-blue spots on a light-blue background. Maximum color within 1-2 min.</td>
<td>---</td>
<td>---</td>
<td>Least practical because color is lost too quickly after plate removed from NH₃ vapor and blue color too transparent for reliable densitometric scanning. If use AL-backed plate retain color longer, however. Underestimates L/S ratio at higher maturity. Demonstrates independence of the degree of phospholipid saturation (Saturation-insensitive).</td>
<td>Densitometry/4</td>
</tr>
<tr>
<td>Molybdenum Blue in Conc. H₂SO₄</td>
<td>Room temperature</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Blue spots</td>
<td>---</td>
<td>---</td>
<td>Reacts with specific functional group of phospholipid molecule.</td>
<td>Densitometry/6</td>
</tr>
<tr>
<td>Brilliant Sulfate and Potassium Iodide (Dragendorff stain)</td>
<td>Air dried several min and destained until L and S stds. appear as bright yellow-orange spots.</td>
<td>&lt;2 mg/dL L stains slightly more than S; not necessary for detection. Limits are a little lower.</td>
<td>Specially reacts with compounds containing a choline residue (i.e., L, S, and lysolecithin) (darker shade). No other phospholipids are therefore made visible. Most specimens from mature fetuses do not show S by this stain.</td>
<td>Visual assessment because spots fade/718</td>
<td></td>
</tr>
<tr>
<td>Staining/charring reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response</td>
<td>Specific usage/comments</td>
<td>Quantitating procedure utilized/reference</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------------------------</td>
<td>----------</td>
<td>------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>BISMUTH SUBNITRATE (continued)</td>
<td></td>
<td>Linear</td>
<td>Highest standard</td>
<td>Many specimens from immature fetuses show only the faintest traces of both L and S. Gradual background color develops on sheet. Distinguishes dipalmitoyl lecithin from the serum lecithin and erythrocytes, and will therefore indicate if there is significant contamination of the sample with blood.</td>
<td></td>
</tr>
<tr>
<td>IODINE (I₂ VAPOR)</td>
<td>Room temperature, sheets placed in a desiccator containing iodine crystals. All phospholipids stain yellow to brown spots.</td>
<td>Yes</td>
<td>100 μg, S.L.</td>
<td>Less specific because all phospholipids are stained, but has increased sensitivity as a stain because can detect 0.2 μg/dL of S or 0.5 μg dipalmitoyl L per dL of amniotic fluid (than bismuth spray). Greatest use in confirming an interpretation of immaturity or transitional maturity. Does not distinguish the various forms of lecithin. Nondestructive stain. After iodine-stained spots fade, the same sheet can be utilized for visualization by other techniques.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 μg/dL S.L.</td>
<td>Densitometry/2</td>
<td>Visual assessment because spots fade.</td>
</tr>
<tr>
<td>Staining/charing reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response</td>
<td>Specific usage/comments</td>
<td>Quantitating procedure utilized/reference</td>
<td>Highest standard</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------------------------</td>
<td>----------</td>
<td>------------------------</td>
<td>------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>PHOSPHOMOLYBDATE-</td>
<td>10 min at 180°C</td>
<td>--</td>
<td>--</td>
<td>Densitometry/4</td>
<td>--</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td></td>
<td>Results demonstrate independence of the degree of phospholipid saturation (Saturation-insensitive). Band color develops more readily and over-darkening of the background is more easily avoided. The technique is still subject to heating-dependent variability similar to that experienced with cupric acetate. The stability of color of molybdate stained chromatographic plates after development allows convenient review and comparison of past results.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHOSPHOMOLYBDATE-</td>
<td>30 min at 105°C</td>
<td>Yes</td>
<td>20 mg S.L</td>
<td>Densitometry/9</td>
<td>5 μg S.L</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td></td>
<td>No</td>
<td>Technique was very convenient, however, spots did not appear in 3-5 min, but developed after 30 min at 105°C (further time in the oven caused spots to fade).</td>
<td>Densitometry/2</td>
<td></td>
</tr>
<tr>
<td>Staining/chanting reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response Linearity</td>
<td>Specific usage/comments</td>
<td>Quantitating procedure utilized/reference</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------------------------------------</td>
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<td>------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>PHOSPHORUS/CHLORIDE-</td>
<td>30 s dip at room temperature then 30 min wash</td>
<td>Yes 75 mg S,L</td>
<td>Detects choline moiety of both L and S. Chromatograms are stable unless exposed to direct sunlight. Spot area measurements and densitometer readings remain constant for seven days after staining.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stannous chloride</td>
<td>(wash determines contrast between spots and background), then stannous chloride dip.</td>
<td>No 75 mg S.L</td>
<td></td>
<td>Densitometry/3 Planiometry/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spray room temperature hot plate until blue spots appear. Phospholipids appear as dark-blue spots against a lighter blue background.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHOSPHORUS/CHLORIDE-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentachlorophenol-azoxylylide</td>
<td></td>
<td></td>
<td>Specific detection of phospholipid on TLC. Better than usual blue molybdenum blue reagent due to its greater sensitivity and its ability to eliminate the problem of the development of background blue color on prolonged exposure.</td>
<td>/11</td>
<td></td>
</tr>
<tr>
<td>PHOSPHORUS/CHLORIDE-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium salt</td>
<td>Air dried/room temperature</td>
<td></td>
<td>Use of a long-wavelength ultraviolet lamp allowed visual inspection. Very low background fluorescence in contrast to 2,7 dichlorofluorescin (which is used by others).</td>
<td>Fluorescence densitometry/4</td>
<td></td>
</tr>
<tr>
<td>of ANS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staining/charring reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response Linearity</td>
<td>Specific usage/comment</td>
<td>Quantitating procedure utilized/reference</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------------------------------------</td>
<td>--------------------</td>
<td>-----------------------</td>
<td>-------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AUS - Magnesium salt, of AUS (continued)</td>
<td>Air dried/room temp. Spray --- lipids fluoresce a bright blue under UV light immediately after spraying.</td>
<td>---</td>
<td>Fluroescene less stable than molybdate stain, although protecting plates from light increases the stability considerably. Demonstrates independence of the degree of phospholipid saturation (Saturation-insensitive). The interfering effect of blood would be minimized by usage of this saturation-insensitive technique, especially when a slightly bloody specimen cannot simply be discarded and replaced.</td>
<td>Visual assessment/0</td>
<td></td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>Room temperature Yes ---</td>
<td>0.2 μg/dl S, L</td>
<td>This spray makes visible equal quantities of L and S. The fluorescence begins to fade perceptibly within 12 hours.</td>
<td>Planimetry/0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromatograph sprayed --- then view under ultraviolet light at 30 min intervals. Lipids appear as bright-orange spots against a pink background about 60 min after spraying.</td>
<td>20 μg L 10 μg S 0.2 μg/dl S, L</td>
<td>The fluorescence is stable for at least 2 weeks.</td>
<td>Visual assessment/0</td>
<td></td>
</tr>
<tr>
<td>Staining/charing reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response</td>
<td>Specific usage/comments</td>
<td>Quantitating procedure utilized/reference</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------------------------------------------</td>
<td>---------</td>
<td>------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>ANTHOCYANIN SULFATE (continued)</td>
<td>L/S ratio increased with gestational age, then they began to fall to progressively lower percentages of the L/S ratio values as determined by recovered lipid weights, with a notable decline beginning at 35 weeks of gestation. This excellent correlation between increasing L/S ratios and fetal pulmonary maturity reported by Gluck and Kulovich appears to have been a reflection of increasing unsaturated lecithin with maturation.</td>
<td>---</td>
<td>---</td>
<td>Densitometry/14</td>
<td></td>
</tr>
</tbody>
</table>

Results in incomplete lecithin charring because of additional time and heat required to volatize ammonia before residual sulfuric acid can carbonize the organic compounds.

Comparing the density of L spot to the density of the S spot, the values are considerably lower if the ammonium sulfate charring method is used rather than the sulfuric acid technique.
### Table XIV (Continued)

<table>
<thead>
<tr>
<th>Staining/charring reagent</th>
<th>Conditions for color development: clime/temperature (°C)</th>
<th>Linearity</th>
<th>Highest standard</th>
<th>Specific usage/comments</th>
<th>Quantitating procedure utilized/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACID SPRAY OR DIP, THEN HEAT CHIAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>5 min over a hot plate set on high heat (700°C) until fuming ceased.</td>
<td>0.2 mg/dL S</td>
<td>L/S ratios measured after spraying with sulfuric acid are about double L/S ratios obtained after an ammonium sulfate spray.</td>
<td>Densitometry/8</td>
<td></td>
</tr>
<tr>
<td>Dacron/ Dacron sulfuric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>0.5 mg/100 ml/L</td>
<td></td>
<td>This technique gives blacker and somewhat larger spots for S than for equal concentrations of L, and lower ratios for a given L/S mix.</td>
<td>Densitometry/8</td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>15-20 min at 160°C</td>
<td></td>
<td>Responds with less intensity as the degree of saturation increases (Saturation-sensitive).</td>
<td>---/4</td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>15-20 min at 160°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid: dichromate</td>
<td>Spray until edges of the silica gel become overloaded and began to look wet. Plate placed on preheated hotplate and maintained at about 200°C until fuming ceased.</td>
<td></td>
<td></td>
<td>At increased temperatures, both the sulfuric acid spray and the potassium dichromate sulfuric acid spray make spots apparent with intensities that accurately reflect the number of carbon atoms in L and S.</td>
<td>Visual assessment/2</td>
</tr>
<tr>
<td>Staining/charing reagent</td>
<td>Conditions for color development: time/temperature (°C)</td>
<td>Response Linearity</td>
<td>Highest standard</td>
<td>Specific usage/comments</td>
<td>Quantitatively utilized/references</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Potassium dichromate (1/1) in sulfuric acid (70ml/dl)</td>
<td>Same as above mentioned method, except for chemicals utilized.</td>
<td>---</td>
<td>---</td>
<td>Subjectively this potassium dichromate-sulfuric acid spray treatment produced quicker, more even charring, as reflected in the greater precision of the standard points.</td>
<td>Visual assessment/2</td>
</tr>
</tbody>
</table>

**References to Table XIV:**


*Saturation-inosensitive and sensitive.* Spillman et al. (4A above) compared seven techniques commonly utilized for detection of amniotic fluid phospholipids in TLE with respect to their sensitivity to saturation of the fatty acid carbon chain of lecithin. The results revealed two saturation classes: sensitive were 10-12% sensitive to fully saturated lecithin as to lecithin with singly unsaturated acid-endpoints. Classes are sensitive: sulfuric acid; sulfuric acid-dichromate mixture; ammonium sulfate; and cupric acetate. Insensitive: ANS; phosphonolphthalein; and bromothymol blue. These seven techniques gave widely differing values for the L/S ratio for actual specimens of amniotic fluid.
result in incomplete staining. A uniformly low background color is the most desirable.

It has been found that dipping of the TLC plate into the staining reagent generally results in a more uniform response than spraying of the plate.

In conclusion, the amount of lecithin and sphingomyelin present on the TLC plate should be directly proportional to the intensity of color obtained from the staining procedure utilized. This would require linearity to approximately 35 μg of phospholipid for acetone precipitated samples and 70 μg of phospholipid for non-acetone precipitated samples (based on a 2 mL amniotic fluid sample, and a lecithin concentration of 35 mg/L as a cut-off value) (139).

2. Step One: Charring

Charring simply involves spraying the TLC plate with sulfuric acid or ammonium sulfate (unless the plate has ammonium sulfate incorporated into the gel), then heating it at 280°C until the phospholipid spots blacken. However, as with the type of gel, the choice of spray affects the densitometric ratio obtained for different concentrations of lecithin and sphingomyelin. Charring techniques involve highly corrosive, noxious chemicals, and high temperatures to make the spots visible. In the case of the phospholipids, the intensities of the spots probably reflect a balance between the ease of hydrolysis and the volatility of hydrolysis products versus the rate of carbonization of organic components.

Plates can be charred after spraying with sulfuric acid or directly
if ammonium sulfate is incorporated in the silica gel. However the latter technique produces lower densities. In 1974, Olson and Graven investigated charring techniques of the following three chemicals and reported their respective results (140):

a. Charring with Sulfuric Acid (50 mL/dL)

This method is the same as the Gluck et al. original method and visualization method originally proposed to gather data for L/S ratio usage of silica gel.

- 8 x 10 cm glass TLC plates (200 mm of silica gel) were sprayed with solution of sulfuric acid/water (1:1 by volume) until edges of silica gel become overloaded and looked wet.
- Plate placed on preheated hotplate and maintained at about 280°C until fuming ceased.
- Figure 46A illustrates the standard curve obtained using 50% sulfuric acid and charring.

Spraying at high temperatures makes spots apparent with intensities that accurately reflect the number of carbon atoms in lecithin or sphingomyelin.

b. Charring with Potassium Dichromate (1 g/dL) in Sulfuric Acid (70 mL/dL)

- Same method as above (charring with sulfuric acid) except solution sprayed on plates was a 1 g/dL solution of potassium dichromate in diluted sulfuric acid (70 mL/dL).
- Figure 46B illustrates the standard curve obtained using potassium dichromate-sulfuric acid and charring.
FIGURE 46

STANDARD CURVE FOR LECITHIN AND SPHINGOMYELIN SPOTS
WITH SULFURIC ACID CHARRING

Legend

A. Figure illustrates standard curves for lecithin and sphingomyelin spots made visible by a 50% sulfuric acid/water (1:1 by volume) spray and charring.

B. Figure illustrates standard curves for lecithin and sphingomyelin developed by spraying the TLC plate with potassium dichromate (1 g/dL) in sulfuric acid (70 mL/dL) and charring. This spray treatment produced quicker, more even charring, as is reflected in the greater precision of standard points.

C. Figure illustrates standard curves for lecithin and sphingomyelin chromatographed on silica gel prepared in a slurry with ammonium sulfate (5 g/dL) and made visible by charring. This method resulted in incomplete lecithin charring and good sphingomyelin charring.

(Brackets show 95% confidence limits, and best-fit lines were determined by reciprocal linear-regression analysis.)

FIGURE 46

A.

B.

C.
Spraying at high temperatures makes spots apparent with intensities that accurately reflect the number of carbon atoms in lecithin and sphingomyelin.

This spray treatment produced quicker, more even charring, as is reflected in the greater precision of standard points (Fig. 46B).

c. Charring with Ammonium Sulfate

In this method ammonium sulfate (5 g/dL) is used instead of water in preparing the silica gel slurry for the plates, resulting in approximately 0.12g of NH₄SO₄ per gram of silica gel on the plate.

Plates are spotted and chromatographed, then placed on a preheated hotplate until fuming ceases.

Gluck and Kulovich switched to this method and their interpretation of a L/S ratio of ≥2 as mature was unchanged (118).

Figure 46C illustrates the standard curve obtained using ammonium sulfate charring. The ammonium sulfate with silica gel resulted in incomplete lecithin charring. Olson and Graven explained this in that, additional time and heat are required to volatilize ammonia before the residual sulfuric acid can carbonize the organic compound and the fatty acids may be partly hydrolyzed and vaporized during this time (140). Therefore, the reason why sphingomyelin is charred well when this method of generating sulfuric acid is utilized can be attributed to the fact that the bonding of fatty acids to sphingomyelin differs chemically to the nature of the fatty acid esterification to the glycerol backbone of lecithin (140). It is known that unsaturated compounds such
as sphingomyelin are charred more readily with sulfuric acid than
are saturated compounds such as dipalmitoyl lecithin (140).

In comparing the three standard curves (Fig. 46A, B, and C), Olson
and Graven concluded that an L/S ratio obtained by comparing the
densities of the lecithin sphingomyelin spots would be considerably lower
if the ammonium sulfate charring method is used rather than the two
sulfuric acid charring techniques (140).

In 1973, Dubbeldam and Gebhardt, agreed with Olson and Graven, by
showing that L/S ratios measured densitometrically after spraying with
sulfuric acid are about double the L/S ratios obtained after an ammonium
sulfate spray (141).

In concluding this initial step of the direct quantitation, in
general it has been found, that stains are more specific than charring
because they react with phosphorus or choline. However, stains
frequently encounter difficulties with background discoloration or color
stability. On the other hand, charring can crack the glass
chromatographic plate, and some commercial plates are made with organic
binders in the gels, which also darken the background. Also charring
results in a lower L/S ratio than does staining because sphingomyelin
contains more carbon than does lecithin.

3. Methods to Quantitate Staining and Charring

The second step of direct quantitation of lecithin and
sphingomyelin is determining the ratio of lecithin to sphingomyelin by
one of the following methods: densitometry; planimetry; gravimetry; or
visual assessment.
a. Densitometry

The chromatogram may be evaluated by reflectance or transmission densitometry, which involves the ratio of the appropriate peak area values giving the densitometric L/S ratio.

Over the past several years reflectance densitometric scanning has become the most popular method of quantitation. Gluck strongly recommended the use of reflectance densitometry, for this technique is much less sensitive to variations in the thickness of the plate or plate support and to irregularities in the surface being scanned (128), and therefore possibly offers an advantage in the case of handmade plates. However, nonuniformity of plates is not a problem when commercially prepared plates are used. Reflectance densitometry is the most accurate method, but the result is again considerably influenced by the staining procedure or method of charring utilized.

Transmission densitometry is performed on an integrating recording densitometer. Appropriate complementary filters are used to achieve maximum sensitivity for different stains. The areas under the peaks are automatically integrated and recorded by the machine and expressed as densitometer units. The lecithin/sphingomyelin ratio (L/S ratio) is calculated by dividing the respective peak area integrator values.

Also, another quantitation of a densitometer tracing is available. This is obtained by measuring the areas under the curve using a direct method, such as cutting out and weighing, rather than utilizing instrument integrations. Some investigators say this method is a more accurate quantitation, because most instruments do not allow for changing
background absorbance or do not allow the baseline to be accurately set to zero.

In conclusion, both the procedure used for scanning (reflectance or transmission), the TLC plate, and the interpretation of the scan, can affect the value obtained in the densitometric quantitation of the L/S ratio. Shellard and Alan recommend that the sample should be applied to the plate as a band rather than a spot and then the narrowest practicable slit width should be used and for maximal linear response, the slot should be no longer than the band width (142).

b. Planimetry

The amount of phospholipid on a chromatograph may also be assessed by planimetry. The product of the length and width of individual spots provides a spot area measurement. The ratio of these values for lecithin and sphingomyelin being referred to as the L-S spot ratio. Planimetry has the advantage of not requiring a scanning densitometer, and results reportedly correlate fairly well with those by densitometry (143), and with gravimetric measurements for L/S ratios <2.0 (128), but are subject to more variability. Planimetry is subject to variability because it is dependent on many factors which influence spot size, such as: \( R_f \) value of the compound (because spots tend to spread as they move up the plate and the \( R_f \) value is affected by tank equilibration and weather conditions), sample application technique, the presence of other components in the extract and diffuseness of the spot edge after separation and staining (144).

Planimetry can give accurate results if conditions are carefully
controlled and standard curves are used. However, planimetric L/S ratios are usually determined without reference to standard curves for each phospholipid, therefore the result would be influenced by the amount of each phospholipid applied to the TLC plate. In conclusion, the planimetric method is not ideal due to the many variables although it is preferable to visual assessment if a densitometer is not available.

c. Gravimetry

The phospholipids can be eluted from the chromatograms and assessed by direct weighing of the eluent residue, which is the gravimetric L/S ratio. The amount of the phospholipid in each spot can be measured by elution of the lipid off the gel, but gravimetry of the eluted lipid is usually inaccurate because of the small amount involved.

d. Visual Assessment

A fourth method used rarely, but must be mentioned, is visual assessment, which is highly subjective. Visual assessment's usefulness is limited to distinguishing between very immature and very mature samples. Visual assessment cannot be considered a satisfactory method for measuring the L/S ratio, because it lacks the sensitivity required for the most critical borderline samples. Also, visual assessment of the spot density is not recommended because the appearance and area of the spots depend on many variables such as the distance traveled, depth of silica gel, the developing system, temperature of charring or sensitivity of the staining reagent.
(d). Variations in the L/S Ratio Procedure

Since the concept of the L/S ratio was first introduced, numerous variations in the assay procedure have appeared. Several investigators have raised certain issues regarding techniques, while others have designed procedures to decrease assay time and allow easier estimation of the ratio in laboratories that do not possess densitometric scanning equipment. Presently, many modified methods, as well as the original method of Gluck and associates (114), are being successfully used to determine fetal lung maturity.

Initially any variations to the original Gluck L/S ratio procedure involved specific variations in the procedural steps such as elimination of the acetone wash step, usage of different TLC plates, substitution of different stains, charring steps, and the method of direct quantitation of lecithin and sphingomyelin. Table XV includes a list of some of the various modified L/S ratio procedures developed since Gluck's original L/S ratio method. The table lists the reference, modifications, findings, advantages, and disadvantages.

(e). The L/S Ratio's Clinical Interpretation and Usefulness

The developing fetal lung secretes surfactant phospholipids into amniotic fluid and the L/S ratio test conveniently relates the phospholipid concentration to the maturity of the fetal lung, for the L/S ratio's rapid widespread acceptance reflects the major clinical need it suffices.

Originally Gluck and Kulovich (114) categorized the L/S ratio clinical interpretations as follows: 2.0 or greater as pulmonary
<table>
<thead>
<tr>
<th>Reference</th>
<th>Amount anesthetic fluid utilized/sonication</th>
<th>Lipid extraction method: chloroform</th>
<th>Acetone/ethanol</th>
<th>TLC</th>
<th>State/condition</th>
<th>Test time</th>
<th>Quantitation</th>
<th>Findings/constituents</th>
<th>Advantages/disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluck &amp; Kulovich, et al. (1978)</td>
<td>1.5 mL</td>
<td>Chloroform spotting on heated activated TLC silica gel (5X)</td>
<td>1:2</td>
<td>5% sulfuric acid spraying 15 min at 200°C</td>
<td>2 h</td>
<td>Reflection densitometry</td>
<td>Original procedure</td>
<td>L/S &gt; 2 pulmonary maturity, L/S ≥ 1.5, transitional</td>
<td>L/S ≥ 1.5, pulmonary immaturity, L/S ≥ 1.0 markedly immature</td>
</tr>
<tr>
<td>Sarkari et al. (1979)</td>
<td>12.000 x g for 10 min</td>
<td>No (by vol)</td>
<td>Acidified ammonium sulfate (modified Zimniak) reagent: 100 mL of aqueous solution of ammonium sulfate (300 g/L) acidified with 10 mL of conc. sulfuric acid</td>
<td>No</td>
<td>2 h</td>
<td>Transmission densitometry</td>
<td>L/S &gt; 2 pulmonary maturity, L/S ≥ 2 greatest risk of developing BES</td>
<td>The relative densities of L and S were determined on the densitometer with a 1.0 x 0.4 cm slit and a of 600 nm. The plates were scanned at 254 nm for 10 sec</td>
<td>This modification of Gluck procedure (1) was designed to increase the speed and efficiency of the analysis. A) Shortened procedure by omitting acetone step. B) Dipping the plates in the modified...</td>
</tr>
</tbody>
</table>
### TABLE IV Continued

<table>
<thead>
<tr>
<th>Reference</th>
<th>Amount amniotic fluid utilized/ contribution</th>
<th>Lipid extraction solvent: chloroform</th>
<th>Lipid fraction</th>
<th>Reaction/ quantitation</th>
<th>Test time</th>
<th>Findings/ comments</th>
<th>Advantages/ disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>(continued)</td>
<td></td>
<td></td>
<td></td>
<td>lower of the charring temperature produces linear calibration curve without serious background interference.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contributed to remove any cells present.</td>
<td></td>
<td></td>
<td>Binucleic substrate ~1 1/2 h reaction (bright yellow-orange spots against white background) specific for L and S and an iodine vapor method to confirm the L/S ratio (yellow to brown spots)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Advantages/ disadvantages:**

- Zinamid reagent eliminated the 5% sulfuric acid spray and obviated the need for an exhaust system.

- **A)** Rapid reliable indicator of the L/S ratio especially in labs that are not equipped with densitometric scanning.

- **B)** Binucleic substrate spray specific for L and S, lysolecithin. Will distinguish the L from AP from that in erythrocytes and plasma.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Amount amniotic fluid utilized/centrifugation</th>
<th>Lipid extraction method: chloroform</th>
<th>Acetone: PPN</th>
<th>TLC</th>
<th>Stain/charing</th>
<th>Test time</th>
<th>Quantitation</th>
<th>Findings/comments</th>
<th>Advantages/disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coch et al. (1973) Clin. Obstet. Gynecol. 19, 987.</td>
<td>1.5 ml/400 g</td>
<td>No</td>
<td>1/2-3/2 by vol.</td>
<td>by this method for a given L/S mixture then by acid spray heat char method</td>
<td>Intense stain less specific because all phospholipids are made visible, but is much more sensitive (1-2x) than the binacillin spray</td>
<td>2 mg/L of S; 5 mg/L of L.</td>
<td>by this method for a given L/S mixture then by acid spray heat char method.</td>
<td>Intense stain less specific because all phospholipids are made visible, but is much more sensitive (1-2x) than the binacillin spray</td>
<td>2 mg/L of S; 5 mg/L of L.</td>
</tr>
<tr>
<td>Blaxx, K. G. et al. (1973) Clin. Obstet. Gynecol. 19, 199.</td>
<td>1.5 ml/400 g</td>
<td>No</td>
<td>1/2-3/2 by vol.</td>
<td>by this method for a given L/S mixture then by acid spray heat char method</td>
<td>Intense stain less specific because all phospholipids are made visible, but is much more sensitive (1-2x) than the binacillin spray</td>
<td>2 mg/L of S; 5 mg/L of L.</td>
<td>by this method for a given L/S mixture then by acid spray heat char method.</td>
<td>Intense stain less specific because all phospholipids are made visible, but is much more sensitive (1-2x) than the binacillin spray</td>
<td>2 mg/L of S; 5 mg/L of L.</td>
</tr>
<tr>
<td>Reference</td>
<td>Amount anodic fluid utilized/ contribution</td>
<td>Lipid extraction solvent: methanol: chloroform</td>
<td>Acetone ppm</td>
<td>TLC</td>
<td>Stain/ charring</td>
<td>Test time</td>
<td>Quantitation</td>
<td>Findings/ comments</td>
<td>Advantages/ disadvantages</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------------</td>
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<td>Blaser, E. G. et al. (1973) Clin. Oncol. 19 1276.</td>
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<td></td>
<td>Both L and S appear as bright pink spots on blue bkg. Laser detection limit of 1 μg for both L &amp; S. Spots remain visible under ultraviolet light for weeks.</td>
<td>values to convert to area. Divide the area of L spots by the area of S spots to obtain L/S ratio.</td>
<td></td>
<td>A) Punched disc system allows acids to be prepared on discs, which are stored frozen to prevent spoilage of standard solution because of solvent evaporation and also saves time.</td>
<td></td>
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<tr>
<td>Harrison et al. (1974) Am. J. Obstet. Gynecol. 120, 1087.</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
<td>Solvent system included: CHCl₃ (90:5:3 by vol.) and distilled water. Samples were applied to absorbent layers TLC plates (silica gel G and water). The plates were heated activated at 105°C for 10-15 min. and then cooled for 5 min at 37°C to develop for 1-2 h developing chamber for 30 min. Then plates were sprayed with 1% sulfuric acid and heated at 300°C for 10 min to locate the tryptophan content of interest.</td>
<td>Determined visually or densitometrically. L/S 2 pulmonary maturity. L/S 1.5-1.9 transitional. L/S 1.5 pulmonary immaturity.</td>
<td></td>
<td>A) Solvent system gives very good separation of L and S. Another advantage is that solvent system can be utilized several times without an observable difference in results.</td>
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</tr>
<tr>
<td>Reference</td>
<td>Amount, anesthetic fluid utilized/centrifugation</td>
<td>Lipid extraction solvent</td>
<td>Acetone</td>
<td>TLC</td>
<td>Stained/cheruing</td>
<td>Test time</td>
<td>Quantitation</td>
<td>Findings/Comments</td>
<td>Advantages/Disadvantages</td>
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<tr>
<td>Morrison et al. (1974)</td>
<td>40°C. This final step removes moisture (moisture affects migration and separation of L and S) that may have accumulated on the silica gel plates during sample application.</td>
<td>chloroform</td>
<td>PBTN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A) Sensitive, reproducible, and economical procedure for fetal mortality determination. A) No modifications in this procedure reduce the number of extraction steps and decreases humidity complications in the chromatographic portion of the assay. A) This modified method has been shown to be simple, rapid, and a reproducible semi-quantitative method for the determination of L and S.</td>
</tr>
<tr>
<td>Reference</td>
<td>Amount amniotic fluid utilized/centrifugation</td>
<td>Lipid extraction solvent: chloroform</td>
<td>Acetone pH 7</td>
<td>TLC</td>
<td>Stain/developing</td>
<td>Test time</td>
<td>Quantitation</td>
<td>Findings/Comments</td>
<td>Advantages/disadvantages</td>
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<tr>
<td>Mann, L. T. Jr. (1978) 1-5 ml/5,429 rev/min for 5-10 min</td>
<td>3 different volumes (0.25, 0.50, and 1.25 ml) of amniotic fluid are extracted and applied. The two smaller volumes are brought to 1.0 ml by adding sodium chloride (0.5%) solution. Measured in liters for extraction.</td>
<td>No</td>
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<tr>
<td>Reference</td>
<td>Amount anesthetic fluid utilized/ centrifugation</td>
<td>Lipid extraction method: chloroform</td>
<td>Acetone</td>
<td>TLC</td>
<td>Stain/ charting</td>
<td>Test time</td>
<td>Quantitation</td>
<td>Findings/ comments</td>
<td>Advantages/ disadvantages</td>
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<tr>
<td>Henn, L. T. Jr. (1978)</td>
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<td>contains: 100 ml. of chloroform, 50 ml. of methanol, 10 ml. of acetone, 1 ml. of glacial acetic acid and 6 ml. of water is prepared immediately before use and may be reused once or twice.</td>
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<td></td>
<td>they show a regular and reproducible progression that permits interpolation of the values of the unknown.</td>
</tr>
<tr>
<td>Cotelli et al. (1978)</td>
<td>3000 rpm</td>
<td>(800 x g) for 10 min</td>
<td>Yes (3 ml supernatant of AF 1:2)</td>
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<td></td>
<td></td>
<td></td>
<td>A) This single dimension thin-layer chromatographic technique also detects PC, The densitometric</td>
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<td>Chin, Oxen. 26, 116,</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>A) Utilized an improved molybdenum-blue spray reagent. The</td>
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<thead>
<tr>
<th>Reference</th>
<th>Amount antibiotic fluid utilized/centrifugation</th>
<th>Lipid extraction solvent: chloroform</th>
<th>Acetone: FFPN</th>
<th>TLC</th>
<th>Staining/developing</th>
<th>Test time</th>
<th>Quantitation</th>
<th>Findings/remarks</th>
<th>Advantages/disadvantages</th>
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<tbody>
<tr>
<td>Geall et al. (1978)</td>
<td></td>
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<td></td>
<td></td>
<td>Edges and allow the plate to remain at room temperature for 15 min to develop maximum color. The stained phospholipids reach maximum density in 10 min and remain stable for at least 90 min after spraying.</td>
<td>1 h</td>
<td></td>
<td></td>
<td>Are ratios of standards were 15-25% greater than the weight ratios. Therefore all unknown L/S ratios were corrected to the average standard L/S area ratio. B) Lengthy procedure.</td>
</tr>
<tr>
<td>Bang et al. (1981)</td>
<td>0.1 mm g for 10 min, then filtered with a 1000 g filter to remove tissue fragments and cells.</td>
<td>From 110% substitute chloroform in the extraction solvent.</td>
<td>Solvent system modified to chloroform/methanol/1.11 mol/L NaOH (26:50:1:6 by vol).</td>
<td>1.11 mol/L NaOH results in best separation of four phospholipids L, S, R, and P.</td>
<td>1 h</td>
<td></td>
<td></td>
<td>This modified method of Glick et al. (1974) Clin. Perinatal, 1, 125. This modified procedure for the L/S ratio is more specific, valid (excellent).</td>
<td></td>
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<tr>
<td>Reference</td>
<td>Amount amniotic fluid utilized/centrifugation</td>
<td>Lipid extraction method: methanol; chloroform</td>
<td>Acetone</td>
<td>TLC</td>
<td>Staining/charging</td>
<td>Test time</td>
<td>Quantitation</td>
<td>Findings/remarks</td>
<td>Advantages/disadvantages</td>
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<tr>
<td>Hurst et al. (1981) Cite. Nov. 21, 1976. (continued)</td>
<td>ration because of the differences in chromatographic systems (see advantages column). Therefore this modified procedure predicts fetal maturity earlier than the original. Also, this modified procedure has been shown to be more specific for the I/S ratio as it has increased validity by significantly reducing the number of false negatives obtained with the original procedure.</td>
<td>resolution of L and S1, and allows for determination of PI and PG. A) Eastern chromatographic plate is less expensive than either glass or high-performance plates. A) No altering of the plates is necessary nor is a densitometer required. A) One-dimensional easier to perform. A) Substitution of Frenon 113% for chloroform in extraction step because Frenon is a non-toxic, non-flammable and has lower boiling point (47°C).</td>
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<td>Reference</td>
<td>Amount amniotic fluid utilized/centrifugation</td>
<td>Lipid extraction method: chloroform</td>
<td>Acetone</td>
<td>Lipid</td>
<td>TLC</td>
<td>Stain/charring</td>
<td>Test time</td>
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<tr>
<td>Brown et al. (1982)</td>
<td>3 ml/250 x g for 5 min</td>
<td>CMM 1:1, 1% by vol.</td>
<td>No</td>
<td>Solution of 39 g capric</td>
<td>1 h</td>
<td>L/S ratio determined by cutting out</td>
<td>L/S &gt; 5 pulmonary</td>
<td>L/S &gt; 5 pulmonary</td>
<td>A) Good separation of L,</td>
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<td>acetate monohydrate per</td>
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<td>the appropriate portions of the</td>
<td>of L/S, L/S &gt; 5</td>
<td>L/S &gt; 5 pulmonary</td>
<td>S, F, and P</td>
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<td></td>
<td>liter of diethyl</td>
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<td>densitometer chart paper</td>
<td>Indeterminate</td>
<td>quantitation</td>
<td>B) Including ethanol into the</td>
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<td>ether acetophosphoric acid</td>
<td></td>
<td>corresponding to L and S</td>
<td>10 µg of</td>
<td>Indeterminate</td>
<td>B) Including ethanol into the</td>
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<td>200 µL</td>
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<td>phospholipid</td>
<td>Quantitation</td>
<td>B) Including ethanol into the</td>
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<td>(200:2:1 by vol.)</td>
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<td>equivalent</td>
<td>findings</td>
<td>B) Including ethanol into the</td>
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<td>Two-dimensional</td>
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<td>weights of L and</td>
<td>A) This procedure improved</td>
<td>B) Including ethanol into the</td>
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<td>TLC utilized.</td>
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<td>S stained less</td>
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<td>B) Including ethanol into the</td>
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<td>intensity than</td>
<td>false predictions</td>
<td>B) Including ethanol into the</td>
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<td>L</td>
<td>A) This procedure improved</td>
<td>B) Including ethanol into the</td>
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<td>linear stain</td>
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<td>B) Including ethanol into the</td>
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<td>at greater</td>
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<td>amount of</td>
<td>A) This procedure improved</td>
<td>B) Including ethanol into the</td>
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<td>A) This procedure improved</td>
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<td></td>
<td>intensity than</td>
<td>false predictions</td>
<td>B) Including ethanol into the</td>
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**Abbreviations:** C = chloroform, M = methanol, W = water, L = lecithin, S = sphingomyelin, vol = volume, AF = amniotic fluid, bg = background, stds = standards, A) = advantages, B) = disadvantages, min = minutes.
maturity; 1.5-1.9 transitional; 1.0-1.49 immature; and 1.0 or less markedly immature.

Although a large number of reports confirm the usefulness of the L/S ratio, there also have been other less enthusiastic reports. When these latter have been evaluated, the technique had been altered without adjustment of the end point to express maturity by the altered technique, and thereby the authors had introduced serious errors in their changing of the procedure, or else a totally different measurement was being made that may have had little or no relationship to the L/S ratio and may not have been a valid measurement of fetal lung maturity.

Over the last several years there have been two controversial problems concerning the clinical value of the L/S ratio. The first problem involves the high incidence of false negative results obtained, i.e., predicted immaturity (L/S < 2.0) when newborn respiratory function was normal. The second problem, concerns a mature L/S ratio (> 2.0) not guaranteeing pulmonary maturity in complicated pregnancy, for there is a higher incidence of RDS in maternal diabetics than in normal pregnancies. These two problems and associated theories will now be discussed.

(i). High Incidence of False Negative Results

In general, the predictive value of a mature result is high, 98 to 100%. However, the predictive value of an immature result ranges from 8 to 96%. This variability can occur apparently independently of the presence of interfering phospholipids in the lecithin-sphingomyelin region of the TLC plate or of the inclusion of acetone precipitation in the method. Any method for assessing fetal lung maturity may be expected
to give some false immature predictions, for there must be a time lag between the synthesis and secretion of surfactant in the lung and its appearance in the amniotic fluid. Therefore the surfactant content of amniotic fluid collected soon after the onset of lung maturation may not truly reflect the state of maturity of the lungs. Also in a few cases the lung may mature during the time between sampling and delivery of the infant. False immature predictions due to such causes probably only arise if the lungs are on the point of maturing at the time of sampling. Therefore in such cases, the observed L/S ratio would probably be in a borderline zone just below the cut-off value for maturity.

The value obtained by a specific L/S ratio procedure is influenced by many factors. The need to standardize the critical steps in the technique may well account for some of the false predictions reported by a specific laboratory. Differences in the clinical performance of the L/S ratio test between laboratories is greatly influenced by the following two steps of the procedure: preliminary centrifugation; and separation of phospholipids by TLC. Excessive centrifugation removes large amounts of lecithin-rich surfactant therefore causing a high incidence of false predictions of immaturity. Moderate centrifugation may cause a L/S ratio to be underestimated if the surfactant is present in a highly sedimentable form. Also a high percentage of false immature predictions could be expected for procedures in which PI and PS tend to co-chromatograph with sphingomyelin. For all samples would not be affected uniformly because the contribution of these phospholipids to total phospholipid is highly variable.
The factors mentioned above influence both the cut-off values to be utilized for clinical interpretation and the order of ranking of L/S ratios in a given sample population. However, other changes in the procedure are more apt to change the cut-off value rather than the ranking of the L/S ratios, such as omitting the acetone precipitation step or varying the method utilized for quantitation after TLC.

Another important point to consider in evaluating the L/S ratio in a study would be the gestational age of the amniotic fluid, for predictive value of an immature result would tend to be reduced if the population contained few preterm infants. However, if the population studied contained a large number of samples from 30 weeks of gestation or less, the incidence of RDS at this stage is relatively high. Similarly, both the specificity of the method and the predictive value of a mature L/S ratio are dependent on the proportion of near-term pregnancies in the population, for beyond 37 weeks' gestation the incidence of RDS is very low.

The most critical assessment of a test's predictive value is between 30 and 37 weeks' gestation, when there is the greatest doubt about the state of maturity of the fetal lungs.

Recent studies expressed satisfaction with the clinical reliability of the L/S ratio in uncomplicated pregnancies and have provided the following guidelines: the L/S ratio utilized end-point must be properly expressed (i.e., with reflectance densitometry 2.0, and 4.0 with gravimetry); when the L/S ratio exceeds 2.0 the newborn is highly unlikely to die from RDS (on the average of about 36 weeks gestation);
when the L/S ratio is between 1.8-2.0 it is labeled transitional; and when the L/S ratio is <1.8 there is a high likelihood of RDS development. This immature value obtained gives a reliable prediction of the severity of RDS; for the lower the L/S ratio, the more severe the RDS (in normal and complicated pregnancies).

In conclusion, this problem of a high incidence of false negativity (predicted immature L/S ratio <2.0, even though newborn respiratory function is normal) could probably be related to inter-individual physiological variability, for it has been found that the patient-to-patient variation of the L/S ratio is large at all gestational ages.

Therefore, because of the various modifications to the Gluck L/S ratio procedure, each laboratory should determine its own mature value of the L/S ratio, based on its own experience. In some laboratories a L/S ratio of 2.0 may not be indicative of pulmonary maturity, and a ratio as high as 4.0 may be necessary to ensure the adequacy of the surfactant system. Thus, although a mature L/S ratio predicts that RDS will not occur with 98% certainty, an immature ratio by no means ensures that RDS will develop.

(ii). L/S Ratio's Value in Complicated Pregnancies

In normal pregnancies the L/S ratio changes and follows a developmental pattern for it is directly related to gestational age. Functional maturity of the surfactant system usually occurs between 33 and 37 weeks gestation in normal pregnancy, the mean being at about 35 weeks. The contribution made to the management of high-risk pregnancies by the L/S ratios cannot be overstated. RDS due to iatrogenic
premature delivery is no longer an everyday concern.

Some complicated pregnancy conditions may cause normal, enhanced or delayed maturation. In abnormal pregnancies the lung can mature (L/S ratio >2.0) independently of the gestational level. Gluck et al. reported that patients with pregnancies complicated by such conditions as pre-eclampsia, diabetes mellitus with severe vascular disease (classes F and R, and some class D) chronic hypertension, prolonged rupture of membranes, and chronic placental separation exhibited accelerated neonatal pulmonary maturity by 2 to 6 weeks (145). These findings were interpreted as suggesting that fetal stress may accelerate fetal pulmonary maturity. A mature L/S ratio prior to 33 weeks gestation constitutes acceleration of maturation of the fetal lungs. In chronic abruptio placentae, almost whenever the amniotic fluid is examined from 26 weeks, the L/S ratio is mature. Acuteness with which a rise in L/S ratio occurs may follow known stress, such as premature rupture of membranes and within one day to a few days a mature L/S ratio may be seen. Also severe chronic toxemia, diabetes mellitus (type D and F), and renal or cardiac hypertension are associated with small infarcted placentas which will stimulate early maturation of the fetal lungs at 30-32 weeks.

Conversely, surfactant maturation may be delayed (beyond 37 weeks) in some diabetics, especially those in classes A-C, and in some fetuses with erythroblastosis fetalis. Furthermore, especially in diabetics, RDS may occur despite L/S ratios indicative of fetal lung maturity, for an L/S ratio > 2.0 and incidence of RDS is higher in maternal diabetics than
normal. An L/S ratio of 2.0 has been reported to have a 2% false positive rate. About half of these false positives occur in infants of diabetic mothers and the other half in severely asphyxiated infants with borderline L/S ratios or in those severely involved with erythroblastosis fetalis.

Curet et al. suggest that the degree of diabetic control may be an important factor (146). For there is evidence that fetal insulin, responding to high concentrations of circulating glucose, may retard the maturation process by competing with cortisol for tissue receptor sites (147). It is worth noting that obtaining a result indicating lung maturity does not necessarily result in the amniotic fluid having a high concentration of surfactant, for many factors contribute to the results obtained. As in diabetic pregnancies, there may be changes in the contribution of nonsurfactant lecithin or other phospholipids which may lead to a false positive result. Bouthon et al. examined the lungs of infants (3 infants of diabetic mothers delivered at 35-38 weeks gestation) who died from RDS and found that the lecithin content of the lungs and the surface activity of their lung homogenates was within the normal range (148). Therefore they suggested that RDS may occur in infants of diabetic mothers who have adequate quantities of surfactant, however, the surfactant is immature because it only contains PI and no PG (148). Also, Oulton has presented data consistent with this immature theory, for she demonstrated that in diabetic pregnancies the appearance of PG lags behind the increase in total phospholipid (149). However, many infants of diabetic mothers who do not develop RDS have sufficient
amounts of surfactant and do not contain PG in their surfactant. Therefore, perhaps the lungs of such infants are primed, ready to turn on PG production at delivery, while those infants who develop RDS are not primed. The possible mechanism for the increased incidence of respiratory distress among infants born to diabetic mothers remains a mystery and if found will answer many of these perplexing questions (Diabetes and complicated pregnancies discussed in detail under lung profile section).

(f). Sources of Contamination

Contamination of an amniotic fluid sample is a problem if the contaminant either interferes with the measurement of the L/S ratio from the chromatograms or if it contains factors which because of their behavior on chromatography or surface active properties artificially elevates the L/S ratio. Various possible natural and chemical contaminants effects on amniotic fluid L/S ratios have been investigated with the following results:

(i). Blood

Both maternal and fetal serum contain significant amounts of lecithin and sphingomyelin, therefore contamination of amniotic fluid with blood usually affects the L/S ratio. The effect is variable depending on the concentration of blood in the amniotic fluid sample and on the L/S ratio of both the original amniotic fluid and contaminating serum. Buhi; and Spellacy (150), found that fetal and maternal serum have L/S ratios from 1.3 to 3.1. Investigators have found that adding increasing amounts of blood to amniotic fluid lowers an originally mature
L/S ratio and raises an originally immature L/S ratio (151). Therefore, if a blood-stained sample gives a value greater than the highest L/S ratio attributable to serum alone and an L/S ratio much greater than the cut-off value for maturity, the result is still valid. However, each laboratory must establish their own highest L/S ratio for serum alone, because it depends on the method used for quantitation. In conclusion, the L/S ratio should not be regarded as valid when a borderline or just mature result is obtained for a blood-stained sample, for a small concentration of blood can change an immature result to a borderline or mature result. The actual percentage of blood contamination to change an immature result is not consistent between investigators and needs further study.

It has been found that a hematocrit of over 1% yields unreliable results and falsely lowered L/S values even when there is no visible hemolysis and when the red cells are removed. Hemolyzed samples should also be interpreted with great caution, particularly in borderline mature cases. This is because the L/S ratio of plasma is about 2.0. Obviously mature ratios of 3.0 or more may still be useful in the presence of small amount of blood or hemolyzed blood contaminants.

(ii). Meconium

The effect of meconium contamination on the L/S ratio has been investigated by several groups with the following variable results:

- Gerbie et al. found that meconium does not affect the L/S ratio (151).
- Buhi and Spellacy found that meconium decreases the L/S ratio (151).
- and Wagstaff et al. found that meconium increases the L/S ratio (121). Wagstaff et al. most significant finding was that when meconium was added in amounts not detectable by the eyes, it did not give chromatographic evidence of its presence, however it raises the L/S ratio (121). Finally, Wagstaff et al. found that gross contamination of the amniotic fluid with meconium usually seen is characterized by a conspicuous lyssolecithin spot and the presence of large amounts of material in advance of the lecithin spot on the chromatograph (121). For meconium interferes with extraction of the lipids and specimens containing meconium should not be utilized.

(iii). Miscellaneous Sources of Contamination

Wagstaff et al. studied the following sources effect on influencing the L/S ratio, with their corresponding results (121).

1. Bilirubin—With increasing increments of bilirubin equivalent to 49 to 580 mg per 100 mL added to amniotic fluid with an L/S ratio of 1.3 no changes were observed.

2. P- Aminobenzoic Acid (PAH)—The measurement of amniotic fluid volume involves the introduction of 20% PAH by injection into the amniotic cavity and after allowing a suitable time for equilibration a sample of fluid is withdrawn for spectrophotometric estimation of PAH in the fluid. The L/S ratio was unaltered by various concentrations of PAH.
3. Lack of Refrigeration-L/S ratios determined from fresh specimens of amniotic fluid were allowed to stand overnight at room temperature and the L/S ratios determined the next morning showed a significant drop.

4. Fetal and Maternal Urine—The urine may be obtained inadvertently at the time amniocentesis; however, no lecithin or sphingomyelin is detected on the chromatograph.

5. Fetal and Maternal Ascitic Fluid—The ascitic fluid may also be obtained inadvertently at the time of amniocentesis. The ascitic fluid analyzed had the following two prominent chromatographic features: there is always a very conspicuous lysolecithin spot following the sphingomyelin component, and the actual quantity of lecithin present was greatly in excess of the amount normally encountered for the period of gestation.

6. Vaginal Samples—Samples collected vaginally may be contaminated with mucus, and bacteria that contain phosphodiesterases and contaminant lipids. Only free flowing or carefully tapped vaginal samples should be used.

7. Serum—Serum contributes more phospholipids and therefore, will result in false elevations.

(g). Summary

The L/S ratio has become the standard test for determining fetal lung maturity through wide usage and long experience. The critical value of the L/S ratio in relation to RDS depends on the procedure and technique utilized in individual laboratories. Each laboratory must establish the
critical values used in interpreting test results. The critical values of the L/S ratio as related to fetal lung maturity are divided into three categories: immature; transitional (borderline); and mature. No exact borders exist between these phases and test results must be interpreted carefully. The results of any test of this type are dependent upon good technique. Also considerable patience and careful handling of materials are essential in obtaining accurate results by this procedure.

2. Lung Profile

(a). Principle

The lung profile consists of determining the presence or absence, and concentration of the following phospholipids: lecithin (L); sphingomyelin (S); (L/S ratio); phosphotidylglycerol (PG); phosphotidylinositol (PI); and disaturated lecithin function. These above phospholipids in amniotic fluid enhance the accuracy of the diagnosis of the fetal lung maturity and greatly extends the information about lung development of the physician managing the pregnancy. The measurement of surfactant phospholipids in amniotic fluid reflects the various stages of lung maturation and correlates with neonatal pulmonary surfactant's stability, and hence can be useful in predicting RDS. The principle phospholipids which lower surface tension in the alveoli are L, PG, PI, and S. The basis for the amniotic fluid lung profile test is the change that occurs in the concentration of these various phospholipids during development.

The lung profile methods represent an extension of the L/S ratio procedure. Since 1971, the simple L/S ratio has been applied as a useful
clinical laboratory method, however, false alarms are fairly frequent in normal pregnancies; immature patterns of the L/S ratio (those < 2/1) are not followed by signs of newborn RDS. On the other hand, some infants (of diabetic mothers) may show respiratory distress when the L/S ratio appears mature (false-negative tests). A study of surfactant phospholipids other than the L/S ratio reduces the false error rate. Lecithin, sphingomyelin, PI, and PG have been found to be most helpful in eliminating errors.

Also another important practical problem is the unreliability of the L/S ratio in amniotic fluid contaminated with blood or meconium, for PG and PI are present in very small quantities in blood products, whereas lecithin and sphingomyelin found in blood interfere with the L/S ratio measurement. Therefore, blood contamination of the amniotic fluid proves to be a particular indication to use the acidic phospholipids for evaluation of fetal lung maturity.

Numerous methods have been applied to study lung phospholipid profiles, which will now be discussed.

(b). Procedure:

(i). Two-Dimensional Thin-Layer Chromatography (TLC) Procedures

Several procedures were experimented with between 1976 through 1979, however they were tedious and required refinement. Then in 1979, Gluck et al. developed a lung profile procedure run by two-dimensional TLC which included the L/S ratio, and the percentages of disaturated acetone-precipitated lecithin, PI, and PG (130). The
procedure involved the following (130):

- Five mL or more of amniotic fluid was centrifuged at 800 x g for 5 minutes to remove cellular debris. The lipids were extracted with chloroform/methanol (2:1, by volume), and then cold-acetone precipitated.

- The studies were run by spotting the precipitated fraction on 20 by 20-cm Pyrex plates coated with silica gel H containing 5% ammonium sulfate for two-dimensional TLC.

- After developing the ammonium sulfate-containing plate in one direction with chloroform/methanol/water/glacial acetic acid (65:25:4:8 by vol) as solvent, the plate is dried, turned 90° and developed in tetrahydrofuran/methylal/methanol/2N ammonium hydroxide (40:28.5:7.8:4.2 by vol).

- Finally, the phospholipid spots were made visible by heating the plate and quantitated by reflectance densitometry.

Figure 47 shows three two-dimensional thin-layer Pyrex plates and separation of the phospholipids into immature (A), mature suitable for delivery if necessary (B), and term mature lung (C). The changes during gestation in the L/S ratio, the percentages of disaturated acetone-precipitated lecithin, and PG were evaluated by an analysis of covariance with which the regression coefficients of the L/S ratio, disaturated acetone-precipitated lecithin, and PG for gestational age were obtained. These changes were linear by computer plotting. PI could not be evaluated similarly, since initially the concentration rises and then falls with advancing gestation. Evaluations were done principally from 33


FIGURE 47
TWO-DIMENSIONAL TLC
SHOWING SEPARATION OF PHOSPHOLIPIDS

Legend

Figure shows two-dimensional thin-layer Pyrex plates (silica gel H with 5% (NH₄)₂SO₄) separation of the phospholipids:

A. Shows an immature plate with an L/S ratio <2.0, <50% disaturated lecithin, low PI, and no PG.

B. Shows a mature lung profile suitable for delivery, if necessary, of pregnancies other than diabetic with a L/S ratio >2.0, >50% disaturated lecithin, high level of PI, and no PG.

C. Shows a term or nearterm mature lung with a L/S ratio >2.0, >50% disaturated lecithin, decreased levels of PI, and a significant percentage of PG; this patient may be delivered whether diabetic or not without fear of RDS.

weeks, since the majority of amniocenteses customarily are done from this time and their numbers allowed valid comparisons for the analysis of regression coefficients (130).

1. Results of Two-dimensional Thin-layer Chromatography Procedure

The results obtained from Gluck et al. two-dimensional TLC procedure on 200 amniotic fluid samples were the following (130):

a. L/S ratios

Figure 48A is a curve derived by computer and regression coefficients for the mean L/S ratios of normal pregnancy patients as a function of the weeks of gestation. The correlation coefficient is 0.986 with a high significance (p <0.0001).

b. Percentage of PG

Figure 48B is a curve derived by computer and regression coefficients for the mean percentages of PG in total phospholipid during gestation. The correlation coefficient of the curve is 0.943 with very high significance (p <0.0001).

c. Percentage of PI

Figure 49A is a curve showing the rise and fall in percentage concentration of PI in normal pregnancy during gestation. As seen in the curve, the PI concentration rises until about the 35th to 36th week of gestation and then declines.

d. Disaturated Lecithin Fraction

Figure 49B is a curve showing the mean changes in percentage of acetone-precipitated disaturated lecithin with gestational.
FIGURE 48
TWO-DIMENSIONAL TLC
PROCEDURE RESULTS FOR NORMAL PREGNANCY L/S RATIO
AND PERCENTAGE OF PHOSPHATIDYLGLYCEROL

Legend

A. Figure illustrates two-dimensional TLC procedure results for L/S ratio in normal pregnancy. This curve was derived by computer and regression coefficients for the mean L/S ratio of normal pregnancy patients as a function of the weeks of gestation. The correlation coefficient is 0.986 with a high significance (p < 0.0001).

B. Figure illustrates regression curve for percentage of PG during gestation in normal pregnancy. The curve was derived by computer and regression coefficients for the mean percentages of PG in total phospholipid during gestation. The correlation coefficient of the curve is 0.943 with very high significance (p < 0.0001).

**FIGURE 48**

**NORMAL PREGNANCY**

LECHTIN/SPIGOHYEGLIN (L: S) RATIO S

![Graph showing Lecithin/Sphingomyelin (L: S) ratios over weeks of gestation.]

\[ y = c + ax \\ r = 0.961 \]

\[ n = 200, p < 0.001 \]

**NORMAL PREGNANCY**

PERCENT PHOSPHATIDYL GLYCEROL

![Graph showing percent phosphatidyl glycerol over weeks of gestation.]

\[ y = d + bx \\ r = 0.945 \]

\[ n = 200, p < 0.001 \]
FIGURE 49
TWO-DIMENSIONAL TLC PROCEDURE RESULTS
FOR PERCENTAGE OF PHOSPHATIDYLINOSITOL
AND DISATURATED LECITHIN

Legend
A. The figure illustrates the curve showing the rise and fall in
percentage concentration of PI in normal pregnancy during gestation. As
shown in the curve the PI concentration rises until about the 35th to
36th week of gestation and then declines.
B. Figure illustrates curve showing the mean changes in percentage of
acetone-precipitated disaturated lecithin with gestational age. The
correlation coefficient of the curve is 0.948 with a high significance
(p <0.0001).

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Figure 49

NORMAL PREGNANCY

PERCENT PHOSPHATIDYL INOSITOL

30 20 10

30 31 32 33 34 35 36 37 38 39 40

N = 200

NORMAL PREGNANCY

PERCENT DISULFATED (ACETONE PRECIPITATED) LEICHTIN

30 40 50 60 70 80

30 31 32 33 34 35 36 37 38 39 40

MEAN ± SEM

\[ y = 53.291 + 3.10x \]

\[ r = 0.040 \]

\[ n = 200, \ p < 0.001 \]
age. The correlation coefficient of the curve is 0.948 with a high significance ($p < 0.0001$).

e. The Lung Profile

Figure 50 shows the form used to report the lung profile data. The four determinations are plotted on the ordinate and the weeks of gestation on the abscissa (as well as the L/S ratio as an "internal standard"). When these are plotted, they fall with a very high frequency into a given grid that then identifies the stage of lung development, as designated in the upper part of the form. The designation of "mature (caution)" refers to the patients other than those with diabetes who can be delivered if necessary at this time; if the patient is diabetic she can be delivered with safety when the values fall into the mature grid (130).

2. Summary of the Lung Profile Compared to the L/S Ratio

Table XVI shows the predictability of the lung profile compared to the low (<2.0) L/S ratio alone in pregnancies including placenta previa, mild pre-eclampsia, mild Rh incompatibility and repeat Cesarean section (all of these are free of those major high risk conditions known to accelerate or delay fetal lung maturation). Line 2g of Table XVI shows that newborns of 57 pregnancies (2a) with low L/S ratios (<2.0) had RDS, 69% were diagnosed correctly by the L/S ratio, whereas the lung profile extends the ability to diagnose unsuspected maturity in infants with low L/S ratios detected by the presence of PG to 93% (130).
FIGURE 50
LUNG PROFILE FORM

Legend

Figure illustrates form utilized to report the lung profile data. The four determinations are plotted on the ordinate and the weeks of gestation on the abscissa (use L/S ratio as an internal standard). When these are plotted, they fall with a very high frequency into a given grid that then identifies the stage of lung development, as designated in the upper part of the form. The designation of "mature (caution)" refers to the patients other than those with diabetes who can (i.e., delivered if necessary at this time; if the patient is diabetic she can be delivered with safety when the values fall in the mature grid.).

FIGURE 50

[Graph showing data with axes labeled for L/S, HEC, and PI. The graph includes curves for different measurements across different stages of development.]
<table>
<thead>
<tr>
<th></th>
<th>L/S ratio</th>
<th>Lung profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. &quot;Mature lung, % predicted mature, no RDS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2. &quot;Nonmature&quot; lung (L/S ratio &lt; 2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. No. of low L/S ratios (&lt; 2.0)</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>b. No. of infants, RDS + low L/S ratio</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>c. No. of infants, no RDS + low L/S ratio</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>d. No. of infants, no RDS diagnosed mature prenatally</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>e. No. of indeterminant diagnoses: low L/S ratio or nonmature lung profile + no RDS</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>f. % &quot;nonmature&quot; lung without RDS (L/S ratio &lt; 2.0)</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>g. % accuracy when &quot;nonmature&quot; (L/S ratio &lt; 2.0)</td>
<td>69</td>
<td>93</td>
</tr>
</tbody>
</table>

The Gluck et al. study concluded the following factors (130):

- The L/S ratio alone is sufficient to monitor most normal pregnancies, however, the four lung parameters measured are key fractions of surfactant that together help to form a clearer picture of fetal lung development.
- With the lung profile most of the noninformative low or intermediate L/S ratio values are eliminated.
- The lung profile provides the ability to detect and evaluate abnormal pregnancy by alterations from the expectant patterns of maturity.
- Amniotic fluid phospholipid profile, particularly the presence or absence of PG, gives an accurate assessment of fetal lung maturation.
- The profile may provide a useful adjunct to the management of high-risk pregnancies, especially after premature membrane rupture, and perhaps also when the mother is diabetic.

The results and conclusions of Gluck et al. proved that the lung profile was very diagnostic and the acidic phospholipids of PG and PI needed further investigation as biochemical markers of fetal lung maturity (130).

Since this time, several methods have been reported for separating the various phospholipids by two-dimensional TLC, which have generally been modifications of the Gluck procedure, with changes in stain, chromatographic absorbent material, and in solvent.

Recently several investigators have been studying the usage of one-dimensional TLC in separating the lung profile phospholipids because they
found the two-dimensional TLC procedure too cumbersome, complex, expensive, and too time consuming. These one-dimensional TLC procedures will now be discussed.

(ii). One-Dimensional Thin-layer Chromatography

In 1978, Gottelli et al. (152) described one-dimensional TLC procedures for simultaneous determination of the L/S ratio and PG in amniotic fluid. However the PI and PS phospholipids were unresolved with this procedure.

Since then other methods have arisen for evaluating the L/S ratio and concurrently separate PG, PI, PS, and PE by one-dimensional TLC which involve: stepwise development, continuous development, simultaneous development, and improved one-dimensional TLC. These four one-dimensional procedures will be reviewed briefly.

1. Stepwise Development Procedure

In 1980, Mitnick et al. described a stepwise development one-dimensional TLC method which required 2.5 hours to complete, allowed simultaneous analysis of up to eight samples per plate, and eliminated the need for a phospholipid phosphorous determination because sphingomyelin was used as an internal standard (PG and PI are expressed as ratios to it) (153). This method (for procedural details see reference 153) proved to be extremely reproducible; the intra-assay variance was 0.004 and the inter-assay variance was 0.009 for both PI/S and PG/S. However, the disadvantages of this method were that it was time consuming and the large (10 x 20 cm) TLC plates utilized could not be accommodated by many densitometers in clinical laboratories.
a. Results

Even though the procedure had two minor technical disadvantages, the study analyzed amniotic fluid specimens from 409 normal pregnant women and 88 mildly diabetic pregnant women (Classes A, B, C) with the following results and conclusions (153): Resolution of all detectable components was complete (Fig 51A). This one-dimensional technique allowed for as many as eight samples at a time for analysis and required only 2.5 hours. In normal pregnancies, the clinical results were similar to those of Gluck et al. (130). For when the L/S ratio was <1.0 the PG and PI ratios were absent or in low concentration. PI did not appear until about the 30th week then PI/S increased gradually to 36 weeks and declined (Fig. 51B). PG/S was low until the 36th week and then increased (Fig. 52A). When the L/S ratio was 2.0, PI/S was 0.8 to 0.9. PG was almost absent when L/S ratio was <2.0, however PG/S increased with increasing L/S ratios. If antepartum amniocentesis resulted in a L/S ratio of >2.0 no respiratory distressed developed. In five normal pregnancies the antepartum L/S ratio was transitional with three newborns resulting in RDS and two with transient tachypnea. Three infants developing RDS had PG/S ratios well below 0.5. Eighty-eight mildly diabetic pregnancies (Class A, B, C) were studied. It was found that the PI and PG ratios varied in relation to the L/S ratio (Fig. 52B) and age of gestation. Appearance of PI and PG was delayed in mild diabetes and in severe diabetics and hypertension it was accelerated.

2. Continuous Development Procedure

In 1980, Kolins et al. described a short-bed continuous
FIGURE 51
STEPWISE DEVELOPMENT
ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY
AND PHOSPHATIDYLINOSITOL RESULTS

Legend
A. Figure illustrates stepwise development thin-layer chromatography of eight samples of amniotic fluid and a reference sample. The figure shows that resolution of all detectable components was complete.
B. Figure illustrates the amniotic fluid PI/S ratio during late gestation in normal pregnancies. For PI does not appear until about the 30th week and then PI/S increases gradually to 36 weeks and then declines.

Abbreviations: LPC lysophosphatidylcholine and the others are for the standard lung phospholipids.

FIGURE 52
STEPWISE DEVELOPMENT ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY PHOSPHATIDYLGLYCEROL RESULTS AND THE PHOSPHATIDYLINOSITOL/PHOSPHATIDYLGLYCEROL RESULTS IN MILD DIabetics

Legend

A. Figure illustrates the amniotic fluid PG/S ratio during late gestation in normal pregnancies. For PG/S is low until the 36th week and then continues to increase.

B. Figure illustrates PI/S and PG/S ratios as a function of the L/S ratio in mild (Classes A, B, C) diabetics. The figure shows that PI/S and PG/S vary in relation to the L/S ratio and age of gestation. Appearance of PI and PG was delayed in mild diabetes and in severe diabetes and hypertension it was accelerated.

development and high-performance (HP) one-dimensional TLC method, which involved the usage of a commercially available 10 x 10 cm plates, however the method still took longer than 2 hours to complete (154). The following phospholipids PC, PI, PS, PE, PG, and sphingomyelin were detected with an antimony molybdate staining reagent and quantitated by transmittance densitometry (see reference 154 for procedural details).

\[ \text{a. Results} \]

Results of this study were:

- All six of the major phospholipids were separated clearly (Fig. 53A).
- Kolins et al. (154) compared their HP-TLC (high-performance thin-layer chromatography) method to the Borer method (one-dimensional TLC) of Gluck et al. (155) for the L/S Ratio: Figure 53B shows the distribution of the L/S ratios in 30 paired specimens run by the Borer's method (left-hand side) and that by the HP-TLC method (right-hand side). As shown in Fig. 53B there was clustering of the ratios by the Borer's method in contrast to the broader distribution of the HP-TLC method, which suggests that the latter method discriminates better (154).
- Also Fig. 53C shows the sensitivity of the Kolins et al. (154) HP-TLC method and that of the Gluck et al. (155) Borer method compared by utilization of commercially prepared L/S mixtures. The greater sensitivity of the HP-TLC method as compared with commonly used planimetric evaluation of L/S ratios is emphasized for the slope of the HP-TLC method is nearly three times that for
FIGURE 53

KOLIN’S ET AL. CONTINUOUS-DEVELOPMENT ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY AND ITS L/S RATIO DISTRIBUTION AND ITS SENSITIVITY COMPARISON TO THE BORER METHOD

Legend

A. Figure illustrates separation of the amniotic fluid phospholipids from amniotic fluid obtained at Cesarean section. As shown in the figure, all six of the major phospholipids were separated clearly.

B. Figure illustrates distribution of L/S ratios in 30 paired specimens run by the Kolin et al. HP-TLC (right-hand ‘side’) method and that of Gluck et al. Borer’s method* (left-hand side). The figure shows that there was clustering of the ratios by the Borer’s method* in contrast to the broader distribution of the HP-TLC method, which suggests that the latter method discriminates better.

C. Figure illustrates sensitivity of Kolin et al. HP-TLC method and that of the Gluck et al. Borer’s method* compared by use of commercially prepared L/S mixtures. The greater sensitivity of the HP-TLC method as compared with commonly utilized planimetric evaluation of L/S ratios is emphasized, for the slope of the HP-TLC method is nearly three times that for the Borer’s method*, indicating the HP-TLC method’s greater sensitivity to changes in lecithin/sphingomyelin concentration.

Abbreviations: X unidentified and the other are for the standard lung phospholipids.


the Boron's method, indicating the HP-TLC methods's greater sensitivity to changes in lecithin/sphingomyelin concentration.

- Kolins et al. densitometric quantification of the phospholipids enables more rapid assessment than planimetric quantification (154).

- In conventional TLC the silica gel utilized has a particle distribution in the range of 5 to 30 μm, therefore this less-dense adsorbent layer results in larger and more diffuse spots. However, in the Kolins et al. HP-TLC system silica gel, a particle size of about 7 μm is utilized, which allows for a density packed thin layer and results in better resolution between similar phospholipids (154).

- The short-bed continuous-development chamber operates on the following principles: it can better resolve substances with similar Rf's because the TLC plate protrudes out of the otherwise sealed chamber, allowing the solvent to proceed up the plate and evaporate outside the chamber. Therefore the continuous movement of the solvent results in resolution equal to that of a much longer chromatographic column or plate, and the increased solvent velocity shortens the time required (154).

3. Simultaneously Determined Procedure

In 1980, Painter described a one-dimensional TLC method by which the ratios and proportions of lecithin, sphingomyelin, PG, PI, PE, and PS in amniotic fluid were simultaneously determined (137). Commercial chromatographic plates (10 x 7.5 cm), pretreated with cupric
chloride (for about 4 s) were used. The plates were sprayed with a cupric acetate (350 g/L)/phosphoric acid (80 mL/L) reagent and charred on a hot plate to make the phospholipids visible. Then the percentage of each phospholipid present was determined by direct reflectance or transmission densitometry. Figure 54A shows the phospholipid separation for a mature amniotic fluid and working phospholipid standards. The correlation coefficients for the L/S, PI/S and PG/S ratios of the two scanning methods were 0.971, 0.980 and 0.955, respectively, for between-day analysis (137).

This one-dimensional TLC method utilizing direct transmission densitometry is more rapid than other methods and makes use of short commercially available plates, which can be used with most clinical densitometers. The directly determined percentage areas of each phospholipid scanned can then be expressed in terms of ratios to sphingomyelin, making the assay insensitive to variations in sample volume and to the number of phospholipids scanned. This method described by Painter should make routine analysis for amniotic fluid phospholipids, including but not limited to lecithin and sphingomyelin, much more practicable to the clinical laboratory (137).

a. Results

The clinical results from this study were (137):

- Figure 54B shows the change in the L/S ratio with gestational age in 25 normal pregnancies. The L/S ratio was 2.0 at 35 weeks of gestation and increased steadily to ratios exceeding 4.0 at term (40 weeks).
FIGURE 54
SIMULTANEOUS DETERMINATION OF ONE-DIMENSIONAL THIN-LAYER
CHROMATOGRAPHY, THE L/S RATIO CHANGE WITH GESTATIONAL AGE,
AND THE CHANGE IN PI/S AND PG/S RATIOS VERSUS THE L/S RATIO

Legend
A. Figure illustrates thin-layer chromatogram for simultaneous
determination of a mature amniotic fluid (channel 2) and working
phospholipid standards (channels 1, 3, 4, and 5).
B. Figure illustrates change in L/S ratio with gestational age in 25
normal pregnancies. The L/S ratio was 2.0 at 35 weeks of gestation, and
increased steadily to ratios exceeding 4.0 at term (40 weeks).
C. Figure illustrates change in PI/S and PG/S ratio versus L/S ratio in
50 normal pregnancies. The L/S ratio ranged from 0.9 to 5.0. The PI/S
ratio increased from 0.70 at an L/S ratio of 2.0 up to peak values at an
L/S ratio of 3.0, followed by a gradual decline. The PG/S ratio remained
at zero until the L/S ratio was 2.0. For a L/S ratio between 3.0 and
3.5, the majority of PG/S ratios exceeded 0.45.

Chem. 26, 1148 and 1150.
- Figure 54C shows the change in PG/S and PI/S ratio versus L/S ratio in 50 normal pregnancies. The L/S ratio ranged from 0.9 to 5.0. The PI/S ratio increased from 0.70 at an L/S ratio of 2.0 up to peak values at an L/S ratio of 3.0, followed by a gradual decline. The PG/S ratio remained at zero until the L/S ratio was 2.0. For a L/S ratio between 3.0 and 3.5, the majority of PG/S ratios exceeded 0.45.

- In studying the results of the general population (75 amniotic fluid samples), if the L/S ratio was >1.5 and PG/S exceeded 0.45, then the newborn did not develop RDS.

- In 11 amniotic fluid samples from diabetics (4-Class A, 3-Class B, 3-Class C, and 1-Class F), none of the newborns delivered by Cesarean section developed RDS if the L/S ratio was >2.0, and the PG/S ratio was >0.45.

4. Improved One-dimensional Thin-Layer Chromatography Procedure

In 1982, Pappas et al. described an improved one-dimensional TLC method with which all the amniotic fluid phospholipids (L, S, PS, PI, PE, and PG) are completely resolved in <70 minutes (from the time specimen is received) on 7.5 x 10 cm commercially available silica gel plates. (156).

In this procedure the phospholipids are made visible by immersing the plate in cupric acetate/phosphoric acid and the phospholipids are quantitated by transmission densitometry (L/S ratio calculated by the relative area as integrated and four other phospholipids reported as
Figures 55 A and B illustrate typical chromatographs of standards, controls, and a patient sample. The data reflected good $R_f$ reproducibility for the phospholipids tested. This method has the definite advantages of simplicity and speed of extraction and subsequent chromatography.

a. Results

The clinical results from this study was that the L/S ratio progressively increases during gestation. Three premature infants developed RDS at birth with a L/S ratio $< 1.5$, and no PG present. PG was detected at 38-40 weeks gestation and if present the neonate did not develop RDS (156). In conclusion, the definite advantages of this method are its simplicity, and speed of extraction, and subsequent chromatography. This method would be extremely useful when prompt clinical decisions regarding acute cases was required (156).

In summary, these four one-dimensional TLC methods are still considered experimental and would require additional and adequate gestational sampling to validate their clinical usefulness. Also the procedure would have to be adaptable to the clinical laboratory on a routine basis.

(iii). Comparison of Two- and One-dimensional Thin-layer Chromatography Procedures for the Phospholipids of Amniotic Fluid.

Besides the methods just mentioned, several other methods have been reported for separating the various phospholipids in amniotic
FIGURE 55
TYPICAL THIN-LAYER CHROMATOGRAPHY PLATE
FROM IMPROVED ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC METHOD

Legend
A. Figure illustrates typical TLC plates with resolution of amniotic fluid phospholipids. 1, L/S control; 2, PS plus PG; 3, PI plus PE; 4, phospholipid composite standard; 5, PI, PS, PE, and PG; 6, S only.
B. Figure illustrates typical TLC plate for clinical samples, 1 and 6, L/S control; 2, PS and PG; 3, PI, PE, and PG; 4 and 5, patient samples.

The data reflected good reproducibility for the phospholipids tested.

fluid, including either two-dimensional or one-dimensional thin-layer chromatographic (TLC) systems. These methods have generally been modifications of the work of Gluck et al., two-dimensional system, with changes in stain, chromatographic absorbent material, and in solvent (114). Also several one-dimensional thin-layer chromatographic techniques have been developed by making modifications to the two-dimensional techniques with considerable savings in time and reagents (as discussed previously). Table XVII lists a comparison by Gross et al. (157) of the two-dimensional (modified Hallman procedure 159) and one-dimensional (modified Tsai and Marshall procedure 136) thin-layer chromatographic procedures, concerning the following technical factors: principle; resolution; quantitation of the spots; precision/reproducibility; and timing.

The results of the comparison by Gross et al. (157) and Tsai (160) reveal the following:

- The two-dimensional TLC procedure gives complete resolution of all six phospholipids, separates compounds from PE and PS on the second migration, gives better precision (CV for PG 15% versus 21% for one-dimensional), and gives clearer results when relatively little PG is present (157). Gross et al. feel that even though the one-dimensional procedure is faster and less expensive, the two-dimensional procedure (Hallman et al.) (159) would better suit the clinical laboratory because it is more unequivocal (157).

- The one-dimensional TLC procedure is simpler, faster, less expensive, and separates the clinically important phospholipids of
### TABLE XVII

**COMPARISON OF TWO-DIMENSIONAL TO ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY PROCEDURE FOR THE PHOSPHOLIPIDS OF AMNIOTIC FLUID**

<table>
<thead>
<tr>
<th>TWO-DIMENSIONAL TLC</th>
<th>ONE-DIMENSIONAL TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRINCIPLE</strong></td>
<td></td>
</tr>
<tr>
<td>- Simple and longer procedure.</td>
<td>- Simpler, faster, and less expensive procedure.</td>
</tr>
<tr>
<td></td>
<td>- Gross et al. feel that unreliable when blood or meconium present (see resolution for explanation)</td>
</tr>
<tr>
<td></td>
<td>- Tsai et al. feel blood and meconium contamination effects occur in both procedures.</td>
</tr>
<tr>
<td><strong>RESOLUTION</strong></td>
<td></td>
</tr>
<tr>
<td>- Complete resolution of L, S, PG, PI, PE, and PS.</td>
<td>- Resolves L, S, PG, and PI.</td>
</tr>
<tr>
<td>- Separates compounds from PE and PS on second migration.</td>
<td>- Does not give complete resolution of PS and PE.</td>
</tr>
</tbody>
</table>
| - Combine one-dimensional TLC, PE, and PS spot is separated only in second migration. | - Interfering compounds co-migrate with PG, PE, and PS: 
  - combined PS and PG peaks contain interfering compound. 
  - meconium compound comigrates with L, S, PI, PS, PE, and obscures L/S ratios. 
  - blood compound migrates near PG (causing a false (+) PG spot). |
| - Clear results when little PG is present. | - Combined PE and PS spot. |
| - Easier to detect and quantitate because complete resolution. | - Does not always detect small amounts of PG. |
|                     | - Gross et al. find PG spot diffuse and often difficult to quantitate. |
TABLE XVII Continued

<table>
<thead>
<tr>
<th>TWO-DIMENSIONAL TLC</th>
<th>ONE-DIMENSIONAL TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Tsai et al. observe PG at low concentration.</td>
<td></td>
</tr>
<tr>
<td>- Tsai et al. results in better less diffuse PG spot and higher PG values (attribute this to increased diffusion after the second chromatograph, and is unlikely due to co-migrating substances).</td>
<td></td>
</tr>
</tbody>
</table>

**PRECISIÓN/REPRODUCIBILITY**

- Reproducibility better for PG.
- Standard deviation and coefficient of variation of PG, due to diffuseness of PG spot and difficulty in detecting small amounts.

- Comparison of $R_f$ values for phospholipids in 10 different amniotic fluid samples with two- and one-dimensional TLC procedures.* $R_f$ Value (x100) (Mean ± standard deviation)

**Direction**

<table>
<thead>
<tr>
<th>1st</th>
<th>2nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 ± 2</td>
<td>Does not migrate</td>
</tr>
<tr>
<td>22 ± 2</td>
<td>Does not migrate</td>
</tr>
<tr>
<td>33 ± 2</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>48 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>50 ± 3</td>
<td>Does not migrate</td>
</tr>
<tr>
<td>62 ± 3</td>
<td>52 ± 4</td>
</tr>
</tbody>
</table>
TABLE XVII Continued

<table>
<thead>
<tr>
<th>TWO-DIMENSIONAL TLC</th>
<th>ONE-DIMENSIONAL TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X (SD)</td>
<td>CV%</td>
</tr>
<tr>
<td>63.50 (4.41)</td>
<td>7</td>
</tr>
<tr>
<td>7.00 (1.07)</td>
<td>15</td>
</tr>
<tr>
<td>12.25 (1.67)</td>
<td>14</td>
</tr>
<tr>
<td>5.40 (1.35)</td>
<td>21</td>
</tr>
<tr>
<td>10.80 (2.10)</td>
<td>19</td>
</tr>
<tr>
<td>2.28 (0.15)</td>
<td>7</td>
</tr>
</tbody>
</table>

Mean standard deviation and coefficient of variation for 10 replicate determinations of the individual phospholipid components in percentage of total phospholipid components in percentage of total phospholipid in amniotic fluid. *

Data based on 10 replicate determinations of the same AF specimen in one- and two-dimensions.

Represents combined PE and PS spot.

TIME

- 150 minutes
- 90 minutes

CONCLUSIONS

- Separates all phospholipids L, S, PG, PE, PI, and PS.
- Advantage of separating compounds from PE and PS on second migration.
- Separates clinically important phospholipids: L, S, PI, and PG.
- Has disadvantage of interference of compounds co-migrating with important phospholipids.

L, S, PI, and PG. However, Gross et al. felt that it can produce problems of interference by other compounds co-migrating with important phospholipids (PG, PE, and PS) and blood and meconium may obscure the lecithin and sphingomyelin spots (method is unreliable when blood or meconium are present) (157). On the other hand Tsai feels that his one-dimensional procedure can detect PG at lower concentrations and results in better less diffuse PG spot and higher PG values (attribute this to increased diffusion after the second dimension of chromatography and is unlikely due to co-migrating substances) (160).

In conclusion, after reviewing several one- and two-dimensional TLC procedures and their outcomes, I have concluded that the differences in precision and resolution are often small and could be attributable to a technical portion of the procedure (i.e., changes in solvent, chromatographic absorbent material or phospholipid stain). Therefore it depends on the laboratory for choice of one- and/or two-dimensional TLC, for the one-dimensional is faster, simpler and less expensive, while the two-dimensional is longer, more complex, and more costly.

(c). The Clinical Usefulness and Interpretation of the Lung Profile

Determination of the acidic phospholipids PI and PG is a major advance in the diagnosis of fetal lung maturity, although the exact function of these substances in surfactant is not totally determined. It is known that PI and, especially, PG are necessary to stabilize lecithin in surfactant; without these acidic phospholipids, lecithin apparently
forms inactive liposomes and does not lower surface tension into the biologic range, except at high temperatures. Hallman and Gluck have postulated that CDP-diglyceride serves as a common precursor for both PI and PG, and the latter compound seems to be the end product rather than a precursor (161). During the course of gestation the two acidic phospholipids undergo unique concentration changes in amniotic fluid.

Therefore, following is a summary of the collective advantages the lung profile offers over the L/S ratio alone and over the presently known procedures for assessing the maturity of the developing fetal lung. With the lung profile most the noninformative low or intermediate L/S ratio values are eliminated. Gluck et al. reported that detection of the presence of PG in cases where the L/S ratio was < 2.0 dramatically reduced the incidence of false negatives (130). Measurement of PG eliminates the false mature L/S ratios. This means that with mature L/S ratios there is a risk of RDS only when PG is absent, for PG appears usually when the L/S ratio exceeds 2.0. Therefore, substantial quantities of PG indeed, indicate lung maturity.

However, PG's absence does not necessarily mean that RDS is inevitable. Hallman et al. have documented cases where PG first appeared within two hours after birth with no symptoms of RDS, but with mature L/S ratios (158). On the other hand, Hallman et al. also found that surfactant with prominent PI sometimes was recovered from the lung effluent at birth but PG did not appear and RDS developed (159). Hallman et al. also found that birth may affect the normally gradual development of the acidic phospholipids in the fetus, (159). In some healthy premature PG appeared
rapidly in lung effluent, within the first neonatal hour (162). However, in RDS, PG gradually developed only during recovery. The failure of induction of PG with prominent PI at birth is remarkable, and suggests that some regulatory factor(s) may be defective. Therefore, PG as a component of the surfactant complex serves as a biochemical marker. In its absence there is a significant risk for developmental lung disease; the presence of PG suggests biochemical maturity of the surfactant.

The present evidence thus far suggests that PG improves the quality of surfactant that may be critical in stabilizing the alveoli. Measurements on a surface balance suggest that surfactant with PG as compared to that without PG has better physicochemical characteristics to guarantee alveolar stability. Therefore, absent PG in amniotic fluid indicates that the surfactant may not be quite mature. The results of many of the studies suggest that the presence of PG is a clearcut indicator of lung maturity and that PG is always absent when RDS occurs (163, 164). However, the situation is very complex, for RDS does occur even when low levels of PG are present in amniotic fluid (153) or in tracheal fluid collected soon after birth (165). It has been suggested that RDS will not occur if the percentages of PG in the amniotic fluid is 3% or more (130) or greater than 1% (136). As mentioned, the feasibility of measuring such low levels of PG accurately is questioned. This entire situation is further complicated by the fact that the absence of PG does not mean that RDS will inevitably occur (136, 163, 164).

Golde and Mosley found that the incidence of RDS in cases lacking PG was only 12% (163). In their study, the infants were delivered within 3
days of sampling. It has been shown that infants who do not develop RDS, even though PG is absent from their tracheal fluid at delivery, begin to produce surfactant containing PG within the first neonatal hours, while those who develop RDS do not (162). Therefore, these data suggest that the appearance of PG in amniotic fluid is a late marker for fetal lung maturity, in the sense that it lags behind the critical event(s) which prime the lung for the production of surfactant containing PG.

PI may be valuable when PG is absent, for high PI values suggest that the lungs are about to be mature, whereas low PI values indicate immature lungs (before 38 weeks). Also PI appears to be more informative the younger the fetus is, since the tubular alveolar system of a tiny premature baby may remain stable even without PG.

Studies by Hallman et al. have suggested that PE and PS make up a significantly higher percentage of total phospholipids in infants who develop RDS (162). Isolation and quantification of these phospholipids from amniotic fluids may prove to be an indicator of immature fetal lung status, in contrast to PG which, when present, indicates mature fetal lung status (130, 166). The converse, however, is not true: the absence of PG does not necessarily indicate an immature lung. Therefore, PE and PS may yield useful information in complicated pregnancies, when the L/S ratio is frequently high despite immature lung status.

The lung profile provides the ability to detect and evaluate abnormal pregnancy by alterations from the expected patterns of maturity. As reported by Kulovich and Gluck an L/S ratio of 2.0 with no PG is characteristic of delayed maturation as seen in gestational, and class A
diabetes mellitus (166). Kulovich and Cluck (166) also found that early appearance of PG may occur in pregnancies that are significantly immature by gestational age, and that actually may have immature L/S ratios, but these infants have no RDS, once 3% or more PG is present, and this represents accelerated fetal lung maturation as reported also by Butos and associates (167). Also as previously mentioned, Painter reported that if the L/S ratio was 1.5 and PG/S exceeded 0.45 then the newborn did not develop RDS (137).

Very recently (1985), Hallman et al. conducted an animal study to investigate the possibility that PG-deficient pulmonary surfactant might have inferior surface properties when compared to normal pulmonary surfactant (168). This specific PG-deficient surfactant was obtained from adult rabbits by withholding glucose and giving them an excess of myoinositol (myoinositol in vitro excess has the potential side effect of promoting the growth of malignant as well as normal cells) by mouth and intravenously for 4 days (controls given a similar quantity of glucose) (168). The myoinositol treatment resulted in the following: increased the alveolar surfactant pool size by 32% (p < 0.01) (by 5 yet unknown mechanism); a drastic reduction of surfactant PG (from 7.2 to 0.3%); a corresponding increase in PI (from 4.8 to 11.3%), and no detectable effect on the relative concentration of the other surfactant components (168).

Hallman et al. study resulted in the following conclusions (168):

- The myoinositol treatment increased the saturated fatty acid moieties specifically palmitate from 18.5 to 27.3% (p < 0.05) of PI (structure of PI approached that of PG) and this is in
accordance with the proposal that PG and PI derive the fatty acid moieties from a common CDP-diacylglycerol pool.

- Hallman et al. also conducted a comparison study of these surface properties of PG and PI surfactants originating from adult lungs, with the following results (summarized in Table XVII) (168): 1) a wide hysteresis loop observed with the Whelmy balance, 2) a rapid adsorption from subphase to surface (PG 35 versus PI 32 mN/m after 30s), 3) a slow collapse of the maximally compressed film toward the equilibrium surface tension, 4) a minimal surface tension of 0 in 4 mN/m on compression of the surface, 5) a compressibility considerably less than the 0.09 mN/m at low surface tension (<15 mN/m), 6) similar ability of PG and PI surfactants to improve lung stability of 27-day old rabbit fetuses, and 7) PI surfactant had a slightly higher recruitment index (PI 10.0 versus PG 13.2), which was the only difference observed for all the parameters analyzed.

- Hallman et al. theorize that PI can substitute for PG without affecting normal surfactant function, for the pressure-volume relationship was equally improved by the two types of surfactant (168).

It still is not clearly understood what the role the acidic phospholipids really is, but present evidence suggests that PG improves the function of saturated PC, for surfactant PG improves the stability of surfactant PC at low surface tension, because the collapse of a DPPC film occurring during compression can be prevented by the presence of PG in the monolayer (168). Also, the poor adsorption of a liposome
<table>
<thead>
<tr>
<th></th>
<th>Phosphatidylglycerol-surfactant</th>
<th>Phosphatidylinositol-surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wheele balance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum surface tension (mN/m)</td>
<td>2.0±0.2 (7)</td>
<td>2.0±0.6 (7)</td>
</tr>
<tr>
<td>Lowest compressibility at 10 mN/m (mN/m)</td>
<td>0.03±0.012</td>
<td>0.02±0.001</td>
</tr>
<tr>
<td>Surface concentration of phospholipid at 10 mN/m (mol/cm²)</td>
<td>1.0±0.4</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>Recruitment index</td>
<td>13.2 ± 1.2</td>
<td>16.0 ± 1.9</td>
</tr>
<tr>
<td>Collapse rate at 10 mN/m (1/min)</td>
<td>1.9±0.4</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Decrease in surface tension during adsorption from subphase to surface</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mL/m² after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 s</td>
<td>12(3)F 35(2)c</td>
<td>14(3)F 32(2)c</td>
</tr>
<tr>
<td>2 min</td>
<td>25, 37</td>
<td>20, 37</td>
</tr>
<tr>
<td>5 min</td>
<td>32, 32</td>
<td>34, 30</td>
</tr>
<tr>
<td>Pulsating bubble surfactometer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum surface tension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mL/m² after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st pulse 15 s</td>
<td>6(3) F 14(2)F</td>
<td>15(2) F 17(2)F</td>
</tr>
<tr>
<td>30s</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Ability of surfactant to improve lung stability of 27 day rabbit fetus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air retention at 35 cm H.O.</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Air retention at 0 cm H.O.</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*a* Mean ± SD (number of assays).

|                                 |                               |                                |
|                                 | b The concentration of surfactant phospholipid 25 mol/mL. |
|                                 | c The concentration of surfactant phospholipid 100 mol/mL. |
|                                 | d The concentration of surfactant phospholipid 2 mol/mL. |
|                                 | e The concentration of surfactant phospholipid 10 mol/mL. |
|                                 | f p < 0.01                     |

suspension prepared from DPPC only, is effectively improved by the inclusion of PG. However, other lipids, especially PI also improve the function of the major surfactant phospholipid, and therefore, the current evidence indicates that PG is not superior to PI as a component of an artificial surfactant. However, one difference noted between PI and PG is their ability to affect the synthesis, as well as the intracellular transport and secretion of surfactant (168). Also Hallman et al. results do not exclude the possibility that PG might have an important role in lipid metabolism (168).

(d). Problems Associated With Phosphatidylglycerol and Phosphatidylinositol Levels in Amniotic Fluid as Indices of Fetal Lung Maturation

The following problems are associated with the utilization of PG and PI levels in amniotic fluid as indices of fetal lung maturation. PG and PI levels are clearly dependent on the amounts of the six phospholipids present in the sample, for they are expressed as a percentage of the sum of these six phospholipids. For example, if a sample has unusually large amounts of nonsurfactant phospholipids present, then PG or PI would be underestimated (125). Some investigators consider 3% of PG present as diagnostic, while others considered just the presence of PG as diagnostic for fetal lung maturity. This data emphasizes the importance of PG detection in the clinical laboratory. For PG is usually measured as a percentage of total phospholipid, however, there are variations in the procedure utilized such as amount of phospholipid applied, type of spray, type of plate, developing solvent,


...charring interval or staining reagents, which may all significantly affect the percentage of PG, and whether or not it is detected at all (125). Therefore, these technical issues must be standardized so that a clinical cut-off value for PG as an index for fetal lung maturity can be established.

(e). Complicated Pregnancies in Relation to the Lung Profile

Some prediction of PG by gestation is desirable because maturation of the L/S ratio and appearance of PG may be either accelerated or delayed with certain pregnancy complications. Table XIX lists the maternal and fetal complications affecting maturation of the fetal lung.

Accelerated lung maturation occurs before 33 weeks gestation. The most frequently encountered complications which cause accelerated fetal lung maturation are: degenerative diabetes mellitus (classes D, E, F); prolonged rupture of membranes (PROM 48-72 hrs); and hypertensive syndrome (severe toxemia, cardiovascular, renal). Delayed fetal lung maturation occurs beyond 37 weeks gestation. The most frequently encountered complications which cause delayed fetal lung maturation are: gestational diabetes mellitus (class A, B, C); hydrops fetalis; and isoimmunization syndrome.

In 1979, Gluck and Kulovich were the first to study the lung profile in amniotic fluid in relation to the various classes of diabetes, to PROM, and to hypertension in pregnancy (166). The procedure utilized for determination of the lung profile was the previously reported two-dimensional TLC method by Gluck et al. (130). The following results show the effects the disease has on the amniotic fluid phospholipids.
TABLE XIX
MATERNAL AND FETAL COMPLICATIONS
AFFECTING MATURATION OF THE FETAL LUNG

<table>
<thead>
<tr>
<th>Acceleration (Mature fetal lungs before 33 weeks gestation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerative diabetes mellitus (classic D, E, FR)</td>
</tr>
<tr>
<td>Prolonged rupture of membranes (PROM)</td>
</tr>
<tr>
<td>Hypertensive syndrome (severe toxemia, cardiovascular, renal)</td>
</tr>
<tr>
<td>Heroin/morphine addiction</td>
</tr>
<tr>
<td>Hemoglobinopathy (Sickle-C)</td>
</tr>
<tr>
<td>Hyperthyroidism</td>
</tr>
<tr>
<td>Maternal Infections (amnionitis)</td>
</tr>
<tr>
<td>Donor parabiotic twin</td>
</tr>
<tr>
<td>Placental insufficiency (infarction)</td>
</tr>
<tr>
<td>Chronic retroplacental bleeding (chronic abruptia placenta)</td>
</tr>
<tr>
<td>Intrauterine growth retardation</td>
</tr>
<tr>
<td>Delay (Mature fetal lungs beyond 37 weeks gestation)</td>
</tr>
</tbody>
</table>

| Diabetes mellitus (gestational, class A, B, C.)            |
| Hydrops fetalis                                           |
| Maternal hepatic disease                                  |
| Maternal systemic autoimmune disease                      |
| Maternal anemia (various)                                 |
| Isoimmunization syndrome (hemolytic disease of the newborn) |
| Polyhydramnios                                            |
appearance and concentration (similar results were obtained by Mitnick et al. (154) in 1980 with 88 patients) (166).

(i). Diabetes Mellitus

Sample size included 220 amniotic fluid samples from 187 pregnant diabetics with the following classification: A-43, B-76, C-46, D-39, F and R-16 (166).

1. L/S Ratio

Figure 56A shows the regression curves for L/S ratios during gestation by class of diabetes. The slopes of the curve do not differ from normal nor from each other. No significant differences in L/S ratios according to gestation were seen among the classes and none of the classes differed significantly from the normal pregnancy curves. Seven of the 43 class A, and a few patients of classes B and C had delayed maturation of the L/S ratio which did not reach 2.0 or greater until 37 weeks or beyond (normal L/S ratio 2.0 at 35 weeks). However, these values were obscured by the much larger number of L/S ratios that were essentially normal.

2. Disaturated Lecithin

Figure 56B shows the percentages of disaturated acetone precipitated lecithin by class of diabetes during gestation. These did not differ from normal (166).

3. Phosphatidylinositol (PI)

Figure 57A shows the percentages of PI during gestation by class of diabetes. Note the prolonged elevation of high percentages of PI in class A diabetes with very late fall (mean of 37 to 38 weeks) as
FIGURE 56

L/S RATIO AND PERCENTAGE OF DISATURATED ACETONE-PRECIPITATED LECITHIN

BY CLASS OF DIABETES DURING GESTATION

Legend

A. Figure illustrates regression curves for L/S ratios during gestation by class of diabetes. The slopes of the curves do not differ from normal nor from each other.

B. Figure illustrates the percentages of disaturated acetone precipitated lecithin by class of diabetes during gestation. These did not differ from normal.

FIGURE 57

PERCENTAGE OF PHOSPHATIDYLINOSITOL AND
PHOSPHATIDYLGLYCEROL DURING GESTATION
BY CLASS OF DIABETES

Legend

A. Figure illustrates percentages of PI during gestation by class of diabetes. Note the prolonged elevation of high percentages of PI in class A diabetes with very late fall (37 or 38 weeks), as compared to normal and to other classes of diabetes (fall about 35 to 36 weeks).

B. Figure illustrates PG percentages during gestation by regression analysis which shows a significant delay (37 to 39 weeks) \( (p < 0.01) \) in appearance of PG in class A diabetes as compared to normal (36 weeks) and to other classes of diabetes.

compared to normal and to other class of diabetes (fall about 35 to 36 weeks) (166).

4. Phosphatidylglycerol (PG)

Figure 57B shows the PG percentages during gestation by regression analysis and the significant delay (p <0.01) in appearance of PG in class A diabetes as compared to normal or other diabetic classes. The PG rose progressively from 36 weeks onward, but did not reach a low mean until 37 weeks as compared to 36 weeks in normal patients. The mean time of appearance of PG in class A diabetes is at 37 to 39 weeks of gestation. Group D, F, R (mainly class D patients) regression curve is in the normal range. The relatively few patients with F and R diabetes showed accelerated maturation, PG appearing as early as 29 or 30 weeks, long before a mature L/S ratio was seen. This was a consistent finding, and in the patients with F and R diabetes L/S ratios of 1.6 to 1.8 were never exceeded; for maturation of the lung was seen before L/S ratios reached 2.0 or more (166).

It has also been found that some infants of diabetic mothers have sufficient amounts of surfactant, however, the surfactant may be immature, in that it contains PT instead of PG (125). Conversely, many infants of diabetic mothers do not develop RDS, who have sufficient amounts of surfactant with no PG. Then maybe these infants, lungs are primed to switch on PG production at delivery and those infants that develop RDS are not (125). These many conflicting situations add to the already complex condition of fetal lung maturation in diabetic pregnancies, which will now be discussed.
5. Disease Relationship to Fetal Lung Maturation

Diabete mellitus is the most complex complication of pregnancy, for it is not one but many syndromes, with only one feature common to all, a high blood sugar. Therefore, it is not surprising, that there are so many different and conflicting statements regarding pregnancy risk, management, and assessment of fetal lung maturity in diabetic pregnancy, since the diabetic syndrome in pregnancy represents so many states. However, regardless of these complexities and confusions, the physician managing a pregnant patient with diabetes, whether mildly insulin dependent, or associated with significant vascular disease, or induced by pregnancy, requires some guidelines for delivery. Traditional management of these patients had been either to induce labor or perform a Cesarean section around 36 or 37 weeks' gestation, therefore balancing the potential risk to the newborn infant of too early delivery resulting in RDS against the potential risk to the fetus of intrauterine death in prolonging the pregnancy. However, management of the pregnant diabetic patient has changed markedly in recent years, with the introduction of the L/S ratio in assessing fetal lung maturity. Although the L/S ratio alone is sufficient to monitor most normal pregnancies, the lung profile, consisting of L/S ratio, disaturated lecithin, PI, and PG determinations, gives the developmental measurements of key fractions in surfactant that together help to form a clearer picture of fetal lung development for the clinician.

In the Kulovich and Gluck study, they found that, no RDS ever was seen when 3% or more PG was present (166). Therefore, it appears that the
presence of PG heralds the final important step in the biochemical maturation of surfactant. Therefore, Kulovich and Gluck suggest that, the presence of 3% or more PG in surfactant in amniotic fluid is the guideline for delivery of the diabetic pregnancy, since it appears to eliminate the risk of RDS regardless of class of diabetes (166).

Currently, the incidence of RDS in diabetic pregnancies appears to be decreasing with improved maternal care during pregnancy. However, in pregnancies with gestational diabetes, RDS continues to occur (with significantly greater frequency), because this class of diabetes is associated with delayed fetal lung maturation, therefore these patients are not being as carefully managed as those known to be diabetic prior to pregnancy (169). In these diabetic pregnancies, their diabetic state could possibly be less well controlled or disclosed too late in pregnancy for optimal treatment (169). Diabetic conditions without severe complications, (i.e., class A and sometimes classes B and C) are associated with the increased appearance of RDS due to delayed fetal lung maturation, whereas longstanding more complicated diabetic states, especially those accompanied by vascular disease (i.e., classes D through R or classes B and C with chronic intrauterine stress), appear more often associated with accelerated fetal lung maturation (169). Therefore, maternal hyperglycemia (elevated blood glucose) is the sole pathological feature which causes delay of fetal pulmonary functional maturation.

The most prominent feature of fetuses of diabetic mothers is the unique association between hyperglycemia and hyperinsulinemia which seems to be involved in the process leading to the potential delay of fetal
lung maturation by some biochemical disturbances— for both of the major surface active constituents of surfactant, DPPC and PG appear to be present in inadequate amounts at birth.

Figure 58 illustrates the metabolic changes in diabetic pregnancies which cause the putative mechanisms of abnormal fetal lung development (169). The impairment may be either indirect, i.e., being the result of an inadequate substrate availability or utilization for surfactant synthesis, or direct, i.e., at the level of DPPC and PG biosynthetic pathway and/or secretion (169).

The understanding of this mechanism is only at its very beginnings, for studies must be conducted to determine more precisely what fetal alterations—(hyperinsulinism, hyperglycemia, increased blood free fatty acids, or other metabolic or hormonal abnormalities) cause the delay in fetal lung maturation, and if several alterations are involved simultaneously, what are their relative importance and function (169).

Following are a few of the currently theorized biochemical mechanisms for diabetic pregnancies resulting in delayed fetal lung maturation. One defect is the effect insulin has on palmitate incorporation into PC which takes place at the level of the phosphatidylcholine-lysophosphatidylcholine remodeling mechanism (169). Also insulin is known to stimulate the uptake of free fatty acids from fetal blood and their incorporation into adipose tissue triglycerides (169). Therefore possibly the high fetal blood insulin levels in the diabetic pregnancy favors incorporation of free fatty acids into a fetal lung pool of triglycerides, but in opposing lipolysis, could actually decrease the availability of diglycerides for PC
FIGURE 58

POSSIBLE MECHANISMS OF THE IMPAIRMENT OF FETAL LUNG
FUNCTIONAL MATURATION IN THE DIABETIC PREGNANCY

Legend

Figure illustrates the possible mechanisms of the impairment of fetal lung functional maturation in the diabetic pregnancy. The impairment may be either indirect, i.e., being the result of an inadequate substrate availability or utilization for surfactant syntheses, or direct, i.e., at the level of DPPC and PG biosynthetic pathway and/or secretion.

Transacylation.

Hyperinsulinism may reduce glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP) production (necessary for biosynthesis of complex lipids, glucose major source of these two) and therefore impair phospholipid synthesis and surfactant production in the lung (Fig. 59) (170). Thus in the hyperinsulinemic and possibly hypoglycemic infant of the diabetic mother, there will be decreased glycerol production resulting in decreased surfactant production and the onset of RDS (170).

To date, with the experimental in vivo and in vitro studies, it is clear that hyperglycemia and fetal reactive hyperinsulinism are both involved in the processes of delaying fetal pulmonary maturation. However, much more research is required to establish the mechanism of surfactant deficiency in fetuses of diabetic mothers.

Bourbon and Farrell conducted research and have reviewed all the currently known knowledge concerning fetal lung development in the diabetic pregnancy (169). They concluded that only the surface has been scratched in understanding the mechanisms involved in this complex disease condition. Basically speaking, one of the major limitations in this research has been the problem of creating appropriate animal models that can replicate the characteristic features of human diabetic pregnancies. Therefore, according to Bourbon and Farrell, after an appropriate model of a diabetic pregnancy is determined (monkey models of current interest), the following questions could possibly be answered (169): Are the surfactant phospholipid biosynthetic pathways impaired? What molecular mechanism is involved? Is the availability of
POSSIBLE EFFECTS OF HYPERINSULINISM ON THE PRODUCTION OF SURFACTANT

Legend

Figure illustrates glycolysis and the possible effects of hyperinsulinism on the production of surfactant. Hyperinsulinism may reduce glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP) production (which are necessary for the biosynthesis of complex lipids) and therefore impair phospholipid synthesis and surfactant production in the lung.

FIGURE 59

Glucose
   ↓
Glucose-6-phosphate
   ↓
Fructose 1,6-diphosphate
   ↓
D.H.A.P. ↔ Glycerol-3-phosphate
   ↓
Pyruvate
   ↓
Hyperinsulinism
   ↓
Acetyl-CoA
   ↓
Krebs' Cycle
   → Pyruvate dehydrogenase
   ↓
Surfactant
   ↓
Phospholipid
substrates for phospholipid biosynthesis insufficient, and what precursor is involved? Is surfactant secretion into the fetal respiratory spaces impaired (169)?

(ii). Premature Rupture of Membrane (PROM)

Sample size included 104 amniotic fluid samples from 92 women with PROM more than 24 hours (60% of whom had PROM longer than 18 hours) (166).

1. L/S Ratio

Figure 60A shows the regression curves for L/S ratios which are significantly accelerated \((p < 0.01)\) with PROM. The figure shows accelerated maturation both with higher mean L/S ratios, and mature L/S ratios earlier in pregnancy than normal.

2. Disaturated Lecithin

Figure 60B shows the regression curve for percentages of disaturated acetone precipitated lecithin. The figure shows that in PROM there is more than 50% disaturated lecithin from as early as 31 or 32 weeks gestation.

3. Phosphatidylinositol

Figure 61A shows that the percentage of PI in PROM is essentially the same as that for normal pregnancy.

4. Phosphatidylglycerol

Figure 61B shows that the slope of the curve in PROM is significantly different \((p < 0.01)\) from that of normal pregnancies. The mean appearance of PG is earlier by 1.5 weeks than normal.
FIGURE 60
L/S RATIO AND PERCENTAGE OF DISATURATED LECITHIN
CURVES IN PROLONGED RUPTURE OF MEMBRANES AND
HYPERTENSION CONDITIONS DURING GESTATION.

Legend

A. Figure illustrates regression curves for L/S ratios in prolonged rupture of membrane (PROM) and hypertension (HTN). No differences from normal during gestation are seen for hypertension, whereas there is a clear (p < 0.01) acceleration with PROM. With PROM there is accelerated maturation both with higher mean L/S ratios and mature L/S ratios earlier in pregnancy than normal.

B. Figure illustrates that the slopes of the curves for disaturated lecithin shown here are not significantly different from each nor from those for normal patients, although in PROM there is more than 50% disaturated lecithin from as early as 31 or 32 weeks gestation.

FIGURE 60

LECHTHIN/SPIKING/MELN RATIONALS

30 31 32 33 34 35 36 37 38 39 40
WEEKS GESTATION

PERCENT DISATURATED (ACETONE PRECIPITATION)

30 31 32 33 34 35 36 37 38 39 40
WEEKS GESTATION
FIGURE 61

PERCENTAGE OF PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLGLYCEROL IN PROLONGED RUPTURE OF MEMBRANES AND HYPERTENSION CONDITIONS DURING GESTATION

Legend

A. Figure illustrates the curves for percentage of PI in PROM and hypertension, which are essentially the same as those for normal pregnancy.
B. Figure illustrates regression curves for percentages of PG in abnormal pregnancy. The slope of the curve in PROM is significantly different (p < 0.01 and 1.5 weeks earlier appearance) from that in normal patients and from the curve for patients with hypertension.

5. Disease Relationship to Fetal Lung Maturation

This Kulovich and Gluck study showed unequivocal biochemical acceleration of fetal lung maturation with early elevated L/S ratios and early appearance of PG. Both are statistically significant (L/S $p < 0.01$; PG, $p < 0.01$) when compared to curves for normal development (166).

Premature rupture of membranes (PROM) is leakage of amniotic fluid prior to the onset of labor from the membranes and its cause is unknown. PROM accelerates lung maturation when the membranes have been ruptured for 48-72 hours or longer to show mature lungs and no RDS. The acuteness with which lungs can mature perhaps is best documented by how quickly the L/S ratio becomes mature after premature rupture of membranes. There is little difference how low the L/S ratio was or how early in gestation it was when the membranes ruptured, for in 3-4 days after rupture the L/S ratio approached or equaled the mature ratio in most pregnancies. There are exceptions, however, and not all infants born after PROM show acceleration of pulmonary maturation, although the incidence of RDS goes down markedly the longer the membranes have been ruptured. The risks of maternal infection from PROM must not be minimized. The dangers of infection, however, may be less from rupture of membrane, than from repeated examination. Once a diagnosis of ruptured membranes is made, the pregnant woman should be put to bed and monitored.

(iii). Hypertension

Sample size included 116 amniotic fluid samples from 89 women with hypertension owing to any cause (166). These hypertensive
patients included anyone with a diastolic pressure greater than 85 mm Hg. The majority of these patients had terminal pre-eclampsia with mild to moderate hypertension, mild proteinuria, and mild ankle and pedal edema. Fewer than one fourth had severe chronic toxemia where illness began in early pregnancy with significant hypertension and proteinuria; a few included pregnancies with cardiovascular and renal hypertension (166).

1. L/S Ratio, Disaturated Lecithin, PI, and PC.

The regression curves and regression coefficients for L/S ratios and percentage of disaturated acetone precipitated lecithin are shown on Fig. 60 A and B; and PI and PC are illustrated of Fig. 61 A and B, respectively. These figures do not differ significantly from the normal pregnancy figures.

2. Disease Relationship to Fetal Lung Maturation

Most of the patients represented in this group with hypertension had terminal pre-eclampsia, with mild elevation of blood pressure, some albuminuria and some pedal edema. This group had normal lung maturation. Also a few patients had chronic toxemia which showed dramatic acceleration of maturation with appearance of PC as early as 29 and 30 weeks. As with class F and R diabetes L/S ratios of 1.5 to 1.8 in these patients were seldom exceeded.

Hypertension, like diabetes, in pregnancy is a syndrome resulting from many causes, ranging from severe pregnancy-induced hypertension to very mild terminal pre-eclampsia, which shortly appears before term, to chronic toxemia, which encompasses hypertensive disorders with cardiovascular and renal problems. The lung profiles of patients with
severe chronic toxemia or other hypertensive disorders when associated with significantly infarcted placentas usually show clear acceleration of maturation. Accelerated maturation in severe chronic toxemia may show early elevated L/S ratios in most cases with early appearance of PG, but in others there may be very early appearance of PG before the L/S ratio becomes 2.0 (as in Class F and R diabetes). However, in patients with mild hypertensive problems, there is usually normal lung maturation.

(f). Sources of Contamination

PI and PG are present only in very small quantities in blood products, therefore blood contamination of an amniotic fluid sample does not interfere with PI and PG determinations.

(g). Summary

Because PI and PG are involved in the dynamics of surfactant maturation, the lung profile represents an advancement intended to reduce the incidence of false negativity that plagues other surfactant assays, and reduces the incidence of false positives in association with maternal diabetes.

The lung profile not only increases the predictability of lung maturity in the fetus to close to 100%, but also may signal an abnormal pregnancy. It gives the physician monitoring the pregnancy reliable information about the status of the maturing lung, when to expect maturity, and when, if necessary, to do a repeat study of fetal lung maturity on amniotic fluid. Also postnatal monitoring of the acidic phospholipids, PG and PI, in lung effluent is useful in diagnosis, and follow-up of RDS as well as in evaluation of various therapies.
The following factors require additional investigation:

- Understanding of the predictive value of the lung profile is somewhat preliminary, therefore, more insight is required into the incidence of RDS occurring in infants delivered after establishment of laboratory evidence of pulmonary maturity (L/S ratio >2.0 and PG present).

- Investigate the mechanism of secretion of PG and the factors that affect it and into the clinical events that may influence the production of PG in utero and the subsequent relationship to the development of RDS, for PG needs to be further defined as an index of fetal maturity, and its concentration value for fetal lung maturity must be established.

- Continue to answer the many questions concerning the mechanisms involved in fetal lung development in the diabetic pregnancy.

3. Optical Density (OD)

(a). Principle

Based on the observation that amniotic fluid becomes progressively more turbid as gestation proceeds, the measurement of the optical density (OD) of amniotic fluid has been investigated in hopes of providing a simple method for assessing fetal lung maturity. The test is based on the principle that the presence of suspended particles of surfactant lamellae in the amniotic fluid increases the turbidity of mature specimens.

Initially attempts to correlate the spectrophotometric analysis of amniotic fluid with fetal maturation were made by measuring changes in
bilirubin concentration by means of optical density readings at 450 nm (170). Further observations agreed with and concluded that the disappearance of the bilirubin peak was a rather constant finding in gestations beyond the 36th week. However, other studies have cast serious doubts on the significance of the correlation between bilirubin disappearance in amniotic fluid and gestational age (171). The measuring of the optical density at 400 nm and 650 nm was another attempt in the same direction and according to their proponents the method evaluates turbidity changes of the total phospholipid concentration (172).

(b). Procedure

In an effort to develop a truly simple, economical, and accurate procedure to replace or compliment the L/S methodology, an empirical observation that amniotic fluids at term were turbid and those at 28 to 30 weeks gestation were generally clear was investigated, and thus the optical density test was developed. Several variations of the optical density procedure have been published, however, the following are the most commonly utilized steps:

- Fresh amniotic fluid is centrifuged in a refrigerated centrifuge either immediately or within 2-3 hours after collection (must be refrigerated till centrifuged) at 2000 x g for 10 minutes.
- An aliquot (3-5 mL) of the fluid is placed in a test tube and the absorbance is measured in a centimeter light path cuvette against distilled water (blank) at 650 nm in a double beam spectrophotometer within two hours of centrifugation. Previous studies would also measure absorbance at 400 nm (172).
However, potential interferences due to absorption of bile pigments at 400 nm were found, therefore the measurement of absorbance was changed to 650 nm. Sbarra et al. found that pigmented amniotic fluid samples generally contain bilirubin, meconium, and/or hemolyzed blood and can absorb significantly at 400 nm, therefore higher than expected absorbancies were noted (172). In order to circumvent this, optical density readings of amniotic fluid were measured at 650 nm, for at this wavelength minimal absorbance of known biologic pigments occurs and the readings would more closely parallel L/S ratios (especialy fluids with some pigments).

(c). Results

Studies have suggested that OD measurements at 650 nm and L/S ratios showed good, fair, and poor correlation, as shown in Table XX, which gives the studies and respective results.

The following comments on results of the reliability of OD$_{650}$ as a fetal lung maturity test and correlation with L/S ratio test are taken in sequential order from Table XX:

(i). Sbarra et al.

Sbarra et al. found that optical density readings of 0.15 or greater at 650 nm correlates with L/S ratios of 2.0 or greater (173). No false positive results were noted, however 6% of the total fluids studied were false negative. A reason why six false negative results were encountered could be explained by some masked dilutional effect. Additionally cells can absorb surfactant and an increased number of cells in the fluid could result in a lower OD reading.
<table>
<thead>
<tr>
<th>Study/reference No.</th>
<th>Amniotic fluid sample size</th>
<th>OD readings of &gt; 0.15 compared to L/S ratio of 2.0</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) SBARRA ET AL. (1977) Obstet. Gynecol. 50, 723./#173</td>
<td>100</td>
<td>6% 0</td>
<td>Good correlation. Six false negative results could be some masked dilutional effect.</td>
</tr>
<tr>
<td>(11) COPELAND ET AL. (1978) Am. J. Obstet. Gynecol. 130, 225./#174</td>
<td>87</td>
<td>41% 0</td>
<td>Predict FPM with OD, but does not predict pulmonary immaturity. Suggest that with low OD readings L/S ratio should also be determined to guide further in therapeutic measures.</td>
</tr>
<tr>
<td>(111) MOODLEY ET AL. (1978) Obstet. Gynecol. 51, 490./#175</td>
<td>127</td>
<td>32% 3%</td>
<td>Same comments as Copeland above.</td>
</tr>
<tr>
<td>Study/reference No.</td>
<td>Amniotic fluid sample size</td>
<td>OD readings of ≥ 0.15 compared to L/S ratio of 2.0</td>
<td>Comments</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------</td>
<td>--------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>(iv). ARIAS ET AL. (1978) Obstet. Gynecol. 51, 152-176</td>
<td>102</td>
<td>False - Negative 34.6% False + Positive 11%</td>
<td>OD not acceptable substitute for L/S ratio in predicting FFM 11% false positive could be explained by at least 4 of the fluids being meconium stained. See Fig. 98 for scatter diagram of correlation.</td>
</tr>
<tr>
<td>(v). SPELLACY ET AL. (1979) Am. J. Obstet. Gynecol. 134, 528-178</td>
<td>78</td>
<td>False - Negative 14.1% False + Positive 3.8%</td>
<td>False positive rate low (3.8%), however false negative rate (14.1%) still very high. Suggest use as a screening procedure, for test is inexpensive.</td>
</tr>
<tr>
<td>(vi). CETRULO ET AL. (1980) Obstet. Gynecol. 55, 263-179</td>
<td>300</td>
<td>True/False negative rate unknown</td>
<td>OD ≥ 0.15 FFM (131 of 131; 100%; 33-42 wks gestation) OD &lt; 0.15 RDS incidence was 8.3% (14 of 169) (24-40 wks gestation). OD measurement at 650nm on centrifuged pigment-free amniotic fluid provide a rapid simple and reliable indicator of fetal pulmonary maturity.</td>
</tr>
</tbody>
</table>

Abbreviations: OD = optical density; FFM = fetal pulmonary maturity.
(ii). Copeland et al.

Copeland et al. found that they could predict pulmonary maturity with OD test but could not predict pulmonary immaturity (174). They suggested that with low optical density readings, L/S ratios should also be determined to guide further therapeutic measures.

(iii). Moodley et al.

Moodley et al. had the same comments as Copeland et al above (175).

(iv). Arias et al.

Arias et al. found that the optical density test was not an acceptable substitute for the L/S ratio in predicting fetal lung maturity (176). Also in an overall analysis, (as shown in Fig. 62) they found that the L/S ratio determination was a more accurate predictor (79% correct), of neonatal outcome than OD550 (only 57% correct). Arias et al. (176) results are at variance with the findings of Sbarra et al. (177) and demonstrate a modest correlation and a high number of false positive and false negative results when comparing the spectrophotometric analysis of amniotic fluid with the L/S ratio. The 11% false positive could possibly be explained by at least four of the fluids being meconium stained (176).

(v). Spellacy et al.

Spellacy et al. showed that prolonged refrigeration could lower OD results (see next section on OD important facts) (178). With the OD reading of 0.15 the false positive rate for predicting a mature L/S ratio remained low (3.8%), however, the false negative rate was still
FIGURE 62

CORRELATION BETWEEN AMNIOTIC FLUID RESULTS OF

OPTICAL DENSITY (OD650) AND THE L/S RATIO

Legend

Figure illustrates scatter diagram of L/S ratio versus optical
density (OD650) measurements. The optical density measurements
corresponding to false negative (34.6%) (upper left quadrant) and false
positive (11%) (lower right quadrant) are indicated in full circles (●).
Those values where both determinations were in agreement are indicated by
open circles (○). The correlation coefficient was 0.52.

Reproduced without permission from: Arias, F., Andrinopoulos, G., and
disturbingly high (14.1%). These figures suggest that the OD test cannot be the sole fetal maturation test, but it can be used as a preliminary screening procedure. For if a fetus is kept in utero during a high risk pregnancy when it is really mature it may be damaged or die. In order to circumvent this problem (14.1% false negative results), all fluids with a negative OD reading must be verified with an L/S ratio test.

(vi): Cetrulo et al.

Cetrulo et al. showed that RDS did not develop in any infant whose mother's amniotic fluid OD was $\geq 0.15$ (gestation ages of amniotic fluid 33-42 weeks) (179). In 14 of 169, (8.3%) cases in which the OD reading was $<0.15$, RDS developed; two infants died in the neonatal period and the remaining 12 survived (gestation age of amniotic fluid 24-40 weeks, majority being $< 35$ weeks). In this study the true incidence of RDS is unknown, for in most cases the mothers received steroid treatment. Cetrulo et al. felt that the optical density test could be utilized in cases of fetal jeopardy, for the obstetrician could rapidly obtain an accurate measurement of fetal pulmonary maturity which to guide the management of his patient (179). With an OD reading of $<0.15$, immaturity is present and inhibition of labor and steroid treatment are considered with fetal condition (179).

(vii): Sbarra et al.

Recently, in 1982, Sbarra et al. conducted a study which revealed that the optical density measurement was potentially a more accurate predictor of fetal pulmonary development than the L/S ratio and that amniotic fluid turbidity was significantly influenced by surfactant and
phospholipids (180). In their study, Sbarra et al. added phospholipid surfactants (L, S, PI, and PG) to the supernatants of centrifuged (16,000 x g, for 60 minutes) amniotic fluids to a desired concentration (i.e., obtain L/S ratio of 2.0, 10 μg/mL of L and 10 μg/mL of S) (180). The following conclusions were obtained.

Centrifuging amniotic fluid at 16,000 x g for 60 minutes significantly reduces both turbidity and phospholipids. Thus the initial OD at 650 nm decreased from 0.40 to 0.001 and the resultant supernatant revealed no detectable phospholipid spots on TLC (180).

Figure 63A shows that the addition of PG to amniotic fluid containing varying amounts of lecithin and sphingomyelin results in a further increase in OD at 650 nm (at all ratios). Also the addition of PI plus PG (Fig. 99) results in an increase in OD that indicates fetal pulmonary maturity (≥ 0.15) (180).

Figure 63B shows the changes in different absolute concentrations of lecithin and sphingomyelin with a constant L/S ratio altering the OD. The greater the number of particles, the greater the optical density. Figure 63B shows that the optical density of fluids increased with increasing concentration of surfactants despite the constant L/S ratio. Therefore, the actual concentration of lecithin rather than the L/S ratio, would be predictive, making OD measurements more valid than the L/S ratio. This observation could account for the many false-positive and false-negative L/S ratios reported (180).

Optical density absorbance by a fluid is dependent on the total size, shape, and number of particles in the fluid; therefore, this method
FIGURE 63
EFFECT OF ADDED PHOSPHOLIPIDS ON AMNIOTIC FLUID OPTICAL DENSITY
AT 650 NM AND OF DIFFERENT ABSOLUTE CONCENTRATIONS OF
LECITHIN AND SPHINGOMYELIN WITH A CONSTANT L/S RATIO OF OD 650 NM

Legend
A. Figure illustrates effect of added lecithin (L), sphingomyelin (S),
phosphatidylglycerol (PG), and phosphatidylinositol (PI) on amniotic
fluid optical density at 650 nm. The addition of PG to amniotic fluid
containing varying amounts of lecithin and sphingomyelin results in a
further increase in OD at 650 nm (at all ratios). Also the addition of
PI plus PG results in an increase in OD that indicates fetal pulmonary
maturity (≥ 0.15).
B. Figure illustrates effect of different absolute concentrations of
lecithin and sphingomyelin with a constant L/S ratio at optical density
650 nm. For the greater the number of particles the greater the optical
density. The figure shows that the optical density of fluids increased
with increasing concentration of surfactants despite the constant L/S
ratio. Therefore, the actual concentration of lecithin rather than the
L/S ratio would be predictive, making OD measurements more valid than the
L/S ratio.

Reproduced without permission from: A. and B. Sbarra, A. J., Cetrulo, C. L.,
FIGURE 63

A.

B. The Effect of Different Absolute Concentrations of Lecithin and Sphingomyelin with a Constant L/S Ratio on OD 650 nm
measures all known phospholipids (L, S, PG, and PI), as well as unidentified surfactants.

From their study, Sbarra et al. concluded that OD measurements of amniotic fluids may be a more meaningful parameter for studying fetal pulmonary maturity than the L/S ratio for OD determinations approximate more closely Gluck's lung profile analysis of total surfactant measurement (180).

(d). Factors Which Affect the Optical Density Test

Optical density studies conducted by investigators revealed that the following factors affect the optical density measurement:

(i). Refrigeration

Spellacy et al. studied the effects of prolonged refrigeration of amniotic fluids on the initial optical density (178). Four amniotic fluids were refrigerated centrifuged at 2000 g for 10 minutes (standard for OD procedure), and the OD at 650 nm was read. Then the fluids were placed in a refrigerator at 4°C and a repeat centrifugation and OD reading at 650 nm was done daily over a five-day time period. Figure 64 depicts the effect that prolonged refrigeration has on amniotic fluid, which was a decrease in the optical density during the first two days and probably due to the flocculation of the fluid proteins. This decrease could bring a mature fluid to an immature OD reading.

(ii). Centrifugation, Blood, and Meconium

Arias et al. found that OD determinations were profoundly influenced by the speed of centrifugation (higher centrifugation speeds
FIGURE 64

THE EFFECT REFRIGERATION HAS ON AMNIOTIC FLUIDS OPTICAL DENSITY

Legend

Figure illustrates the effect of prolonged refrigeration of amniotic fluids on the optical density reading at 650 nm (N=4). Fluids were exposed to five days of repeated centrifugation, and OD_{650 nm} measurements, and refrigeration at 4°C. The effect the prolonged refrigeration had on the amniotic fluids was a decrease in the optical density during the first two days and this is probably due to the flocculation of the fluid proteins. This decrease could bring a mature fluid to an immature OD reading.

gave lower absorbance), and the presence of blood or meconium in the amniotic fluid (177). These variables also have a profound effect on the L/S ratio test, but in the opposite direction, for blood and meconium will tend to decrease the L/S ratio, while they will cause increases in the OD₅₅₀ measurement (181).

(iii). Gestational Age and Polyhydramnios

Cetrulo et al. found low OD readings with low L/S ratios in some patients near term (179). Also others have observed that L/S ratios do not necessarily correlate with gestational age, which Cetrulo et al. also found true of OD readings (179).

In all the studies conducted, the patient population included a wide variety of maternal complications with no apparent discrepancies, except for polyhydramnios. Sbarra et al. found polyhydramnios to have a dilutional effect, which caused a falsely depressed OD measurements in a number of Class B diabetics, where absorbances at 650 nm below 0.15 were seen in association with L/S ratios greater than 2.0 (174).

(e). Contamination

Specimens containing gross meconium, blood, bilirubin or frozen and thawed fluids should be excluded, since these substances are known to effect the OD measurement results because they absorb at this wavelength.

(f). Summary

Basically the optical density test should be utilized as a screening procedure. For the OD₅₅₀ test is simple, inexpensive, can be completed in approximately 15 minutes in any laboratory on a 24-hour basis, and the information derived is valuable if the results are
positive.

Measurement of amniotic fluid OD$_{650}$ has been proposed as a simple alternative to the L/S ratio. Several of the studies reviewed have given conflicting results. The last two studies (Table XX) demonstrated a good correlation between an L/S ratio $> 2.0$ and OD$_{550} > 0.15$ for fetal pulmonary maturity. However, other studies have exhibited high false negative rates with the OD$_{550}$ test, which can be explained by the effects of refrigeration, storage (progressively declines), and centrifugation can have. All OD$_{550}$ samples with values below 0.15 must also be tested for their L/S ratio, because some infants with such values may be mature. A correction of the high false negative rates with the OD test must be investigated and also an all inclusive pregnancy population (both gestational age 24-40 weeks, and complicated pregnancy conditions) must be further tested to determine if the $> 0.15$ value is the clinical cut-off value for fetal pulmonary maturity interpretation.

Also some investigators have noted that even though surfactant undoubtedly contributes to the optical density of amniotic fluid, its contribution relative to other components of amniotic fluid at various stages of gestation is really unknown. Therefore, the optical density test lacks specificity, for it may work at an overall level reflecting fetal maturity rather than fetal lung maturity. The OD measurement may not be much more useful than the experienced obstetrician's visual assessment of the amniotic fluid status of mature or immature.
4. Cortisol

(a) Principle

There is evidence that fetal production of cortisol (hydrocortisone), which parallels lung maturation, is reflected by the appearance and increase of cortisol in amniotic fluid. Fencel and Tulchinsky measured amniotic fluid cortisol levels at different stages of gestation and found a progressive increase in term, a finding consistent with the theory that lung maturation is initiated as fetal glucocorticoid production increases (182). They concluded that the amniotic fluid cortisol level may provide an indication that lung maturation has been initiated. However, they warned against using this measurement instead of the L/S ratio to monitor fetal lung maturity, since a time lag is likely to occur between the rise in cortisol and the achievement of lung maturation (182).

However, the following evidence has been reported in support of using cortisol in measuring fetal lung maturity. Glucocorticosteroid receptors have been demonstrated in fetal lung tissue of several species including the human (76). Administration of glucocorticosteroids to pregnant sheep or to their fetuses, accelerated the appearance of pulmonary surfactant and enhanced fetal lung maturation (78). Also, cortisol levels in umbilical-cord blood were lower in newborns in whom RDS subsequently developed than in those of comparable gestational age without the syndrome (183). Currently administration of glucocorticosteroids to mothers whose infants are to be born prematurely is being investigated with very promising results. It has been found that amniotic fluid
cortisol level rises around the 20th week of gestation, remains at a plateau until at least the 35th week and then rises rapidly in the 2 weeks before the onset of spontaneous labor. However, findings from studies suggest that although there is a positive correlation between amniotic fluid lecithin and cortisol, the amniotic fluid cortisol cannot be used with confidence to predict lung maturation as indicated by lecithin production.

(b). Different Procedures and Their Corresponding Results

Because of the differences of opinion in utilizing amniotic fluid cortisol levels in assessing fetal lung maturity, several different procedures with their corresponding results have arisen and will now be reviewed.

(i). Fenc1 and Tulchinsky-Total Cortisol

In 1975, Fenc1 and Tulchinsky determined total cortisol (conjugated and unconjugated) by a modification of the radioimmunoassay for plasma cortisol, at various stages of gestation and correlated it with the L/S ratio (182). Their procedure involved the following steps (182):

- 0.1 mL amniotic fluid was precipitated and centrifuged. Then 0.03-0.04 mL of the supernatant was removed and diluted with assay buffer (0.1 M sodium phosphate). To the unknowns and standards 0.1 mL of antibody (diluted 1:5000 in assay buffer) was added.
- The samples were incubated overnight at 4°C and separation of the free and unbound cortisol was done with charcoal-dextran suspension.
The tubes were then centrifuged and decanted into counting vials containing scintillation solution.

And finally the radioactivity was counted long enough to give a counting error of less than 2%.

The major disadvantage of this procedure was the very time-consuming overnight incubation step, and also only 48 samples were tested.

The important factors which this study of Fencel and Tulchinsky did reveal are the following (182). A sharp increase in total amniotic fluid cortisol occurs after the 34th week of gestation as shown in Fig. 65. This rise in amniotic fluid cortisol after the 34th week, continues as pregnancy progresses and may well reflect a rise in cortisol production by the fetal adrenal glands. The increase in cortisol production by the fetal adrenal glands could in turn be responsible for the induction of the process of fetal lung maturation. Clinical observations have shown that maturation of the fetal lung and production of surfactant can be accelerated by administration of corticosteroids directly to the fetus, or to the mother (87). Also no occurrence of RDS in newborns was observed when, 48 hours before labor, total amniotic fluid cortisol was higher than 60 ng/mL. The association between cortisol values and the L/S ratios as evaluated by rank correlation was highly significant (r = 0.83, p <0.001). Fencel and Tulchinsky felt that this observation should not be interpreted to mean that the information provided by the amniotic fluid cortisol determinations may be substituted for that based on L/S ratios (182). The level of amniotic fluid cortisol may predict that the process inducing fetal lung maturation has been initiated, whereas the
FIGURE 65

TOTAL CORTISOL CONCENTRATION IN AMNIOTIC FLUID AT VARIOUS STAGES OF PREGNANCY

Legend

Figure illustrates sharp increase in total amniotic fluid cortisol after the 34th week of gestation. This rise continues as the pregnancy progresses and may well reflect a rise in cortisol production by the fetal adrenal glands. The increase in cortisol production by the fetal adrenal glands could in turn be responsible for the induction of the process of fetal lung maturation. The middle line in each bar represents the mean value for the group; the shaded areas represent the skewed 95% confidence limits of normal values.

Figure 65
L/S ratio in amniotic fluid reflects what has been accomplished, and there may be a time lag between rise of the cortisol and the rise of the ratio (182).

This procedure, by Fencel and Tulchinsky determined many important factors concerning the role of cortisol, however, the procedure was much too time consuming because of the overnight incubation step and also only 48 samples were tested. Therefore, the appearance of the following procedure resulted.

(ii). Tan et al. - Unconjugated RIA Cortisol

In 1976, Tan et al. developed a specific and thoroughly validated competitive protein-binding radioassay for the measurement of unconjugated cortisol in human amniotic fluid (184). The procedure involved the following steps (184):

- Amniotic fluid cortisol was extracted (from 83 normal pregnancy samples) with ethyl acetate, followed by chromatographic purification and assay. The dried organic extract to be chromatographed was then applied to a 16 x 1-cm column packed with 1 g of Sephadex LH-20 after redissolving in a solvent mixture consisting of cyclohexane/benzene/methanol (60:40:10 by volume).
- The first 16 mL containing other steroids was discarded, and the next 10 mL containing cortisol was collected for assay (recovery = 75 ± 1% SE).
- Unconjugated cortisol was then assayed using a competitive protein binding radioassay utilizing human transcortin (interassay variability = 18%). A 10% aliquot was taken for estimation of
recovery and 2 x 40% aliquot for assay. Eight columns were usually packed and run at a time, the procedure took about 2 hours.

Figure 66A shows the cortisol values of the 83 amniotic fluid samples analyzed for the different weeks of gestation (183). Cortisol levels rose progressively from 8.6 ng/mL at less than 20 weeks to 11.4 ng/mL between 20–25 weeks and to 19.8 ng/mL at 30–40 weeks gestation (183).

Tan et al. observed the following important factors from their study (184). Cortisol values were no higher in samples of over 40 weeks of gestation than those between 30–40 weeks (22.9 versus 19.8 ng/mL respectively, p = 0.5) (Fig. 66A). The rise in cortisol levels with gestational age noted in this study is in general agreement with the observations of other investigators (185). In pregnancies of 30 weeks or more, amniotic fluid cortisol levels were significantly elevated in those samples with L/S ratios of more than 2 compared to those with ratios of less than 2 (22.2 versus 15.4 ng/mL, p <0.02). Figure 66B shows the good correlation obtained between individual cortisol values and L/S ratios (r = 0.79; p <0.001). Also, when only those specimens of greater than 30 weeks were analyzed, similar significance was obtained (r = 0.45; p <0.005) (184).

However, several differences exist when compared to Fencel and Tulchinsky, such as: Fencel and Tulchinsky (182) noted significantly higher cortisol values in gestation of 40 weeks or more, a finding not reproduced in the Tan et al. (184) study and perhaps explained by differences in methodology or by the small number of samples studied.
FIGURE 66
AMNIOTIC FLUID CORTISOL VALUES
FROM COMPETITIVE PROTEIN-BINDING RADIOASSAY PROCEDURE
AND THE CORRELATION OF AMNIOTIC FLUID CORTISOL
AND THE L/S RATIO VALUES

Legend
A. Figure illustrates cortisol values in 83 amniotic fluid samples for the different weeks of gestation. The cortisol level rose progressively from 8.6 ng/mL at less than 20 weeks to 11.4 ng/mL between 20-25 weeks to 19.8 ng/mL at 30-40 weeks, and 22.9 ng/mL at greater than 40 weeks gestation (mean ± SE; p < 0.05 for all groups except between 30-40 and >40 weeks).

B. Figure illustrates the correlation of amniotic fluid cortisol and L/S ratio in uncomplicated pregnancy. It shows good correlation between individual cortisol values and L/S ratios (r = 0.79; p < 0.001). When only those samples of greater than 30 weeks were compared similar significance was obtained (r = 0.45; p < 0.005).

FIGURE 66

A. Amniotic fluid cortisol (ng/mL)

- <20 weeks (n=26)
- 20-25 weeks (n=16)
- 30-40 weeks (n=36)
- >40 weeks (n=5)

B. Amniotic fluid cortisol (ng/mL)

- 30-40 weeks
- Indicative abortion (15-24 weeks)

LECITHIN/SPIRIGOMYELIN RATIO

*p < 0.79, p < 0.01
(excluding indicative abortions) p < 0.001
Also FencI and Tulchinsky noted no differences in cortisol values when samples of less than 20 weeks were compared with those of 20-34 weeks gestation (182). Tan et al. noticed a small, but significant difference in their study, in which a larger sample size was analyzed (184).

In conclusion, Tan et al. results support the hypothesis that cortisol plays a role in fetal lung maturation (184). This study indicates the correlation between amniotic fluid cortisol level and the state of pulmonary maturation is reflected in various surfactant indices (Unconjugated and therefore biologically active cortisol was measured). The correlation represents evidence that the normal mechanism of turning on surfactant production involves endogenous fetal corticoid production. Because of methodologic difficulties, Tan et al. doubt that measurement of amniotic fluid cortisol will ever replace the simpler tests now in use (such as the L/S ratio); nevertheless, these data are important physiologically (184).

Meanwhile the following method was being investigated to decrease cortisol assay time.

(iii). McCann et al.-RIA Cortisol

In 1977 McCann et al. radioimmunoassayed amniotic fluid cortisol (186). The procedure involved the following steps (186):

- Utilized a commercially available immobilized antibody produced against the 21-oxy-succinyl-derived conjugate of this steroid.
- The sample volume was 0.1 mL of either maternal plasma or amniotic fluid.
- A heat denaturation step freed the cortisol from binding proteins.
Before the current study, McCann et al. had validated the radioimmunoassay procedure by comparing results with those of the classical fluorometric technique for estimation of free 11-
hydroxycorticoids in human plasma (186). Values for the cortisol measured in the 50 amniotic fluid samples ranged from 15 to 127.5 µg/L, with a mean and standard deviation of 56.3 ± 30.3 µg/L. The L/S ratios ranged from 1.1 to 5.9, with a mean of 3.02 ± 0.97. Figure 67 shows that the linear coefficient of correlation between the two parameters was statistically significant (r = 0.489, P < 0.01 with n = 50) (186).

Further inspection of McCann's et al. data suggests that in no instance was an amniotic fluid cortisol value of more than 50 µg/L associated with a low L/S ratio (186). However, as many as 18 amniotic cortisol concentrations below 50 µg/L were associated with L/S ratios greater than 2.0. In this study deliveries were based on L/S ratios and because no problems were encountered in any infants delivered after the L/S ratio exceeded 2, some apparently low amniotic fluid cortisol levels were associated with healthy infants born without signs of respiratory distress.

McCann et al. also investigated the potential interference of maternal cortisol into amniotic fluid samples (186). In 1974, Murphy et al. reported the conversion of maternal cortisol to cortisone by the fetoplacental unit (187). Although most of the available evidence suggests that very little cortisol finds its way into the amniotic fluid from maternal circulation, either as such or as cortisone, McCann et al. decided to investigate the interference from this metabolite in the
FIGURE 67
AMNIOTIC FLUID CORTISOL CONCENTRATION
VERSUS L/S RATIO VALUES

Legend

Figure illustrates the amniotic fluid cortisol concentration (µg/L) versus the L/S ratio value. The linear coefficient of correlation
between the two parameters was statistically significant (r = 0.489, P
≤0.01 with n = 50).

Reproduced without permission from: McCann, D. S., Kirkish, L. S.,
FIGURE 67

\[ r = 0.682, \quad n = 5, \quad p < 0.01 \]

\[ y = 1.4 + 15.1x \]
following way. Trace amounts of tritiated cortisol and cortisone were added to several specimens of amniotic fluid; the two steroids were then separated on a Sephadex LH-20 column (186). Then the cortisone and cortisol containing peaks were submitted separately to cortisol RIA. Their results were not unexpected for most of the available evidence suggests that amniotic fluid cortisol reflects fetal adrenal activity and not maternal cortisol concentrations (as evidenced by the low correlation between maternal plasma and amniotic fluid cortisol values) (186).

In conclusion, McCann et al. procedure is quick, simple, reproducible, and used as described, measures total cortisol (186). However, the population sample size was small (50 amniotic fluid samples) and the amniotic fluid cortisol correlated fairly well with the L/S ratio, but the latter is the better diagnostic test for assessing fetal lung maturity.

Meanwhile the Fencl and Tulchinsky original method was being modified to reduce assay time.

(iv). Doran et al. Total Cortisol

In 1979, Doran et al. (188) determined the total cortisol (conjugated and unconjugated) level in amniotic fluid by a modification procedure of the radioimmunoassay for plasma cortisol as initially utilized by Fencl and Tulchinsky in 1975 (182). The assay was carried out as follows (188):

- Amniotic fluid was pipetted into a test tube containing 500 μL of borate buffer (0.05 M, pH = 8.0). These contents were heated at 65°C for 30 minutes and then 100 μL of 3H-cortisol (2,500 d.p.m.)
in buffer was added. The mixture was incubated at room temperature for 60 minutes (original 1975 procedure required overnight incubation at this step) and free and antibody-bound cortisol were separated using dextran-coated charcoal.

- After a 10-minute setting period, the tubes were centrifuged at 3,000 r.p.m. for 10 minutes and the supernatant was decanted into counting vials containing 10 mL of Biofluor.
- Then the samples were counted on a Beckman LS-230 liquid scintillation counter.

This procedure is a recent modification of the original, for it required only about 2 1/2 hours to perform, while the original required overnight incubation.

Doran et al. study concluded the following observations (188). Doran et al. found the L/S ratio to be a good indicator of fetal maturity because the method had a 52% differential percentage, indicating the accuracy of assessing fetal maturity. On the other hand, total cortisol is a less useful test for assessing fetal maturity because of the low differential percentage of 12%, see Table XXI (188). Doran et al. (188) agreed with Fencel and Tulchinsky (182) that total cortisol levels in amniotic fluid rose toward term. However, Doran et al. (188) found, as did Murphy et al. (185), that amniotic fluid cortisol was a poor indicator of fetal maturity (37 weeks' gestation) as the levels started to rise at about 30 weeks gestation compared to 35 to 36 weeks for L/S ratio.

In conclusion, Doran et al. found the cortisol test to be 94% specific and the predictive value of an immature (positive) result was only 33%
### TABLE XXI:

**Critical Values and Differential Percentages**

*Indicating Accuracy of Assessment of Fetal Maturity*

<table>
<thead>
<tr>
<th>Parameter and critical value</th>
<th>31 - 36½ weeks</th>
<th>37 - 42 weeks</th>
<th>Differential percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>No. above critical value/total</td>
<td>% Total</td>
<td>No. above critical value/total</td>
</tr>
<tr>
<td>L/S ratio (3:1)</td>
<td>142</td>
<td>16/64</td>
<td>25</td>
</tr>
<tr>
<td>Total cortisol (30 ng/mL)</td>
<td>108</td>
<td>33/42</td>
<td>79</td>
</tr>
</tbody>
</table>

and in assessment of sensitivity (positivity in disease) total cortisol had a low 50%, however, the sample size was small (188). Therefore, total cortisol is not as useful as the L/S ratio in assessing fetal lung maturity, because of the above mentioned low sensitivity and accuracy.

(c). Pregnancy Disease Conditions Which Affect the Amniotic Fluid Cortisol Concentrations

Tan et al. investigated the following pregnancy associated disease conditions and their affect on the amniotic fluid cortisol concentration (189):

- Amniotic fluid volume may be important in determining cortisol concentrations, for results obtained showed a significantly lower amniotic fluid cortisol in diabetic pregnancies which were unrelated to gestational age. This could be interpreted as being consistent with the relative polyhydramnios frequently associated with this clinical condition. Another explanation could be a dilutional effect secondary to the mild to marked polyhydramnios often noted in late diabetic pregnancies; this would result in lower than normal amniotic fluid cortisol values.

- Elevated amniotic fluid cortisol values were found in patients with toxemia (with immature L/S ratio), early severe Rh incompatibility, and with impending intrauterine death, as well as in isolated cases of PROM and infection. All of these conditions represent a fetal response to prenatal stress.

(d). Contamination

Studies suggest that gross contamination with meconium probably
introduces undetermined cross-reacting steroids, or other substances that interfere with measurements of cortisol. Therefore, all amniotic fluid samples grossly stained with meconium should be excluded.

Also samples contaminated with blood should also be excluded.

(e). Summary

Several groups have measured amniotic fluid cortisol levels in an attempt to predict fetal lung maturity. The results have been compared and correlated to L/S ratio values. The findings suggest that there is a fairly good correlation between the two, however, amniotic fluid cortisol levels cannot be utilized with confidence in predicting lung maturation, as indicated by lecithin production. The correlation represents evidence that the normal mechanism of turning on surfactant production involves endogenous fetal corticoid production. However, because of methodologic difficulties and inadequate sample size, the current amniotic fluid cortisol tests will not replace the simpler, widely accepted and utilized L/S ratio. On the other hand, the results and observations obtained are important physiologically.

In all the studies conducted the amniotic fluid cortisol values rose progressively with increasing gestational age in both normal and high-risk pregnancies followed with serial amniocentesis. These findings provide evidence of the important role cortisol has in fetal lung maturation. However, the studies have revealed that stress and dilutional factor may influence amniotic fluid cortisol levels, and therefore these additional factors need further clarification.

The precise role that cortisol plays in fetal lung maturation and its
corresponding gestational appearance, must be determined before the
amniotic fluid cortisol level can be utilized as a clinical index.

5. Palmitic Acid

(a). Principle

Surfactant lecithin obtained from mature lungs is esterified (two
palmitic acid moieties) with about 70% palmitic acid, therefore, attempts
have been made to assess fetal lung maturity by measuring the palmitic
acid content of amniotic fluid. For with advancing gestation and
increasing pulmonary maturity there is an increase in the proportion of
palmitic acid (C₁₂₅) and palmitoleic acid (C₁₃₁₈) in amniotic fluid
lecithin. The proportion of all other fatty acids tends to fall.

(b). Different Procedures and Their Corresponding Results

Many different procedures have been utilized to measure amniotic
fluid palmitic acid concentration. The chloroform/methanol extraction
method (Folch) followed by gas-liquid chromatography (GLC) has been
utilized by several investigators.

Following is a summary of the most important methods utilized.

(i). Gas-Liquid Chromatography (GLC)

In 1975, Schirar et al. developed the palmitic/stearic acid
ratio (P-S ratio) which appeared to correlate well with gestation and
neonatal performance (190). Zuspan et al. (191) also confirmed the
observations of Schirar et al. (190). Both investigators utilized the
following procedure. The lipids were extracted and hydrolyzed, and the
fatty acids released were methylated and determined by gas-liquid
chromatography (GLC) from the total lipids extract of amniotic fluid.
Unfortunately the Zuspan (191) and Schirar (190) results were not in agreement (Table XXII). Schirar's data would suggest that a P-S ratio in excess of 3.5 indicates the presence of lecithin of a predominantly mature configuration and they postulated that as such it could be utilized as a reliable index of respiratory status at birth (190). However, due to the differences obtained in the results, further evaluation was required; Schirar et al. did show the variations of several saturated or unsaturated fatty acids from amniotic fluid (190). As Table XXIII shows, all fatty acids have a decreasing slope during gestation except palmitic (C_16) and palmitoleic (C_16:1) acids.

Several others, also utilized the GLC method to determine palmitic acid content of amniotic fluid lecithin. Russell et al. utilized a GLC method which they felt had diagnostic value for assessing fetal maturity. However, two major disadvantages of the method were: the length of time to perform the analysis (about 4 h elapsed time; 2.5 h technician time; a maximum of six samples per day per technician); and the technical expertise to measure fatty acids by gas chromatography (192).

(ii). Thin-Layer Chromatography (TLC)

Others have determined the palmitic acid content or the palmitic/stearic acid ratio of lecithin by thin-layer chromatography (TLC). A major disadvantage of the above mentioned TLC method is that the measurement of the palmitic acid content of amniotic fluid is a very nonspecific method for monitoring its surfactant content. It has been found that phospholipids from other sources and neutral lipids, including mono-, di-, and triglycerides, free fatty acids, and cholesterol esters
### TABLE XXII
MEAN PALMITIC/STEARIC ACID RATIOS IN LECITHIN
(AMNIOTIC FLUID IN NORMAL PREGNANCY)

<table>
<thead>
<tr>
<th>Weeks gestation</th>
<th>Palmitic/stearic acid ratio (Zuspan et al., 1975)</th>
<th>Palmitic/stearic acid ratio (Schirar et al., 1975)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 - 18</td>
<td>3.0</td>
<td>---</td>
</tr>
<tr>
<td>25 - 26</td>
<td>4.3</td>
<td>---</td>
</tr>
<tr>
<td>33 - 34</td>
<td>6.9</td>
<td>4.0</td>
</tr>
<tr>
<td>36 - 37</td>
<td>8.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Term</td>
<td>10.7</td>
<td>7.5</td>
</tr>
</tbody>
</table>

## TABLE XXIII
FATTY ACID VARIATION IN CONCENTRATION WITH GESTATION

<table>
<thead>
<tr>
<th>Weeks</th>
<th>0.8 (9)**</th>
<th>29 to 30 (9)</th>
<th>31 to 32 (16)</th>
<th>33 to 34 (27)</th>
<th>35 to 36 (38)</th>
<th>37 to 38 (33)</th>
<th>39 to 40 (36)</th>
<th>41 to 42 (34)</th>
<th>43 (5)</th>
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<td>16:0 palmitic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27.48</td>
<td>33.71</td>
<td>30.26</td>
<td>34.37</td>
<td>41.36</td>
<td>53.01</td>
<td>51.53</td>
<td>52.42</td>
<td>55.00</td>
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<tr>
<td>2</td>
<td>34.14</td>
<td>47.96</td>
<td>30.26</td>
<td>41.95</td>
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</tr>
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<td>3</td>
<td>32.1 to 35.9</td>
<td>37.5 to 41.3</td>
<td>32.8 to 43.0</td>
<td>41.9</td>
<td>38.0 to 60.1</td>
<td>60.6 to</td>
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<td></td>
</tr>
<tr>
<td>16:1 palmitoleic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>6.50</td>
<td>5.12</td>
<td>6.44</td>
<td>6.69</td>
<td>5.46</td>
<td>7.51</td>
<td>10.46</td>
<td>13.97</td>
<td>14.75</td>
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<tr>
<td>2</td>
<td>± 4.0</td>
<td>± 3.78</td>
<td>± 2.01</td>
<td>± 1.82</td>
<td>± 0.83</td>
<td>± 2.07</td>
<td>± 1.86</td>
<td>± 2.23</td>
<td>± 8.02</td>
</tr>
<tr>
<td>3</td>
<td>12.1 to 6.7</td>
<td>8.7 to 15.0</td>
<td>8.3 to 22.2</td>
<td>21.2 to 27.7</td>
<td>17.9 to</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>13.07</td>
<td>13.25</td>
<td>12.48</td>
<td>11.75</td>
<td>10.48</td>
<td>8.25</td>
<td>6.89</td>
<td>6.18</td>
<td>5.88</td>
</tr>
<tr>
<td>2</td>
<td>± 3.95</td>
<td>± 2.09</td>
<td>± 1.71</td>
<td>± 0.86</td>
<td>± 0.66</td>
<td>± 0.76</td>
<td>± 0.54</td>
<td>± 0.42</td>
<td>± 1.10</td>
</tr>
<tr>
<td>3</td>
<td>16.6 to 14.0</td>
<td>14.6 to 14.5</td>
<td>13.0 to 11.0</td>
<td>9.6 to 4.6</td>
<td>4.8</td>
<td>4.2</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 oleic</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.62</td>
<td>29.11</td>
<td>28.78</td>
<td>27.85</td>
<td>24.05</td>
<td>19.65</td>
<td>20.16</td>
<td>18.65</td>
<td>18.27</td>
</tr>
<tr>
<td>2</td>
<td>± 6.65</td>
<td>± 6.84</td>
<td>± 1.71</td>
<td>± 2.00</td>
<td>± 2.57</td>
<td>± 1.81</td>
<td>± 1.05</td>
<td>± 1.76</td>
<td>± 1.51</td>
</tr>
<tr>
<td>3</td>
<td>35.0 to 31.7</td>
<td>30.6 to 35.5</td>
<td>34.7 to 29.9</td>
<td>25.5 to 28.4</td>
<td>19.2 to</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 linoleic</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>14.44</td>
<td>13.95</td>
<td>15.78</td>
<td>13.24</td>
<td>12.31</td>
<td>7.13</td>
<td>7.14</td>
<td>5.55</td>
<td>4.38</td>
</tr>
<tr>
<td>2</td>
<td>± 3.25</td>
<td>± 4.41</td>
<td>± 8.84</td>
<td>± 1.16</td>
<td>± 1.85</td>
<td>± 1.37</td>
<td>± 0.93</td>
<td>± 0.89</td>
<td>± 0.50</td>
</tr>
<tr>
<td>3</td>
<td>16.2 to 16.0</td>
<td>34.5 to 22.5</td>
<td>19.7 to 12.7</td>
<td>15.5 to 10.5</td>
<td>4.6 to</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.8</td>
<td>12.9</td>
<td>2.5</td>
<td>7.2</td>
<td>6.6</td>
<td>3.0</td>
<td>4.1</td>
<td>1.8</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

* Fatty acids are designated by their chemical characteristics (number of carbons: number of double bonds); 1 mean value in per cent; 2, ±2 standard errors of mean; 3, range.

** Number of samples in parentheses.

all contribute to palmitic acid (193). It has been shown that 72 to 98% of the palmitic acid of amniotic fluid is derived from lecithin, however, amniotic fluid collected near term was utilized in these experiments and the contribution of lecithin palmitate to total palmitate at earlier stages of gestation is really unknown (194). Therefore, all of these previously described methods would have increased palmitic acid values, because the nonphospholipid substances (triglycerides and free fatty acids) contain palmitic acid residues and are present in significant amounts in amniotic fluid.

(iii). Ip et al. Modified Extraction Gas-Liquid Chromatography Procedure

In 1977, Ip et al. described a method in which a modified extraction procedure was utilized to separate the phospholipids from the neutral lipids prior to fatty acid analysis (193). In this procedure the measured palmitic acid appears to be derived from lecithin, principally dipalmitoyl lecithin, because the nonphospholipid sources (triglycerides and free fatty acids) of palmitic acid are removed by the hexane/2-propanol/sulfuric acid solvent extraction. This procedure's details include the following (193):

- Amniotic fluid samples are centrifuged at 250 x g for 5 minutes and the resulting supernatant fluid which is free from cell debris and sediment, is kept frozen at -20°C until analyzed.
- To 2 mL of the centrifuged amniotic fluid sample, a hexane/2-propanol/sulfuric acid solvent extraction mixture is added. In this step the phospholipid is extracted and a linoleic acid
internal standard is added to the mixture.

- The residue from the extraction step is reconstituted with two drops of methanol and the palmitic acid ester concentration of this solution was analyzed by gas-liquid chromatography.

- The phospholipid palmitic acid content of amniotic fluid is expressed in mg/L from a linear calibration curve of palmitic acid concentration (in micrograms) versus the ratio of the palmitic acid and linoleic acid peak areas.

Following is a summary of the Ip et al. study's results (193):

- Figure 68 illustrates a typical gas-liquid chromatogram of the amniotic fluid phospholipid fatty acid esters obtained by the Ip et al. procedure (193).

- Table XXIV shows the amount of selected lipid components present in human amniotic fluid at 40 weeks gestation of normal pregnancy in seven healthy patients (no complications or previous high-risk pregnancies) (193). The mean values obtained for phospholipids, triglycerides, and free fatty acids from these seven patients are $51.0 \pm 16.2 \text{ mg/L}$, $68.6 \pm 25.0 \text{ mg/L}$, and $0.40 \pm 0.21 \text{ mmol/L}$, respectively (as shown in the right-hand mean value column) (193).

- Ip et al. (193) compared their GLC method (extraction removal of nonphospholipid sources of palmitic acid) to a selected GLC method, by Warren et al. (194) where both triglycerides and free fatty acids were extracted with the phospholipids. The two methods are compared in Table XXV which shows a remarkable
FIGURE 68
GAS-LIQUID CHROMATOGRAM OF THE AMNIOTIC FLUID
PHOSPHOLIPID FATTY ACID ESTERS

Legend

Figure illustrates typical gas-liquid chromatogram of the amniotic fluid phospholipid fatty acid esters as determined by the Ip et al. method. Arrow indicates position of the reference standard.

<table>
<thead>
<tr>
<th>Patient</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
<td>VI</td>
<td>VII</td>
</tr>
<tr>
<td>Phospholipids (mg/L)</td>
<td>43.8</td>
<td>37.5</td>
<td>35.0</td>
<td>61.3</td>
<td>40.3</td>
<td>46.3</td>
<td>82.0</td>
</tr>
<tr>
<td>Mono-, di- and triglycerides (mg/L)</td>
<td>81.7</td>
<td>42.6</td>
<td>62.5</td>
<td>74.3</td>
<td>103.1</td>
<td>31.2</td>
<td>64.6</td>
</tr>
<tr>
<td>Free fatty acids (mMol/L)</td>
<td>0.73</td>
<td>0.21</td>
<td>0.18</td>
<td>0.44</td>
<td>0.37</td>
<td>0.26</td>
<td>0.62</td>
</tr>
</tbody>
</table>

*The results are the mean of two gas-chromatographic measurements made on each sample.

*b Estimated as total phospholipid phosphorus x 25.

*c The modified method of Rafter and Ko as described by Moses et al. (1970) was used, except used 3.0 mL of amniotic fluid instead of 0.3 mL of serum. The volume of 2-propanol and "lipo-Frax" columns used was increased by 10-fold to adjust for the larger sample size. The 2-propanol eluate was concentrated to 3.7 mL for color development.*


TABLE XXV
AMNIOTIC FLUID PHOSPHOLIPID PALMITIC ACID,
AS DETERMINED BY TWO GAS–CHROMATOGRAPHIC METHODS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Measured palmitic acid (mg/L)</th>
<th>Warren et al. composition method b</th>
<th>Ip et al. present method</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10.0 ± 0.6</td>
<td>6.7 ± 0.3</td>
<td>33.0</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>11.2 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>10.9 ± 0.4</td>
<td>8.8 ± 0.3</td>
<td>19.3</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>19.1 ± 0.5</td>
<td>12.5 ± 0.3</td>
<td>34.6</td>
<td></td>
</tr>
</tbody>
</table>

a Data presented represents mean of duplicates ± SD; each measurement was made in duplicate.


difference in the measured palmitic acid concentrations, for in-
patient II there was 42.9% palmitic acid difference—this
represents phospholipid and the nonphospholipid fraction (193).
The other three patient samples studied all showed a decrease of
at least 19.3% by the Ip et al. (193) (specific) method as
gcompared to the Warren et al. (194) (nonspecific) method.

- They also studied the correlation between palmitic acid and the
  L/S ratio in 60 different samples with a correlation coefficient
  of \( r = 0.96 \) (193). At this coefficient the palmitic acid is 8.0
  mg/l while the L/S ratio equals 2.0 (8.0 mg of palmitic acid
  per liter corresponds to 11.5 mg of dipalmitoyl lecithin per
  liter). Infants are unlikely to develop RDS when the measured
  palmitic acid in amniotic fluid exceeds 8.0 mg/l.
  (corresponding to an L/S of 2.0) (193). Figure 69 shows the
  significant changes in phospholipid palmitic acid concentration at
  different stages of gestation with 59 samples. As depicted in
  Fig. 69, the palmitic acid in amniotic fluid increases
  significantly after 37 weeks gestation, for palmitic acid values
  of <4.5 mg/l correspond to gestation periods of <35 weeks.

- Additionally Ip et al. studied the correlation of palmitic acid
  concentration with that of total phospholipid phosphorus with a
  resulting correlation coefficient of \( r = 0.92 \) (193). The 8.0 mg/L
  concentration of palmitic acid is equivalent to 1.6 mg of total
  phospholipid phosphorus per liter, which is close to the 1.4 mg/L
  value reported by Nelson & Lawson (195).
FIGURE 69
PALMITIC ACID LEVELS VERSUS GESTATIONAL PERIODS

Legend

Figure illustrates scatter diagram of the measured palmitic acid levels versus gestational periods with 59 samples. The palmitic acid in amniotic fluid increases significantly after 37 weeks gestation, for palmitic acid values of < 4.5 mg/L correspond to gestation periods of < 35 weeks.

In conclusion, the Ip et al. specific method appears to be a reliable index for fetal pulmonary status, for it actually measures palmitic acid of dipalmitoyl lecithin, and eliminates the nonphospholipid sources of palmitic acid by solvent extraction (193). However, its major disadvantages are length of test time (3 hours) and laboratory expertise required. Also additional thorough sample testing of this method would be required to give a total palmitic acid concentration graph versus gestation age and various high-risk and complicated pregnancy conditions would have to be included so that a standardized cut-off value for fetal pulmonary maturity could be established.

(c). Contamination

The effect blood components have on the results of phospholipid palmitic acid was studied by Ip et al. and is shown in Table XXVI (193). Blood components were added to two different specimens of amniotic fluid and resulted in no appreciable increase in measured palmitic acid when serum or hemolyzed serum components were present in the amniotic fluid. However, the presence of whole blood (25% by volume) in amniotic fluid greatly increased the values of phospholipid palmitic acid. This increase in measured palmitic acid is attributed to the presence of large amounts of palmitic acid in components of cellular constituents, and these must be eliminated from the amniotic fluid sample before analysis (193). Therefore, samples contaminated with blood should be excluded.

The effect meconium has on palmitic acid has not been documented.

(d). Summary

Theoretically, it may be concluded that the palmitic acid
TABLE XXVI

EFFECT OF PRESENCE OF BLOOD COMPONENTS ON
RESULTS OF PHOSPHOLIPID PALMITIC ACID IN AMNIOTIC FLUID

<table>
<thead>
<tr>
<th>Blood component</th>
<th>Palmitic acid measured</th>
<th>% change</th>
<th>Palmitic acid measured</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/L</td>
<td></td>
<td>mg/L</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.8</td>
<td>---</td>
<td>21.5</td>
<td>---</td>
</tr>
<tr>
<td>Whole blood (25%)</td>
<td>23.0</td>
<td>95.0</td>
<td>46.5</td>
<td>116.3</td>
</tr>
<tr>
<td>Serum (25%)</td>
<td>12.3</td>
<td>4.5</td>
<td>21.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Serum from hemolyzed blood (25%)</td>
<td>12.7</td>
<td>8.3</td>
<td>23.0</td>
<td>6.9</td>
</tr>
</tbody>
</table>

a Results represent the average of duplicate determinations.
b Blood components used on patient I and II were from different sources.
c Blood caused to hemolyze by forcing it through a syringe.

concentration in amniotic fluid is perhaps as reliable a "variancy of RDS as is the L/S ratio, but the latter can be estimated by much simpler methods, which many laboratories have found easy to establish as routine procedure. Only more extensive investigations will show if measurement of palmitic acid will have value as a second-line test when an equivocal intermediate L/S ratio has been found. Furthermore, in some cases of RDS that the L/S ratio has failed to predict (false positive), the seemingly adequate amounts of lecithin may consist largely of a less strongly surface active variety than that with the mature and highly effective dipalmitoyl configuration; therefore in such cases a misleading adequate L/S ratio would result in a relatively low palmitic acid determination.

The Ip et al. procedure, which is specific for dipalmitoyl lecithin determination, because they extract the nonphospholipid sources of palmitic acid, is very promising, however has the disadvantages of length of test time and laboratory expertise required for GLC (193). Therefore this lengthy procedure seems to offer no advantage over the determination of disaturated lecithin by the L/S ratio, a method which is likely to be more specific for surfactant.

6. Lamellar Body Analysis

(a). Principle

Production of surfactant in the lungs begins before birth. Lamellar bodies, present in the type II cells of the alveolar epithelium are the major source of pulmonary surfactant phospholipid. As the fetal lung matures, the membranous content of the lamellar bodies is secreted into the potential air spaces of the lung and passes into the amniotic
fluid, where it accumulates as gestation proceeds. Various methods for assessing fetal lung maturity depend on the accumulation of the lamellar body-derived phospholipid in amniotic fluid.

(b). Procedures

Two procedures, the 10,000 x g pellet method and the LB-PL method (lamellar body phospholipid) have been developed for the assessment of fetal pulmonary maturity by phospholipid analysis of amniotic fluid lamellar bodies and are routinely utilized. A third experimental method, called the fluorometric procedure for determining lamellar body phospholipids in the particulate fraction of amniotic fluid after filtration, will also be discussed.

(i). 10,000 x g Pellet Method

In 1980, Oulton et al. (150) developed the 10,000 x g pellet method and in 1982 adopted the method for routine usage at their center, replacing the L/S ratio method (196). When present in amniotic fluid, lamellar body structures can be quantitatively harvested as a pellet fraction by centrifugation for 20 minutes at 10,000 x g. Phospholipid analysis of this fraction obtained from human amniotic fluid at various gestational ages revealed a developmental profile which correlated with individual stages in fetal pulmonary maturation.

1. Procedure

Oulton et al. procedure consists of the following steps which were utilized on 940 samples of amniotic fluid (196):

- Initially samples were centrifuged at 140 x g for 5 minutes to remove cellular debris. This supernatant was then centrifuged at
10,000 x g for 20 minutes and the resulting supernatant discarded.
- The remaining 10,000 x g pellet was suspended in sodium chloride and the lipids were extracted and the following analysis were performed on the resultant chloroform layer.
- The lipids were separated on silica gel G plates by two-dimensional TLC, and then were visualized by exposure of the plates to iodine vapor.
- Analysis of the phospholipid composition consisted of scraping each individual phospholipid from the plate and quantitating it by measurement of its phosphorus content. The results for each was expressed as a percentage of the total lipid phosphorus. Then each sample was assigned to one of the five stages of lung maturity on the basis of phospholipid composition. The PCh value was defined as the lecithin phosphorus content of the 10,000 x g pellet fraction obtained from 10.0 mL of amniotic fluid (corrections were made for <10 mL volumes).

2. Results

Analysis of the phospholipid composition resulted in a derivation of the following stages of a developmental profile (Table XXVII) (196):

Stage I is the earliest stage (14-18 weeks), termed presurfactant because it contains nonlamellated structures. The most characteristic features of the phospholipid composition at this stage are that sphingomyelin is the most abundant phospholipid (38.9%), PG is absent, and PI is present in low proportion (2.3%). Figure 70A depicts this
### TABLE XVII
CONCENTRATION AND COMPOSITION OF STRUCTURES PRESENT IN 10,000 x g PELLET AT VARIOUS DEVELOPMENTAL STAGES

<table>
<thead>
<tr>
<th>Electron Microscopy/Development stage</th>
<th>Gestation (Week)</th>
<th>Phospholipid concentration (μg/mL)</th>
<th>PC</th>
<th>PG</th>
<th>PI</th>
<th>LPS</th>
<th>PE</th>
<th>Palmitic acid (% of lecithin fatty acid)</th>
<th>Respiratory status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Nonlamellated vesicles/presurfactant</td>
<td>14-18</td>
<td>(1) ≤5 (2) (4.0) (28.1) (0) (10) (2.3) (38.9) (13.5) (17.3)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>No Infants Delivered</td>
</tr>
<tr>
<td>II No electron microscopy performed/Onset of surfactant synthesis</td>
<td>30</td>
<td>(1) ≤25 (5) SPH (0) (10) (2.3) (13.5) (17.3)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>100% RDS</td>
</tr>
<tr>
<td>III Lamellar bodies/early lamellar body</td>
<td>34-36</td>
<td>(1) ≤75 (2) (52.3) (72.8) (0) (13.5) (4.2) (3.2) (5.3) (80.0)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>75% RDS 84.6% RDS and TRD</td>
</tr>
<tr>
<td>IV Lamellar bodies—appearance of PG/biochemical maturation of lamellar bodies</td>
<td>36-37</td>
<td>(1) ≤50 (2) (52.3) (72.8) (0) (13.5) (4.2) (3.2) (5.3) (80.0)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0% RDS in ODM 50% RDS in GDM</td>
</tr>
<tr>
<td>V Lamellar bodies—accumulation of PG/biochemical maturation of lamellar bodies</td>
<td>38-40</td>
<td>(1) ≤50 (2) (52.3) (72.8) (0) (13.5) (4.2) (3.2) (5.3) (80.0)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0% RDS 1.7% TRD</td>
</tr>
</tbody>
</table>

---

*Values shown for the phospholipid concentration and composition represent a summarization of data obtained from 6 to 117 specimens at each stage. Electron microscopy was performed on 1 to 5 individual specimens for each stage indicated. The gestations represent the approximate time which each stage was first observed. The stage of development of the fetal lung surfactant system was assigned on the basis of these analyses.

*Electron microscopy was performed on five specimens at 14 to 18 week gestation and 10 specimens from 36 week to postterm. Chemical analysis represent results obtained from one specimen at each stage indicated. Those of 36 and 42 week gestation were from the same patient.

Abbreviations: PC, lecithin; PI, phosphatidylinositol; PG, phosphatidylglycerol; SPH, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; TRD, transient respiratory distress; ODM, overt diabetics.

FIGURE 70.

STAGE I AND V DEVELOPMENTAL PROFILE AND CHARACTERISTIC

CHROMATOGRAMS FOR THE DEVELOPMENTAL CHANGES IN STAGES I, III, AND V.

Legend

A. Electron micrograph of early gestation pellet (14 to 18 weeks).

Stage I). Membrane-bound vesicles (V) present, but absence of lamellated structures. (x 15,000)

B. Electron micrograph of late gestation pellet (36 weeks Stage V) depicting lamellar bodies (LB). (x 7,200)

C. Figure illustrates representative chromatograms at the stages of development indicated by Roman numerals. The spot labeled GL is a glycolipid. The unlabeled spot at Stage I is tentatively identified as a glycolipid.


Stage II is the stage when the onset of surfactant synthesis begins (≈ 30 weeks). The most characteristic features of the phospholipid composition of this stage are the significant increase in PC, and PL, while PG is still absent and sphingomyelin has significantly decreased in value.

Stage III is the stage termed early lamellar body, since it represents the stage at which lamellar bodies were first identified by electron microscopy (34–36 weeks). The most characteristic features of the phospholipid composition of this stage are that both PC (72, 87) and PL (13, 77) have approached their maximum values, and PG is still absent.

Stage IV and V are the final stages (36–37 weeks) and are termed biochemical maturation of the lamellar bodies and are characterized by the appearance and accumulation of PC. As PC accumulates, there is a concomitant decrease in PI. See Fig. 70B depicting Stage V.

Serial analysis on individual patients revealed developmental progression of the phospholipid composition that fit into the five stages of the profile. Oulton found that PG never appeared until PC represented approximately 80% of the total phospholipid, and PI reached its maximum value (196). It was also observed that there was considerable variation in the gestational age at each of the stages (i.e., Stage IV was detected as early as 20 weeks, and Stage III lasted as long as 40 weeks gestation).

Figure 70C, depicts Stages I, III, and V characteristic chromatograms for the developmental changes in phospholipid concentration.
with progression through these stages.

Table XXVII depicts the respiratory status of infants delivered at various stages of development and the corresponding incidence of RDS. Oulton found that with delivery at increasingly mature developmental stages, not only the incidence of RDS, but the severity of the disease decreased (196).

3. Comments

Technically, the most critical aspect of this procedure is strict adherence to the centrifugation conditions recommended for isolating the 10,000 x g pellet. Possible sources of nonsurfactant phospholipid in this procedure are the sphingomyelin-rich granules which have been found at 14 to 18 weeks gestation, plasma membrane fragments, or other membranous cellular debris. However, some of these should be removed at the initial 140 x g centrifugation.

Oulton et al. did not utilize the cold acetone precipitation step because the resultant phospholipid analysis of the precipitate did not reflect the true composition of surfactant and the step added additional test time (196). Therefore, they utilized the isolated lamellar body fraction for analysis because it provides more precise information.

Concerning the topic of one-dimensional versus two-dimensional thin-layer chromatography for lipid separation, Oulton utilized the two-dimensional system because it clearly separated all the lipid components, even though it took two hours (196).

Oulton et al. utilized chemical analysis for quantitation of the phospholipids because it provided them with the most reliable results.
<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Intervals 0 - 2 days</th>
<th>Incidence of RDS 3 - 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4/4</td>
<td>1/1</td>
</tr>
<tr>
<td>II</td>
<td>11/13*</td>
<td>6/9*</td>
</tr>
<tr>
<td>III</td>
<td>12/23</td>
<td>3/22</td>
</tr>
<tr>
<td>IV</td>
<td>3/45+</td>
<td>1/32</td>
</tr>
<tr>
<td>V</td>
<td>4/274</td>
<td>0/164</td>
</tr>
</tbody>
</table>

*No RDS: All cases had prematurely ruptured membrane with preterm labor, steroids >24 hours, and were vaginal deliveries.
+ RDS: All were infants of diabetic mothers (two class C, one class R).

4. Contamination Effects

This method is not subject to interference by phospholipids present in blood since whole cells are removed at 140 x g and serum lipoproteins do not sediment at 10,000 x g. Meconium, if present in high concentration in a sample containing little surfactant, may contribute significantly to the 10,000 x g pellet phospholipid, but does not contribute enough phospholipid to interfere when the surfactant concentration is high.

(ii). The Lamellar Body Phospholipid (LB-PL) Method

In 1979, Duck-Chong (197) developed this LB-PL method and in 1981 Duck-Chong et al. (198) adopted this method for usage in a routine clinical laboratory. The basis for most tests for assessing fetal lung maturity is the detection of a sufficiently high concentration of surfactant phospholipid in amniotic fluid. However, interpretation of such tests may be complicated by the fact that amniotic fluid contains phospholipid from sources other than the lamellar bodies of the fetal lung. This method to be described here, was devised for evaluating fetal lung maturity by measuring the concentration of lamellar body phospholipid (LB-PL) in amniotic fluid, after separating it from other sources of phospholipid by isopycnic density-gradient centrifugation (198). Because surfactant has a high phospholipid/protein ratio, it has an unusually low density. Therefore the surfactant cannot pass through the Ficoll cushion, so it collects at the interface between the sample and the Ficoll layer, forming a clearly visible band. This LB-PL method
by Duck-Chong et al. correctly predicted lung maturity in cases which the L/S ratio assessed as immature (198). Also Duck-Chong et al. have assessed this LB-PL method and clinically tested 451 samples of amniotic fluid, which were collected within two days of delivery.

1. Procedure

The LB-PL content of the amniotic fluid was determined by the following procedure (198):

- Whole amniotic fluid is mixed before sampling and then 100 µL of fluid is carefully layered over 75 µL of Ficoll solution and centrifuged for 15 minutes at 173 kPa in the A-100 rotor of a Beckman Airfuge.

- The supernate is separated by discarding the upper portion, and removing the interface lamellar body fraction. Then the lamellar body fraction is washed, mixed, and centrifuged twice with chloroform/methanol/saline and the aqueous upper layer is removed.

- Then the chloroform layer is placed in a test tube containing Mg(NO₃)₂ solution and evaporated in a water bath at 85-100°C.

- At this point, phospholipid phosphorus was converted to inorganic phosphate and determined.

- Also, simultaneously the determination of total phospholipid was done, by adding 50 µL of amniotic fluid to 3 mL of chloroform/methanol at the time of sampling for LB-PL determination and processed along with the chloroform/methanol extracts of the lamellar body fraction. Duck-Chong et al.
standard practice was to include this assessment of total phospholipid with every LB-PL determination because it required only a little additional work, and served to confirm the LB-PL results (198).

2. Results

This three-year clinical trial of the LB-PL method, involving 451 pregnancies, revealed that the incidence of RDS is inversely related to the concentration of the lamellar body phospholipid (LB-PL) in the amniotic fluid.

The results reveal that RDS is associated with a low concentration of LB-PL, <35 mg/L. Tables XXIX, XXX, and XXXI summarize the results, which are the following (198):

- Among infants delivered within two days of sampling, there were thirteen cases of RDS with LB-PL value ranging from 10 to 47 mg/L (Table XXIX A).
- In infants delivered three to seven days after sampling there were four cases of RDS, with LB-PL values of <35 mg/L (Table XXIX B).
- Also, two infants with LB-PL values of <35 mg/L suffered serious respiratory problems which were related to lung immaturity (Table XXIX C).
- Table XXX shows the 422 pregnancies (out of 427), where the infants were free of respiratory problems with LB-PL values of >35 mg/L. 90 of these infants were delivered preterm at 33-37 weeks gestation, when the risk of RDS is high.
- Table XXXI shows that nine infants developed transient tachypnea
TABLE XXII
THE LAMELLAR BODY PHOSPHOLIPID CONTENT OF AMNIOTIC FLUID FROM ALL PREGNANCIES
RESULTING IN SERIOUS RESPIRATORY PROBLEMS

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Gest. age, weeks</th>
<th>Appear at 1.5 min</th>
<th>LB/pl. mg/L</th>
<th>Days to delivery</th>
<th>Maternal condition</th>
<th>Steroid given</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M</td>
<td>32</td>
<td>4.8</td>
<td>10</td>
<td>0</td>
<td>Toxemia</td>
<td>22 h</td>
</tr>
<tr>
<td>2F</td>
<td>28</td>
<td>1.7</td>
<td>15</td>
<td>0</td>
<td>Toxemia</td>
<td>20h</td>
</tr>
<tr>
<td>3F</td>
<td>30</td>
<td>3.2</td>
<td>16</td>
<td>0</td>
<td>Toxemia</td>
<td>17 days &amp; 43 h</td>
</tr>
<tr>
<td>4F</td>
<td>31</td>
<td>6.6</td>
<td>18</td>
<td>1</td>
<td>Toxemia</td>
<td>4 days</td>
</tr>
<tr>
<td>4M</td>
<td>33</td>
<td>6.9</td>
<td>18</td>
<td>2</td>
<td>RH incompatibility</td>
<td>45 h</td>
</tr>
<tr>
<td>6M</td>
<td>34</td>
<td>6.9</td>
<td>18</td>
<td>0</td>
<td>Diabetes</td>
<td>17 h</td>
</tr>
<tr>
<td>7M</td>
<td>32</td>
<td>5.8</td>
<td>20</td>
<td>2</td>
<td>Essential hypertension</td>
<td>--</td>
</tr>
<tr>
<td>8M</td>
<td>30</td>
<td>7.8</td>
<td>21</td>
<td>0</td>
<td>Toxemia</td>
<td>14 h</td>
</tr>
<tr>
<td>9M</td>
<td>34</td>
<td>6.9</td>
<td>22</td>
<td>0</td>
<td>Toxemia</td>
<td>4 days</td>
</tr>
<tr>
<td>10M</td>
<td>34</td>
<td>5.7</td>
<td>23</td>
<td>24</td>
<td>Essential hypertension</td>
<td>--</td>
</tr>
<tr>
<td>11M</td>
<td>33</td>
<td>4.8</td>
<td>27</td>
<td>0</td>
<td>Toxemia</td>
<td>--</td>
</tr>
<tr>
<td>12M</td>
<td>28</td>
<td>3.6</td>
<td>38</td>
<td>0</td>
<td>Toxemia</td>
<td>4 days</td>
</tr>
<tr>
<td>13F</td>
<td>37</td>
<td>3.4</td>
<td>47</td>
<td>0</td>
<td>Diabetes, gestational</td>
<td>--</td>
</tr>
<tr>
<td>B. RMD, delivered more than two days after sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14F</td>
<td>38</td>
<td>6.9</td>
<td>11</td>
<td>5</td>
<td>Essential hypertension</td>
<td>--</td>
</tr>
<tr>
<td>15M</td>
<td>36</td>
<td>6.9</td>
<td>18</td>
<td>7</td>
<td>Growth retardation</td>
<td>--</td>
</tr>
<tr>
<td>16F</td>
<td>35</td>
<td>-7.9</td>
<td>20</td>
<td>3</td>
<td>Placenta previa</td>
<td>--</td>
</tr>
<tr>
<td>17F</td>
<td>35</td>
<td>9.9</td>
<td>33</td>
<td>3</td>
<td>Premature labor</td>
<td>16 days &amp; 15 h</td>
</tr>
<tr>
<td>C. Other serious respiratory problems</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18M</td>
<td>36</td>
<td>9.9</td>
<td>9</td>
<td>0</td>
<td>RH incompatibility</td>
<td>--</td>
</tr>
<tr>
<td>19M</td>
<td>35</td>
<td>2.5</td>
<td>32</td>
<td>1</td>
<td>Premature labor</td>
<td>--</td>
</tr>
</tbody>
</table>

M. male; F, female. Indicates time between commencement of glucocorticoid administration and delivery. Infant died 8-72 h after delivery; hyaline membranes present in lungs (postmortem examination). In these cases, betamethasone was used, as recommended by Liggins et al. (11). In the remaining cases, the mothers were given in intravenous infusion of 1 g of hydrocortisone in 50 g/L dextrose during 12 h. Fetal heart rate dropped to 70 beats/min after amniocentesis; delivered by emergency cesarean section, severely asphyxiated. Exhibited all the clinical symptoms of RMD, but showed only minor changes on chest roentgenogram. Died of bilateral pleural effusion at 8 h.

<table>
<thead>
<tr>
<th>No. cases</th>
<th>LB-PL, mg/L</th>
<th>Days to delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 15 weeks gestation or less:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>127 (56-280)</td>
<td>1-2</td>
</tr>
<tr>
<td>B. 15-37 weeks gestation:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>38 (38)</td>
<td>0.2</td>
</tr>
<tr>
<td>9</td>
<td>45 (40-49)</td>
<td>0-2</td>
</tr>
<tr>
<td>30</td>
<td>120 (52-325)</td>
<td>0-2</td>
</tr>
<tr>
<td>C. &gt;37 weeks gestation:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>44 (40-49)</td>
<td>0-2</td>
</tr>
<tr>
<td>316</td>
<td>128 (51-614)</td>
<td>0-2</td>
</tr>
</tbody>
</table>

aSee reference for definition of minimal respiratory distress.

bWhere data have been pooled, the value represents the mean; the range of values is given in parentheses.

### TABLE XXXI

**LAMELLAR BODY PHOSPHOLIPID (LB-PL) CONTENT OF AMNIOTIC FLUID FROM ALL PREGNANCIES RESULTING IN TRANSIENT TACHYPNEA**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Gest. age, Weeks</th>
<th>Apgar at 1,5 min.</th>
<th>LB-PL, mg/L</th>
<th>Days to delivery</th>
<th>Maternal condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>5, 10</td>
<td>27</td>
<td>0</td>
<td>Toxemia</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>8, 9</td>
<td>35</td>
<td>0</td>
<td>Elective C.S.</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>2, 9</td>
<td>38</td>
<td>1</td>
<td>Toxemia</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>9, 9</td>
<td>45</td>
<td>0</td>
<td>Elective C.S.</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>6, 9</td>
<td>45</td>
<td>2</td>
<td>Growth retardation</td>
</tr>
<tr>
<td>6</td>
<td>33(\frac{1}{2})</td>
<td>7, 7</td>
<td>49</td>
<td>1</td>
<td>Premature labor</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>4, 7</td>
<td>52</td>
<td>1</td>
<td>Elective C.S.</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>5, 8</td>
<td>55</td>
<td>0</td>
<td>Diabetes, gestational</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>5, 4</td>
<td>77</td>
<td>2</td>
<td>Femoral thrombosis with heparin therapy</td>
</tr>
</tbody>
</table>

\(^a\)All infants were male.

\(^b\)C. S. = Cesarean section.

with LB-PL values ranging from 27 to 77 mg/L. Duck-Chung et al. concluded that there was a marked tendency for transient tachypnea to be associated with LB-PL values between 25 and 50 mg/L, suggesting that this condition may occur when the fetal lungs are on the borderline between immaturity and maturity (198).

- Figure 71 summarizes the incidence of respiratory problems at various LB-PL concentrations which should be used in diagnosis. The LB-PL concentrations are the following: <25 mg/L indicates a very high risk for RDS or other serious respiratory problems and delivery should be avoided if possible; a 50% risk of RDS exists between 25 and 35 mg/L; and a slight risk between 35 and 50 mg/L exists. A LB-PL value of 50 mg/L or more can be regarded as a clear indication of lung maturity and that the infant will be free of RDS. Figure 71B depicts the data for the 112 preterm infants (37 weeks of gestation or less).

- One of the major disadvantages of the L/S ratio method is its assessment of fetal lung maturity in pregnancies complicated by maternal diabetes. However, the LB-PL method seems to have given accurate predictions of fetal lung status in the presence of maternal diabetes (Table XXXII). More data is required for LB-PL values of 50 mg/L or less to totally confirm this impression.

3. Comments

Technically, the following points are very critical to the LB-PL method: Before sampling, the amniotic fluid should be mixed well to prevent aggregates of lamellated structures from settling to the
FIGURE 71

RELATIONSHIP BETWEEN RESPIRATORY PERFORMANCE OF THE NEWBORN AND THE LAMELLAR BODY PHOSPHOLIPID CONCENTRATION

Legend

Figure illustrates relationship between respiratory performance of the newborn and the LB-PL concentration of amniotic fluid collected within 2 days of delivery. Figure summarizes the incidence of respiratory problems at various LB-PL concentrations which should be used in diagnosis, such as: <25 mg/L indicates a very high risk for RDS or other serious respiratory problems and delivery should be avoided if possible; a 50% risk of RDS exists between 25 and 35 mg/L and a slight risk between 35 and 50 mg/L exists; and a LB-PL value of 50 mg/L or more can be regarded as a clear indication of lung maturity and that the infant will be free of RDS. Figure A illustrates all data; Figure B depicts the data for the 112 preterm infants (37 weeks of gestation or less). Black area, HMD and cases 18 and 19, hatched area, transient tachypnea; open area, no respiratory problems or minimal respiratory distress.

FIGURE 71

[Graph showing incidence as a function of LB PL (mg/L)]


<table>
<thead>
<tr>
<th>No. cases&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gest. age, weeks</th>
<th>LB-PL, mg/L</th>
<th>Days to delivery</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>18</td>
<td>0</td>
<td>HMD, severe</td>
</tr>
<tr>
<td>1 (1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37</td>
<td>47</td>
<td>0</td>
<td>HMD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (1)</td>
<td>36, 37</td>
<td>49</td>
<td>1-2</td>
<td>No RD&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 (1)</td>
<td>36</td>
<td>55</td>
<td>0</td>
<td>Transient tachypnea</td>
</tr>
<tr>
<td>3 (2)</td>
<td>38-40</td>
<td>51-56</td>
<td>2</td>
<td>No RD</td>
</tr>
<tr>
<td>4 (1)</td>
<td>36-40</td>
<td>60-65</td>
<td>0-2</td>
<td>No RD</td>
</tr>
<tr>
<td>10 (2)</td>
<td>35-40</td>
<td>70-99</td>
<td>0-2</td>
<td>No RD</td>
</tr>
<tr>
<td>27 (11)</td>
<td>34-41</td>
<td>102-232</td>
<td>0-2</td>
<td>No RD</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of gestational diabetics indicated in parentheses.

<sup>b</sup> See case 6, Table XXIX.

<sup>c</sup> Severely asphyxiated; see case 13, Table XXIX.

Abbreviation: RD = respiratory distress.

bottom of the tube. Because of its critical density, molecular weight and viscosity, Ficoll 70 should be utilized as the cushioning material in the Airfuge procedure. Also, the amniotic fluid must be layered carefully over the Ficoll to give a sharp interface. A reliable inorganic phosphate standard is essential. Also, because of the sensitivity of the inorganic phosphate method utilized, all glassware must be acid-washed and care must be taken to avoid contamination.

Two potential limitations of the procedure were cited by Duck-Chong et al., which are the following (198). Theoretically, the LB-PL procedure is not as specific as the original large-scale procedure, which involved a continuous sucrose density gradient (197). Any phospholipid-containing material with a density between that of the Ficoll layer and amniotic fluid will collect at the Ficoll/sample interface during centrifugation, and therefore contribute to the LB-PL value. However, this potential limitation does not appear to cause problems in practice, for the LB-PL value obtained by the Airfuge procedure reflects that determined by the original density-gradient technique over a wide range of LB-PL concentrations (197).

In addition, non-sedimentable phospholipid may contribute to the LB-PL value, because a small volume of the upper layer inevitably is removed when sedimented material is collected from the interface. However, this potential limitation does not appear to cause problems in practice for non-sedimentable phospholipid in amniotic fluid is exceptionally rich in sphingomyelin (123), but lamellar body fractions isolated from amniotic fluid by the Airfuge procedure and conventional density gradient
techniques contain little sphingomyelin relative to lecithin (199).

4. Contamination Effects

Currently the effects of blood and meconium contamination on the LB-PL method are being investigated. The following contamination effects have been determined: 1% blood concentration contributes negligible phospholipid to the LB-PL value; and it has been found that amniotic fluid samples heavily contaminated with meconium resulted in LB-PL values as low as 30 to 40 mg/L therefore, indicating that meconium does not contribute more than this amount of phospholipid to the LB-PL value.

However, both the effects of blood and meconium must be further investigated for their clinical interpretation on the low and borderline LB-PL values.

(iii). Fluorometric Procedure

In 1983, Egberts et al. described a rapid (30 min) semiautomated continuous-flow procedure for use in assessing the phospholipids of the lamellar body fraction of human amniotic fluid (200). The method is based on measuring the difference in fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH) added to amniotic fluid before and after micropore filtration (200). The filtration step removes lamellar body particles, which are considered to contain the fetal lung surfactant. The phospholipid values for the filtered particles are independent of background fluorescence, which increases when amniotic fluid is contaminated by bilirubin pigments or blood components. In the Egberts et al. procedure the fluorescence is measured before and after
filtration, so that the fluorescence derived from the LP fraction can be calculated (200). To shorten the time needed for determination, the fluorescence procedure was automated and the LP values obtained by fluorometry were compared with the corresponding values obtained by colorimetry and L/S ratios (Merkus et al., (201) with acetone precipitation).

1. Procedure

The procedure involves the following steps (200).

- Amniotic fluid samples were promptly centrifuged for 10 minutes at 1500 x g. Then the fluid was analyzed colorimetrically and fluorometrically.

- The method of Bhagwanani et al. was utilized for the colorimetric determination of the total phospholipid content (TPL) of amniotic fluid in terms of phosphorus (202).

- The fluorometric analysis utilized 0.3 mL of unfiltered amniotic fluid or 0.6 mL of the filtrate which was diluted to 3 mL with saline and introduced into the automatic sampler (Fig. 72A). The automatic procedure's arrangement included: a four-channel roller pump, a thermostated mixing coil (43°C); and a fluorometer fitted with a mercury lamp and filters at 365nm and 460 nm. The sampling rate was 30 per hour with alternate saline washings interspersed between samples. The emission signal was recorded within 12 minutes of sampling. The fluorescence intensity of the phospholipid standard was utilized to determine the phospholipid content of the filtered and unfiltered amniotic fluid samples.
FIGURE 72

FLUOROMETRIC AUTOMATED PROCEDURE FOR THE LAMELLAR BODY FRACTION
AND THE RELATIONSHIP BETWEEN THE FLUOROMETRIC AND COLORIMETRIC RESULTS

Legend

A. Figure illustrates the manifold diagram of the automated method for determining phospholipid content. The automatic procedures arrangement includes: a four channel roller pump; a thermostated mixing coil (43°C); and a fluorometer fitted with a mercury lamp and filters at 365 nm and 460 nm. The sampling rate is 30 per hour with alternate saline washings interspersed between samples. The emission signal was recorded within 12 minutes of sampling. The fluorescence intensity of the phospholipid standard was utilized to determine the phospholipid content of the filtered and unfiltered amniotic fluid samples.

B. Illustrates relationship between the fluorometric and colorimetric method for estimating the lamellar body phospholipid (LP) concentration in amniotic fluid (left-hand side).

C. Illustrates the ratio between lamellar body phospholipids and total phospholipids (LP/TPL) as determined by both methods (right-hand side).

FIGURE 72

A. Flow 3.9

B. \( \mu \text{mol/L} \)

C. Fluorometric

D. Recorder

E. \( \text{prim: } 365 \text{ nm} \), \( \text{sec: } 400 \text{ nm} \)

\( r = .89 \)

\( r = .88 \)
The phospholipid concentration of the filtrate was subtracted from the total phospholipid value of the amniotic fluid before filtration to determine the concentration of the particulate lamellar body phospholipids for both procedures.

2. Results

Figure 72B shows the relationship between the fluorometric and colorimetric methods (left-hand graph) for estimating the lamellar body phospholipids (LP) concentration in amniotic fluid. The right-hand graph (Fig. 72C) shows the ratio between the lamellar body phospholipids and total phospholipids (LP/TPL) as determined by the two methods. Egberts et al. found good correlation between results of the fluorometric and colorimetric method for determining phospholipids for the estimation of LP concentration \((r = 0.89, n = 70)\) and the LP/TPL ratio \((r = 0.88)\) \((200)\). The LP/TPL ratio was included because, like the L/S ratio, it is independent of the amniotic fluid volume.

Table XXXIII shows the correspondence between the L/S ratio and fluorometric phospholipid values for amniotic fluid sampled from 30-37 weeks of gestation. There was disagreement between the L/S ratio and the LP or LP/TPL values observed in five of the 51 samples analyzed. The equivalent value of an L/S ratio of 2.0 was estimated by linear regression, and for the LP it was 18 \(\mu\)mol/L and for the LP/TPL ratio it was 0.35.

3. Comments

With Egberts et al. modification of the DPH phospholipid method, the phospholipid results are obtained 30 minutes
### TABLE XXXIII

**CORRESPONDENCE BETWEEN L/S RATIOS AND FLUOROMETRIC PHOSPHOLIPID VALUES FOR AMNIOTIC FLUIDS SAMPLED FROM 30-37 WEEKS OF GESTATION**

<table>
<thead>
<tr>
<th>L/S ratio</th>
<th>LP, mol/L</th>
<th>LP/TPL ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;16</td>
<td>16 - 20</td>
</tr>
<tr>
<td>&lt; 1.8</td>
<td>17</td>
<td>---</td>
</tr>
<tr>
<td>1.8-2.2</td>
<td>---</td>
<td>10</td>
</tr>
<tr>
<td>≥2.2</td>
<td>2</td>
<td>---</td>
</tr>
</tbody>
</table>

Abbreviations: L/S = lecithin/sphingomyelin, LP = lamellar body phospholipids, TPL = total phospholipids.

after amniocentesis and the procedure is relatively simple and quickly learned. The DPH method gives at least as good results as the usual colorimetric determination of phospholipid phosphorus because of the low CV and linearity even at low concentrations (200). However, the following factors affect the DPH method.

- Does not always give correct values for total phospholipid (TPL), because it is affected by such factors as intrinsic fluorescence and phospholipid class and degree of dispersion (200).
- Meconium and blood contaminated samples, and the yellow in amniotic fluid of patients with Rh-factor complications affect the DPH method by giving overestimated values, the result of high background fluorescence both in the filtered and unfiltered fractions of the amniotic fluid.
- This method is not specific for lamellar body phospholipids, for any particle of appropriate size could be retained in the LP fraction. However, it seems that most of the phospholipids in these fractions are of lamellar body origin, at least in amniotic fluid collected after the fetal lungs are mature (150).

The DPH fluorescence method was applied to amniotic material that was used for estimation of the L/S ratio. Oulton (196) determined the phospholipid content in the 10,000 x g pellet of amniotic fluid (lamellar body fraction) and found the same rate of disagreement between the LP and L/S ratio results as Egberts et al. did (200). However, the cut-off value between the two studies differed; Oulton et al. (196) was 53 μmol/L and Egberts et al. (200) had a much smaller value of 18 μmol/L.
Egberts et al. had lower values probably because their centrifugation of amniotic fluid samples was at 1500 x g, which decreases the amount of phospholipids in the supernatant fluid to 30-50% of the original quantity (200). This centrifugation speed was utilized because it was also part of the L/S ratio method that Egberts et al. routinely used (200). Optimal conditions of centrifugation remain to be determined because reduction of centrifugation speed to 140 x g would increase the amount of lamellar body material in the LP fraction, but could also increase the risk of contamination of the supernatant fluid, and hence the LP fraction, with other particulate matter.

4. Contamination Effects

As mentioned above, blood, meconium, and yellow-pigmented (samples from patients with Rh complications) amniotic fluid samples affect this DPH method by giving overestimated values, which is the result of high background fluorescence in both the unfiltered and filtered fractions.

(c). Summary

The 10,000 x g pellet procedure and the LB-PL method are both utilized by their perceptive investigators in their hospitals to measure fetal lung maturity. However, both appear to have disadvantages and discrepancies to prevent them from becoming clinically accepted.

The phospholipid composition of the 10,000 x g pellet provides precise information about the biochemical maturation of the fetal pulmonary surfactant system, for results can be fit into one of the five developmental profile stages, and the risk of developing RDS can be
fit into one of the five developmental profile stages, and the risk of
developing RDS can be determined. Also, it is important to emphasize
that, by measuring PC on the 10,000 x g pellet, only surfactant PC of the
lamellar bodies is being measured and when lamellar bodies are absent the
PC value reflects this. Also the presence or absence of detecting PG
(reported as percentage of PG) is greatly increased by performing this
analysis on the quantitatively isolated lamellar body fraction. The
presence of PG in amniotic fluid clearly indicates that the fetal lung
surfactant system is in its final stages of maturation. The major
disadvantage of this procedure is the length of time required to run it
(3 1/2 hrs). Also there was some discrepancy concerning its false
negative rate (absence of PG) which was higher when compared to others.
However, Oulton et al. explained that its false negative rate was mostly
confined to Stage III, and since the gestational time overlap of stages
varied, those infants at risk of developing RDS had barely reached Stage
III (196). Additional testing and adequate population sampling would
clarify this issue.

The LB-PL₄ is currently being used routinely for the evaluation of
fetal lung maturity at the King George V Hospital by Duck-Chong et al.
(198). This micro-procedure for isolating the lamellar body phospholipid
(LB-PL₄) fraction of amniotic fluid has proved useful in comparing it to
the L/S ratio, and for predicting fetal lung maturity in normal and
diabetic pregnancies. However, the limitations and technical critical
points must be considered in applying this method in the clinical
laboratory. Also its acceptance as an index of fetal lung maturity in
its own right must await the collection of more data in the critical preterm period (before the 35th week of gestation) and in high-risk pregnancy conditions.

Finally the DPH experimental fluorometric phospholipid estimation, combined with the step for lamellar body separation (which increases the specificity for estimating surfactant phospholipids) needs further evaluation and resolution of some factors before being utilized routinely. Also it must be established whether the LP and LP/TPL values are better than the L/S ratio in predicting RDS by additional clinical testing.

Even though the first two methods described are utilized in a clinical laboratory, much more extensive testing would need to be conducted for these lamellar body procedures to be adopted in a clinical laboratory setting.

7. Lecithin

(a) Principle

Phospholipids are the primary surface active components of the lung. It has been shown that the most abundant of these are lecithins (3-sn-phosphatidylcholine species). Because of variations in fatty acid composition, there are several types of lecithin in the lung. Total lecithin, however, is not surface active, and only the disaturated fraction particularly dipalmitoyl lecithin (DPL) displays this property. Studies have shown that the surface active material of the fetal lung reaches amniotic fluid late in gestation leading to an increase in the concentration of lecithin. In one study, an infant developed typical RDS
despite the normal total lecithin concentration of amniotic fluid at birth. However, fatty acid analysis revealed that the surfactant lecithin concentration was extremely low, showing a great discrepancy between total lecithin, and surfactant lecithin concentrations. Therefore, the determination of disaturated lecithin in amniotic fluid appears to be the most precise sign of potential risk of RDS.

Even though disaturated lecithin is a more specific marker of pulmonary surfactant than total lecithin, the procedures for measuring this fraction in the past have been tedious, extremely time consuming, and not applicable to the clinical laboratory (gas-liquid chromatography). This previous procedure did not provide direct information on the concentration of disaturated lecithin, but has revealed that palmitic acid is the predominant fatty acid in amniotic fluid during late gestation, after fetal pulmonary maturity is attained. Therefore, investigators pursued more convenient analytical techniques in an attempt to devise a method for routinely measuring this constituent as an index of fetal pulmonary maturation.

(b). Procedure

After several investigations with animal species, Torday et al. utilized the following procedure on human amniotic fluid for disaturated lecithin (203).

- The sample was centrifuged at 500 x g for 10 minutes to remove cellular debris and then the standard methanol/chloroform (1:2 by vol) lipid extraction was performed.
- Then the chloroform extract was evaporated under nitrogen and
resuspended in 0.5 mL of carbon tetrachloride containing 3.5 mg of osmium tetroxide... The osmium tetroxide is a powerful oxidizing agent which oxidizes the susceptible unsaturated fatty acids at the carbon-carbon double bonds (destroys or modifies the bond), however, the disaturated fatty acids resist oxidation and are preferentially spared from destruction, and can be separated by TLC for quantitation.

- Samples were applied to a silica gel H thin-layer chromatogram and developed in a chloroform/methanol/water (65:25:4 by vol) solution.
- The developed chromatogram was dipped in bromothymol blue stain for 15 seconds, and then blotted and dried at 100° for 5 minutes. Then the saturated phosphatidylcholine (SPC) was measured by means of reflectance densitometry. The standard curve to quantitate SPC was linear from 200 to 1500 mg when dipalmitoyl lecithin was utilized as a standard. Torday et al. determined from their study that an SPC value greater than 500 μg/dL was positive for fetal lung maturity (203).

In approximately 1% of samples an alumina column chromatography was required prior to TLC to eliminate interfering substances, for a black residue (reaction product) would obscure the SPC spot.

(c). Results

Figure 73 illustrates the effect of reacting the powerful oxidizing agent osmium tetroxide with various phospholipids. Dioleic lecithin, an unsaturated lecithin (Fig. 73A), is eliminated by osmium
FIGURE 73
EFFECT OF REACTING OSMIUM TETROXIDE WITH VARIOUS PHOSPHOLIPIDS

Legend
The figure is a chromatogram illustrating the effect osmium tetroxide has on phospholipid standards and various lipid extracts. The first and last lanes show a standard mixture of lecithin (L) and sphingomyelin (S). The other pairs of lanes (A to E) show the substrate before (left) and after (right) treatment with osmium tetroxide. The following letters denote: A = dioleic lecithin, B = dipalmitoyl lecithin, C = amniotic fluid, D = meconium, and E = blood.

tetroxide, while saturated dipalmitoyl lecithin (Fig. 73B) is unaffected. Torday et al. chromatographed extracts of blood, meconium, and amniotic fluid, and found that both L and S were detected; however, after osmium tetroxide treatment only SPC was detectable in the extract from amniotic fluid. (203). Fatty acid analysis (GLC) revealed good recovery (97%) of saturated lecithin, for more than 93% of the unsaturated fatty acids were removed by osmium treatment.

In reviewing their data Torday et al. have concluded the following results (203). In uncontaminated samples from uncomplicated pregnancies there was no differences in the results from the L/S ratio and SPC determination. However, in the total population (n = 322; 38 infants developed RDS) which included 75% of samples which were contaminated or from complicated pregnancies or both, the SPC procedure was far better in predicting fetal pulmonary maturation, for the reliability of a positive result showed increased sensitivity (LS ratio 66% vs SPC 92%) and predictive value (L/S ratio 56% vs SPC 83%). Also there was a slight improvement in the reliability of a negative result, for the specificity slightly increased (L/S ratio 93% vs SPC 98%) and predictive value slightly increased (L/S ratio 95% vs SPC 99%). The overall accuracy of the SPC procedure 97% as compared to 90% for the L/S ratio.

In conclusion, Torday et al. felt that their procedure for determining SPC was practical and was a more reliable predictor of RDS than the L/S ratio (203). However, the following two disadvantages prevail with this procedure: the utilization of osmium tetroxide, which is a toxic substance has some accompanying hazards, such as, it vaporizes readily
at room temperature, therefore must be utilized in a well-ventilated area and continued exposure may cause skin problems or problems with vision (due to black oxide deposition in the eyes), therefore skin and eye contact must be avoided, secondly, the amount of nonsurfactant disaturated lecithin present in the amniotic fluid must be determined. Disaturated lecithin is not unique to pulmonary surfactant, for it is found in the spleen, brain and blood (all contain 30% SPC). Also the fetal urine and membranes are two other sources of amniotic fluid phospholipid, which may contain as much as 50% SPC. Therefore, the success and reliability of the Torday et al. method is dependent on the fact that disaturated lecithin in amniotic fluid be derived from surfactant (203).

Also, recently, enzymatic procedures have arisen (phospholipase D) which are utilized to measure choline content. The advantage of these procedures are that they provide specificity of the reagent for the lecithin substrate in amniotic fluid, however, without prior isolation of surfactant lecithin, therefore somewhat negating the advantage.

(d). Contamination

The presence of blood (cells removed at centrifugation if hemolyzed affect) and meconium (unsaturated L) do not affect the measurement of SPC (markedly alter L/S ratio).

(e). Summary

Overall this SPC procedure appears better than the L/S ratio, however, due to its disadvantages it has not been accepted as routine in the clinical laboratory. The question concerning the quantitative
importance of nonsurfactant disaturated lecithin relative to surfactant disaturated lecithin in amniotic fluid must be answered before this procedure would be accepted as routine in the clinical laboratory.

8. Enzymatic Procedures

(a). Principle

The previously mentioned procedures utilized in assessing the lecithin and sphingomyelin concentrations have involved either thin-layer, or gas-liquid chromatography. These procedures are either tedious, time-consuming, and consequently expensive, or semiquantitative. Therefore, in 1978 Anoohar et al. developed an enzymatic procedure for lecithin in amniotic fluid (204). Initially this enzymatic procedure appeared quite lengthy, however, recently several new enzymatic procedures have appeared which are simpler and faster than the original one. These procedures are based on the enzymatic reaction sequence of hydrolysis of the phospholipids.

(b). Procedures

The four most recently published enzymatic procedures will now be reviewed.

(i). McDonald et al. - L/S Enzymatic Radiochemical Method

McDonald's et al. method determines lecithin, lyssolecithin, and sphingomyelin in amniotic fluid (205). The only phospholipids in amniotic fluid that contain a choline moiety are lecithin, lyssolecithin, and sphingomyelin.

1. Procedure

McDonald et al. procedure (flow chart shown in Fig.
is based on the alkaline hydrolysis of the lecithins, because sphingomyelin is not degraded by alkali, and therefore, choline generated by alkaline hydrolysis is derived solely from lecithin (205). Sphingomyelin is converted to choline by the following enzymatic hydrolysis:

\[
\text{Sphingomyelin} \xrightarrow{\text{sphingomyelinase}} \text{phosphorylcholine} + \text{ceramide} \\
\text{Phosphorylcholine} \xrightarrow{\text{alkaline phosphatase}} \text{choline} + \text{inorganic phosphate}
\]

Then the choline formed in each hydrolysis is enzymically phosphorylated with \( \gamma^{32}\text{P} \) ATP to yield \( \gamma^{32}\text{P} \) phosphorylcholine which is isolated by anion-exchange chromatography. McDonald et al. demonstrated that the \( \gamma^{32}\text{P} \) phosphorylcholine was stoichiometrically related to sphingomyelin, lecithin, and lysolecithin and that other phospholipids did not interfere in the procedure (205).

2. Results

With this procedure McDonald et al. compared the standard chromatographic L/S ratio to three indices total lecithin concentration, the ratio of lecithin plus lysolecithin (total lecithin) to sphingomyelin, and total lecithin as percentage of total amniotic fluid phospholipid with the following results (205).

Figure 75A, B, and C shows the results of the three indices investigated for assessment of fetal lung maturity. The data suggests that measuring the percentage of total lecithin provided the best assessment of fetal lung maturity. Figure 75C shows the comparison of L/S ratio to percentage of total lecithin. McDonald et al. hypothesized
FIGURE 74

ENZYMATIC RADIOCHEMICAL METHOD FLOW CHART

Legend

Figure illustrates protocol for quantitation of total phospholipids and choline-containing phospholipids in amniotic fluid.

Figure 74

McDonald et al. Flow chart of their procedures' step

ANASTOTIC FLUID

Extract phospholipids with CHCl₃:MeOH (2:1)

Aqueous MeOH layer
Discard

CHCl₃ layer

Evaporate under N₂

OR

Assay for total phospholipid phosphorous

Hydrolyze with alkalis

19/20

Extract sphingomyelin with CHCl₃:MeOH (2:1)

Aqueous MeOH layer
Discard

CHCl₃ layer

Wash out choline with alkaline MeOH

Alkaline MeOH layer
Discard

Evaporate under N₂

Hydrolyze with Sphingomyelinase + Alkaline Phosphatase

Assay for choline derived from sphingomyelin

1/20

Assay for choline derived from lecithin and lyssolecithin
FIGURE 75

STANDARD CHROMATOGRAPHIC L/S RATIO

COMPARED TO THREE INDICES

AND THE PERCENTAGE TOTAL LECITHIN

Legend

A., B. and C. Figure illustrates relationships between the chromatographic L/S ratio, and (A) total L/S ratio, (B) total lecithin concentration, and (C) % total lecithin.

D. Figure illustrates dependence of % total lecithin on fetal lung maturity as estimated by the total L/S ratio.

that pulmonary maturity is accompanied by a progressive increase in the proportion of lecithin in the total phospholipid pool (205). Therefore, the relationship between the \((L + Ly)/S\) ratio and total lecithin (Fig. 75D) was examined and proved that the proportion of lecithin in the total phospholipid pool paralleled fetal lung maturity, for the percentage of total lecithin levels off after a relatively sharp increase in spite of an increasing \((L + Ly)/S\) ratio. The fact that the percentage total lecithin increased abruptly just before, and leveled off immediately after the transitional zone defined by the \((L + Ly)/S\) ratio (2.0-4.0) therefore, suggested that the percentage of lecithin may be an accurate measure of fetal lung maturity (205).

Both \((L + Ly)/S\) ratio and percentage of total lecithin are independent of changes in amniotic fluid volume.

(ii). Siegel et al. - Phosphatidylglycerol (PG) Enzymatic Radiochemical Method

Siegel et al. enzymatic radiochemical method determines phosphatidylglycerol (PG) in amniotic fluid (206).

l. Procedure

This procedure for PG is similar to the previously mentioned assay for lecithin, for PG is hydrolyzed in alkali and the resulting glycerol is then enzymatically phosphorylated with adenosine 5-[\(\tau\-^{32}P\)] triphosphate to yield glycerol \(\left[{^{32}}P\right]\) phosphate. The extent of hydrolysis is dependent on both concentration of alkali and temperature. Charcoal is utilized to remove the excess \(\left[{^{32}}P\right]\) ATP and then the radioactivity of the glycerophosphate is measured in a liquid
scintillation counter. Triglyceride present in the amniotic fluid does not interfere with this PG analysis, because it is hydrolyzed by lipase before extraction. This method has the distinction of being the first quantitative chemical method for measurement of PG in amniotic fluid which avoids chromatographic separation and staining techniques.

2. Results

Siegel et al. obtained the following results in their study (206). Siegel et al. evaluated this procedure with seven normal and 20 complicated pregnancies and also ran corresponding L/S ratios (Gluck method) for comparison (Fig. 76A) (206). A weak correlation ($r = 0.550$) was revealed on regression analysis. However, Siegel et al. established a PG concentration of $10 \, \mu\text{mol/L (n mol/mL)}$ or greater to relate to a 2.0 or greater L/S ratio cut-off value for fetal lung maturity (206).

Siegel et al. also evaluated the PG concentration corresponding to weeks of gestation (206). As shown in Fig. 76B little or no PG ($<2 \, \mu\text{mol/L}$) was found in the amniotic fluid prior to 34.5 to 35 weeks, and then at about 36 weeks it increases significantly.

(iii). McGowan et al.-L/S Enzymatic Colorimetric Method

The McGowan et al. method determines lecithin and sphingomyelin in aqueous solution using enzymatic colorimetry (207).

1. Procedure

In this procedure, initially the phospholipids are dissolved in chloroform/methanol (2:1 by vol), the solvent is evaporated, and the residue redissolved in an aqueous zwitterionic detergent solution
FIGURE 76

ENZYMATIC RADIOCHEMICAL PHOSPHATIDYLGLYCEROL DETERMINATION COMPARED TO THE CHROMATOGRAPHIC L/S RATIO AND THE CONCENTRATION CORRESPONDING TO WEEKS OF PHOSPHATIDYLGLYCEROL GESTATION

Legend

A. Figure illustrates relationship between PG concentration in amniotic fluid and the chromatographic L/S ratio results. A weak correlation ($r = 0.550$) is revealed on regression analysis. Established a PG concentration of 10 mol/L (n mol/mL) or greater to relate to a 2.0 or greater L/S ratio cut-off value for fetal lung maturity.

B. Figure illustrates relationship between PG concentration in amniotic fluid and weeks of gestation. Little or no PG ($< 2 \mu$mol/L) is found in the amniotic prior to 34.5 to 35 weeks, and then at about 36 weeks it increases significantly.

FIGURE 76

- Infant born within 48 h of analysis
- Infant born after 48 h of analysis
(compatible with both L and S and because it solubilizes phospholipids) (207). Both assays involve enzymatic hydrolysis of the phospholipids to produce choline, which is then oxidized to betaine, thus generating hydrogen peroxide (Fig. 77). Then the hydrogen peroxide is enzymatically coupled with sodium 2-hydroxy-3,5-dichlorobenzenesulfonate and 4-aminoantipyrine to form a red chromogen (Fig. 77). In this study McGowan et al. determined that the presence of non-reacting phospholipid enhanced the hydrolysis of the reacting phospholipid, therefore, lecithin was added to the sphingomyelin standards and sphingomyelin to the lecithin standards (207).

2. Results

McGowan et al. assayed 10 amniotic fluid samples by their enzymatic procedure and compared them to the standard L/S ratio - TLC procedure (207). The comparison of the results are in Table XXXIV, which shows that the lecithin concentration varies over a much wider range than does the sphingomyelin concentration. The enzymatic L/S ratio parallels the standard TLC - L/S ratio.

(iv). Artiss et al. - Phosphatidylglycerol (PG) Enzymatic Colorimetric Method

The Artiss et al. method determines PG in amniotic fluid by enzymatic colorimetry (208).

1. Procedure

This procedure for PG determination is similar to the lecithin and sphingomyelin enzymatic procedure just discussed. Therefore, enzymatic determination for PG, L, and S may provide accurate
FIGURE 77
REACTION SEQUENCE FOR McGOWAN ET AL. ENZYMATIC DETERMINATION
OF LECITHIN AND SPHINGOMYELIN

Legend
Figure illustrates reaction sequences for enzymatic determination of
lecithin and sphingomyelin.

Abbreviations: SMase = sphingomyelinase, AP = alkaline phosphatase,
COD = choline oxidase, POD = peroxidase, PL - Do = phospholipase D from
cabbage.

Reproduced without permission from: McGowan, M. W., Artiss, J. D., and
FIGURE 77

REACTION SEQUENCE FOR McGOWAN ET AL. ENZYMATIC DETERMINATION

OF LECITHIN AND SPHINGOMYELIN

SPHINGOMYELIN $\xrightarrow{\text{SNase}}$ CFRAMIDE + PHOSPHORYLCHOLINE

PHOSPHORYLCHOLINE $\xrightarrow{\text{AP}}$ CHOLINE + $\text{PO}_4^{-3}$

LECITHIN $\xrightarrow{\text{PL-Dc}}$ PHOSPHATIDIC ACID + CHOLINE

CHOLINE $+$ $\text{O}_2$ $\xrightarrow{\text{COD}}$ BETAIN + $2\text{H}_2\text{O}_2$

$2\text{H}_2\text{O}_2$ $+$ 2 HYDROXY-3,5-DICHLOROBENZENESULFONATE $\xrightarrow{\text{POD}}$ RED

$+$ 4-AMINOANTIPYRINE $\xrightarrow{\text{CHROMOGEN}}$
TABLE XXXIV

L/S RATIOs IN 10 SPECIMENS OF AMNIOTIC FLUID AS DETERMINED BY
THE PROPOSED ENZYMATIC METHOD AND BY THIN-LAYER CHROMATOGRAPHY.

<table>
<thead>
<tr>
<th>Lecithin (μmol/L)</th>
<th>Sphingomyelin</th>
<th>Enzymatic-L/S</th>
<th>TLC-L/St</th>
</tr>
</thead>
<tbody>
<tr>
<td>93.4</td>
<td>8.3</td>
<td>11.3:1</td>
<td>&gt; 4:1</td>
</tr>
<tr>
<td>134.0</td>
<td>11.5</td>
<td>11.7:1</td>
<td>17:1</td>
</tr>
<tr>
<td>17.8</td>
<td>11.3</td>
<td>1.6:1</td>
<td>0.6:1</td>
</tr>
<tr>
<td>117.3</td>
<td>16.8</td>
<td>7.0:1</td>
<td>&gt; 4:1</td>
</tr>
<tr>
<td>248.6</td>
<td>20.8</td>
<td>12.0:1</td>
<td>&gt; 4:1</td>
</tr>
<tr>
<td>40.8</td>
<td>20.1</td>
<td>2.0:1</td>
<td>3.4:1</td>
</tr>
<tr>
<td>155.6</td>
<td>17.9</td>
<td>8.7:1</td>
<td>&gt; 4:1</td>
</tr>
<tr>
<td>7.2</td>
<td>18.8</td>
<td>0.4:1</td>
<td>1.1:1</td>
</tr>
<tr>
<td>77.4</td>
<td>13.1</td>
<td>5.9:1</td>
<td>&gt; 4:1</td>
</tr>
<tr>
<td>166.4</td>
<td>24.2</td>
<td>6.9:1</td>
<td>&gt; 4:1</td>
</tr>
</tbody>
</table>

and precise lung profile information for assessment of fetal lung maturity. This PG procedure is similar to the previously described L and S procedure, for the samples are extracted in chloroform/methanol (2:1 by vol). (extraction required because of the presence of relatively large quantities of reaction intermediates), evaporated, and then the phospholipid-containing residue is redissolved in a non-ionic detergent. The enzymic reaction sequence (Fig. 78) involves phospholipase-catalyzed hydrolysis of glycerol from its phospholipid (L and S procedures involves hydrolysis of the phospholipids to produce choline, which is oxidized to betaine, thus generating hydrogen peroxide) (Fig. 78). The remaining enzyme-catalyzed reactions phosphorylate the glycerol and oxidize the resulting glycerol phosphate to produce hydrogen peroxide, which is reacted to produce an intense red chromogen (510 nm) in the peroxidase-catalyzed coupling of 4-aminonitriptive and 2-hydroxy-3,5-dichloro-benzenesulfonate (208).

2. Results

Artiss et al. investigated the following conditions in their study with their corresponding results (208):

- The source of phospholipase D (PL-D) is very important for obtaining the correct products in enzymatic reactions. For PL-D from Streptomyces chromofuscus catalyzes the hydrolysis of choline from lecithin, lyssolecithin, and sphingomyelin and additionally they found it to quantitatively hydrolyze the glycerol base from PG (208). As illustrated in Fig. 79A by Artiss et al., the optimum conditions for PL-D assay for PG were: pH 7.6; ATP
FIGURE 78

REACTION SEQUENCE FOR ENZYMIC PHOSPHATIDYLGLYCEROL DETERMINATION

Legend

Figure illustrates reaction sequence for the enzymic determination of PG.

Abbreviations: PL-D = phospholipase D, GK = glycerol kinase, GPO = L-α-glycerophosphate oxidase, POD = peroxidase.

FIGURE 78

REACTION SEQUENCE FOR ENZYMIC PG DETERMINATION

\[ \text{Phosphatidylglycerol} \xrightarrow{\text{PL-D}} \text{Phosphatidic Acid} + \text{Glycerol} \]

\[ \text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP} \]

\[ \text{Glycerol 3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetonephosphate} + H_2O_2 \]

\[ 2H_2O + 2\text{-hydroxy-3,5-dichlorobenzenesulfonate} \xrightarrow{\text{POD}} \text{Red chromogen} \]

+ 4-aminocoumarine
FIGURE 79.

OPTIMUM CONDITIONS FOR PHOSPHOLIPASE D (PL-D) ASSAY FOR
PHOSPHATIDYLGLYCEROL AND THE COMPARISON OF COLORIMETRIC
ENZYMIC TO THIN-LAYER CHROMATOGRAPHIC RESULTS
FOR PHOSPHATIDYLGLYCEROL

Legend

A. Figure illustrates optimization data for the change in absorbance at 510nm as pH and concentrations of ATP, MgCl₂, and CaCl₂ are varied.
B. Figure illustrates comparison of results by the enzymic procedure (Artiss et al.) with those by the thin-layer chromatographic (TLC) procedure (Gluck et al.). Comparison of these two procedures produced lower enzymatic values and poor correlation (y = 2.07X + 8.22, r = 0.5956, n = 53).

FIGURE 79

A.

B.
concentration should be at least 500 μmol/L for the concentration of PG tested; magnesium, which activates glycerol kinase is optimum at 5 mmol/L (exhibits inhibitory effect at greater concentrations); and calcium, which activates hydrolysis of PG by PL-D is optimum at 2 mmol, however, 10 mmol/L were utilized to eliminate competitive interference from lecithin (208).

- Triton X-100 is the non-ionic detergent utilized in preparing the standards and dissolving the samples, because it has no noticeable effect on the assay.

- In 53 samples Artiss et al. (208) compared their enzymatic PG procedure results to those of Gluck et al. (130) thin-layer chromatographic (TLC) procedure (Fig. 79B). Comparison of these two procedures produced lower enzymatic values and poor correlation (y = 2.07 + 0.22, r = 0.3956), which is probably due to the following factors.

  - The TLC procedure utilized the cold-acetone precipitation step and the enzymatic did not utilize it because of the controversy (208).

  - A lecithin:sphingomyelin densitometric ratio of 2:1 corresponds to a concentration ratio of from 4:1 to 6:1 as reported by Gluck and Kulovich (118).

- As illustrated in Fig. 80 Artiss et al. also compared the PG concentration versus gestational age for both procedures (208). Just as in Fig. 79B the enzymatic values were consistently lower, however, both procedures produced the same
**FIGURE 80**

COMPARISON OF PHOSPHATIDYLGLYCEROL CONCENTRATIONS VERSUS GESTATIONAL AGE BY THE THIN-LAYER CHROMATOGRAPHY AND ENZYMIC PROCEDURES

**Legend**

Figure illustrates comparison of PG concentrations by the thin-layer chromatographic (TLC) procedure (Gluck et al.) and the enzymic colorimetric procedure versus gestational age. Just as in the previous figure (123), the enzymic values were consistently lower, however, both procedures produced the same general distributions of concentrations with gestational age.

general distributions of concentrations with gestational age.

(c). Contamination

Blood and meconium contamination have similar effects, as with the lung profile indices. L and S are affected and PG is not affected by blood.

(d). Summary

All four of these methods are still in the experimental phases, however, they appear very promising for the future in predicting fetal lung maturity rapidly and with reliable results (especially in complicated pregnancies—these are two disadvantages of the L/S ratio procedure).

The first two radiochemical enzymatic methods (i and ii) were precise and accurate. The McDonald's et al. procedure (i) results in improved accuracy and reproducibility for assessing lecithin and sphingomyelin because it eliminates the L/S ratios steps of chromatographic separation, development, and quantitation of these lipids (205). The Siegel et al. procedure (ii) is the first quantitative chemical analysis for PG in amniotic fluid and has the advantage of no interference with a blood contaminated sample (206). These two above mentioned procedures have the following disadvantages: it requires approximately 2½–3 hours to complete, inadequate population sampling and size utilized, and the fact that the procedures are not totally enzymatic and, therefore must deal with the expense and waste disposal associated with radiolabeled compounds. Additional clinical testing would be required for acceptance.

The latter two enzymatic colorimetric methods (McGowan et al. iii...
and Artiss et al. iv) could replace the thin-layer chromatographic lung profile procedure, its shortcomings and controversial technical steps (separation, development, quantitation, centrifugation, cold-acetone precipitation). This enzymatic colorimetric procedure for lecithin, sphingomyelin, and PG is accurate, precise, and additionally is quick (1 h) simple, and inexpensive. The procedure (iv) appears to be somewhat more time consuming because of the extraction step required to eliminate the relatively large quantities of reaction intermediates for sphingomyelin and PG, however, the three phospholipids may be determined from this single extraction (similar to TLC procedure).

This enzymatic colorimetric procedure (iv) could be utilized as a tool in investigating the L/S ratio's controversial steps involved in the chromatographic determination of phospholipids. Then, after some of the controversies have been settled, this procedure could be tested for adequate population sampling and size and then be adapted on the clinical laboratory for routine assessment of fetal lung maturity.

(a). Principle

In the past few years several problems have arisen in the measurement of the L/S ratio for predicting fetal lung maturity. The problems include: the high incidence of false negatives; unpredictable pulmonary maturity in poorly controlled diabetic mothers; and the indiscriminant measurement of disaturated lecithin (PC), because all disaturated phosphatidylcholines are measured not just the highly surfactant dipalmitoyl species. Therefore, many workers have tried
quantifying only lecithin (PC) rather than the L/S ratio.

1976 was the first time field desorption mass spectrometry was utilized to quantify dipalmitoylphosphatidylcholine (DPPC). However, it was found that the field desorption technique irreproducibly generated a mixture of molecular ion species protons or sodium cations on the phosphate moiety.

In 1983, Ho et al. decided to evaluate FAB (Fast-Atom Bombardment) mass spectrometry in analysis of DPC in amniotic fluid (209). Ho et al. utilized a commercially available solid-phase desorption FAB technique, which is highly effective for desorption of compounds such as lecithin and it apparently is not as sensitive to cationic (Na⁺, K⁺, etc.) contamination (209). The basic principle of the Ho et al. procedure is that DPC is quantified by taking advantage of stable-isotope labeled d₅-DPC as an internal standard, and a mass spectrometer is utilized to measure the ratio of d₅/DO (209). The d₅-DPC was synthesized by refluxing dipalmitoylethanolamine with d₅-methyl iodide in methanol in the presence of sodium bicarbonate for 26 hours. The FAB was utilized to desorb the preformed phosphatidylcholine ions in a mass spectrometer of the Nier-Johnson geometry (209).

(b). Procedure

The procedure developed by Ho et al. is relatively simple, rapid, has a nearly quantitative yield, and involves the following steps (209): Add d₀-DPC in chloroform, to each amniotic fluid sample to obtain an estimated 1:1 ratio of d₀/DO. Then vortex for 5 minutes and sonicate for 30 minutes. PC is then extracted into chloroform, the extract
evaporated, and then this residue is redissolved with chloroform to give a concentration of approximately 2 $\mu$g of total $d_9$-DPC plus $d_9$-DPC per 10 $\mu$L. Then the mass-spectrum determinations are conducted in the following manner: ratios of the intensities of molecular ion species from the stable-isotope-labeled internal standard and from the analyte. For each sample five scans were averaged by computer through the mass range of m/z 700 to 800.

(c). Results

The following results were obtained by Ho et al. (209). Figure 81 shows the partial positive-ion FAB mass spectrum of a chloroform extract of amniotic fluid to which $d_9$-DPC was added as an internal standard. The most prominent peaks are $d_9$-DPC and $d_9$-DPC corresponding to m/z 744 and 735, respectively. Also peaks are present at m/z 761 and 707 corresponding to palmitoyl oleoyl, and palmitoyl tetradecanoyl phosphatidycholines, respectively. The intensities of these two latter peaks (761 and 707) relative to that at m/z 735 is not an indication of the relative concentrations of the phospholipid in amniotic fluid. The quality of the spectrum obtained in Fig. 81 reflects DPC and $d_9$-DPC as major components and was very reproducible throughout many samples.

Table XXXV shows the results obtained from three normal pregnancies, three complicated pregnancies, and six mid-term pregnancies for comparison of mass spectrometry, TLC, and L/S ratio. There is a range of DPC concentrations in the normal (18, 55, 150), and complicated (2.2, 6.3, 9.5) amniotic fluid samples. The mid-term DPC values were low (0.52 - 0.80). When comparing the DPC values to the L/S ratio there is poor
FIGURE 81

PARTIAL POSITIVE-ION FAST-ATOM BOMBARDMENT MASS SPECTRUM

Legend

Figure illustrates partial positive-ion fast-atom bombardment mass spectrum of a chloroform extract of amniotic fluid to which dq-DPC (dipalmitoylphosphatidylcholine) was added as an internal standard. The peaks and corresponding m/z are: dq-DPC/744, do-DPC/735, palmitoyloleoyl/761, and palmitoyltetradecanoylphosphatidylcholine/707. The intensities of the two latter peaks (761 and 707) relative to that at m/z 735 is not an indication of the relative concentrations of the phospholipids in amniotic fluids. This spectrum reflects the DPC and dq-DPC as major components and was very reproducible throughout many samples.

### TABLE XXXV

**DIPALMITOYL PHOSPHATIDYLCHOLINE (DPC) CONTENT OF AMNIOTIC FLUIDS**

<table>
<thead>
<tr>
<th>Total DPC, mg/L</th>
<th>Mass spec.</th>
<th>TLC</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>From three normal pregnancies (all at term)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.51</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>370</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>260</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>From three complicated pregnancies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 (36 wk)</td>
<td>4.6</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>9.5 (38 wk)</td>
<td>16.0</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>6.3 (40 wk)</td>
<td>13.0</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>Mid-term (20 to 22 wk), n = 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.69</td>
<td>7.1</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>0.64</td>
<td>6.5</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>0.80</td>
<td>6.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>0.66</td>
<td>8.0</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>0.68</td>
<td>7.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>0.52</td>
<td>4.7</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

---

*a* Infant respiratory distress did not eventuate in any of these births.

*b* Result of an inadequate concentration of sphingomyelin rather than an excessive concentration of lecithin.

correlation. Ho et al. were not sure what concentrations of DPC were adequate and inadequate for establishing a cut-off value for fetal pulmonary maturity (209).

Also Table XXXV indicates that DPC makes up less than 16% of the total PC mixture. Differing desorption efficiency, plus such temporal fractionation for saturated and unsaturated fatty acids results in the quantification of relative amounts of saturated and unsaturated PC, difficult with FAB. The isotope-labeled analog is readily detected, with a quantitative relationship and therefore, the isotope discrimination based only on mass difference does poor at these high masses (209). However, this isotope-ratio method could be easily adapted to quantitate two or more compounds in amniotic fluid (i.e., DPC and PG).

Ho et al. also studied and evaluated the following parameters and their corresponding results (209).

- Studied feasibility of quantifying DPC by FAB mass spectrometry. This was accomplished by determining the relative concentrations of deuterated DPC and deuterated DPC by measuring the relative intensities of the molecular ion peaks at m/z 735 and 744, respectively. Figure 82 illustrates that a log-log plot of the ratios of intensities of the peaks at 735 and 744 versus the ratios of the amounts of deuterated DPC and deuterated DPC in the test mixture (do/d0) was linear (slope 0.871, r = 0.994).

- Evaluated the accuracy of amniotic fluid extracts and chemical standards at various times throughout the study. The extracts and standards were introduced independently and consecutively into the
FIGURE 82

LOG-LOG PLOT OF THE RATIO INTENSITIES OF PEAKS VERSUS RELATIVE AMOUNTS OF D₃ AND D₉-DIPALMITOYLPHOSPHATIDYLCOLINE

Legend

Figure illustrates that a log-log plot of the ratios of intensities of the peaks at 735 and 744 versus the ratios of the amounts of do-DPC and d₉-DPC in the test mixture (do/d₉) was linear (slope 0.871, r = 0.9994).

FIGURE 82.

![Graph showing a linear relationship between two variables on a log scale. The graph includes a correlation coefficient of 0.9994.](image)
mass spectrometer and the standard deviations were <1 and ± 0.6, respectively.

- Assessed accuracy as a function of sample size by introducing varying µg concentrations (i.e., 1, 0.5, 0.25, and 0.1) of do-DPC and d9-DPC onto the mass spectrometer probe and obtained ratios of m/z 735 to 744 (100/99, 100/97.2, 100/102.5, and 100/94.3, respectively). These results reveal that accuracy is lowered for samples of less than 1 µg. Also they found that the best accuracy is obtained when internal standard and sample are present in equal amounts.

- Evaluated DPC concentration as a function of centrifugal force applied. This was accomplished by monitoring the PC concentration by TLC, and DPC was analyzed by adding d9-DPC after each 15-minute centrifugation and measuring the isotope ratio. The results reveal that more than 50% of both PC and DPC were lost near 1000 x g, and greater loss accompanied increased g force (Table VI).

Summary

This procedure of Ho et al., which utilizes an isotope-labeled analog as an internal standard for FAB mass spectrometry appears to provide a very promising technique for quantifying DPC (209). Ho and associates evaluated and assessed various parameters concerning this technique's fate in various laboratory manipulations (209). This technique could be adapted to the clinical laboratory environment for testing fetal pulmonary maturity, however, the following must be determined:
TABLE XXXVI
EFFECT OF CENTRIFUGAL FORCE ON CONCENTRATION OF PHOSPHATIDYLCHOLINE (PC) AND DIPALMITOYLPHOSPHATIDYLCHOLINE (DPC) IN AMNIOTIC FLUID

<table>
<thead>
<tr>
<th></th>
<th>Percent retention at g force of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PC (by TLC)</td>
<td>100</td>
</tr>
<tr>
<td>DPC (by mass spec.)</td>
<td>100</td>
</tr>
</tbody>
</table>

• Must establish what DPC value is the cut-off value for fetal pulmonary maturity. This must include evaluation of a large sample size of various pregnancy conditions and simultaneously comparing L/S ratios and DPC values on these amniotic fluid samples and correlating the fetal outcome.

• Must determine if this isotope-ratio method could be expanded to measure both DPC and PG.

• A major disadvantage is the availability and expensive cost of an FAB mass spectrometer. Currently the FAB mass spectrometer is utilized for research in University affiliated hospitals and research laboratories. However, the common hospital laboratory could not incur the cost or have usage of an FAB mass spectrometer. This technique has passed the initial phase of laboratory adaptability and appears very promising as being applied to the clinical laboratory in DPC determination, however, much more work must be completed to establish its acceptance. Also in the future if this procedure proves diagnostic, possibly the cost of the FAB mass spectrometer may be more affordable.

B. BIOPHYSICAL METHODS

The biochemical procedures discussed in the previous section depended upon the measurement in the amniotic fluid of chemical compounds, which in varying degrees are associated with the surface active compounds derived from the lung.

The biophysical methods discussed in this section, include surface tension, shake (bubble) test, and fluorescence polarization. These
methods are termed biophysical methods for they measure the functional activity of surfactant in amniotic fluid.

Surfactant, as the name implies is a surface active material which by its presence in the alveolar lining film stabilizes the alveolus. During expiration, the area of the alveolar film is diminished and collapse of the alveolus is prevented by a corresponding drop in the surface tension of the film. This property is a measure of the capacity of the polarized molecules at the interface to pack as the area of the film is progressively diminished.

The basis of these three biophysical procedures are the following:

1. The surface tension lowering ability procedure utilizes a surface balance to measure the surface tension/surface activity of amniotic fluid lipid extracts.

2. The shake test relies on the characteristic ability of surfactant phospholipids to form highly stable surface films that can support the structure of foam for a period of time.

3. The fluorescence polarization procedure is based on the theory that viscosity and surface tension of fluids are determined by the intermolecular forces of the fluid and are, therefore, interrelated, so that the surface tension of the pulmonary surfactant can be translated into terms of microviscosity. The lipid microviscosity is determined by usage of a lipid-soluble fluorescent probe which polarizes incident radiation.

Each of these biophysical procedures will now be discussed in detail.
1. Surface Tension Lowering Ability of Amniotic Fluid

Surfactant

(a). Principle

Historically, as early as 1929, Von Neergaard noted the effect of a liquid monolayer on lowering surface tension to facilitate the air inflation of pulmonary alveoli (11). However, this concept was not demonstrated till 1967 when Scarpelli found for the first time that amniotic fluid at term and lung extracts from diseased newborn infants with fully developed lungs, showed similar surface properties when examined with the surface balance (15). Since Scarpelli’s demonstration, several different procedures have been utilized in measuring the surface tension lowering ability of amniotic fluid. Most of these procedures involve the direct measurement of surface tension utilizing a surface balance, however, some use amniotic fluid while others prepare a surface film from lipids extracted from amniotic fluid. These various procedures will now be discussed.

(b). Different Procedures and Their Corresponding Results

(i). Shelley et al. Method

In 1973, Shelley et al. realized the importance of trying to separate the surface-active fraction from the amniotic fluid before assessing its activity by developing a simple method of treating amniotic fluid with the gel-filtration medium Sephadex, which consistently yields surface-active fractions from amniotic fluid of normal term pregnancies (210). The procedure involved the following steps (210):

- Five mL of amniotic fluid (preserved in 0.02M sodium citrate) was
gently centrifuged at about 500 rpm (less than 100 xg) for 3 minutes with 0.15 g superfine Sephadex (gel-filtration medium) G-100.

The supernatant was introduced into the trough of a cycling surfactometer (as described by Greenfield and Kimmell (211)) containing 25 ml of Ringer-lactate solution, for a compression and expansion cycling time of 3 minutes (surface film was allowed to age for 10 minutes before starting cycle). The cycling was continued until no change was observed in 3 consecutive cycles.

This treatment of the amniotic fluid with Sephadex allowed for removal of cellular component and debris with very little centrifugation and, therefore, resulted in slight loss of lung surfactant.

Although the phospholipid concentration of the samples was being estimated for the purpose of comparison, the authors failed to substantiate this assumption by presenting figures for total phospholipid concentration before and after treatment with Sephadex (210). Nevertheless, hysteresis was demonstrated in the samples analysed. The minimum surface tension ($\gamma_{\text{min}}$) on compression of the film, area of the hysteresis loop, and stability index, $\overline{S} = 2 (\gamma_{\text{max}} - \gamma_{\text{min}})/\gamma_{\text{max}} + \sigma_{\text{min}}$ were recorded. Figure 83A shows the surface tension area diagram of normal term amniotic fluid treated with Sephadex, and Fig. 83B shows the surface tension area diagram of amniotic fluid from delivery of an infant with RDS. In 6 infants with RDS $\gamma_{\text{min}}$ was significantly raised compared with controls, as seen in Table XXXVII compared to normal in Table XXXVIII. The stability index ($\overline{S}$) was also significantly reduced.
FIGURE 83
SURFACE TENSION AREA DIAGRAMS

Legend
A. Illustrates surface tension area diagram of normal term amniotic fluid treated with Sephadex.
B. Illustrates surface tension area diagram of Sephadex treated amniotic fluid from delivery of an infant with RDS (Case No. 183).

TABLE XXXVII
SURFACE TENSION MEASUREMENTS AND PHOSPHOLIPID VALUES OF AMNIOTIC FLUID
SAMPLES COLLECTED AT DELIVERY OF INFANTS WHO DEVELOPED RESPIRATORY DISTRESS

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Gestational age (wk.)</th>
<th>Birth weight (g)</th>
<th>Phospholipids (mg/mL)</th>
<th>Phospholipids on surfactometer (µg/mL)</th>
<th>Y min</th>
<th>S</th>
<th>Area</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>215</td>
<td>32</td>
<td>1,450</td>
<td>2.11</td>
<td>181</td>
<td>25</td>
<td>0.61</td>
<td>0.30</td>
<td>Mild RDS</td>
</tr>
<tr>
<td>177</td>
<td>34</td>
<td>1,840</td>
<td>2.39</td>
<td>210</td>
<td>19</td>
<td>0.88</td>
<td>0.68</td>
<td>Mild RDS</td>
</tr>
<tr>
<td>214</td>
<td>39</td>
<td>2,640</td>
<td>2.66</td>
<td>208</td>
<td>23</td>
<td>0.63</td>
<td>0.31</td>
<td>Mild RDS</td>
</tr>
<tr>
<td>183</td>
<td>38</td>
<td>1,960</td>
<td>1.34</td>
<td>318</td>
<td>25</td>
<td>0.72</td>
<td>0.49</td>
<td>Severe RDS</td>
</tr>
<tr>
<td>153</td>
<td>32</td>
<td>1,610</td>
<td>2.58</td>
<td>307</td>
<td>20</td>
<td>0.81</td>
<td>0.62</td>
<td>RDS-Died</td>
</tr>
<tr>
<td>240</td>
<td>33</td>
<td>1,450</td>
<td>3.96</td>
<td>326</td>
<td>21</td>
<td>0.43</td>
<td>0.30</td>
<td>RDS-died</td>
</tr>
</tbody>
</table>

*More than 4.8 mL of amniotic fluid was tested on the surfactometer in all cases.

*Infants in whom cyanosis, grunting, and intercostal retractions were present for periods of less than 48 hours were designated as having mild RDS.

*Severe RDS were characterized by symptoms noted after persisting for longer than 48 hours, and confirmed by radiologic evidence.

*Diagnosis of RDS was confirmed by the autopsy findings.

### TABLE XXVIII

**SURFACE TENSION MEASUREMENTS AND PHOSPHOLIPID VALUES OF AMNIOTIC FLUID**

Specimens collected at delivery of five healthy premature infants and two infants of diabetic mothers.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Gestational age (wk.)</th>
<th>Birth weight (g)</th>
<th>Phospholipids (mg/100 mL)</th>
<th>Phospholipids on surfactometer a</th>
<th>( \gamma ) min</th>
<th>( S )</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>151</td>
<td>32</td>
<td>1,920</td>
<td>8.02</td>
<td>7/1</td>
<td>353</td>
<td>7.5</td>
<td>1.52</td>
</tr>
<tr>
<td>228</td>
<td>35</td>
<td>2,680</td>
<td>21.18</td>
<td>10/1</td>
<td>720</td>
<td>8.0</td>
<td>1.58</td>
</tr>
<tr>
<td>156</td>
<td>36</td>
<td>2,500</td>
<td>2.39</td>
<td>5/1</td>
<td>105</td>
<td>6.0</td>
<td>1.61</td>
</tr>
<tr>
<td>228</td>
<td>36</td>
<td>2,560</td>
<td>3.53</td>
<td>3/1</td>
<td>95</td>
<td>12.0</td>
<td>1.17</td>
</tr>
<tr>
<td>209</td>
<td>37</td>
<td>2,540</td>
<td>10.83 b</td>
<td>10/1</td>
<td>496</td>
<td>7.0</td>
<td>1.54</td>
</tr>
<tr>
<td>242</td>
<td>36</td>
<td>3,100 c</td>
<td>10.58</td>
<td>5/1</td>
<td>508</td>
<td>11.0</td>
<td>1.26</td>
</tr>
<tr>
<td>165</td>
<td>37</td>
<td>4,390 c</td>
<td>19.52</td>
<td>10/1</td>
<td>644</td>
<td>6.0</td>
<td>1.57</td>
</tr>
</tbody>
</table>

a More than 4.8 mL of amniotic fluid was tested on the surfactometer in all cases.

b Samples contained blood.

c Samples collected from diabetic patients.


The area of the hysteresis loop in these cases was of less prognostic significance, the results overlapping with normal values. The six cases which developed RDS lacked surface activity, however, not due to inadequate amounts of phospholipids tested on the surfactometer, for one hundred micrograms of phospholipid was sufficient to show surface activity of the phospholipids which were from active lung surfactant (210).

(ii). Muller-Tyl et al. Method

In 1975, Muller-Tyl et al. used a commercially available modified Wilhelmy surface balance (Fig. 84) with a small teflon trough (15 mm x 20 mm x 105 mm) (212). The procedure involved the following (212):

- Fresh, uncentrifuged amniotic fluid was used. The sample was placed directly in the trough and cycling commenced after 30 minutes of aging with a duration for each cycle of 4 minutes at room temperature.
- Surface tension area diagrams (SAD or some abbreviate STD) were recorded on a x/y plotter.
- The following parameters were also evaluated: surface tension at 100 per cent surface (y-max); and at 20 per cent of initial surface (y-min) and hysteresis area (cm²). Also the stability index was calculated:

\[
\overline{S} = \frac{2(y_{\text{max}} - y_{\text{min}})}{y_{\text{max}} + y_{\text{min}}}
\]

Figure 85 shows the results graphically of the above-mentioned surface tension parameters during the 23rd to 42nd weeks of gestation and
FIGURE 84
SURFACE BALANCE AND SURFACE TENSION AREA DIAGRAMS

Legend
A. Illustrates schematic representation of measurements with the surface balance*: In the trough (1) place amniotic fluid and the platinum plate (2), connected to a force recorder (3). Barrier (4) alters the surface area in a cyclic manner. The surface tension area diagrams (STD) are recorded with a x/y recorder (5).
B. Illustrates typical surface tension area diagrams (STD) of amniotic fluid from: 23-26 weeks gestation (1); 31-34 weeks gestation (2); and 39-41 weeks gestation (3).

*This figure is just a schematic for illustration of the trough's principle, and is not the modified surface balance utilized.

Reproduced without permission from: Müller-Tyl, E., Lempert, J. (1975)
FIGURE 85
SURFACE TENSION PARAMETERS OF AMNIOTIC FLUID

Legend

Figure illustrates the following surface tension parameters of amniotic fluid: \( y_{-\text{max}}, y_{-\text{min}} \) (dyn/cm), hysteresis area (cm\(^2\)), and stability index (\( S \)), during the 23rd to 42nd weeks of gestation (mean \( \pm 1 \) SD). Figure shows that during the second half of pregnancy there is a continuous rise of surface activity of amniotic fluid.

the fact that during the second half of pregnancy there is a continuous rise of surface activity of amniotic fluid. On the basis of this study, an exact assessment of the amniotic fluid surface activity is possible, for it undergoes a substantial change during pregnancy and has a minimal scatter; however, no conclusions as to the degree of fetal lung maturity could be established, because there was no correlation between the surface activity value of amniotic fluid and the clinical condition of the newborn infant lungs' maturity.

A comparison between the amniotic fluid y-min, the L/S ratio, the lecithin content, and dilution titer curve of the semiquantitative bubble stability test, is shown in Fig. 86. Figure 86 illustrates that the difference between the three other techniques and the y-min curve is the gradual drop of the y-min values after week 32 of pregnancy (212). This drop of the y-min values could be due to the fact that the surface balance measures the total activity of surfactant, whereas surfactant specific individual components are measured by the three other techniques.

(iii). Goldkrand et al. Method

In 1977, Goldkrand et al. developed a procedure for the direct measurement of the surface tension lowering properties of an amniotic fluid lipid extract (213). The procedure involved the following:

1 to 2-mL aliquot of amniotic fluid was transferred to a 15-mL glass-stoppered conical centrifuge tube for methanol-chloroform extraction of lipids.
FIGURE 86
COMPARISON BETWEEN SURFACE TENSION \( \gamma \)-MIN,
THE L/S RATIO, THE LECITHIN CONTENT, AND
BUBBLE STABILITY TEST

Legend

Figure illustrates comparison between the amniotic fluid (X/dyn/cm) surface tension \( \gamma \)-min\(^a\), (L:S) the L/S ratio\(^b\), (L) the lecithin content (mg/100 mL)) and (T) dilution titer of the semiquantitative bubble stability test\(^c\) during the second half of gestation (mean \( \pm \) 1 S.D.). The difference between the three other techniques and the \( \gamma \)-min curve is the more gradual rise of the \( \gamma \)-min values after the 32nd week of pregnancy, which could be attributable to the fact that the surface tension measures the total activity of surfactant, while the other three tests measure specific individual components of surfactant.


- The extracts were analyzed on a Du Nouy tensiometer with the use of a platinum ring of 5.992 cm circumference (213). The surface tension measurements utilized a standard 60 by 15 mm glass Petri dish cover filled with 15 mL of twice-distilled water (used as standard to check instrument, 72.0 dyn/cm) and having the platinum ring submerged in the water.

- 20 μL of the lipid extract was layered on the surface of the water and after all visible droplets of extract spread over the water surface, the surface tension was measured by withdrawing the ring. For subsequent volume additions, the ring was submerged and the above procedure was repeated (213).

Pulmonary maturity by this lipid extract surface tension measurement is less than 56 dynes per centimeter at 120 μL of extract and less than 46 dynes per centimeter at 220 μL of extract. Also, surface-tension values greater than 56 dynes per centimeter at 120 μL, and greater than 46 dynes per centimeter at 220 μL are considered transitional, and would require a second measurement before pulmonary maturity could be predicted. Values exceeding these limits at each extract were classified as immature (213). Figure 87 is a graphic representation of the mean surface tension values ± standard error of the mean (SEM) for amniotic fluid lipid extracts from infants with pulmonary maturity and immaturity.

From the above-mentioned Goldkrand et al. study (213) and later studies (214) in 1977, they found that when the surface tension values of both mature and immature amniotic fluids reached a plateau, a subsurface globule formed in the system. In the later study, Goldkrand
**Figure 87**

**Mean Surface Tension Values**

**Legend**

Figure illustrates mean surface tension values ± standard error of the mean (SEM) for amniotic fluid lipid extracts from babies with pulmonary maturity and immaturity.

Figure 87

Graph showing the relationship between volume of extract and surface tension for mature and immature samples.
et al. found that the volume of extract required to produce this subsurface globule varied with the degree of fetal pulmonary maturity and immaturity (214). For this procedure, Goldkrand et al. utilized the same extraction and tensiometer parameter as previously mentioned (213), but after the droplets were allowed to disperse and a surface tension measurement was made and recorded, the amniotic fluid extract was continuously layered until the addition of more extract did not disperse on the surface (214). The added extract remained as a globule on the surface and then sank below the water, and at this point the volume of extract was recorded.

The amount of required extract to achieve globule formation varied with the degree of fetal pulmonary maturity and the volume required was used to differentiate pulmonary maturity (≤ 320 μl), transitional status (340 to 440 μl), and pulmonary immaturity (> 460 μl). Globule measurements of 74 amniotic fluid samples were compared with their corresponding L/S ratios with the following results (shown in Table XXXIX) (214): 22 of 24 (91.7%) samples termed mature had a L/S ratio > 2.0, while 32 of L/S (76.2%) with a value termed immature had a L/S ratio ≤ 1.9. Chi-square analysis of these relations yielded a P < 0.001. These results compared favorably with the L/S ratio and the results only errored on a predicted immaturity, and then no RDS developed.

The Goldkrand et al. procedure for measuring the surface tension lowering properties of surfactant is dependent on the establishment of a monolayer of amniotic fluid lipid extract on the water surface (214). The surface tension decreases as the monolayer is complete, however, the
**TABLE XXXIX**

COMPARISON OF THE AMNIOTIC FLUID LIPID EXTRACT

GLOBULE FORMATION AND THE L/S RATIO

<table>
<thead>
<tr>
<th></th>
<th>Mature &lt;320μl</th>
<th>Transitional 340 - 440μl</th>
<th>Immature &gt;460μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/S mature (&gt;2.0)</td>
<td>22 (of 24)</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(91.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/S transitional</td>
<td>1</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>(1.0-1.9)</td>
<td></td>
<td></td>
<td>(32 of 42) = (76.2%)</td>
</tr>
<tr>
<td>L/S immature &lt;1.0</td>
<td>1</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

X' yields P <0.001

surface tension does not decrease indefinitely, but reaches a plateau in fluids for immature and mature samples at about the same surface tension, but the volume of extract required varies. When the monolayer is packed, after plateau formation occurs subsequently the subsurface globule appears. Figure 88A is a diagram illustrating the formation of this subsurface globule from an amniotic fluid extract, which represents an outer chloroform (a nonpolar solvent) layer surrounding the surfactant phospholipid interior. Addition of more phospholipid in chloroform onto the tightly packed surface favors globule formation versus the spreading of the phospholipid chloroform solution on the surface (214). The chloroform obeys the law of gravity and falls into the liquid phase because chloroform is more dense than water, and is in the globule formation instead of spreading out over the surface (214).

The amount of surfactant in the amniotic fluid lipid extract is directly related to the appearance of the subsurface globule, for mature fluids have more surfactant in each volume of extract, and therefore, pack the surface monolayer, and allow globule formation to occur at lower volumes, while immature fluids on the other hand have less surfactant in each volume of extract, and therefore, pack the surface monolayer, and allow globule formation to occur at higher volumes.

(iv). Golkrand and Slattery

In 1979, Gölkbrand and Slattery studied 211 amniotic fluid samples from 123 patients with normal and abnormal pregnancies (55 normal, 23 diabetics, 19 Rh sensitization, and 26 preeclampsia) by measuring surface tension values of extract and the volume of extract
FIGURE 88
FORMATION OF THE 'SUBSURFACE GLOBULE' AND THE COMPARISON
OF THE SURFACE TENSION VALUES AND VOLUME REQUIRED
FOR GLOBULE FORMATION

Legend

A. Figure illustrates the formation of the subsurface globule formation
from an amniotic fluid extract, which represents an outer chloroform (a
nonpolar solvent) layer surrounding the surfactant phospholipid interior.
Addition of more phospholipid in chloroform onto the tightly packed
surface favors globule formation versus the spreading of phospholipid-
chloroform solution on the surface. The chloroform obeys the law of
gravity and falls into the liquid phase, because chloroform is more dense
than water, and is in the globule formation instead of spreading out over
the surface.

B. Figure shows comparison of the surface tension values at 220 l of
extract with the volume of extract required for globule formation. This
regression analysis yielded a coefficient of $r = 0.9368$ and $P < 0.001$.
This correlation shown demonstrated both methods to be essentially
interchangable and both provide for direct antenatal assessment of the
risk of neonate developing RDS.

Reproduced without permission from: A. Goldkrand, J. W., Varki, A., and
required for globule formation (215). The procedures and cut-off values were identical to those used in the two previously mentioned studies (213, 214).

Figure 88B shows the results obtained by Goldkrand and Slattery from 63 amniotic fluid samples of normal patients, comparing the surface tension (dyn/cm) reading at 220.μL of amniotic fluid lipid extract with the volume of extract required to induce globule formation (215). This regression analysis yielded a coefficient of correlation of r = 0.9368 and P < 0.001. The correlation shown, demonstrated both methods to be essentially interchangeable and both provide for direct antenatal assessment of the risk of a neonate developing RDS.

Figure 89A shows the pattern of pulmonary maturation in normal pregnancy and the marked changes in surface activity during the last half of pregnancy. Normal patients approach pulmonary maturity at about 29-30 weeks' gestation, however, at approximately 36 weeks instead of progressive maturity, there is an abrupt but brief return to a less mature pattern before final maturity is reached and these changes from less mature to a mature pattern may correspond with the biochemical surfactant changes in quality and quantity as the type II pneumocyte of the fetal lung shifts lecithin synthesis from the immature trimethylation pathway to the major choline incorporation pathway of the mature fetus (215).

Goldkrand and Slattery also studied abnormal pregnancy states and the results are the following (215):

- Diabetes mellitus. Class A, B, and C pregnancies are shown in Fig.
FIGURE 89
PATTERN OF PULMONARY MATURATION IN NORMAL PREGNANCY
AND IN DIABETES MELLITUS

Legend
A. Figure shows the pattern of pulmonary maturation in normal pregnancy and the marked changes in surface activity during the last half of pregnancy. Normal patients approach pulmonary maturity at about 29-30 weeks' gestation, however, at approximately 36 weeks instead of progressive maturity, there is an abrupt but brief return to a less mature pattern before final maturity is reached, and these changes from less mature to a mature pattern may correspond with the biochemical surfactant changes in quality and quantity as the type II pneumocyte of the fetal lung shifts lecithin synthesis from the immature trimethylation pathway to the major choline incorporation pathway of the mature fetus.

B. Figure illustrates pattern of pulmonary maturation in diabetes mellitus classes A, B, and C (mean ± SEM). Values for these diabetic classes (23 pregnancies) are superimposed over the normal curve and show that at 36 weeks' gestation there is significantly less maturity in infants of diabetic mothers than their corresponding normal infants and by 37 weeks' gestation the two groups are indistinguishable.

Normal pattern of pulmonary maturation is _______________.
Diabetic pattern of pulmonary maturation is _______________.

89B. Values for these diabetic classes (23 pregnancies) are superimposed over the normal curve and show that at 36 weeks' gestation there is significantly less maturity in infants of diabetic mothers than their corresponding normal infants and by 37 weeks' gestation the two groups are indistinguishable.

- Rh mildly to moderately sensitized pregnancies are shown on Fig. 90A. Values for nineteen Rh pregnancies shows a difference from normal in the mean ± SEM values at 35 and 37 weeks gestation.
- Chronically stressed preeclampsia pregnancies are shown on Fig. 90B. Values for the twenty-six preeclampsia pregnancies overlap the normal values during the critical period of 35-37 weeks' gestation.

These surface tension results compare very similarly to results obtained by the L/S ratio test for these abnormal pregnancy states. All of these changes correspond to pulmonary development found in the clinical setting.

However, Goldkrand and Slattery did experience five false mature results. These amniotic fluid samples were analyzed between 22 and 24 weeks' gestation and showed changes in 5 cases (4 normal and 1 Rh-sensitized), in which 3 infants delivered within 24 hours of amniocentesis died of pulmonary immaturity and RDS, yet the results predicted maturity. These five false-mature results may be attributed to surface active compounds present but not related to the lung, or its surfactants, for after 28 weeks' gestation Goldkrand and Slattery found their results to accurately predict neonatal outcome (215).
FIGURE 90

PATTERN OF PULMONARY MATURATION IN RH-SENSITIZATION AND PRE- ECLAMPSIA

Legend

A. Pattern of pulmonary maturation in mild to moderately Rh-sensitized pregnancies. Values for nineteen Rh pregnancies shows a difference from normal in the mean ± SEM values at 35 and 37 weeks' gestation.

B. Pattern of pulmonary maturation in chronically stressed preeclampsia pregnancies. Values for twenty-six preeclampsia pregnancies overlap the normal values during the critical period of 35-37 weeks' gestation.

Normal pattern of pulmonary maturation is ________________:
Rh-sensitized (A) and preeclampsia (B) patterns of pulmonary maturation is ____________.

(c). Contamination

Contamination with blood moved the mature value toward immature, and the immature toward the mature. This effect of blood contamination of the amniotic fluid on surface tension parallels the effect bloody fluid has on the L/S ratio.

(d). Summary

Shelly et al. (i) utilized surface tension area diagrams and their data revealed that surface activity was a reliable method for estimating fetal pulmonary maturity (210). The amniotic fluid samples were treated with Sephadex and surface activity measured. Six infants tested lacked surface activity and developed RDS. In these cases of RDS, 181 μg or more of phospholipids were measured on the surfactometer and the values were clearly outside the normal ranges for $\gamma_{\text{min}}$ and $\overline{\gamma}$. However, the normal area varied over a wide range and even two RDS cases values fell within this range. Therefore, the area appeared to be the least reliable parameter probably because it was susceptible to varying concentration changes of neutral lipids, proteins, meconium or blood. This test for surface activity only required small amounts (<5mL) of amniotic fluid, took less than one hour to complete, and was very simple to perform. However, due to the problems associated with clinical outcome and variance, others tried to improve on the basic theory of measuring surface tension for predicting fetal pulmonary maturity.

Muller-Tyl et al. (ii) utilized the Wilhelmy balance and noted a progressive increase in surface activity and decrease in surface tension with increasing gestational age (212). In their study they assessed the
amniotic fluid surface activity, however, no conclusions as to the degree of fetal lung maturity could be established because there was no correlation between surface activity value of amniotic fluid and the clinical condition of the newborn infant lungs' maturity. Also Muller-Tyl et al. compared surface tension activity to the L/S ratio and found that in the y-min curve there was a gradual drop of the y-min values after the 32nd week of pregnancy (212). This drop of the y-min values could be due to the fact that the surface balance measures the total activity of surfactant, whereas, the L/S ratio measures surfactant specific individual components. Since this study did not establish the correlation of surface activity of amniotic fluid with a cut-off value for assessing the degree of fetal lung maturity, the procedure was further investigated and developed by others.

Goldkrand et al. (iii) developed the procedure for the direct measurement of the surface tension lowering properties of an amniotic fluid lipid extract (213). The extracts were analyzed on a Du Nouy tensiometer with the use of a platinum ring. The lipid extracts were layered on the surface of the water and after all visible droplets of the extract spread over the water surface, the surface tension was measured by removing the ring. Goldkrand et al. found that the surface tension values for both mature and immature amniotic fluids reach a plateau and a subsurface globule is formed in the system (213). Therefore, after the droplets were allowed to disperse and the surface tension measurement made, the extract was continuously layered, until the addition of more extract did not disperse on the surface, but rather remain as a globule
on the surface and then sank below the water, at this point the volume of extract was recorded (iv). The amount of required extract to achieve globule formation varied with the degree of fetal pulmonary maturity. Globule measurements were compared to the L/S ratio and found to correlate closely. Also studied were normal and abnormal pregnancy amniotic fluids for globule surface tension measurement. For normal pregnancies globule measurement approached pulmonary maturity at about 29-30 weeks gestation, however, at approximately 36 weeks there was an abrupt but brief return to a less mature pattern before final maturity was reached. These changes corresponded with the biochemical surfactant changes in quality and quantity. For the normal and abnormal pregnancy conditions (diabetes, Rh, and preeclampsia), the surface tension results compared similarly with the L/S ratio results. However, they did encounter five false-mature results (4 normal and 1 Rh pregnancy), between 22 and 24 weeks, which may be attributed to surface active compounds present but not related to the lung or its surfactants. For after 28 weeks gestation the results accurately predicted neonatal outcome. These results indicate that the surface tension of amniotic fluid lipid extracts is a reliable and rapid (1 hr) means of antenatally predicting fetal pulmonary maturity.

The surface tension globule formation measurement of amniotic fluid lipid extract is a relatively simplified procedure, which demonstrated clinically and statistically its predictability of fetal pulmonary maturity, and had good correlation with the L/S ratio.

However, this procedure has not gained much popularity in the
clinical laboratory because large aliquots of extract are needed, it requires approximately 1 hour completion time, and the presence of other surface active compounds (i.e., proteins, bile salts, and salts of free fatty acids) may affect the results obtained. Therefore, when comparing this procedure to the L/S ratio physicians favor the L/S ratio acceptability in predicting fetal pulmonary maturity.

2. Shake Test (Foam Stability Test, Bubble Stability Test (BST), Rapid Surfactant Test (RST))

(a). Principle

The shake test concept is based on the characteristic ability of surfactant phospholipids to form highly stable surface films that can support the structure of foam for a period of time. The complex phenomenon of air trapped in a quasi-stable envelope, is the process of foam formation (158), for the escape of a bubble above the meniscus is dependent upon the surface tension of the solution itself (158). Bile salts, proteins, salts of free fatty acids, and other constituents of amniotic fluid will also cause the formation of foam, but if ethanol is added to the system, it acts as a nonfoaming competitive inhibitor, by competing with the other substances for a position in the surface film (158). As Clements et al. (216) found, most biochemical compounds are unable to maintain a bubble with lower surface tension than exists at the air-solvent interface when ethanol is present, as shown in Fig. 91.

The surface tension of an ethanol-water mixture falls as the proportion of ethanol increases. When the proportion of ethanol at 25°C is 47.5% then the surface tension is 29 dynes/cm², a level below that not
FIGURE 91.
DECREASE IN SURFACE TENSION AT AN AIR-LIQUID INTERFACE
WITH INCREASING VOLUME FRACTION OF ETHANOL

Legend

Figure illustrates decrease in surface tension at an air-liquid interface with increasing volume fraction of ethanol. The biological surfactants are positioned on the vertical axis according to the surface tension above which they are able to form stable foams. The dashed line represents a surface tension of 29 dynes/cm at an ethanol volume fraction of 0.475.

normally achieved by proteins, fatty acids, and lysophosphatides (Fig. 91) (158). Addition of phosphatidylcholine will further depress the surface tension producing a stable foam after agitation, the effect of other agents having been competitively eliminated by the ethanol. Both saturated and unsaturated lecithins will generate foam in such a system, but that formed with unsaturated phosphatidylcholine breaks down in a short time, whereas, that formed by lecithin, the fatty acids of which are saturated (myristic, palmitic, stearic), will persist for a few hours at room temperature. Therefore, by mixing equal volumes of amniotic fluid and 95% ethanol, a system is produced in which the volume fraction of ethanol is 47.5%. If the sample is agitated, then a persistent foam indicates some factor which is reducing the surface tension below 29 dynes/cm² in amniotic fluid; this factor is principally surfactant phospholipids.

(b). Different Procedures and Their Results

Following is an account of the procedures utilized for the shake test.

(i). Clements et al. Original Shake Test

In 1972, Clements et al. introduced the shake test for assessing fetal pulmonary maturity and designed it to measure functionality, the presence or absence of surfactant phospholipids (216). The shake test procedure involved utilizing uncentrifuged amniotic fluid diluted with 0.9% saline (NaCl) and 1-mL portions of a 1:1 and 1:2 dilution, were tested by adding 1 mL of 95% ethanol, shaking vigorously for 15 seconds and observing for the presence or absence of a stable ring.
of foam in the tube at the end of 15 minutes. The results were interpreted in the following manner: negative (no foam) at a 1:1 dilution indicating immaturity and a high risk that the infant will develop RDS; intermediate, positive (with foam) at a 1:1 dilution; but negative at a 3:2 dilution; and positive (with foam at both dilutions) indicating maturity and that the infant was unlikely to develop RDS.

This procedure is semi-quantitative because of the two dilutions and was found to be comparable to the L/S ratio in predicting fetal pulmonary status (216).

(ii). Edwards and Baillie-Modified Shake Test

In 1973, Edwards and Baillie (217) described a modification of the original Clements shake test (216). They recommended using absolute (100%) ethanol rather than 95% ethanol as Clements did. This modification was a change in the final ethanol volume-fraction of the mixture from 0.475 for the Clements test (0.95 mL ethanol in 2.00 mL total volume) to 0.500 (1.00 mL ethanol in 2.00 mL total volume) (158). This change in final ethanol volume-fraction from 47.5% to 50% amounts to reducing the surface tension by perhaps 1 dyne/cm², as shown in Fig. 91. This modification will significantly affect the results as shown in Fig. 92A, which is a graph depicting the threshold of foam formation for a given amount of dipalmitoyl phosphatidylcholine in an ethanol-aqueous mixture dependent on the final volume fraction of ethanol (158). This observation suggests that there is an operational difference in the threshold value of a positive test, for the Clements test value is about 30 mg/L and the Edwards and Baillie modification test value is about 45
FIGURE 92

FOAM AS A FUNCTION OF ETHANOL VOLUME FRACTION AND CONCENTRATION OF LECITHIN AND THE COMPARISON OF FOAM STABILITY INDEX VALUES WITH NEONATAL OUTCOME

Legend

A. Figure illustrates the presence or absence of foam as a function of ethanol volume fraction and concentration of dipalmitoyl lecithin in the final assay mixture.

B. Figure illustrates comparison of Foam Stability Index (FSI) values with neonatal outcome in 208 cases when amniotic fluid was obtained within 72 h of delivery. Also the figure shows that a FSI value of ≥0.48 indicates fetal pulmonary maturity (analogous to a L/S ratio of 2.0), whereas FSI values <0.44 suggest a high risk of RDS, and FSI values from 0.44 to 0.46 represent a transition region.

Solid bars represent 27 patients with RDS, grey bars represent 181 infants with normal respiratory function.

mg/L (158).

Based on the information in Fig. 92A a semi-quantitative foam-stability assay called the Foam Stability Index (FSI) test was developed to provide more information about the quantity of surfactant present than the simple shake test did.

(iii). Sher et al.-Foam Stability Index (FSI) Test

In the FSI test Sher et al. (218) added undiluted amniotic fluid to tubes containing varying volumes of 95% ethanol, resulting in final ethanol volume fractions of 42 to 52% as shown in Table XL. In this procedure, the tubes are shaken vigorously for 30 seconds and allowed to stand for 15 seconds before evaluation. Data presented by Sher et al. suggests that the short standing time of 15 seconds compared with the 15 minutes in Clements procedure is sufficient to allow any foam generated by unsaturated lecithin to disperse (219), for the highest ethanol volume fraction which permits the formation of a stable ring of foam is designated the foam stability index for that sample and this provides an indication of fetal lung maturity. This assay differs from the Clements test in that it measures surfactant in concentrations ranging from 15 to 30 mg/L in the assay mixture, while the Clements test is positive for surfactant values greater than about 30 mg/L. To quantitate in this lower concentration range provides the clinician with significant information (158). Figure 92B shows that an FSI value of >0.48 indicates fetal pulmonary maturity (analogous to a L/S ratio of 2.0), whereas FSI values <0.44 suggest a high risk of respiratory distress syndrome, and FSI values from 0.44 to 0.46 represent a
### TABLE XL

RELATIONSHIP OF ETHANOL VOLUME FRACTION IN FINAL ASSAY MIXTURE TO VOLUME OF 95% ETHANOL ADDED TO 0.05 ML OF AMNIOTIC FLUID

<table>
<thead>
<tr>
<th>Ethanol volume fraction</th>
<th>Volume of 95% ethanol/(mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.42</td>
<td>0.395</td>
</tr>
<tr>
<td>0.43</td>
<td>0.415</td>
</tr>
<tr>
<td>0.44</td>
<td>0.430</td>
</tr>
<tr>
<td>0.45</td>
<td>0.450</td>
</tr>
<tr>
<td>0.46</td>
<td>0.470</td>
</tr>
<tr>
<td>0.47</td>
<td>0.490</td>
</tr>
<tr>
<td>0.48</td>
<td>0.510</td>
</tr>
<tr>
<td>0.49</td>
<td>0.530</td>
</tr>
<tr>
<td>0.50</td>
<td>0.550</td>
</tr>
<tr>
<td>0.51</td>
<td>0.580</td>
</tr>
<tr>
<td>0.52</td>
<td>0.605</td>
</tr>
<tr>
<td>0.53</td>
<td>0.630</td>
</tr>
<tr>
<td>0.54</td>
<td>0.660</td>
</tr>
<tr>
<td>0.55</td>
<td>0.685</td>
</tr>
</tbody>
</table>

This was achieved by preparing a series of 14 tubes measuring 12 by 75 mm with different volumes of 95% ethanol. The amniotic fluid supernatant was dispersed in 0.50 mL aliquots into each of the prepared test tubes containing graded volumes of ethanol (left-hand column).

transition region (158).

(iv), Schlueer et al.-Improved Shake Test

In 1979, a refinement of the above Clements procedure (i) was utilized in an attempt to increase the predictive value of the shake test. The refined procedure by Schlueer et al. is similar to Clements, however, it tests three dilutions of amniotic fluid and the interpretation of the bubble patterns is much more precisely defined (220). In this procedure, saline and alcohol are added to test tubes containing amniotic fluid to give the following three dilutions:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution (mL)</td>
<td>1:1</td>
<td>1:1.3</td>
<td>1:2</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>1.0</td>
<td>0.75</td>
<td>0.5</td>
</tr>
<tr>
<td>Saline (0.9% NaCl)</td>
<td>---</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Alcohol (95% ethanol)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The tubes are capped, shaken vigorously by hand for 15 seconds, placed on a rack, and left undisturbed for 15 minutes. The air-liquid interface of each tube is then examined for the presence of stable bubbles. Contents of the tube are recorded as being positive when there are enough bubbles present to form a complete ring around the tube, intermediate when small bubbles are present but not in sufficient numbers to form a complete ring around the tube, and negative when there are no bubbles. Figure 93 is a chart which corresponds to the bubble patterns observed for each of the three dilutions, and Table XLI assigns the results to one of the five categories with respect to the risk of RDS. Because the test has three possible reactions in each of three dilutions, the results are ranked
FIGURE 93
EIGHT POSSIBLE RESULTS OF THE FOAM TEST

Legend

Figure is a schematic representation of eight possible results of the foam test and their interpretation: Contents of each tube are recorded as being positive when there are enough bubbles present to form a complete ring around the tube (A–E), intermediate when small bubbles are present, but not in sufficient numbers to form a complete ring around the tube (F and G), and negative when there are no bubbles (H).

TABLE XLI
RISK FOR DEVELOPING HMD IF DELIVERY IS WITHIN
24 HOURS OF AMNIOTIC FLUID FOAM TEST

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Amniotic fluid dilution</th>
<th>HMD/total</th>
<th>HMD</th>
<th>Infants status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>1:1</td>
<td>1/205</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>1:1.3</td>
<td>1/207</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>1:2</td>
<td>1/205</td>
<td>25</td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>+</td>
<td>1/205</td>
<td>44</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>+</td>
<td>1/205</td>
<td>79</td>
</tr>
</tbody>
</table>

according to eight degrees of reaction as a titer of amniotic fluid surfactant. A progressive increase in incidence of RDS was found with decreasing reaction, with the infants falling into the five groups shown in Table XLII. Schlueter et al. found the following results in the 410 infants tested within 24 hours of delivery (220):

- 205 had positive tests in all three dilutions, and only one developed RDS.
- In the other 205 infants the reaction was intermediate or negative in one or more dilutions; RDS developed in 64 of these infants.
- Infants in groups I and II were heavier, and more mature than those in groups III to V. However, among infants of equivalent gestational age or birth weight, the incidence of RDS still correlated significantly with the foam test results.
- Table XLII shows that at a given level of foam test reaction, the risk of RDS decreased as length of gestation increased, but at a given gestational age, the risk of RDS decreased as surfactant titer increased.
- There was no false negative tests among the less mature infants, and all infants born before 33 weeks' gestation in Group V had RDS.

Schlueter et al. final conclusion was that the foam test could be used to accurately predict a graded risk of development of RDS in an infant delivered within 24 hours after the test. For the eight levels of foam test reaction appear to span most of the range of surfactant concentration from the immature to the fully matured lung, thus giving a
TABLE XLIII
INDEPENDENT ASSOCIATIONS OF FOAM TEST AND
GESTATIONAL AGE WITH THE RISK OF HMD

<table>
<thead>
<tr>
<th>Risk group</th>
<th>20</th>
<th>30-32</th>
<th>33-35</th>
<th>36-38</th>
<th>39-41</th>
<th>42</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.1</td>
<td>0.4</td>
<td>0.4</td>
<td>1.5</td>
<td>0.102</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(27)</td>
<td>(27)</td>
<td>(27)</td>
<td>(27)</td>
<td>(27)</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>1.1</td>
<td>0.14</td>
<td>0.26</td>
<td>1.24</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>(87)</td>
<td>(87)</td>
<td>(87)</td>
<td>(87)</td>
<td>(87)</td>
<td>(87)</td>
</tr>
<tr>
<td>III</td>
<td>0.8</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>0.78</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
</tr>
<tr>
<td>IV</td>
<td>2.2</td>
<td>2.3</td>
<td>2.5</td>
<td>3.6</td>
<td>0.75</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
</tr>
<tr>
<td>V</td>
<td>0.6</td>
<td>0.102</td>
<td>0.78</td>
<td>1.21</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
<td>(107)</td>
</tr>
</tbody>
</table>

semi-quantitative measurement of surfactant concentration (220).

(c). Sources of Error and Interference

Following are several factors important in the performance of the various shake tests.

Accurate reproduction of the initial 47.5% ethanol mixture. As small volumes are involved care with titration is essential. Similarly, the 95% ethanol stock solution must be accurate for ethanol at this concentration is hygroscopic and will tend to deteriorate, becoming progressively dilute (125).

All glassware must be free of soap, serum, or biological fluids to eliminate interference, and rubber stoppers should be avoided as material leached from these can also interfere (125).

When the ethanol is added to the tube a precipitate may form and this must not be confused with the presence of foam (221).

The test tube may have several affects on the test, such as the diameter of the tube may affect the characteristics of foam formation, any movement of the tubes during the waiting period could disrupt the foam, therefore affecting the results, and finally if the tubes are not read at the proper time, the test must be set up again (125).

Keston et al. studied the affect of temperature on the shake test and found that foam generated by disaturated lecithins becomes less stable as the temperature rises above 25°C (222).

Thorough mixing of the amniotic fluid sample before assay is essential. However, there is controversy on utilization of uncentrifuged samples versus centrifuged samples with some loss of
(d). Contamination

False positive and false intermediate results are obtained with blood and meconium contaminated amniotic fluid samples. The rule in most laboratories is to exclude all visibly contaminated samples. However, low meconium concentrations not detected by visible inspection can produce false positive results. Also the presence of certain obstetric creams, vaginal secretions, and ascitic fluid can produce false positive results. (121).

(e). Summary

The Clements, and Edward and Baillie shake tests are excellent screening methods and their primary advantage are the ease with which the test can be performed and speedy results (less than 1/2 hour to complete). However, both tests have two major deficiencies. First, the interpretation of the meniscus for the presence of bubbles is relatively subjective, for there are only two possible results—foam (a positive result), or no foam (a negative result) and accordingly, the assay is not sufficiently quantitative. Secondly, they often produce false negative results (suggesting insufficient surfactant, with normal neonatal pulmonary status in association with no foam). As a screening procedure, while the absence or rarity of false positive results is essential, the occurrence of some false negative results is not a major disadvantage. Several reports support the view that the shake test provides a reliable simple screening procedure, provided negative results (which should include Clements' intermediate result) are taken to indicate the need for
one of the more complex and precise predictive tests, such as the L/S ratio or FSI. When performing the shake test, it should be taken into consideration that the results are affected by centrifugation speed, and the presence of contaminants in the amniotic fluid. Additionally, extreme care to the technical detail of pipetting in the ethanol volume fraction in the final assay mixture is critical and may account for the occasional reports of this test's unreliability.

The FSI test, in contrast to the two shake tests, quantifies the surface tension lowering ability of surfactant in terms of 44 possible values (e.g., 0.42 through 0.55 inclusive). Therefore, this test gives a semiquantitative measurement of surfactant concentration for the 8 levels of reaction span from immature to fully matured lung. Also, the FSI assay has been shown to be consistently reproducible; replicate value on the same specimen agree within 0.01 (219). The FSI assay is simple, rapid, and requires no expensive equipment or special expertise. The FSI test is readily available to most laboratories at a minimal cost. The reliability of the FSI test does, however, depend on the ability to pipette accurately. Accordingly, this test should not be considered a bedside procedure.

Finally, when comparing the shake test results with the L/S ratio results there appears to be good correlation, for these two test procedures in theory are both detecting the absence or presence of lecithin rich in saturated fatty acids in the amniotic fluid. These two procedures are comparable in the following ways, for both require careful technique and only a small aliquot of amniotic fluid, and both
are susceptible to interferences by certain contaminants (i.e., blood and meconium). However, these two procedures differ in the following ways. The shake test requires only test tubes, pipettes and ethanol, is easily taught to an unskilled person, and requires only 1/2 hour for analysis time. On the other hand, the L/S ratio procedure requires isolation and quantification of lecithin and sphingomyelin with relatively sophisticated and expensive equipment, skilled technical personnel and 1½ hours for analysis.

With Clements' initial concept of foam formation in the presence of surfactant phospholipids, the foam test was utilized by physicians because of its ease of performance and speedy results. However, since the L/S ratio's appearance and acceptability as an indicator of fetal pulmonary maturity, the shake test performance has rapidly declined.

3. Fluorescence Polarization
   (a). Principle

   The physical parameters of viscosity and surface tension of fluids are determined by the intermolecular forces of the fluid and are, therefore, interrelated, so that the surface tension of the pulmonary surfactant can be translated into intrinsic viscosity which, as for other lipid systems, may be expressed in terms of microviscosity (223). The phospholipid and neutral lipid composition are the two elements which the microviscosity of the hydrophobic interior of the lipid membrane are dependent on. It has been shown that microviscosity is related to the length and degree of saturation of phospholipid acyl side chains, the mole ratio of different phospholipids, and the presence of cholesterol in
the lipid aggregates (125).

One of the most efficient techniques for the determination of lipid microviscosity is by usage of a lipid-soluble fluorescent probe, such as 1,6-diphenyl-1,3,5-hexatriene (DPH). The DPH probe polarizes incident radiation (365 nm) in an aqueous environment, emitting light at a wavelength greater than 418 nm (158).

Figure 94 illustrates the measurement of microviscosity by fluorescence depolarization (125). Depending on the fluidity of the environment, the probe (dissolved in the interior of the lipid micelle) undergoes rotational motion (125). Then polarized incident radiation (365 nm) is directed on to the sample and only those probe molecules which are in a suitable orientation relative to the incident light are excited. Movement of the molecules results in fluorescence depolarization (P value) which is determined by measuring the intensities of emitted light in planes parallel to and perpendicular to the plane of polarization of the excitation light (125). If the molecules are fixed the light emitted is polarized in a plane parallel to that of the incident light (125).

The detected degree of fluorescence polarization (P) directly relates to the rotational motion of the probe and, therefore, to the microviscosity of the hydrocarbon region of the lipid assembly (223). For the greater the viscosity, the more effectively the liposome opposes the rotation of the probe molecule. Therefore, depolarization of the incident light is a result of restricted rotation of the probe (158).

Fluorescence polarization decreases with advancing gestational age.
FIGURE 94
MEASUREMENT OF MICROVISCOITY BY FLUORESCENCE DEPOLARIZATION
AND AMNIOTIC FLUID MICROVISCOITY CHANGES DURING GESTATION

Legend
A. Figure is a schematic illustrating measurement of microviscosity by fluorescence depolarization.

(A) Phospholipid micelles or bilayers are shown diagramatically, the circles representing the polar head groups of the phospholipid molecules whose fatty acid side chains form the hydrophobic region of the micelle or bilayer (stippled area). (B) DPH molecules are shown randomly oriented in a hydrophobic region. Those molecules which are in the correct orientation to fluoresce in response to the polarized excitation light are indicated in black. (C, D, and E). Varying degrees of movement of the molecules between the time of excitation and fluorescence, and the corresponding effect on the degree of depolarization of the emitted light are shown. If the microviscosity of the hydrophobic region is so high that no movement of the molecules occurs between excitation and fluorescence, the emitted light is completely polarized (C). In contrast, probe molecules in a region of low microviscosity may have time to become randomly rearranged, resulting in complete depolarization of the emitted light (E).

B. Change in mean and standard deviation of amniotic fluid microviscosity during gestation (from 116 samples): Graph illustrates the fact that amniotic fluid microviscosity begins to decrease at a mean gestational age of 31.4 weeks and continues to do so until term (prior to 31.4 weeks it remains high).

FIGURE 94

At time of emission of fluorescence:

Incident light

A

or

B

C

or

Polarized fluorescence

D

Partially depolarized fluorescence

or

E

Completely depolarized fluorescence

At time of excitation with polarized light

AMNIOTIC FLUID RELATED TO GESTATIONAL AGE

AMNIOTIC FLUID MICROBACILITY (X 1000)

Gestation Age (Weeks)

-5 SD

Mean

+5 SD
As shown in Fig. 94B Stark et al. found that amniotic fluid microviscosity begins to decrease at a mean gestational age of 31.4 weeks and continues to do so until term (before 31.4 weeks it remains high).

(b) Procedure

In 1976, Shinitzky et al. reported on the usage of a fluorescent hydrocarbon probe to assess the microviscosity of amniotic fluid (225). A freshly drawn sample of amniotic fluid was first centrifuged at about 1000 rpm (g force unspecified) for 10 minutes to remove tissue fragments and cells. The supernatant fluid (1 ml) was mixed with 4 ml of the DPH probe in a phosphate-buffered saline dispersion and incubated at 37°C for at least 20 minutes. After incubation the sample was allowed to cool to room temperature (24 ± 1°C) after which it was ready for measurement of the P value (225).

The P values are measured and recorded with a specially designed instrument, the Elscint Fetal Lung Maturity Analyzer (FELMA) (Fig. 95). Figures 95A and B illustrate the fetal lung maturity analyzer and its schematic flow process, which is (225):

- The wavelength necessary to excite the molecular probe is produced by the light emitted by a high intensity air-cooled mercury light source which is passed through a quartz optics and a narrow bandwidth filter.
- A Glan-Thompson polarizer polarizes the monochromatic light and excites the molecular probe dissolved in the amniotic fluid contained in the quartz cuvette.
FIGURE 95
ELSCINT FETAL LUNG MATURITY ANALYZER (FELMA)

Legend
A. Illustrates the Elscint Fetal Lung Maturity Analyzer (FELMA) instrument.
B. Illustrates the FELMA schematic flow process.

- The cuvette is placed in a thermoelectric temperature stabilized compartment (+ 0.2°C), for the microviscosity of amniotic fluid is temperature dependent.

- Then the emitted light is depolarized as a function of the microviscosity of the amniotic fluid, and the depolarization is detected by measuring the intensity of the light with two perpendicular channels, measuring the intensity of the light with two photomultipliers and feeding the amplified and balanced outputs to a calculating unit.

- And finally the calculated depolarization value is digitized and displayed on the front of the instrument panel.

(c). Modifications of the Original Procedure

In the few years the test has been utilized, several modifications have been introduced into the method and investigated. These modifications include the following:

(i). Centrifugation

The centrifugation time and speed initially varied between laboratories; for example, Stark et al. (226) centrifuged the amniotic fluid sample for 10 minutes at 1000 x g or 1500 x g, while Golde et al. (227) centrifuged for 10 minutes at 1000 r/min.

However, most recently, in 1981, Simon et al. studied in detail the significant effect centrifugation has on the fluorescence polarization results of amniotic fluid (228). In this study, fluorescence polarization was measured on amniotic fluids of different gestational ages before and after centrifugation and at relative centrifugal forces of 34 to 4955 x g
for 10 minutes at room temperature. Simon et al. reported the following results (Fig. 96A and B) (228):

- Centrifugation significantly increased the FP (fluorescence polarization) values over those of uncentrifuged fluid.
- The greatest increases were observed in the most mature fluids centrifuged at 34 to 230 x g. Simon et al. explained this effect by the presence of increasing amounts of cells and other materials in the amniotic fluid as gestation nears term (228).
- Almost without exception, use of centrifugal forces greater than 1239 x g did not further increase FP values.
- The fact that centrifugation increases the FP values of most amniotic fluids is explained by the precipitation of lipids associated with debris and cells and originating from other sources than surfactant (228). Therefore, the increase in FP values represents the differences between the composition of these lipids removed in the supernatant and composition of surfactant.

For as mentioned previously, the amniotic FP values are affected by the following factors: degree of saturation of fatty acid; nature of phospholipid; chain-length; cholesterol content; and protein content of lipid aggregates (229).

In conclusion, Simon et al. recommend centrifugation as a prerequisite to reproducible FP values (228). Also they recommend that centrifugation be performed at an RCF (relative centrifugal force) of at least 1500 x g before FP is measured, because of the observed increases in FP values with RCF's between 230 and 1239 x g.
FIGURE 96
EFFECT OF CENTRIFUGATION ON FLUORESCENCE POLARIZATION OF AMNIOTIC FLUID

Legend
A. Graph depicts amniotic fluid fluorescence polarization as a function of relative centrifugal force. Four (A, B, C, E) of the five fluids showed no further significant effect of centrifugation at higher relative centrifugal forces (RCF). Only fluid D displayed a further increase in FP value with an increasing RCF (up to 1239 x g).

B. Graph depicts effect of low relative centrifugal forces on fluorescence polarization of near-term amniotic fluids. Four (G-K) of the five fluids had FP values which increased when centrifuged as slowly as 34 x g, and which stabilized between 102 and 230 x g. In these four no further significant increase was observed with additional centrifugation. Fluid F exhibited no centrifugation effect.

(ii). Incubation Conditions

Various incubation conditions have been employed, such as 10 minutes at room temperature (230), and 30 minutes at 30°C followed by 10 minutes at 25°C (224). In 1983, Barkai et al. investigated the effect the physiological temperature of 37°C would have on increasing the specificity of the P value obtained with amniotic fluid fluorescence polarization measurements (231). For microviscosity of lipids is inversely related to temperature and since 90% of the alveolar surfactant is lipids, temperature does affect the measured P value (231). As noted above in previous studies, the P value was determined at room temperature (25°C), however, Barkai et al. investigated the effect 37°C has on the P value, because this temperature reflects the physiological state of the lung surfactant at body temperature (231). Therefore, Barkai et al. compared the discriminative ability of the P value measured at both temperatures in relation to clinical outcome in 288 consecutive cases at risk for preterm delivery (288 samples at 25°C = P25, and 112 of these were measured concurrently at 37°C = P37 as well) (231). Simultaneous evaluation of the predictive value of P37, P25, and gestational week in the subgroup of 112 samples, revealed that P37 was the best predictor and was sufficient to discriminate between RDS and the non-RDS cases (231). At 37°C, when sensitivity was fixed at 100%, the specificity is significantly improved to 97%, while P25 was associated with low specificity of 79% when sensitivity was fixed at 100%, as shown in Table XLIII. The results revealed the following:

- The P37 results resemble the specificity achieved by the lung
**TABLE XLIII**

SENSITIVITY AND SPECIFICITY OF FLUORESCENCE

POLARIZATION VALUES MEASURED AT 25 AND 37°C

<table>
<thead>
<tr>
<th>Temperature</th>
<th>25°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>288/Total Group</td>
<td>112/Subgroup</td>
</tr>
<tr>
<td>Cut-off Value for lung maturity</td>
<td>≤0.316</td>
<td>≤0.286</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100% (27/27)</td>
<td>100% (9/9)</td>
</tr>
<tr>
<td>Yielding</td>
<td>55</td>
<td>---</td>
</tr>
<tr>
<td>Misdiagnosed no-HMD</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td>Yielded specificity</td>
<td>79% (206/261)</td>
<td>97% (100/103)</td>
</tr>
</tbody>
</table>

\[ \text{Sensitivity} = \frac{\text{HMD correctly classified as immature}}{\text{Total HMD}} \]

\[ \text{Specificity} = \frac{\text{no-HMD correctly classified as mature}}{\text{Total no-HMD}} \]

profile test suggested by Gluck et al. (130).

- Also Barkai et al. felt that the FP method at 37°C more accurately predicted RDS than the L/S ratio (231).

- A false positive problem was encountered with toxemic samples at both temperatures and this group was the only one that failed to improve specificity up to 100% at 37°C. The situation is currently being investigated and might be explained by some peculiar concentration of phospholipids in pregnancies complicated by toxemia.

In conclusion, Barkai et al. stated that since only nine cases of RDS were detected at 37°C, more RDS cases would have to be studied before the lower limit of the P value for immature lungs at 37°C is conclusively known and the specificity estimated with satisfactory precision (231): Therefore, they suggest readings at both temperatures to be taken.

(iii). FP Cut-off Value and Its Relationship to Neonatal RDS.

The different laboratories investigating the FP procedure from 1978-1981 utilized various cut-off values for clinical interpretation of the results. In 1981, Cheskin and Blumenfeld compiled table XLIV of all the previous, studies showing recommended cut-off values relating FP value to neonatal RDS and calculations of sensitivity, specificity and predictive value of positive results (232). As seen in Table XLIV the cut-off values range from >0.320 to >0.345. The lower cut-off values give increased sensitivity and the higher cut-off values give increased specificity and predictive value. A
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
</tr>
<tr>
<td>FP value indicating high risk of RDS</td>
<td>&gt;0.320</td>
<td>&gt;0.320</td>
<td>&gt;0.320</td>
<td>&gt;0.320</td>
<td>&gt;0.330</td>
<td>&gt;0.340</td>
<td>&gt;0.345</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>4</td>
<td>102</td>
<td>56</td>
<td>186</td>
<td>161</td>
<td>40</td>
<td>83</td>
</tr>
<tr>
<td>No. of RDS cases</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>86%</td>
<td>86%</td>
<td>85%</td>
<td>85%</td>
<td>79%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Predictive value of a positive result</td>
<td>50%</td>
<td>50%</td>
<td>32%</td>
<td>31%</td>
<td>67%</td>
<td>180%</td>
<td>100%</td>
</tr>
</tbody>
</table>

---

**Table IIV**

SENSITIVITY, SPECIFICITY, AND PREDICTIVE VALUE OF FLUORESCENCE POLARIZATION

---

definitive quantitative statement relating the risk of RDS to FP value is undetermined, due to the small number (40) of RDS pregnancies determined by FP (Table XLIV). However, Cheskin and Blumenfeld found that in all samples measured within 48-72 hours of delivery, if the FP value was <0.320 no RDS resulted, whereas a very high risk of RDS was associated with FP values >0.336 (232).

Cheskin and Blumenfeld also compared the FP method to the L/S ratio method and found the definite relationship of a FP value >0.336 corresponding to a ratio of <2 (232). However, they also found that the two methods do not relate totally, for neither the FP value nor the L/S ratio is a linear scale when evaluating regression equations and correlation coefficients. Also they noted that the FP value is greatly influenced by the phospholipids PG and PI which are not involved in the L/S ratio.

Another study, conducted by Cheskin et al., investigated the microviscosity effect of prepared phospholipid mixtures of PG and PI at varying gestational phases as shown in Fig. 97 (229). This figure is evidence that during advancing gestation, the amniotic fluid microviscosity measurements reflect the presence and quantitative changes of PG and PI. Therefore, microviscosity measurements could be very helpful in determining maturity of amniotic fluid from a diabetic mother, for it is common for some infants of diabetic mothers to develop RDS when their amniotic fluid L/S ratios indicate maturity and PG is absent. Based on this study and others, an amniotic fluid with PG absent, a borderline mature L/S ratio, and mean physiological amounts of
FIGURE 97
MICROVISCOSITY OF PHOSPHOLIPID MIXTURES
REPRESENTING THREE PHASES OF GESTATION

Legend
In this graph, the horizontal dashed lines indicate the microviscosities of mixtures containing mean phospholipid percentages corresponding to the composition at each period. Within each gestational period PG or PI was varied as noted on the x coordinate.

other phospholipids would have a microviscosity much higher than if PG were present in normal quantities (229). Therefore, amniotic fluid microviscosity determination may be a reliable index of the risk of RDS in infants of diabetic mothers, when PG is absent. Cheskin et al. felt that the measurement of amniotic fluid FP was precise and correlated well with the L/S ratio, however, they point out that the L/S ratio only measures L and S, and not the other important surfactant phospholipids of PG, and PI, as FP measurement does (229).

From their study Cheskin et al. concluded the following points (229):

- During gestation the FP measurements parallel the development of the fetal pulmonary surfactant system, and reflect the varying concentrations in all the amniotic fluid phospholipids (L, S, PG, PI, PS, and PE).
- The FP measurements are easy and fast to perform and can reliably predict fetal lung maturity and the risk of RDS in the newborn.
- Finally, the FP measurements have a lower coefficient of variation than the L/S ratio and the two methods correlate well. Therefore, the FP measurement appears to have diagnostic as well as technical advantages over the L/S ratio.
- Volume of amniotic fluid sample can be varied from 0.25 mL to 1.0 mL without affecting the FP value.

(d). Contamination

Contamination by bilirubin or related pigments within the normal range were found to have only a negligible effect on P. However, both blood and meconium contamination interfere in the determination of the P
value of amniotic fluid. Their effect is variable depending on their lipid composition, as well as their concentration in the amniotic fluid (231). Generally, such contamination will reduce the P value and may lead to false positive results.

(e). Summary

This FP method is quite new in the medical field and is based on the following advantages and disadvantages.

Advantages of the fluorescence polarization method are its speed, simplicity, technical accuracy, and reproducibility. This method requires only limited skill on the part of laboratory personnel and is quite rapid, the test time only being dependent upon the incubation of the dye probe with the amniotic fluid (30 minutes). This method employs the use of the highly specialized instrumentation, which simplifies the procedure to such an extent that the obstetrician can have the results within less than two hours after amniocentesis. Some clinicians feel that by employing this method it is possible to follow up the progress of lung maturation at short time intervals. Also they feel that it would be an ideal screening method. The determined P values are highly accurate and reproducible even when tested with different microviscosimeters. This FP method should be reproducible between laboratories, provided that the following four test conditions are controlled and standardized: 1) preliminary centrifugation of amniotic fluid; 2) incubation conditions; 3) temperature at which the P value is measured; and, 4) usage of specified cut-off values for clinical interpretation. Changes in FP measurements in amniotic fluid during gestation parallel the development
of the surfactant system. These measurements reflect the relative amounts of lecithin, as well as the other phospholipids (PG, PI, PS, and PE) important for fetal lung maturity. On the other hand, the L/S ratio just measures lecithin and sphingomyelin and not the other amniotic fluid phospholipids (PG, PI, PS and PE) important for fetal lung maturity.

The primary disadvantage of this method is the cost of the specialized instrumentation required. Also only a small population of amniotic fluids have been tested by this FP method, therefore, more data and much more detailed reporting of clinical trials of this method are required to allow its clinical performance to be evaluated. Relevant information for each case should be recorded such as: P value, time between sampling and delivery, gestational age, and respiratory outcome of the neonate. Also one standardized procedure must be utilized with specified centrifugation of sample, incubation time, and temperature.

Then data from the different laboratories could be pooled for evaluation and the establishment of a generally acceptable cut-off value.

(f). Items for Future Investigation

It has been noted previously that the fluorescence polarization method is better than the L/S ratio because it measures all the phospholipids of surfactant, however, some have noted that other phospholipids that are not surfactant are also present in amniotic fluid. For, Shinitzky et al. theorize that the measured microviscosity represents the weighted average of the microviscosities of all lipid domains accessible to the DPH fluorescent probe, therefore, the microviscosity of amniotic fluid would only reflect the surface tension
of surfactant, if surfactant was the main source of amniotic fluid at all
gestational phases (233). Amniotic fluid contains variable and
significant amounts of nonsurfactant phospholipid and neutral lipids,
including cholesterol and cholesterol esters (233). Therefore, to some,
it is questionable whether the fluorescence polarization method is really
measuring the surface tension of the pulmonary surfactant.

Since 1980, Gebhardt has mentioned several factors that may influence
the microviscosity of amniotic fluid, but which are probably independent
of the production of lung surfactant (234). These factors which
influence microviscosity of amniotic fluid are (234):

- Amniotic fluid contains high-density lipoproteins and these
  proteins have their own microviscosity, which will be superimposed
  upon that of the lung phospholipids.
- Amniotic fluid contains cholesterol and this will cause a decrease
  in the FP of the high-density lipoproteins.

Therefore, Gebhardt suggests that the microviscosity be determined
for the high-density lipoproteins to see their effect on the total
microviscosity (234).

Also in 1982 (235) and 1983 (236), Gebhardt suggested that the FP be
determined by isolating the lamellar bodies of amniotic fluid to predict
fetal lung maturity, for it was found that the lamellar body phospholipid
content of amniotic fluid was a more specific measure of fetal lung
maturity than the L/S ratio. Therefore, measuring the FP of lamellar
bodies also would better indicate fetal lung maturity than would the FP
of whole amniotic fluid. However, a discrepancy has arisen in this
theory, for investigators have recently shown that the FP of the lamellar body fraction does not differ significantly from the non-lamellar FP of amniotic fluid (236). This discrepancy requires some further investigation and explanation. Another discrepancy concerning FP has arisen, for Cox et al. findings indicate that endogenous fluorescence in clinical samples exhibits significant variability and can represent between 5-50% of the total fluorescence (237). It was demonstrated that this endogenous fluorescence increases with gestational age and in many cases has the effect of lowering the total polarization (237). Studies conducted on aqueous lipid dispersions indicate that the biochemical composition of the lipids is an essential determinant for the motion of the DPH probe. Therefore, several factors can contribute to the degree of polarization for a specific amniotic fluid.

In final conclusion, this FP procedure has the simplicity and speed for clinical application, however the instrument is costly. But the unanswered questions of endogenous fluorescence, and the nonsurfactant high-density lipoproteins' affect on the microviscosity FP value obtained, must answered, before its clinical usefulness in predicting fetal lung maturity is accepted. Also the procedure's steps must be standardized and the predictive cut-off value for fetal lung maturity determined with a larger RDS population.

C. COLLECTION AND HANDLING OF SPECIMENS FOR FETAL PULMONARY MATURITY ASSESSMENT

1. Collection

The amniotic fluid sample can be obtained either by amniocentesis or
vaginally after rupture of membranes (this can be contaminated). The standard procedure for amniocentesis is transabdominal perforation of the amniotic sac by a physician. Vaginally obtained samples are satisfactory only if care is taken to obtain a clean sample. It should be obtained if the fluid is freely flowing and carefully tapped. Vaginal samples should be utilized with caution because they are easily contaminated with mucus and bacteria that may contain phosphodiesterases. In general, hospital obstetric services, usually perform transabdominal amniocentesis during the third trimester for determining fetal pulmonary maturity. Amniotic fluid surfactant evaluation is helpful in cases with obstetrical conditions that warrant early delivery, such as obstetrically-complicated cases of undetermined gestational age, and in cases with obstetrical complication that may alter surfactant production.

2. Handling

The following are the standard universally accepted policies for handling of the amniotic fluid specimen for fetal pulmonary assessment:

- If specimen cannot be processed immediately it should be refrigerated at 4°C, for if specimen is left at room temperature for any length of time, any phosphodiesterase present may destroy the lecithin.
- For storage longer than 24 hours the specimen should be frozen at -20°C.
- Avoid repeated freezing and thawing.

In 1981 Schwartz et al. conducted a study to evaluate the stability of phospholipids in amniotic fluid as a function of temperature and time.
They determined optimum conditions for storage and for transport of specimens to centralized laboratories. Schwartz et al. determined the following optimum conditions (238):

- Room temperature storage is acceptable for periods up to 24 hours only (then a significant decrease in values occurs).
- When longer delays are anticipated before lipid extraction, the specimens must be frozen, or refrigerated, or wet-ice storage (4°C) should be utilized.
- Amniotic fluid samples which were stored frozen and strict quality control was maintained in analytic procedures; only minimal changes occurred in phospholipid concentrations over 12 months.
- This study demonstrated the remarkable stability of PC, SPC, and PG over a range of temperatures (4°C, 22°C, and -20°C) and times (2, 24, 48, and 72 hours; 1, 3, 6, and 12 months).

In conclusion, these standardized handling policies must be followed, for even a simple modification in handling could have a significant effect on the results obtained.
CHAPTER VI
SURVEYS OF CURRENT CLINICAL METHODS AND
PHILOSOPHIES ON FETAL LUNG MATURITY ASSESSMENT

During my extensive literature search for this critique, I encountered some variations and discrepancies concerning respiratory distress syndrome (i.e., radiographic diagnosis, treatment, prevention, and terminology) and the methods utilized to evaluate fetal lung maturity. Therefore, I decided to survey the Metro Detroit area hospitals' clinical laboratories and physicians to determine the actual philosophies, and practices in current usage.

A. FETAL LUNG MATURITY CLINICAL TESTING SURVEY

As illustrated in CHAPTER V, (review of the methods utilized to evaluate fetal lung maturity) numerous variations were encountered in the procedures utilized and results obtained. There variations included the reliability of specific tests, predictive values of results obtained, and lack of standardization of procedures utilized. Also many investigators correlated their procedure's results with the L/S ratio (universally accepted standard test); even though their correlation was good, their sample size was inadequate and population tested not inclusive (complicated pregnancies and RDS cases omitted). Therefore, I surveyed the Metro Detroit area hospital clinical laboratories to determine which procedures are currently being utilized by physician's to monitor and diagnose fetal lung maturity.

APPENDIX A is the questionnaire utilized in the clinical laboratory survey. The questionnaire reviewed the methods utilized in the last 5-15
years in each specific laboratory. Then, since the L/S ratio has been labeled the universally accepted standard test for fetal lung maturity determination, the questionnaire was broken down into each procedural step for specific questions. For each of the steps of the L/S ratio procedure have been subjected to innumerable variations to improve, or simplify the test. These variations are very controversial and directly affect the tests' analytical and/or clinical significance. The questions included specimen handling and contamination, centrifugation, lipid extraction, acetone precipitation, thin-layer chromatography, staining, charring, quantitation, reproducibility, cut-off values, quantity of tests run, physicians attitude and trust of the procedure, and new procedures being investigated. The questionnaire was sent out to four hospital laboratories, and I surveyed six clinical chemists of area hospitals with the questionnaire.

A summary of the ten area hospitals clinical laboratory procedures for evaluating fetal lung maturity is on Table XLV, which describes the following: hospital; procedure; test time; quantity of tests run; handling of sample and contamination; the procedural steps including centrifugation, lipid extraction, acetone precipitation, thin-layer chromatography (TLC), staining, charring, quantitation; cut-off values; reproducibility; comments and new procedures being investigated. A brief summarization of the survey will now be discussed (numbering corresponds with tables' column titles).

1. Procedure Utilized

Eight laboratories perform one-dimensional thin-layer chromatography
<table>
<thead>
<tr>
<th>Hospital</th>
<th>L/S Procedure utilized/cast size/quantity</th>
<th>Handling of sample and contamination</th>
<th>Centrifugation</th>
<th>Lipid extraction</th>
<th>Acetone precipitation</th>
<th>Thin-layer chromatography (TLC)</th>
<th>Staining/Quenching</th>
<th>Quantitation</th>
<th>Reproducibility</th>
<th>Cut-off values</th>
</tr>
</thead>
</table>
| Patient 1 | Heme with in-house modifications, acetone spin, and two solvent systems one for L/S and one for R/S | IF contaminated, spin in acetone to remove contamination | 3 min at 8000 G in a 15 ml conical tube. Volume 20 ml, vortex 20 sec, then 5 ml, vortex 5 sec. | 2 ml, vortex 5 sec. | 2 ml, vortex 5 sec. | Solvent-G | Quantitative scanning | Heme stain | L/S 1-2 |.
| Patient 2 | 3 days | Heme with in-house modifications, acetone spin, and two solvent systems one for L/S and one for R/S | 3 min at 8000 G in a 15 ml conical tube. Volume 20 ml, vortex 20 sec, then 5 ml, vortex 5 sec. | 2 ml, vortex 5 sec. | 2 ml, vortex 5 sec. | Solvent-G | Quantitative scanning | Heme stain | L/S 1-2 |.

<insert table data here>

**TABLE IX**

*METRO DEPARTMENT AREA SUMMARY OF THE CLINICAL TESTING SURVEY FOR FETAL LUNG MATUREITY ASSESSMENT*
<table>
<thead>
<tr>
<th>Hospital</th>
<th>L/S Procedure utilized/estimation time/quantity</th>
<th>Handling of sample and contamination</th>
<th>Centrifugation</th>
<th>Lipid extraction</th>
<th>Aqueous precipitation</th>
<th>Thin-layer chromatography (TLC)</th>
<th>Staining/Charring</th>
<th>Quantitation</th>
<th>Reproducibility</th>
<th>Cut-off values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. A. J. Walton Clinical Obstet.</td>
<td>A specimen with a packed RBC volume &gt; 0.5% is regarded as unacceptable for a reliable L/S.</td>
<td>10 ml of specimen placed in a 15 ml central centrifuge tube and centrifuged at 700 × gg for 10 min. The conditions chosen are a safe compromise between over and under centrifugation.</td>
<td>Take 1 ml.</td>
<td>Take 1 ml.</td>
<td>Spotted on glass plate</td>
<td>Spotted on glass plate</td>
<td>Spotted on glass plate</td>
<td>Spotted on glass plate</td>
<td>Visual examination</td>
<td>≥ 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLC- - The specimen cannot be stored in contact with TLC.</td>
<td>1 ml.</td>
<td>1 ml.</td>
<td>TLC-</td>
<td>TLC-</td>
<td>TLC-</td>
<td>TLC-</td>
<td>TLC-</td>
<td>Type I for blood, type I for plasma.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 ml.</td>
<td>1 ml.</td>
<td>TLC-</td>
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<td>Type I for blood, type I for plasma.</td>
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Note: The results of the L/S ratio are questionable. Does not interfere with TLC.

Memorandum:
- Any detected by spectrophotometric scan renders the results of the L/S ratio questionable. Does not interfere with TLC.
<table>
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<th>Hospital</th>
<th>LS Procedure utilized/test time/quantity</th>
<th>Handling of sample and contamination</th>
<th>Centrifugation</th>
<th>Lipid extraction</th>
<th>Acetone precipitation</th>
<th>Thin-layer chromatography (TLC)</th>
<th>Staining/Charring</th>
<th>Quantiﬁcation</th>
<th>Reproducibility</th>
<th>Cut-off values</th>
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If not tested immediately, samples placed in refrigerator at 4°C for 24 hrs. If longer than 24 hrs before analysis, sample is frozen at -20°C. If stored sample obtained, let it thaw to 4°C and then conduct the experiment. A blank sample can give a positive interference. A sample with acetone can give a negative interference. GB is not affected by blood or minerals.

St. (minimum) centrifuge 5 min. at 20 x g. Get rid of debris without loss of liquid. Utilize from 100 ml H:1.1 and add 85% 40% sec and then centrifuge at 1000 x g for 10 min.

Do not utilize because felt to be technically too tricky; thereby liquid could easily be lost. (Found the literature to be no help).

Spots were utilized from methanol (9:1). Plates utilized were Alumina plate 25 cm x 20 cm, activated at 90°C for 30 min.

Plates were utilized because here a separate starting zone for spotting which is prescanned. Method of application was spotting at the starting zone for each channel. This allowed for uniformity in application in all channels.

Migration values were prepared freshly, developing time was 30 min at room T.

A helium stream of cupric acetate (3%) cupric acetate in 8% H2O was utilized.

Plate dipped 10 sec into the reagent and then air dried (1 min) and kept onto the hot plate for 5 min at 80°C.

Densitometry: convenient, reliable, specific, quantitative, easy scan, and record easy. Separation results were best when plates were activated and solvents were fresh. PE and GB never fully separated from each other. Also encountered a slight baseline problem between PE and L but correctable by graphing.
<table>
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<tr>
<th>Hospital</th>
<th>L/S Procedure utilized/test time/quantity</th>
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<tr>
<td>Postac General Hospital</td>
<td>L/S ratio with simultaneous detection of RC and PI</td>
<td>Utilize Helen Fetal Tek-Tek controls, IV, 15-25 month</td>
<td>If not tested immediately should be refrigerated at 4°C, for storage longer than 24 h, specimen is frozen at -70°C. Specimens contaminated with reagents are discarded. Blind in sample may cause univariate results. Any sample with a packed erythrocyte volume over 50 dL/24 h of amniotic fluid is not utilized. Blind conversation with not appreciably interfere with RC and PI.</td>
<td>2 ml supernatant plus 2 ml H2O and mix 10 seconds on Vortex, then add 4 ml of C and mix 30 sec. on Vortex. Then evaporate extract (30 min).</td>
<td>Not because Helen Fetal Tek-Tek laboratories compared results of 150 samples and concluded that acetone precipitation could be eliminated.</td>
<td>Fetal-Tek 2D plate (Helen). Developing solvent must be prepared fresh daily (C, M, Propanol, triethylamine, purified water). Developing chamber (Fetal-Tek 2D) must be saturated with solvent, 50 ml of sample is applied to plate and then allowed to dry. The plate is then placed in the developing chamber and phospholipids allowed to migrate for 70 min. Plate is then removed from chamber and allowed to air dry. Place plate in 100-150°C oven for 5 min.</td>
<td>Spray plate with epic acid acetate until completely set. Place plate in 100-150°C oven and allow bands to dry until they show a maximum blackish-grey color. (7 - 9 min).</td>
<td>Identify the phospholipids present in sample by comparison to controls. Then densitometrically scan the L and S bands. Bands should be scanned within 4 h, after charring because the bands have a tendency to fade.</td>
<td>In house study of 15 samples obtained C.V. = 5% at an L/S ratio of 2.0.</td>
<td>Same as Clock (1): L/S ≥ 2.0</td>
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<td>Thin-layer chromatography (TLC)</td>
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<td>Reproducibility</td>
<td>CUTOFF VALUES</td>
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<tr>
<td>Crittendon</td>
<td>Helen Fetal Tok-300 method for L/S ratio and simultaneous separation of Fg and Fl by TLC.</td>
<td>/3 h.  / 2-3 week</td>
<td>3 mL of supernatant plus 24 mL methanol by vortexing for 10-15 sec.</td>
<td>Add 2 mL of chloroform mix by vortexing for 20 sec. Centrifuge at high speed 300 rpm (1000 x g) for 10 min. Remove 1 mL of chloroform layer and evaporate to dryness (30 min.).</td>
<td>The reconstituted sample with chloroform is strained over the entire cholesterol of the Fetal Tok-300 plate, (in one 50 mL application). When all the samples and controls have been applied the plate is under microfused with blower and it will take approximately 4 min for the plate to dry. Then the plate is placed in the developing chamber for approximately 70 min. The plate is then removed and placed in a 100°C oven for 2-3 min to dry.</td>
<td>Scanning densitometer. The plate is then scanned for the L, completely wet. Then it is placed in a 100°C oven and allowed to dry. The bands will appear blue-grey color (7-10 min). Identify the phospholipids present in the sample by comparison to Fetal Tok-300 marker.</td>
<td>Good</td>
<td>L/S ≥ 2.0</td>
<td>Sensitivity: L/S 1.5 - 1.9</td>
<td>Insensitivity: L/S ≤ 1.5</td>
</tr>
<tr>
<td>Hospital</td>
<td>Lipid Extraction</td>
<td>Acute Precipitation</td>
<td>Thin-Layer Chromatography (TLC)</td>
<td>Staining/Charring</td>
<td>Qualification</td>
<td>Reproducibility</td>
<td>Cut-off Values</td>
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<tr>
<td>Hotel</td>
<td>Run three simultaneous procedures for determination of L/S ratio and TG. s/p extraction and total cholesterol. Phospholipid (PL) ratio and TG. Total phospholipid (PL) ratio.</td>
<td>Utilize sample with blood and/or serum but note on report. Hem Ultra will retard migration on the plate. If blood or serum present, spin at low speed to remove. Receive aliquot of washed pool samples.</td>
<td>L/S ratio and PL-1st of well-mixed nonfat-skimmed milk. Add 1 ml of 2% CM and vortex for 1 min to separate layers. Evaporate solvent to dryness at 70°C.</td>
<td>L/S ratio and TG- following removal of plate and evaporation of solvents, plate is heated at 180°C for 30 min to visualize areas containing migrated phospholipid material.</td>
<td>Utilize Sigma L/S, TG, PL, and TG controls. (L/S ratio control: 1.6 ± 0.2)</td>
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<tr>
<td>Hotel</td>
<td>If spectrum cannot be processed immediately freeze. No initial centrifugation done for the three procedures.</td>
<td>No because with it bone serum of the phospholipids.</td>
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<td>Hotel</td>
<td>HTL-1st of well-mixed nonfat-skimmed milk. Add 1 ml of 2% CM and vortex for 1 min to separate layers.</td>
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</table>
| Dr. E. Szekeres | | | | | | | }

<table>
<thead>
<tr>
<th>Hospital</th>
<th>Procedure utilized/test time/quantity</th>
<th>Handling of sample and concentration</th>
<th>Centrifugation</th>
<th>Lipid extraction</th>
<th>Aqueous precipitation</th>
<th>Thin-layer chromatography (TLC)</th>
<th>Scanning/reading</th>
<th>Quantitation</th>
<th>Reproductibility</th>
<th>Quenching values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrel Hospital Continued</td>
<td>Reference for lecithin</td>
<td>1 ml samples of well mixed</td>
<td>Sample not centrifuged; however, if it is not feasible to carry out the extraction of 1 ml aliquots of fluid, then fluid should be centrifuged at 1,000 rpm for 10 min., the supernatant discarded, and stored under refrigeration.</td>
<td>Pipet 1 ml (2 ml if TTP 0.14 mg/100 ml or 0.5 ml if TTP is 0.20 mg/100 ml)</td>
<td>The uncentrifuged well mixed amniotic fluid is placed into tube and added 4 ml of 2.1 CM and vortexed for 30 sec, then centrifuged at 2,000 rpm to separate layers (if at this point the analysis can not continue, stopper tube and refrigerate).</td>
<td>Column chromatography. The solvent extracted is passed through a hydroxyapatite (HAP) column which retains all of the phospholipids except L and S, dimethylaminoethyl cellulose (DEAE) is utilized to pack the column and HPT is the adsorbent. The solvent extract of the fluid is placed in the HAP column and then drawn through the column collected in a 2 ml sample with sterile water at 45 min.</td>
<td>The phosphate ion are assayed utilizing molybdate and reducing agent. (hydroxide sulfate/ stannous chloride/dihydrate/water/sulfuric acid).</td>
<td>Utilize LS analog control. A 0.1 solution of lecithin is hydrolyzed with the samples. The lecithin value of this solution is 0.16 ± 0.02 mg/100 ml. If the control value is out of range, make new control and repeat samples. If control is still out make new digestion reagents and repeat test.</td>
<td>Any value &gt;3.5 mg/100 ml lecithin indicates a low risk for RB.</td>
<td></td>
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<tr>
<td>Hospital</td>
<td>L/S Procedure utilized/ test time/quantity</td>
<td>Handling of sample and contamination</td>
<td>Centrifugation</td>
<td>Lipid extraction</td>
<td>Apo A-2 precipitate</td>
<td>Thin-layer chromatography (TLC)</td>
<td>Scanning/ charting</td>
<td>Quotation</td>
<td>Reproducibility</td>
<td>OD-off values</td>
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<tr>
<td>Reference</td>
<td>Protein procedure are:</td>
<td></td>
<td>Simple not</td>
<td>4 ml of 0.2 M</td>
<td>N/A</td>
<td>Digestion. The residue</td>
<td>Measure all</td>
<td>0.25 low risk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>Nelson, C., Lamson, S. (1973) 1.4–2.6</td>
<td>centrifuged</td>
<td></td>
<td>solution</td>
<td></td>
<td>containing all the</td>
<td>the tubes</td>
<td>of NSS</td>
<td>moderate risk</td>
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<tr>
<td>Hospital</td>
<td>C. Olsen, G. (1971) 1.4–2.6</td>
<td>unless fluid</td>
<td></td>
<td>unsedimented</td>
<td></td>
<td>phospholipids. The</td>
<td>absorbing</td>
<td>of RIS (0.16)</td>
<td>high risk RIS</td>
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<td></td>
<td>contains FSC</td>
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<td>solution</td>
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<td>residue digested with</td>
<td>against the</td>
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<td>or meconium, then</td>
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<td>acid to inorganic</td>
<td>reagent blank</td>
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<td></td>
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<td>centrifuge</td>
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<td>at 600 rpm for 15 min.</td>
<td>at 5 min.</td>
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<td>at 1000 rpm for 10 min.</td>
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<td>the tubes should not stand</td>
<td>for more</td>
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<td>for 10, 20 min.</td>
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<td>for more than 15 min.</td>
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<td>for 10, 20 min.</td>
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<td>before</td>
<td>measuring the</td>
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<td>for 10, 20 min.</td>
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<td>the absorbance, the</td>
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<td>for 10, 20 min.</td>
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<td>test is</td>
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<td>for 10, 20 min.</td>
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<td>linear to 1.0</td>
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<td>for 10, 20 min.</td>
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<td>standard</td>
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<td>for 10, 20 min.</td>
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<td>solution to 10</td>
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<td>for 10, 20 min.</td>
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<td>ml with</td>
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<td>for 10, 20 min.</td>
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<td>chloroform-acid</td>
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<td>for 10, 20 min.</td>
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<td>ethanol solution</td>
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<td>for 10, 20 min.</td>
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<td>by 1 ml of the primary</td>
<td>0.24 ± 0.03</td>
<td>0.5±0.03 mg/dL</td>
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<td>for 10, 20 min.</td>
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<td>solution and digest with</td>
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<td>for 10, 20 min.</td>
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<td>fluid samples as a control.</td>
<td>0.24 ± 0.03</td>
<td>0.5±0.03 mg/dL</td>
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<td>for 10, 20 min.</td>
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<td>the value will be</td>
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<td>for 10, 20 min.</td>
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<td>0.24 ± 0.03 mg/dL. P.</td>
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<tr>
<th>Hospital</th>
<th>L/S Procedure utilized/last time/quantity</th>
<th>Handling of sample and contamination</th>
<th>Centrifugation</th>
<th>Lipid extraction</th>
<th>Nature precipitation</th>
<th>Thin-layer chromatography (TLC)</th>
<th>Staining/charing</th>
<th>Quantitation</th>
<th>Reproducibility</th>
<th>On-off values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premont Hospital</td>
<td>Glicks original L/S ratio test</td>
<td>Same as Glick's method</td>
<td>Centrifuge at 500 rpm for 10 min.</td>
<td>Standard HCl, 1:2 lipid extraction</td>
<td>Yes</td>
<td>Silicon nitride TLC plates, heat</td>
<td>Reflectivity</td>
<td>L/S &gt; 2.5</td>
<td>mature, if.</td>
<td>PC present.</td>
</tr>
<tr>
<td>Royal Oak</td>
<td>L/S ratio test and PC done with two-dimensional TLC, Borer method</td>
<td>Hospital above.</td>
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<td></td>
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<td>very mature.</td>
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<td>Dr. M. Epstein</td>
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<tr>
<td>Clinical Chemistry</td>
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<td>(126 total = 172)</td>
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</table>

| St. Joe Mercy Hospital | Modified Glick L/S ratio determined by one-dimensional TLC, send out PC to Pontiac General | Same as Glick's method              | Centrifuge at low speed for 10 min. | Standard HCl, 1:2 lipid extraction | No                   | Utilize commercially available plates, visual assessment (L, W) | Utilize one control/sample and immature. | L/S > 2     | mature.         | L/S 1.5-1.9    | transitional |
| Clinical Chemistry  |                                          | Pontiac General.                    |               |                 |                     |                                 |                 |              | immature.      | L/S ≤ 1.5    | immature.      |
|                   |                                          | /1/3 h                               |               |                 |                     |                                 |                 |              |                 |               |
|                   |                                          |                                       |               |                 |                     |                                 |                 |              |                 |               |
|                   |                                          | /1 every 3 months.                   |               |                 |                     |                                 |                 |              |                 |               |


Abbreviations include: L = lecithin, S = sphingomyelin, C = chloroform, M = methanol, HC = red blood cells, TLC = thin-layer chromatography, T = temperature, lab = laboratory, N/A = not applicable.
(TLC) procedures for the L/S ratio and PG determination. Two laboratories perform a one-dimensional TLC procedure for L/S ratio and a two-dimensional TLC for PG. Also, one laboratory performs a lecithin determination, and total phospholipid phosphorus in addition to its one-dimensional TLC procedure for L/S ratio and PG, for these two other tests have a high predictive accuracy of 98% for appearance of RDS.

The specific procedural breakdown and variations include the following. One procedure was the Gluck (114) original one-dimensional TLC procedure, and five were modified Gluck procedures. The Gluck original method utilized acetone precipitation, ammonium sulfate incorporated into the silica TLC plates, heated charring for visualization, and quantitated the L/S ratio by reflectance densitometry. The five modified Gluck procedures consisted of the following:

- Two utilize cupric acetate, and charring for visualization, densitometric scanning for quantitation and omit acetone precipitation.
- Two other procedures utilize bromothymol blue stain for visualization, and one scans densitometrically, and the other uses visual assessment for quantitation (one uses acetone precipitation and the other omits it, respectively).
- The remaining procedure utilizes ammonium sulfate with charring for visualization, and visual assessment for quantitation (with acetone precipitation).

The other four procedures were the Helena Fetal Tek-200 method from Helena Laboratories (one was an in-house Helena modification).
Helena Fetal Tek-200 procedure is a one-dimensional TLC technique for determining the lecithin to sphingomyelin ratio (L/S) in amniotic fluid with simultaneous separation of PG and PI by TLC. In this Helena Fetal Tek-200 procedure the phospholipids (L, S, PG, and PI) are extracted from the sample with a chloroform/methanol mixture and streaked onto a TLC plate. Then the phospholipids are separated using a solvent system containing chloroform, methanol, 2-propanol, triethylamine, and water. The phospholipids are made visible using a dilute cupric acetate-phosphoric acid reagent and charring at 180°C until a bluish-grey color appears. The phospholipids are identified by comparison to the Fetal Tek-200 marker. The lecithin and sphingomyelin bands are quantitated in a scanning densitometer, and the relative intensity of the two bands are expressed as a ratio. The presence or absence of PG is noted. Acetone precipitation is omitted because Helena laboratories compared results of 100 samples. They concluded that acetone precipitation could be eliminated because it is just a laborious step which frequently introduces error in recovery of the phospholipids.

(a). Test Time

The average test time for all ten of these procedures was three hours.

(b). Quantity of Tests Performed

Of the ten hospital clinical laboratories surveyed, six of them averaged four fetal lung maturation evaluations per week, two laboratories averaged ten to twelve requests per week, and of the remaining two, one laboratory had one request every three months, and the
remaining hospital eliminated the fetal lung maturity evaluation due to staffing problems.

2. Handling of the Sample and Contamination

All laboratories immediately refrigerated (4°C) the amniotic fluid sample if not tested, and for storage longer than 24 hours the specimen is frozen at -20°C. Concerning contamination, all laboratories agreed that blood and meconium affect the L/S ratio; however, all had specific methods for handling of the specimen (see Table XLV). Also all laboratories agreed that blood and meconium do not appreciably interfere with PG analysis; however, all had specific methods of handling the specimen (see Table XLV). Additionally, laboratories commented that samples obtained from vaginal pools might be contaminated and, therefore, could affect the results.

3. Centrifugation

This step of the procedure is very controversial and varied. Seven of the clinical laboratories centrifuged at slow (500 x g or 1000 rpm) speeds, from 200 x g through 500 x g. However, the time of centrifugation of these seven slow speeds varied with four at 10 minutes, and the remaining three at 3, 5, and 15 minutes. Two laboratories centrifuged at considerably higher speeds: 1000 x g for 10 min, and 2000 x g for 5 min. The remaining laboratory did not utilize centrifugation because of the possibility of lowering the phospholipid concentration. Basically, the laboratories which spin at low speeds (500 x g) do so just to get rid of debris without loss of liquid and it is a safe compromise between over and under centrifugation. The Helena Fetal
Tek-200 procedure comments that excessive speed or prolonged 
centrifugation may sediment phospholipids and incomplete centrifugation 
may cause false results.

4. Lipid Extraction

Six clinical laboratories utilize the standard 1:2 chloroform/methanol lipid extraction step. Four others utilize a 1:1 chloroform/methanol ratio except one substitutes Freon 113 for chloroform. The vortexing and centrifugation times and speeds all varied.

5. Acetone Precipitation

Four clinical laboratories utilize this controversial step, with the following explanations: results in cleaner chromatography; used for the rare falsely elevated L/S ratio that drops drastically when only acetone precipitation is utilized; and utilized in modified Gluck procedure, because included in original Gluck procedure.

Six clinical laboratories omit this acetone-precipitation step, with the following explanations: step is technically too tricky, whereby liquid could be easily lost; and this step adds another laborious step which frequently introduces error in recovery of the phospholipids. Several laboratories commented that the literature was of no help in determining whether to utilize or omit this step.

6. Thin-Layer Chromatography (TLC)

Five clinical laboratories utilize the commercially available Helena Fetal Tek-200 plates which consist of two different surface areas: one has a 3-cm predsorbent strip across the lower edge of the plate and, the second area has the silica gel analytical layer. The remaining five
laboratories utilized the following plates: three a standard plate (silica H) with ammonium sulfate incorporated, and the other two utilized a standard TLC plate for the L/S ratio and one utilized a high-performance 2-dimensional PG plate and the other a longer plate for the PG determination.

7. Staining/Charring

Five of the clinical laboratories utilize the Helena plates with cupric acetate reagent (spray 3% cupric acetate in 8% H₃PO₄ till completely wet) and charring (160°-180°C oven for 7-10 min) for bluish-grey color visualization (one lab dips the plates with reagent and chars on the hotplate for 6 min). Three laboratories utilize ammonium sulfate impregnated TLC plates and charring (220°-240°C for 30-45 min) for visualization. The two remaining laboratories utilize bromothymol blue stain and one develops with ammonia fumes.

8. Quantitation

Eight of the clinical laboratories densitometrically scan and two utilize visual assessment for quantitation. Most of the laboratories said they utilize densitometric scanning because of consistency, speed, reliability, convenience and it is semi-quantitative. Most of the laboratories also visually assess the L/S ratio by comparing it to the controls. Phosphatidylglycerol (PG) was assessed by all laboratories as either absent or present (except for two which give a % PG). Note quantitation column for specific separation problems.

9. Cut-off Values/Reproducibility

The L/S ratio cut-off values vary with the following ranges:
immature <1.0–1.5, and two at <2.0; transitional, most at 1–2, with one
at 2.5–2.0, and another at 3.0–3.5; mature, most at >2.0; a couple at
>2.5, one at >3.5 and two at >4.0. Eight clinical laboratories report PG
as present for maturity and absent for immaturity. The two remaining
laboratories give a % PG value, both with a cut-off value for maturity of
PG >1%. All the laboratories run controls to monitor the procedure (see
reproducibility column for details).

10. Comments

All the clinical laboratories stated that the physician's attitudes
to L/S ratio was good and has been enhanced immensely by the PG
assessment.

11. New Procedures Being Investigated

The clinical laboratories were questioned as to what new if any
procedures they were investigating for fetal lung maturity assessment.
The Amniostat-FLM and Lumadex-FSI were the two frequently mentioned, and
will now be discussed.

(a). Amniostat-FLM

The Amniostat-FLM (APPENDIX B) is a rapid agglutination test for
PG in amniotic fluid manufactured by Hana Biologics (239). Amniostat-FLM
is an immunologic semi-quantitative (2 quantified positive controls)
agglutination test for determining the presence of PG in amniotic fluid
at concentrations indicative of fetal lung maturity. The test is
specific, sensitive (detects PG at 2 µg/mL or >), rapid (15 min)
requires less than 0.1/mL of specimen (amniocentesis or vaginal pool) and
is not affected by blood or meconium contamination. This immunologic
agglutination test performs as follows (APPENDIX B pg.634): PG in amniotic fluid is incorporated into lipid particles formed by adding reagent A (contains lecithins and cholesterol in ethanol). After dilution, the suspension of particles is mixed with reagent B (anti-PG serum fraction with 0.02% sodium azide) which contains antibodies that react with particles containing PG. The presence of PG in the sample is indicated by the agglutination of the microscopic particles to form macroscopic clusters with clearing (decrease in turbidity) in the background. The PG concentration present in the sample can be estimated by comparison of the two positive controls (assure assay validity). The positive results obtained are reliable, however, negative results require a supporting assay to be performed for further confirmation. Several clinical laboratories have investigated this procedure and all concluded it has two disadvantages: 1) experienced discrepancies in end-point interpretations (for it is difficult to read), and 2) the cost per kit is high (12 test kit is $300.00). However, others have found that it could possibly be utilized as a screening test on amniocentesis samples (vaginal pool samples resulted in several false positive results), for there was good correlation with positive result, and if results is negative a supporting procedure should be performed (TLC). One clinical laboratory will be utilizing this Amniostat-FLN as a routine procedure in the near future.

(b). Lumadex-FSI

The second new procedure being investigated is the Lumadex-FSI (foam stability index) (APPENDIX C) test for lung maturity by Beckman
(240). This test is based on the same principle as the manual FSI test of Sher and Statland (218). There is a test cassette (APPENDIX C pg. 640) with premeasured controls and differing volumes of ethanol in six test wells to determine the foam stability index (FSI). Supernatant amniotic fluid is added to the six test wells to the upper marked lines, then the cassette is shaken vigorously for 30 seconds. The cassette is allowed to stand for 1 minute to permit any foam to rise to the surface and then visually examined for the presence of a stable ring of foam. The addition of equal amounts of amniotic fluid to the differing amounts of ethanol, results in a range of ethanol volume fractions equivalent to the range of 44% to 50%. The lower the value (≤46) the greater the probability of immature fetal lung development. If foam is visible in well 47 or above, it indicates that the lungs of the fetus are mature, and that it is very unlikely that RDS will occur if the baby is delivered. However, Lumadex-FSI was taken off the market because the positive control could not be made to foam. Beckman explained that the original positive control was made weak, because they did not want to miss a borderline positive. Now they have changed the positive control concentration so that a positive is obtained by shaking very hard (sometimes even have to reshake). Beckman commented that they are thinking of developing an automatic shaker for this procedure to eliminate this shaking variable.

Several laboratories have investigated this Lumadex-FSI test and found two major disadvantages: 1) the interpretation of results is subjective and 2) 3 mL of amniotic fluid is required. This Lumadex-FSI
test is a rejuvenation of the shake test which is a biophysical test for phospholipid surfactants, measuring all phospholipids present.

B. RESPIRATORY DISTRESS SYNDROME (RDS): NEONATOLOGIST SURVEY

During my literature search of respiratory distress syndrome I encountered several variations, including, disease terminology, radiographic diagnostic timing, usage of antibiotics, and follow up studies on RDS infants. Therefore, I conducted a telephone survey of seven Metro Detroit area neonatologists to obtain an updated philosophical picture of RDS. The survey is summarized in Table XLVI and includes questions concerning the following: predisposing factors, terminology utilized, physical and radiographic findings, differential diagnosis, treatment, complications, survivor follow up, and comments. Each of these specific topics concerning RDS will now be briefly discussed (will follow order of Table XLVI).

1. Predisposing Factors (CHAPTER IV C. 1)

All seven neonatologists agreed that prematurity was the single most important factor predisposing an infant to RDS. Also it was agreed that an infant's birth weight is associated with prematurity, for a weight <2500 g has increased chances of RDS development, and a weight >2500 g has lower chances of RDS development. Additionally all neonatologists agreed with the other predisposing risk factor for RDS development listed in Fig. 36.

2. Terminology (CHAPTER IV B.)

Two of the seven neonatologists utilize RDS to identify the disease with surfactant deficiency. These two feel that RDS is more widely
# Table IIIV

**PITFALL DEBTOR AREA SURVEY OF NEONATOLOGISTS CONCERNING RESPIRATORY DISTRESS SYNDROME**

<table>
<thead>
<tr>
<th>Hospital/Neonatologist</th>
<th>Predisposing factors</th>
<th>Terminology</th>
<th>Physical findings</th>
<th>Radiographic findings</th>
<th>Differential diagnosis</th>
<th>Treatment</th>
<th>Complications</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pratip General Hospital/ Dr. Rao</td>
<td>Low birth weight (less than 2500 g), prematurity, severe</td>
<td>Utilize IVD, because has specific symptoms; may be caused by type I or II sepsis</td>
<td>Standard findings are confirmed in text. Symptoms can occur anywhere from first hour to 24 h.</td>
<td>Immediate x-ray will be hallmark of disease but may not be diagnostic. The 6-8 h after birth is most sensitive.</td>
<td>Differential diagnosis occurs frequently, especially in the first 12 h of life. IHD can be diagnosed with 95% certainty.</td>
<td>Depending on severity of IHD, utilize the standard form of treatment received in text. Antibiotics are not routinely given and are administered only with the following IHD conditions: male babies (more susceptible), amnionitis, infected lower, PDA, or if the infant is in distress.</td>
<td>Infants experience complications depending on their condition and severity. Usually difficult to wean very small infants off oxygen. The smaller the infant, the greater the prematurity, the more chance for later complications.</td>
<td>Follow standard treatment mentioned in text.</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: IVD = interventional delivery, PDA = patent ductus arteriosus.*
<table>
<thead>
<tr>
<th>Hospital/</th>
<th>Predisposing</th>
<th>Physical</th>
<th>Radiographic</th>
<th>Differential</th>
<th>Treatment</th>
<th>Complications</th>
<th>REE surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>neonatologist</td>
<td>factors a</td>
<td>findings b</td>
<td>findings c</td>
<td>diagnosis d</td>
<td></td>
<td></td>
<td>follow-up/</td>
</tr>
<tr>
<td>Dominent Hospital/</td>
<td>Infant weighs 700-2000 g and</td>
<td>&amp; become diseased</td>
<td>&amp; difficult to differentiate from</td>
<td>&amp; especially in the</td>
<td>&amp; depending on</td>
<td>Infants experience</td>
<td>for 2 yrs.</td>
</tr>
<tr>
<td>Dr. Nuria</td>
<td>prematurity (Cm6 wk)</td>
<td>with specific symptoms</td>
<td>pneumonia or aspiration</td>
<td>first 12 h of life.</td>
<td>severity of HD,</td>
<td>complications</td>
<td>HD infants are</td>
</tr>
<tr>
<td></td>
<td>Agreed with other predisposing factors in text</td>
<td>&amp; early x-ray may be difficult</td>
<td>&amp; antibiotics given</td>
<td>&amp; treated as</td>
<td>&amp; standard forms of</td>
<td>dependent on their</td>
<td>have a 75-85% chance of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>to differentiate from pneumonia or aspiration.</td>
<td>&amp; low birth weight</td>
<td>treatment</td>
<td>rarely used in text.</td>
<td>conditions and severity.</td>
<td>survival.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&amp; HD is rare in text.</td>
<td>&amp; blood cultures are being run.</td>
<td>&amp; HD is</td>
<td>&amp; treatment mentioned in text.</td>
<td>&amp; All premature HD infants</td>
<td>HD weights &gt;1500 g have a 90-95% chance of survival.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&amp; HD is rare in text.</td>
<td>&amp; HD is rare in text.</td>
<td>&amp; HD is</td>
<td>&amp; treatment mentioned in text.</td>
<td>&amp; HD infants are</td>
<td>HD weights &gt;1500 g have a 90-95% chance of survival.</td>
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<td>&amp; HD is rare in text.</td>
<td>&amp; HD is rare in text.</td>
<td>&amp; HD is</td>
<td>&amp; treatment mentioned in text.</td>
<td>&amp; HD infants are</td>
<td>HD weights &gt;1500 g have a 90-95% chance of survival.</td>
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<tr>
<td></td>
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<td>&amp; HD is rare in text.</td>
<td>&amp; HD is rare in text.</td>
<td>&amp; HD is</td>
<td>&amp; treatment mentioned in text.</td>
<td>&amp; HD infants are</td>
<td>HD weights &gt;1500 g have a 90-95% chance of survival.</td>
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<tr>
<td></td>
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<td>&amp; HD is rare in text.</td>
<td>&amp; HD is rare in text.</td>
<td>&amp; HD is</td>
<td>&amp; treatment mentioned in text.</td>
<td>&amp; HD infants are</td>
<td>HD weights &gt;1500 g have a 90-95% chance of survival.</td>
</tr>
</tbody>
</table>

Note: a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, AA, AB, AC, AD, AE, AF, AG, AH, AI, AJ, AK, AL, AM, AN, AO, AP, AQ, AR, AS, AT, AU, AV, AW, AX, AY, AZ, BA, BB, BC, BD, BE, BF, BG, BH, BI, BJ, BK, BL, BM, BN, BO, BP, BQ, BR, BS, BT, BU, BV, BW, BX, BY, BZ.
<table>
<thead>
<tr>
<th>Hospital/</th>
<th>Predisposing factors</th>
<th>Terminology</th>
<th>Physical findings</th>
<th>Radiographic findings</th>
<th>Differential diagnosis</th>
<th>Treatment</th>
<th>Complications</th>
<th>EES survivor follow-up comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henry Ford Hospital/ Dr. E. Z. Hatcher</td>
<td>Infant weight and prematurity. Agreed with other predisposing factors in text.</td>
<td>Utilize EES because disease with specific symptom.</td>
<td>Standard findings as mentioned in text.</td>
<td>If newborn has low birth weight and very sick (has symptoms) x-ray in first hour to see of EES.</td>
<td>Differential diagnosis occurs frequently especially in the first 12 h of life. Group B streptococcus has similar x-ray to EES, other diseases not the sure.</td>
<td>Depends on severity of EES. Utilize the standard form of treatment mentioned in text. Antibiotics and vitamin E not routinely utilized with EES.</td>
<td>Infants experience complications depending on their condition and severity. Percentage of complications occurring: IHR 25%; pneumonia 25-33%; and RIA is common in small birth weight baby.</td>
<td>Have a follow-up program. EES infants seem to be more susceptible to viral infections and experience a slower development course but catch up by about 6 yrs. of age. For diabetic pregnancies utilize delivery criteria use L/S; ratio ≥3.0-3.5 and βG present. Betamethasone surfactant replacement has potential for future usage however must overcome problems and be studied.</td>
</tr>
<tr>
<td>Hospital/</td>
<td>Precluding factors</td>
<td>Terminology</td>
<td>Physical findings</td>
<td>Radiographic findings</td>
<td>Differential diagnosis</td>
<td>Treatment</td>
<td>Complications</td>
<td></td>
</tr>
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</tr>
<tr>
<td>University of Michigan/ Dr. Surn</td>
<td>Low birth weight and prematurity prior precluding factors. Agreed with other precluding factors in test.</td>
<td>IHX is opened with BES. Closely monitor IHX or BES as mild, moderate, and severe.</td>
<td>Standard findings as mentioned in test.</td>
<td>Immediately after a newborn displays respiratory distress an x-ray is taken depending on course of disease and severity will take follow up x-rays.</td>
<td>Pneumonia most difficult to differentiate from BES especially in very small preterms.</td>
<td>Depends on severity of BES. Utilize standard form of treatment as mentioned in text. Do not routinely utilize antibiotic except if can not differentiate between BES and pneumonia or if sepsis complications occur.</td>
<td>Infant experience complications depending on their conditions and severity. Follow standard treatment as mentioned in text.</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

RMS survivor follow-up comments: Have had a follow up developmental clinic for these infants for 15 yrs. Feels it is difficult to comment on survivors because of the many variables involved in follow up study and wide spectrum of variants in RMS. Feels that small preterms 750 g are being saved but the complications and development of infant are problems. Infants weigh 1000 g have good prognosis. Feels surfactant substitution has great potential and research and many human trials are required to evaluate the risk versus potential utilization.
<table>
<thead>
<tr>
<th>Hospital / \nPractitioner</th>
<th>Preceding Factors \n(Ch. 1)</th>
<th>Terminology \n(Ch. 1)</th>
<th>Physical \nFindings \n(Ch. 1)</th>
<th>Radiographic \nFindings \n(Ch. 1)</th>
<th>Differential \nDiagnosis \n(Ch. 1)</th>
<th>Treatment \n(Ch. 1)</th>
<th>Complications \n(Ch. 1)</th>
<th>\nEFS survivor \nfollow-up/ \ncurrent</th>
</tr>
</thead>
</table>
| Providence \nDr. Greg | Prematurity (Ch. 1), low birth weight, Apley, \nwith other \npreadjusting \nfactors in text | IND is utilized \nfor specific \nsurface \ndysfunction syndrome. \nPULM is utilized in \na broader general \nclassification of \nrespiratory \nproblems. | Standard findings \nas mentioned in text. | Standard findings \nas mentioned in text. | Differential \ndiagnosis occurs \nfrequently especially \nin the first 12-24 h. \nFind that pneumonia \nhave similar \nclinical and \nradiographic \nfindings as IND. | Depending on \nseverity of IND. \nUtilize standard \nform of treatment \nmentioned in text. \n Routinely \nutilize \nan antibiotic \n
to prevent infections. | Infant experience \ncomplications \ndepending on severity \nand condition. \nFolloowup \ntreatment \nmentioned in text. \n\nInfant pneumonia \ncan cause \nproblems for up to \ntwo years. | \nEFS survivor \nfollow-up/ \ncurrent |
<p>| Hazel Hospital \nDr. Black | Low birth weight \nand prematurity \n(Ch. 1), \nApley, \nwith other \npreadjusting \nfactors in text | Standard findings \nas mentioned in text. | Initially \nstable newborn \nwith respiratory \ndistress \nx-ray. \nFindings \ndependent on \nseverity and \ntiming of \ndischarge. \nRepeat \nx-ray \ndepending on \nprevious \nfindings. | Differential \ndiagnosis occurs \nin the first 12 h \nof life. \nFind pneumonia \ndifficult to \ndistinguish from RDS. | Depends on \nseverity of RDS. \nUtilize standard \nform of \ntreatment \nmentioned in text. \nDo not routinely \nadminister \nan antibiotic \nunless \nindicated (e.g., \nPneumonia). | Infant experience \ncomplications \ndepending on condition \nand severity. \nFollowup \ntreatment \nmentioned in text. \n\nFuture needs \nmore research \nto address \nquestions. | \nLow birth \nweight \ninfants \nfollowed \nup for 3 yrs. \nNeeds \nsurfacetant \nsubstitution \nas a \npossible \nform of \ntreatment \nfuture. \nHowever, \nmore \nresearch \nand \nclinical \ntrials \nare \nrequired. |</p>
<table>
<thead>
<tr>
<th>Hospital/Center</th>
<th>Preceding factors</th>
<th>Terminology</th>
<th>Physical findings</th>
<th>Radiographic findings</th>
<th>Differential diagnosis</th>
<th>Treatment</th>
<th>Complications</th>
<th>Follow-up comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>University Hospital/Dr. Roberts</td>
<td>Prematurity and low birth weight</td>
<td>IDH infant with extreme prematurity</td>
<td>Standard findings mentioned in text</td>
<td>Initial x-ray can differentiate bronchopulmonary dysplasia</td>
<td>Infant experience complications depending on severity</td>
<td>Infant experience complications depending on severity</td>
<td>Infant experience complications depending on severity</td>
<td>Infant experience complications depending on severity</td>
</tr>
</tbody>
</table>

Letters denote part of text which corresponds to column titles:

- a Chapter IV C. 1.
- b Chapter IV B.
- c Chapter IV E: 1-2.
- d Chapter IV E. 1.
- e Chapter IV F.
- f Chapter IV C. 1-7.
- g Chapter IV H. 1 and 2.
- h Chapter IV I.

Abbreviations: IDH = infantile disease, RDS = respiratory distress syndrome, BD = bronchopulmonary dysplasia, PDA = patent ductus arteriosus.
accepted and utilized. The other five neonatologists utilize HMD to identify surfactant deficiency, because the disease has specific symptoms. They classify HMD by types (type I being the most severe). These five neonatologists utilize RDS to identify the wide spectrum of newborn respiratory problems (respiratory frequency >60/min).

3. Physical Findings (CHAPTER IV E. 1. a-e)

All seven neonatologists agreed on the physical and clinical findings, which include, tachypnea, chest wall retractions, flaring of the nares, cyanosis, and mixed metabolic and respiratory acidosis (see Fig. 37 for the remainder of the findings). Currently there is no scoring of chest wall retractions as previously done (Silverman scoring method), for the HMD or RDS is classified as mild, moderate, or severe. One of the neonatologists feels that the clinical symptoms are more diagnostic initially than the radiographic findings.

4. Radiographic Findings (CHAPTER IV E. 3)

There are many variations of the radiographic findings because they are dependent on the infants severity, complication, and timing after respiratory problems initiated. If newborn is very sick (has symptoms of RDS) premature and/or low birth weight (<1500 g) and is having respiratory problems, an x-ray is taken within first hour. If RDS is of mild to moderate severity, the x-ray may not be diagnostic till 6-8 hours after birth. If severe RDS is present, the initial x-ray can be immediately diagnostic. Most of the neonatologists feel the 6-8 hour after birth x-ray is the most diagnostic and usually can differentiate RDS from other diseases (especially pneumonia).
5. Differential Diagnosis (CHAPTER IV F)

Most of the neonatologists feel that RDS can be differentially diagnosed in the first twelve hours of life. Also most of the neonatologists find pneumonia the most difficult to differentiate (especially in very small preterm newborns) from RDS because of some similar clinical and radiographic findings. RDS can be diagnosed with about 90% certainty.

6. Treatment (CHAPTER IV G, 1-7)

All the neonatologists agreed that treatment is dependent on severity, complications, and clinical findings manifested by the newborn. All the neonatologists followed the different forms of treatment discussed in the text such as ventilation support, oxygen therapy, acid-base balance, body temperature and blood volume regulation, and nutrition. The only aspect the neonatologists differed on was antibiotic administration, for two of the physicians routinely administer antibiotics, and five do not. The antibiotics that are administered routinely are utilized to prevent infections. The physicians who do not routinely administer antibiotics do administer them in the following situations: if one cannot differentiate between pneumonia and RDS (given while urine and blood cultures being run, because infant with Group B Streptococcus pneumonia can die within 4-6 hours without antibiotics); if sepsis complications occur; and with the following RDS associated conditions, i.e., male babies (more susceptible), amniotitis, maternal fever, PROM, or change in white blood cell count.

7. Complications (CHAPTER IV H, 1 and 2)
All the neonatologists commented that infants experience complications depending on their severity and conditions. All the neonatologists agreed on the complications of RDS and specific forms of treatment. The complications include disease and prematurity associated ones, such as pneumothorax, pneumomediastinum, PDA, BPD, and metabolic disturbances. There are also intensive care associated complications such as endotracheal intubation, umbilical vessel catheterization, infection, fluid administration, intracranial hemorrhage, and oxygen therapy. One of the neonatologists commented on the percentage of occurrence of specific complications: BPD 25%, and pneumothorax 25-30%. PDA occurrence is common in very small birth weight babies (very premature), and can sometimes cause problems for up to two years, and therefore medication must be given to close the patent ductus arteriosus (PDA).

8. RDS Survivor Follow-up (CHAPTER IV K)

All seven hospitals have a developmental follow-up clinic (one just started, others have existed) for premature and low birth weight infants. Generally speaking the neonatologists agreed that <1500 g weight newborns have a 75-80% chance of survival, and >1500 g weight newborns have a 90-95%, chance of surviving RDS. They also commented that RDS survivors are more susceptible to viral infection and experience a slower development, however, catch up by 4 years of age (especially the >1500 g RDS infants). Basically speaking the smaller infant (<1500 g) has a higher risk (50%) for cranial hemorrhage development, while the larger infants (>1500 g infant) have a much better prognosis. One physician commented that it is
difficult to comment on survivors because of the many variants involved in follow-up studies and wide spectrum of variants in RDS. This is why there has not been any summary or conclusions of follow-up studies because of the multitude of variants involved.

9. Comments

I discussed the recent developments in synthetic surfactant substitution as a form of treatment in RDS infants. All the neonatologists commented that synthetic surfactant substitution has great potential, however, much research and many human trials are required to evaluate the risk versus potential utilization. Many questions must be answered concerning this synthetic surfactant substitution and many problems overcome, such as antigen-antibody interactions.

C. FETAL LUNG MATURITY ASSESSMENT: HIGH RISK OBSTETRICIAN SURVEY.

During my literature search of respiratory distress syndrome I encountered several variations concerning complicated pregnancies fetal lung maturity assessment, such as L/S ratio cut-off value, PG presence, and utilization of steroids to enhance fetal lung maturation. Therefore, I conducted a telephone survey of five area high risk obstetricians (three others contacted, but unavailable for comment) to obtain a updated philosophical picture of assessing and treating complicated pregnancies fetal lung maturation. The survey is summarized in Table XLVII and includes questions concerning the following: prematurity prevention; diagnostic criteria utilized in complicated pregnancies; and utilization of glucocorticoids (steroids) to enhance fetal lung maturity. Each of these specific topics concerning complicated pregnancies will now be briefly
TABLE XVII
METRO DETROIT AREA SURVEY OF HIGH RISK OBSTETRICIANS
CONCERNING HIGH RISK PREGNANCIES FETAL LUNG MATURITY

<table>
<thead>
<tr>
<th>Hospital/obstetrician</th>
<th>Prematurity prevention</th>
<th>FIM assessment in complicated pregnancies</th>
<th>Glucocorticoid (steroid) treatment utilized to enhance FIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of Michigan/Evelyn Rashil, RN Clinical Specialist</td>
<td>Utilize ritodrine to prevent prematurity if &lt;36 weeks gestation.</td>
<td>Utilize L/S ratio and BSEF for FIM assessment. Normal pregnancy L/S &gt;2.5 for FIM. In complicated pregnancy (diabetic) do amniocentesis at 34 weeks and no complications 36 weeks and determine L/S ratio. Diabetic pregnancy L/S ratio must be &gt;3.0 for FIM.</td>
<td>Yes, if need to enhance fetal lung maturation, give 12.5 mg betamethasone IM two times per week (total 25 mg/wk) at least 24 hours apart.</td>
</tr>
<tr>
<td>Oakwood Hospital/Dr. Schoen</td>
<td>&lt;36 weeks gestation utilize ritodrine to stop premature labor.</td>
<td>Diabetic pregnancy (class B and D) will run amniocentesis about 34 weeks to determine L/S ratio. Diabetic L/S ratio must be &gt;3.25-3.5 to have a low risk of RDS development. Most current philosophies wait for the presence of RDS in amniotic fluid for assessment of FIM. However, this physician had a difficult situation occur, because he waited for RDS presence and lost a baby, therefore he no longer waits for the presence of RDS as long as the L/S ratio is mature he will deliver the baby.</td>
<td>Utilize steroid very selectively in low gestational patients (&lt;34 wk) with inadequate surfactant. Have only utilized steroids 4 times in the last 4 years, because feels not enough is known about side effects. 12 mg betamethasone is given at zero time and 12 mg repeated at 12 hours and by 48 hours baby delivered. Dr. Kazarz (neonatologist) commented that 54 yrs. ago utilized steroids however today they are not utilized because can cause maternal side effects (no fetal side effects encountered). The external side effects include pulmonary edema, congestive heart failure, and infection caused by drug interactions (steroid + methyl xanthine-ritonine).</td>
</tr>
<tr>
<td>Hospital/obstetrician</td>
<td>Prematurity prevention</td>
<td>RLU assessment in complicated pregnancies</td>
<td>Glucocorticoid (steroid) treatment utilized to enhance RLU</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------------</td>
<td>--------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Hazel Hospital/Dr. Kozzi</td>
<td>Ritodrine and magnesium sulfate utilized to prevent premature delivery to 36 weeks gestation. Utilize L/S ratio, RC bilirubin and TTP TTP (total phospholipid phosphorus), for evaluating RLU. A L/S ratio of 4/1 indicates a low risk of RDS developing. In the majority of cases of at least 36 weeks gestation, the presence of PG indicates a low risk of RDS. Cut-off value of L is ( \geq 3.5 \text{ mg/dL} ).</td>
<td>Utilize steroids from 28-34 weeks gestation. Utilize betamethasone: 12 mg/24 hr IM for 2 days (Liggins regimen). Continue giving steroids weekly until 32 or 34 weeks of gestation. Utilize steroids because Liggins did a follow-up study, which showed that infants whose mothers were given steroids experienced no fetal side effects.</td>
<td></td>
</tr>
<tr>
<td>Bennington Hospital/Dr. Leung</td>
<td>Utilize ritodrine to prevent premature labor (36 weeks).</td>
<td>Utilize L/S ratio and PG for RLU assessment. In diabetic pregnancy the L/S ratio must be ( \geq 3.0 ) and PG present for very low risk of RDS developing.</td>
<td>Yes, if need to enhance fetal lung maturation give betamethasone and follow Liggins regimen: 12 mg betamethasone given IM 24 hrs for 2 days. Utilize steroids because 3 year follow-up studies show no adverse fetal side effects and feels that if can prevent RDS is better than newborn developing RDS.</td>
</tr>
<tr>
<td>Hospital/obstetrician</td>
<td>Prematurity prevention</td>
<td>FIM assessment in complicated pregnancies</td>
<td>Glucocorticoid (steroid) treatment utilized to enhance FIM</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------------</td>
<td>-------------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Henry Ford Hospital/Dr. Lee</td>
<td>Utilize ritodrine to prevent premature labor (CRH wk).</td>
<td>Utilize L/S ratio and BC's presence to assess FIM. For diabetic pregnancy run a baseline ultrasound early in pregnancy and then at 36 weeks (if not complicated) run an amniocentesis. If L/S ratio is &lt;3.0 and BC present then deliver if required. However, depending on the situation and complications cannot always wait for BC to appear.</td>
<td>Randomly utilize steroids to enhance fetal lung maturation. Follow Liggins regimen of 12 mg betamethasone/24 every 24 hours for 2 days.</td>
</tr>
</tbody>
</table>

Abbreviations: FIM = fetal lung maturity, BC = decreased phosphatidylcholine, DI = intramuscularly, L = lecithin
discussed (will follow order of Table XLVII).

1. Prematurity Prevention

All five obstetricians utilize ritodrine to prevent premature labor (till approximately 36 weeks gestation). One of the obstetricians also utilized magnesium sulfate. All obstetricians agreed that the longer (gestationally) prematurity is prevented the lower the risk of RDS development.

2. Fetal Lung Maturity Assessment in Complicated Pregnancies

All five obstetricians feel that diabetic pregnancies (Classes A, B, and C) are five times more susceptible to RDS development. All stated that the L/S ratio had to be >3.0 and PG present for a low risk of RDS development. However, several commented that they do not always wait for PG to appear because several infants have died while waiting for PG's appearance. Most of the obstetricians run a baseline ultrasound early in diabetic pregnancies (establish correct due date). Then, if no complications arise, the diabetic mother has an amniocentesis performed at 36 weeks (if complications present, 34 weeks) to determine the L/S ratio and PG. Then depending on mature or immature results obtained the baby is delivered or mother monitored and a repeat amniocentesis and L/S ratio and PG determination performed, respectively.

3. Glucocorticoid (Steroid) Treatment to Enhance Fetal Lung Maturation

Three of the obstetricians routinely utilize steroid treatment (between 28-34 weeks gestation) and the other two obstetricians very selectively utilize steroids. Two obstetricians, who routinely utilize steroids, and one who randomly utilizes steroids, follow the Liggins (87)
regimen (12 mg betamethasone given intramuscularly every 24 hours for 2 days, and continue giving weekly (till 34 weeks or till delivery). The other obstetrician has modified the Liggin (87) regimen slightly and gives 12.5 mg betamethasone intramuscularly two times per week (24 hours apart - total = 25 mg/week) till 34 weeks or delivery. The final obstetrician, who very selectively (only 4 times in 4 years) utilizes steroids, gives 12 mg betamethasone at zero time and 12 mg repeated at 12 hours and by 48 hours delivers the baby. This obstetrician is skeptical about utilizing steroids because not enough is known about fetal and maternal side effects. Also the neonatologist from this same hospital commented that approximately five years ago steroids were routinely utilized to enhance fetal lung maturation, however, are not today because of the maternal side effects encountered (no fetal side effects encountered to date). The maternal side effects experienced were pulmonary edema, congestive heart failure, and infection caused by drug interactions (steroids + methyl xanthine-ritodrine). The other obstetricians which routinely utilize steroid treatment to enhance fetal lung maturity do so because the follow-up studies of infants whose mothers were given the steroid experienced no adverse fetal side effects. These obstetricians feel that if they can prevent RDS by steroids, thus enhancing lung maturity, that this is better than a newborn developing RDS and its complications.
CHAPTER VII

SUMMARY AND CONCLUSIONS

The thirty-five years since the first clinical description of infants with respiratory distress caused by respiratory distress syndrome (RDS) have been marked by extensive research. Currently the pathophysiology of RDS has been carefully documented; there have been major advances in fetal lung development, surfactant biochemistry, RDS diagnosis and therapy, and prenatal prediction and prevention of RDS are now possible in many instances.

In my opinion, fetal lung development requires coordination of anatomic, physiologic, and biochemical processes, the timing of which must be carefully regulated. The ultimate product of these changes in structure, function, and metabolism is a lung with alveoli having adequate surface area and capable of sustained ventilatory excursions for efficient gas exchange. Currently the characterization of the components of pulmonary surface active material is far from complete. Generally, the nature of the surfactant composition is agreed upon, however, a precise stoichiometry for the surfactant components remains to be determined. The key to unlocking all the surface active material unknowns is the lamellar inclusion bodies of the type II alveolar cells, where surfactant is synthesized, stored, and extruded. Clearly much progress has been made, however, much remains to be learned about the composition and mechanisms of these various components of alveolar froth. Following are some of the items which require investigation: delay between the time of secretion of surfactant in the fetal lung and its
appearance in amniotic fluid; rate of removal of surfactant from amniotic fluid due to degradation or fetal swallowing; phosphatidylglycerol's synthesis and function as the late biochemical fetal lung maturity marker; specific function and mechanism of synthesis for phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin (theorized that these helper phospholipids adsorb the film onto the alveolar surface when it is first secreted or they respread the film during respiratory changes of alveolar surface area); in amniotic fluid throughout the stages gestation critical for fetal lung assessment is surfactant the major source of disaturated lecithin or does disaturated lecithin arise from sources other than lecithin; when surfactant synthesis is being rapidly turned on or off it appears that there may be times when the surfactant content of amniotic fluid does not reflect the state of the fetal lung; and finally determine accelerated or delayed maturation mechanism of specific complicated pregnancies conditions (diabetes, PROM, hypertension, etc.).

Immaturity of fetal lung development is regarded as the reason for poor surfactant production and, therefore, the term respiratory distress syndrome does not describe a disease but rather a phenomenon of development. Infants who develop RDS are those whose lungs are not biochemically mature at birth and this lung immaturity is usually dependent on the two primary predisposing factors of gestational prematurity and low birth weight (however, there are other predisposing factors involved CHAPTER IV C. 1).

In theory, RDS in newborns may be caused by failure to elaborate a
sufficient amount of pulmonary surfactant or failure to manufacture a qualitatively normal complex. The two hypotheses are not mutually exclusive, for there actually appears to be both deficient production and abnormal phospholipid content of surfactant of newborns with RDS.

A survey of the Metro Detroit area's neonatologists on their current philosophies and practices in treatment of RDS was quite similar to the literature. The literature and survey were in agreement on the predisposing factors, and the diagnostic criteria utilized in RDS (CHAPTER IV C, and D. 1-3). However, the neonatologists' theory on radiographic findings and differential diagnosis of RDS differed from the literature. The neonatologists stated that the radiographic findings vary considerably because they are dependent on the infants severity, complications, and timing after respiratory problems begin. Concerning differential diagnosis the neonatologists felt that pneumonia was the most difficult to differentiate from RDS because of similar clinical and radiographic findings; however, RDS can usually be diagnosed in the first twelve hours of life with about 90% certainty.

The neonatologists agreed with the forms of treatment and associated RDS complications found in the literature. Treatment and management of RDS has improved greatly with the birth of the neonatal intensive care unit. When RDS cannot be prevented, respiratory support takes over. Recognition of early signs of anoxia has led to rapid correction of metabolic acidosis and to mechanical ventilation before irreversible damage occurs. Constant positive airway pressure now gets many babies through respiratory difficulties without endotracheal intubation. These
recent improved forms of treatment have increased the survival rate of
RDS. In 1965 only 10% of RDS infants weighing less than 1500 g
survived, in 1979 the figure rose to 80%, and today an infant weighing
750 g has a greater than 50% survival (cannot improve survival much
beyond 750g) and an RDS infant weighing 1000 g has 80% survival. All the
neonatologists felt that today’s saves were tomorrows problems, due to
complications encountered in these low birth weight babies and that their
goal was to continue to improve the quality and percentage of survival
for these babies. Additionally the neonatologists are conducting
follow-up studies on these RDS survivors and find it difficult to make
general conclusions on these follow-up studies due to the multitude of
variants involved, such as the wide spectrum of variants in RDS infants
and in the developmental studies. Basically the neonatologists agreed
that the smaller the infant (<1500 g) the more complications encountered
such as more susceptible to viral infections and experience slower
development; however, usually these infants catch-up to normal by four
years of age, while the larger RDS infant (>1500 g) has a much better
prognosis.

Finally, the most recent development in RDS treatment is surfactant
substitution after birth. All the neonatologists felt that this
surfactant substitution has great potential, however, much research and
many human trials are required to evaluate the risk versus potential
utilization. Many questions concerning this synthetic surfactant’s
substitution must be answered and many problems overcome, such as,
antigen-antibody interactions. Also recently, human lung surfactant has
been isolated from amniotic fluid of term infants delivered by Cesarean section. Therefore, it is postulated the human fetus may become the best surfactant donor.

In this author's opinion the key to RDS prevention is to prevent prematurity. For mothers-to-be should be educated to recognize risk factors and either change them or obtain expert prenatal care. Education may aid some women avoid premature labor; however, there are thousands of mothers-to-be who have no known risk factors. Therefore, much research must be conducted to prevent prematurity, and understand the complex mechanism of keeping babies in the womb till they are mature, and what actually triggers premature labor.

During my literature search of RDS I encountered several variations concerning complicated pregnancies and utilization of glucocorticoids to enhance fetal lung maturation, so I surveyed several Metro area high-risk obstetricians to obtain their updated philosophical views. All the obstetricians utilize the new beta-mimetic agent, ritodrine, to suppress uterine contractions in premature labor. However, ritodrine has metabolic and cardiovascular effects which generally render them off limits to women with diabetes, vaginal bleeding, cardiac disease, hypertension, or preeclampsia.

All the obstetricians feel that diabetic pregnancies (classes A, B, and C) are five times more susceptible to RDS development. The reasons for this are that hyperglycemia and fetal reactional hyperinsulinism are both involved in the processes delaying fetal lung maturation and in turn delay or impair the appearance of PG and lecithin (169). Further studies
on the understanding of the molecular and cellular mechanisms leading to delayed fetal lung maturation will be conditional on the availability of animal models reproducing the hormonal and metabolic environments of human fetuses in diabetic pregnancies.

Presently, in assessing a diabetic mother, the obstetrician will run a borderline ultrasound in early pregnancy to establish a correct due date. Then if complications arise the diabetic mother will have an amniocentesis performed at 36 weeks (if complications present 34 weeks) to determine fetal lung maturity (assessed by L/S ratio and PG). Depending on mature or immature results obtained, the baby is delivered or mother monitored and repeat amniocentesis with L/S ratio and PG determination are performed.

Another recent obstetrical complication in premature pregnancies is the utilization of the controversial procedure of glucocorticoids (steroids) treatment to enhance fetal lung maturity. Three of the five surveyed obstetricians routinely utilize steroid treatment and the other two very selectively utilize steroids. All the obstetricians follow the regimen of Liggins (87) or a modified version of steroid administration. The obstetricians who utilize steroid treatment do so because the follow-up studies (animals only) of infants whose mothers were given steroids experienced no adverse fetal side effects. These obstetricians feel that prevention of RDS by steroid administration enhancing fetal lung maturation is better than a newborn developing RDS and its complications. On the other hand the obstetricians who very selectively utilize steroids do so very skeptically, for they feel not enough is
known about fetal and maternal side effects. In one hospital in the Metro Detroit area, obstetrician staff utilized steroids about five years ago to enhance fetal lung maturation, but experienced maternal complications (pulmonary edema, congestive heart failure, and infection caused by drug interactions of steroids, ritodrine, methylate). This author feels that in theory the steroids do enhance fetal lung maturity, and no fetal side effects have been encountered in human infants, however, there have been some maternal side effects experienced which need clarification (because only select conditions have warranted side effects), and if selectively utilized steroids can enhance fetal lung maturation without any fetal or maternal side effects.

Prenatal fetal breathing movement promotes the passage of surfactants from the fetal airways into the surrounding amniotic fluid. Approximately 150 mL of tracheal contents per day reaches the amniotic fluid in this manner. Amniocentesis, consequently, provides a proper specimen for measuring the amount and/or functionality (i.e., surface tension lowering capability) of fetal lung surfactant. Since the production, expulsion into the amniotic fluid, and degradation of amniotic fluid is rapid, the measurement of amniotic fluid surfactant provides useful information regarding fetal lung maturity. This information is valuable in helping assess high-risk pregnancies, and the measurement of amniotic fluid surfactant has assumed an important role in the management of pregnancy by the obstetrician. Therefore, the demonstration of quantitative or qualitative deficiency of phospholipids in fetal pulmonary extract (i.e., amniotic fluid) can predict RDS before
its onset following delivery.

Surfactant is composed of six major phospholipids (PC, S, PG, PI, PS, and PE). The basis for amniotic fluid surfactant testing is the gestational change that occurs in the concentration of these various phospholipids during prenatal development. Therefore, the availability of a truly unambiguous indice of fetal pulmonary maturity would be of immense clinical value. The considerable effort expended toward this end has resulted in numerous biochemical and biophysical, assays purporting to solve the obstetricians dilemma. However, as illustrated by their bewildering numbers, each assay has its own shortcomings.

My literature search resulted in the review of twelve methods utilized to assess fetal lung maturity. Also, I surveyed the Metro Detroit area clinical laboratories and physicians to determine what procedure(s) and current philosophies concerning RDS and fetal lung maturity assessment were being utilized. Table LIV summarizes each of these twelve procedures with respect to principle, critical cut-off value, advantages, disadvantages, and interferences. From the survey it can be concluded that the majority of Metro Detroit area clinical laboratories are utilizing one-dimensional thin-layer chromatography (TLC) methods for the L/S (lecithin/sphingomyelin) ratio determination and simultaneous separation of PG and PI (several others perform two-dimensional TLC for PG and PI). These specific one-dimensional TLC methods include: one Gluck original procedure (114), five modified Gluck procedures, and four Helena Fetal Tek-200 procedures (commercially available kit) (Table XLV). The majority of these procedures omit
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<td>Lecithin/Sphingomyelin Ratio (L/S Ratio)</td>
<td>Universally accepted procedure and most widely utilized today. Critical cut-off value of L/S ratio in relation to RDS depends on procedure utilized. Therefore, each laboratory must establish critical values used in interpreting test results.</td>
<td>Two controversial problems concerning L/S ratios' clinical value: high incidence of false negative results and value of L/S ratios in complicated pregnancy. Controversial steps of procedure which need standardization: centrifugation, acetone precipitation, staining or charring for visualization and quantitation of spots. Reason for controversy concerniung procedure in the numerous variations developed with lack of standardization.</td>
<td>Blood will effect L/S ratio by: decreasing an originally mature result and increasing an originally immature result. (Effect is variable depending on blood concentration in amniotic fluid sample). Meconium will usually increase the L/S ratio, for meconium interferes with extraction of lipids.</td>
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<td>Most abundant lipids prior to term are lecithin and sphingomyelin for their concentration in equal prior to 35 weeks gestation when lecithin concentration sharply rises to four times that of sphingomyelin. Then lecithin continues to increase while sphingomyelinc decrease. With L/S ratio procedure extract and precipitate surface active phospholipids, then apply to TLC plate which is developed in solvent and quantitate the spots.</td>
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<td>2. Lung Profile/ Determine presence or absence of the following phospholipid indices: L, S, (L/S ratio), PG, PI, and disaturated lecithin fraction.</td>
<td>Lung profile reduces false error rate. Eliminates L/S ratio high incidence of false-negative results (that plague other assays), and complicated diabetic pregnancy false-positive results. Most noninformative low or intermediate L/S ratio values eliminated. These four parameters help form clearer picture of fetal lung development (especially in complicated pregnancy). Increases predictability of lung maturity in fetus to close to 100%, but may also signal an abnormal pregnancy. Also perinatal monitoring of PG, and PI in lung effluent is useful in diagnosis and follow-up of RDS as well as in evaluation of various therapies.</td>
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<td>Since PG and PI are present in very small quantities in blood products (whereas L and S found in blood interfere with results) blood contaminated amniotic fluid sample will not interfere with PG or PI results.</td>
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<td>3. Optical Density (OD)/ Method evaluates turbidity changes of the total phospholipid concentration. Test based on the principle that presence of suspended particles of lamellae in amniotic fluid increases turbidity of specimens with gestation.</td>
<td>Utilize as screening test because fast, inexpensive, and information derived is valuable if the results are positive.</td>
<td>Correlation with L/S ratio was good, fair, and poor. Many false negative and false positive results because OD absorbance by a fluid is dependent on total size, shape, and number of particles in the fluid. Therefore this method measures all phospholipids as well as unidentified surfactants and possibly nonsurface active substances. Test lacks specificity, because even though surfactant contributes to the optical density of amniotic fluid its contribution relative to other components of amniotic fluid at various stages of gestation is unknown. Therefore, OD may reflect overall fetal maturity rather than fetal lung maturity.</td>
<td>Prolonged refrigeration decreases the OD probably due to flocculation of the fluid proteins. Higher centrifugation will give lower absorbance readings. Blood and meconium contamination will increase OD₀₀₅₀ measurement (because absorb at wavelength). Polymidrmasae has dilutional effect causing falsely lowered results.</td>
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6. Cortisol
Evidence shows that fetal production (adrenal glands) cortisol of (hydrocortisone) parallels lung maturation and is reflected by the appearance and increase of cortisol in amniotic fluid. Cortisol levels rise around the 28th week of gestation, remain at a plateau until at least the 39th week and then rise rapidly in the 2 weeks before the onset of spontaneous labor.

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<td>4. Cortisol/ Evidence shows that fetal production (adrenal glands) cortisol of (hydrocortisone) parallels lung maturation and is reflected by the appearance and increase of cortisol in amniotic fluid. Cortisol levels rise around the 28th week of gestation, remain at a plateau until at least the 39th week and then rise rapidly in the 2 weeks before the onset of spontaneous labor.</td>
<td>The precise role that cortisol plays in fetal lung maturation and its corresponding gestation appearance, must be determined before an amniotic fluid cortisol level can be utilized as a clinical index.</td>
<td>Amniotic fluid cortisol level may provide an indication that lung maturation has been initiated, however, there is a time lag which occurs between the rise in cortisol and the achievement of lung maturation (L/S ratio in amniotic fluid reflects what has been accomplished). Because of methodologic difficulties most investigators doubt that measurement of cortisol will ever replace the simpler tests in use. Nevertheless, the data obtained was important physiologically. Evidence that normal mechanism of turning on surfactant production involves endogenous fetal cortisol production. Total cortisol is not as useful as the L/S ratio in assessing PLR because has low sensitivity and accuracy.</td>
<td>Both blood and meconium contaminated samples should be excluded. Meconium introduces undetermined cross-reacting steroids that interfere with cortisol levels. Polyhydramnios lowers amniotic fluid cortisol levels. Conditions representing a fetal response to prenatal stress elevated the cortisol levels (i.e., toxaemia, early severe Rh-incompatibility).</td>
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<td>5. Palmitic Acid/Surfactant lecithin obtained from mature lungs is esterified (two palmitic acid moieties) with about 70% palmitic acid; therefore, assess fetal lung maturity by measuring palmitic acid content of amniotic fluid. With advancing gestation and increasing pulmonary maturity there is an increase in the proportion of palmitic acid (C₁₆:0) and palmitoleic acid (C₁₆:1) in amniotic fluid lecithin (all other fatty acids have decreasing slope during gestation). Palmitic acid in amniotic fluid is increased significantly after 37 weeks gestation.</td>
<td>Palmitic acid in amniotic fluid is as reliable a variance of RDS as in the L/S ratio; however, the latter can be estimated by such simpler methods. Only more extensive investigations will show if measurement of palmitic acid will have value as second-line test when an equivocal intermediate L/S ratio has been found. Ipek et al. (1981) procedure which is specific for dipalmitoyl lecithin (extract the nonphospholipid sources of palmitic acid) is very promising. However, has the disadvantage of length of test time and laboratory expertise required for test.</td>
<td>Previous methods (to Ipek et al. 1981) all have increased palmitic acid values because nonphospholipid sources (triglycerides and free fatty acids) contain palmitic acid residues and are present in amniotic fluid. Therefore, this procedure does not seem to offer any advantage over the L/S ratio.</td>
<td>Presence of whole blood greatly increases the value of phospholipid palmitic acid. This is attributed to the presence of large amounts of palmitic acid in components of cellular constituents which must be eliminated from amniotic fluid sample before analysis. (Serum and hemolyzed serum components resulted in no appreciable increase in palmitic acid). Meconium effect has not been documented.</td>
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<td>6. Lamellar body analysis</td>
<td>Phospholipid composition of the 10,000 x g pellet provides precise information about biochemical maturation of the fetal pulmonary surfactant system. Results fit into one of the five developmental stages and risk of developing RDS determined (utilized as test time 3 h). Clarify issue of considerable variation in gestational age at each stage, by additional testing.</td>
<td>Not subject to interference by phospholipids present in blood since whole cells are removed at 140 x g initial centrifugation (serum lipoproteins do not sediment at 10,000 x g). Meconium if present in high concentrations may contribute to 10,000 x g</td>
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<td>(10,000 x g pellet method continued.)</td>
<td>routine procedure. Measuring lecithin PC on the 10,000 x g pellet, only surfactant PC of the lamellar bodies is being measured. Also presence or absence of detecting PC is greatly increased by this method.</td>
<td>Two technically critical points which must be watched are: amniotic fluid must be layered carefully over the ficoll to give sharp interface. Because of sensitivity of inorganic phosphate method utilized all glassware must be acid-washed and contamination avoided. Its acceptance as an index of fetal lung maturity in its own right must await the collection of more data in the critical preterm</td>
<td>pellet, if sample contains little surfactant however, it does not contribute enough phospholipid to interfere when the surfactant concentration is high.</td>
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<td>(b). Lamellar body phospholipid method (LB-PL). Evaluates fetal lung maturity by measuring the concentration of LB-PL in amniotic fluid, after separating it from the other sources of phospholipid by isopycnic density-gradient centrifugation. Because surfactant has a high phospholipid/protein ratio, it has an unusually low density, therefore surfactant cannot pass through the</td>
<td>Currently routinely utilized for this micro-procedure for isolating the LB-PL fraction of amniotic fluid has proved useful in comparing it to the L/S ratio and for predicting fetal lung maturity.</td>
<td>Current information states that: C/PL blood concentration contributes negligible phospholipid to the LB-PL value and it has been found that amniotic fluid samples heavily contaminated with meconium resulted in LB-PL values as low as 30 to 40 mg/L therefore indicating that meconium does not contribute more than this amount of phospholipid to the LB-PL value.</td>
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<td>Ficoll layer, forming a clearly visible band</td>
<td>Rapid (30 min) microdialyzers continuous flow procedure utilized in measuring phosphatidyl choline (PC) of the lamellar body fraction of amniotic fluid. This method gives at least as good results as usual colorimetric determination of phospholipid phosphorus because of low CV and linearity.</td>
<td>Does not always give correct value for total phospholipid, because affected by intrinsic fluorescence and phospholipid class and degree of dispersion. This method not specific for L/S, for any particle of appropriate size could be retained in this LP fraction, however, it seems that most of the phospholipids in these fractions is of the lamellar body origin, at least in amniotic fluid collected after fetal lungs mature. Most established whether LP and EID/EID values are better than the L/S ratio in predicting RDS.</td>
<td>Meconium and blood contaminated samples, and the yellow in amniotic fluid of patients with Rh factor complications affects the DPH method by giving overestimated values, the result of high background fluorescence both in the filtered and unfiltered fractions of amniotic fluid.</td>
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<td>(c). Fluorometric method Method based on measuring difference in fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH)-added to amniotic fluid before and after micropore filtration. The micropore step removes lamellar bodies particles. Fluorescence is measured before and after filtration so that the fluorescence derived from the LP fraction can be calculated.</td>
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<td>2. Lechthin/ Determination of disaturated lecithin in amniotic fluid appears to be more precise sign of potential risk of RDS.</td>
<td>This procedure for determining saturated phosphatidylcholine (SPC) was a more reliable predictor than L/S ratio. (Overall accuracy of this SPC procedure was 92% compared to 90% for the L/S ratio). Reliability of this method dependent on the fact that disaturated lecithin in amniotic fluid derived from surfactant.</td>
<td>Utilization of sodium dithionite which is a toxic substance and has some accompanying hazards; use in well-ventilated area because contaminates readily at room temperature, and skin and eye contact must be avoided. Amount of non-saturating disaturated lecithin present in amniotic fluid must be determined because disaturated lecithin is not unique to pulmonary surfactant (found in spleen, heart, blood all contain 30% SPC) and in fetal urine and amniotic fluid (about 3%). Over all this SPC procedure appears better than the L/S ratio; however, due to its disadvantages it has not been accepted as routine.</td>
<td>Presence of blood (cells removed at centrifugation if hemolyzed affect) and meconium (unsmutated L) to not affect the measurement of SPC (markedly alter L/S ratio).</td>
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<td>(a). Enzymatic assay/</td>
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<td>(b). L/S and PG</td>
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<td>chloroform/methanol</td>
<td>determination is</td>
<td></td>
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<tr>
<td>(2:1 by vol), then</td>
<td>accurate, precise, quick</td>
<td></td>
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<tr>
<td>solvent is evaporated,</td>
<td>(15 min), simple and</td>
<td></td>
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<tr>
<td>and the residue</td>
<td>inexpensive. Add this</td>
<td></td>
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<tr>
<td>Test/principle</td>
<td>Advantages</td>
<td>Disadvantages</td>
<td>Contamination and other effects</td>
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8. (b), continued

...redissolved in an aqueous detergent solution (LVS switzenol) (compatible with L and S and because solubilizes phospholipids). L and S assays involve enzymatic hydrolysis of the phospholipids to produce choline, which is then oxidized to betaine, then generating hydrogen peroxide which is enzymatically coupled to form a red chromogen. Enzymatic colorimetric determination of PG similar to the above for L and S. Samples are extracted in chloroform/methanol (2:1 by vol.) extraction required because of the presence of relatively large quantities of reaction intermediates and...
TABLE IlVII Continued

<table>
<thead>
<tr>
<th>Test/principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Contamination and other effects</th>
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<tbody>
<tr>
<td>8. (b). continued</td>
<td>evaporated and then the residue is redissolved in a non-ionic detergent (Triton X-100). The enzymatic reaction involves phospholipase catalyzed hydrolysis of glycerol from its phospholipid and then the glycerol is phosphorylated and oxidized to produce hydrogen peroxide which in reacted to produce an intense red chromogen.</td>
<td>Procedure appears to be somewhat more time consuming because of extraction step required to eliminate relatively large quantities of reaction intermediates for sphingomyelin and KG, however, the three phospholipids may be determined from the single extraction. This procedure could be utilized as a tool for investigating the L/S ratio controversial steps and after standardization of steps, this procedure could be tested for adequate population sampling and then adopted for routine clinical usage.</td>
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</table>
TABLE XLVIII Continued

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<thead>
<tr>
<th>Test/principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Contamination and other effects</th>
</tr>
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<tbody>
<tr>
<td>9. Fast-Atom Bombardment (FAB) Mass Spectrometry</td>
<td>Procedure relatively simple, rapid, and has a nearly quantitative yield. Looks promising. However, the following must be determined: Can this isotope-ratio method be expanded to measure both DPC and HG? Must establish DPC cut-off value for fetal pulmonary maturity. Must include evaluation of a large sample size of various pregnancy conditions and simultaneously compare I/S ratio and DPC values on amniotic fluid samples and correlate the fetal outcome.</td>
<td>Availability and expensive cost of FAB mass spectrometer.</td>
<td>Undetermined.</td>
</tr>
<tr>
<td>Dipalmityl phosphatidylcholine DPC is quantified by taking advantage of stable-isotope labeled-d₂-DPC as an internal standard, and a mass spectrometer (Nier-Johnson geometry is utilized to measure ratio of d₂/DPC. The d₂-DPC is synthesized by refluxing dipalmityl ethanolamine with d₂-methyl iodide in methanol in the presence of sodium bicarbonate for 26 hours. The FAB mass spectrometer is to detect the formed PC ions.</td>
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<td>Test/principle</td>
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<td>Disadvantages</td>
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<tr>
<td>10. Surface tension lowering ability of amniotic fluid surfactant</td>
<td>Surface tension globule formation measurement of amniotic fluid lipid extract is relatively simplified procedure which has good correlation with L/S ratio.</td>
<td>Surface balance measures the total activity of surfactant while L/S ratio measures surfactant specific individual components. This procedure has not gained much popularity in the clinical laboratory because large aliquots of extract are required, it takes 1 h completion time and the presence of other surface active compounds (i.e., bile salts, proteins, and salts of free fatty acids) may affect results obtained. When comparing to L/S ratio physicians favor the L/S ratio acceptability in predicting fetal pulmonary maturity.</td>
<td>Contamination with blood moved the mature value towards immature and the immature towards the mature (similar to L/S ratio).</td>
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</tbody>
</table>

Goldbrand et al. (211) found that the surface tension values for both mature and immature amniotic fluids reach a plateau.
TABLE ILVIII Continued

<table>
<thead>
<tr>
<th>Test/principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Contamination and other effects</th>
</tr>
</thead>
</table>

10. continued

and a subsurface globule is formed in the system. Therefore, after the extract was made, the extract was repeatedly layered, until the addition of more extract did not disperse on the surface, but rather remain as a globule on the surface and then sink below the water, at this point the volume of extract was recorded.

11. Shake Test (from Bubble Stability Test)/
Based on characteristic ability of surfactant phospholipids to form highly stable surface film that can support structure of foam for a period of time.

Reliable screening test provided negative result using L/S ratio. Ease at which test can be performed and speedy results (<1/2 h).

Procedure semi-quantitative and was

Since L/S ratio appearance and acceptability the shake test performance has rapidly decreased. Has two major deficiencies: Interpretation of results is relatively

Blood and meconium contaminated amniotic fluid sample can cause false positive and false intermediate results. Prescence of certain obstetric cream, vaginal secretions and amniotic fluid can
<table>
<thead>
<tr>
<th>Test/principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Contamination and other effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 (a). Original Shake Test</td>
<td>found comparable to L/S ratio. Measure surfactant concentrations. (&gt;30 mg/L).</td>
<td>subjective-only two possible results, and often produces false negative results.</td>
<td>produce false positive results. Other interference, or sources of error include for all shake procedures: accurate reproduction of initial 47.5% ethanol mixture; glassware free of soap, and serum; test tube movement during foam evaluation period and reading of tube at proper time. Results affected by centrifugation speed and presence of above contaminants. Care must be taken in the detail of pipetting the ethanol volume fraction.</td>
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<tr>
<td>Make 1:1 and 1:2 dilution of amniotic fluid plus 95% ethanol (volume fraction 47.5%), shake for 15 s and observe for presence or absence of filmy ring of foam in 15 min.</td>
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<td>Advantages</td>
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<tr>
<td>(b). Foam Stability Index (FSI) Test. Add undiluted amniotic fluid to tubes containing varying volumes of 95% ethanol resulting in final ethanol volume fractions of 42 to 55%. Tubes shaken for 30 s and allowed to stand for 15 s. For highest concentration of ethanol volume fraction which permits stable ring of foam to form in FSI.</td>
<td>Test simple and rapid. Measures lower concentration range of surfactant (range from 15 to 30 mg/L) which provides clinicians with significant information. Shown to be consistently reproducible; replicate values on the same specimen agree within ±0.01.</td>
<td>Reliability of test depends on ability to pipette accurately.</td>
<td>See above.</td>
</tr>
<tr>
<td>(c). Improved Shake Test. Similar to Clements (a) procedure except have three dilutions (1:1, 1:1.3, and 1:2) and bubble pattern interpretation is more precisely defined. Because has 3 possible reactions in each of 3 tubes therefore have 8</td>
<td>Gives semi-quantitative measurement of surfactant concentration. Eight levels of this foam test span most of the range of surfactant concentration from immature to the fully matured lung. Test simple and rapid.</td>
<td></td>
<td>See above.</td>
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<td>Test/principle</td>
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<tr>
<td>11 (c), continued</td>
<td>degrees of reaction as a titrator of amniotic fluid surfactant.</td>
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<tr>
<td>12. Fluorescence polarization (FP)/</td>
<td>The physical parameters of viscosity and surface tension of fluids are determined by intermolecular forces of the fluid and are interrelated so that surface tension of pulmonary surfactants translated into intrinsic viscosity (in terms of microviscosity).</td>
<td>Procedure has speed (10 min), simplicity, technical accuracy and reproducibility. Ideal screening method.</td>
<td>Primary disadvantage is cost of the specialized instrumentation. Also, only small population have been tested for this FP method, therefore, requires additional clinical trials so that clinical performance can be evaluated. Also, one standardized procedure must be utilized with specified centrifugation of sample incubation time and temperature so that results could be pooled for establishment of an acceptable cut-off value.</td>
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<td></td>
<td>Determine lipid micro-viscosity by usage of lipid-soluble fluorescent probe (DPH).</td>
<td>Changes in FP measurements in amniotic fluid during gestation parallel surfactant systems' development (measures all phospholipids important for surface tension).</td>
<td>Both blood and meconium contamination interfere in FP value determination, and their effect is variable depending on their lipid composition, as well as concentration in amniotic fluid (usually will reduce FP value leads to false positive results).</td>
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<td>12), continued</td>
<td>365 nm in an aqueous environment, and emits light at 460 nm.</td>
<td>Unanswered questions of endogenous fluorescence, lower total polarization and nonsurfactant high-density lipoprotein affect on the microviscosity FF value obtained must be answered before its clinical usefulness in predicting fetal lung maturity is accepted.</td>
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acetone precipitation, utilize cupric acetate reagent and charring for visualization, and densitometrically scan the phospholipids for quantitation (a couple utilize visual assessment).

The literature stated repeatedly that the L/S ratio of amniotic fluid was the first method proposed for evaluation of fetal lung maturity and that it was still widely utilized. However, it has been acknowledged that this procedure's steps have been subjected to innumerable variations to improve or simplify the test. These variations are very controversial and directly affect the test's analytical and/or clinical significance. Also, the lack of standardization of the L/S ratio procedure between laboratories or even between operators within the same laboratory has greatly influenced the results obtained which affect the cut-off values utilized in clinical interpretation. The most important controversial steps of the L/S ratio procedure will now be briefly summarized.

The most controversial step of the L/S ratio (or any other fetal maturity assessment test) is the speed and duration of centrifugation in which cells and cellular debris are removed from the amniotic fluid. This was demonstrated by the extreme variations in the literature and survey. This author feels that regardless of the centrifugation force and time, some phospholipids are precipitated (including those adsorbed to cells) and that more phospholipids are lost at higher g-forces. For example, the L/S ratio is reduced as the centrifugal force is increased as a result of a disproportionate loss of lecithin. This author feels that thorough testing should to be conducted to determine the effect centrifugation force and time have on amniotic fluid phospholipid
concentration at specific gestational ages and population samplings (inclusive complicated and normal pregnancies). It is generally agreed that centrifuging at 250 x g virtually removes whole cells from the whole amniotic fluid sample and corresponding supernate. Therefore in this authors opinion all clinical laboratories should establish a standardized centrifugation speed of 250 x g (the time must be established through testing).

Another controversial step of the L/S ratio is whether or not to utilize cold-acetone precipitation of the phospholipids. The survey revealed that 6 out of 10 laboratories omit this acetone-precipitation step (4 utilize). This author agrees with the omission of this step, because it is a laborious step which frequently introduces error in recovery of the phospholipids.

A third controversial problem of the L/S ratio procedure is the high incidence of false-immature ratios obtained, and the increase in false-mature ratios obtained (from 1 to 15%) in complicated pregnancies, particularly diabetes mellitus. In pregnancies complicated by diabetes mellitus some investigators have questioned the predictive value of mature results, while others have found no difference in the predictability of results in diabetic or other pregnancies particularly with the L/S ratio test. Possible explanations for this contradictory results include methodologic differences in patient populations (i.e., inclusion of class A diabetic) and difference in criteria utilized to define RDS. Nonetheless, there is general agreement that in diabetic pregnancies, there is continued risk of RDS even with mature L/S ratio
results...This risk has been related to fetal hyperinsulinemia. Because of the controversy over the incidence of RDS associated with a mature surfactant value in the pregnancies, a better predictive value was sought. This perplexing problem of false-positive L/S ratios in diabetic pregnancies (between 35-37 weeks gestation) was solved by the development of the lung profile determination: L/S ratio; phosphatidylglycerol (PG); phosphatidylinositol (PI); and disaturated lecithin. Initially delayed appearance of PG was taken as an indication of immaturity even with a mature L/S ratio. Since phosphatidylglycerol's determination in the lung profile, there have been conflicting results with respect to predicting neonatal outcome (PG present, L/S ratio mature, infant developed RDS; PG absent, L/S ratio mature, infant did not develop RDS). All the obstetricians surveyed utilize the L/S ratio (>3.0) and PG (present) as factors in assessing the fetal lungs as mature, however, two of the physicians do not always wait for PG's presence, because they have lost babies in waiting for PG to appear. The other three obstetricians have not experienced any RDS in infants whose amniotic fluid L/S ratio was >3.0 and PG was present. Therefore, phosphatidylglycerol's and phosphatidylinositol's syntheses, gestational appearance, and mechanism must be further defined and clarified in normal and diabetic (complicated) pregnancies to obtain reliable indices to base fetal lung maturity assessment on. This author feels that the lung profile procedure (one-dimensional TLC) is currently the most complete evaluation method which can clinically predict fetal maturity in normal pregnancies and most diabetic pregnancies. Although lung profile estimations prove
very helpful particularly in diabetic pregnancies, there is an obvious need for more accurate means of evaluating pulmonary maturation at the earlier stages of gestation before PG appears (36 weeks in normal pregnancies). Therefore, further studies on enzymatic assays and the fluorescence polarization method for assessing subtle changes in amniotic fluid surfactant levels should be conducted.

The recent enzymatic assays for lecithin, sphingomyelin, and PG look very promising for the future because they are relatively quick, simple, and inexpensive. Also the fluorescence polarization method has the following advantages over the current methods: speed (40 min); simplicity; technical accuracy; and reproducibility. The primary disadvantages of this method is the cost of the specialized instrumentation required, and whether the variable and significant amounts of nonsurfactant phospholipids and neutral lipids are being measured with the surfactant phospholipids in this method. Therefore, much more detailed testing of the enzymatic and fluorescence polarization methods are required to determine their potential in assessing fetal lung maturity.

One of the major problems with all the methods utilized in predicting fetal lung maturity, is that none of the methods has complete reliability, for some infants develop RDS in spite of normal pulmonary maturity test, whereas other are free from disease in spite of an immature test result. The incidence of false immature L/S ratios as well as other amniotic fluid tests results is dependent upon patient variability, on the method employed, the threshold value taken for
distinguishing a normal from an abnormal condition, and on the fact that few authors report their results in terms of sensitivity (percentage of sick newborn infants with a true immature-test result), and specificity (percentage of a healthy infants with true mature test result). Therefore, all methods have more false immature than false mature results. The following factors may explain the discrepancy between neonatal outcome and results for immature values in amniotic fluid tests: mode of delivery; fetal distress (chronic fetal hypoxia); small-for-date infants; prolonged rupture of fetal membranes; premature delivery; and birth asphyxia. Another variable is represented by the fact that there are extrapulmonary sources for amniotic fluid surfactant constituents, although the relative contribution from each are not known precisely and they may vary with gestational age.

In conclusion, this author feels that these various methods of assessing fetal lung maturity have undoubtedly helped to reduce perinatal mortality and morbidity due to RDS. The utilization of these tests is recommended for: timing of delivery prior to elective Cesarean section; complicated pregnancy monitoring; premature labor; complications and institution of pharmacologic prevention of RDS in utero or at delivery. No single method has achieved the destination of reliability and universal applicability. A mature value in most tests is almost 99% (predictive value of positive test), while an immature value has very low accuracy.

Where laboratory facilities are minimal, it is advisable to perform the shake test (or new test cassette: Lumadex-PSI (foam stability index))
or phosphatidylglycerol rapid agglutination slide test as screening procedures (not affected by blood or meconium). However, when these tests indicate immaturity the L/S ratio or lung profile determination must be carried out. If laboratory facilities are sufficient, then the L/S ratio (one-dimensional TLC) procedure with simultaneous PG determination or the lung profile determination should be performed. The new cassette Lumadex-FSI (foam stability index) or PG rapid agglutination slide test could be utilized in these laboratories as screening procedures on weekends and on shifts not manned to perform the longer procedures.

The various approaches and extensive literature on fetal lung maturity assessment have led to much confusion, causing some investigators to opt for new analyses or for more rapid methods. This is a questionable approach, for each laboratory should evaluate the following points in choosing a technique for determining amniotic fluid phospholipid maturity: clinical laboratory applicability of procedure including cost, speed, simplicity and the ability of the method to cope with contaminated samples; and predictive potential of the test for development of RDS by the neonate. After choosing their best-suited technique, the laboratory should establish and validate this analytical procedure, and the critical values utilized for clinical interpretation.

Finally, it should be stressed that no method can predict RDS with 100% accuracy, probably because fetal lung maturity is a very complex process that involves more than the ability of the developing lung to synthesize and secrete surfactants. Therefore, only after the mysteries
of the developing lung have been solved, will an accurate test be developed for fetal lung maturity assessment.
APPENDIX A

TO Director of Clinical Chemistry Department

DATE May 17, 1984.

SUBJECT: Laboratory procedures utilized for Amniotic Fluid determination of Fetal Lung Maturity.

I'm conducting this questionnaire survey concerning the procedure(s) employed by our laboratory for amniotic fluid determination of Fetal Pulmonary maturity.

I'm currently writing my Master Thesis Major Paper on "Pulmonary Surfactants and Respiratory Distress Syndrome." The paper discusses in detail the following areas: process of fetal lung development, pulmonary surfactant physiology and biochemistry, Respiratory Distress Syndrome (RDS): etiology, diagnostic criteria, differential diagnosis, treatment, complications and prevention, and lastly reviews the methods of evaluating fetal pulmonary maturity. The methods which I investigated include: the L/S ratio, the Lung Profile, Cortisol, Palmitic Acid, Lamellar Body, Static(Bubble) Test, Optical Density, Surface Tension, Fluorescence Depolarization, and Enzymatic assays of Choline-containing Phospholipids.

In my intensive literature search of these various methods, I encountered numerous variations in the procedures utilized and results obtained. These variations included, the reliability of specific tests, predictive values of results obtained, and lack of standardization of procedures utilized. Also many investigators correlated their procedures' results with the L/S ratio standard test, even though their correlation was good their sample size was inadequate and population tested not inclusive (complicated pregnancies and RDS cases omitted). Therefore, I've decided to survey the Metro Detroit and Windsor area Hospitals to determine which procedures are being utilized by Physicians to monitor and diagnose fetal pulmonary maturity.

The questionnaire survey is enclosed and the results will be presented in my paper in a chart and written format. If you wish to obtain a copy of the results, please request so when returning the questionnaire (enclosed is a self-addressed stamped envelope). Also if you have any additional comments or questions, please contact me. Please return no later than June 11th.

Your detailed input will be greatly appreciated, and I Thank You for your cooperation and time.

Sincerely,

Patricia K. Kanga
Clinical Chemist
Master's Candidate

627
QUESTIONNAIRE CONTINUED

F) Thin-layer chromatography (TLC).
   State: spotting solvent utilized volume and why.
   -plates utilized and why
   -method of application of sample to plate
     (i.e., streak, spot, narrow channels or cut narrow strips)
   -chromatographic tank and development time and temperature

G) Staining
   State: staining reagent and why (i.e., specific for certain phospholipids)
   -method of staining and quantity of reagent
   -rate of color development (time and temperature)

H) Charring
   State: charring reagent and why
   -method of charring
   -heating temperature and time utilized

I) Method of quantitation utilized: densitometric scanning,
   planimetry, gravimetric or visual assessment.
   State: -method utilized and why
   -any problems encountered with separation of phospholipids.

J) State reproducibility of method and standard controls utilized.

K) State cut-off values utilized for clinical interpretation
   and correlation of results with fetal outcome or HDS or not.

L) State number of L/S ratios run weekly/monthly.

M) Comment briefly on physicians' attitude and trust of methods available for both normal and complicated pregnancies.

6) Recently, enzymatic assays for determining lecithin, PG,
   and total choline-containing phospholipids in amniotic fluid are being investigated. Also an immunoassay kit for PG is currently being marketed. Has your laboratory been exposed to either or are you investigating either procedure.
2) Procedure utilized by your laboratory in the last 5-10 years.
   If different from question 1, answer A - I used question 1 for this procedure below. OR IF IT IS THE L/S RATIO TEST SEE QUESTION 5 A) - I).

3) Procedure utilized by your laboratory in the last 10-15 years.
   If different from questions 1 or 2, answer A - I used question 1 for this procedure below. OR IF IT IS THE L/S RATIO TEST SEE QUESTION 5 A) - I).

4) Comment if your laboratory ever utilized two methods, such as, optical density as a screening procedure and then the L/S ratio.

5) Over the years the determination of the L/S ratio of amniotic fluid has been widely accepted as the procedure utilized in evaluation of fetal lung maturity. However this procedure has encountered the following difficulties:
   - The test may give an unacceptable proportion of false predictions for lung immaturity.
   - Each of the steps of the procedure has been subjected to innumerable variations to improve or simplify the test. These variations are very controversial and directly affect the tests' analytical and/or clinical significance. Also the lack of standardization of the procedure between laboratories or even between operators within the same laboratory has greatly influenced the results obtained which affect the cut-off values utilized in clinical interpretation.

Please answer each of the following questions and be specific:
A) Reference L/S ratio procedure utilized.

B) Handling of sample and contamination of sample with blood or meconium.

C) Centrifugation of sample (i.e. volume of sample used, speed, time, and dimensions of tube) and specify why speed and time chosen.

D) Lipid extraction. State chemicals, volumes, time of vortex mixing, centrifugation speed and time utilized.

E) Acetone precipitation. State whether utilized or not and why. This step is very controversial in the literature.
FETAL PULMONARY MATURITY METHODS EVALUATION QUESTIONNAIRE

Please review the following questions and answer in detail:

1) State the current (last 5 years) method utilized by your laboratory. IF IT IS THE L/S RATIO TEST SEE QUESTION 5. If it is some other procedure answer the following:

   A) Procedure and reference of test.

   B) Handling of sample (i.e. refrigerated or frozen if not processed immediately for testing). Comment on bleed or mecunium contamination of sample.

   C) Centrifugation of sample (i.e. volume of sample, speed and time) and specify why this centrifugation was chosen.

   D) Controls and standardization utilized in procedure.

   E) Actual time of analysis from time sample obtained in laboratory.

   F) Special instrumentation utilized.

   G) Any factors affecting the procedure or results.

   H) Predictive and cut-off values utilized for clinical interpretations.

   I) Results and false predictions in correlating to RDS.
APPENDIX B

The Rapid Immunologic Screen for Fetal Lung Maturity

Agglutination Test for Phosphatidylglycerol in Human Amniotic Fluid

For In Vitro Diagnostic Use

Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intended Use</td>
<td>2</td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Summary and Explanation of Test</td>
<td>3</td>
</tr>
<tr>
<td>Principle of the Procedure</td>
<td>5</td>
</tr>
<tr>
<td>Reagents</td>
<td>6</td>
</tr>
<tr>
<td>Specimen Collection and Preparation</td>
<td>7</td>
</tr>
<tr>
<td>Procedure</td>
<td>8</td>
</tr>
<tr>
<td>Schematic of Procedure</td>
<td>10</td>
</tr>
<tr>
<td>Procedure (Continuation)</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>15</td>
</tr>
<tr>
<td>Expected Values</td>
<td>16</td>
</tr>
<tr>
<td>Limitations of Procedure</td>
<td>17</td>
</tr>
<tr>
<td>Specific Performance Characteristics</td>
<td>18</td>
</tr>
<tr>
<td>Bibliography</td>
<td>18</td>
</tr>
</tbody>
</table>

NOTICE: Failure to follow instructions as set forth in this labeling can adversely affect performance characteristics and stated or implied label claims.
Intended Use

Ammosat FLM™ is an immunologic semi-quantitative agglutination test for determining the presence of phosphatidylglycerol in human amniotic fluid at concentrations indicative of fetal lung maturity. Ammosat FLM™ is specific sensitive, and rapid. Results are not affected by blood, meconium contamination of amniotic fluid samples. It requires less than 0.1 ml of specimen which can be obtained by transabdominal amniocentesis or from a vaginal pool. The assay can be performed in about 15 minutes.

Ammosat FLM™ may be used as a late screening test in which positive results correctly predict the absence of Respiratory Distress Syndrome or as one in a series of tests performed to obtain a more thorough assessment of fetal lung maturity.

Introduction

The ability to determine antenatal pulmonary maturity is of great importance in the timing of delivery for pregnant in high-risk pregnancies, such as diabetes mellitus, Rh immunization, toxemia, premature rupture of membranes, premature labor, uncertain gestational age, intrauterine growth retardation, and repeat cesarean section.

Summary and Explanation of Test

Proper function of the pulmonary system is essential for the fetus to survive in an extrauterine environment. Infants born before the lung has reached maturity are said to have Respiratory Distress Syndrome (RDS). The primary physiological defect in RDS is a deficiency of surfactant, a complex mixture of lipids, proteins, and carbohydrates which reduces the surface tension of the alveolar lining and prevents collapse of the lung.

The most direct means of prenatal assessing fetal pulmonary maturity is to analyze the components of surfactant present in amniotic fluid. Of these, lecithin (L), sphingomyelin (S), and phosphatidylglycerol (PG) are the most significant.

One of the first diagnostic tests that was developed to assess maturity depends upon thin layer chromatography (TLC) of amniotic fluid to obtain an L/S ratio. Although this test has proved to be popular and reliable, it requires technical expertise and understanding to successfully execute the time-consuming TLC procedure and to correctly interpret the results.

The L/S ratio determined by TLC is also adversely affected by contamination of the specimen with blood, meconium or vaginal secretion. Therefore, certain maternal conditions such as diabetes mellitus, hypertension, severe anemia, and intrinsic renal disease are known to lead to a high incidence of false predictions with this test. As a consequence, other parameters have been investigated and related to maturity.

Clinical studies have repeatedly shown that the presence of PG in amniotic fluid predicts that the lung is sufficiently mature to allow that the neonate will be free of RDS.

In cases involving certain maternal complications, notably diabetes mellitus, the presence of PG even with an immature L/S ratio predicts the neonate will be free of RDS, whereas the absence of PG with a mature L/S ratio indicates a substantial risk of RDS.

Ammosat FLM™ is a rapid, reliable immunologic agglutination test which produces a clearly positive result when PG is at a concentration of approximately 2 mg/ml or greater.

Ammosat FLM™ is useful as an early diagnostic tool for assessing fetal lung maturity when indicated.

Principle of the Procedure

Ammosat FLM™ is an immunologic agglutination test in which PG in amniotic fluid is incorporated into lipoparticles labeled by adding Reagent A in a prescribed manner. After dilution, the suspension of particles is mixed with Reagent B which contains antibodies that react with particles containing PG.

The presence of PG in the specimen is indicated by the agglutination (clumping) of the microscopic particles to form macroscopic clusters with a visible increase in turbidity in the background. The degree of agglutination is roughly proportional to the amount of PG present.

The concentration of PG in the specimen can be estimated by comparing the test reaction with control reactions prepared in an identical manner using control reagents. The control reactions also assure assay reliability. If the expected results are not obtained, faulty technique and/or contaminated reagents are to be suspected.
Reagents

Yellow NEGATIVE (+) CONTROL
Butter with 0.02% sodium azide containing no PG

Orange POSITIVE (++) CONTROL
Contains 2 µg PG per ml Butter with 0.02% sodium azide

Red STRONG POSITIVE (++++) CONTROL
Contains 4 µg PG per ml Butter with 0.02% sodium azide

Green REAGENT A. Contains optimized concentrations of lecithin and cholesterol in ethanol
CAUTION: Solvent is volatile and flammable, cap tightly after use and keep away from flames
Components may crystalize at low temperatures, ensure complete solubilization before use

Blue REAGENT B. Anti-PG serum fraction with 0.02% sodium azide

White BUFFER. Phosphate dilution butter with 0.02% sodium azide

- Store all reagents in tightly capped vials at 2-4°C (refrigerated)
- Equilibrate reagents to 20-25°C (room temperature) and shake gently before use
- Use a clean sterile pipette tip for each aliquot to prevent contamination

For In Vitro Diagnostic Use

Specimen collection and Preparation

The specimen of choice is fresh, uncontaminated amniotic fluid obtained by transabdominal amniocentesis. However, amniotic fluid obtained from a vaginal pool or a sample contaminated with blood or meconium can be used. The volume of fluid actually utilized in the test is 0.025 ml.

Immediately upon receipt, the specimen should be centrifuged at 500 x g for 5 minutes to remove cells and debris which could interfere with sample retrieval or produce artifacts during the assay.

Caution: Excessive centrifugation speed and time will sediment PG-containing material. Note: The RPM equal to 500 x g is calculated by this formula

\[ \text{RPM} = \frac{\sqrt{44.76 \times \text{radius in cm}}} {1000} \]

After centrifugation, the supernatant liquid must be decanted completely and mixed thoroughly. The specimen should be analyzed immediately to avoid possible degradation of PG.

If necessary, the supernate may be stored at 2-4°C for up to 4 hours or at about minus 20°C for longer periods. Before analysis, the specimen must be mixed thoroughly by vigorous manual or mechanical means.

Procedure

Reagents and Materials Provided

Controls
- Negative, Positive and Strong Positive Reagents A and B
- Butter
- Disposable Agglutination Slides
- Viewing Mirror

Materials needed but not Provided
- Disposable glass round-bottom 12x75 mm test tubes
- Test tube rack
- Micropipetters for delivering 10, 25, and 250 µl
- Sterile disposable plastic pipette tips
- Serological rotator (60-80 rpm speed)
- Centrifuge (low speed) and centrifuge tubes

Available from Hance Biological 60 rpm fixed speed rotator, 110 V, 60Hz. Catalog number D100-900

Preliminary Preparations

The "AmnioStat FLA" reagents must be at room temperature for proper function of the assay. As soon as you receive the specimen, remove the kit from the refrigerator, then remove the reagents and one slide from the carton. The bottle of Butter may be placed in a small container of warm (30-35°C) water for a brief period of time to help bring it to the required temperature. To avoid spilling the reagents, return them to the carton before beginning the assay.

Assemble the necessary equipment and mark four test tubes for identification.
Schematic of Procedure

CAUTION: Equilibrate reagents to room temperature and shake gently before use.

Check Reagent A for complete solubility.

Use a new, separate pipet for each sample.

A Preparation of
Lipid Particles
(On a 12 x 75 mm glass tube)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Negative</th>
<th>Positive</th>
<th>Strong Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 25 µl Specimen</td>
<td>25 µl Control</td>
<td>25 µl Reagent A</td>
<td>25 µl Reagent A</td>
</tr>
<tr>
<td>(Mix well)</td>
<td>(Mix well)</td>
<td>(Mix well)</td>
<td>(Mix well)</td>
</tr>
<tr>
<td>2. 250 µl Buffer</td>
<td>250 µl Buffer</td>
<td>250 µl Buffer</td>
<td>250 µl Buffer</td>
</tr>
<tr>
<td>(Mix well)</td>
<td>(Mix well)</td>
<td>(Mix well)</td>
<td>(Mix well)</td>
</tr>
</tbody>
</table>

B Preparation of
Agglutination Reactions
(On a glass slide)

C Incubation of
Reactions Mixtures
Cover and rotate
at 60 rpm for 5 min

D Examination of
Completed Reactions
Place slide on mirror

Detailed instructions for use begin on page 12

10

Appearance of Controls

Confirm assay validity by examining the control reactions which should appear as follows:

Negative (-) Control: Uniform, grey-white, slightly granular-looking appearance, possibly with a few white specks.

Positive (+) Control: Clumps of white particles of varying size with obvious clearing (decrease in turbidity) in the background.

Strong Positive (+++) Control: Large, clumps with a very clear background.

The photographs below illustrate the appearance of typical Negative, Positive, and Strong Positive Controls.

Results

If the controls meet the criteria noted above, examine the specimen reaction and score as follows:

- "Negative" similar to the Negative Control, i.e., uniform, slightly grey-white looking, possibly with a few white specks.
- A "Positive" result is obtained when PG is absent or present at concentrations associated with a significant risk of RDS.
- "Strong Positive" similar to or stronger than the Positive Control, i.e., obvious clumps of agglutinated particles of varying size with distinct clearing in the background.
- A "Weak Positive" result is obtained when PG is present at approximately 2 µg/ml or greater, which is reported to be 100% predictive of maturity.

"Weak Positive" significantly smaller clumps than present in the Positive Control, but distinct from the Negative Control with definite clearing in the background.

- A "Negative" result is obtained when the trace presence of PG has been associated with a low risk of RDS.

For a specimen to be scored positive, an agglutination pattern with a distinct clearing in the background is different from the Negative Control reaction and must be observed.

If viewed under the microscope, the Negative Control will display a uniform field of small, irregular-shaped particles. The Strong Positive Control will have large masses of agglutinated particles with few individual particles in the background. The Positive Control will display a pattern in between these two.
Expected Values

Ammiostat-FLM™ produces a positive result with amniotic fluid containing PG at concentrations indicative of fetal lung maturity. Studies have shown that maturation of the lung normally occurs around the 35th or 36th week of gestation, although this process can occur at considerably earlier or later times.\(^\text{1-5,19}\)

PG has been reported to be present at a concentration of 1.2 \(\mu\text{g/ml}\) or greater in about 50% of the amniotic fluid specimens obtained at gestational week 35.\(^\text{1,19}\) However, it has been shown to be present as early as the 28th or 29th week without incidence of RDS.\(^\text{10,19}\) and to be absent as late as the 36th or 39th week with or without incidence of RDS.\(^\text{4,15}\)

The presence of PG in a specimen with an immature L/S ratio has been reported to correctly predict maturity.\(^\text{1,12}\)

Limitations of Procedure

Ammiostat-FLM™ is intended to be used as an indicator of the presence of PG in human amniotic fluid. It is also intended for use as a semi-quantitative test, based on the use of two qualified positive controls.

Ammiostat-FLM™ will register a positive agglutination reaction which is distinct from a negative reaction. However, not all specimens will produce agglutination patterns identical to the equivalent control reaction.

Ammiostat-FLM™ is not subject to artefacts associated with other tests for PG; however, a false value could be obtained if the instructions are not followed. A false positive interpretation would occur if one did not look for “clearing in the background” of the agglutination reaction. Excessive centrifugation will sediment PG and lead to a low value.

Equivocal results should be scored negative. If the criteria described for the agglutination patterns of the three control reactions are not met, the assay is to be considered invalid. Under certain conditions (such as low temperature), the agglutination reaction may proceed slowly and additional rotation time will be required for optimum results. If the proper patterns are not obtained after 18 minutes of rotation, the entire test should be repeated with the specimen and the controls. Reagent instability, as a consequence of improper storage or contamination, should be considered if the test is again invalid.

Specific Performance Characteristics

Ammiostat-FLM™ produces a clearly positive result with specimens containing PG at a concentration of approximately 2 \(\mu\text{g/ml}\) or greater. A “weak positive” result is obtained with specimens containing PG at lower concentrations considered to be indicative of a rapidly maturing lung. A “negative” result is associated with a significant risk of RDS but does not necessarily indicate that the condition is inevitable.

The assay can be performed on specimens contaminated with blood, meconium, or vaginal secretion.

Each Ammiostat-FLM™ kit contains a matched set of reagents which are quality tested to give standardized agglutination reactions with control solutions. Each assay must include control reactions which serve as internal standards and assure assay validity.

Bibliography

Lumadex-FSI Fetal Lung Maturity Test

Now there's a decisive, 10-minute test for fetal lung maturity that lets the doctor know if a baby's lungs are ready for the outside world.

Lumadex is FAST
- Immediate results in 10 minutes.

Lumadex is CONVENIENT-TO-PERFORM
- Involves only a few steps.

Lumadex is PORTABLE
- Can be done anywhere—in the laboratory, clinic, operating room or at bedside in the labor or delivery room—day or night, any time of the day, any day of the week.

Lumadex is ESSENTIAL
- As an aid in the management of high-risk pregnancies.
- When the physician has a question as to the degree of fetal lung maturity.
The Lumadex®-FSI Test Procedure

With the Lumadex-FSI Test a significant breakthrough has been realized. The development of an ingenious test “cassette” has provided the means to make the determination of fetal lung maturity immediately accessible and easier to perform than all other previously used methods.

As in other methods for determining fetal lung maturity, the Lumadex-FSI Test is performed using amniotic fluid from the sac surrounding the fetus. First, you centrifuge the amniotic fluid. Then you add the supernatant amniotic fluid to the six test wells of the cassette, using the syringe provided, filling to the upper marked lines on the cassette.

Agitate the cassette vigorously for 30 seconds, allow it to stand for one minute to allow any foam to rise to the surface, and, finally, visually examine the cassette for the presence of a stable ring of foam.

With the addition of equal amounts of amniotic fluid to the differing amounts of ethanol, you will end up with a range of ethanol volume fractions equivalent to the range of 44% to 50%. Each test well of the Lumadex-FSI Test cassette is labeled with the equivalent ethanol fraction number or FSI (Foam Stability Index) Value for that well — 44, 45, 46, 47, 48 and 50. (The control well is labeled CTL.)

The lower the value (46 or below) the greater the probability of immature fetal lung development. If foam is visible in well 47 or above, it indicates that the lungs of the fetus are mature and that it is very unlikely that RDS will occur if the baby is delivered.
Lumadex-FSI
Fetal Lung
Maturity Test

A fast, convenient-to-perform, portable test for the determination of fetal lung maturity that offers you these exclusive benefits:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>FSI (Foam Stability Index) Value pre-printed on the outside of each test well...</td>
</tr>
<tr>
<td></td>
<td>• No need for labeling individual test tubes.</td>
</tr>
<tr>
<td></td>
<td>• Eliminates labeling errors.</td>
</tr>
<tr>
<td>2.</td>
<td>Sealed test cassette...</td>
</tr>
<tr>
<td></td>
<td>• No evaporation or leakage.</td>
</tr>
<tr>
<td></td>
<td>• Minimizes test variations and spillage.</td>
</tr>
<tr>
<td>3.</td>
<td>Built-in control well (CTL)...</td>
</tr>
<tr>
<td></td>
<td>• No guesswork regarding cassette operation.</td>
</tr>
<tr>
<td></td>
<td>• Indicates that temperature of the cassette is correct and agitation has been adequate, and provides an example of a stable and persistent ring of foam at the air-liquid interface.</td>
</tr>
<tr>
<td>4.</td>
<td>Blue dye added to the ethanol...</td>
</tr>
<tr>
<td></td>
<td>• The foam is extremely visible at the air-liquid interface.</td>
</tr>
<tr>
<td></td>
<td>• Assures you of easy-to-read test results.</td>
</tr>
<tr>
<td>5.</td>
<td>Consistent well diameter...</td>
</tr>
<tr>
<td></td>
<td>• Minimum test-to-test and lab-to-lab variation.</td>
</tr>
<tr>
<td></td>
<td>• Gives you a high degree of confidence in your test results.</td>
</tr>
<tr>
<td>6.</td>
<td>Six test wells with differing amounts of ethanol...</td>
</tr>
<tr>
<td></td>
<td>• Various ethanol volume fractions are part of a single test.</td>
</tr>
<tr>
<td></td>
<td>• Produces a degree measure of surfactant functionality and lung maturity.</td>
</tr>
<tr>
<td>7.</td>
<td>Pre-measured ethanol...</td>
</tr>
<tr>
<td></td>
<td>• No ethanol measuring and dispensing steps.</td>
</tr>
<tr>
<td></td>
<td>• Insures accurate reagent volume required.</td>
</tr>
<tr>
<td></td>
<td>• Uniformity in the concentration of the ethanol in the azeotropic mixture.</td>
</tr>
<tr>
<td></td>
<td>• Assures the cassette-to-cassette reproducibility you require.</td>
</tr>
<tr>
<td>8.</td>
<td>Lower set of horizontal guidelines on test wells...</td>
</tr>
<tr>
<td></td>
<td>• A visual indicator that the cassette contains the appropriate amount of ethanol reagent.</td>
</tr>
<tr>
<td></td>
<td>• Assures you that the room temperature cassette is ready-to-use.</td>
</tr>
<tr>
<td>9.</td>
<td>Upper set of horizontal guidelines on test wells...</td>
</tr>
<tr>
<td></td>
<td>• A visual filling level for the accurate addition of amniotic fluid.</td>
</tr>
<tr>
<td></td>
<td>• Minimizes dispensing errors.</td>
</tr>
</tbody>
</table>
The Clinical Studies

Clinical studies show a direct relationship between the FSI value and the probability of neonatal respiratory distress syndrome (RDS), a leading cause of perinatal mortality and morbidity in most countries of the world today.

The data collected from 462 cases studied indicates the following:

<table>
<thead>
<tr>
<th>FSI Value</th>
<th>Total # of RDS</th>
<th># of RDS</th>
<th>Likelihood of RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;44</td>
<td>31</td>
<td>18</td>
<td>~60%</td>
</tr>
<tr>
<td>45</td>
<td>16</td>
<td>7</td>
<td>~45%</td>
</tr>
<tr>
<td>46</td>
<td>26</td>
<td>5</td>
<td>~22%</td>
</tr>
<tr>
<td>47</td>
<td>46</td>
<td>2</td>
<td>~4%</td>
</tr>
<tr>
<td>48</td>
<td>67</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>50</td>
<td>276</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

Thus, out of 389 cases where the FSI Value was greater than or equal to 47, only two cases of RDS occurred. The excellent diagnostic utility of the Lumadex-FSI Test was demonstrated in a study of 94 pregnancies in which both the L/S ratio and the Lumadex-FSI Values were determined. Nineteen cases of RDS occurred. Out of these 19, all but one had a Lumadex-FSI Value under 47. Five of the RDS cases had what is considered a mature L/S ratio of 2.0 or greater. In four of those cases, the Lumadex-FSI Foam Stability Index Test correctly predicted that RDS would occur. The authors of this study concluded that in the critical diagnostic range (L/S of 1.6-2.0 and a Lumadex-FSI Value of 45-46), the Lumadex-FSI Test more consistently predicted RDS than did the L/S ratio.

Preliminary studies of Lumadex-FSI Values for low birthweight babies indicate that the test may be useful in discriminating between babies of Appropriate for Gestational Age (AGA), and those that are Small for Gestational Age (SGA). A clinical study of 38 such babies having a biparietal diameter of ≤8.4 cm showed that 16 of 19 babies having FSI Values ≥47 were independently categorized as being SGA while all 19 babies having FSI Values <47 were independently categorized as being AGA. None of the babies with FSI ≥47 developed RDS, while 12 of the 19 babies with FSI Values <47 developed the disease. A second study has verified this conclusion.
The Test Concept

The Lumadex-FSI Fetal Lung Maturity Test Provides a Measurement of the Functionality of All Lung Surfactants in the Amniotic Fluid and the Degree of Lung Maturity.

During the last trimester of pregnancy, various enzyme systems in the fetal lungs mature, initiating the production and secretion of surfactant by the type II alveolar pneumocytes. Surfactant, which is essential for the proper operation of the lungs of the neonate, acts in vivo to modulate the surface tension of the air-liquid interface on the inner surface of the alveolar sacs. Inadequate surfactant production will lead to a tendency for these alveolar sacs to collapse, producing Respiratory Distress Syndrome.

Prenatal fetal breathing movements promote the passage of surfactant substances from the fetal airways into the surrounding amniotic fluid. Approximately 150 mL of tracheal contents per day reaches the amniotic fluid in this manner. Amniocentesis provides a proper specimen for measuring the amount and/or functionality (i.e., surface tension lowering capability) of fetal lung surfactant.

The concept of a functional test to measure the surface tension properties of amniotic fluid began with the work of J. Clements et al. (5) and, subsequently, J. Edwards and P. Ballie (6) who introduced the Simple Shake Test, where one part of uncentrifuged amniotic fluid was combined with an equal part of ethanol in a test tube. They were making use of the principle of the antifoaming properties of ethanol to assess surfactant functionality.

The mixture in the tube was then agitated vigorously, allowed to settle for a stated period of time and evaluated for the presence or absence of stable foam on the surface of the liquid. The presence of an adequate amount of surfactant in the amniotic fluid stabilized the foam and gave a positive result for the test. The absence of foam indicated a negative result.

This test provided a black-or-white, simple yes-or-no assessment of lung maturity.

G. Sher and B. Statland (7) introduced the Manual Foam Stability Index (FSI) Test as a significant refinement of the Simple Shake Test. The Manual FSI Test provided a series of graduated ethanol-amniotic fluid mixtures containing varying ethanol volume fractions (EVF) ranging from 0.42 to 0.55 (i.e., 42% to 55%), inclusive. The highest EVF which would permit a stable ring of foam to persist following vigorous agitation was defined as the FSI Value. This value was described in papers published by Sher and Statland (8) as being a useful tool in assessing the subsequent risk of RDS occurring in the neonatal period and with a better clinical reliability than the conventional L/S ratio (9).

The Lumadex-FSI Fetal Lung Maturity Test is based on the Manual FSI Test principle. With the introduction of the Lumadex-FSI Fetal Lung Maturity Test, a significant breakthrough has been realized. The development of an ingenious test "cassette" has provided the means to make the determination of fetal lung maturity immediately accessible and easier to assess than all other previously used methods including the Manual FSI Test.
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BIBLIOGRAPHY


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