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ISOLATION AND CHARACTERIZATION
OF J-CHAIN FROM BOVINE COLOSTRAL IgM
AND NASAL SECRETORY IgA

By
RON KOMAR

A Thesis
Submitted to the Faculty of Graduate Studies through
the Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

WINDSOR, ONTARIO, CANADA
1974
Ron Komar 1974
ABSTRACT

J-chain was found to exist in two forms. One form free in the colostrum and the other covalently bound to colostral IgM. The molecular weight of the free and bound forms of the fast component was determined to be 16,555 by SDS-PAGE. In addition, the free form of the fast component was found to be antigenically identical to the bound form. The free form sedimented as a single peak to 1.56S. Antiserum against the fast component (containing both the bound and the free forms) precipitated neither bovine colostral IgG nor μ-chains and BSA, but precipitated native or denatured intact IgM (devoid of the free form of the fast component) and human J-chain. All criteria therefore indicated that the fast component be classified as J-chain. The radioalkylation experiments revealed the presence of 9.4-10 sulfhydryl groups per mole for both the free and bound forms of bovine J-chain. The stoichiometry of J-chain determined from the densitometric tracing of the reduced and alkylated bovine colostral IgM (devoid of the free J-chain) in stained analytical polyacrylamide gels revealed the presence of one J-chain per IgM molecule.
IgA was found to exist in two polymeric forms (15.3S and 12.2S) in bovine nasal secretions. The 15.3S secretory (S-IgA) dissociated into 12.2S and 7S components and a trailing edge upon treatment with 4M guanidine hydrochloride. The partial specific volume ($\bar{\nu}$) and molecular weight of the 12.2S S-IgA were calculated to be 0.721 g per cc and 430,380 g per mole respectively. The molecular weight of the 15.3S polymer was determined to be 641,900 g per mole assuming a $\bar{\nu}$ of 0.721 g per cc. The 12.2S S-IgA was found to show complete identity with the 15.3S S-IgA and in addition shared antigenic determinants with human colostral IgA.

J-chain was released from both species of S-IgA following treatment with 2-mercaptoethanol. The molecular weight of J-chain from S-IgA ($J_\alpha$) was determined to be 17,040 by SDS-PAGE. Antigenic analysis showed $J_\alpha$ and $J_\mu$ (bound-form) to be identical. Stoichiometric studies of J-chain determined from densitometric scans of polyacrylamide gels of reduced and alkylated bovine 12.2S S-IgA revealed the presence of one J-chain per S-IgA molecule.
ACKNOWLEDGEMENTS

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For their critical reviews of this thesis, my sincere appreciation is extended to Dr. L. R. Sabina, Department of Biology, and Dr. D. E. Schmidt, Department of Chemistry, both of the University of Windsor.

My gratitude is extended to Mrs. Beth Abson and Miss Usha Tewari for their able assistance.

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DEDICATION

To My Parents and Wendy.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
</tbody>
</table>

Chapter

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>MATERIALS AND METHODS</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>ISOLATION AND CHARACTERIZATION OF J-CHAIN FROM BOVINE COLOSTRAL IgM</td>
<td>16</td>
</tr>
<tr>
<td>IV</td>
<td>ISOLATION AND CHARACTERIZATION OF NASAL SECRETORY IgA</td>
<td>38</td>
</tr>
<tr>
<td>V</td>
<td>SUMMARY</td>
<td>66</td>
</tr>
<tr>
<td>APPENDIX A</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>APPENDIX B</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>APPENDIX C</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>REFERENCES</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>VITA AUCTORIS</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Results of Precipitation Reactions</td>
<td>24</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ouchterlony analysis of IgM</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Immunoelectrophoretic analysis of IgM</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Schleiren pattern of IgM</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Analytical PAGE of IgM, reduced and alkylated IgM, sulphonated IgM, J-chain reduced and alkylated IgM (devoid of free J-chain), bound J-chain, and redissolved precipitates</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Ouchterlony analysis of free and bound J-chain</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>Ouchterlony analysis of human J-chain and bovine bound J-chain</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Molecular weight determination of J-chain</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Schleiren pattern of free J-chain</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Elution profile of reduced and alkylated IgM (devoid of free J-chain) on Bio-Gel P-200 equilibrated with 1M acetic acid</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>Densitometric scan of reduced and alkylated IgM (devoid of free J-chain)</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>Elution profile of (NH₄)₂SO₄ precipitate of nasal secretions on Bio-Gel P-300</td>
<td>40</td>
</tr>
<tr>
<td>12</td>
<td>Schleiren patterns of Peak I and Peak II (Fig. 11)</td>
<td>41</td>
</tr>
<tr>
<td>13</td>
<td>Elution profile of Peak I (NH₄)₂SO₄ precipitate of nasal secretions off Bio-Gel P-300 on Bio-Gel Λ 0.5-M</td>
<td>43</td>
</tr>
<tr>
<td>14</td>
<td>Schleiren pattern of purified peak I nasal</td>
<td>45</td>
</tr>
</tbody>
</table>
15. Schleiren patterns of Peak I and Peak II in 4M Gd-HCl, pH 8.0 .......................... 45
16. Ouchterlony analysis of 15.3S S-IgA and 12.2S S-IgA .................................. 48
17. Ouchterlony analysis of 12.2S S-IgA with IgM and IgG ................................. 48
18. Ouchterlony analysis of human IgA and 12.2S S-IgA .................................. 50
19. Immunoelectrophoretic patterns of 15.3S and 12.2S S-IgA ........................... 50
20. Sedimentation equilibrium data for 12.2S S-IgA .......................................... 52
21. Analytical polyacrylamide gel electrophoresis of reduced and alkylated 15.3S S-IgA, reduced and alkylated 12.2S S-IgA, reduced and alkylated IgM and purified J-chains from 12.2S S-IgA ........................................ 55
22. SDS-polyacrylamide gel of reduced and alkylated 15.3S S-IgA ........................ 55
23. Molecular weight determination of S-IgA components ................................. 57
24. Ouchterlony analysis of J\u and J\a ................................................................. 58
25. Localization of J-chain on the elution profile of reduced and alkylated S-IgA passed on a column of Sephadex G-100 equilibrated with 0.5M propionic acid ........................................... 61
26. Densitometric scanning pattern of reduced and alkylated 12.2S S-IgA .......... 63
CHAPTER I
INTRODUCTION

Immunoglobulins are composed of two basic types of polypeptide chains, heavy (H) and light (L). A third chain referred to as secretory component (SC) is associated with IgA in external secretions (S-IgA) (1). Recently, the existence of a fourth polypeptide component, designated J-chain, was described in polymeric forms of human and rabbit IgA (2) and human IgM (3) but absent from monomeric IgA, IgG, IgD, or IgE (4). Since J-chain was found to be associated only with the polymeric immunoglobulins, it was postulated that it might play an important role in the assembly of these polymers (5). Parkhouse and Della Corte have studied the biosynthesis of IgA and IgM in mouse myeloma tumor cells (6,7,8). They have reported that J-chain and a disulphide-exchanging enzyme are required for polymerization of the monomeric subunits of IgM and IgA.

A variety of methods have been used to isolate J-chain. Morrison and Koshland (9) have used anti-light chain immunoadsorbants to isolate human J-chain from IgM and IgA while Tomasi et al. (10) isolated J-chain by
dialysis of the L-chain fraction against distilled water. Under these conditions the J-chain was precipitated whereas the L-chain remained in the supernatant. Mestecky et al. (3) used ion-exchange chromatography of the light chain fraction on a column of DEAE Sephadex equilibrated with a buffer containing 8M urea to isolate J-chain. Other investigators purified this protein by elution from polyacrylamide gels (10,11). In all cases, the L- and J-chain fraction was separated from the H-chains before subsequent isolation of J-chain.

Physico-chemical properties of human J-chains isolated from polymeric IgA and IgM revealed the following information:

a) That J-chain was readily detectable as a fast anodal band following alkaline-urea polyacrylamide gel electrophoresis (2,3).

b) The molecular weight of J-chain varied from 6,400 to 48,000 as determined by analytical ultracentrifugation (10,12,13,14), polyacrylamide gel electrophoresis in sodium dodecylsulfate (3,14,15,16,17) and gel filtration (3,15).

c) J-chain had an unusually high half-cystine content (3,9,12,13,15,17,18,19).

d) J-chain was covalently linked by disulfide bonds most probably to the Fc portion of these immunoglobulins (20).
e) There was a J-chain per dimer of IgA (9) or pentamer of IgM (3, 6).

Amino acid analysis indicated that the compositions of J-chains from IgM and IgA were quite similar or identical (3, 9). Heavy chains, light chains and secretory component, however, had strikingly different amino acid compositions than J-chain (3, 9, 12, 13, 15, 17, 18, 19, 21). J-chain was also found to contain twice as many cysteine residues per thousand residues when compared to L-chains.

Peptide maps revealed complete identity of J-chains from IgM and IgA (3, 21). Maps of J-chains were different from those of H-chains, L-chains from IgG and L-chains from IgM and IgA after removal of J-chains.

The antigenic properties of J-chain have also been investigated. Morrison and Koshland found that J-chains isolated from polymeric human IgA and IgM were identical by the criterion of antigenicity (9).

Recently, Kobayashi et al. (22) showed that rabbit antisera, rendered specific for human J-chains cross-precipitated extensively with J-chains of reduced and alkylated polymeric IgA and IgM from the dog, cat, cow, goat, sheep, pig, horse, hedgehog, guinea pig, rat, mouse, and chicken. The significance of this work was that it established the presence of a protein homologous to human J-chain in polymeric immunoglobulins from many different species.
Isolated J-chain has been shown to have an $S_{20,w}$ from 1.28 (12) to 1.4 (10) and a half-cystine content of 7-10 residues/mol (12).

Since the size and half-cystine content of J-chain has been well established, two linkage models were postulated (12). In the "bracelet" model, J-chain is extended around the polymer so that disulfide bonds can be formed between J and each heavy chain and the polymer is closed by an intra-J disulfide bond. In the "clasp" model, J-chain is linked only to two heavy chains in adjacent monomer units with its remaining half-cystines forming intra-J disulfide bonds. Furthermore, the polymer is completed by disulfide bonding between monomer units.

Although considerable information has now been accumulated in the literature concerning the antigenic and physico-chemical properties of J-chains from human (2,3,9,10,11,12,14,15,16,17,18), canine (19), mouse (6,7,8,23) and porcine polymeric immunoglobulins (21), there is practically no information about the identification and physico-chemical properties of J-chains isolated from bovine macroglobulins. This investigation describes the identification and some of the physico-chemical properties of J-chains isolated from bovine colostral IgM and nasal secretory IgA.
CHAPTER II
MATERIALS & METHODS

Purification of Bovine Colostral IgM

Bovine colostrum was obtained from cows within 3 days post-partum. The colostrum was defatted by centrifugation at 12,100 x g and 4°C for 30 minutes. It was then diluted 1:10 with 0.15 M NaCl and the casein was precipitated by adjusting the pH to 4.75 with 6 N HCl. The casein was removed by centrifugation at 4,080 x g and 4°C for 30 minutes in a Sorvall RC-2B centrifuge. The colostral whey was adjusted to pH 6.5 and fractionated essentially according to Mukkur and Froese (24). The globulin fractions were precipitated with saturated (NH₄)₂SO₄ at a final concentration of 50 percent. The precipitate was dissolved in a buffer containing 0.01 M Tris-HCl and 0.32 M NaCl, pH 8.0 (0.32 ST) and dialyzed exhaustively against 0.01 M KH₂PO₄ (pH 4.75) in the cold in order to precipitate the euglobulins. The latter were obtained by centrifugation at 12,100 x g and 4°C for 30 min. in a Sorvall RC-2B centrifuge. The euglobulins were dissolved in 0.32 ST and subjected to gel filtration on columns of Bio-Gel P-300 (2.5 cm x 90 cm) equilibrated with 0.32
ST. The leading peak was subjected to ion-exchange column chromatography on TEAE-cellulose (1.2 cm x 40.0 cm) using a discontinuous gradient (1st buffer: 0.01 M Tris-HCl + 0.13 M NaCl, pH 8.6; 2nd buffer: 0.01 M Tris-HCl + 0.32 M NaCl, pH 8.6). The IgM eluted with the second buffer and was checked for purity by immunoelectrophoresis, Ouchterlony's gel diffusion analysis and analytical ultracentrifugation.

Isolation of Bovine Colostral IgG

Bovine colostral IgG was isolated by gel filtration on Bio-Gel P-300 followed by ion-exchange chromatography and recycling chromatography on Bio-Gel P-150 according to Tewari and Mukkur (25).

Isolation of Bovine IgA from Nasal Secretions

Nasal secretions were collected by washing the nostrils of 30 cows with 0.15 M NaCl. The secretions were homogenized in a waring blender and filtered immediately using Whatman MM 3 filter paper. Since no previous reports were available in the literature on the isolation of immunoglobulins from bovine nasal secretions, a procedure described under results, was developed.

Isolation of Human Colostral IgA

Purified human colostral IgA which was isolated by molecular sieve chromatography of the 50 percent
\((\text{NH}_4)_2\text{SO}_4\) precipitate of human colostrum on Bio-Gel A 15-M was kindly provided by Mrs. E. C. Abson of this laboratory.

Reduction and Alkylation

Reduction of bovine IgM was carried out in a buffer containing 5 M urea and 0.2 M Tris-HCl, pH 8.0 using 0.2M 2-mercaptoethanol (2-ME) (Aldrich Chemical Co., Milwaukee, Wis., U. S. A.) at 37°C in a water bath for 2 hours under a nitrogen atmosphere. Alkylation was accomplished using a 100 percent molar excess of sodium iodoacetate. (Sigma Chemical Company, St. Louis, Mo., U. S. A.).

Human IgM (a gift from Dr. F. P. Inman) was reduced with a 0.024 M mercaptoethylamine (MEA) followed by alkylation as described above.

Bovine IgA was reduced with 0.2 M 2-ME at 25°C for 12 hours and alkylated with 0.3 M sodium iodoacetate.

Sulphitolyis of Bovine Colostral IgM

IgM suspended in 0.5 M NH\textsubscript{4}Cl pH 8.6 was treated with 0.015 M Na\textsubscript{2}SO\textsubscript{3} in the presence of 0.004 M CuSO\textsubscript{4} for 30 minutes at 30°C in a water bath according to Franek and Żikan (26). The reaction mixture was then dialyzed against borate-saline buffer (0.16 M NaCl - 0.001 M Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}·10H\textsubscript{2}O pH 8.0) and stored frozen until ready for use.
Alkaline - urea Polyacrylamide Gel Electrophoresis (PAGE)

The procedure of Reisfeld and Small (27) was followed with the modifications of Raam and Inman (23):

a) Cyanogum 41 at a gel concentration of 5 percent was used instead of acrylamide and bis-acrylamide.

b) The urea concentration was 5M.

c) Only the lower gel composition was used.

d) Tris-glycine buffer Tris (5.16g/liter) - glycine (4g/liter) was used with 5 M urea and the pH was 9.0.

e) Electrophoresis was carried out at 3 milli-amperes per tube for 75 minutes. Coomassie blue (1%) in 12.5 percent trichloroacetic acid was the stain. Destaining was accomplished in 7 percent acetic acid. Each gel was loaded with 400 to 500 ug of protein. The composition of various stock solutions used in the preparation of the gel are mentioned in Appendix A.

Polyacrylamide Gel Electrophoresis in SDS

PAGE in SDS was performed exactly according to the method of Weber and Osborn (28). A standard curve for molecular weight determination was constructed using bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700) (Worthington Bio-chemical Corp., Freehold, N. J., U. S. A.) as standards. Mobility was calculated.
according to the following formula.

\[
\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}
\]

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale. The details on the electrophoretic procedure and solution composition are described in Appendix B.

**Preparative Polyacrylamide Gel Electrophoresis**

Preparative PAGE was carried out in a 7.5 percent gel in 6 M urea at a pH of 9.5 using a Shandon Southern preparative polyacrylamide apparatus (Mark-II-SAE-2782). The buffer employed contained Tris-HCl (15g/2.5 liters) - glycine (72g/2.5 liters). The stock solution composition and electrophoresis procedure is described in Appendix C.

**Isolation of J-chain**

Reduced and alkylated bovine colostral IgM, nasal IgA and human IgM were subjected to preparative PAGE as described above. Both bovine and human J-chains were found to elute after electrophoresis for 240 to 270 minutes.
Reduction and Radioalkylation of J-chain

Reduction of the isolated J-chain was carried out in 0.2 M Tris-HCl: pH 8.0 containing 5 M urea, using 0.2 M 2-ME in a 37°C water bath under a N₂ atmosphere for 2 hours followed by alkylation with³H-Iodoacetic acid (61.9 mCi/m M, New England Nuclear, U.S.A.).

The samples were exhaustively dialyzed against 0.32ST until only a background radioactivity was detected in the dialyzing buffer. Then an aliquot was counted in 10.0 ml of Aquasol (New England Nuclear, U. S. A.) using a Nuclear Chicago Liquid Scintillation Counter (Mark II). The number of sulfhydryl groups per J-chain was then calculated assuming a mole to mole ratio with iodoacetic acid. All samples were adjusted for quenching.

Protein Determination

While the concentration of IgM was calculated using E₁cm, 280 nm of 12.6 (24), that of J-chain was calculated using the Lowry modification of the Folin-Ciocalteu test (29). A standard curve was constructed by the use of bovine serum albumin at various concentrations.

Preparation of Antisera

a) Anti-bovine-J-chain antisera

400 µg of J-chain obtained from reduced and alkylated IgM in 0.5 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.)
U. S. A.) and 0.5 ml *Hemophilus pertussis* vaccine (Connaught Medical Laboratories, Toronto, Ontario, Canada) was injected intradermally at multiple sites along the back of a rabbit. Two booster injections were given at 14 and 28 days followed by bleeding at various intervals.

b) **Anti-human J-chain antiserum**

Anti-human-J-chain antiserum was kindly donated by Dr. F. P. Inman, Department of Microbiology, University of Georgia, Athens, Georgia, and was raised in the following manner. J-chain (300 ug) in Freund's complete adjuvant was distributed subcutaneously in several areas along the back of a rabbit. Four weeks later a booster injection of 300 ug was given intramuscularly in Freund's incomplete adjuvant. The rabbit was bled three weeks after the booster injection and then once a week for the next three weeks.

c) **Anti-bovine colostrum**

Colostrum (1 O.D./ml) in complete Freund's adjuvant was injected subcutaneously at several sites along the back of a rabbit. Booster injections were given at 3 week intervals and the animal was bled periodically.
d) **Anti-bovine nasal secretions**

   Anti-bovine nasal secretions were prepared by immunizing rabbits with whole nasal secretions (1 O.D./ml) in complete Freund's adjuvant.

e) **Anti-bovine colostral IgM**

   Rabbit anti-bovine IgM was prepared by injection of one milligram of purified colostral IgM in complete Freund's adjuvant, followed by booster injections at monthly intervals.

f) **Anti-bovine colostral IgG**

   Anti-colostral IgG was produced in rabbit by immunizations with this antigen (1mg/ml) in complete Freund's adjuvant.

g) **Anti-bovine nasal secretory IgA (S-IgA)**

   Anti-bovine nasal IgA was elicited in rabbit by subcutaneous injections of IgA in complete Freund's adjuvant.

h) **Anti-human IgA**

   Specific rabbit anti-human secretory IgA antisera was kindly donated by Dr. J. Bienenstock, Dept. of Medicine, McMaster University, Hamilton, Ontario, Canada.

**Separation of Heavy (H) and Light (L) Chains**

An extensively reduced and alkylated sample of IgM was passed through a column of Bio-Gel P-200
(2.0 cm x 35.0 cm) equilibrated with 1 M acetic acid with resultant separation of the polypeptide chains.

**Analytical Ultracentrifugation**

Sedimentation coefficients for all proteins were measured from the schleiren patterns obtained using a Beckman Model E analytical ultracentrifuge equipped with schleiren optics and electronic speed control. All samples were analyzed at 56,000 rpm and 20°C in an An-H rotor. S_{observed} was corrected to S_{20,w} according to Schachman (30).

The molecular weights of the IgA polymers were determined using the meniscus depletion method of Yphantis (31). The partial specific volume of the 12.2S IgA was determined by the H_2O - D_2O method of Edelstein and Schachman (32).

**Immunologic Tests.**

The purity of IgM, IgG and IgA were tested by immunoelectrophoresis according to Scheidegger (33) and Ouchterlony's gel diffusion (34). The latter method was modified for the gel diffusion analysis of J-chain. The gel diffusion slides were allowed to incubate overnight at room temperature in a humid atmosphere followed by incubation at 4°C for 3-4 days. That the precipitin lines thus observed were not artifacts was shown by the fact that the use of BSA in one of the
wells against anti bovine-J antiserum did not show any precipitin line. A precipitation reaction in a test tube could be obtained within 24 hours when 0.1 ml. of J-chain mixed with 0.1 ml of anti J-chain antiserum was incubated at 37°C for 1 hour and then over night at 4°C. The presence of J-chain in the precipitate was verified by dissolving it in Tris-glycine buffer containing 5 M urea and subjecting it to analytical PAGE when a fast, anodally moving component equivalent to J-chain was observed.

**Stoichiometry of J-chain**

IgM devoid of the free form of J-chain was reduced extensively in the presence of 4 M Guanidine hydrochloride (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) pH 8.0 (Gd-HCl) alkylated as described above. It was then dialyzed against Tris-glycine buffer containing 5 M urea and subjected to analytical PAGE stained with Coomassie Blue and destained in 7 percent acetic acid.

Reduced and alkylated 12.2 S nasal S-IgA was also subjected to analytical PAGE. The gels were then scanned using a Beckman Microzone Densitometer (R-110) equipped with a gel scanning attachment and an integrator-recording system. The areas under the respective peaks were calculated by counting the number of integrations and the percentage of a particular component was calculated according to the formula:
\[ \text{\% component} = \frac{\text{area of component}}{\text{sum area of all components}} \times 100 \]

Separation of Heavy (H) and Light (L) Chains

S-IgA which had been reduced and alkylated with 0.2 M 2-ME was passed through a column of Sephadex G-100 (2 cm x 40 cm) equilibrated with 0.5 propionic (M) acid to achieve dissociation of the polypeptide chains.
CHAPTER III
ISOLATION AND CHARACTERIZATION OF J-CHAIN FROM BOVINE COLOSTRAL IgM

Results

Bovine colostrum IgM, isolated according to Mukkur and Froese (24), was judged pure by immuno-electrophoresis, Ouchterlony's gel diffusion analysis and analytical ultracentrifugation (Fig. 1-3). The pure IgM was subjected to analytical PAGE in urea and gave a fast-moving band which migrated anodally (Fig. 4a). This protein band will be referred to as the free-form. A similar band was also observed when IgM which had been sulphonated or reduced with 0.2 M 2-ME followed by alkylation was subjected to PAGE (Fig. 4b,c). In order to differentiate between the fast-moving band detected before and after the reduction of IgM, a sample (80 mg) was reduced and alkylated followed by dialysis against Tris-glycine buffer. The dialyzed sample was subjected to preparative PAGE and the fast-moving band isolated. After concentration the fast band was run on analytical PAGE to check its purity and only a single band was obtained (Fig. 4d). A portion of this material was used to obtain anti-fast component antiserum as described in Chapter II.
Fig. 1. Ouchterlony's gel diffusion of colostral IgM (right well) and colostral IgG (left well) against rabbit anti-bovine colostrum.

Fig. 2. Immunoelectrophoretic patterns of colostral IgM (top) and intermediate peak of euglobulin (bottom). The trough contained rabbit anti-bovine colostrum.
Fig. 3. Schleiren pattern of bovine colostral IgM obtained 9 min. after reaching a speed of 56,000 rev/min.
Fig. 4. Analytical polyacrylamide gel electrophoresis of a) Unreduced, immuno-electrophoretically pure intact IgM; b) Reduced and alkylated IgM. Both a and b show the presence of a single, fast, anodally moving component representing in a, free J-chain and in b a mixture of free and bound J-chain; c) Sulphitolised IgM showing the presence of J-chain; d) Isolated J-chain; e) Reduced and alkylated IgM (devoid of the free-form); f) Isolated bound J-chain; g) Urea-dissolved precipitate of rabbit anti-J-chain antisera and bound J-chain; h) Urea-dissolved precipitate of rabbit anti-J-chain antisera and free J-chain.
The intact IgM was then subjected to preparative PAGE to obtain IgM devoid of the free-form of the fast-moving band. Since intact IgM penetrates 7.5% gel to only a small extent, the former was obtained by stirring the entire gel in Tris-glycine buffer followed by centrifugation at 20,200 x g for 1 hour (Sorvall RC-2B centrifuge). The supernatant fluid was concentrated to obtain IgM devoid of the free-form. A sample (40 mg) of this IgM was reduced and alkylated and subjected to preparative PAGE to isolate the fast-moving band referred to as the bound-form, since its isolation was dependent on reduction of disulfide bonds.

Analytical PAGE of the reduced and alkylated IgM (devoid of the free-form) and the isolated bound-form of the fast component is shown in Fig. 4e and 4f.

When the precipitation test was performed it was found that rabbit anti-fast-component antiserum did not yield a precipitate with μ-chains, colostral IgG or BSA but precipitated with native IgM, IgM devoid of the free-form (whether the IgM was isolated by preparative PAGE using Tris-glycine buffer with or without urea) and both the bound- and the free-form of the fast component. Confirmation that the fast components from both the bound- and free-form were precipitated was obtained using analytical PAGE (Fig. 4g,h). Conversely anti-colostral IgG did not precipitate either the
bound- or the free-form of the fast component. Finally, anti-bovine J-chain antiserum was found to precipitate human J-chain and this was confirmed by analytical PAGE. The results of the precipitation reactions are summarized in Table 1. These data suggest that the fast moving components (both bound- and free-form) are J-chains.

In order to determine the antigenic relationship between the free- and bound-form of J-chain and bound and human J-chain, two Ouchterlony's gel diffusion tests were set as described under Materials and Methods. Both the free- and bound-form of J-chain showed lines of complete identity. Thus pointing to their antigenic similarity (Figure 5). Human J-chain and bovine-bound J-chain showed a line of partial identity when developed against anti-human J-chain antiserum (Figure 6).

The molecular weight of the bound- and the free-form of J-chain was determined by PAGE in SDS and was found to be 16,555 ± 560 grams per mole regardless of whether the J-chain was isolated from the reduced and alkylated or sulphotolylzed preparations (Figure 7). The sedimentation coefficient of the free J-chain was found to be 1.56S when determined at a concentration of 1.0 mg per ml (Figure 8).

In order to localize the position of elution of J-chain, 20 mg of IgM devoid of the free J-chain was extensively reduced with 0.2M 2-ME in the presence of
<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen</th>
<th>Precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Anti-J-chain antiserum</td>
<td>Free J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bound J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>μ-chain</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Native IgM</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>IgM minus free J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reduced and alkylated IgM (minus free J-chain)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>colostral IgG</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.32 S.B.</td>
<td>-</td>
</tr>
<tr>
<td>b) Anti-native IgM</td>
<td>Human J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Free J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bound J-chain</td>
<td>+</td>
</tr>
<tr>
<td>c) Anti-IgM minus free J-chain</td>
<td>Free J-chain</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bound J-chain</td>
<td>+</td>
</tr>
<tr>
<td>d) Anti-bovine colostral IgG</td>
<td>Free J-chain</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bound J-chain</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 5. Ouchterlony's gel diffusion of bound J (left well) and free J (right well) developed against rabbit anti-bovine J-chain antiserum.

Fig. 6. Ouchterlony's gel diffusion of human J-chain (left well) and bovine bound J (right well) against rabbit anti-human J-chain antiserum.
Fig. 7. Molecular weight determination of J-chain by analytical polyacrylamide gel electrophoresis in SDS. Log molecular weight is plotted versus mobility.
Fig. 8. Schleiren pattern of free J-chain obtained 66 minutes after reaching a speed of 56,000 rev/min.
4M guanidine-hydrochloride followed by alkylation. This preparation was then extensively dialyzed against 1M acetic acid and subjected to gel filtration through Bio-Gel P-200 equilibrated in the same buffer. The pooled fractions, obtained from the column were dialyzed against distilled water, then against Tris-glycine buffer containing 5 M urea and subjected to analytical PAGE. The majority of the J-chain eluted with the light chain fraction (Fig. 9). The number of sulfhydryl groups present in the free- and bound-form of J-chain was deduced from radioalkylation studies using $^3$H-iodoacetic acid. J-chain was found to contain 9.4 sulfhydryl residues per mole for the bound-form and 10 sulfhydryl residues for the free-form.

The densitometric tracings of a PAGE gel obtained from a sample of extensively reduced (2-ME) and alkylated IgM (minus the free-form) revealed that the J-chain constituted 1.6 percent of the total IgM molecule (Figure 10).

Discussion

We have presented evidence to suggest that J-chain exists in two forms. One form is free in the colostrum and a second form covalently bound to IgM apparently holding the subunits (IgMs) together. The designation of the anodally fast moving component as
Fig. 9. Elution profile of extensively reduced and alkylated IgM (devoid of free J-chain) obtained by gel-filtration through Bio-Gel P-200 (2.0 cm x 35.0 cm) equilibrated with 1 M acetic acid. The bar indicated the region of localization of J-chain in the elution profile as judged by analytical polyacrylamide gel electrophoresis in urea.
Fig. 10. Densitometric scanning pattern of reduced and alkylated colostral IgM (devoid of free J-chain) on polyacrylamide disc electrophoresis stained with Coomassie blue.
J-chain was based on the following criteria:

1) Non-precipitability of anti-fast component antisera with \( \mu \)-chain, colostral IgG and BSA.

2) The non-precipitability of the free-form with anti-colostral IgG antisera.

3) The precipitability of the free- and the bound-form by anti-fast component and anti-IgM antisera.


5) The partial identity observed between human J-chain and bovine-bound J-chain when developed against anti-human J-chain antiserum.

The fact that anti-bovine J-chain did not precipitate BSA indicates that J-chain is distinct from fast moving protein reported recently to be present in rabbit and human serum. This protein has been reported to show a line of partial identity upon gel diffusion analysis with homologous serum albumins (35).

The molecular weight of 16,555 for both the free and the bound forms of J-chain is slightly higher than that reported for the human counterpart (12,13,14). The fact that the bound-form of J-chain elutes on the dissociating column with the light chains and also in the area preceding the light chain (MW = 22,500) peak suggests that J-chain is aggregated under these conditions.
Since the free form of J-chain showed a precipitin line of complete identity with the bound form, the sedimentation coefficient of the bound form was also assumed to be 1.56S. The sedimentation coefficient of 1.56S is slightly higher than the value reported for human J-chain (10,12). The higher molecular weight and sedimentation coefficient for bovine J-chain is not surprising in view of the fact that bovine IgM has a higher molecular weight compared to human IgM (24).

The stoichiometry of J-chain in the IgM molecule was evaluated from the densitometric tracing and was based on the assumption of uniform staining affinity of proteins by Coomassie Blue. J-chain was found to contribute 1.6% of the total weight of reduced and alkylated IgM devoid of free J-chain. Based on a molecular weight of 1,030,000 grams per mole for intact bovine IgM (24), J-chain would contribute 16,480 grams per mole to the total weight of the protein. Therefore the experimentally determined value of 16,555 for bovine J-chain suggests that there is one bound J-chain per bovine IgM. Human IgA and IgM have also been reported to contain one J-chain per molecule (3,36).

The number of sulfhydryl groups calculated from the radio alkylation data were found to be 9.4 per mole of bound and 10 per mole of the free form of J-chain. This is also in agreement with the human
counterpart (2,3). Although we do not have any direct evidence to support the linkage model recently proposed by Wilde and Koshland (12), the fact that the IgM devoid of the free form of J-chain is precipitable by antibovine J-chain antisera and vice-versa does favor the bracelet model for the attachment of J-chain to IgM.
CHAPTER IV

THE ISOLATION AND CHARACTERIZATION OF NASAL SECRETORY IgA (S-IgA)

Results

The ammonium sulfate precipitate of whole nasal secretions was subjected to gel filtration on a column of Bio-Gel P-300 (2.5 cm x 90 cm) equilibrated with 0.32M NaCl-0.01M Tris-HCl, pH 8.0 (0.32ST), when two peaks were realized (Fig. 11). These peaks were collected, concentrated and subjected to analytical ultracentrifugation.

While Peak I was found to contain a major faster moving (15.3S) component, Peak II was free of any faster moving material and had a sedimentation coefficient of 12.2S (Fig. 12). Peak I was further purified on a column of Bio-Gel A 0.5-M (2 cm x 33 cm) (fig. 13) and upon ultracentrifugation was found to be free of the 12.2S contaminant (Fig. 14).

In order to examine the structure of the 15.3S polymer, the immunoglobulin was subjected to analytical ultracentrifugation at 56,000 rpm in 4M Gd-HCl, pH 8.0, where it was shown to be composed of two major peaks and a trailing edge. The sedimentation coefficient of the faster peak (4.21S) was the same as
Fig. 11. Gel filtration profile of \((NH_4)_2SO_4\) precipitate of nasal secretions on Bio-Gel P-300 (2.5 cm x 95 cm) equilibrated with 0.32ST.
Fig. 12. Schleiren patterns of Peak II nasal (top) and Peak I nasal (Fig. 11) obtained 18 min. after reaching a speed of 56,000 rev/min.
Fig. 13. Elution profile of Peak I (NH₄)₂SO₄ precipitates of nasal secretions of Bio-Gel P-300 passed on Bio-Gel A 0.5-M (2.0 cm x 33 cm) equilibrated in 0.32 M.
Fig. 14. Schleiren pattern of purified Peak I nasal (Fig. 13) obtained 14 min. after reaching speed of 56,000 rev/min.

Fig. 15. Schleiren patterns of 12.2S S-IgA (top) and 15.3S S-IgA (bottom) in 4 M guanidine hydrochloride obtained 51 min. after reaching a speed of 56,000 rev/min.
that for the 12.2S material (4.21S). The slow moving peak had a $S_{20,w}$ of 3.8S when run in 4M Gd-HCl, pH 8.0 (Fig. 15). Then the 12.2S and 15.3S peaks were further examined by gel-diffusion analysis using either anti-nasal or anti-12.2S antiserum, a reaction of complete identity was observed (Fig. 16). The 12.2S peak showed a reaction of partial identity with bovine colostral IgG and IgM (Fig. 17) using rabbit anti-whole colostrum, anti-nasal or anti-12.2S antisera. In addition, a reaction of partial identity was observed with human IgA on gel diffusion analysis using specific rabbit anti-human IgA antiserum (Fig. 18), thus satisfying Vaerman's first order criterion (37). Further purity of the 12.2S and 15.3S polymers was ascertained by immunoelectrophoretic analysis when a single arc was observed (Fig. 19).

The partial specific volume ($\bar{v}$) and the molecular weight of the 12.2S S-IgA were calculated to be 0.721 g per cc and 430,380 g per mole respectively from sedimentation equilibrium data obtained in buffer (0.32ST) and D$_2$O-buffer (Fig. 20). The 15.3S S-IgA had a molecular weight of 641,900 g per mole assuming a partial specific volume of 0.721 g per cc.

Alkaline-urea disc electrophoresis of the reduced and alkylated 12.2S IgA showed the presence of a fast moving band comparable in mobility to that of
Fig. 16. Ouchterlony's gel diffusion of 15.3S S-IgA (left well) showing complete identity with 12.2S S-IgA (right well) when developed against anti-whole nasal secretion antiserum (centre well).

Fig. 17. Ouchterlony's gel diffusion of 12.2S S-IgA (2,5) showing partial identity with colostral IgM (1) and colostral IgG (3,4) when developed with rabbit anti-whole colostrum, anti-nasal or anti-12.2S S-IgA antisera (6).
Fig. 18. Quchterlony's gel diffusion of human IgA (left well) and bovine nasal 12.2S S-IgA (right well) against specific rabbit anti-human IgA antiserum (centre well).

Fig. 19. Immunoelectrophoretic pattern of 15.3S S-IgA (top) and 12.2S S-IgA (bottom). The trough contained rabbit anti-bovine nasal secretions.
Fig. 20. Sedimentation equilibrium data for 12.2S S-IgA in buffer (0.32ST) and D_{2}O-buffer. Ln y displacement in microns is plotted versus the radius squared in centimeters.
J-chain isolated from bovine IgM (Fig. 21).

When the 12.2S and 15.3S IgA which had been reduced and alkylated were subjected to PAGE in SDS, four distinct bands were observed (Fig. 22), the molecular weights of which were calculated to be 78,000, 62,750, 24,000 and 17,040 probably corresponding to secretory component, α-chain, light chain and J-chain respectively (Fig. 23).

In order to isolate J-chain, a sample of reduced and alkylated S-IgA (12.2S) was subjected to preparative PAGE. After concentration the J-chain was run on analytical PAGE when only a single band was observed (Fig. 21).

When a precipitation test was performed antobody J-chain antiserum was found to precipitate the J-chain obtained from S-IgA. To determine the antigenic relationship between J\( _\mu \) and J\( _\alpha \), an Ouchterlony's gel diffusion test was set as described under materials and methods. J\( _\mu \) (bound form) showed a line of complete identity with J\( _\alpha \) showing their antigenic similarity (Fig. 24).

To localize the position of elution of J-chain a sample of IgA (15.3S) was reduced with 0.2M 2-ME followed by alkylation. The preparation was then dialyzed against 0.5 M propionic acid and filtered
Fig. 21. Analytical polyacrylamide gel electrophoresis of (from left to right) reduced and alkylated 15.3S S-\(\text{IgA}\), reduced and alkylated 12.2S S-\(\text{IgA}\), reduced and alkylated colostral IgM and purified J-chain from 12.2S S-\(\text{IgA}\).

Fig. 22. SDS-polyacrylamide gel of reduced and alkylated 15.3S S-\(\text{IgA}\). The 12.2S S-\(\text{IgA}\) banded identical to this.
Fig. 23. Determination of molecular weight of S-IgA components (both polymers) by SDS polyacrylamide gel electrophoresis.
Fig. 24. Ouchterlony's gel diffusion of J-chain (bound) from colostral IgM (left well) and J-chain from 12.2S S-IgA developed against rabbit anti-bovine J-chain antiserum (centre well).
through Sephadex G-100 equilibrated in the same buffer. The column fractions obtained were then pooled as shown in Fig. 25, dialyzed extensively against distilled water, then against Tris-glycine buffer and subjected to analytical PAGE. The majority of the J-chain eluted with the light chain fraction.

The densitometric tracings of analytical PAGE of extensively reduced (12-ME) and alkylated S-IgA (12.2S) preparation revealed that J-chain constituted 4.6 percent of the total IgA molecule (Fig. 26).

Discussion

Nasal secretory IgA was found to exist in two forms (15.3S and 12.2S). The fact that the 15.3S IgA dissociated in Gd-HCl into 4.21S and 3.8S components suggests that the components of the molecule were held by non-covalent bonding. The S values observed in Gd-HCl suggests that the 15.3S S-IgA is composed of a 12.2S component and a 7S component (38). The molecular weight analysis of the two polymeric forms of IgA further supports this theory.

When S-IgA which had been reduced and alkylated was subjected to SDS-PAGE, four bands were observed. The first band had a molecular weight of 78,000 and most probably corresponded to secretory component which has been reported by Vaerman et al. (39). The second
Fig. 25. Elution profile of reduced and alkylated 15.3S S-IgA obtained by gel filtration through Sephadex G-100 (2.0 cm x 33.0 cm) equilibrated with 0.5M propionic acid. The bar indicates the region of localization of J-chain in the elution profile as judged by analytical polyacrylamide gel electrophoresis in urea.
Fig. 26. Densitometric scanning pattern of reduced and alkylated nasal 12.2S S-IgA on polyacrylamide disc electrophoresis stained with Coomassie blue.
band (M. W. = 62,750) corresponds to α-chain (40) while the third and fourth bands are L-chains and J-chains respectively.

J-chain was released from the S-IgA following reduction and alkylation and had a molecular weight of 17,040 (average of two determinations on SDS-PAGE). This value is comparable with that reported for the J-chain associated with bovine colostral IgM. The fact that the J-chain elutes with the light chains and also in the area preceding the light chain peak on the dissociating column suggests that J-chain is aggregated under these conditions.

By the criterion of antigenicity the J-chains were found to be indistinguishable from each other whether they were derived from colostral IgM or nasal S-IgA. This data supports the hypothesis of Morrison & Koshland (9), suggesting that the same J-chain joins the monomeric units of IgA and IgM to form their respective polymeric molecules.

The stoichiometry of J-chain from nasal S-IgA (12.2S) was evaluated from the densitometric tracing of a PAGE gel tube. The area under the J-chain peak in the tracing of reduced and alkylated IgA constituted 4.6% of the total molecule. Using a molecular weight of 430,380 g per mole for IgA and 17,040 g per mole
for J-chain, the theoretical value of 4% is quite in agreement with the experimentally observed value. This datum suggests that there is one J-chain per IgA molecule and is in agreement with that reported in the literature (3, 9).
CHAPTER V
SUMMARY

1. J-chain was found to exist in two forms. One form is free in the colostrum and the other covalently bound to IgM.

2. Both the bound and free forms of J-chain had molecular weight of 16,555 ± 560.

3. The $S_{20,w}$ of J-chain was determined to be 1.56S.

4. The stoichiometry of J-chain in the IgM molecule suggested that there was one J-chain per molecule of IgM.

5. The bound-form of J-chain was found to be antigenically identical to the free-form of J-chain.


7. There were 9.4 sulfhydryl residues per mole of bound J-chain and 10 sulfhydryl residues per mole of free J-chain.

8. S-IgA was found to exist in two polymeric forms (15.3S and 12.2S) in bovine nasal secretions.

9. The 15.3 S S-IgA dissociated into 12.2S and 7S components and a trailing edge upon treatment with
4M guanidine-hydrochloride.

10. The partial specific volume and molecular weight of the 12.2S S-IgA were calculated to be 0.721 g per cc and 430,380 g per mole respectively.

11. The molecular weight of the 15.3S polymer was determined to be 641,900 g per mole assuming a \( V \) of 0.721.

12. The 12.2S S-IgA and the 15.3S S-IgA were antigenically identical.

13. The 12.2S S-IgA shared antigenic determinants with human colostral IgA.

14. J-chain was released from both species of S-IgA following treatment with 2-mercaptoethanol.

15. The molecular weight of J, was 17,040 on SDS-PAGE.

16. Antigenic analysis showed J, and J, (bound form) to be identical.

17. Stoichiometric studies of reduced and alkylated 12.2S S-IgA revealed the presence of one J-chain per IgA molecule.
Reagents and Solutions for Analytical PAGE in Urea

<table>
<thead>
<tr>
<th>No. of Volumes</th>
<th>Substance</th>
<th>Components per 100 ml.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyanogum 41</td>
<td>16.8 g</td>
<td>to volume</td>
</tr>
<tr>
<td></td>
<td>5M urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* 1</td>
<td>Tris</td>
<td>18.15 g</td>
<td>24.0 ml.</td>
</tr>
<tr>
<td></td>
<td>IN HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.24 ml.</td>
<td>to volume</td>
</tr>
<tr>
<td></td>
<td>5M urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ammonium persulfate</td>
<td>0.14 g</td>
<td>to volume</td>
</tr>
<tr>
<td></td>
<td>5M urea</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TENED: N,N,N,N-tetramethylethlenediamine.

* pH 9.4, measured at 25°C without urea.
APPENDIX B

Procedure and Solution Composition for SDS-PAGE

Gel Buffer

\[
\begin{align*}
\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} & \quad 7.8 \text{ g} \\
\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O} & \quad 38.6 \text{ g} \\
\text{SDS} & \quad 2.0 \text{ g} \\
\text{Dist. Water} & \quad \text{to 1,000 cc}
\end{align*}
\]

10% Acrylamide Solution

\[
\begin{align*}
\text{Acrylamide} & \quad 22.2 \text{ g} \\
\text{Methylene bis-acrylamide} & \quad 0.6 \text{ g} \\
\text{Dist. Water} & \quad \text{to 100 cc}
\end{align*}
\]

Preparation of Gel

1. 15 ml. gel buffer – deaerate.
2. Add 13.5 ml. acrylamide solution to gel buffer and deaerate.
3. Add 1.5 ml. ammonium persulfate (15 mg/ml) and 0.045 ml. N,N,N,N tetramethylethylenediamine.
4. Fill gel tubes.

Electrolyte Buffer

Gel Buffer diluted 1:1 with Dist. water.

Current

8 milliamps per tube.

Sample is prepared in 0.01M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% 2-mercap- toethanol.
APPENDIX C

Reagents and Solution Composition for Preparative Polyacrylamide Gel Electrophoresis

<table>
<thead>
<tr>
<th>No. of volumes</th>
<th>Components per 100 ml</th>
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</thead>
<tbody>
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<td>10N HCl</td>
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</tr>
<tr>
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<td>Tris</td>
<td>36.3 g</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.46 ml.</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>36.0 g</td>
</tr>
<tr>
<td></td>
<td>Dist. H₂O</td>
<td>to 100 ml.</td>
</tr>
<tr>
<td>2</td>
<td>Cyanogum 41</td>
<td>30.8 g</td>
</tr>
<tr>
<td></td>
<td>Potassium ferricyanide</td>
<td>0.015 g</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>36.0 g</td>
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<tr>
<td></td>
<td>Dist. H₂O</td>
<td>to 100 ml.</td>
</tr>
<tr>
<td>4</td>
<td>Ammonium persulfate</td>
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</tr>
<tr>
<td></td>
<td>Urea</td>
<td>36.0 g</td>
</tr>
<tr>
<td></td>
<td>Dist. H₂O</td>
<td>to 100 ml.</td>
</tr>
<tr>
<td>1</td>
<td>Urea</td>
<td>36.0 g</td>
</tr>
<tr>
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<td>Dist. H₂O</td>
<td>to 100 ml.</td>
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Elution Buffer

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<td>Glacial Acetic Acid</td>
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<td>Dist. H₂O</td>
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Electrolyte Buffer

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<tr>
<td>Tris</td>
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</tr>
<tr>
<td>Glycine</td>
<td>72 g</td>
</tr>
<tr>
<td>Dist. H₂O</td>
<td>to 2.5 liters</td>
</tr>
</tbody>
</table>

Current

30 mA for the first hour and then increase the current to 80 mA.
REFERENCES


VITA AUCTORIS

1950: Born in Windsor, Ontario, April 14.

1963: Graduated - Grade 8 - St. Gabriels Grade School.


1972: Accepted into the Faculty of Graduate Studies at the University of Windsor, Windsor, Ontario.