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A SPECTROPHOTOMETRIC EVALUATION OF ONE NON-IRON AND THREE IRON TYPE REACTIONS USED FOR THE ENDPOINT DETERMINATION OF SERUM CHOLESTEROL

BY

JOSEPH DONALD ARTISS

A Thesis
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

1977
ABSTRACT.

A spectrophotometric evaluation of one non-iron and three iron procedures for the determination of serum cholesterol was carried out.

The non-iron procedure, formerly commercially available in kit form, used a modified Liebermann-Burchard endpoint reaction, which utilized the oxidizing agent chloramine-T on a serum blank to correct for bilirubin interference. Quantitative and qualitative spectral studies have shown this to be a doubtful correction technique.

Spectral studies of three iron procedures for the determination of serum cholesterol were undertaken, on a comparative basis, to study differences in the reactions. It was found that the three procedures are basically the same and that spectral differences encountered are at least partly due to solvent and anion effects.
DEDICATION

To

MOM and DAD
ACKNOWLEDGEMENTS

I gratefully acknowledge my co-advisors, Dr. R. J. Thibert, and Dr. B. Zak who have so generously given of their time and assistance.

I would also like to acknowledge the financial support given me by the University of Windsor and the National Research Council of Canada.
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LIST OF ABBREVIATIONS

GAA = Glacial Acetic Acid
DES = Diethylstilbestrol
LB = Liebermann-Burchard
CT = Chloramine-T
CHAPTER I

INTRODUCTION

The development of the basic strong acid colour reactions for cholesterol determinations (1 - 3) immediately lead to a proliferation of modifications and sundry combinations of reagents and reaction conditions most often by direct determinations. A number of these variations are in use today, even though it has been reported that most of them will consistently yield high results while others carried out under less than ideal conditions may yield anything but correct values (4). It is not always an inherent methodological error which effects these anomalies but rather our own lack of understanding as to what is measured and as to how the procedure is carried out.

Before any pertinent conclusions can be made concerning the validity of a procedure a thorough study of the factors affecting the spectrophotometric variability must be made. Single wavelength measurements are helpful and indeed necessary for quantitative determinations, however, a qualitative study of the total spectrum of the chromogen is extremely helpful in the study of the chemistry of the colour reaction.

Many manual determinations for total serum cholesterol are
both lengthy and difficult as tedious preliminaries such as saponification, extraction, and digitonide or tomatide precipitation are often required before the colour reaction is performed. Conversely some procedures are quite simple, involving only the addition of serum to a single reagent or reagent mixture. In the routine laboratory, the lengthy procedures often prove too involved, and subject to serious errors due to the tedium which they cause, while the simplest procedures may produce results which have been subjected to interference by either naturally occurring serum constituents (5-7), in vitro additions to the sample (5, 8), browning reactions (7, 9), or the in vivo addition of drugs and/or their metabolites (10-12).

Since the introduction of enzymes as chemical reagents (13-17) their clinical use has grown immensely. Although the strong acid reagents will probably be replaced by enzymatic type reagents, because of the numerous advantages of the latter, the former will no doubt remain in use for some time purely on the basis of cost. For this reason the following spectrophotometric studies, involving a modified Liebermann-Burchard (LB) and three iron type reactions were carried out.
CHAPTER II

A SPECTROPHOTOMETRIC STUDY OF A REAGENT REPORTED TO CORRECT FOR BILIRUBIN INTERFERENCE IN A MODIFIED LIEBERMANN-BURCHARD CHOLESTEROL REACTION

INTRODUCTION

A simple direct procedure has been marketed in the form of a kit, by J. T. Baker Chemical Company (18). This kit is based on the Pearson, Stern and McGavack (19) modification of the Liebermann-Burchard reaction (1, 2). Our reason for studying this kit was, however, not due to its cholesterol methodology but rather for the oxidative reagent substitute in a system utilized to blank out interferences caused by abnormally high bilirubin levels.

The common Liebermann-Burchard reaction utilizes the addition of sulphuric acid to cholesterol in the presence of acetic anhydride to form a green colour. When performed directly on serum without prior extraction it yields comparatively high results due to the development of nonsteroidal chromogens (18, 19). The Chole-Tech® Kit (20) is a modification of the direct method of Pearson, et al. (21) which incorporates p-toluenesulphonic acid as a means of improving the photometry relative to the Liebermann-Burchard reaction. J. T. Baker
claims that the Chole-Tech® kit corrects for the chromagenic influence of elevated bilirubin. If this claim were true the method would probably be not only rapid and simple but also free of one of the major interferences in cholesterol determinations.

EXPERIMENTAL

Materials

Cholesterol standard solution (5000 mg/l, 12.93 mM): Pierce Chemical Company, Box 117, Rockford, Illinois 61105, Solution 121-A, aqueous cholesterol standard (experimental).

Cholesterol standard solution (5.14 mM): Prepared from reagent grade cholesterol, J. T. Baker available through Canadian Laboratory Supplies, Toronto, Ont., recrystallized four times from absolute ethanol, in absolute ethanol.

Working cholesterol reagent: This reagent was prepared fresh according to the instructions contained on the package insert. The working reagent was prepared in the ratio of 4.6 ml of Reagent A; 70.1% w/w acetic anhydride, 28.0% w/w glacial acetic acid, 1.3% w/w p-toluenesulphonic acid monohydrate and 0.6% w/w sodium bisulfite, to 0.9 ml of Reagent B; sulphuric acid (95-98%). The appropriate amount of Reagent A was swirled in an ice bath while Reagent B was slowly added and mixed. This solution was allowed to reach room temperature
prior to use.

Icterus working reagent: "To 5.0 ml of Reagent E; 26.0 gm/l p-toluenesulphonic acid in glacial acetic acid, 0.5 ml of Reagent F; 5.6 gm/l chloramine-T trihydrate in water, was added and mixed.

Bilirubin stock solution (1.03 mM): Prepare by dissolving 3.0 mg of bilirubin, Sigma Chemical Company, St. Louis, MO., in 0.1 ml of 0.1M Na₂CO₃ and 0.225 ml of 0.1M NaOH and then diluting to 5.0 ml with 70.0 gm/l bovine serum albumin.

Alcoholic potassium hydroxide solution: Prepare by adding 6 ml of 33% (W/V) aqueous KOH to 94 ml of absolute ethanol immediately prior to use.

Petroleum ether: Reagent grade B.P. 60–90°C.

All other reagents or chemicals were analytical grade.

Apparatus

Micropipettors: Oxford, available through Canadian Laboratory Supplies Ltd., Toronto, Ont.

Spectrophotometer: Beckman Acta MVI, Beckman Instruments, Inc., Analytical Instruments Sales & Service Division, Toronto, Ont.
Methods

The procedure as described in the package insert (13) for serum was followed implicitly. That is 0.2 ml of serum or standard was added with mixing to 5.5 ml of working reagent. After a five or ten minute incubation at room temperature the colour was read, at 625 nm, or scanned in a double-beam recording spectrophotometer.

The icterus working reagent was also used in the prescribed manner, that is, 0.2 ml of serum or standard was added to 5.5 ml of icterus working reagent.

Pure extracts of serum were obtained by saponification and subsequent extraction with petroleum ether, as described (22). A 2.0-ml aliquot of the extract, representing 0.2 ml of serum, was evaporated to dryness and the residue dissolved in working reagent.

RESULTS AND DISCUSSION

The spectrum generated by both pure standard and normal serum by the Liebermann-Burchard reagent exhibits a maximum near 610 nm and a minimum between 500 and 550 nm (Fig. 1) which is in agreement with early studies (21, 23). At 625 nm the absorptivity of pure cholesterol is about 2315 L mole⁻¹ cm⁻¹
FIGURE 1

SPECTRUM OF CHOLESTEROL WITH THE LIEBERMANN-BURCHARD REAGENT
AND BILIRUBIN WITH THE LIEBERMANN-BURCHARD AND
CHLORAMINE-T REAGENTS

Legend

The direct reaction with cholesterol (6.46 mM) in the presence of the Liebermann-Burchard reagent is illustrated in the upper curve marked LB. Bilirubin (0.39 mM) reacted directly with the Liebermann-Burchard reagent, middle curve marked LB, and the chloramine-T reagent, lower curve marked CT.
FIGURE 1

6.46 mM Chol.
0.39 mM Bili.

ABSORBANCE

NANOMETERS

LB
LB
CT
for this reaction, while the absorptivity for bilirubin, on a molar basis, with this same reaction is 7.5 to 9 times greater, at 625 nm. Bilirubin in the presence of the LB reagent generates a spectrum with a maximum at 590 nm and a minimum at 410 nm. The chloramine-T (CT) reagent system exhibits no reaction with cholesterol at 625 nm but has an absorptivity of about 700 L mole\(^{-1}\) cm\(^{-1}\) for bilirubin at this wavelength. A typical spectrum for bilirubin with chloramine-T has a maximum at 375 nm, 600 nm and 700 nm with a minimum at 465 nm (Fig. 1). A standard curve for cholesterol in the presence of the LB reagent is illustrated in Figure 2.

Both the original authors (21) and an evaluator (23) recommend the absorption minimum of 550 to 560 nm for routine determinations as they feel the colour at the 625 nm maximum is not stable for more than a few minutes. However, Baker states that the sensitivity of the reaction is increased by reading at 625 nm, but that the samples must be read within ten minutes of complete colour development. For this reason a time development experiment was performed and as can be seen in Figure 3 after 5 or 6 minutes of development the colour is stable for at least 20 minutes.
FIGURE 2

A TYPICAL STANDARD CURVE FOR CHOLESTEROL WITH
THE LIEBERMANN-BURCHARD REAGENT

Legend

The direct reaction of pure cholesterol standards with
the Liebermann-Burchard reagent is illustrated.
FIGURE 3

TIME DEVELOPMENT CURVE OF CHOLESTEROL WITH
THE LIEBERMANN-BURCHARD REAGENT

Legend

A time development curve of 7.1 mM cholesterol with
the Liebermann-Burchard reagent. The reaction was placed
in the spectrophotometer after 30 seconds of pre-incubation;
therefore, the first thirty seconds of the curve were
interpolated.
FIGURE 3

TIME (min.)

ABSORBANCE
If the chloramine-T reaction were a perfect blank for bilirubin interfering with the LB reaction it would generate the same spectrum with bilirubin as does the LB reaction. Obviously from Figure 1 the spectra are not identical. Since the spectra were not identical, standard curves with pure bilirubin standards for both reactions were generated in an attempt to find a relationship between the two reactions. As can be seen in Figure 4A, after 5 minutes of incubation in accordance with the manufacturers directions, both reactions deviate from linearity at concentrations greater than 0.34 mM, and in fact plateau between 0.34 and 0.77 mM. This experiment was repeated allowing a 10 minute colour development and as can be seen in Figure 4B both reactions are linear to a concentration of at least 1 mM bilirubin. It is obvious from Figure 4B that the chloramine-T reaction yields only 45 to 50 percent of the colour, at 625 nm for bilirubin, that the Liebermann-Burchard reaction does.

In order to obtain a better understanding of the two reactions with a pure bilirubin standard the colour reactions were slowed by reducing the incubation temperature to 7°C while repetitive scans were performed over about one hour. Unfortunately due to the viscosity of the reagents and the heat generated by the reactions it is impossible to get a good
FIGURE 4

STANDARD CURVES FOR BILIRUBIN WITH THE LIEBERMANN-BURCHARD
AND CHLORAMINE-T REAGENTS

Legend
A) Both reactions after a 5 minute colour development
B) Both reactions after a 30 minute colour development
zero time reference. However, in comparing Figure 5 to Figure 6 one might conclude that the products of the two reactions are completely different. The spectrum of the Liebermann-Burchard reaction with bilirubin is relatively complex, while that of the chloramine-T reaction is comparatively uncomplicated. Figure 5 shows that as the specie(s) which have absorption maxima at 455 nm and 520 nm decrease, the specie(s) which absorb at 385 nm and 610 nm are increasing. Figure 6, the chloramine-T reaction, shows peaks at 370 nm, 600 nm and 700 nm, none of which seem to correspond to the Liebermann-Burchard reaction. It is also obvious that the chloramine-T reaction requires much less time and heat to reach completion.

Merely as a matter of interest, the same technique as the above was employed with cholesterol and the Liebermann-Burchard reagent. Figure 7 shows that the absorbance between 395 nm and 750 nm increases with time, the only maxima occurs at 610 nm.

Figure 8 illustrates the type, and to some degree the extent of the error which might be expected in the determination of cholesterol in an icteric serum, with this kit. The assayed total bilirubin for the serum was 0.39 mM. When reacted directly with the Liebermann-Burchard reagent the apparent
FIGURE 5
BILIRUBIN REACTED WITH THE LIEBERMANN-BURCHARD
REAGENT AT 7ºC

Legend
The arrows indicate whether the peaks are increasing
or decreasing with time. The bilirubin concentration is
0.42 mM and the temperature of the cell chamber was 7ºC.
Prior to addition of the sample the reagents were kept in
an ice bath.
FIGURE 6

BILIRUBIN REACTED WITH THE CHLORAMINE-T REAGENT AT 7°C

Legend
The arrows indicate whether the peaks are increasing or decreasing with time. The bilirubin concentration is 0.42 mM and the temperature of the cell chamber was 7°C. Prior to addition of the sample the reagents were kept in an ice bath.
FIGURE 7

CHOLESTEROL REACTED WITH THE LIEBERMANN-BURCHARD REAGENT AT 70°C

Legend

The cholesterol concentration is 6.46 mM and the cell chamber temperature was 70°C. Prior to sample addition the reagents were kept in an ice bath.
FIGURE 7

6.46 mM Chol.
7°C

ABSORBANCE

NANOMETERS
FIGURE 8

THE LIEBERMANN-BURCHARD AND CHLORAMINE-T REACTIONS
WITH AN ICTERIC SERUM

Legend

The spectrum of the direct reaction on an icteric serum, 0.39 mM bilirubin, with the Liebermann-Burchard reaction is designated as LB. The apparent cholesterol concentration, as measured at 625 nm, is 5.8 mM. The spectrum of the extract, designated Ex, suggests the actual cholesterol concentration is 2.6 mM. The contribution of bilirubin to the Liebermann-Burchard reaction is shown as LB-Ex and the chloramine-T reaction for bilirubin is designated as CT.
FIGURE 8

Icteric Serum
0.39 mM Bili.

ABSORBANCE

LB
LB-Ex
Ex
CT

400 500 600 700
NANOMETERS
total cholesterol value was 5.8 mM, which is slightly more than twice the 2.6 mM value of the extract. If the extract is scanned against the direct reaction, the bilirubin spectrum resulting from the direct reaction is obtained. Of the apparent total 5.8 mM cholesterol, 3.23 mM of that value is contributed by bilirubin. Applying the CT correction which is equivalent to 2.35 mM cholesterol a value of 3.5 mM is obtained which is 137% of the extract value.

Sommers et al. (24) have proposed the use of double-wavelength measurements to correct for icteric interference. With reference to Figure 8 if this correction is applied the apparent cholesterol value obtained, 3.7 mM, is 142 percent of the extract value. Careful examination also shows that in this particular serum 29% of the absorbance at 730 nm is contributed by cholesterol.

SUMMARY AND CONCLUSIONS

A spectrophotometric study of some aspects of a modified Liebermann-Burchard reaction for serum cholesterol as well as a chloramine-T serum blank reagent system to blank out bilirubin interference has been presented.

It has been shown, contrary to previous reports, that the colour developed at 625 nm is stable long enough to be easily
read. Therefore, because of the enhanced sensitivity, 625 nm is the preferred wavelength of measurement.

Spectral studies of the Liebermann-Burchard and chloramine-T reactions for bilirubin have suggested that the reaction products are not the same and therefore the chloramine-T reaction is not an ideal blank. Single wavelength measurements at 625 nm have shown that the LB reaction for bilirubin is more sensitive (absorptivity is 2 - 3 times greater) at 625 nm, than is the CT. It would also appear that there is no simple relationship between the colour intensities of the two reactions at the wavelength of measurement.

Corrections by double-wavelength spectrophotometry have also proven unsatisfactory in this case. The colour produced by bilirubin at 730 nm was shown not to be equal to that produced by this species at 625 nm and that a substantial portion of the colour at 730 nm was due to the cholesterol reaction.
CHAPTER III
A SPECTROPHOTOMETRIC EVALUATION OF THREE IRON TYPE REACTIONS FOR THE DETERMINATION OF SERUM CHOLESTEROL

INTRODUCTION

Parekh and Jung (25) first reported, in late 1970, an iron type reagent system for the determination of total serum cholesterol which they proposed to be superior to all previously reported cholesterol methodologies including those iron procedures they are modifications of. This reagent system is composed of a ferric acetate-uranyl acetate mixture in glacial acetic acid, and a ferrous sulfate glacial acetic acid mixture in concentrated sulfuric acid. It was proposed that the incorporation of uranyl acetate promotes complete protein precipitation and that the ferrous sulfate enhances and stabilizes colour production. Although they claim that the procedure is a direct method, they state that their ferric acetate-uranyl acetate reagent not only serves as a precipitant but also to extract the cholesterol, an apparent anomaly.

The rationale proposed by the authors for the use of ferric acetate, rather than the previously reported ferric chloride (3), is that the presence of chloride ion imparts a
yellow colour to the blank due to the partial evolution of HCl gas (25) which supposedly also causes variability in reaction temperature and therefore colour production.

A second publication by these authors (26) is basically an evaluation of their own method (25). However, one very interesting point is made by the authors, i.e. use of concentrated sulfuric acid rather than the sulfuric acid-ferrous sulfate reagent yields falsely elevated values. Yet in the first paper they claim that ferrous sulfate is only added to enhance and stabilize the colour development. It is difficult to understand just how the colour can be enhanced while the apparent concentration of cholesterol is depressed, and it is not explained by the authors.

A comparison of reaction specificity, with respect to numerous steroids, between the proposed method and an iron chloride reaction (27) was then published in 1975 (28). It was concluded by the authors that the ferric acetate reagent was more specific toward cholesterol than the iron chloride reaction, and that this added specificity could not be explained by the reaction mechanism as proposed by Burke et al. (29). The authors also modified an earlier claim that the assay is not affected by icteric sera, up to 15 mg bilirubin/dl, to a
blanket statement that there is no interference due to bilirubin.

As a "Selected Method" in Clinical Chemistry, Jung et al. (30) the sample-to-reagent ratio was modified from 1:200 to 1:100. The earlier publications (25, 26, 28) used the 1:200 dilution as the 1:100 ratio supposedly yielded too much colour to be measured at higher cholesterol concentrations. This in itself is acceptable, however, they also claim linearity to 16 gm/L (41.3 mM) whereas before (26), with the greater dilution, linearity was reportedly only to 10 gm/L (25.8 mM). The authors also studied bromide interference and have stated that the apparent cholesterol concentration is increased by 0.43 mg/L (1.1 x 10^{-2} mM) for each milligram of sodium bromide per liter in the range of 200-2000 mg/L (1.9 - 19.4 mM) sodium bromide.

The most recent paper (31) involving the iron acetate reagent system involves a comparison of this reaction with two iron chloride procedures (27, 32) and the iron perchlorate reaction of Wybenga et al. (33) with respect to the effects of sodium azide. As with previous papers (25, 26, 28) the authors increase the serum to reagent ratio to 1:200 for the most part. The obvious explanation for this is to dilute the interference(s) so that there is actually less of the interfering substance(s) in their system than in those which they
are comparing it to.

Apart from the above-mentioned studies numerous other reports of bromide (34-38), 2-thiouracil (37-40), nitrate (41,42), and other sterols (43) have been published elucidating the effects of these interferences on iron type reactions for serum cholesterol determinations. Therefore, it was the purpose of this study to evaluate and compare three common iron reactions (30, 33, 44) with respect to the effects of these interferences and to determine if the procedure as proposed by Parekh and Jung (26, 30) is indeed unique as is claimed.

EXPERIMENTAL

Materials

Cholesterol standard solution (10.34 mM): Prepared from reagent grade cholesterol, recrystallized four times from absolute ethanol, and dissolved in glacial acetic acid (GAA).

Cholesterol standard solution (5.14 mM): Prepared from reagent grade cholesterol, recrystallized four times from absolute ethanol, and dissolved in absolute ethanol.

Ferric perchlorate colour reagent: Obtained from Dow Diagnostics, Dow Chemical U.S.A., Indianapolis, IN.

Ferric acetate-uranyl acetate colour reagent: prepared according to Jung et al. (30). One gram of ferric chloride
hexahydrate was dissolved in 80 ml of water and split into two 50-
ml conical centrifuge tubes with teflon-lined screw caps. Am-
monium hydroxide (3 ml) was added to each tube, mixed well and
centrifuged for 5-10 min at about 3000 rpm. The supernatant was
washed with distilled deionized water, and centrifuged again.
This procedure was repeated until a negative silver nitrate test
for chloride was obtained. All of the supernatant except for
about 1 ml was aspirated. This differs from the original proce-
dure—and about 40 ml of GAA was added. Mixing was continued
until all of the precipitate was dissolved and the solution was a
clear dark red-brown colour. This solution was transferred to
a two-liter volumetric flask, 200 mg of uranyl acetate added,
Pfaltz and Bauer, Inc., Flushing, N.Y., and dissolved with about
1.2 l of glacial acetic acid. This often took in excess of six
hours. The reagent was diluted to volume with GAA and stored
in a brown glass bottle.

Ferric acetate-sodium acetate colour reagent: This re-
gent was prepared in the same manner as the ferric acetate-
uranyl acetate colour reagent except that the reagent was
prepared to contain 0.237 mM sodium acetate in place of the
uranyl acetate.

Sulfuric acid-ferrous sulfate reagent: In a two-liter
volumetric flask 200 mg of anhydrous ferrous sulfate was
dissolved in a mixture of 200 ml of GAA and 200 ml of concentrated sulfuric acid. About 1.4 l of concentrated sulfuric acid was added and mixed until the solution had cooled to room temperature. The reagent was diluted to volume with concentrated sulfuric acid and stored in a brown bottle.

Ferric ammonium chloride colour reagent: Prepared exactly according to Zak (44). Ferric ammonium chloride (FeCl$_3$·2NH$_4$Cl·H$_2$O) (2.12g), Matheson Coleman and Bell Division, The Matheson Company Inc., Norwood, Ohio, was dissolved in 100 ml of 80% (v/v) acetic acid. This reagent was initially turbid but cleared on standing overnight.

Thiouracil stock solution: Prepare this solution to contain 3.1 mM/L in distilled deionized water. Thiouracil was purchased from J. T. Baker Chemical Co., Philipsburg, N.J.

Sodium azide stock solution: Prepare this solution to contain 500 mM/L in distilled deionized water. Sodium azide was purchased from Fisher Scientific Co., Ltd., Toronto, Ont.

Sodium nitrate stock solution: Prepare to contain 50 mM/L nitrate in distilled deionized water. Sodium nitrate was purchased from Fisher Scientific Co., Ltd., Toronto, Ont.

Sodium bromide stock solution: Prepare this to contain 50 mM/L bromide in glacial acetic acid. This solution is not stable and should be prepared immediately prior to use. Sodium
bromide was purchased from Fisher Scientific Co., Ltd.,
Toronto, Ont.

Steroid stock standard solutions: Prepare these solutions
to contain 10.34 mM/L $\Delta^5$-pregnenolone, corticosterone, dihydro-
cholesterol, estriol, and pregnanetriol in GAA. $\Delta^5$-Pregnenolone,
corticosterone and estriol were purchased from Sigma Chemical
Co., St. Louis, MO. Dihydrocholesterol was purchased from Cal-
biochem, Los Angeles, CA. and the pregnanetriol was obtained from
Mann Research Laboratories Inc., New York, N.Y.

Diethylstilbestrol stock solution: Prepare to contain 10.34
mM/L diethylstilbestrol (DES) in a 1:1 (v/v) mixture of GAA and
absolute ethanol. Diethylstilbestrol was purchased from Sigma
Chemical Co., St. Louis, MO.

Alcoholic potassium hydroxide solution: Prepare by adding
6 ml of 33% (w/v) aqueous KOH to 94 ml of absolute ethanol im-
mEDIATELY prior to use.

Petroleum ether: Reagent grade B.P. 60-90°C.

All other reagents or chemicals were analytical grade.

Apparatus

Micropipetters: Oxford, available through Canadian Labora-
tory Supplies Ltd., Toronto, Ont. Gilson, available through
Mandel Scientific Co., Ltd., Montreal, Que.
Dispensors: Repipets manufactured by Labindustries, available through Canadian Laboratory Supplies Ltd., Toronto, Ont.

Heating Block: Dow heating block, Dow Chemical Co., Indianapolis, Indiana, calibrated to 100°C.

Spectrophotometer: Beckman Acta MVI, Beckman Instruments, Inc., Analytical Instruments Sales and Service Division, Toronto, Ont.

Methods

Ferric acetate: Where raw serum was used or where calibration standards for these sera were run the procedure according to Jung et al. (30) was followed. Fifty microliters of serum were added and mixed well with 5.0 ml of ferric acetate-uranyl acetate colour reagent in teflon lined screw cap culture tubes. Each sample was allowed to stand for about five minutes before centrifugation at about 3000 rpm for 5-10 min. Three milliliters of supernatant were transferred to a second teflon lined screw cap culture tube. Sulfuric acid-ferrous sulfate reagent was added to each tube which was then mixed immediately. Once the tubes cooled to room temperature, 10-15 min, they were either read at 560 nm, or scanned, against a reagent blank.

In those instances where the same sample concentration was
required for all three iron colour reactions fifty microliters of standard were added to 3 ml of iron reagent. Sulfuric acid-ferrous sulfate reagent (2 ml) was added and the remainder of the procedure, as above, was carried out.

Ferric perchlorate: In all instances the originally described procedure for serum (33) was followed implicitly. Fifty microliters of serum or standard were added to 5.0 ml of the reagent solution mixed well and incubated for 90 sec in a heating block at $100^\circ$ C. This mixture was contained in tightly closed screw-capped 1.25 x 10 cm tubes, as supplied by Dow. After incubation the mixture was cooled in a water bath, less than $17^\circ$ C, for 5 min. The samples were then either read at 560 nm or scanned against a reagent blank.

Ferric ammonium chloride: In all instances the originally described procedure for serum extracts (44) was followed. Fifty microliters of standard were added to 3 ml of GAA mixed well and remixed upon the addition of 0.1 ml of ferric ammonium chloride reagent. Concentrated sulfuric acid (2 ml) was added and mixed well. This solution was contained in teflon lined screw cap culture tubes. Once the samples cooled to room temperature, 5-10 min, they were either read at 560 nm or scanned against a reagent blank.

Pure extracts of serum were obtained by saponification and
subsequent extraction with petroleum ether, as described (22). A 1.0-ml aliquot of the extract, representing 0.05 ml of serum was evaporated to dryness and the residue dissolved either with colour reagent or GAA.

In order to study the effect of 2-thiouracil 0.025 ml of the appropriate standard was added in addition to 0.05 ml of cholesterol standard. The various colour reactions were carried out as described above.

The effects of sodium-azide were studied upon addition of 0.025 ml of the appropriate standard solution in addition to 0.05 ml of cholesterol standard. The various colour reactions were carried out as described above.

Nitrate was studied by adding 0.005 ml of the appropriate standard in addition to 0.05 ml of cholesterol standard. The various colour reactions were carried out as described above.

Bromide enhancement was studied by the addition of 0.025 ml of the appropriate standard solution with and without 0.05 ml of cholesterol standard. The various colour reactions were carried out as described above.

The necessity of ferrous sulfate in the sulfuric acid reagent was studied by dispensing about 3.05 ml of a cholesterol colour reagent mixture (1.9 ml of a 7.8 mM cholesterol standard
to 135 ml of reagent to yield a final reaction mixture concentration equivalent to 6.56 mM/L cholesterol) into 2 ml of either sulfuric acid-ferrous sulfate reagent or concentrated \( \text{H}_2\text{SO}_4 \). Following the appropriate colour development period the samples were read at 560 nm against a reagent blank.

RESULTS AND DISCUSSION

Perhaps the best place to start the evaluation of the three iron reactions (30, 33, 44) is with a qualitative examination of the spectra of the three reactions. With reference to Figure 9, one can readily see that all three spectra have maxima at about 560 nm and that the general shape of the curves are the same. One might also notice that the absorbance for the iron acetate reaction is somewhat lower relative to the iron perchlorate and iron chloride reactions, when all three reactions have the same concentration of cholesterol in the final reaction mixture.

Figure 10 illustrates a series of standard curves for the iron reactions. Jung et al. (30) claim that their reagent is stable indefinitely. The ferric chloride reagent used to generate the line identified as "Old FeCl\(_3\)" was 15 years old at the time this study was made. Fifteen years may not be indefinitely, but it is obviously a long time. Repeated analyses indicate that the absorptivity of the ferric perchlorate and
FIGURE 9

SPECTRA OF THREE COMMON IRON REACTIONS FOR THE DETERMINATION OF CHOLESTEROL

Legend

Shown are the visible spectra of the ferric perchlorate (A), ferric acetate - uranyl acetate (B) and ferric chloride (C) reactions for cholesterol. Pure cholesterol standards were employed at three concentrations. Each spectrum is displaced one inch on the chart paper.
FIGURE 10

STANDARD CURVES OF THREE COMMON IRON REACTIONS FOR THE DETERMINATION OF CHOLESTEROL

Legend

Typical absorbances, as measured at 560 nm, for the iron reactions at various concentrations of cholesterol are illustrated.
FIGURE 10

ABSorbance

Old FeCl₃
New FeCl₃
Fe(ClO₄)₃
FeOAc
FeOAc 1:100
FeOAc 1:200

CHOLESTEROL (mM)
ferric chloride reactions is roughly 11,000 L mole^{-1} cm^{-1} while for the iron acetate-uranyl acetate reaction it is about 9,500 L mole^{-1} cm^{-1}. The lower two lines in Figure 10 are indicative of the absorbances obtained with the iron acetate-uranyl acetate reaction when the recommended serum to reagent dilutions of 1:100 and 1:200 are employed.

The necessity of ferrous sulfate in the sulfuric acid reagent is studied in Table I and Figure 11. Table I lists the absorbances obtained when repeated analyses with an iron acetate-uranyl acetate colour reagent—cholesterol standard mixture were made with either sulfuric acid-ferrous sulfate reagent or concentrated sulfuric acid. Calculations performed on this data show that there is no significant difference in either the means or standard deviations of the two sets of analyses. Therefore, the addition of ferrous sulfate apparently does not enhance the colour of pure standards at 560 nm. See page 47.

Figure 11 suggests that ferrous sulfate does not impart any apparent stability to the ferric acetate-uranyl acetate reaction, as both reactions are still parallel after three hours. It also illustrates that at three hours there is only a slight decrease in the colour produced by the iron chloride reaction.

Although Parekh and Jung (25, 26) claim to have developed a new direct method for the determination of total serum
Table I
Absorbances Obtained in the Ferric Acetate-Uranyl Acetate Procedure With and Without the Sulfuric Acid-Ferrous Sulfate Reagent

<table>
<thead>
<tr>
<th>H₂SO₄ - FeSO₄ Reagent</th>
<th>Concentrated H₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.550</td>
<td>0.565</td>
</tr>
<tr>
<td>0.542</td>
<td>0.558</td>
</tr>
<tr>
<td>0.573</td>
<td>0.563</td>
</tr>
<tr>
<td>0.555</td>
<td>0.553</td>
</tr>
<tr>
<td>0.553</td>
<td>0.561</td>
</tr>
<tr>
<td>0.578</td>
<td>0.566</td>
</tr>
<tr>
<td>0.568</td>
<td>0.571</td>
</tr>
<tr>
<td>0.572</td>
<td>0.564</td>
</tr>
<tr>
<td>0.563</td>
<td>0.558</td>
</tr>
<tr>
<td>0.578</td>
<td>0.567</td>
</tr>
<tr>
<td>0.575</td>
<td>0.568</td>
</tr>
<tr>
<td>0.568</td>
<td>0.560</td>
</tr>
<tr>
<td>0.563</td>
<td>0.545</td>
</tr>
<tr>
<td>0.566</td>
<td>0.556</td>
</tr>
<tr>
<td>0.566</td>
<td>0.566</td>
</tr>
<tr>
<td>0.566</td>
<td>0.557</td>
</tr>
<tr>
<td>0.561</td>
<td>0.553</td>
</tr>
<tr>
<td>0.565</td>
<td>0.562</td>
</tr>
<tr>
<td>0.572</td>
<td>0.560</td>
</tr>
</tbody>
</table>
FIGURE 11

A COLOUR STABILITY STUDY

Legend

The upper portion illustrates the colour development, beginning at 30s. The lower portion illustrates a study initiated at 20 min. following normal colour development procedures.

Ferric chloride reaction
Ferric acetate with FeSO₄
Ferric acetate without FeSO₄
"F" Test

\[
\frac{S_1^2}{S_2^2}
\]

Where \( S_1 > S_2 \)

"t" Test

\[
\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1 + n_2 - 2} \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}
\]
cholesterol, it bears a great deal of resemblance to previously reported extraction techniques (33, 45-47). As with the earlier reports, Parekh and Jung (25, 26), Parekh and Creno (48), Jung et al. (30) and Parekh et al. (28, 31) employ the iron color reagent to precipitate the serum proteins and extract the cholesterol from its binding sites. It is apparent that this is certainly not a direct procedure nor is it therefore a new idea or concept.

Parekh and Jung (25, 26) incorporate uranyl acetate to afford complete protein precipitation. According to these authors the results obtained, without the uranyl salt in the reagent, do not compare well with the Abell (22) extraction technique. In order to examine this claim, ferric acetate reagents were prepared incorporating: (a) uranyl acetate, (b) sodium acetate, and (c) no metal salt other than the iron. Table II presents the data obtained from an icteric sera, with a bilirubin concentration of 0.44 mM/L and a triglyceride of 1.7 mM/L. From this data calculations show that the standard deviations of the ferric acetate-uranyl acetate reagent and the ferric acetate-sodium acetate reagent compare well with the results obtained when the ferric acetate-uranyl acetate reaction was performed on the Abell type extract. However, when no metal salt, other than the iron acetate, is employed the standard deviations do not compare
Table II

Replicate Analyses of an Icteric Serum With Ferric Acetate Reagents

<table>
<thead>
<tr>
<th>Ferric Acetate - Uranyl Acetate</th>
<th>Ferric Acetate - Sodium Acetate</th>
<th>Ferric Acetate</th>
<th>Abell Type Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.95</td>
<td>3.15</td>
<td>3.44</td>
<td>2.76</td>
</tr>
<tr>
<td>3.02</td>
<td>3.39</td>
<td>3.36</td>
<td>2.82</td>
</tr>
<tr>
<td>3.05</td>
<td>3.39</td>
<td>3.39</td>
<td>2.84</td>
</tr>
<tr>
<td>3.00</td>
<td>3.36</td>
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<td>3.82</td>
</tr>
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<td>2.97</td>
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<td>3.00</td>
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<td>2.92</td>
<td>3.44</td>
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<td>3.00</td>
<td>3.72</td>
<td>3.28</td>
<td>2.82</td>
</tr>
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<td>2.97</td>
<td>3.36</td>
<td>3.36</td>
<td>2.79</td>
</tr>
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<td>2.97</td>
<td>3.33</td>
<td>3.33</td>
<td>2.71</td>
</tr>
<tr>
<td>3.00</td>
<td>3.36</td>
<td>3.31</td>
<td>2.82</td>
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<td>2.79</td>
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<td>3.31</td>
<td>2.74</td>
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<td>2.61</td>
<td>3.23</td>
<td>3.36</td>
<td>2.71</td>
</tr>
<tr>
<td>2.97</td>
<td>3.43</td>
<td>3.26</td>
<td>2.89</td>
</tr>
<tr>
<td>2.97</td>
<td>3.28</td>
<td>3.23</td>
<td>2.71</td>
</tr>
<tr>
<td>2.97</td>
<td>3.36</td>
<td>3.23</td>
<td>2.74</td>
</tr>
<tr>
<td>2.87</td>
<td>3.36</td>
<td>3.20</td>
<td>2.79</td>
</tr>
<tr>
<td>2.92</td>
<td>3.26</td>
<td>3.31</td>
<td>2.71</td>
</tr>
<tr>
<td>2.89</td>
<td>2.89</td>
<td>2.61</td>
<td>2.79</td>
</tr>
</tbody>
</table>

Mean 2.92 3.31 3.31 2.76
with the petroleum ether extract. In all cases the means do not compare with the ether extract value. This data suggests that the incorporation of uranyl acetate improves the accuracy of the procedure, relative to a reagent without a second metal ion present, if one accepts the ether extraction value as the "true" value. However, the ferric acetate - uranyl acetate reagent system does not yield values comparable with the Abell type extraction procedure when the sample is very icteric.

Figures 12, 13 and 14 illustrate the visible spectrum of the three reactions with a known interferent (37-40), 2-thiouracil. Note that apart from the depression of colour intensity there is little, if any, spectral distortion in either the ferric acetate - uranyl acetate or ferric chloride reactions. However, a prominent shoulder appears at about 635 nm in the ferric perchlorate spectra. Although it is not evident from these spectra it was found that 2-thiouracil does not give a blank reaction, which is in agreement with previous studies (37, 38). The ferric perchlorate reaction is affected most, at 560 nm, by 2-thiouracil, the ferric chloride reaction the least and the ferric acetate - uranyl acetate reaction only slightly more than the iron chloride (Fig. 15). The data as presented in Figure 15 is an average of 5 determinations at 5 different thiouracil concentrations.
FIGURE 12

SPECTRA OF THE IRON PERCHLORATE REACTION SHOWING
THE EFFECTS OF THIOURACIL

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and 2-thiouracil contaminated standards (T) when reacted with the iron perchlorate reagent.
FIGURE 12

$\text{Fe(ClO}_4\text{)}_3$
2.6, 5.2, 7.8 mM Chol.
1.56 mM Thiouracil

ABSORBANCE

NANOMETERS
FIGURE 13

SPECTRA OF THE FERRIC ACETATE – URANYL ACETATE REACTION
SHOWING THE EFFECTS OF THIOURACIL

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and 2-thiouracil contaminated standards (T) when reacted with ferric acetate – uranyl acetate reagent.
FeOAc
2.6, 5.2, 7.8 mM Chol.
1.56 mM Thiouracil

ABSORBANCE

C
T
C'
T

NANOMETERS

400 550 675
FIGURE 14

SPECTRA OF THE IRON CHLORIDE REACTION SHOWING THE EFFECTS OF THIOURACIL

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and 2-thiouracil contaminated standards (T) when reacted with ferric chloride reagent.
FeCl₃
2.6, 5.2, 7.8 mM Chol.
1.56 mM Thiouracil

FIGURE 14

Absorbance vs. Nanometers

400 550 675
FIGURE 15

COLOUR SUPPRESSION BY THIOURACIL

Legend

Shown are the effects of 2-thiouracil, on the three iron reactions.
FIGURE 15

% OF STANDARD

5.2 mM Chol.

THIOURACIL (mM)
Kitamura and Arimatsu (42) reported that nitrate contamination of commercially available sulfuric acid, in Japan, lead to depression of colour formation in an iron chloride (32) procedure for serum cholesterol determinations. Rice (41) has confirmed this report, but pointed out that the levels of nitrate necessary for noticeable colour suppression far exceed American Chemical Society standards. Figures 16, 17 and 18 show the spectra characteristics for the ferric perchlorate, ferric acetate - uranyl acetate and ferric chloride reactions, respectively, for cholesterol standards and cholesterol standards contaminated with sodium nitrate. Qualitatively there are no apparent spectral changes apart from an increase in absorbance around 380 nm in the ferric acetate - uranyl acetate reaction. There is a suppression of colour development in the ferric acetate - uranyl acetate and ferric chloride reactions (Fig. 19). It is apparent from Figure 19 that the ferric chloride reaction is severely suppressed by the nitrate contaminant while there is little or no interference in the ferric perchlorate reaction and only slight inhibition of the ferric acetate. It should be remembered, however, that these levels are higher than those accepted by the ACS.

Although azide has lost popularity as a serum preservative because of its potential explosive properties it has recently,
FIGURE 16

SPECTRA OF THE IRON PERCHLORATE REACTION SHOWING THE EFFECTS OF NITRATE

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and standards contaminated with nitrate (N), when reacted with the ferric perchlorate reagent.
FIGURE 16

Fe(ClO)₃
2.6, 5.2, 7.8 mM Chol.
5 mM NO₃⁻
FIGURE 17

SPECTRA OF THE FERRIC ACETATE - URANYL ACETATE REACTION,
SHOWING THE EFFECTS OF NITRATE.

Legend

Shown are the spectra obtained with pure cholesterol
standards (C) and standards contaminated with nitrate (N), with the
ferric acetate - uranyl acetate reagent.
FIGURE 1B

SPECTRA OF THE IRON CHLORIDE REACTION SHOWING THE EFFECTS OF NITRATE

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and standards contaminated with nitrate (N), with the ferric chloride reagent.
FeCl₃,
2.6, 5.2, 7.8 mM Chol.
5 mM NO₃⁻
FIGURE 19

COLOUR SUPPRESSION BY NITRATE

Legend

Shown are the average results of 4 determinations at 5 concentration levels of sodium nitrate.

\[ \text{ClO}_4^- = \text{ferric perchlorate} \]
\[ \text{OA}_c = \text{ferric acetate} - \text{uranil acetate} \]
\[ \text{Cl}_3 = \text{ferric chloride} \]
FIGURE 19

% OF STANDARD

NITRATE (mM)

5.2 mM Chol.

ClO₃⁻ OAc

Cl₃
been reported (31) to cause a negative interference in the three iron reactions. For this reason a study of the effects of azide on the three reactions was carried out. As may be seen in Figures 20, 21 and 22 the shape of the cholesterol spectra with the azide contamination was not altered, however, the colour development was depressed considerably. Figure 23 is a quantitative study of this interference. The curves presented are the average of six determinations at 5 concentrations of sodium azide. As can be seen the ferric perchlorate reaction suffers the most inhibition followed by the ferric chloride and the ferric acetate - uranyl acetate reactions. The effects of azide appear to plateau at about 100 mM for all three reactions. It is difficult to state whether or not the data is consistent with that of Khayan - Bashi et al. (31), as their axes are mislabelled. Empirically, however, we do seem to agree as to the order of magnitude of the interference.

Enhancement, due to bromide contamination, of the ferric chloride (34-36) and ferric perchlorate (37, 38) reaction has been well documented. As may be seen in Figures 24, 25 and 26 bromide gives a blank reaction, however this reaction alone does not account for the enhanced spectra of a contaminated sample. Figures 27, 28 and 29 illustrate an increase in
FIGURE 20

SPECTRA OF THE IRON PERCHLORATE REACTION
SHOWING THE EFFECTS OF AZIDE

Legend

Shown are the spectra, in ascending order, obtained with pure cholesterol standards and with azide contaminated standards (A, B, C) when reacted with the ferric perchlorate reagent.
Fe(ClO₄)₂
2.6, 5.2, 7.8 mM Chol.
31 mM NaN₃
FIGURE 21

SPECTRA OF THE FERRIC ACETATE - URANYL ACETATE REACTION
SHOWING THE EFFECTS OF AZIDE

Legend

Shown are the spectra, in ascending order, of pure cholesterol standards and azide contaminated standards (A, B, C) when reacted with ferric acetate - uranyl acetate reagent.
FeOAc
2.6, 5.2, 7.8 mM Chol.
31 mM NaN₃
FIGURE 22
SPECTRA OF THE IRON CHLORIDE REACTION
SHOWING THE EFFECTS OF AZIDE

Legend
Shown are the spectra obtained, in ascending order, obtained with pure cholesterol standards and azide contaminated standards (A, B, C) when reacted with the ferric chloride reagent.
FIGURE 23

COLOUR SUPPRESSION BY AZIDE

Legend

Shown are the averaged results of 6 determinations at 6 different concentrations of sodium azide for the three iron reactions.
FIGURE 24

SPECTRA OF THE IRON PERCHLORATE BLANK
REACTION WITH BROMIDE

Legend

Shown are the spectra obtained with pure cholesterol standard (C), bromide standard (Br) and a mixture of these two with the ferric perchlorate reagent (upper curve).
Fe(ClO₄)₃
5.2 mmol l Chol.
15 mmol l Br⁻
FIGURE 25

SPECTRA OF THE FERRIC ACETATE – URANYL ACETATE BLANK REACTION WITH BROMIDE

Legend

Shown are the spectra obtained with pure cholesterol standard (C), bromide standard (Br) and a mixture of these two with the ferric acetate – uranyl acetate reaction (upper curve).
FeOAc
5.2 mmol/l Chol.
15 mmol/l Br⁻
FIGURE 26

SPECTRA OF THE IRON CHLORIDE BLANK
REACTION WITH BROMIDE

Legend

Shown are the spectra obtained with pure cholesterol
standard (C), bromide standard (Br) and a mixture of these
two with the ferric chloride reagent (upper curve).
FeCl₃·NH₄Cl·H₂O
5.2 mmol/l Chol.
15 mmol/l Br⁻
FIGURE 27
SPECTRA OF THE IRON PERCHLORATE REACTION SHOWING
THE EFFECTS OF BROMIDE

Legend
Shown are the spectra obtained with pure cholesterol
standards (C) and bromide contaminated standards (Br)
when reacted with ferric perchlorate reagent.
FIGURE 27

Fe(ClO₄)₃
5.2 mmol/l Chol.
1,5,... 25 mmol/l Br⁻
FIGURE 28

SPECTRA OF THE FERRIC ACETATE - URANYL ACETATE REACTION
SHOWING THE EFFECTS OF BROMIDE

Legend

Shown are the spectra obtained with pure cholesterol standards (C) and bromide contaminated standards (Br) when reacted with ferric acetate - uranyl acetate reagent.
FeOAc
5.2 mmol/1 Chol,
5, 10, ... 25 mmol/1 Br⁻
FIGURE 29
SPECTRA OF THE IRON CHLORIDE REACTION SHOWING
THE EFFECTS OF BROMIDE

Legend
Shown are the spectra obtained with pure cholesterol
standards (C) and bromide contaminated standards (Br) when
reacted with ferric chloride reagent.
Figure 29

FeCl$_2$·NH$_3$Cl·H$_2$O
5.2 mmol/l Chol.
5, 10, 15, 20 mmol/l Br$^-$

Absorbance

Nanometers
enhancement concomitant with an increase in bromide contamination. All three reactions show different characteristic spectral aberrations when bromide is present. Bromide contamination is responsible for the prominent shoulders, to the 560 nm maxima, at 475 nm and 620 nm in the ferric perchlorate reaction. While for the ferric acetate - uranyl acetate reaction a 500 nm shoulder increase until at 25 mM/L bromide it is almost as intense as the 560 nm maxima. Perhaps the most interesting change occurs with the ferric chloride reaction. There is little spectral change, apart from the obvious enhancement, except for the peak at 410 nm. As may be seen in Figure 26 this peak also occurs in the blank reaction and is supposedly due to the formation of some sort of ferric - chloro- bromo complex. This is of interest because even at low levels of contamination this bright yellow colour is noticeable to the unaided eye before the initiation of the colour reaction. Therefore, bromide poisoned samples are easily recognized when the ferric chloride procedure is utilized. In fact, of the several contaminants studied, bromide is the most likely to be encountered as a contaminating interference whereas the others are not encountered or their levels are too low to affect the reaction.
Figure 30 illustrates the enhancement produced by bromide, as sodium bromide, on a 5.2 mM cholesterol standard. The lines as drawn are the averaged result of 8 determinations at 6 levels of bromide. It must be pointed out, however, that the reproducibility of these reactions when contaminated with bromide, is very poor. Note that the ferric perchlorate reaction is enhanced considerably more (20-40%) than either of the other two reactions. It is also apparent that the enhancement plateaus between 20 and 25 mM/L bromide in all three reactions. Due to the poor reproducibility, as mentioned above, there is no easy way to include bromide for the purpose of enhancing the sensitivity of these reactions.

Parekh et al. (28) then presented a comparative study of steroid interferences using the ferric chloride and ferric acetate - uranyl acetate reactions. They, however, were unfair in their comparison as they diluted their samples 1:200, as before (25, 26), but left the ferric chloride samples undiluted. It was decided, therefore, that some of this work should be repeated, in a manner such that the concentration of steroid was the same in the final reaction mixtures for the three iron reactions. Two criteria were used to select the steroids to be examined: (a) those steroids which reportedly
FIGURE 30

COLOUR ENHANCEMENT BY BROMIDE

Legend

Shown are the averaged results, of 8 determinations at 6 levels of bromide, for the three iron reactions.
gave the greatest interference in the work of Parekh et al. (28); and (b) those steroids which were readily available to us. It should be pointed out that the concentration of the steroids used are absurdly high, relative to serum levels, but it was felt that the levels previously used (28), 1.03 mM, were too low to elicit any meaningful data. It is presumed that 1.03 mM is the sort of level one might expect in rat adrenal tissue as that is what the authors (28) were going to study.

Figures 31, 32 and 33 illustrate the spectra produced by 5 steroids and diethylstilbestrol. By making single wavelength readings (560 nm), Parekh et al. (28) concluded that the ferric acetate - uranyl acetate reaction (25) was more specific than the ferric chloride reaction (32) and that the reaction mechanism as proposed by Burke et al. (29) again could not explain this increased specificity. However, a qualitative study of the spectra presented in Figures 31, 32 and 33 suggest that the three iron reactions produce similar, although not identical, spectra for the compounds studied. It is a commonly accepted phenomenon that modification of the basic structure of a chromogen produces spectral changes.
FIGURE 31

SPECTRA OF THE IRON REACTIONS WITH
DIHYDROCHOLESTEROL AND CORTICOSTERONE

Legend

Shown are the spectra of the three iron reactions with
5.2 mM cholesterol (C) and 10.3 mM dihydrocholesterol (S) on
the left, and 10.3 mM corticosterone (S) on the right.
FIGURE 32

SPECTRA OF THE IRON REACTIONS WITH PREGNANETRIOL AND DIETHYLSTILBESTEROL

Legend

Shown are the spectra of the three iron reactions with 5.2 mM cholesterol (C) and 10.3 mM pregnanetriol (S) on the left and 10.3 mM diethylstilbesterol (S) on the right.
FIGURE 33

SPECTRA OF THE IRON REACTION WITH 
$\Delta^5$-PREGNENOLONE AND ESTRIOL

Legend

Shown are the spectra of the three iron reactions with 5.2 mM cholesterol (C) and 5.2 mM $\Delta^5$-pregnenolone (S) on the left and 10.34 mM estriol (S) on the right.
It is, therefore, unreasonable to dispute the proposed reaction mechanism (29) on the basis of single wavelength readings.

Standard curves for these compounds, as measured at 560 nm, are presented for the three reactions in Figures 34, 35 and 36. Generally, the response is linear, with the exception of estriol by the ferric perchlorate and ferric chloride reactions, but not all of the curves pass through the origin. These deviations from the ideal may be explained by the location on a given spectrum where the measurement is made. Table III presents the data as the percent of a cholesterol standard (5.2 mM) on an equal molar basis. As may be seen the ferric acetate—uranyl acetate reaction is probably no more specific to cholesterol, and perhaps even a bit less specific, than either the ferric perchlorate or ferric chloride reactions when measured at 560 nm.

SUMMARY AND CONCLUSIONS

A spectrophotometric and chemical evaluation of three common iron reactions for the determination of serum cholesterol has been presented. It has been shown that all three reactions are effected in a similar manner by various interfering substances; such as 2-thiouracil, nitrate, azide,
FIGURE 34

STANDARD CURVES FOR STEROIDAL INTERFERENCES WITH THE
IRON-PERCHLORATE REACTIONS

Legend

Typical absorbances, as measured at 560 nm, with various
steroids and diethylstilbestrol compounds.

Cholesterol
Δ5-Pregnenolone
Diethylstilbestrol
Estriol
Pregnanetriol
Dihydrocholesterol
Corticosterone
FIGURE 35

STANDARD CURVES FOR STEROIDAL INTERFERENCES WITH THE FERRIC ACETATE - URANYL ACETATE REACTION

Legend

Typical absorbances, as measured at 560 nm, with various steroids and diethylstilbestrol.

- Cholesterol
- Δ⁵-Prenenolone
- Diethylstilbestrol
- Estriol
- Pregnanetriol
- Dihydrocholesterol
- Corticosterone
FIGURE 36

STANDARD CURVES FOR STEROIDAL INTERFERENCES WITH THE IRON CHLORIDE REACTION

Legend

Typical absorbances, as measured at 560 nm, with various steroids and diethylstilbestrol.

- Cholesterol
- Δ⁵-Pregnenolone
- Diethylstilbestrol
- Estriol
- Pregnanetriol
- Dihydrocholesterol
- Corticosterone
Table III
Colour Production by Various Compounds with the Iron Reagents

<table>
<thead>
<tr>
<th>Compound (5.2 mM)</th>
<th>Percent of Standard at 560 nm</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iron Perchlorate</td>
<td>Iron Acetate-Uranyl Acetate</td>
<td>Iron Chloride</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(\Delta^5)-Pregnenolone</td>
<td>68</td>
<td>46</td>
<td>37</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Dihydrocholesterol</td>
<td>15</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Estriol</td>
<td>13</td>
<td>49</td>
<td>29</td>
</tr>
<tr>
<td>Pregnanetriol</td>
<td>9</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>26</td>
<td>46</td>
<td>32</td>
</tr>
</tbody>
</table>
bromide, diethylstilbestrol and other steroidal compounds. Spectral differences between the reactions are probably due to solvent and anion effects.

The incorporation of uranyl acetate as a precipitating agent in the ferric acetate - uranyl acetate procedure does not make the results obtained comparable with the Abell type extraction. Incorporation of ferrous sulfate does in no manner effect the intensity or stability of colour development.

As proposed, the ferric acetate - uranyl acetate procedure for the determination of cholesterol is neither new nor direct. The use of ferric acetate as opposed to ferric chloride, ferric ammonium chloride or ferric perchlorate appears to have no real advantages. In fact the reaction is less sensitive while the preparation of the reagent is tedious and time consuming. Although no quantitative studies were performed, the only effects that chloride had on the reactions were in the region of 400 nm sufficiently far enough away from the 560 nm peak not to affect single wavelength determinations. The concept that the acetate reagent contains only acetate and sulfate anions is nullified as soon as one adds a serum aliquot to the colour reagent.
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