PHOTOMETRIC DETERMINATION OF TOTAL CHOLESTEROL IN PLASMA OR SERUM BY A MODIFIED LIEBERMANN-BURCHARD REACTION

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The wide-spread use of photoelectric colorimeters in the field of quantitative clinical chemistry has brought with it the realization that many of the visual colorimetric methods in use are grossly inaccurate and that their use may be justified only on the basis of an arbitrary range of normal values for the given procedure. This is particularly true in the case of total cholesterol values which have a reported normal range from about 100 to 400 mg. per cent. The principal sources of the inaccuracies for the cholesterol methods employing the Liebermann-Burchard reaction are well known and have been recently discussed in some detail in a paper by Sperry and Brand (1). They will be mentioned here only because in the procedure presented by the authors the experimental studies of the Liebermann-Burchard reaction were run under conditions somewhat different from those of most other investigators (2–4).

Instead of the usual organic solvents, e.g. chloroform, alcohol-ether, alcohol-acetone, etc., used for extracting cholesterol and cholesterol esters from biological fluids, the authors have followed the lead of Drekter (5) who used acetic anhydride as the medium for the simultaneous precipitation of the proteins and the extraction of the total cholesterol present in these fluids. Because of the extreme rapidity of development of the maximal color density, pure acetic anhydride was unsuited for the photometric determination of total cholesterol. It was found experimentally that a mixture of acetic anhydride and 1,4-dioxane (3:2 by volume) not only would extract cholesterol and cholesterol esters from biological fluids with the simultaneous precipitation of proteins, but in addition would reduce the rate of development of maximal color density to a point at which the Liebermann-Burchard reaction might be used to obtain accurate photometric readings.

The method presented here is unlike any other found in the literature in that no attempt is made to remove the water from the biological fluid;

A major portion of this problem was completed overseas while the authors were members of the Chemistry Section of the Seventh Medical Laboratory of the Third United States Army.
instead, an amount of water equivalent to that present in the biological fluid is added to the cholesterol standards. In the course of the procedure, the water present in the standard or the unknown is converted into acetic acid when the tube containing the water and acetic anhydride is heated in a boiling water bath for a specified time. It is a known experimental fact that the Liebermann-Burchard reaction takes place in acetic acid solution as well as in chloroform, although the rate of reaction may vary with the different solvents (6).

Many investigators have shown that the rate of reaction for pure cholesterol with the Liebermann-Burchard reaction is much slower than that for a corresponding amount of cholesterol ester (as total cholesterol). It is mainly for this reason that the more reliable methods for total cholesterol require the conversion of the cholesterol ester present into the alcohol form previous to the addition of the colorimetric reagents. However, for the routine clinical analysis of total cholesterol in biological fluids it would be most desirable if this step could be omitted, provided that it was accomplished without any corresponding reduction in the accuracy of the procedure. This problem was solved by developing a new total cholesterol standard composed of equal parts of cholesterol and cholesterol ester which are dissolved in a solution of acetic anhydride-dioxane (3:2). Since this standard solution is 50 per cent cholesterol ester, it more closely approximates the actual conditions found in most sera or plasma. Moreover, in the setting up of the standard curve, these standards are run (with the addition of an equivalent amount of water, heating, filtration, etc.) in exactly the same manner as are the unknowns. Since a valid objection might be raised with respect to the variability of the ratio of cholesterol esters to total cholesterol as found in biological fluids, separate studies were made for both pure cholesterol and cholesterol esters in acetic anhydride-dioxane solution with the Liebermann-Burchard reaction. Although cholesterol ester develops color at a somewhat higher rate than does a corresponding amount of pure cholesterol, the maximal density values reached are about the same for both. Furthermore, these maximal values remain constant over a sufficient time interval so that various mixtures of cholesterol and cholesterol ester can be determined, without conversion of the esters into the alcohol form, from the same curve. This experimental work also indicates the importance of using the maximal density value rather than a given time interval for the photometric reading if consistent results are to be obtained.

In agreement with Sperry and Brand (1) it has been found that relatively slight changes in temperature produce significant changes in the maximal density and in the rate at which those values are reached. In this procedure the temperature is rigidly controlled at 37.5°, which is a
higher temperature than that advocated by most other investigators. The reason for this is that the addition of dioxane to the acetic anhydride reduces the rate at which the green color develops in the Liebermann-Burchard reaction to such a marked degree that a higher temperature is required to obtain the maximal color density within a reasonable time interval.

Many investigators (1, 7) have stressed the advantage of such experimental factors as the mixing of acetic anhydride and sulfuric acid previous to its addition to a chloroform or acetic acid solution of total cholesterol, the control of the temperature at which the acetic anhydride and sulfuric acid are mixed and added to the total cholesterol solution, the need for developing the color in total darkness, etc. In the method as given in this paper, although the concentrated sulfuric acid is added directly from a micro burette to the total cholesterol solution at room temperature and no special precautions are taken to allow the color development to take place in complete darkness, highly reproducible results have been obtained for standards, unknowns, and recoveries of added cholesterol and cholesteryl ester. The fact that no special precautions are required for the addition of the Liebermann-Burchard reagents or for the color development, except for temperature control, further enhances the simplicity of this method compared to other procedures.

EXPERIMENTAL

A. Reagents and Apparatus—

1. Standard cholesterol solution. Dissolve 0.1000 gm. of pure cholesterol in 100 ml. of pure dry chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 1.00 mg. of cholesterol.)

2. Dilute cholesterol standard. Dilute the above standard 1:5 with chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 0.20 mg. of cholesterol.)

3. Standard cholesterol ester solution. Dissolve 0.1105 gm. of pure cholesteryl acetate (Eastman Kodak, No. 2391) in 100 ml. of pure, dry chloroform in a 100 ml. volumetric flask. (1 ml. is equivalent to 1.00 mg. of total cholesterol.)

4. Dilute cholesterol ester standard. Dilute the above standard 1:5 with chloroform in a volumetric flask. (1 ml. is equivalent to 0.20 mg. of total cholesterol.)

5. Standard cholesterol solution (new). Weigh 25 mg. of pure dry c.p. cholesterol and 27.7 mg. of cholesteryl acetate into a 500 ml. Erlenmeyer flask. Add 240 ml. of acetic anhydride-dioxane solution; heat the flask for approximately 15 minutes in a boiling water bath until all the solid is in solution. Cool to room temperature and wipe the outside of the flask to remove any moisture. Transfer the solution quantitatively to a 250 ml.
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volumetric flask and dilute to mark with acetic anhydride-dioxane solution. Mix and transfer to a clean, dry, glass-stoppered, brown bottle. (1 ml. is equivalent to 0.20 mg. of total cholesterol.)

6. Acetic anhydride-dioxane solution. Mix 3 volumes of reagent grade acetic anhydride with 2 volumes of 1,4-dioxane (Eastman Kodak, No. P2144). Transfer to a clean, dry, glass-stoppered, brown bottle. The mixture is stable in the absence of moisture. Dioxane vapor is toxic and all work with this reagent should be done in a hood.

7. Sulfuric acid, concentrated, reagent grade.

8. Micro burette, 2.00 or 5.00 ml., graduated in 0.05 ml. or in smaller divisions.

9. Photoelectric colorimeter, Evelyn type, with selected Pyrex test-tubes graduated at 5 and 10 ml.

All reagents used in this procedure should be of reagent grade and free from water. All glassware used should be chemically clean and dry.

B. Selection of Color Filter—0.20 mg. and 0.50 mg. of total cholesterol standards were prepared, as described under the preparation of the standard curve in (D) below, and the data for spectral transmission for the modified Liebermann-Burchard reaction were obtained with a Coleman Junior spectrophotometer. The readings were taken at room temperature and between 25 and 35 minutes after the addition of the colorimetric reagents. The data obtained are illustrated in Fig. 1 and show a minimum transmittance at about 650 mμ. This wave-length corresponds approximately to a combination of Corning glass Filters 241, 4 mm., and 397, 2 mm., for filter type photometers.

C. Studies of Factors Affecting Color Development with Modified Liebermann-Burchard Reaction

1. Effect of Variation of Amount of Acetic Acid from Water. Preparation of Samples—To two sets of graduated colorimeter tubes, each containing five tubes, were added 1.00 ml. (0.20 mg.) and 3.00 ml. (0.60 mg.) of the dilute cholesterol standard in chloroform, respectively. The tubes were placed in a boiling water bath and the solvent evaporated off until no odor of chloroform was detectable. To each pair of standards, one containing 0.20 mg. and the other 0.60 mg. of cholesterol, were added the various amounts of water shown in Fig. 2. The contents of each tube were diluted to the 5.00 ml. mark with the acetic anhydride-dioxane solution; the tubes were heated in a boiling water bath for 30 minutes and then cooled to room temperature.

Development of Color—At this point each pair of tubes was handled separately. To the first tube was added 0.25 ml. of concentrated sulfuric acid from a micro burette. Immediately after, an interval timer which had
previously been set for 5 minutes was started up. The contents of the tube were well mixed, care being taken to see that unmixed acid remained on the sides of the tube. The tube was then left in a rack at room temperature. About \( \frac{1}{2} \) minute before the 5 minute interval elapsed, 0.25 ml. of concentrated sulfuric acid was added to the second tube. At the expira-

![Fig. 1. Spectrophotometric curves for total cholesterol](http://www.jbc.org/)

![Fig. 2. Effect of variation of acetic acid obtained from water and acetic anhydride on color development](http://www.jbc.org/)

tion of the first 5 minute interval, the timer was reset for the same interval and the first tube was placed in a water bath maintained at 37.5°. The contents of the second tube were then well mixed and the tube allowed to remain at room temperature. At expiration of the second 5 minute interval, the timer was again reset, the second tube was placed in the 37.5°
water bath, and the first tube was removed, wiped dry, and read in the
photoelectric colorimeter with a 650 mμ filter against a reagent blank set
at 100 per cent transmission. In this manner a series of readings was
taken at 5 minute intervals until a maximal density value was reached, when
the readings began to increase again. The second tube was read exactly
5 minutes after the first one. All other pairs of tubes were run exactly
in the same manner.

The variation of density values with time for cholesterol standards con-
taining various amounts of water is shown in Fig. 2. From these data
the following conclusions may be drawn: that the presence of small amounts
of water has the greatest proportional effect on decreasing the rate of color
development as well as the maximal density value for a fixed amount of
cholesterol; that, as the water content increases, the further addition of
water causes progressively smaller changes in the rate of reaction and in
the maximal density value for a fixed amount of cholesterol.

These experimental data prove that, if accurate results are to be ob-
tained, the volume of water added to the standards, to compensate for the
amount present in serum or plasma, should be maintained within fairly
narrow limits. Since it was intended to use a 0.20 ml. sample in running
the unknowns, and since 90 per cent of serum or plasma is actually water,
0.18 ml. of water was added to each tube in performing all the subsequent
experimental runs and in setting up the standard curves.

2. Effect of Varying Ratio of Acetic Anhydride to Dioxane—The samples
were prepared in the manner described above except that 0.50 and 1.00 mg.
of cholesterol standard were used and 0.18 ml. of water was added to each
tube, the final volume being the same. The color was developed as above.

The maximal density obtained in these runs was plotted against the
various acetic anhydride to dioxane ratios for the two cholesterol standards
and the curves obtained are shown in Fig. 3. These results indicate that
the maximal density increases continuously as the acetic anhydride to
dioxane ratio increases for a given cholesterol value. It is possible to obtain
almost any density value desired for a fixed amount of cholesterol by choos-
ing the proper ratio of acetic anhydride to dioxane. A value of 3 volumes
of acetic anhydride to 2 volumes of dioxane was chosen for two reasons.
First, because the maximal density values for standards ranging from 0.20
to 1.00 mg. of cholesterol fall somewhere between 20 and 80 per cent trans-
mision, which is the most desirable range for colorimetry. The second
reason is shown in Fig. 4, which gives the variation of density values with
time for the various acetic anhydride to dioxane ratios. The density values
for the 3:2 ratio reach a maximum in about 15 minutes and there is only a
slight variation in the maximal value between the 15 and 25 minute in-
tervals.
3. Effect of Variation of Temperature and Time of Reading—The standards for this run were prepared exactly as given above except that a constant volume of 0.18 ml. of distilled water was added to each tube.

![Graph showing effect of variation of ratio of acetic anhydride to dioxane on maximal density value.](image1)

**Fig. 3.** Effect of variation of ratio of acetic anhydride to dioxane on maximal density value.

![Graph showing variation of transmission values with time for varying acetic anhydride ratios.](image2)

**Fig. 4.** Variation of transmission values with time for varying acetic anhydride ratios.

The color was developed exactly as described above except that instead of the tubes being placed in a bath at only one temperature (37.5°), the
color development took place in baths maintained at the various tempera-
tures shown in Fig. 5.

Fig. 5 shows the relationship between the density values obtained for a
given cholesterol value and the time of reading at the various temperatures
at which color is developed. From the results shown in Fig. 5, it can be
concluded (1) that as the temperature increases the time at which the
maximal density is reached decreases; (2) that as the temperature increases
the maximal density value obtained with a fixed amount of cholesterol
increases; (3) that for a constant temperature, the variation of the density
value at ±5 minutes from the maximal density value is about ±1 scale
division of the colorimeter.

In agreement with the results of many previous investigators, the data

Fig. 5. Effect of variation of temperature and time of reading on color develop-
ment of cholesterol with the modified Liebermann-Burchard reaction.

obtained show the need for rigid temperature control if accurate cholesterol
values are to be obtained. The temperature of 37.5° was chosen because
it is a conveniently available laboratory temperature, and because the
maximal color at this temperature develops somewhere between 15 and 20
minutes; so that the time required for each determination is considerably
reduced. However, it should be pointed out that any other constant
temperature below 40° may be used provided that both standards and
unknowns are run at the same temperature.

4. Effect of Temperature at Which Colorimetric Reagents Are Mixed—
Sperry and Brand (1), Shapiro et al. (7), and other investigators lay stress
on the technique employed in adding the acetic anhydride-sulfuric acid
mixture to the chloroform solution of cholesterol as a potential source of
error in the Liebermann-Burchard reaction. Shapiro et al. have recommended using a mixture of acetic anhydride and sulfuric acid prepared at room temperature to avoid any errors due to the difficulty of measuring accurately small quantities of sulfuric acid and to reduce the temperature changes due to variable heating effects. Sperry and Brand carry this procedure a step further and recommend that the reagents be mixed in an ice bath and maintained at that temperature until added to the cholesterol solution in chloroform. Since the matter is still controversial, and since it would be desirable to avoid the temperature effect of adding a solution at 0° to one at the bath temperature, this problem was investigated for the acetic anhydride-dioxane-water solution of cholesterol.

A series of standards was prepared containing 0.20 and 0.60 mg. of cholesterol in the manner given above which included the addition of the

<table>
<thead>
<tr>
<th>Temperature of mixing</th>
<th>Total cholesterol</th>
<th>Color density (Evelyn colorimeter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>15 min. 20 min. 25 min. 30 min.</td>
</tr>
<tr>
<td>Ice bath, 0°</td>
<td>0.20</td>
<td>0.080 0.088 0.092* 0.091</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.258 0.284 0.292* 0.288</td>
</tr>
<tr>
<td>Water, 15°</td>
<td>0.20</td>
<td>0.680 0.081 0.089* 0.086</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.268 0.288 0.290* 0.278</td>
</tr>
<tr>
<td>Room temperature, 19.5°</td>
<td>0.20</td>
<td>0.088 0.092* 0.092 0.089</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.276 0.292* 0.292 0.278</td>
</tr>
</tbody>
</table>

* Maximal density value.

0.18 ml. of distilled water and the use of the 3:2 solution of acetic anhydride-dioxane.

Each pair of standards was adjusted to the temperatures given in Table I previous to the addition of the concentrated sulfuric acid from the micro burette. The rest of the procedure is exactly as given above.

The maximal density values obtained in this run, as is shown in Table I, prove that the temperature of the solution at the time of addition of the concentrated sulfuric acid has no effect on the maximal density value obtained and that the acid may be added at room temperature. The maximal temperature developed by the addition of concentrated sulfuric acid to the acetic anhydride-dioxane-water solution of cholesterol was about 32.5° for the color development; the heat generated in this step does not affect the color development.

5. Effect of Varying Time of Heating Acetic Anhydride-Dioxane-Water Solution of Cholesterol—A series of 1.00 mg. of cholesterol standards was
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prepared exactly as given above, including the addition of 0.18 ml. of distilled water and the use of the 3:2 acetic anhydride-dioxane solution, except that the tubes were heated for 15, 30, and 45 minutes in the boiling water bath.

Development of color was carried out by the usual procedure.

The results obtained in this run are given in Table II and show that continuing to heat the tubes in a boiling water bath for more than 15 minutes has a negligible effect on the color development.

D. Preparation of Standard Curve for Cholesterol—The experimental data obtained above indicate that reproducible cholesterol values can be obtained only if rigid control is maintained over such factors as the temperature of color development, ratio of acetic anhydride to dioxane, amount of water present in the sample, and if the maximal density value rather than a specific time interval is used for the colorimetric readings. Less control

Table II
Effect of Variation of Time of Heating

<table>
<thead>
<tr>
<th>Cholesterol standard, 1.00 mg.</th>
<th>Time of heating</th>
<th>Color density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
<td>15 min.</td>
</tr>
<tr>
<td>15 min.</td>
<td>0.409</td>
<td>0.456</td>
</tr>
<tr>
<td>30 min.</td>
<td>0.447</td>
<td>0.472*</td>
</tr>
<tr>
<td>45 min.</td>
<td>0.414</td>
<td>0.456*</td>
</tr>
</tbody>
</table>

* Maximal density value.

is required over such factors as the temperature at which the colorimetric reagents are mixed and the time of heating the tubes in the boiling water bath. These data were then applied to the preparation of a standard curve for pure cholesterol.

Preparation of Samples—Into a series of graduated calorimeter tubes were pipetted, in duplicate, amounts of the cholesterol standards in chloroform equivalent to 0.20, 0.40, 0.60, 0.80, 1.00, and 1.20 mg. of cholesterol. The rest of the procedure was run exactly as given under (C, 2) except that the 3:2 acetic anhydride-dioxane solution was used for all the samples.

Color was developed by the usual procedure.

The curve obtained by plotting the average maximal density values against the cholesterol standards is shown in Fig. 6.

E. Effect of Variation of Temperature and Time of Reading on Values for Cholesterol Ester—Since many investigators have called attention to the more rapid rate of color development with cholesterol esters compared to
pure cholesterol in chloroform, or as found experimentally, in acetic acid solution, it was decided to investigate this matter for acetic anhydride-

dioxane water solutions of cholesterol. It was assumed that there would not be any marked difference in the results obtained with cholesterol ester
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standards for factors such as the ratio of acetic anhydride to dioxane, the amount of water present, the temperature at which the colorimetric reagents are added, or the time of heating the tubes in the boiling water bath; therefore these factors were not reinvestigated for cholesterol ester. However, it was considered desirable to reinvestigate for cholesterol ester such factors as the effect of the variation of temperature at which color development takes place, and the time of reading the samples upon the maximal density values obtained for a fixed amount of the standard (calculated as total cholesterol). From these data it could be ascertained whether, at any particular temperature or reading time, the same density values could be obtained for cholesterol ester as for an equivalent amount of cholesterol, thus avoiding the necessity of converting the ester into the alcohol form to obtain accurate results for the total cholesterol value.

The run was performed exactly in the same manner as is given under (C, 3) for pure cholesterol except that cholesterol ester (cholesteryl acetate) standards in chloroform corresponding to 0.20 and 0.60 mg. of total cholesterol were used. The results obtained in this run are shown in Fig. 7.

It is concluded from these experimental data that the effect of temperature on the maximal density values is exactly the same as with pure cholesterol. A comparison of Figs. 5 and 7 leads to an even more important conclusion; namely, that for the most part the maximal density values for both cholesterol and cholesterol ester fall within the same time interval, and if the temperature remains constant, the maximal density values for a constant amount of cholesterol ester are practically identical with those obtained for an equivalent amount of pure cholesterol.

F. Preparation of Standard Curve for Cholesterol Ester—This was prepared in the same manner as the curve for pure cholesterol, except that cholesterol ester standards containing the equivalent amount of total cholesterol were used. The results obtained for the average maximal density values are plotted against the total cholesterol values, and the curve obtained is illustrated in Fig. 6. The data indicate that, while the two curves are not identical, they are sufficiently close together, particularly in the lower range of values, where the large majority of unknowns will fall, to warrant the use of a common curve for both cholesterol and cholesterol esters, thus avoiding the involved process of converting one form into the other, previous to the color development.

G. Determination of Maximal Density Values for Known Mixtures of Cholesterol and Cholesterol Ester—As further proof that the use of a common curve for both cholesterol and cholesterol ester is warranted in the acetic anhydride-dioxane-water procedure, a series of known mixtures of cholesterol and cholesterol ester was prepared in such ratios as are likely to occur in pathological human sera or plasma. These mixtures were
then run exactly in the same manner as is described for the cholesterol standards in (D). The maximal density values obtained for these various known mixtures are given in Table III together with the average maximal density values taken from the cholesterol and cholesterol ester curves in Fig. 6.

The data in Table III prove that the results obtained for these mixtures are generally within ±0.02 mg. of total cholesterol of the theoretical value taken from the curve and furnish conclusive evidence of the validity of employing a single curve for the determination of the total cholesterol content of an unknown sample regardless of the ratio of cholesterol to cholesterol ester present.

### Table III

**Determination of Total Cholesterol Content of Known Mixtures of Cholesterol and Cholesterol Ester**

<table>
<thead>
<tr>
<th>Cholesterol added mg.</th>
<th>Cholesterol ester added mg.</th>
<th>Total cholesterol added mg.</th>
<th>Ratio, cholesterol ester to total cholesterol</th>
<th>15 min.</th>
<th>20 min.</th>
<th>25 min.</th>
<th>Color density</th>
<th>Average color density from curve</th>
<th>Average error per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>1.00</td>
<td>50</td>
<td>0.456</td>
<td>0.462*</td>
<td>0.450</td>
<td>0.469</td>
<td></td>
<td>-1.50</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>1.00</td>
<td>50</td>
<td>0.459</td>
<td>0.462*</td>
<td>0.453</td>
<td>0.469</td>
<td></td>
<td>-1.50</td>
</tr>
<tr>
<td>0.70</td>
<td>0.30</td>
<td>1.00</td>
<td>30</td>
<td>0.435</td>
<td>0.453*</td>
<td>0.456</td>
<td>0.466</td>
<td></td>
<td>-3.42</td>
</tr>
<tr>
<td>0.70</td>
<td>0.30</td>
<td>1.00</td>
<td>30</td>
<td>0.438</td>
<td>0.456*</td>
<td>0.456</td>
<td>0.466</td>
<td></td>
<td>-2.77</td>
</tr>
<tr>
<td>0.30</td>
<td>0.70</td>
<td>1.00</td>
<td>70</td>
<td>0.401</td>
<td>0.447*</td>
<td>0.447</td>
<td>0.469</td>
<td></td>
<td>-4.70</td>
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<tr>
<td>0.30</td>
<td>0.70</td>
<td>1.00</td>
<td>70</td>
<td>0.423</td>
<td>0.465*</td>
<td>0.459</td>
<td>0.469</td>
<td></td>
<td>-0.85</td>
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<tr>
<td>0.50</td>
<td>0.20</td>
<td>0.70</td>
<td>28.5</td>
<td>0.328</td>
<td>0.335*</td>
<td>0.325</td>
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<td>+1.52</td>
</tr>
<tr>
<td>0.50</td>
<td>0.20</td>
<td>0.70</td>
<td>28.5</td>
<td>0.328</td>
<td>0.330*</td>
<td>0.323</td>
<td>0.330</td>
<td></td>
<td>±0.00</td>
</tr>
<tr>
<td>0.20</td>
<td>0.50</td>
<td>0.70</td>
<td>71.4</td>
<td>0.319</td>
<td>0.321*</td>
<td>0.312</td>
<td>0.330</td>
<td></td>
<td>-2.73</td>
</tr>
<tr>
<td>0.70</td>
<td>0.50</td>
<td>1.20</td>
<td>41.7</td>
<td>0.542</td>
<td>0.557*</td>
<td>0.553</td>
<td>0.569</td>
<td></td>
<td>-2.11</td>
</tr>
<tr>
<td>0.70</td>
<td>0.50</td>
<td>1.20</td>
<td>41.7</td>
<td>0.534</td>
<td>0.549*</td>
<td>0.542</td>
<td>0.569</td>
<td></td>
<td>-3.52</td>
</tr>
<tr>
<td>0.50</td>
<td>0.70</td>
<td>1.20</td>
<td>58.2</td>
<td>0.549</td>
<td>0.553*</td>
<td>0.538</td>
<td>0.569</td>
<td></td>
<td>-2.81</td>
</tr>
</tbody>
</table>

* Maximal density.

**H. Method for Photometric Determination of Total Cholesterol in Serum or Plasma**—Into test-tubes (150 X 15 mm.) containing approximately 5 ml. of acetic anhydride-dioxane solution was pipetted, in duplicate, 0.20 ml. of clear, unhemolyzed serum or plasma. The contents were mixed well by shaking, care being taken that no unmixed serum or plasma remained on the sides of the tube. The tubes were heated in a boiling water bath for 30 minutes with occasional shaking, and the moisture was wiped off the outside. The contents were filtered through Whatman No. 42 filter paper (7 cm.) into graduated colorimeter tubes. The original tubes were washed with 0.25 ml. portions of acetic anhydride-dioxane solution, these washings...
being poured through the filter paper until the total volume of the filtrate in each tube was 5.00 ml.

The development of color was carried out exactly as given under (C, 1).

The average maximal density value obtained by this procedure is used to find the total cholesterol value from the standard curve prepared as given in the following section.

I. Preparation of Standard Curve for Use with Unknown Samples—In order to overcome the effect of the slight variation noted above between the curves for pure cholesterol and cholesterol ester and to prepare a total cholesterol curve which more closely simulated the actual conditions found in the case of human sera or plasma, the new cholesterol standard which has already been described under (A) was used in this run. In addition, while it was not desirable to go through the tedious processes of filtration, washing, making up to volume, etc., in establishing the theoretical concepts of the method, it was desirable that these processes be included in the preparation of a standard curve for actual use with unknown samples, so that any changes in the density values due to these additional steps would be compensated for.

Into a series of the test-tubes (150 × 15 mm.) were pipetted, in duplicate, amounts of the new cholesterol standard equivalent to 0.20, 0.40, 0.60, 0.80, and 1.00 mg. of total cholesterol. The total volume in each tube was then brought up to about 5 ml. with acetic anhydride-dioxane solution. 0.18 ml. of distilled water was added to each tube and the contents well mixed so that no unmixed water remained on the sides of the tube. The remainder of the procedure for the unknown samples was carried out exactly as described under (II).

Color was developed by the usual procedure.

J. Determination of Total Cholesterol by Visual Colorimetry—If a photoelectric colorimeter is not available, the new cholesterol standard can also be used for visual colorimetry and is prepared for the color development exactly as described under (I) above, with 2.00 and 4.00 ml. aliquots of the cholesterol standard corresponding to 0.40 and 0.80 mg. of total cholesterol, respectively. The unknown samples of serum or plasma are prepared simultaneously for the color development exactly as described for the photoelectric method in (II) above. The color development is performed by the usual procedure except that the reading of the unknown against the standard is performed in the visual colorimeter between the 15 to 20 minute time interval since, as is shown in Table IV, the maximal density values usually fall in this interval for serum or plasma.

K. Determination of Normal Serum Values and Recoveries of Added Cholesterol—Some 50 samples of clear, unhemolyzed sera, sent in for serological examination, were analyzed by the procedure as given under (II).
A typical set of results, to illustrate the importance of using the maximal density value rather than a constant time interval, is given in Table IV.

**Table IV**

*Photometric Determination of Total Cholesterol Content of Normal Human Serum and Recoveries of Added Cholesterol, Cholesterol Ester, and Mixtures of Both*

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Color density</th>
<th>Total cholesterol in serum</th>
<th>Total cholesterol added</th>
<th>Total cholesterol recovered</th>
<th>Standard used</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min.</td>
<td>15 min.</td>
<td>20 min.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.141</td>
<td>0.168*</td>
<td>0.166</td>
<td>0.58</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.246</td>
<td>0.301*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.194</td>
<td>0.282*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.367</td>
<td>0.372*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.194*</td>
<td>0.364*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.238</td>
<td>0.347*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
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<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
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</tr>
<tr>
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<td>0.57</td>
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<td>0.19</td>
</tr>
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<td>0.19</td>
</tr>
<tr>
<td>1</td>
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<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
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<td>0.57</td>
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<td>0.19</td>
</tr>
<tr>
<td>1</td>
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<td>0.166</td>
<td>0.57</td>
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</tr>
<tr>
<td>1</td>
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<td>0.166</td>
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<td>0.19</td>
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<td>0.174*</td>
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<td>0.166</td>
<td>0.57</td>
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<tr>
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<td>0.166</td>
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<td>0.19</td>
</tr>
<tr>
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<td>0.174*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
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<td>0.19</td>
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<tr>
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<td>0.347*</td>
<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
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<td>0.166</td>
<td>0.57</td>
<td>0.20</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* Maximal density value.
† The new standard is 50 per cent cholesterol and 50 per cent cholesterol ester.

Recoveries of total cholesterol added to sera, including that added as pure cholesterol, cholesterol ester, and mixtures of both (new standard), are also included in Table IV.

**L. Preparation of Total Cholesterol Standard Curve from Digitonin Choles-**
DETERMINATION OF CHOLESTEROL

teride—Into a series of 15 ml. graduated centrifuge tubes were pipetted, in duplicate, amounts of the pure cholesterol standard in chloroform ranging from 0.20 to 1.00 mg. of total cholesterol. The solvent was evaporated off by placing the tubes in a boiling water bath until no odor of chloroform could be detected. 3 ml. of alcohol-acetone (1:1) solution were added to each tube and the cholesterol dissolved by warming the tubes gently in the water bath. The cholesterol was then converted into digitonin cholesteride by the procedure of Schoenheimer and Sperry (8). To the pure, dry digitonin cholesteride precipitate contained in the centrifuge tubes were added 0.18 ml. of distilled water and 5 ml. of the acetic anhydride-dioxane solution. The tubes were heated for 30 minutes in a boiling water bath and the precipitate dissolved with the aid of occasional stirring with a glass rod placed in each tube. The tubes were removed from the bath and the contents filtered into the graduated colorimeter tubes through Whatman No. 42 filter paper (7 cm.). The centrifuge tubes were washed with 0.25 ml. portions of acetic anhydride-dioxane, these washings being poured through the filter paper until the total volume of the filtrate in each tube was 5.00 ml.

The development of color was carried out exactly as described under (C, 1).

The average maximal density values in this run were plotted against the cholesterol values and the curve obtained is shown in Fig. 6. This curve is practically identical with that of the cholesterol ester standard curve.

M. Comparison of New Procedure with Digitonin Cholesteride Procedure for Normal Sera—The following experiments were intended mainly to furnish additional proof of the reliability of the new procedure and to explore the possibility of using it for the separate determination of cholesterol and cholesterol esters. (1) The total cholesterol content of a number of normal sera was determined exactly as given in (H) above. (2) An alcohol-acetone (1:1) extract of the same sera was prepared as for the Schoenheimer-Sperry (8) method and the total cholesterol content determined as follows:

A 5.00 ml aliquot of the alcohol-acetone filtrate of the sera (1:25 dilution) was pipetted into a test-tube (150 X 15 mm.) and the contents evaporated just to dryness, care being taken not to char the residue in this step. The residue was dissolved in a few ml. of chloroform and the contents again evaporated just to dryness. The chloroform evaporation was repeated once again, but this time the heating was continued until no further odor of chloroform could be detected. 0.18 ml. of distilled water and 5 ml. of acetic anhydride-dioxane solution were added to each tube. The tubes were then heated in a boiling water bath for 30 minutes with occasional shaking, removed from the bath, etc., the remainder of the procedure being
performed exactly as described under (H) above. The results for this run are given in Table V.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Serum or plasma No.</th>
<th>Color density</th>
<th>Total cholesterol (mg. per cent)</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>0.135, 0.168, 0.177*</td>
<td>0.176, 0.169, 0.163</td>
<td>200 New 5 ml. alcohol-acetone (1:1) extract, evaporated with CHCl₃</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>0.155, 0.182, 0.183*</td>
<td>0.181, 0.171, 0.153</td>
<td>205 Digitonin cholesterol, 3 ml.</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>0.066, 0.065, 0.065*</td>
<td>0.086, 0.086, 0.082</td>
<td>210 Same as (2) New</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>0.147, 0.181, 0.184*</td>
<td>0.176, 0.168, 0.168</td>
<td>205 Same as (2) New</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>0.138, 0.176*</td>
<td>0.174, 0.168, 0.155</td>
<td>210 Same as (2) New</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>0.061, 0.080, 0.085*</td>
<td>0.082, 0.080</td>
<td>216 Same as (2) New</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>0.115, 0.155, 0.166*</td>
<td>0.166, 0.160, 0.152</td>
<td>190 Same as (2) New</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>0.141, 0.165</td>
<td>0.168, 0.157, 0.149</td>
<td>190 Same as (2) New</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>0.068, 0.082*</td>
<td>0.078, 0.069</td>
<td>204 Same as (2) New</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>0.174, 0.229, 0.235*</td>
<td>0.227, 0.215, 0.199</td>
<td>265 Freshly drawn normal plasma; new</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>0.149, 0.218, 0.237*</td>
<td>0.233, 0.224, 0.208</td>
<td>270 Same plasma 1 hr. after drawing; new</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>0.204, 0.238, 0.242*</td>
<td>0.229, 0.220</td>
<td>275 Same plasma 9 hrs. after drawing; new</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>0.172, 0.218, 0.227*</td>
<td>0.222, 0.208, 0.191</td>
<td>260 Plasma from blood after standing 1 hr.; new</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>0.174, 0.222, 0.229*</td>
<td>0.222, 0.208, 0.191</td>
<td>260 Plasma from blood 9 hrs. after standing; new</td>
</tr>
</tbody>
</table>

* Each value is the average of two or more determinations.

The total cholesterol content of the alcohol-acetone filtrate of the same sera as above was then determined, in duplicate, by the Schoenheimer-Sperry method (8), except that instead of dissolving the digitonin cholesterol in concentrated acetic acid, the precipitates were treated exactly as were the standards in (L). The results obtained in this run are also given in Table V.
The remaining data in Table V were all obtained with the new procedure and tend to confirm the findings of Kraus and Kalal (9) that the total cholesterol content of human serum or plasma does not change on standing, and that these determinations need not be done immediately after the blood is drawn.

DISCUSSION

The results obtained in the studies of the effect of the variation of the amount of acetic acid derived from water on the color development with the modified Liebermann-Burchard reaction show the decided effect which even traces of moisture have on the maximal density values obtained and on the time in which these values are reached for a fixed amount of cholesterol. Unless all water is completely removed from the unknown in the treatment of the sample previous to the color development, it is to be expected that the standard being made up in the pure solvent will develop more color in the same time interval than will the unknown, and thus low results will be obtained. In the procedure presented in this paper, the small amounts of acetic acid derived from water present both in the standards and in the unknowns actually have a beneficial effect on the method. Its presence prolongs the time in which maximal color is reached, allowing the use of higher bath temperatures; it stabilizes the color formation so that accurate readings of the maximal density value may be obtained over a 10 minute or longer interval; and the presence of traces of moisture, in the reagents and on the glassware, does not have the same detrimental effect on color formation as in the more commonly used procedures.

Several different samples of acetic anhydride and dioxane from different manufacturers were used. While the maximal density values obtained for a constant amount of cholesterol were about the same for acetic anhydride-dioxane solutions obtained from different sources, the time in which this value was reached varied markedly with different products. It was for this reason as well as for the greater reproducibility of values that the maximal density reading, rather than the time after mixing, was selected as the criterion for the total cholesterol values. The importance of this factor has also been stressed in a recent paper by Clarke and Marney (10) which deals with a modification, for use with the photoelectric colorimeter, of the Schoenheimer-Sperry method. Since the acetic anhydride-dioxane solution is stable over long periods of time, it is best to prepare a batch of the reagent large enough to set up all the necessary standard curves and time and temperature studies, and to have enough left over for a large number of unknowns.

In these experiments, identical results were obtained whether standards were run in a dark incubator with a thermostatic control, a water bath in a lighted room, or in an incubator heated by carbon filament bulbs.
The results obtained for normal human sera and plasma (Tables IV and V) by the new procedure indicate that while some of the recoveries of added total cholesterol are somewhat low the maximal error obtained for the recovery of 0.20 mg. of cholesterol is -15 per cent and the average error for all the recovery values is about ±5 per cent. They further indicate that good results are obtained for the recoveries whether the total cholesterol is added in the pure form, as the ester, or as a mixture of both forms. Results given in Table V prove that the same values are obtained by the acetic anhydride-dioxane-water procedure whether the extraction of the serum is performed directly or whether the cholesterol is first extracted with an alcohol-acetone (1:1) solution. These latter results are important when both cholesterol and cholesterol ester determinations are contemplated. The final and most conclusive proof of the validity of the method is that it gives results which check experimentally with those obtained by the digitonin cholesteride method if these latter values are read from a digitonin cholesteride curve.

The 50 normal sera used in these determinations were obtained from the serology department of this laboratory and the total cholesterol content was determined in from 4 to 48 hours after the blood had been centrifuged and the serum removed. This meant that in some instances the serum was removed almost immediately after clotting had occurred and in other instances only after serum had been standing in contact with the blood clot for 10 to 12 hours. Kraus and Kalal (9) have shown that even under these conditions the total cholesterol values do not change, and the values shown in Table V substantiate their findings and justify the inclusion of these results for serum as normal findings. Considering the random method by which these normal sera were selected, the most surprising thing about the values obtained is the relatively small range which they cover, i.e. from about 190 to 270 mg. per cent, and that the large majority of the values falls at about 210 mg. per cent. Although this range may be considered representative for male adults from 20 to 35 years of age, many more determinations will have to be run by the procedure for other segments of the population before it can be stated that this smaller normal range is due to the better reproducibility and greater accuracy of the method compared to other procedures.

There has been some scepticism concerning the reliability of the usual clinical laboratory tests for cholesterol and cholesterol esters and of their value in diagnostic work (11, 12). While it is generally conceded (11) that the Schoenheimer-Sperry (8) method or some of its modifications (13, 10) are the most reliable procedures, their very complexity makes them unsuited for routine clinical work. This investigation was started because the many requests for cholesterol determinations from hospitals in the Third United States Army area, in cases such as infectious jaundice, acute
DETERMINATION OF CHOLESTEROL

epidemic hepatitis, etc., indicated a need for such a procedure. Since the method presented in this paper is simple, highly reproducible, and gives results of the same order of accuracy as does the Schoenheimer-Sperry method, the authors believe that it will satisfy the need for a good clinical method for the photometric determination of total cholesterol.

SUMMARY

A simple, reproducible, and accurate method is presented for the photometric or visual determination of total cholesterol in human sera or plasma. The principle of the method depends upon the experimental fact that when a mixture of serum or plasma and acetic anhydride-dioxane (3:2 by volume) is heated in a boiling water bath for 30 minutes there occur simultaneously an extraction of cholesterol and cholesterol esters, precipitation of proteins, and conversion of water present into acetic acid.

A new total cholesterol standard consisting of a solution of equal parts of cholesterol and cholesterol ester in acetic anhydride-dioxane is used in this procedure. In the preparation of the standard curve an amount of water is added to the standards equivalent to that contained in the serum or plasma.

Extensive experimental studies were made of all the factors affecting the color produced with this modified Liebermann-Burchard reaction, including variations in the amount of water present, temperature, time of reading, ratio of acetic anhydride to dioxane, time of heating, method of adding reagents, and ratio of cholesterol to cholesterol ester. These studies furnished conclusive proof that by the use of this procedure conversion of the cholesterol ester into cholesterol previous to the addition of the Liebermann-Burchard reagents is not required for accurate results.

Some 50 sera obtained from normal male adults were examined by this procedure and gave a normal range from about 190 to 270 mg. per cent of cholesterol, with the large majority of the values falling at about 210 mg. per cent. Recoveries of total cholesterol added as cholesterol, cholesterol ester, or a mixture of both, averaged about ±5 per cent, and the serum values by this procedure checked with those obtained with a digitonin cholesteride method.

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PHOTOMETRIC DETERMINATION OF TOTAL ChOLESTEROL IN PLASMA OR SERUM BY A MODIFIED LIEBERMANN-BURCHARD REACTION
Abraham Saifer and Otto F. Kammerer


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