THE COLORIMETRIC DETERMINATION OF CHOLESTEROL

BY WARREN M. SPERRY AND FLORENCE C. BRAND

(From the Departments of Biochemistry, New York State Psychiatric Institute and Hospital and the College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, July 20, 1943)

It is widely recognized that most of the methods in general use for the determination of cholesterol, based on the Liebermann-Burchard color reaction, are unsatisfactory, and the principal sources of inaccuracy are known; but it does not appear to be realized that large errors may result from relatively small variations in procedure. Recently we were stimulated to investigate the colorimetric estimation of cholesterol by a request that we recommend a procedure for inclusion in the "Technical manual of methods for laboratory technicians of the War Department." Over 550 known and unknown samples were read under various conditions. Some of the findings are presented here primarily to direct attention to the potential inaccuracies in cholesterol methods as they are ordinarily used. A procedure with which good analyses can be obtained will be summarized and the sources of error will then be discussed in relation to the steps of that procedure.

Procedure

Pipette 0.4 cc. of blood serum into approximately 5 cc. of alcohol-acetone (1:1) in a 10 cc. volumetric flask (or 1 cc. of serum in a 25 cc. flask if replicate determinations are desired) with swirling of the solution, bring the solvent to a boil on the steam bath, cool the flask, make up to the mark with alcohol-acetone, mix, filter, and pipette 5 cc. of the filtrate into a 25 cc. Erlenmeyer flask to which 0.15 cc. of potassium hydroxide solution (10 gm. in 20 cc. of water) has previously been added. Swirl the liquid gently at intervals until the alkali has mixed completely with the alcohol-acetone, place the flask in an incubator at 37-40° for 40 minutes, add 1 drop of phenolphthalein solution, titrate with 10 per cent acetic acid in absolute alcohol (about 0.6 cc. should be required¹), add 1 drop excess, and evaporate just to dryness on the steam bath with the aid of a stream of air applied by means of suction through a glass tube, bent to avoid contamination from the rubber connection and clamped about 2 cm. above the surface of the solution. Cool the flask, add 0.1 cc. of 50 per cent alcohol without delay,

¹ The solution of acetic acid slowly loses strength through the formation of ethyl acetate.
DETERMINATION OF CHOLESTEROL

and wash down the walls of the flask with about 3 cc. of petroleum ether. Swirl gently at intervals until the salt dissolves and a clear separation of the two liquids occurs. If the salt does not dissolve completely within 10 minutes, add 0.05 cc. more 50 per cent alcohol. Decant the petroleum ether through a small funnel into a dry \( \frac{1}{2} \) or 1 ounce bottle, equipped with a well fitting glass stopper, with care that none of the aqueous layer is carried over. Repeat the washing with small portions of petroleum ether five times more and evaporate the combined extracts to complete dryness by placing the bottle in a small beaker of cold water which is heated on the steam bath while a stream of air is applied by means of a suction line as before. If there is any doubt that the residue is dry, add a few drops of absolute alcohol, rotate the bottle so the walls are wet, and repeat the drying process.

Development of Color and Reading—Adjust to 24° a water bath placed in a dark cabinet (a packing box, equipped with a door, will serve) and maintain the temperature at this point throughout the development of color by adding hot or cold water as needed. Pipette 5 cc. of acid-free chloroform into each bottle containing dried serum extract and 5 cc. portions of standard solutions containing 0.24, 0.4, and 0.6 mg. of pure cholesterol in chloroform into three similar bottles. Stopper and place the bottles in the water bath, using a support, such as a wire basket, so that they will not tip over. Measure 20 cc. of pure acetic anhydride into a suitable glass-stoppered container and chill in an ice bath. Add 1 cc. of concentrated sulfuric acid with shaking while the container is kept in the ice bath.² Start a stopwatch. At about 9 minutes remove one of the standard samples from the 24° bath, wipe the bottle dry, add 2 cc. of the cold reagent, shake the bottle for 10 seconds, and return it to the bath. The time of starting this process will depend on the time it requires (speed of the pipette, etc.); it should be so timed that the bottle is returned to the bath at 10 minutes ±5 seconds. The interval between samples will be determined by the time required for making a reading, to be established by practice. The color must be developed in all samples with exactly the same technique and with the same timing. Read in a colorimeter between 17 and 18 minutes after the bottle is returned to the bath against a solution containing 14 mg. of naphthol green B per 100 cc. The dye solution is standardized against each of the three standard cholesterol solutions and the unknown solutions are read against the “standard setting” of the dye with which they are in closest agreement. If a light filter with maximal transmission at about 625 mp is

² This mixed reagent is now being used in the method of Schoenheimer and Sperry (1). A description of this procedure with some modifications (2, 3), which have been adopted since the original publication, is being prepared and will be available on request.
available, an ink standard as described by Shapiro, Lerner, and Posen (4) or neutral gray filters may be used in place of the dye solution.

The degree of variation among replicate determinations carried out with this procedure on 50 cc. extracts of 2 cc. portions of serum is shown in Table

**TABLE I**

_Variation among Replicate Determinations by Proposed Method_

2 cc. portions of serum were extracted in 50 cc. volumetric flasks. 5 cc. portions of the extracts were analyzed by the proposed method and 1 cc. portions by a modification of the method of Schoenheimer and Sperry (1). All sera, except Sera 7, 8, 10, 13, and 15, were pooled samples.

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Proposed method</th>
<th>Method of Schoenheimer and Sperry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate determinations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mg. per 100 cc.</td>
<td>Average mg. per 100 cc.</td>
</tr>
<tr>
<td>1</td>
<td>232, 228, 233, 228, 234, 230, 231, 232</td>
<td>231</td>
</tr>
<tr>
<td>2</td>
<td>230, 233, 234, 231, 234, 230, 231</td>
<td>232</td>
</tr>
<tr>
<td>3</td>
<td>227, 206*, 228, 227, 226, 228, 234, 235</td>
<td>229</td>
</tr>
<tr>
<td>4</td>
<td>231, 233, 231, 242, 237, 234, 240, 229</td>
<td>235</td>
</tr>
<tr>
<td>5</td>
<td>221, 218, 221, 222</td>
<td>221</td>
</tr>
<tr>
<td>6</td>
<td>226, 217, 222, 213</td>
<td>220</td>
</tr>
<tr>
<td>7</td>
<td>242, 244, 241, 249, 245, 244, 239</td>
<td>243</td>
</tr>
<tr>
<td>8</td>
<td>184, 183, 185</td>
<td>184</td>
</tr>
<tr>
<td>9</td>
<td>233, 230, 233, 233</td>
<td>232</td>
</tr>
<tr>
<td>10</td>
<td>180, 178, 180, 182, 180</td>
<td>180</td>
</tr>
<tr>
<td>11</td>
<td>256, 250, 240, 256, 243</td>
<td>249</td>
</tr>
<tr>
<td>12</td>
<td>220, 227, 227, 217</td>
<td>223</td>
</tr>
<tr>
<td>13</td>
<td>220, 219, 218, 216</td>
<td>218</td>
</tr>
<tr>
<td>14</td>
<td>238, 234, 239, 235</td>
<td>237</td>
</tr>
<tr>
<td>15</td>
<td>216, 216, 221, 219</td>
<td>218</td>
</tr>
</tbody>
</table>

* Probably not dry; omitted from the average.

I. 1 cc. portions of the same extracts were analyzed by a modification of the method of Schoenheimer and Sperry. Dimter (5) reported recently that a substance which gives a positive Liebermann-Burchard reaction, but which is not precipitated by digitonin, is present in the unsaponifiable fraction of human serum. The close agreement (Table I) of values yielded by a method in which the color reaction is applied directly with those obtained with a procedure in which the cholesterol is first isolated as the digitonide is not in accord with this finding unless the substance occurs in very small quantity, within the limits of error of the methods. Dimter gives no information concerning the quantity of the unknown substance, or substances, in serum, though he implies that it is present in considerable amount. In one of his fractions, the Liebermann-Burchard reaction developed very slowly and it is possible that the color had not yet developed under the conditions employed in the present investigation.
DETERMINATION OF CHOLESTEROL

DISCUSSION

Necessity of Saponification—In 1917 Bloor (6) reported that the Liebermann-Burchard color developed more rapidly with blood extracts than with pure cholesterol. The difference became larger as the temperature was decreased. A hint at the reason for this effect was given by Gardner and Williams (7) in their incidental observation that cholesterol benzoate and acetate gave values which were 5 and 11 per cent, respectively, too high with the colorimetric procedure. Many years later the explanation became clear with the finding, made independently by Reinhold (8), Yasuda (9), Noyons (10), and Kelsey (11), that esterified cholesterol develops color at a more rapid rate, or to a greater extent, than does free cholesterol. The difference was found by Reinhold to be so large at 0–2° as to permit the differential estimation of combined cholesterol. Kelsey reported an experiment in which he measured the course of color development in solutions of cholesterol and of cholesterol palmitate at 23°. With this exception, no systematic study of the magnitude of the effect under the conditions ordinarily employed for cholesterol determination has been reported.

In a series of experiments the color was developed with the technique described above, or in some cases with separate addition of acetic anhydride and sulfuric acid, in solutions of recrystallized cholesterol (m.p. 147.5–148°, uncorrected) and of cholesterol palmitate (m.p. 78–79°, uncorrected), containing equivalent quantities of cholesterol. Sufficiently large volumes were employed so that a series of readings could be carried out on the same solution over a period of time. Portions were withdrawn with a transfer pipette without removing the solutions from the bath. In each experiment the cholesterol and the ester were carried through side by side under conditions as nearly identical as could be maintained. The two solutions were read alternately in a photoelectric photometer (12) with Corning light filters No. 244 and No. 978. Typical results of two such experiments are shown in Fig. 1. It will be seen that there was a considerable difference between cholesterol and the palmitate, not only in the density of color at the maximum, but also in the rate of color development. With the ester more color was produced and the maximum was reached earlier than with cholesterol. The findings are in general agreement with those of previous investigators, but they differ in detail from the results reported by Kelsey (11) who found that the curves yielded by cholesterol and its palmitate at 23° were approximately parallel, with a maximum at about 15 minutes in each case, and that the difference between maxima was about 30 per cent. In accord with the results of Bloor (6) and of Reinhold (8) our experiments show a considerable temperature effect. The percentage difference be-
tween maxima was about the same at 20° and at 24°, but there was a much larger difference in the time at which the maximum was reached at the lower temperature.

Since in most colorimetric methods in general use a saponification step is not included, and since blood serum contains a high proportion of cholesterol esters, this factor represents a source of considerable error and, as stressed by Kelsey (11), probably accounts, at least in part, for the high values usually obtained by these methods. It is possible that the unsaturated esters which characterize blood serum (13) may not behave like

![Graph showing color development in solutions containing cholesterol](http://www.jbc.org/)

**Fig. 1.** Curves showing the rate of color development in solutions containing equivalent quantities of cholesterol in the free and combined forms. • free cholesterol; ○ cholesterol palmitate; solid lines, color developed at 24°; broken lines, color developed at 20°.

the palmitate, but there is strong evidence against this assumption: (a) the observations reported by Bloor (6); (b) the findings by Noyons (10) of 17 per cent lower values with the digitonin method than with the colorimetric method applied to unsaponified serum extracts, and of 24 per cent lower values following saponification than without saponification with the colorimetric method; and (c) Yasuda's report (9) that the oleate gives about the same high result as the palmitate and stearate.

*Extraneous Color—Most workers with colorimetric methods for cholesterol determination have been troubled by off shade colors, occurring more or less at random. In recent years the difficulty has been overcome to a
considerable extent by the use of filters which mask out the extraneous yellow or brown colors. We have carried out no systematic study of this factor, but some observations are pertinent. In one instance a series of samples was allowed to stand overnight at room temperature after incubation without acidification. A brownish yellow color developed in all samples. With the dry extraction procedure then in use some of this color was carried into the final chloroform solutions and it was difficult or impossible to obtain satisfactory readings. Bloor, Pelkan, and Allen (14) called attention to the danger of heating with strong alkali. For this reason we recommend the use of the mild conditions for saponification employed in the method of Schoenheimer and Sperry (1), and acidification without delay.

During the early part of this study an alcoholic solution of hydrochloric acid was used for acidification. In some cases a brown color was seen to develop during evaporation just before dryness was reached and almost immediately the solvent refluxing in the suction tube became deeply yellow, while the color in the residue decreased. Apparently some partially volatile pigment was formed by action of hydrochloric acid. This difficulty was avoided by the use of acetic acid.

With the method as described no trouble with extraneous colors has been experienced. We attribute this result not only to the factors mentioned, but also to the wet extraction procedure. Fairly good results were obtained by extracting the dry residue with petroleum ether or chloroform, but off shade colors were sometimes encountered and the values were more variable than those obtained with extraction from water and alcohol. The yellow color which is usually present appears to be retained in the aqueous alcohol layer.

Extraction after Saponification—After the development of color during evaporation in the presence of hydrochloric acid was discovered, two procedures of neutralization were investigated: (a) titration with hydrochloric acid followed by alkalization with a drop or two of dilute potassium hydroxide solution, and (b) titration with acetic acid. In addition in some experiments sodium hydroxide was used as the saponifying agent and titrated with acetic acid. All of these procedures were studied in conjunction with dry extraction with various solvents (petroleum ether and chloroform for the most part) and with petroleum ether extraction after the addition of water. Aside from the variation already noted dry extraction has the disadvantage of requiring filtration. Petroleum ether extracts from samples to which water alone was added tended to be cloudy and the results were usually low. The addition of alcohol facilitates the separation; it may be used under the conditions described because potassium acetate is very soluble in alcohol. This salt has the disadvantage of being deliquescent, but this gives no trouble if the extraction is carried out immediately after drying.
Development of Color.  (a) Method of Adding Reagents—In practically all methods for the colorimetric estimation of cholesterol acetic anhydride and sulfuric acid are added separately to the chloroform solution. It is difficult to measure accurately the small quantity of sulfuric acid and this has been recognized as a potential source of error. Furthermore, since heat is produced rapidly after addition of the acid, and since the development of color is greatly affected by temperature, variations in the amount of acid, in the speed of addition, and in the speed of mixing may have a marked effect on the final result. That this is the case is indicated by the data presented in Fig. 2. Equal volumes of a solution of cholesterol in chloroform were placed in glass-stoppered cylinders such as are used in most methods for the development of color. After the temperature had been adjusted to 24°, acetic anhydride was added and mixed well with the chloroform. Sulfuric acid was then added from a micro burette, as described by Schoenheimer and Sperry (1), the solution was mixed thoroughly, and the cylinder was returned to the bath. Samples were read in the photoelectric photometer at the times after addition of the acid indicated in Fig. 2. Although every attempt was made to maintain exactly the same conditions throughout the procedure, in only two of the eight samples did the curves of color development coincide almost exactly. At the extreme there was a difference of 11 per cent between maxima. We are convinced from the foregoing experiments and from much experience with the Liebermann-Burchard reaction that the whole course of color development and the density of color produced at the maximum are determined, other factors being constant, by the events occurring immediately after the addition of sulfuric acid.

To avoid these difficulties Shapiro, Lerner, and Posen (4) employed a mixture of acetic anhydride and sulfuric acid. These substances react rapidly to form acetyl sulfuric acid with the evolution of considerable heat, and this reaction is followed by the much slower formation of sulfoacetic acid (15). Shapiro et al. cautioned that the mixed reagent is stable for 1 hour only. With a reagent mixed at room temperature according to their directions we obtained constant readings up to 35 minutes after mixing, but there was evidence of some change at 45 minutes (Table II). Constant results were obtained up to 1 hour after mixing with a reagent mixed and kept at ice bath temperature (Table II), and in other experiments only a small change was observed at 110 minutes under these conditions. With this reagent satisfactorily consistent results have been uniformly obtained.2

(b) Effect of Temperature—Although it has been known for many years that the development of color in the Liebermann-Burchard reaction is influenced by temperature (6), in most methods little attention is paid to the control of this variable. Ranges of 20–25° are specified in some procedures. An indication of the variation which may occur within this range
may be obtained by comparing the curves for free cholesterol at 20° and 24° in Fig. 1. The color developed much more slowly at the lower temperature.

**Fig. 2.** Variation in development of color in the Liebermann-Burchard reaction with separate addition of acetic anhydride and sulfuric acid. All samples contained the same amount of cholesterol and were treated under conditions as nearly identical as possible.

**TABLE II**

*Stability of Acetic Anhydride-Sulfuric Acid Reagent*

The data represent color density as read in a photoelectric photometer.

<table>
<thead>
<tr>
<th>Time of reading after adding reagent</th>
<th>Reagent mixed at room temperature</th>
<th>Time of reading after adding reagent</th>
<th>Reagent mixed in ice bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>Time after mixing reagent</td>
<td>min.</td>
<td>Time after mixing reagent</td>
</tr>
<tr>
<td></td>
<td>15 min.</td>
<td>25 min.</td>
<td>35 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.276</td>
<td>0.261</td>
<td>0.265</td>
</tr>
<tr>
<td>14</td>
<td>0.326</td>
<td>0.319</td>
<td>0.316</td>
</tr>
<tr>
<td>16</td>
<td>0.368</td>
<td>0.368</td>
<td>0.367</td>
</tr>
<tr>
<td>18</td>
<td>0.415</td>
<td>0.409</td>
<td>0.389</td>
</tr>
<tr>
<td>20</td>
<td>0.444</td>
<td>0.439</td>
<td>0.425</td>
</tr>
</tbody>
</table>
and reached a higher maximum. Except at the point where the curves cross (19 minutes) comparisons between two solutions in which color was developed at these two temperatures would be grossly in error. When the acetic anhydride and sulfuric acid are added separately, the effect of temperature may be even greater; in one experiment a difference of 35 per cent between the maxima developed at 20° and 25° was observed.

It may be argued that the effect of temperature is not important because standards and unknowns are treated side by side under the same conditions. This is true to a certain extent if a bath is used, but when the containers are allowed to stand in the air during color development, as in many methods, there is no assurance that the temperature is the same for all samples. The development of heat at the start may vary, as already pointed out, and the rate at which the heat is dissipated may vary with the thickness of the glass walls of the containers and perhaps with other factors. In some methods the use of a bath is recommended, but no particular temperature is specified. This is not sufficient, because for good results the temperature and time must be so controlled that the reading is taken at the point of maximal density where the rate of change is at a minimum. Measurements during the period when color is developing rapidly are particularly subject to error (cf. Fig. 1).

(c) Effect of Time—The importance of developing the color for the same length of time in all samples is apparent from the foregoing discussion. The common practice of reading several unknown samples against a single standard cholesterol solution introduces a large potential source of error, since the color of the standard is changing during the time the readings are carried out.

It will be noted (Fig. 1) that a much broader maximum is obtained at 20° than at 24°. By working at the lower temperature considerably more latitude in timing would be permissible. However, it is difficult to maintain a bath at 20° during warm weather and for this reason we selected 24°. At higher temperatures the color develops too rapidly.

Brown Color—After several hours the green color of the Liebermann-Burchard reaction fades out and a yellowish brown color supervenes. Whereas the development of green color is entirely empirical and is influenced by several factors as discussed above, it seemed possible that the density of the brown color, which appears to be an end-result of the reaction, might be proportional to the amount of cholesterol without regard to the conditions under which the color was developed. Hence in a number of the foregoing experiments and others not reported here the residual solution was retained overnight and the color was measured in the photoelectric photometer with filters having a maximal transmission in the violet, where the brown color shows maximal absorption. A wide variation
DETERMINATION OF CHOLESTEROL

was found. Moreover, it was impossible to read the color accurately in a colorimeter.

SUMMARY

Under the conditions ordinarily employed for the determination of cholesterol by means of the Liebermann-Burchard color reaction large errors may occur.

Saponification is necessary before the estimation of total cholesterol in blood serum or tissues containing esterified cholesterol, because in the combined form it develops considerably more color at a faster rate than does free cholesterol.

Interference by extraneous colors may be avoided by the use of mild conditions for saponification and a weak acid (acetic) for acidification, and by extraction from a water-alcohol solution of the acidified residue.

The development of color may be influenced to a considerable extent by small and uncontrollable variations in the procedure of adding acetic anhydride and sulfuric acid. This source of error may be avoided by mixing these reagents beforehand (4).

Small variations in the temperature at which color is developed have a large effect on the time at which the maximum is reached and on the height of the maximum. The temperature and time of reading should be selected so that the reading is carried out at the maximum, and should be the same for each sample in a series of determinations. The common practice of reading several unknown samples against one cholesterol standard violates this requirement.

A procedure which avoids these sources of error is described.

BIBLIOGRAPHY
