THE COLORIMETRIC ESTIMATION OF CHOLESTEROL IN BLOOD, WITH A NOTE ON THE ESTIMATION OF COPROSTEROL IN FECES.*

BY VICTOR C. MYERS AND EMMA L. WARDELL.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital.)

(Received for publication, August 31, 1918.)

During the past 6 years a number of different procedures have been utilized in the extraction of cholesterol from small quantities of blood for its ultimate colorimetric estimation. From the multiplicity of methods employed for the extraction process one would infer that they were not entirely satisfactory. Since 1913 a number of these methods have been tried out in our laboratory. In connection with a study of the blood lipoids in obesity, carried out in collaboration with Dr. Kast, it seemed necessary to investigate further the question of a suitable cholesterol method. For some time now, however, we have employed a comparatively simple and very satisfactory method,1 which is described in the present paper.

Grigaut2 was apparently the first to attempt the colorimetric estimation of cholesterol, using the Liebermann-Burchard reaction. 2 years later, Weston3 made use of the Salkowski reaction for a similar purpose. Since these reactions are very delicate they at once afforded a means of estimating the cholesterol in small amounts of blood, thus furnishing an impetus to this type of

*This study was made possible by funds contributed to the Laboratory through Professor Ludwig Kast of the Department of Medicine.

1 A preliminary report of these observations was presented before the Society for Experimental Biology and Medicine, October 17, 1917; see Kast, L., Myers, V. C., and Wardell, E. L., Proc. Soc. Exp. Biol. and Med., 1917–18, xv, 1.


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investigation. Of the two reactions, the Liebermann-Burchard appears to have found somewhat greater favor, although both have been extensively employed. In addition to Grigaut and Weston, Autenrieth and Funk, Henes, Myers and Gorham, Bloor, Csonka, Gettler and Baker, Bernhard, and others have described methods of cholesterol extraction in which these color reactions are used.

In the case of the excellent but laborious gravimetric digitonin method of Windaus, for the estimation of total cholesterol, saponification of the cholesterol esters is necessary, since only the free cholesterol is precipitated by the digitonin. Cholesterol esters give the color reaction as well as does the free cholesterol. This fact does not appear to have been recognized until very recently, since the directions for the colorimetric estimation have almost invariably called for a preliminary saponification. As pointed out by Bloor, this saponification is unnecessary and the colorimetric estimation of the cholesterol thus becomes further simplified.

Bloor has suggested a method of extraction for the cholesterol which is very simple and would appear to be complete, but the results obtained with the method as finally carried out are higher than those by the older methods, and rather irregular, owing, apparently, to the presence in the extracts used of substances interfering with the Liebermann-Burchard color reaction for cholesterol. Other workers in this field, notably Mueller and Weston, have criticized the high results obtained with the Bloor method. They are of the opinion that these results are due in part to the admixture of brownish tints frequently obtained in the final development of the color. Luden obtained similar high

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5 Henes, E., Jr., Proc. N. Y. Path. Soc., 1913, xiii, 155.
6 Myers, V. C., and Gorham, F. D., Post-Graduate, 1914, xxix, 938.
9 Gettler, A. O., and Baker, W., J. Biol. Chem., 1916, xxv, 211.
results, but believed that these resulted from a combination of bile pigments and bile acids. Her data bearing on this point are very interesting. Our own observations regarding the Bloor method have likewise led us to the conclusion that its results are too high, and Baumann\textsuperscript{15} has come to similar conclusions.

A description of the proposed method is given below, together with an adaptation to the estimation of coprosterol (?) in feces.

\textit{Methods.}

\textit{Cholesterol Estimation in Blood.}\textsuperscript{16}

1 cc. of blood, plasma or serum, is pipetted into a porcelain crucible or small beaker containing 4 to 5 gm. of plaster of Paris, stirred, and dried, preferably in a drying oven. It is now emptied into a small extraction shell (4 cm. long) and then inserted in a short test-tube (2.5 \times 6 cm.), in the bottom of which are a number of small holes (Fig. 1).\textsuperscript{17} This is now attached to a large cork on a small reflux condenser and the tube and cork are inserted in the neck of a 150 cc. extraction flask containing about 20 to 25 cc. of chloroform. Extraction is continued for 30 minutes on an electric hot plate, the chloroform made up to some suitable volume, such as 15 cc., filtered if necessary, and colorimetric estimation carried out as follows: 5 cc. of the chloroform extract are pipetted into a dry test-tube, and 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid (best with 0.1 cc. pipette) are added. After thorough mixing, the solution is placed in the dark for exactly 10 minutes\textsuperscript{18} to allow the color to develop and then compared with a standardized aqueous solution of naphthol green B in a Duboscq or Kober colorimeter. The dye excellently matches the cholesterol color and appears to be permanent.

\textsuperscript{15} Baumann, L., Personal communication.

\textsuperscript{16} In testing out various points in connection with the method we have been indebted to several individuals working in this laboratory during the past five years, Dr. F. D. Gorham, Mr. A. Bernhard, Dr. R. L. Kahn, and Dr. A. J. P. Pacini. The use of the dye was suggested to us by Mr. Bernhard, and Dr. Pacini made valuable suggestions in connection with the extraction.

The general problem of the cholesterol content of human blood has already been discussed, see Gorham and Myers, \textit{Arch. Int. Med.}, 1917, xx, 599.

\textsuperscript{17} We have generally used three extractors simultaneously on the same hot plate.

\textsuperscript{18} In order to get the proper temperature for color development in warm weather it is advisable either to keep the reagents in a cool place or to insert the tubes in water during the development of the color.
Regarding the use of the plaster of Paris, it would seem that, in addition to putting the blood into a finely divided and readily extractable condition, this calcium salt holds back substances which add to the color development with the Bloor technique. Chloroform appears to be a most excellent selective extractive here, and, in addition, is the solvent in which the color reaction must be carried out.

In conducting the extraction and developing the color it is important that the reagents should be perfectly anhydrous. The chloroform is best redistilled over calcium chloride while the acetic anhydride and sulfuric acid should be of known purity. On a number of occasions weak color development has been traced to acetic anhydride. For this reason we never develop a series of unknown solutions without first checking the quality of our reagents by developing a solution of pure cholesterol. With the method of extraction outlined above, brownish shades do not appear in the development of color. The use of the aqueous naphthol green B would appear to offer several advantages over the use of chloroform solutions of cholesterol as a standard. It appears to be rather more stable than the cholesterol in chloroform and does not evaporate as readily. With the dye it is not necessary to prepare continuously new standards to allow for the constant change in color.

Since the wedges and cups of the Hellige colorimeter are unaffected by chloroform, our first analyses were made with this instrument, though more recently the Duboscq has been used, mounting the cup employed for the chloroform in plaster of Paris, as suggested by Bloor. Still more recently we have had the clear glass cylinders of our Duboscq colorimeter replaced with black glass by the Klett Manufacturing Co. This removes the necessity for a light shield and renders the colors more easily matched. The present Kober instrument, in addition to the black cylinders has its clear glass bottoms fused on, a great advantage when using chloroform.

With our present lot of acetic anhydride, it has been found that when an 0.005 per cent solution of naphthol green B is used as a standard and set at 15.5 mm. on the Duboscq or Kober instrument, 0.4 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric
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acid will read 15 mm. The color curve for both the cholesterol and naphthol green B appears to fall in a straight line so that readings somewhat above or below the standard are accurate.

Coprosterol (?) Estimation in Feces.

To 2 or 3 gm. of well mixed moist feces, accurately weighed in a porcelain casserole, are added 1 gm. of calcium hydroxide which is thoroughly mixed in with the feces. To this is then added 10 cc. of a 20 per cent solution of sodium hydroxide, and well stirred with a small glass rod. The casserole is now placed on the water bath and heated for about 2 hours with frequent stirring. When the mixture has evaporated almost to dryness, it is removed from the water bath, 3 to 4 gm. of finely powdered plaster of Paris are added, the mass thoroughly mixed, and dried in an oven at a temperature of 95° for 2 hours. The extraction and subsequent colorimetric estimation are carried out exactly as for cholesterol. A moisture determination should be made on another fraction of the same specimen so that the value of the coprosterol can be calculated in terms of percentage of the dry specimen. The calcium hydroxide is used to hold back any bile pigments. Saponification would, likewise, appear to be necessary here on account of the high fat content of feces. As we have found, liquid petroleum, now so commonly used therapeutically, may introduce a very disturbing factor in the determination.

Inasmuch as we have not yet been able to obtain coprosterol in a state known to be perfectly pure, it has been thought best to record the values in terms of cholesterol. Employing the usual technique of isolating coprosterol, we have been able to obtain an unsaponified light amber residue, which showed the needles supposedly typical of coprosterol (but no cholesterol plates). So far we have not been successful in isolating the needles. The colorimetric value of this dried residue, which is ordinarily weighed up as coprosterol, was found to be about one-third that of cholesterol, but identical in appearance.

The coprosterol content of dry feces, calculated as cholesterol, has been found to vary from 0.5 to 1.5 per cent. As yet we have not been able to note any correlation with the clinical findings in the cases.

We had planned a rather extensive study of the coprosterol content of the feces, but difficulties in obtaining entirely satisfactory quantitative specimens, as well as of ascertaining the exact color value for pure coprosterol have greatly delayed us. We have not,
however, given up hope of carrying out this work. Such a study
would appear to be of considerable interest in certain of the
obscure liver conditions, as well as in supposed disorders of
cholesterol metabolism.

DISCUSSION.

As will be noted in the tables given below, this method fulfils
three obviously necessary requirements. It gives consistent du-
plicates, completely recovers known amounts of added choles-
terol, and satisfactorily checks with a recognized reliable method
based on an entirely different principle (method of Windaus).
From our data this does not seem to be true in the case of the
Bloor method.

Table I presents a series of determinations with the method
described above on normal human blood and blood to which
either cholesterol or cholesterol palmitate was added. These
were paralleled by similar determinations with the Bloor method.
The duplicates and recoveries of added cholesterol would seem
to be satisfactory with the method described, but this was not the
case with the method of Bloor. It may be noted, however, that
the data on the cholesterol palmitate with the proposed method
bear out Bloor's contention that saponification of cholesterol
esters is unnecessary for color development. With the Bloor
method the cholesterol in alcohol-ether solution was added to the
alcohol-ether to be used for the extraction, but with the pro-
posed method a chloroform solution was added to the blood and
plaster of Paris and the three were thoroughly mixed before
drying. It may be stated that in the latter case the cholesterol
was never intimately mixed with the blood, but we believe that
such criticism is not valid.

Data are presented in Table II giving parallel determinations
on the same samples with the Bloor method and the proposed
method in comparison with the digitonin method. The checks
of the proposed method with the Windaus gravimetric method
are satisfactory, but this was not the case with the Bloor method.

Mueller12 has made some very valuable suggestions regarding
the Fraser and Gardner19 technique of carrying out the digitonin.

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TABLE I.
Comparative Cholesterol Estimations.

<table>
<thead>
<tr>
<th>Colorimetric readings.</th>
<th>Bloor method.</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Cholesterol added</td>
</tr>
<tr>
<td>mm.</td>
<td>mg. per cc.</td>
<td>mg. per cc.</td>
</tr>
<tr>
<td>7.1</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>2.15</td>
<td></td>
</tr>
</tbody>
</table>

1 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.

<table>
<thead>
<tr>
<th></th>
<th>Bloor method.</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.7</td>
<td>2.33</td>
<td>0.8</td>
</tr>
<tr>
<td>5.9</td>
<td>2.26</td>
<td>0.8</td>
</tr>
<tr>
<td>5.7</td>
<td>2.33</td>
<td>0.8</td>
</tr>
<tr>
<td>6.1</td>
<td>2.18</td>
<td>0.8</td>
</tr>
</tbody>
</table>

2 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.

<table>
<thead>
<tr>
<th></th>
<th>Bloor method.</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>2.89</td>
<td>1.6</td>
</tr>
<tr>
<td>4.6</td>
<td>2.89</td>
<td>1.6</td>
</tr>
<tr>
<td>4.6</td>
<td>2.89</td>
<td>1.6</td>
</tr>
<tr>
<td>4.8</td>
<td>2.78</td>
<td>1.6</td>
</tr>
</tbody>
</table>

1 cc. of 0.2 per cent cholesterol palmitate solution per 1 cc. of blood.

<table>
<thead>
<tr>
<th></th>
<th>Bloor method.</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>2.72</td>
<td>1.24</td>
</tr>
<tr>
<td>4.8</td>
<td>2.77</td>
<td>1.24</td>
</tr>
</tbody>
</table>

At the time this series of observations was carried out our dye standard was set at 9.3 mm., which corresponded exactly with the color development of 0.4 mg. of cholesterol at 10 mm.

All observations in this series were on fractions of the same specimen of blood. Two estimations grouped together indicate the results of two colorimetric developments on the same extract.

determination, most of which we have followed. In view of the importance of this determination as a check on our own method, it may be well to outline the procedure as we have carried it out. It is as follows:
10 cc. of blood are saponified for 2 hours on the water bath with 100 cc. of 25 per cent aqueous potassium hydroxide. They are then shaken out first with 250 cc. of chloroform, and later with 125 cc., three times. The extracts are now combined and evaporated to dryness. The residue is dissolved in alcohol, and to the boiling alcoholic solution a 1 per cent solution of digitonin in 90 per cent alcohol is added, using about 25 per cent excess. This is allowed to stand over night in an ice box, and then filtered on tared hardened filter papers, washing first with ether and then with boiling water. The filter papers are placed in weighing bottles, dried in an oven, transferred to a desiccator for a short time, and weighed. The digitonin-cholesterol compound multiplied by the factor 0.2431 gives the cholesterol.

**TABLE II.**

Comparative Cholesterol Values with the Digitonin, Bloor, and Proposed Methods.

<table>
<thead>
<tr>
<th>Blood</th>
<th>Digitonin method</th>
<th>Bloor method</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Z.</td>
<td>0.149</td>
<td>0.206</td>
<td>0.139</td>
</tr>
<tr>
<td>2 M.</td>
<td>0.147</td>
<td>0.203</td>
<td>0.141</td>
</tr>
<tr>
<td>3 R.</td>
<td>0.125*</td>
<td>0.181</td>
<td>0.124</td>
</tr>
<tr>
<td>4 Mixed blood</td>
<td>0.158</td>
<td>0.187</td>
<td>0.164</td>
</tr>
<tr>
<td>5 “ “</td>
<td>0.072</td>
<td>0.095</td>
<td>0.077</td>
</tr>
<tr>
<td>6 Guinea pig</td>
<td>0.158</td>
<td>0.158</td>
<td>0.116</td>
</tr>
<tr>
<td>7.</td>
<td>0.216</td>
<td>0.216</td>
<td>0.153</td>
</tr>
<tr>
<td>8.</td>
<td>0.177</td>
<td>0.177</td>
<td>0.150</td>
</tr>
<tr>
<td>10.</td>
<td>0.163</td>
<td>0.163</td>
<td>0.127</td>
</tr>
<tr>
<td>11.</td>
<td>0.238</td>
<td>0.238</td>
<td>0.164</td>
</tr>
</tbody>
</table>

* 20 cc. of blood used.

Mueller has pointed out that tared filter papers permit more rapid filtration than Gooch crucibles, and we can confirm him in this. As indicated by Fraser and Gardner, the use of ether and hot water is preferable for washing to the ether and alcohol used in the original method. Digitonin is not readily soluble in cold alcohol, but is easily soluble in boiling water and the excess of digitonin is thus easily removed. The digitonin-cholesterol compound is slightly soluble in alcohol, so the use of ether and boiling water obviates the slight loss in weight that might follow the use of alcohol for washing.
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SUMMARY.

A method is described for the colorimetric estimation of cholesterol in blood, in which the cholesterol is directly extracted from the blood with the solvent (chloroform) employed in the development of the color reaction, thus rendering the estimation very simple.

Data are presented showing that good duplicates can be obtained with the method and added cholesterol completely recovered. Observations are likewise given in which the estimations excellently check those obtained with the Windaus gravimetric method.

Figures which we have obtained with the Bloor method are higher than those obtained with either the digitonin or the proposed method.

A modification of the method is described whereby it is possible to determine the coprosterol (?) of the feces.
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J. Biol. Chem. 1918, 36:147-156.

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