THE DETERMINATION OF CHOLESTEROL IN BLOOD.

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(Received for publication, February 27, 1917.)

Weston\(^1\) has recently reported results obtained by the use of my method for the determination of cholesterol in blood. These results are inconsistent and justify his finding that in his hands the method was useless. On the other hand, the method has given consistent results in this laboratory in various hands for over a year and is known to be in use in other laboratories where a thorough testing out before adoption would be a matter of course. In published work on the subject Mueller\(^2\) has obtained results similar to mine in a comparison of the method with that of Autenrieth and Funk and has reported the alcohol-ether method of extraction "most excellent." Denis\(^3\) has reported essentially the same values for normal blood as I had obtained. While it could hardly be expected that any method would find acceptance at all hands, this method seemed to have the advantages of simplicity in principle and ease and rapidity in operation, with the further advantage that the same alcohol-ether extract may be used for the determination of other blood lipoids. It was desirable therefore to find out if possible the reason for Weston's inconsistent results, both for the justification of those already using the method and to correct if necessary the directions so as to prevent further trouble with it.

Experience has taught that in cases of this kind the most frequent cause of failure is a use of the method in some other way than that directed or intended. It is a fact well known to chemists that few methods are flexible enough to be used in any

\(^3\) Denis, W., *J. Biol. Chem.*, 1917, xxix, 93.
other way than that worked out by the originator. While Weston's description of his procedure is brief and lacking in essential details, there is sufficient to show that he has probably violated both the principle and the expressed directions. Thus in his version of the method, after describing the extraction with alcohol-ether, bringing to 100 cc., and filtering, the filtrate was divided into two parts, the first part was evaporated to dryness, and the residue "taken up with chloroform." The second was treated with potassium hydrate and then as in the Weston-Kent method. Two points may be noted here; first, that the filtrate is not the whole extract—it is an indefinite volume depending on the amount held back by the precipitate and filter paper, and generally about 90 cc.—so that what is obtained by taking half of it is about 45 per cent of the whole extract; second, that while complete extraction of the residue from half the extract is possible, and is desirable in certain cases, it is a much more difficult and time-consuming procedure than complete extraction of the residue from 10 cc. It does not follow at all that since the cholesterol from a small residue may be extracted by a few minutes' boiling with chloroform the larger residue can be extracted by a similar short treatment. It is evident that failure to get a good extraction is probably an important cause of the inconsistent results, from the fact that lower values are obtained from Solution B which contains 50 per cent more cholesterol than Solution A. Whether the extraction of the serum with alcohol-ether was also at fault is not apparent, but it is evident that it was not always so, for the values which he obtained from the alcohol-ether extract saponified and determined in his own way are consistent and in fair agreement (when increased by 10 per cent to cover the error in aliquot) with those obtained by the various other methods. Thus for Solution A the reported value is 173 mg., and for Solution B 270 mg. These values increased 10 per cent would be 190 and 297 mg., which compare well with the values of 185 and 285 mg. obtained by the other methods. It is perhaps significant that these values obtained by his own method, with which he would presumably be most familiar, are the only consistent results obtained by the use of any part of my method.

My directions for the second part of the procedure, the pro-
duction of the color, call for the cholesterol from 10 cc. of alcohol-ether extract—ordinarily about 0.5 mg. (volumes to contain this amount are specified in later use of the method)—dissolved in 5 cc. of chloroform and compared with 0.5 mg. of cholesterol also in 5 cc. of chloroform; that is, the standard and test solutions are of about the same strength. They are also treated with the same amounts of reagents and are otherwise under parallel conditions—a plan of procedure which has been followed in all the methods for blood lipoids and which is believed to be the safe one for work in a new field. In Weston’s version of my procedure a corresponding amount of serum extract is used for the test solution, but for the standard he used a solution containing 2.7 mg. of cholesterol in 10 cc. of chloroform—a standard approximately two and one-half times as strong as the test. For this strong solution the same concentration of acetic anhydride and sulfuric acid was employed as for the much weaker test solution. While the error introduced by comparing colored solutions so widely different in strength might not be great, it was improbable that either the quality or intensity of the color would be the same under these different conditions of treatment. That this is the case is shown by the following experiment.

Chloroform extracts of blood cholesterol obtained by the Autenrieth and Funk method (Solutions A, B, F, and G, below) were compared with standards prepared (a) according to my directions, standard 0.5 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid; and (b) according to Weston’s modification of my directions, standard 2.7 mg. of cholesterol in 10 cc. of chloroform treated with 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid. All were digested for 15 minutes in the dark at 22°C. The values, calculated in mg. per 100 cc. of blood, are shown in Table I.

The values obtained by Weston’s modification are thus distinctly higher than those obtained by my directions. The colors were quite smoky in tint so that comparison was difficult. The high values may be in part due to the difference in tint, but probably more to the relatively low concentration of acetic

TABLE I.

<table>
<thead>
<tr>
<th>Solution</th>
<th>By my directions</th>
<th>By Weston's modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>218</td>
<td>245</td>
</tr>
<tr>
<td>B</td>
<td>216</td>
<td>240</td>
</tr>
<tr>
<td>F</td>
<td>115</td>
<td>150</td>
</tr>
<tr>
<td>G</td>
<td>113</td>
<td>148</td>
</tr>
</tbody>
</table>

anhydride and sulfuric acid in Weston's standard which resulted in an incomplete development of the color.

Weston's experiment was repeated as follows:

Two samples of 50 cc. each of fresh beef plasma were taken and to one were added 5 cc. of ether containing 50 mg. of cholesterol, and to the other 5 cc. of ether alone (Weston did not add ether to his control serum). The two solutions were well shaken and after standing for about 2 hours with occasional shaking, samples were taken from them as follows.

a. Eight 3 cc. samples were taken from each and extracted with alcohol-ether according to directions in my method. 10 cc. of the extracts were used for the determination.

b. Four 2 cc. samples were taken from each, digested with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk. The chloroform extracts after dehydration and filtering were made up to 100 cc. 15 cc. samples were used for the determinations and were measured into small beakers, evaporated to less than 5 cc., cooled, transferred to the 10 cc. cylinders, and made up to 5 cc. with the washings from the beakers.

c. Four 50 cc. samples of the alcohol-ether extracts from a were evaporated almost to dryness, then treated with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk, using corresponding amounts of alkali and chloroform.

In all determinations the samples and standard were each contained in 5 cc. of chloroform and were treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid in the dark at room temperature (20-22°C.) for 15 minutes. The Duboscq colorimeter was used throughout and there can be no question that it is more accurate and less subject to personal error than the Autenrieth-Königsberger instrument, certainly than the "Hellige" form which appears in this country. The Duboscq has also the advantage that the standard and test solutions are of about the same strength. It may be noted here that Autenrieth and Funk did not use a cholesterol standard, but instead used a permanent standard made of a mixture of metallic salts which was calibrated by the use of known cholesterol solutions, thus avoiding the difficulty mentioned.

above. A permanent standard such as Autenrieth and Funk used has some advantages especially for use with the Autenrieth-Königsberger type of colorimeter, and as they do not give the composition of their solution, an attempt is being made to prepare a suitable solution.

The results of single consecutive determinations on these samples are given in Table II. The values are expressed in mg. per 100 cc. of blood.

<table>
<thead>
<tr>
<th>A. My procedure.</th>
<th>B. Autenrieth and Funk's procedure.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control plasma.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
</tr>
<tr>
<td>3</td>
<td>130</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td>128</td>
</tr>
<tr>
<td>6</td>
<td>129</td>
</tr>
<tr>
<td>7</td>
<td>129</td>
</tr>
<tr>
<td>8</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma plus 1 mg. of cholesterol per 1 cc.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>227</td>
</tr>
<tr>
<td>2</td>
<td>229</td>
</tr>
<tr>
<td>3</td>
<td>229</td>
</tr>
<tr>
<td>4</td>
<td>230</td>
</tr>
<tr>
<td>5</td>
<td>229</td>
</tr>
<tr>
<td>6</td>
<td>235</td>
</tr>
<tr>
<td>7</td>
<td>235</td>
</tr>
<tr>
<td>8</td>
<td>229</td>
</tr>
</tbody>
</table>

These results show that the method will do all that has been claimed for it when used as originally directed. Satisfactory recovery of added cholesterol is obtained either by the use of...
my method throughout or by the application of my procedure for color production to extracts obtained by the Autenrieth-Funk digestion either of plasma direct or of alcohol-ether extracts of it.

The alkali treatment either of plasma or of alcohol-ether extracts of it results in lower values.

The points enumerated below are sufficient to explain the low values reported by Weston but not the very high ones obtained by him in some cases. My method of cholesterol determination is based on the Liebermann-Burchard color reaction as used by Autenrieth and Funk, adapted for use with the alcohol-ether extraction of blood. The only departure from Autenrieth and Funk's directions (aside from the use of a cholesterol standard instead of a salt standard) is that the color production is carried out at room temperature instead of at about 32°C. (obtained by setting the color tubes in a beaker of water at 32-35°—in which the temperature may be 3° or 4° lower by the end of the 15 minutes). The change to room temperature was made as the result of experiments which showed that slightly higher results were obtained at the lower temperature and that the solutions were free from a yellowish tint which developed at the higher temperature. In the published directions no definite temperature was specified and as this seemed to be a possible source of error, temperature conditions were sought other than those in which the reaction was ordinarily carried out. Ordinarily the cylinders in which the color was produced were placed in the cupboard of a table in the middle of the laboratory where the temperature was at least as constant as that of the laboratory which is kept at 20-23°C. for all but the hottest months of the year. On the other hand, the temperature in a partly enclosed window-seat of a north window in the laboratory was found to be 12°C. A series of samples were therefore allowed to digest 15 minutes in a dark box there. The values obtained for blood cholesterol were very high, almost double those obtained at 21°C., and were quite irregular. The color in the standard cholesterol cylinder was obviously only little developed while that in the blood cholesterol cylinder was well developed. These results indicated that the standard cholesterol did not react at the same rate as the blood cholesterol, and other experiments to test this
point were carried out as follows. Solution A was a chloroform extract of residues from evaporation of mixed alcohol-ether extracts of human blood. Solution B was a standard cholesterol (Kahlbaum) solution in chloroform containing 0.5 mg. of cholesterol in 5 cc. Pairs of samples consisting of one each of these solutions were digested at temperatures of 10–34° as follows.

5 cc. of each solution were measured into a 10 cc. flask and 2 cc. of acetic anhydride added. The solution was adjusted to the required temperature by shaking in water at that temperature, 0.1 cc. of concentrated sulfuric acid added, the temperature again quickly adjusted, and the little flasks were placed in water in a beaker thermostat at the desired temperature, covered, and set in the dark for 15 minutes. At the end of that time the reaction was stopped by chilling the solutions in ice water and comparisons were made at once with the standard solution.

As a standard for each of the determinations a 5 cc. sample of the standard cholesterol was digested at 22° in the same way as the test samples, then chilled before use. The results of a typical series are given in Table III.

### Table III

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Solution A (blood cholesterol) mg.</th>
<th>Tint* of color.</th>
<th>Solution B (Kahlbaum's cholesterol) mg.</th>
<th>Tint* of color.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blue.</td>
<td></td>
<td>Trace.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.48**</td>
<td>Blue.</td>
<td>0.21**</td>
<td>Blue.</td>
</tr>
<tr>
<td>14</td>
<td>0.62</td>
<td>Bluish.</td>
<td>0.30**</td>
<td>&quot;</td>
</tr>
<tr>
<td>18</td>
<td>0.62</td>
<td>&quot;</td>
<td>0.50</td>
<td>Matched.</td>
</tr>
<tr>
<td>22</td>
<td>0.68</td>
<td>Matched.</td>
<td>0.52</td>
<td>Faint yellowish.</td>
</tr>
<tr>
<td>26</td>
<td>0.62</td>
<td>Faint yellowish.</td>
<td>0.48</td>
<td>Yellowish.</td>
</tr>
<tr>
<td>30</td>
<td>0.60**</td>
<td>Yellow.</td>
<td>0.40**</td>
<td>Yellow.</td>
</tr>
<tr>
<td>34</td>
<td>0.58**</td>
<td>Deep yellow.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* As compared with the standard.

** These values are approximate because of the difficulty of comparing colors of different tints.

Extracts from other blood samples showed some variations from the above, but in general the results were the same and indicate that the blood cholesterol behaves quite differently from the standard cholesterol. It reacts more readily with the re-
agents, comes to its maximum sooner, and begins to fade sooner
than the standard (gall-stone?) cholesterol. Whether the blood
cholesterol is wholly or in part a different substance from the
standard or whether the difference in speed of reaction is due to
an impurity in the blood extracts which accelerates the reaction
is not clear, but in view of these differences in rate a further study
of the Liebermann-Burchard reaction in its application to blood
cholesterol is desirable and is now being undertaken in this
laboratory.

The results obtained above indicate that values obtained at
22° are more nearly correct than those obtained at either higher
or lower temperatures. At lower temperatures the standard
color has not fully developed while at the higher temperatures
the blood cholesterol color has passed its maximum and has
begun to fade.

The experiments recorded in Table III throw an interesting
light on the question of tints of color in blood cholesterol deter-
minations. Mueller and Weston have both reported yellowish
or brownish tints in the colors obtained from extracts made by
my method. As noted above, the color, whether of blood cho-
lesterol or of the standard cholesterol, passes through the stages
of blue-green, green, yellow-green, and finally yellow, and whether
the standard solution has a bluish or yellowish tint as compared
with the test solution depends on the relative stage of develop-
ment of the two. There is, of course, a considerable personal
factor in judging tints of color, but in the course of a great num-
ber of blood cholesterol determinations carried out by my method
at room temperature (22°) I have found very few in which the
difference of tint was sufficient to make any difficulty in read-
ing. In some cases, most frequently in extracts of whole blood,
the alcohol-ether extract was brown or brownish green, and it
was found that this color carried through into the chloroform.
To get rid of this color Mueller’s directions have been found
useful. After evaporating the alcohol-ether extract and extract-
ing the residue with chloroform (using about 15 cc.), the chloro-
form is washed with water, dried with anhydrous sodium sulfate,
evaporated to small volume, and the color produced in the cy-
inders as usual. The brownish color is soluble in water and is
almost entirely removed by this treatment. A simpler way of
accomplishing the same result (due to Dr. W. E. McEllroy of this laboratory) is to shake the alcohol-ether extract with about 5 gm. of the anhydrous sulfate. Occasionally a sample is obtained (I have had three) in which the alcohol-ether extract is colorless but which develops a brown color in the chloroform on treatment with acetic anhydride and sulfuric acid. Washing or treatment with sodium sulfate was less effective in these cases. Some form of alkali treatment was necessary either with sodium ethylate, as was originally directed, or with strong watery alkali and extraction, as in Autenrieth and Funk's method.

SUMMARY.

Results reported by Weston in the use of my method for the determination of cholesterol in blood are discussed and probable reasons for their inconsistency noted.

Differences in behavior of blood cholesterol and standard (Kahlbaum's) cholesterol are found.

A temperature of 22°C. has been found most suitable for the color production.

Causes of “off color” are discussed and suggestions offered for their correction.

† Bloor, J. Biol. Chem., 1915, xxiii, 317.
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