Simplified Rapid Technic for the Extraction and Determination of Serum Cholesterol without Saponification

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The value of cholesterol determinations as an aid to diagnosis and treatment in a variety of clinical states, such as arteriosclerosis, biliary obstruction, diabetes, liver disease, and nephrosis, is well recognized. However, clinical laboratories are often unable to keep pace with the growing demand for these determinations because of the laborious and exacting nature of the more reliable technics (19, 23, 24). The need existed, therefore, for a simple, rapid, yet accurate technic which could readily accommodate a fairly large number of specimens in a single series.

With a few exceptions (10, 12, 25, 27, 30), colorimetric methods for the determination of cholesterol are based upon the original reaction of Liebermann and Burchard, although they often differ in the method of extraction. Solvent extraction from serum dried on plaster of paris (11) or paper (9, 20) is cumbersome and tedious. Wet-extraction methods employing such solvents as ethanol-ether (2, 3, 5, 7, 16, 28), ethanol-acetone (19, 22-24), and petroleum ether (1, 27) require at least two additional steps for analysis: removal of solvent and its replacement by a medium suitable for color development. Direct extraction by chloroform (8, 31) achieves a considerable simplification in technic. However, ample evidence (5, 7, 8, 15, 23, 26, 29) exists in support of Bloor's (3) finding that cholesterol esters produce greater color intensities than does an equivalent concentration of free cholesterol, when the Liebermann-Burchard reaction is carried out in chloroform. Values for total cholesterol are, therefore, incorrect when the Liebermann-Burchard reaction is applied, without prior saponification, to extracts of serum cholesterol.

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in chloroform. Application of a factor (7, 8, 26) to correct for these differences in color intensity is strictly valid only when an analysis for free cholesterol is also performed, as in the method of Teeri, since it can be shown that a correct factor necessarily varies with the ratio of free to total cholesterol. It has been found by the authors that extraction of serum cholesterol into acetic anhydride, as proposed by Drekter, overcomes these difficulties. We have been able to show that in an acetic anhydride–acetic acid solvent equal maximum color intensities are produced by equimolar concentrations of free and esterified cholesterol, upon treatment with a modified Liebermann-Burchard reagent. A careful survey of the literature has revealed no experimental refutation of this finding.

A method (13) similar to ours has appeared while this work was in progress. The method is quite rapid and convenient, but we find that when color is developed in the presence of serum proteins, as recommended by Pearson, Stern, and McGavack, the values obtained for cholesterol are approximately 10 per cent too low. A second disadvantage is the possibility of explosive reactions (6), which may result from accidental introduction of an excessive quantity of water, when the hygroscopic reagent p-toluenesulfonic acid is used. Finally, in view of the known sensitivity of the Liebermann-Burchard reaction to changes in temperature (5, 8, 18, 19), closer control of temperature is indicated.

The procedure detailed below, embodying extensive modifications of a direct extraction method with acetic anhydride (4), should fulfill the stated need for a simple, rapid, yet thoroughly reliable method for the determination of cholesterol in serum or plasma. In our laboratories this method has given consistently satisfactory performance in more than 13,000 analyses during a 3-year period. Equally good results have been reported to us by other laboratories which have adopted the procedure.

**MATERIALS AND METHOD**

**Reagents**

Glacial acetic acid, reagent grade.

Acetic anhydride, reagent grade. Keep tightly stoppered.

Concentrated sulfuric acid, reagent grade. Keep tightly stoppered.

Cholesterol standard, 200 mg. per 100 ml.: Dissolve 0.2000 Gm. recrystallized cholesterol, m.p. 148°, in approximately 50 ml. glacial acetic acid. Dilute with glacial acetic acid to exactly 100 ml.

Sulfuric acid–acetic acid reagent, 1:1 (v/v): Slowly pour 100 ml. conc. H₂SO₄ into a 500-ml. Pyrex flask containing 100 ml. glacial acetic acid.
Mix the contents by gentle rotation until the addition has been completed. Allow the mixture to cool to room temperature before using. This reagent is stable at room temperature for at least 6 months. Keep well stoppered.

Dehydrating reagent: Mix 10 ml. H₂SO₄-acetic acid reagent (1:1) with an equal volume of glacial acetic acid. Transfer to a dropping bottle. The reagent is stable for at least 6 months.

Instrumentation: Satisfactory results have been obtained with the Klett-Summerson photoelectric colorimeter and with the Junior and Universal models of the Coleman spectrophotometer. Other photometers capable of measuring absorbance at 620 mµ are probably satisfactory.

Procedure

Test Sample

Accurately pipet 0.2 ml. serum into a test tube suitable for centrifugation. Thick-walled, lipless tubes, measuring 125 x 16 mm. are recommended. Add 0.8 ml. glacial acetic acid to the serum, mix gently and allow to stand for 1–2 minutes.

Serum Blank

Repeat the above procedure using a second 0.2-ml. aliquot of serum.

Reagent Blank

Add 0.8 ml. glacial acetic acid to 0.2 ml. distilled water.

Standard

Measure 0.2 ml. cholesterol standard into a fourth tube. Add 0.6 ml. glacial acetic acid and 0.2 ml. distilled water. Prepare this standard for each series of determinations.

Calibration

To calibrate a photometer for this procedure, prepare a series of standards by measuring 0.1, 0.2, 0.3, 0.4, and 0.5 ml. cholesterol standard into separate tubes. Add glacial acetic acid to a final volume of 0.8 ml. Finally, add 0.2 ml. distilled water to each and mix gently. For highest accuracy duplicate standards, accompanying each set of determinations, are more reliable than an occasionally prepared calibration curve.

Extraction and Deproteinization

To each sample, prepared according to the procedures outlined above, add 4 ml. acetic anhydride. Adequate mixing is achieved by allowing the
reagent to flow freely into each sample without contacting the wall of the tube. If necessary, complete the mixing process by imparting a rotary motion to the contents of the tube. Precipitation of serum proteins commences after approximately 2 ml. acetic anhydride have been added. Centrifuge all serum-containing samples for 5 minutes at approximately 2000 rpm. By decantation, transfer the clear supernatant solution as completely as possible into a similar tube. Discard the protein precipitate. Add 1 ml. glacial acetic acid to each serum blank only, and set it aside for photometric reading.

**Dehydration and Development of Color**

Allow 1 drop of dehydrating reagent to fall directly into each solution other than the serum blank. Mix by rotation. This step is accompanied by a marked increase in temperature after a brief induction period. If the solution does not become hot within 1–2 minutes, repeat with not more than 1 additional drop of reagent. Immediately after dehydration, transfer each specimen to a water bath regulated at 25°. A rectangular enameled pot with a capacity of approximately 6 L. is most inexpensive, yet wholly suitable. When the vessel is half-filled with water at 25°, the temperature is usually maintained within satisfactory limits, i.e., 1°, for at least 1 hour without further regulation.

After the contents of the tubes have reached bath temperature (approximately 5–10 minutes), pipet 1 ml. of H$_2$SO$_4$-acetic acid reagent into the tube containing the reagent blank. The reagent should drop directly into the solution without touching the wall of the tube. Promptly remove this tube from the water bath and ensure complete mixing by holding the upper portion of the tube firmly in the left hand while imparting a rotary motion to the contents by tapping the bottom of the tube with the fingers of the right hand. Replace at once in the water bath. Do not permit water to drop into tubes by accidental splashing. With a little practice the addition of reagent and mixing can be completed within $\frac{1}{2}$ minute. At successive 1-minute intervals following the addition of H$_2$SO$_4$-acetic acid reagent to the reagent blank, repeat this process with the standard and with the test sample. By maintaining exact intervals during color development, followed by the same intervals during readings of absorbence, equal periods of color development are assured for all specimens, even when many samples are analyzed in series.

Transfer the reagent blank to a suitable photometer cuvet or cell. Twenty minutes after the addition of H$_2$SO$_4$-acetic acid reagent adjust the photometer to read zero absorbence (or Klett units) against the reagent blank at 620 m$\mu$ (Klett filter No. 62). Read the standard and test
specimens at successive 1-minute intervals. Finally, read the absorbance value of the serum blank. After measurement of their absorbances, reaction mixtures are disposed of by pouring into a large amount of water. Do not add water to tubes containing these mixtures.

Calculation

When photometers which show conformity to Beer's law for this color reaction are employed (e.g., Coleman or Klett), the average absorbance of duplicate standards is used for the following calculation:

\[
\text{mg. total cholesterol per 100 ml. serum} = \frac{D_T - D_b}{D_s} \times C_s \times \frac{100}{0.2} \times 1.01
\]

where \(D_T\) = absorbance of test specimen

\(D_b\) = absorbance of serum blank

\(D_s\) = average absorbance of duplicate standards

\(C_s\) = amount of cholesterol (0.4 mg.) in 0.2 ml. of standard

For a given value of \(D_s\), the expression may be reduced to

\[K(D_T - D_b)\]

where \(K = \frac{202}{D_s}\)

EXPERIMENTAL

Optimum Conditions for Color Development

A 0.4 ml. amount of a solution containing 200 mg. of cholesterol in 100 ml. of glacial acetic acid was pipetted into a Klett cuvet. To this was added 0.4 ml. glacial acetic acid, 0.2 ml. distilled water, and 4 ml. acetic anhydride. Following the addition of 1 drop of dehydrating reagent, the temperature rose spontaneously to 50–60° after a short induction period, usually a few seconds. After the mixture was cooled to 25° in a water bath, 1 ml. of 25 parts by volume of \(\text{H}_2\text{SO}_4\) in 75 parts by volume of glacial acetic acid was introduced. The contents of the tube were promptly mixed and the tube then returned to the water bath. A Klett photoelectric colorimeter was adjusted to zero reading at 620 m\(\mu\) (Klett filter No. 62) against a reagent blank. Periodic readings of color intensity were then recorded at fixed intervals.

Similar studies were conducted by employing 1 ml. of each of the following mixtures as the color-developing agent: 50% \(\text{H}_2\text{SO}_4\) in acetic acid (v/v), 75% \(\text{H}_2\text{SO}_4\) in acetic acid (v/v), and 98–99% \(\text{H}_2\text{SO}_4\) (concen-

\(^1\)Application of the factor 1.01 is discussed in a later section. The factor is valid when the extract is decanted as completely as possible from 125 × 16 mm. tubes.
The variation of color intensity with time of development is shown for each reagent in Fig. 1. Although maximum color intensity is a function of $H_2SO_4$ concentration, it is evident, too, that color stability varies inversely with the concentration of this reagent. It can also be seen that the time required for development of maximum color intensity decreases with increasing concentration of $H_2SO_4$. A reagent consisting of equal volumes of $H_2SO_4$ and acetic acid possesses the advantages of satisfactory sensitivity, adequate color stability, and a reasonable period for development of color (20 minutes). A maximum of 1 per cent variation in color intensity is observed during the 15–25 minute interval following addition of the reagent.

Relation between Cholesterol Concentration and Absorbance

Varying volumes of a standard solution containing 2 mg. cholesterol per ml. glacial acetic acid were measured into tubes. All volumes were adjusted to 0.8 ml. by the addition of glacial acetic acid. Following the addition of 0.2 ml. of water to each tube, each determination was completed in the usual manner. It is evident from Fig. 2 that Beer's law is closely obeyed at 620 m$\mu$, within the useful operating ranges of the Klett
photoelectric colorimeter and of the Coleman Junior spectrophotometer. These curves have been found to be closely reproducible for a given instrument. Although the use of these curves for the calibration of other instruments of the same manufacture is not recommended, they may serve as a guide to expected readings.

**Relative Chromogenicity of Free Cholesterol and Cholesterol Esters**

One hundred milliliters of $5.17 \times 10^{-4} M$ solutions of recrystallized preparations of cholesterol (m.p. 148.5°), cholesteryl acetate (m.p. 113.5°), cholesteryl stearate (m.p. 79.5°), and cholesteryl olate (m.p. 39°) were prepared by dissolving 200.0, 221.8, 337.8, and 336.8 mg., respectively, of these substances in 100 ml. of chloroform. Two-tenths ml. of each solution was treated successively with 0.8 ml. glacial acetic acid, 0.2 ml. water, and 4.0 ml. acetic anhydride. Each analysis was then completed in the usual manner. Samples of the same sterol solutions
Table 1. Comparison of Readings Obtained with Cholesterol and Cholesterol Esters (Klett, Filter 62)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Proposed method</th>
<th>Bloor method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reading</td>
<td>Average</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>199</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>239</td>
</tr>
<tr>
<td>Cholesteryl acetate</td>
<td>198</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>260</td>
<td>268</td>
</tr>
<tr>
<td>Cholesteryl stearate</td>
<td>204</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>303</td>
</tr>
<tr>
<td></td>
<td>205</td>
<td>301</td>
</tr>
<tr>
<td>Cholesteryl oleate</td>
<td>196</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>197</td>
<td>260</td>
</tr>
</tbody>
</table>

Table 2. Recovery of Cholesterol Added to 0.2 ml Serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Present (mg.)</th>
<th>Added (mg.)</th>
<th>Found (mg.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.326</td>
<td>0.300</td>
<td>0.530</td>
<td>100.3</td>
</tr>
<tr>
<td>2</td>
<td>0.326</td>
<td>0.400</td>
<td>0.716</td>
<td>98.6</td>
</tr>
<tr>
<td>3</td>
<td>0.326</td>
<td>0.400</td>
<td>0.728</td>
<td>100.3</td>
</tr>
<tr>
<td>4</td>
<td>0.326</td>
<td>0.400</td>
<td>0.728</td>
<td>100.3</td>
</tr>
</tbody>
</table>

were also treated in chloroform with Liebermann-Burchard reagent according to a modification (14) of the original Bloor technic (2).

From the results shown in Table 1, it can be seen that equivalent concentrations of free cholesterol, cholesteryl acetate, cholesteryl stearate, and cholesteryl olate produce essentially equal color intensities when the technic described by the authors is followed. On the other hand, when color development is carried out in chloroform, the esters of cholesterol produce considerably more color than does the free alcohol.

Recovery of Cholesterol Added to Serum

To individual 0.2-ml. aliquots of a serum sample were added 0, 0.1, and 0.2 ml. of a standard solution containing 0.2 mg. of cholesterol per 0.1 ml. of acetic acid. Following adjustment of the total volume to 1 ml. by the addition of glacial acetic acid, each sample was analyzed for cholesterol in the usual manner. Satisfactory recovery of added cholesterol is shown in Table 2.

Effect of Illumination on Color Development

Five samples of serum were analyzed for cholesterol by the procedure adopted by the authors. During color development, illumination was
Table 3. **Comparison of Cholesterol Values Obtained after Development of Color in the Dark and Under Strong Illumination**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Illuminated development</th>
<th>Dark development</th>
<th>Deviation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>106</td>
<td>260</td>
<td>258</td>
<td>+0.8</td>
</tr>
<tr>
<td>111</td>
<td>260</td>
<td>264</td>
<td>-1.5</td>
</tr>
<tr>
<td>122</td>
<td>220</td>
<td>225</td>
<td>-2.2</td>
</tr>
<tr>
<td>139</td>
<td>151</td>
<td>154</td>
<td>-1.9</td>
</tr>
<tr>
<td>170</td>
<td>290</td>
<td>292</td>
<td>-0.7</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>240</td>
<td>242</td>
<td>±1.5</td>
</tr>
</tbody>
</table>

Table 4. **Serum Cholesterol Values as Obtained by the Proposed Method and by the Schoenheimer-Sperry Method**

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Mg. cholesterol per 100 ml. serum</th>
<th>Deviation (%)</th>
<th>Serum no.</th>
<th>Mg. cholesterol per 100 ml. serum</th>
<th>Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>775</td>
<td>115</td>
<td>109</td>
<td>+5.5</td>
<td>728</td>
<td>241</td>
</tr>
<tr>
<td>101</td>
<td>150</td>
<td>143</td>
<td>+4.9</td>
<td>95</td>
<td>241</td>
</tr>
<tr>
<td>783</td>
<td>152</td>
<td>158</td>
<td>-3.8</td>
<td>196</td>
<td>250</td>
</tr>
<tr>
<td>723</td>
<td>153</td>
<td>154</td>
<td>-0.7</td>
<td>700</td>
<td>262</td>
</tr>
<tr>
<td>709</td>
<td>170</td>
<td>181</td>
<td>-6.1</td>
<td>718</td>
<td>269</td>
</tr>
<tr>
<td>169</td>
<td>176</td>
<td>184</td>
<td>-4.3</td>
<td>726</td>
<td>269</td>
</tr>
<tr>
<td>730</td>
<td>177</td>
<td>171</td>
<td>+3.5</td>
<td>83</td>
<td>280</td>
</tr>
<tr>
<td>193</td>
<td>179</td>
<td>178</td>
<td>+0.6</td>
<td>96</td>
<td>254</td>
</tr>
<tr>
<td>909</td>
<td>183</td>
<td>186</td>
<td>-1.0</td>
<td>758</td>
<td>269</td>
</tr>
<tr>
<td>767</td>
<td>208</td>
<td>198</td>
<td>+5.0</td>
<td>744</td>
<td>316</td>
</tr>
<tr>
<td>162</td>
<td>212</td>
<td>219</td>
<td>-3.2</td>
<td>90</td>
<td>328</td>
</tr>
<tr>
<td>743</td>
<td>221</td>
<td>233</td>
<td>-5.1</td>
<td>761</td>
<td>336</td>
</tr>
<tr>
<td>722</td>
<td>232</td>
<td>236</td>
<td>-1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVERAGE</td>
<td>228</td>
<td>228</td>
<td>±3.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

provided by diffused daylight reinforced by 1120 watts of fluorescent room lighting at a distance of 4 to 8 feet. In a duplicate series the Liebermann-Burchard color was developed in a dark cabinet. Comparative results, as summarized in Table 3, show that even relatively strong lighting exerts no appreciable effect on color intensity.
Comparison of Proposed Method with Schoenheimer-Sperry (19) Procedure

Twenty-five randomly selected samples of serum, obtained from hospitalized patients, were analyzed for cholesterol according to the proposed technic, as well as by the method of Schoenheimer and Sperry. The averages of duplicate values, listed in Table 4, show good agreement between both methods. No trend toward higher or lower values is shown by either procedure. Ten values were higher than the corresponding Schoenheimer-Sperry results by an average of 3.4 per cent; fourteen values were correspondingly lower by an average of 3.1 per cent. The degree of correspondence of values for individual samples, as obtained by each method, as well as the coincidence of the average for each group (228 mg. per 100 ml.), supports the validity of the proposed method. The agreement of results also demonstrates that extraction of serum cholesterol is complete and that both methods are equally specific for cholesterol. The results further confirm the reported finding of equal chromogenicity of free and esterified cholesterol.

DISCUSSION

Functions of Reagents Employed

Although purified free cholesterol and its esters are soluble in the usual lipid solvents (i.e., petroleum ether, diethyl ether, chloroform, etc.), these solvents are not suitable for the direct extraction of esterified and so-called free cholesterol from serum. Both forms must first be freed from their associated lipoproteins before extraction can be effected. Mixtures of ethanol and ether (2, 3, 5, 7, 16, 28) and of ethanol and acetone (19, 23, 24) are effective extracting agents, but these solvents interfere with the Liebermann-Burchard reaction. For example, ethanol, in the presence of H₂SO₄, adversely affects the determination by removing a considerable portion of essential acetic anhydride through esterification. These solvents, when present, must therefore be removed before color development. On the other hand, glacial acetic acid is well suited to a direct technic for cholesterol, since it facilitates quantitative extraction of serum cholesterol, yet it does not interfere with the Liebermann-Burchard reaction. A 4:1 ratio of glacial acetic acid to serum suffices for complete extraction. Lower ratios may result in incomplete extraction and may also cause the mixture to gel. No significant extraction of cholesterol is effected by acetic anhydride alone.

Acetic anhydride, in the presence of acetic acid, fulfills the multiple functions of cholesterol extraction, precipitation of serum protein, and
furnishing a medium suitable for color development. Acetic anhydride also facilitates production of the necessary anhydrous system before addition of color reagent (4). Because the intensity of color produced is a function of the concentration of acetic anhydride, adequate control of this concentration as well as of the total water content is essential.

The deleterious effect on the Liebermann-Burchard reaction of relatively small quantities of water is well known (5, 15, 18). The effect is due to the presence in the same system of two mutually reactive substances: water and acetic anhydride. The addition of a strong acid, as H₃SO₄ in the Liebermann-Burchard reagent, to such a mixture would serve to catalyze the strongly exothermic hydrolysis of acetic anhydride. The temperature would rise so rapidly that its adequate control, an essential condition for reproducibility of absorbence readings (5, 7, 18, 19, 22), could not be maintained. It is therefore necessary to effect dehydration before color development is begun. This may be accomplished by allowing the extract to stand overnight at room temperature, by heating the extract for 30 minutes in a boiling water bath (4), or most simply by means of the dehydrating reagent. The quantity of H₃SO₄ chosen for this purpose is sufficient to catalyze the reaction between water and acetic anhydride, yet it is not adequate for the initiation of color development. After dehydration, a sufficient excess of acetic anhydride is available for the Liebermann-Burchard reaction.

The H₃SO₄-acetic acid reagent is basically a modified Liebermann-Burchard reagent. It is designed to provide adequate sensitivity and stability of color for the conditions of analysis adopted by the authors. Compared with mixed H₃SO₄-acetic anhydride reagents, it possesses important advantages of stability combined with minimum hazard attendant upon its use. While H₃SO₄-acetic anhydride mixtures must be used shortly after they have been prepared (21, 23), the proposed modified reagent has shown no measurable deterioration during long periods of storage at room temperature. Further, contrary to experience with the mixed Liebermann-Burchard reagent, no violent reactions are occasioned by accidental contamination of the modified reagent with water. When the reagent is added to a mixture of acetic anhydride and acetic acid, no appreciable rise in temperature is observed, nearly all of the heat of reaction of H₃SO₄ with these reagents having been previously expended during preparation of the H₃SO₄-acetic acid reagent. Development of color, therefore, proceeds under essentially isothermal conditions.
Advantages of Deproteinization

Although we have been able to apply the proposed technic to the determination of cholesterol without prior removal of protein, this modification is not recommended. If the precipitated protein is not removed by centrifugation, it will eventually redissolve upon addition of the H₂SO₄-acetic acid reagent, with simultaneous development of the characteristic Liebermann-Burchard color. However, this modification is characterized by lower color intensity and higher serum blanks due to protein-associated pigments. Only about 90 per cent of expected Liebermann-Burchard readings for cholesterol are observed in the presence of serum proteins. A similar finding is available in Fig. 1 of the paper by Pearson, Stern, and McGavack.

Use of Decanted Protein-free Extract Without Measurement of Its Volume

Ten samples of serum were treated in weighed tubes with glacial acetic acid and acetic anhydride according to the described procedure. The volume of residue which remained in each tube after centrifugation and decantation, as determined by weighing and correction for density, averaged 0.23 ± 0.02 ml. Thus, whereas the addition of 1 ml. of H₂SO₄-acetic acid reagent to the decanted extract reduces the relative concentration of cholesterol to 82.7 per cent (i.e., 4.77/5.77), the standard, which is not decanted, is correspondingly diluted to 83.3 per cent (i.e., 5/6). For accurate results, therefore, a small correction factor, 1.007 (i.e., 0.833/0.827), may be applied in calculation. The average error attendant upon the use of this factor is less than 0.1 per cent. This device serves to simplify operations and to minimize handling and pipetting of a somewhat unpleasant mixture. When tubes of dimensions other than those recommended are employed, a new correction factor should be determined experimentally. For more critical studies, a correction factor may be dispensed with by mixing 4.0 ml. of the protein-free extract with 0.8 ml. of H₂SO₄-acetic acid reagent.

Determination of Free Cholesterol

The determination of free cholesterol is not described in detail, since technics adequate for the isolation of the free sterol are available (19, 22, 24). In our laboratories the ether-washed precipitate of cholesterol digitonide (22) is dissolved in 0.8 ml. of glacial acetic acid at 70°. After the addition of 0.2 ml. of water and 4.0 ml. of acetic anhydride, the technic described for total cholesterol is followed.
Comparative Chromogenicity of Free and Total Cholesterol

It must be emphasized that all studies of the Liebermann-Burchard reaction which have demonstrated greater chromogenicity for esterified cholesterol were conducted in chloroform solutions (5, 7, 8, 15, 23, 26, 29). Although it may seem reasonable to suppose that similar differences in chromogenicity would also appear in an acetic anhydride–acetic acid medium, the results of our studies, summarized in Table 1, prove equal chromogenicity. Concordant findings have been reported by others (1, 13, 17). Since a side-by-side comparison of both sets of conditions for color development has apparently not been reported, it seemed desirable, also, to apply the Bloor technic (2) to solutions of free and esterified cholesterol in chloroform. The magnitude of the deviations from equal chromogenicity in chloroform solution, as shown in Table 1, is in essential agreement with the reported findings of others (5, 7, 29). The need for saponification of cholesterol esters is, therefore, determined by the nature of the solvent in which the Liebermann-Burchard reaction is to be performed. When the solvent is chloroform, a saponification step is required; for color development in acetic anhydride–acetic acid medium saponification is superfluous.

Specificity

A direct study of the specificity of the proposed method has not been made, but the work of Abell et al. may be cited in this connection. By application of a countercurrent distribution technic, these investigators demonstrated that more than 99 per cent of the material in serum which responded to the Liebermann-Burchard test was actually cholesterol. Excellent agreement with the method of Schoenheimer and Sperry was reported, indicating equal specificity for the latter technic. Table 4 also shows equally good agreement between our proposed method and that of Schoenheimer and Sperry. All three procedures, therefore, appear to be equally specific for cholesterol in serum.

General Considerations

Success in application of the recommended technic is largely dependent upon adequate control of the concentration of water. The same initial volume of water, 0.2 ml., should therefore be present in the standard as well as in the test sample. For this purpose, no significant error is made if the water content of serum is assumed to be 100 per cent. Subsequent accidental inclusion of water is to be scrupulously avoided. Similarly, all stored reagents should be kept in tightly stoppered bottles. The presence
of water produces a change in the proportion of acetic anhydride to acetic acid in the final solution. Since 0.2 ml. of water reacts with 1.05 ml. of acetic anhydride to produce 1.27 ml. of acetic acid, the relative volumes of acetic anhydride to acetic acid are altered from 4.0:0.8 to 2.95:2.07 upon hydrolysis (or dehydration). For best results this final ratio should be maintained constant, because the intensity of color as well as the rate of color development varies with the concentration of acetic anhydride.

It is characteristic of the various modifications of the Liebermann-Burchard reaction that the rate of decomposition of the derivative of cholesterol which is responsible for color is generally quite appreciable in comparison to its rate of development. The existence of a peak, in place of a more suitable level plateau, in the time-absorbence curve necessitates a fixed period of color development prior to absorbence measurement. The method of Schube, which utilizes the presumably more stable "end-product" brown color for measurement has been reported to produce a wide variation in results (23). The degree of stabilization of color which is attained by the present procedure compares favorably with other methods. According to Fig. 1, the greatest variation in absorbence is 1.1 per cent during the 15–25 minute interval following the addition of reagent, and only 0.3 per cent during the 18–22 minute interval. Somewhat better stabilization may be achieved by substituting for the 1:1 reagent an alternate reagent, prepared with 1 volume of concentrated H₂SO₄ and 3 volumes of glacial acetic acid. With this reagent a maximum variation of 0.3 per cent in absorbence was observed during the interval: 45–55 minutes after initiation of color development. This modification would be useful when 20 to 50 specimens are to be analyzed in sequence.

Correction for the blank absorbence of normochromic serums is relatively small. However, inclusion of a serum blank is recommended for improved accuracy. Because the addition of dehydrating reagent occasionally causes turbidity, this reagent is normally omitted from the serum blank. Test samples are not affected by this turbidity, since it disappears readily upon addition of the H₂SO₄-acetic acid reagent. When hyperbilirubinemic serums are analyzed, however, the dehydrating reagent should be added to the serum blank, because H₂SO₄ in the modified color reagent causes a significant alteration in the color of bilirubin. No turbidity has been observed following the addition of dehydrating reagent to hyperbilirubinemic serums.

Some modifications of the basic procedure have been discussed. They
include an extension of the period of color development, if desired, by employing a modified color reagent, prepared with 1 volume of H₂SO₄ and 3 volumes of acetic acid. Also, the correction factor, 1.01, may be eliminated by adding 0.8 ml. of H₂SO₄-acetic acid reagent to 4.0 ml. of serum extract plus 1 drop of dehydrating reagent. Other modifications may readily suggest themselves. When an abnormally high cholesterol value is anticipated, less than 0.2 ml. of serum may be taken for analysis. In this instance, water sufficient to bring the total volume to 0.2 ml. is to be added. Microadaptations may be applied simply by proportional reduction of the standard volumes of all solutions. For example, 0.05 ml. of serum is treated with 0.2 ml. of glacial acetic acid and 1.0 ml. of acetic anhydride in a small tube. After centrifugation, 1.0 ml. of clear supernatant fluid is transferred to a small cuvet, e.g., Coleman No. 6-310; it is then treated with 0.02 ml. of a 5 per cent solution (v/v) of H₂SO₄ in acetic acid, to effect dehydration. Color is developed with 0.2 ml. of H₂SO₄-acetic acid reagent for 20 minutes at 25°. The analysis is completed in the usual manner by measuring absorbences of the sample, a suitable standard, and a serum blank against a reagent blank.

SUMMARY

An accurate yet simple procedure for the determination of total cholesterol, based upon the application of a Liebermann-Burchard color reaction directly in the solvent employed for extraction of cholesterol from serum, has been described. Extraction of cholesterol and removal of protein are accomplished by means of acetic acid and acetic anhydride. Serum water is removed by the acid-catalyzed hydrolysis of acetic anhydride. The Liebermann-Burchard color is then developed with a stable, modified reagent consisting of equal volumes of H₂SO₄ and acetic acid.

Excellent agreement with the technic of Schoenheimer and Sperry is obtained. Equal intensities of color are produced by equivalent concentrations of free and esterified cholesterol. Preliminary saponification of cholesterol esters is therefore not required. Color development may proceed in ordinary room lighting without loss of accuracy.

REFERENCES