29. THE COLORIMETRIC ESTIMATION OF TOTAL CHOLESTEROL IN WHOLE BLOOD, SERUM, PLASMA AND OTHER BIOLOGICAL MATERIAL

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SINCE Autenrieth & Funk [1913] and Weston [1917] first drew attention to its inaccuracies, the colorimetric method for the estimation of cholesterol based on the Liebermann-Burchard reaction [Grigaut, 1910] has undergone many modifications. It has been the author's experience, however, that no method yet published has succeeded in entirely overcoming the difficulties mentioned by these authors.

With the majority of modern photoelectric colorimeters the colour produced in the test solution alone is measured and the required result is obtained by consulting a previously drawn calibration chart, not by comparing the colour with that of a standard simultaneously developed. It is essential therefore that the colour be exactly reproducible. With this in mind a study was made of existing methods of colour development [Bloor, 1913-14; 1914; 1915; 1916; 1928; Autenrieth & Funk, 1913; Cornell, 1928; Schoenheimer & Sperry, 1934; Reinhold & Sheils, 1936], with a view to adapting them for use with the photoelectric colorimeter. Using a standard solution of cholesterol in chloroform, not one of the above methods gave satisfactorily reproducible results, and furthermore, when the standard solution was replaced by a cholesterol solution prepared from blood by existing methods of extraction [Bloor, 1913-14; Sackett, 1925; Myers & Wardell, 1918; Reinhold & Sheils, 1936], the variations were accentuated in all cases.

The object of this investigation was therefore to examine the factors influencing the accuracy of the colorimetric method for the estimation of cholesterol and to utilize the results obtained to devise a more accurate method.

The investigation fell into two sections: the study of (a) methods of extraction of cholesterol from blood; (b) methods for the development of the colour.

Extraction methods. Two general methods exist for the extraction of cholesterol: (1) chloroform extraction of blood dried by heat or by dehydrating agents [Myers & Wardell, 1918; De Toni, 1926; Leiboff, 1924; Ling, 1928; Reinhold & Sheils, 1936]; (2) extraction from fresh blood by means of alcohol, alcohol-ether, acetone etc. These latter methods are generally accepted as being more complete, and that due to Bloor [1913-14] is the one most frequently used. Their great disadvantage lies in the fact that the extract has a yellow brown colour which leads to high and erratic results [Mueller, 1916; Weston, 1917; Luden, 1917]. Various methods have been suggested for the elimination of this interference. Mueller [1916] washed the chloroform extract with water and then dried it with anhydrous sodium sulphate. Stoddart & Drury [1929] and others have advocated saponification before solution in chloroform. Sackett [1925] claimed that the colour

could be avoided if the ether-alcohol extraction was carried out without heating. Cantoni [1930] suggested adding a mixture of oleic and palmitic acids to the standard to compensate for the colour. Bloor [1928] advised the use of a red glass filter in the colorimeter when there was any serious degree of discoloration. Schoenheimer & Sperry [1934] and Kelsey [1939] avoid this difficulty by saponifying the extract and removing the cholesterol as digitonide, which is then purified and analysed.

Methods for the development of the colour. The following are the conditions laid down in existing methods for the control of the development of the green colour produced by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution:

Bloor [1913-14; 1928]. Stand for 15 min. in the dark or in the light in which the comparison is to be made—no temperature given.

Autenrieth & Funk [1913]. 15 min. at 35°.

Weston [1917]. Add H_2SO_4 , mix, stand in dark 30 min., then add the acetic anhydride and stand 15 min.

Cornell [1928]. 15 min. away from light at laboratory temperature.

Schoenheimer & Sperry [1934]. Warm solution to 25° before adding reagents, develop colour in water bath at 25° and read between 27-37 min. The amount of H_2SO_4 added is described as 0.1 ml. (or 4 uniform drops).

Reinhold & Sheils [1936]. 30 min. at 25° in the same light in which the comparison is to be made. The solution is not previously heated but warm acetic anhydride-sulphuric acid mixture is added.

Kelsey [1939]. Set in water bath at 23° for 15 min. under moderate conditions of lighting.

The factors concerned are therefore: light, concentration of reagents and of cholesterol itself, temperature and time, and one other not mentioned above, namely, the condition of the cholesterol whether free or esterified. Some of the above methods use extracts prepared directly from blood and therefore containing both esterified and free cholesterol, others use saponified extracts containing only free cholesterol. Gardner & Williams [1921] found that esterified cholesterol reacted at a greater rate than free cholesterol and that cholesteryl acetate gave an 'apparent cholesterol' 10% greater than an equivalent amount of free cholesterol; cholesteryl benzoate gave values 4.6% greater than free cholesterol. Kelsey [1939] found that the colour produced by cholesteryl palmitate was 130% of that produced by an equivalent amount of free cholesterol. Reinhold & Sheils [1936], using their method of colour development, confirmed the findings of Gardner & Williams [1921] and reported an 'apparent cholesterol' for cholesteryl palmitate of 120%. Yasuda [1936] has reported the palmitate as giving 24.5%, the stearate 25.2% and the oleate 27.6% greater values than an equivalent amount of free cholesterol.

EXPERIMENTAL

The factors controlling the development of the colour were studied first in order to try to evolve a standard method which could be used to compare the various extraction methods. Each factor was investigated in turn while the others were kept as constant as possible.

Light. It was found that light had a marked effect on the colour development, as will be seen in the following experiment, in which two series of exactly similar solutions of cholesterol were treated alike in every respect except that the colour development in one series took place in the dark and in the other in the light.

	Colour developed in light Absorption density 640 m μ	Colour developed in dark Absorption density 640 m μ
1	0.357	0.387
2	0.362	0.387
3	0.355	0.387
4	0.333	0.389
5	0.352	0.382

The series developed in the light, it will be seen, gave paler solutions which varied in intensity, due probably to unequal lighting. Hence in all subsequent experiments the colour was allowed to develop in the dark.

Concentration of reagents. Alteration of the concentration of the reagents, particularly that of H_2SO_4 , gave rise to changes in the final colour.

	ml.	Absorption density 640 m μ
Solution A	4.7 standard cholesterol solution	0.387
	0.2 chloroform	
	0.1 conc. H_2SO_4	
	2.0 acetic anhydride	
Solution B	4.7 standard cholesterol solution	0.373
	0.1 chloroform	
	0.2 conc. H_2SO_4	
	2.0 acetic anhydride	
Solution C	4.7 standard cholesterol solution	0.367
	0.3 conc. H_2SO_4	
	2.0 acetic anhydride	

This difficulty can only be overcome by standardizing the amounts of reagents present. In this investigation 2 ml. of a mixture, prepared not more than 10 min. previously, of acetic anhydride A.R. (20 ml.) and conc. H_2SO_4 A.R. (1 ml.) were added to every 5 ml. of chloroform solution of cholesterol.

The concentration of cholesterol itself is another factor to be considered. Methods quoted above use concentrations varying from 3.0 to 0.2 mg. per 5 ml. chloroform (i.e. amounts roughly equivalent to those present in 1.5–0.1 ml. of normal serum). Concentrations of 0.2–0.6 mg. per 5 ml. were taken in these experiments, largely because the depth of colour produced was convenient for the photoelectric colorimeter used. Later experiments showed that with higher concentrations of cholesterol the colour formation tended to become erratic.

The two remaining factors temperature and time were considered together. Fig. 1 shows the relation between absorption density and time at 18°, 25° and 35°, all other factors being standardized.

Each point was determined under the following conditions. To 5 ml. of a standard solution (=0.289 mg.) of cholesterol in chloroform at 18°, 25° or 35° were added 2 ml. of a mixture of acetic anhydride and conc. H_2SO_4 (20 : 1) freshly prepared and warmed to the same temperature. After careful mixing the solution was placed in a beaker of water maintained at the required temperature in the dark and removed at the expiration of the required time. The same tubes were used throughout, being as far as possible of the same thickness, to prevent irregularities of heating. The readings were taken on a Bolton-Williams type of photoelectric colorimeter, using as the blank 5 ml. of chloroform with 2 ml. of acetic anhydride-sulphuric acid mixture which had been treated in the same way as in the test. The absorption of light (640 m μ) by a

layer of solution 1 cm. in thickness was measured and the result was converted into absorption density (which is proportional to cholesterol content).

It will be seen from the curve that the colour is partially destroyed by heat, the maximum point attained decreasing with temperature. The curve for 35° shows that this temperature is obviously unsuitable for colorimetric purposes, the changes of colour being too fast to give adequate time for a reading to be made.

Fig. 1. Colour development charts for free cholesterol at 18°, 25° and 35°.

The same may be said with less force of the 25° curve, the rate of change corresponding to as much as 2.1 mg./100 ml. in the original blood per min. at 30 min.

Further, 25° is a difficult temperature to maintain exactly in a small laboratory without special apparatus for the purpose. Small differences in heating due to outside factors such as differences in the thickness of the tubes cause appreciable variations. At 25° and 30 min. twenty-five estimations gave: average absorption density $0.209 (145) \pm 0.015$; max. 0.223 (155) and min. 0.190 (131). (The figures in brackets give the approximate equivalent in terms of mg./100 ml. normal serum.)

The optimum temperature proved to be 18° and the time 60 min., the fading of the colour being sufficiently slow to permit an accurate reading to be made, even when a number of estimations are being carried out at the same time. It was also quite easy to maintain this temperature. Results, using these conditions, were exactly reproducible. Twenty-five estimations on the same solution gave an average absorption density $0.234 (145) \pm 0.002$ with max. 0.236 (147) and min. 0.232 (143).

Another factor not yet mentioned is the presence of water in the reagents, particularly the chloroform. Previous methods have insisted on the use of water-free reagents without giving any explanation for this precaution. This is really only another demonstration of the influence of temperature on the reaction. The heat evolved by the addition of conc. H_2SO_4 to chloroform containing only traces of water is sufficient to accelerate the colour development and the subsequent fading and hence lower the final result. All reagents should therefore be completely water-free.

Condition of the cholesterol. The last factor, namely, the state of the cholesterol, was investigated as follows. In each of four 100 ml. conical flasks were placed 30 ml. of chloroform containing 1.794 mg. of cholesterol as (1) free cholesterol, (2) cholesteryl acetate, (3) cholesteryl oleate, (4) cholesteryl stearate. The four flasks were then placed in the dark in a water bath maintained at 18°. To each were added 12 ml. of a freshly prepared mixture of acetic anhydride and conc. H_2SO_4 cooled to 18°. The absorption of light of wave-length $640\text{ m}\mu$ by each of the solutions was measured at frequent intervals and a chart prepared showing the relation between time and absorption density (colour) of the solutions (Fig. 2).

Fig. 2. Colour development charts for cholesterol and its esters (18°). Key: Cholesterol —. Cholesteryl oleate x—x. Cholesteryl acetate — — —. Cholesteryl stearate — · —.

The following points are worthy of note.

- (1) The four curves are similar in shape.
- (2) All esters show a faster colour development, a deeper maximum colour, and a faster colour fading than free cholesterol.
- (3) The difference between cholesterol and its esters is greatest in the region of maximum colour development and diminishes as the colours fade, tending to become constant; readings taken after 24 hr. still showed higher values for esters than free cholesterol.
- (4) Each ester has its own characteristic rate of colour development and also maximum colour. After a certain time the rate of colour fading is approximately the same in all cases.
- (5) This experiment was repeated at various temperatures, but no conditions could be found in which the same amount of cholesterol gave an identical colour in all four forms.

The above facts explain the great diversity in the 'apparent' cholesterol values obtained by previous workers for the various esters. Using the conditions

specified above, the 'apparent' cholesterol values for cholesteryl stearate, oleate and acetate are respectively 119, 103.2 and 109.2 % of that for free cholesterol.

The obvious method of eliminating this difference between the various forms is to convert them into one common form; saponification presents the easiest way of doing this. Difficulties were encountered in adapting existing methods. To test them, two chloroform solutions were prepared, each containing the same weight of cholesterol per unit volume, one as free cholesterol, the other as cholesteryl stearate. 5 ml. portions of the cholesteryl stearate solution were taken and after removal of chloroform subjected to the various procedures suggested for the saponification of blood fats; they were then redissolved in 5 ml. chloroform. The 'apparent' cholesterol content of the saponified extracts and also that of 5 ml. of the original cholesteryl stearate solution were determined colorimetrically (using the procedure developed above) by comparison with the colour simultaneously developed by 5 ml. free cholesterol solution.

The following are some typical results taken at random from a large number of analyses to illustrate the type of results obtained.

Material	Method of saponification	'Apparent' cholesterol mg.		Remarks
		(a)	(b)	
0.386 mg. free cholesterol	None	0.386	0.386	Blank
0.652 mg. cholesteryl stearate	None	0.463	0.463	Actual cholesteryl content = 0.386 mg.
0.386 mg. free cholesterol	Aqueous KOH (10 %)	0.386	0.386	Test of extraction by chloroform (<i>v. infra</i>)
"	"	0.384	0.386	
0.653 mg. cholesteryl stearate	Aqueous KOH (10 %), 3 hr. in sealed flask	0.415	0.422	Chloroform extraction. Extract colourless
0.652 mg. cholesteryl stearate	1.0 <i>N</i> sodium ethylate [Bloor, 1928]	0.360	0.370	Light petroleum extraction. Extract yellow
"	"	0.410	0.418	Chloroform extraction. Extract yellow
"	Alcoholic (70 %) KOH (10 %), 3 hr. at 40°, alcohol evaporated off at 70°	0.431	0.423	Chloroform extraction. Extract yellow
"	Alcohol-acetone KOH (10 %), 3 hr. at 40°, alcohol-acetone evaporated off at 70°	0.425	0.440	Chloroform extraction. Extract yellow
"	2 ml. aqueous KOH (10 %), 1 ml. amyl alcohol, 90 min. at 100° in stoppered flask	0.386	0.386	Chloroform extraction. Extract colourless

A serious difficulty encountered here was the fact that in the majority of the methods, after saponification the cholesterol is not extracted from the aqueous-alcohol media as such but is precipitated as cholesteryl digitonide. Early experiments after the method of Bloor [1928], using light petroleum as solvent, gave rather low results (see table). Light petroleum, while being a good general fat solvent, is not a sufficiently good one for cholesterol. Attempts were therefore made to extract cholesterol quantitatively from strong aqueous alkaline solutions by means of chloroform, which is easily the best cholesterol solvent. As will be seen from the table, 100 % recovery was obtained of cholesterol added to 2 ml. portions of a 10 % aqueous solution of KOH. Details of the actual technique used are given later in the section on method.

The general conclusion to be drawn from the above table is that alcohol is unsuitable as a solvent during saponification for colorimetric analysis, since,

wherever it is used, the final chloroform extract is tinted yellow, and since this colour is increased by heat it is difficult to apply sufficient heat to the system to keep the cholesterol in solution and to obtain complete saponification. Amyl alcohol, on the other hand, although less miscible with water has the advantage that it is less volatile and no colour is produced during saponification. 2 ml. of 10% aqueous KOH and 1 ml. of amyl alcohol at 100° in a closed flask for 90 min. completely saponified 0.652 mg. of cholesterol stearate. Complete saponification of cholesteryl acetate and oleate was also obtained by this same method. The application of the method to cholesterol-containing extracts prepared from blood will be considered after the next section.

Extraction methods. Only the standardized method of colour development evolved above was used in the analysis of the various extracts referred to in this section.

All methods involving the drying of blood gave consistently low results and poor recovery of added cholesterol. It was found difficult, if not impossible, to obtain complete extraction of cholesterol under these conditions, a conclusion agreeing with that of other workers in this field.

As regards 'wet' methods, the yellow colour of the extract already referred to presents a big difficulty. Below are some of the results obtained in experiments carried out to test the suggestions quoted earlier for removing it.

The colours of chloroform extracts prepared from the same serum by the various methods were compared in terms of their absorption of light of wavelength 440 m μ .

Extraction method	Method of purification	Percentage absorption of light, 440 m μ	
		(a)	(b)
Hot ether-alcohol	None	9.0	9.5
Hot ether-alcohol	Mueller's method	9.0	9.0
Hot ether-alcohol	Saponification (sodium ethylate)	11.0	12.5
Cold ether-alcohol	None	7.0	6.5

The remaining methods were found to be equally ineffective in overcoming the difficulty. It will be noted that results from Sackett's method indicate that heating in Bloor's original method increases the colour, as also does saponification with sodium ethylate.

Theoretically the procedure advocated by Schoenheimer & Sperry [1934] and Kelsey [1939], viz. saponification of extract, removal of cholesterol as digitonide, its saponification and finally its analysis, is more accurate, but it is open to criticism from a number of points of view. For clinical purposes it is too long and complicated and the number of stages involved tends to increase the possibility of error. In practice it was found difficult to isolate quantitatively so small an amount of the very light particles of cholesteryl digitonide merely by centrifuging them. The method of colour development is also open to criticism; in addition to the points already noted, i.e. time, temperature, concentration of reagents etc., it was noted that the digitonin present in the solution after the liberation of the cholesterol from the digitonide itself gave a reddish-purple colour with the acetic anhydride-sulphuric acid mixture. To compensate for this Schoenheimer & Sperry advocated the use of colour filters. My experiments on these lines indicated that whatever colour screen was used some light absorption could always be detected.

It was therefore decided to revert to Bloor's original ether-alcohol extract and endeavour to find some method whereby it could be decolorized without loss of cholesterol. This was finally accomplished as follows. When a yellow-brown chloroform extract prepared from blood by Bloor's method was passed through

a column of finely divided silica gel, the issuing fluid was found to be completely colourless. When solutions of pure cholesterol were passed through, followed by chloroform washings, 100 % recovery of cholesterol was obtained in every case.

The following are some typical results.

	Optical colorimeter			Photoelectric colorimeter		
	A	B	C	A	B	C
Bloor's extract untreated, coloured	205	196	220	194	190	216
Ditto after silica treatment, colourless	190	187	210	190	188	212
Ditto plus added cholesterol equivalent to 50 mg./100 ml. after silica treatment, colourless	240	237	260	240	238	262

As would be expected results are lower after the removal of the interfering colour. The fact that the difference is less marked in the case of the photoelectric colorimeter can be accounted for as follows. The green colour was measured in terms of its percentage absorption of light of wave-length 640 m μ (red brown); the percentage absorption of this wave-length by the similarly coloured solutions before colour development averaged only 0.8 %, which is equivalent to an apparent cholesterol of 4 mg./100 ml. original serum. This, it will be seen, corresponds with the average difference between the results before and after silica treatment. The brown pigment is apparently not affected by the sulphuric acid-acetic anhydride mixture [cf. Luden, 1917] and therefore in cases where extreme accuracy is not required it is justifiable, in the case of serum or plasma, to omit the silica treatment provided that the colour is measured in terms of its percentage absorption or transmission of light of wave-length 640 m μ . When estimating the cholesterol content of whole blood or red blood cells the interfering colour is much stronger and should always be removed.

The practical details of the silica treatment are given later in the section on method. In addition to blood the method has proved of even greater value in tissue analysis, since the brown colour which is often present in larger amounts in tissue extracts is completely removed by the silica.

Application of saponification method to blood extracts. The cholesterol content of a number of 2 ml. samples of whole blood and serum was determined by the following procedure: extracting with ether-alcohol, filtering, evaporating off the ether-alcohol, extracting the residue with chloroform, decolorizing the chloroform extract, removing the chloroform, saponifying and finally extracting the cholesterol for colorimetric analysis, according to the conditions already noted. Simultaneously, further ether-alcohol extracts of 2 ml. samples of the same blood and serum were prepared and to these were added known amounts of cholesterol or cholesterol ester. The total cholesterol content was then measured in the same manner as above. The results (below) showed a good recovery of added cholesterol, indicating that the figures for whole blood and serum were also consistent.

Material	Serum A, mg. cholesterol		Serum B, mg. cholesterol		Blood C, mg. cholesterol		Blood D, mg. cholesterol	
	1	2	1	2	1	2	1	2
0.2 ml. blood or serum	0.305	—	0.352	—	0.210	—	0.242	—
0.2 ml. blood or serum + 0.193 mg. free cholesterol	0.500	0.195	0.545	0.193	0.404	0.194	0.435	0.193
0.2 ml. blood or serum + 0.193 mg. cholesterol as stearate	0.498	0.193	0.545	0.193	0.405	0.195	0.435	0.193
0.2 ml. blood or serum + 0.193 mg. cholesterol as oleate	0.498	0.193	0.545	0.193	0.403	0.193	0.432	0.190

The figures in the second columns give the actual cholesterol recovery.

METHOD

Preparation of ether-alcohol extract

2 ml. of whole blood, serum or plasma are run slowly into 60 ml. of freshly mixed ether-alcohol (3/2) in a 100 ml. conical flask, the liquid being gently shaken with a swirling motion. The flask is then immersed in hot (80°) water and rotated continuously to prevent superheating. When the contents of the flask have been brought to the boil it is removed and cooled in running water to room temperature. The solution is next filtered into a dry graduated 100 ml. flask, the conical flask and the residue on the paper being washed several times with further quantities of warm ether-alcohol mixture. When cool the combined filtrates are made up to 100 ml. and well mixed.

Removal of colour

50 ml. of this solution are transferred to a 100 ml. squat-form beaker and evaporated just to dryness on a water bath. The residue is then extracted three times, twice with 5 ml. and once with 3 ml. of warm dry chloroform, the extracts being decanted into a small clean dry separating funnel.

A silica adsorption tube is meanwhile prepared as follows. A clean dry test tube $6 \times \frac{1}{2}$ in. is heated in the bunsen flame about 1 in. from the sealed end and drawn out to a capillary (about $\frac{1}{12}$ in. bore) which is cut 1 in. from the main part of the tube. When cool, a lightly packed plug of cotton wool is placed in the tube at the point where it begins to narrow, and the tube is filled to within $\frac{3}{4}$ in. of the top with finely divided pure silica (see below). A second plug of cotton wool is placed on top of the silica. The tube is fitted by means of a rubber stopper into the neck of a side-arm test tube (30 ml. capacity), or small filter flask, which is connected to the filter pump. 5 ml. of dry chloroform are allowed to flow through the adsorption tube and the residue is removed by suction. The receiver is changed and first the chloroform extract and then 15 ml. of pure dry chloroform are allowed to run through (without suction) at the rate of 15–20 drops per min.; the final portion of the chloroform is removed from the silica by turning on the filter pump, slowly at first, then at full pressure, for 5 min. The chloroform extract and washings are completely transferred to a dry 50 ml. graduated flask and made up to volume; the flask is stoppered and well shaken to ensure thorough mixing.

Saponification of esters

10 ml. of chloroform solution are pipetted into a clean dry 120 ml. pyrex conical flask fitted with a ground glass stopper. The flask, unstoppered, is placed in a steamer (if a bacteriological one is not available one of the household kind can be made to serve the purpose), until all the chloroform has been removed. 1 ml. of amyl alcohol is then pipetted into the flask, followed by 2 ml. of 10% aqueous KOH. After its contents have been well mixed by shaking, the flask is replaced in the steamer. Twenty minutes later the stopper is placed in position and the heating continued for 90 min.: from time to time the flask, without being removed from the steamer, is given a swirling motion to agitate its contents. (Care should be taken that the stopper is not blown out.) At the end of the required time the flask is removed from the steamer and the stopper withdrawn while still hot. When the contents of the flask have cooled to 40°, 5 ml. of chloroform are run in and the flask is then shaken with a vigorous swirling motion, care being taken not to spill any of the contents. After allowing a few

minutes for it to settle, the chloroform-water mixture is decanted into a clean dry test tube (thick walled, 30 ml. capacity), the last few drops being washed in with the aid of a chloroform wash bottle. The stoppered flask is then carefully washed out with two 3 ml. portions of warm chloroform, the washings being added to the main chloroform-water mixture in the tube. The test tube is filled to within $\frac{3}{4}$ in. from the top of the tube with distilled water, tightly stoppered and inverted several times. When the two layers have separated, the tube is given a number of sharp taps at about the level of the water meniscus to dislodge the small drops of chloroform that usually remain in the meniscus, and it is then replaced in the rack for a further 3 min. Having made sure that all the chloroform has settled into the lower layer, the bulk of the upper layer is drawn off by means of gentle suction, using a narrow glass tube drawn to a jet, the tip of which is turned upwards. An aqueous layer about $\frac{1}{8}$ in. deep is left above the chloroform and to this are added 10–12 ml. of *N* HCl; the tube is then stoppered and inverted several times and the contents are allowed to settle as before. The HCl washing is repeated twice, followed by three water washings in the same manner. After the final water washings have been removed the chloroform extract is decanted into a clean dry 50 ml. beaker and the tube is washed out twice with 2 ml. portions of chloroform, the washings being added to the main bulk of the solution. The beaker is then placed on a hot water bath at 80° and the extract evaporated just to dryness. The residue is dissolved by warming with 2 ml. of pure dry chloroform, the solution is decanted with the aid of a glass rod and chloroform wash bottle into a small test tube ($5 \times \frac{1}{2}$ in.) graduated at the 5 ml. mark. The beaker is washed out with further portions of chloroform and when cold the combined extracts are made up to 5 ml. with dry chloroform.

Development of colour

The tube is corked and placed in a water bath maintained at 18° and side by side with it is placed a test tube containing a freshly prepared mixture of 20 : 1 acetic anhydride and H_2SO_4 (both A.R.). After 10 min. the two tubes are withdrawn and 2 ml. of the acetic anhydride-sulphuric acid mixture are pipetted into the extract. The contents of the tube are well mixed and the tube is placed in the water bath at 18° in the dark. After 60 min. the tube is removed and its percentage absorption of light of wave-length 640 μ is measured in the Bolton-Williams type of photoelectric colorimeter, using a blank consisting of 5 ml. of chloroform and 2 ml. of acetic anhydride-sulphuric acid mixture. The quantity of cholesterol per 100 ml. of blood or serum can then be read off from calibration charts previously prepared, using standard quantities of cholesterol under the same conditions.

For photometric measurements, using the Pulfrich photometer, one cell is filled with the blank solution and the unknown is pipetted into the other. The percentage transmission (*T*) is read (see manufacturer's directions).

Observed = $(-\log (T/100))/L$, where *L* = length of cell.

$E_{\text{sp.}}$ = specific extinction coefficient of cholesterol.

Then $E/E_{\text{sp.}}$ = mg. cholesterol in sample.

The amount of cholesterol per 100 ml. blood = 500 times the amount in sample.

For the ordinary optical colorimeter, standards containing 0.5, 0.4 and 0.3 mg. cholesterol per 5 ml. are prepared and the colour is developed simultaneously with the test solutions. The test is compared with the standards and the results are obtained by the usual *S/T* ratios.

Preparation of silica for adsorption tube

The more obviously iron-containing particles are removed by hand from ordinary silica gel as purchased. The remaining particles are crushed with the aid of a mortar and pestle and are then transferred to a large flat-bottomed pyrex flask and more than covered with H_2SO_4 (1 vol. acid to 2 vol. water). The flask is next heated with constant shaking for about 20 min. in the fume cupboard. When cold the H_2SO_4 is decanted off and the silica washed several times with warm water; for the final washing the silica is left standing in contact with the water overnight. The purified silica is dried in a steam-heated oven for several hours. Only those particles of the pure silica which pass through a mesh $\frac{1}{10}$ in. diameter and yet not through one $\frac{1}{32}$ in. are used for the adsorption tube.

SUMMARY

1. It has been demonstrated that existing methods for the development of the green colour by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution do not sufficiently take into account all the factors concerned. These factors have been studied, and from the results obtained an improved method of colour development has been suggested.

2. It has also been demonstrated that the yellow-brown colour in ether-alcohol extracts from biological material, which has been so frequently referred to, can be removed without loss of cholesterol.

3. On the basis of these facts an improved method for the colorimetric estimation of cholesterol in biological material has been described.

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