XL. A CRITICAL STUDY OF THE METHODS OF ESTIMATING CHOLESTEROL AND ALLIED SUBSTANCES.

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In recent years two methods, quite different in type, have come into common use for the estimation of cholesterol and its esters: a gravimetric method based on the formation of a highly insoluble molecular compound of cholesterol with digitonin, and a colorimetric method based on the Borchard-Liebermann reaction with acetic anhydride and sulphuric acid.

Both methods give good results when pure cholesterol is in question, but difficulties arise when mixtures of different sterols, or mixtures of cholesterol and oils, as, for example, the ether extracts of tissues, faeces, etc., have to be dealt with.

Gravimetric method.

In the year 1901 Ransom [1901] made the important discovery that the haemolytic action of saponin on blood corpuscles was prevented by the addition of cholesterol, and concluded that a kind of affinity existed, or a solubility equilibrium was set up, between the saponin and cholesterol. This discovery was of far-reaching interest, and soon led to experiment with other haemolytic materials. Thus it was found that cholesterol had an inhibitory effect on haemolysis by bee poison, garden-spider poison, snake poison, etc. Further investigations by Hausmann [1905] and by Abderhalden and Le Count [1906] proved that whereas cholesterol and phytosterol inhibit the action of saponin their esters do not. In 1909 Fraser and Gardner [1909] successfully made use of these facts to compare the relative cholesterol content of the sera of rabbits in dietetic experiments.

For some time it remained doubtful whether the inhibitory action of cholesterol was due to chemical action or to some physical process such as adsorption. Arrhenius [1907] regarded it as analogous to the neutralisation of toxin by antitoxin. The question was, however, definitely settled in 1909 by Windaus [1909], who made use of digitonin in his experiments, because this saponin was crystalline, had been proved to be a single substance and its

empirical formula had been determined. He found that when digitonin dissolved in 95 % alcohol was mixed with an alcoholic solution of cholesterol a compound of digitonin with cholesterol, insoluble in alcohol, was formed. This had the formula $C_{82}H_{140}O_{29}$. He also found that similar insoluble compounds were formed with sitosterol, β -cholestanol, coprosterol and stigmasterol.

In 1910 Windaus [1910] proposed a method of estimating cholesterol and its esters, based on this observation, which has since come into general use. About the same time Fraser and Gardner [1910] published a similar method, differing somewhat in detail, for attaining the same end, for which they claimed some advantages. In 1914 Thaysen [1914] published a detailed critique of the digitonin method and emphasised the necessity of using $1\frac{1}{2}$ to $2\frac{1}{2}$ % excess of digitonin in order to obtain accurate results. This conclusion we can fully confirm. Thaysen also pointed out the conditions necessary to attain complete hydrolysis of cholesterol esters by sodium ethoxide. In general he completely confirmed the validity of the digitonin method.

Alcohols of the sterol group can be divided into two classes according as they form insoluble digitonides or not, though as yet reliable data are wanting to complete the groups.

Group I. Insoluble digitonides.

The unsaturated alcohols—cholesterol, sitosterols, and probably other phytosterols, stygmasterol.

The saturated alcohols— β -cholestanol, coprosterol.

Group II. No insoluble digitonides.

The saturated alcohols— ψ -coprosterol, ϵ -cholestanol [Windaus 1916].

The unsaturated alcohol ψ -cholesterin [Windaus and Resau 1915].

Also α -cholestanol, though this has been shown by Windaus and Uibrig [1913] to be an amyl derivative of cholesterol.

The amorphous substances which accompany coprosterol in faeces [Gardner 1921] if these really belong to the sterol group.

The esters of Group I are not precipitated by digitonin.

Cholesterol digitonide. According to Windaus this substance is easily soluble in pyridine. He gives the following figures for its solubility in alcohols:

100 cc. of methyl alcohol at 18° dissolve 0.47 g.

100 cc. of 95 % ethyl alcohol at 18° dissolve 0.014 g.

100 cc. of 95 % ethyl alcohol at 72° dissolve 0.16 g.

100 cc. of 50 % ethyl alcohol at boiling point dissolve 0.03 g.

It is insoluble in cold water, acetone, ether, ethyl acetate, and benzene; and gives the Borchard-Liebermann reaction. It dissociates into its constituents on heating in the vapour of boiling xylene, and some slight amount of dissociation also occurs on prolonged boiling with methyl alcohol.

Sitosterol digitonide is quantitatively precipitated in crystalline needles and has similar properties. The stigmasterol compound is still more insoluble.

β -Cholestanol digitonide. β -Cholestanol according to Windaus forms a slightly more soluble compound, 100 cc. of 95 % alcohol dissolving 0.21 g. We found,

by digesting β -cholestanol digitonide for several days with ethyl alcohol of 96 % by weight at temperatures ranging from 10–18° with occasional shaking, values of 0.034 to 0.067 per 100 g. of solvent. At the boiling point 0.27 g. was dissolved. Working in a similar manner the solubility in commercial ether at 10–17° was found as an average of three estimations to be 0.0084 g., and in light petroleum 0.01 g. per 100 g. of solvent.

Coprosterol digitonide we found to be much more soluble in alcohol than the digitonides of other members of the group. Thus 100 g. of 96 % alcohol (by weight) dissolved at 10°, 0.143 g.; at 15°, 0.24 g.; and at 78°, 0.83 g.

In commercial ether at 10–17° the average of three estimations was 0.0077 g., and in light petroleum 0.0025 g. per 100 g. of solvent.

In applying Windaus's procedure for the estimation of any of these sterols a correction may be introduced for the solubility in alcohol. This becomes the more desirable the smaller the quantity of digitonide to be weighed. It is always, however, necessary in the case of coprosterol. In this case also a considerable excess of digitonin should be used. In the procedure recommended by Fraser and Gardner [1910] such a correction is obviated, since the alcohol is evaporated off at a low temperature before washing the precipitated digitonide. In this case any accompanying oils should be washed away with ether or light petroleum, and the excess of digitonin by warm water. The precipitate should not be washed with alcohol.

In both methods a very slight error will be introduced by the ether or light petroleum washing, owing to the slight solubility of the compounds in these solvents, but in most cases the error is negligible. When these methods are used to estimate free sterol in a large excess of fat, or non-sterol unsaponifiable matter, it is possible that the solubility of the compounds in ether in the presence of such substances may be increased and a somewhat greater error introduced. A long experience of the digitonin method as applied to the analysis of ether extracts of faeces, tissues, milks, etc., has however convinced us that such error is not serious, provided the quantities of compound to be weighed are not too small.

As the compound weighed is about four times the weight of the sterol, accurate results are attainable with very small quantities of sterol. In order to illustrate the reliability of the method in the case of the estimation of very small quantities of β -cholestanol and coprosterol we give some results in the following table. The estimations were made by the procedure of Fraser and Gardner, the precipitate being well washed with ether, as would be the case in an actual estimation in a fat, and finally with warm water.

Sterol	Weight of sterol used in g.	Weight of digitonin compound in g.	Calculated sterol (anhydrous)
β -Cholestanol crystals	0.0108	0.0459	0.0112
" "	0.0176	0.0705	0.0172
" "	0.0177	0.0711	0.0174
Coprosterol "	0.1022	0.4190	0.1022
" "	0.0109	0.0451	0.0110

Colorimetric method.

The colour methods usually employed depend either on the Salkowski reaction, as developed by Weston and Kent [1917] and others, or on the Liebermann reaction as originally proposed by Grigaut [1910, 1913].

In general a solution of cholesterol in acetic anhydride gives on addition of a few drops of strong sulphuric acid a succession of colours: red, blue, bluish green, and then a beautiful green. The latter gradually fades and in a few hours leaves a brownish solution. The spectral characters of the different stages of this reaction have been studied by Lifschütz [1913], who also showed that "oxycholesterin" presents the same succession of colours with the same spectral characters.

The Liebermann reaction is also given by coprosterol and the various phytosterols, and in a modified manner by chortosterol [Dorée and Gardner 1908], ischolesterol, and some other members of the sterol group.

This reaction is also given according to Wieland and Weil [1912] by cholatrienic acid and other bile derivatives. Many resinous substances give with acetic anhydride and sulphuric acid a reddish-brown colour, which sometimes develops on standing a green tint, or a green fluorescence. The amorphous substances which accompany coprosterol in faeces also give this reaction in a modified manner [Gardner 1921].

Obviously the presence of these substances would exercise a disturbing effect on the estimation of cholesterol by colour reactions, and various observers have been at pains to show the absence of such disturbing factors from the unsaponifiable matter of tissues. Thus Unna and Goloditz [1909] showed that in skin there is no trace of ischolesterol, or other substance capable of interfering with the reaction. Mauriac and Defaye [1912] showed that the presence of oleic acid has no influence on the reaction, but on the other hand Lifschütz [1908] by oxidation of oleic acid with permanganate obtained a substance which gave the reaction. Grigaut [1911] also was at some pains to show that the cholesterol extracted from serum was sufficiently free from disturbing substances by asserting the constancy of melting point and optical properties of his cholesterol.

Grigaut in his monograph [1913] also recognises that the reaction is a progressive one and would be affected by the mass of various reagents, and mentions the importance of always using in comparative estimations the same quantity of sulphuric acid. Autenrieth and Funk [1913] also lay stress on this point, as affecting the degree and type of colour produced. None of these authors, however, in any way elucidates the effect produced by varying the volume of the acid. With regard to the estimation of the other members of the sterol family, the only mention, as far as we are aware, is that made by Myers and Wardell [1915], who attempted to estimate coprosterol in faeces colorimetrically. They said that coprosterol had a value about one-third that of cholesterol.

The present investigation was, therefore, undertaken with a view to determining whether other members of the sterol group gave the colour reactions in such a way as to be comparable with cholesterol, and how variations of conditions affect the ultimate colour produced.

EXPERIMENTAL.

The production of colour in solutions of cholesterol and coprosterol was first compared.

The conditions used were those recommended by Autenrieth and Funk, *i.e.* 10 cc. of a chloroform solution of the sterol, of the order of $\frac{2}{10000}$ molecular, were treated with 4 cc. of acetic anhydride and 0.2 cc. of conc. sulphuric acid. The colour was developed in the light and at room temperature.

Under these conditions the colour developed slowly in the case of cholesterol, the first trace of colour being green. With coprosterol the development of the colour was still slower, and after half an hour the solution was only a very pale green. The deepening of the tint during $1\frac{1}{2}$ hours was still slight, and the colour was never comparable with that of the equivalent cholesterol solution, but on the addition of more acid a marked increase in depth of colour became apparent.

A detailed study of the effect of acid was therefore undertaken. The results are summarised in the following table:

Volume of sulphuric acid cc.	10 cc. chloroform solution of cholesterol (0.007 g. per 100 cc.) + 4 cc. acetic anhydride	10 cc. chloroform solution of coprosterol (0.007 g. per 100 cc.) + 4 cc. acetic anhydride
0.2	Green colour slowly developed. Maximum depth in 15–20 minutes	Very faint green colour, gradually developing during 2–3 hours
0.4	Blue developed first, and in about 5 minutes the colour was pronounced blue green. No apparent deepening on further standing	Colour began to appear in about 8 minutes, and developed rather more rapidly than above. No further deepening after 1 hour. Final shade not so deep as the cholesterol
0.5	Mauve colour first, changing rapidly to blue, then green. Full development of colour in about 5 minutes	Colour developed more quickly than above. Blue tint first seen, slowly changing to green. Colour in $\frac{1}{2}$ hour was identical with that of the cholesterol, but the latter had by then faded to some extent
0.6	Ditto	Ditto
1.0	Pink → mauve almost at once, then violet, blue, blue green and green. The whole series of changes occupied less than 2 minutes, and full depth of colour appeared to have been produced	Colour appeared in $\frac{1}{2}$ minute—slate-blue first, deepening rapidly, though less quickly than cholesterol. In 5 minutes the colour was slightly paler than the cholesterol, and rather more bluish green in quality
1.5	Changes as above	Pink → mauve colour almost instantly → blue → green. Colour deepened rapidly and rate and depth were about identical with cholesterol
2.0	Changes instantaneous, but sulphuric separated as an oil from solution	As cholesterol

In these reactions the depth of colour was compared by eye only in Nessler glasses, as qualitative results were first required. The reaction was however

clearly a time reaction, the rate depending on the amount of acid. Luden [1917] attempted unsuccessfully to show this sequence of colours by using smaller quantities of reagents, but reached some sign of them in very concentrated cholesterol solutions, though the colours were apparently less distinct than those noted above.

In order to determine whether the addition of the larger quantities of acid has any effect on the *final* depth of colour, readings were taken in a Dubosc colorimeter, using the same standard cholesterol solution—0.0394 in 250 cc. chloroform—in each case.

Two difficulties were encountered: firstly, the colours with greater quantities of sulphuric acid were much more blue-green than the tint with 0.2 cc. sulphuric acid, and secondly the colours were less transparent and therefore more difficult to compare. Much reliance, therefore, could not be placed on the results, but with the standard (containing 0.2 cc. of acid) set at 15 in the scale, the mean of a somewhat irregular series of readings for the solution containing 1.2 cc. acid was 15.1. It appears probable that the final depths of colour in the two cases are not divergent, but the practical difficulties point to the necessity of using the same volume of sulphuric acid throughout any series of estimations.

The influence of temperature was next investigated. The series of reactions quoted above were performed at room temperature, *i.e.* about 10–10.5°. Considerable divergence of opinion, however, seems to be prevalent as to what is the best temperature for the reaction. Luden [1917] uses room temperature, presumably because of the short period of colour induction allowed in her experiments before beginning colorimetric estimation. Autenrieth and Funk [1913] recommend a temperature of 30–35°, as also does Czonka [1916]. Bloor [1916], on the other hand, prefers room temperature. To test the effect of varying temperature duplicate experiments at room temperature (10°) and at 35° were made. In each case 10 cc. of the above chloroform solution and 4 cc. of acetic anhydride were treated with the selected quantity of acid, *e.g.* 0.2 cc., and allowed to stand 15 minutes. In every case, with the smaller amounts of acid, the higher temperature caused the production of a brilliant yellow green colour, as compared with an emerald green at the lower temperature. This made comparison in the colorimeter harder, but the scale readings at the higher temperature were systematically somewhat lower than at room temperature, for instance, using 0.2 cc. sulphuric acid with the scale at room temperature set at 15, the mean of four readings of that of the higher temperature solution was 14, so that a slightly greater depth of colour had evidently been produced. With 1.2 cc. of acid at the higher temperature the colour was again less transparent, but the shade bluer, which made comparison easier.

From our results it is evident that in any series of estimations it is necessary to use sensibly the same temperature throughout.

Experiments with varying quantities of acetic anhydride showed that this factor was unimportant, provided the substance was present in large excess.

It was also found best to use concentrated sulphuric acid, since traces of water, as was also observed by Grigaut, retard the action. To test the accuracy of the estimations under optimum conditions a large number of experiments were made. At first we used a dilution method. The colour was developed in the solutions to be compared and the darker was then diluted with a mixture of chloroform, acetic anhydride and sulphuric acid of the same composition as the fluid to be diluted, until equality of colour with the paler solution was attained. Matching was done with the naked eye. The method proved unsuitable for the following reasons:

- (1) Matching by eye was not sufficiently delicate.
- (2) On dilution the shade of green often became more yellow in quality.
- (3) The diluted solution on standing was often found to darken appreciably in colour, so that the end point was often overshoot, *i.e.* the time factor came in.
- (4) The method was not very sensitive. The addition of 1 cc. or more of the solution appeared to make only a very slight variation in the depth of colour.

Accurate results were obtained only when the solutions to be compared did not differ very much in strength. With solutions of molecular concentration above $\frac{4}{10000}$, which required considerable dilution, grave errors crept in, and the results as a rule were much too high.

This method was therefore discarded and a colorimeter used. A series of known cholesterol solutions were estimated against a standard solution. In each case 10 cc. of chloroform solution plus 4 cc. of acetic anhydride were treated with 1.2 cc. sulphuric acid—*i.e.* the maximum quantity that could safely be added without risk of separation of the acid, and the temperature was 35°. The following figures are typical of the results obtained.

Cholesterol solution taken (g. per cc.)	Strength found (g. per cc.)
0.00371	0.00378
0.00329	0.00310
0.00288	0.00280

Coprosterol. Coprosterol solutions were also investigated quantitatively. 10 cc. of a standard coprosterol solution (0.0658 g. per 100 cc. CHCl_3) and 4 cc. acetic anhydride were used in every case. The following example will suffice. To *A* 0.2 cc. sulphuric acid was added and to *B* 1.2 cc., both at room temperature, and to *C* 0.2 cc. at 35°. At the end of 15 minutes *B* was obviously darker to the eye than *A* or *C*. The solutions were allowed to stand for another 10 minutes and then compared in the colorimeter. With *A* set at 15 on the scale, the reading of *B* was 3.3, and of *C* 8.3. Obviously therefore both volume of acid and temperature are of vital importance.

Estimations of coprosterol against standard solutions of cholesterol, using a Dubosc colorimeter, lead to the same conclusion.

Using a cholesterol solution containing 0.000412 g. per cc. and a coprosterol solution of 0.000658 g. per cc., 0.2 cc. sulphuric acid was added to a

mixture of 10 cc. of each with 4 cc. acetic anhydride, and they were allowed to stand at room temperature for 30 minutes and then compared.

With the cholesterol scale at 5, the mean reading of the coprosterol solution was 10.6, indicating 0.00019 g. per cc. instead of 0.000658.

On repeating the experiment at 35°, with the cholesterol set at 15, the mean coprosterol reading was 13.25, equivalent to 0.000466 g. per cc. instead of 0.000658.

Using, however, 1.2 cc. of sulphuric acid at room temperature a value of 0.000636 was obtained, and at 35° 0.00068. The higher temperature had the advantage of favouring the production of the same quality of colour in each solution.

The time of colour induction, as already indicated, is much longer in the case of coprosterol than in the case of cholesterol. On comparing solutions in the above proportions using 0.2 cc. of acid the colours approximate at the end of about 2.5 hours, but during this period the colour of the cholesterol solution has appreciably faded. We found that in the case of a cholesterol solution, using 0.2 cc. of acid, fading began to be appreciable at the end of half an hour. Consequently in comparisons of cholesterol with coprosterol or mixtures of cholesterol and coprosterol sufficient sulphuric acid should be added to get the maximum colour development in each as rapidly as possible, certainly within 30 minutes. If these conditions are fulfilled accurate estimations can be made.

ψ-Coprosterol. The behaviour of this substance was investigated in a similar manner, and compared with that of coprosterol. With solutions of approximately $\frac{2}{10000}$ molecular concentration the colour of the *ψ*-coprosterol solution was insufficient to make comparison possible. Solutions of *ψ*-coprosterol of $\frac{4}{10000}$ and $\frac{6}{10000}$ molecular concentration were therefore used.

With 0.4 cc. of sulphuric acid (to 10 cc. chloroform sol. + 4 cc. acetic anhydride) no immediate coloration appeared, but after about 25 minutes a faint yellow green was seen. With 0.8 cc. acid the colour was obvious in 5–10 minutes, and was definitely pale green in 25 minutes. Even with 1.2 cc. of acid the colour did not appear immediately. No sign of mauve was noted, but a dirty grey colour came first. After about 5 minutes the green colour developed slowly. Increased temperature was found to speed up the reaction, so that with sufficient acid colour appeared almost at once, but the final shade was never really comparable in quality with that of cholesterol or coprosterol, and certainly not in depth.

A series of readings were however taken, as nearly as we could manage, using optimum conditions with the following results:

<i>ψ</i> -coprosterol weighed	0.017 g.	found	0.0068 g.
„	„	0.023 „	„ 0.0053 „
„	„	0.023 „	„ 0.0062 „

Evidently *ψ*-coprosterol cannot be estimated colorimetrically against either

cholesterol or coprosterol, and if present in a mixture with either of these substances would vitiate any colour estimations of the latter.

β -Cholestanol. According to Willstätter and Mayer [1908], when a chloroform solution of β -cholestanol was treated with acetic anhydride and two drops of sulphuric acid, it remained colourless, but after adding double the mass of reagents the solution, without progressive colour changes, became pale green and showed fluorescence.

A specimen of β -cholestanol prepared by Ellis and Gardner [1918] by reducing until further change in optical rotation no longer took place, gave very faint colour changes with plenty of acid—first blue and then green—but the depth of colour was so feeble as to lead to the conclusion that probably a specimen absolutely free from cholesterol would give no colour.

Esters. The reactions of cholesteryl acetate, benzoate and stearate were examined, and also those of coprosteryl chloroacetate.

The colour reactions in the first three substances were given as easily as with cholesterol itself. The colour developed with either 0.2 cc. acid or 1.2 cc., both in range and final shade, was identical with the cholesterol standard.

With coprosteryl chloroacetate, on the other hand, the colour changes were very slow. The first shade to appear was yellow, changing slowly to a dirty grey-green, and at least 10 minutes elapsed before the normal green shade was apparent. The depth of colour never approximated to that given by a coprosterol or cholesterol solution of equivalent strength. In the case of the cholesteryl esters the quantitative results were invariably rather on the high side. For example, cholesteryl benzoate and acetate tested against cholesterol gave:

Weight benzoate taken 0.001417 g. found 0.001483 g.

„ acetate „ 0.001656 „ „ 0.00184 „

In the case of stearate the figures were generally slightly higher than truth.

Amorphous sterols from faeces [Gardner 1921].

These substances in chloroform solution with acetic anhydride and sulphuric acid give a brown colour, rather like ferric acetate in shade. This gradually changes through a dusky green to a brighter green. The rate of change depends on the quantity of sulphuric acid used and the temperature. The final green colour is however quite different in quality from that of cholesterol or coprosterol, being a yellowish green rather than a bluish green. Consequently it is very difficult to compare the depth of colour in a colorimeter with a standard coprosterol colour, and the results are of little value. Nevertheless we made comparisons as well as we could against both coprosterol and ψ -coprosterol, using a maximum quantity of sulphuric acid, and room temperature. The time factor was more or less eliminated by taking readings first at the end of 30 minutes and again at the end of 60 minutes, but this proved unnecessary as the readings were identical.

Assuming for purposes of calculation that the amorphous sterols had the formula of coprosterol the following results were obtained, using different specimens against a coprosterol standard:

	Composition of sample		Amount weighed in g.	Calculated amounts
(1)	83.75 C	12.01 H	0.0156	0.0087
(2)	84.16 „	12.2 „	0.008	0.004
(3)	83.45 „	12.09 „	0.0075	0.0032
(4)	83.70 „	12.36 „	0.04	0.025
(5)	84.04 „	12.23 „	0.0235	0.012

It will be seen that the calculated quantities are about half the actual.

Using a ψ -coprosterol standard the following figures were obtained:

	Amount weighed g.	Calculated g.
(1)	0.056	0.132
(2)	0.0215	0.032
(3)	0.044	0.071

From these figures the depth of colour produced under optimum conditions by these substances would appear to be about half-way between that of coprosterol and that of ψ -coprosterol, but more nearly allied to the latter in quality. Obviously the presence of such substances in any extract would render the accurate colorimetric estimation of cholesterol and coprosterol in such extract quite impossible.

Examination of the ether extract of faeces.

In the application of these methods to the analysis of the unsaponifiable matter of faeces, which contains coprosterol, β -cholestanol, cholesterol, possibly phytosterols from vegetable food, amorphous sterols and alcohols, such as cetyl alcohol, etc., the digitonin method permits of the quantitative separation of the *total* coprosterol, β -cholestanol, cholesterol and phytosterol from the amorphous sterols and non-sterol alcohols. The precipitated sterols may be extracted from the digitonides by heating in the vapour of boiling xylene as recommended by Windaus. *There is no simple method, however, by which small quantities of these recovered sterols can be analysed.* If sufficient quantity of the material were available the cholesterol (and perhaps any phytosterol) could be approximately separated from the coprosterol and β -cholestanol by precipitating the cholesterol dibromide from an ethereal solution of the sterols by means of a suitable solution of bromine in glacial acetic acid at a low temperature. The cholesterol may be recovered from the dibromide by reduction with zinc dust and glacial acetic acid. The coprosterol may also be separated, after the bulk has been isolated by fractional crystallisation, from β -cholestanol by conversion into ψ -coprosterol and precipitation of the β -cholestanol by digitonin, according to the plan adopted by Windaus [1916] in his work on the separation of the three dihydrocholesterols obtained by the reduction of cholesterol by hydrogen in the presence of nickel. A rough approximation of the amount of β -cholestanol in the mixture could also be

obtained by the colorimetric method, as β -cholestanol either gives no colours or very faint ones.

Colour methods as applied directly to the unsaponifiable matter of faeces are quite useless, as it is impossible to make any accurate comparison of the colour obtained with the unsaponifiable matter with that of a coprosterol standard either by naked eye comparison in reagent glasses or by a colorimeter owing to the quite different quality of the green shades obtained. Even if this could be got over it would be difficult to assign any meaning to the results, since, though cholesterol and coprosterol give comparable colour reactions, β -cholestanol gives only feeble colours, if any, and the amorphous sterols give not only a yellower green but a colour of a lower order of intensity. This will be readily seen from the following estimations by the two methods of the unsaponifiable matter of the faeces of a number of individuals fed on practically the same diet.

	Sterols by digitonin, reckoned as copro- sterol per g. of unsaponifiable matter	Sterols by colour method, reckoned as coprosterol per g. of unsaponifiable matter
(1)	0.511	0.513
(2)	0.575	0.431
(3)	0.606	0.382
(4)	0.632	0.418
(5)	0.696	0.434
(6)	0.629	0.446*
(7)	0.472	0.563
(8)	0.671	0.709*
(9)	0.594	0.678*
(10)	0.517	0.852*

* These samples were especially difficult to compare owing to the marked yellow shade.

The ether extracts of tissues, etc.

With the ether extracts of tissues, animal fluids, such as milk, serum, etc., according to our own long experience and that of numerous other workers, the digitonin method gives accurate results, and has the important advantage that both cholesterol and its esters can be estimated, though where the esters are relatively small in quantity experimental errors are correspondingly larger. It must not be forgotten however that though the quantity of cholesterol in fats may be accurately estimated, the accuracy of the results when expressed as a percentage of the original tissue naturally depends on the assumption of the efficient extraction of the fatty matter from the tissue, but this is a question outside the present inquiry.

The colorimetric methods give satisfactory results on the whole with serum or blood, but with other tissue extracts the estimations are less trustworthy, owing to greater or less differences in quality between the shades obtained with such tissues and the cholesterol standard used, which render the comparison of the colour intensities less precise. When the cholesterol is completely precipitated from the unsaponifiable matter of the fat of a tissue,

such as muscle or liver, or of milk, by digitonin, the unprecipitated portion of the unsaponifiable matter in chloroform solution always gives with acetic anhydride and sulphuric acid a marked brownish-red coloration, which often changes in a few minutes through a dusky green to a yellowish green, and even a bright grass green. Also, when ordinary alcohol is boiled with alkalis, diluted and extracted with ether, some substance is extracted by the ether which in chloroform solution gives with acetic anhydride and sulphuric acid a brown colour, which often becomes more or less green [*vide* Gardner and Fox, 1921]. Whether the green colour observed with the cholesterol-free unsaponifiable matter is due to the inherent properties of this substance, or to traces of cholesterol digitonide remaining in solution, or to the outside cause referred to has not yet been decided. Nevertheless the production of this green introduces an error into the colorimetric results, and in any case the brown colour always obtained modifies the quality of the shade of colour due to the cholesterol. The digitonin method necessitates the use of a certain minimum quantity of material to give an amount of digitonide sufficient for reliable weighing; in the case of blood, for instance, this means at least 30 to 50 cc. The colorimetric method on the other hand is of the nature of a micromethod, and in the case of blood can be carried out on 2 cc. or even less. This, of course, is a very great advantage in the study of the living animal and the human subject. Very fair agreement between the digitonin and colour methods has been recorded by Klinkert in the case of serum [1912, 1913]. In the case of blood, milk, etc., accurate comparative results can, we believe, be obtained in a series of experiments provided *rigidly* comparable conditions are adopted. The results should also at some stage be checked by the digitonin method. Unless these precautions are observed serious errors may arise.

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