tion of the free sugar. Both these interpretations, however, give only a minor significance to the appearance of this ester in the milk.

The demonstration of a lactosephosphate on the other hand, does bring more certain evidence for the view, hitherto held with very slender experimental support, that phosphorylation is of fundamental importance in lactose synthesis. An isolated instance of the occurrence of such an ester in a trichloroacetic acid extract of cow-udder tissue has already been reported (Malpress, 1950), and it seems probable that this compound may prove to be the immediate precursor of lactose in the mammary gland.

SUMMARY

1. Barium fractionation procedures, supplemented by chromatographic analysis, have shown that α -galactose-1-phosphate, α (?)-glucose-1-phosphate, glucose-6(?) -phosphate, a lactosephosphate and phosphopyruvic acid are present in trace amounts in normal milk.

2. The possible significance of these findings in relation to lactose synthesis is discussed.

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The Determination of Cholesterol by the Liebermann-Burchard Reaction

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The methods adopted for the determination of cholesterol in blood can be divided into two main groups. Those in the first group, in which the principal variation is in the manner of extraction, all conclude with the application of the Liebermann-Burchard reaction (Liebermann, 1885; Burchard, 1889) to the final extract and comparison of the colour developed against a standard cholesterol

solution similarly treated. Included in this are the methods of Myers & Wardell (1918), Leiboff (1924) and Sheftel (1944). In these methods the specimen is absorbed on a suitable absorbent and the cholesterol is extracted by means of a continuous extractor. With the methods of Sackett (1925) and Bloor (1928) the blood is extracted directly in a given volume of mixed solvent. Attempts have also

been made to estimate cholesterol directly in acid chloroform in the methods of Sols (1947) and Zuckerman & Natelson (1948).

Into the second group can be gathered all the methods which entail precipitation of the extracted cholesterol as digitonide, which may either be weighed, as in the methods of Gardner & Gainsborough (1927), Boyd (1933) or Man & Gildea (1933), determined gasometrically as in that of Kirk, Page & Van Slyke (1934) or by oxidation as in the method of Okey (1928). The disadvantage of the weighing procedure lies in the relatively large volumes of blood required, and of the gasometric and oxidation procedures in that special equipment and technical skill are essential. Schoenheimer & Sperry (1934) succeeded in applying the Liebermann-Burchard reaction to cholesterol isolated as digitonide from 0.2 ml. of blood.

It has been found that techniques involving the Liebermann-Burchard reaction, like many of the older colorimetric methods, require a more specific set of conditions than was formerly recognized if they are to be adapted to a final absorptiometric determination. The object of this investigation has been to study the factors which influence colour development in chloroform solutions of cholesterol and its esters, with particular reference to the use of selective filters, the effect of time, temperature, exposure to light and the proportions of reagents, and to explain or eliminate the so-called spurious colours which so often develop and interfere with final evaluation. On the basis of these results it was hoped to evolve a method suitable for routine use in clinical laboratories and establish normal limits for such a method. A preliminary communication on part of this work has already been made (Kenny, 1948).

EXPERIMENTAL AND RESULTS

The instrument employed throughout this work was a Hilger Spekker absorptiometer, using spectral filters of the series H 558 and cells of 1 cm. thickness.

Effect of selective filters

The scheme adopted to determine the effect of selective filters upon the extinction value consisted of setting up a series of identical tubes, each containing 1 mg. of cholesterol in 10 ml. CHCl_3 . The tubes were lightly stoppered and placed in the dark in a thermostatically controlled water bath at the temperature being investigated. After the contents of the tube had attained the temperature of the bath, 2 ml. acetic anhydride were added and the tubes shaken, then 0.2 ml. conc. H_2SO_4 was measured in, and the tubes finally inverted and returned to the water bath. At prearranged intervals, the tubes were removed from the bath, and the extinction values of the contents determined on the absorptiometer, a different pair of filters being used for each successive tube. Fig. 1 shows the absorption curve

for the eight filters of the series, the colour having been developed at 20 and 40° for 10 and 30 min. in each case. The curves show two main maxima, one at about 430 $\text{m}\mu$. and the other between 600 and 690 $\text{m}\mu$.

Using a spectrophotometer, Morgareidge (1935) was able to demonstrate three absorption bands in the colour given in the Liebermann-Burchard reaction by cholesterol and certain related sterols, one at 450 $\text{m}\mu$, a second at 620 $\text{m}\mu$. and a third at 675 $\text{m}\mu$. The 600–690 $\text{m}\mu$. maximum shown in the absorptiometric measurements presumably represents the second and third peaks.

Fig. 1. Absorption curves for a solution of 1 mg. cholesterol in 10 ml. CHCl_3 + 2 ml. acetic anhydride + 0.2 ml. conc. H_2SO_4 , for the eight Ilford filters of the series, H 558. Solutions were developed in the dark at 20 and 40° for 10 and 30 min. as indicated.

At 40° the development of colour was found to be more rapid than at 20°, and the absorption at 430 $\text{m}\mu$. had considerably increased, while the 600 and 690 $\text{m}\mu$. readings had fallen. There is, therefore, evidence of a shift in the intensity of the absorption bands. The explanation may be that the colour is due not to a single substance or, more probably, that this substance can be transformed from one form to another, since the loss in the blue-green coloration, absorbing at 690 $\text{m}\mu$., is not paralleled by a loss in the yellow, absorbing at 430 $\text{m}\mu$.

The findings would indicate that in an estimation the choice of filter lies between a red which estimates a thermolabile component of the colour or a blue which is not influenced by this component. Further data on this point are provided in the next section.

Effect of temperature

The importance of the control of temperature has been generally recognized, but the methods suggested by different investigators have shown considerable variation, although later workers have been more precise in this connexion. The following results show that such care is indeed warranted, especially with photometric measurements.

With the effect of filters in mind, a more detailed study of the influence of temperature was made. Series of tubes were set up as previously described and incubated at specified temperatures, one sample tube being removed at each time interval. The graded effect of temperature upon colour

development, using a filter with maximum transmission at 430 $m\mu$., and also the same tubes read against a 690 $m\mu$. filter is illustrated in Fig. 2.

These results have been found to confirm and extend the work of Hoffman (1940). It will be seen that adding the reagents and maintaining at 0° 'freezes' the system, and over a period of 30 min. only a trace of colour develops. Transferring the tube, even after 30 min., to a higher temperature, results in the development of a colour along, but slightly below, the curve obtained if the whole reaction were at the new temperature conditions.

Fig. 2. Absorption curves for 1 mg. cholesterol in 10 ml. CHCl_3 + 2 ml. acetic anhydride + 0.2 ml. conc. H_2SO_4 , developed at temperatures from 0 to 50° and plotted for a period of 30 min. with filters having maximum transmissions at (a) 430 $m\mu$. and (b) 690 $m\mu$. A 'room temperature' development curve is also shown.

The general effect of increasing the reaction temperature with the 430 $m\mu$. filter was to give a more rapid colour development and a greater sensitivity. With the 690 $m\mu$. filter, on the other hand, the maximum absorption was found to reach the same critical level for all temperatures of 20° and over. After this point the absorption at 690 $m\mu$. diminished while, as stated above, that at 430 $m\mu$. continued to increase.

The area enclosed between the temperature curve, the 0.46 abscissa and the 30 min. ordinate beyond the maximum point with filter 690 $m\mu$., was found to be approximately 1.5 times the corresponding area when using the 430 $m\mu$. filter. In the first case the extinction is on the decline and the area is below the 0.46 abscissa, whilst in the second, with the increasing extinction, it is above this line. This would indicate a constant conversion of the green to the yellow component, beyond the point where the extinction-time curve has reached its maximum with filter 690 $m\mu$. The rate of development of the green component and of its conversion to the yellow is dependent principally upon temperature. As a consequence, any reading taken on a 430 $m\mu$. temperature curve at or beyond 0.46 added to 0.67 of the reading on the corresponding 690 $m\mu$. curve gives a constant reading of approximately 0.75, which would represent the

ultimate value of the 430 $m\mu$. reading when complete conversion to the yellow component had taken place, and appears to be a characteristic of the concentration. This point is further illustrated in Table 1 where readings are shown for both filters, the colour having been developed at 20 and 40° for periods up to 3 hr. The continuing rise in the readings with the 430 $m\mu$. filter, and the corresponding fall in the 690 $m\mu$. filter are clearly indicated. An analysis of the results plotted in Fig. 3 also supports this contention but reveals minor discrepancies attributable to deviation from Beer's law.

Variations in the concentration of cholesterol resulted in curves which all showed the same general character, but were set at higher or lower levels as the case may be. It is interesting to note that if colour be developed at room temperature, around 20°, it follows closely the 30° curve.

Table 1. *Effect of time on colour development*

(A continuation of the results plotted in Fig. 2.)

Temperature (°)	Maximum transmission of filter ($m\mu$.)	Spekker readings			
		30 min.	1 hr.	2 hr.	3 hr.
20	430	0.48	0.53	0.56	0.58
	690	0.43	0.40	0.31	0.28
40	430	0.62	0.64	0.69	0.72
	690	0.18	0.12	0.07	0.05

When the 10 ml. of CHCl_3 solution are placed in a test tube in an open rack and treated with 2 ml. acetic anhydride, there is a rise in temperature of 4° and on the addition of 0.2 ml. conc. H_2SO_4 a further rise of 8° occurs, after which the normal cooling curve sets in and the contents of the

tube return to within 1° of room temperature in about 30 min.

From the results it would appear that 30 min. development at 37° would be a satisfactory arrangement when using a filter of maximum transmission at 430 m μ ., since most laboratories possess an incubator or thermostat normally maintained at this temperature.

Reaction of esters

Cholesterol oleate and stearate were examined under the same conditions as outlined above and were found to give the same general pattern as did cholesterol. Several authors (Gardner & Williams, 1921; Reinhold, 1936; Yasuda, 1936; Kelsay, 1939) have reported that increased colour values are obtained from equivalent amounts of the esters as compared with the free sterol, and these enhancements have been reported as ranging from 5 to 30%. In the present experiments these esters were found to give a value 13% higher than cholesterol itself when calculated on an equivalent basis. This is a point which must be borne in mind when dealing with a mixture of free and esterified cholesterol such as exists in a Bloor extract.

The same maximum in the extinction-time curve was found with filter 690 m μ . but occurred at an extinction value of 0.52 in the case of the esters when estimated against a 1 mg. level. The rate of colour development over the range 0–40° was not significantly greater than for cholesterol itself and could not be taken as a basis for differentiating between free and combined cholesterol as in the method of Reinhold (1935).

Effect of time of development

The time recommended in certain of the earlier methods was 15 min. and, as seen from Fig. 2, could be applied with a fair degree of accuracy to a 'room temperature' development, or to temperatures close to 25°. With more precise temperature control, none of the curves reaches a maximum with the 430 m μ . filter even after 30 min., although the gradient of the curve is very small at this point with temperatures above 30°. With filter 690 m μ . the maximum depends upon the temperature, being 2.5 min. for 40° and 19 min. for 20°.

Table 1 shows readings obtained at intervals up to 3 hr. after the addition of the H₂SO₄, and represents an extension of the 20 and 40° results plotted in Fig. 2.

Table 2. *Effect of illumination upon colour development*

(Reagents: 2 ml. acetic anhydride followed by 0.2 ml. conc. H₂SO₄.)

General experimental conditions		Spekker readings		
		Illumination		Dark thermostat
		Filter (m μ .)	Ultraviolet lamp	
(a) 10 ml. CHCl ₃ containing 1 mg. cholesterol in thin-walled soft-glass test tube. Reagents added and maintained at 40° for 30 min.	430 690	0.342 0.174	0.261 0.170	0.621 0.178
(b) As above, but maintained first at 40° for 30 min., then reagents added and developed for a further 30 min. at 40° in dark thermostat	430 690	0.630 0.177	0.632 0.175	0.630 0.170
(c) As in (a), but employing silica test tubes	430 690	0.452 0.179	0.395 0.172	0.620 0.178
(d) As in (b), but employing silica test tubes	430 690	0.611 0.180	0.628 0.168	0.618 0.178

The effect of exposure to light

The deleterious effect of light upon the coloured product was also early recognized, but again the measures taken varied from developing in complete darkness to developing in the illumination existing in that part of the laboratory where the comparison was made.

The gross effect of light is demonstrated in the following experiment. Into three ordinary soft-glass tubes were pipetted 10 ml. CHCl₃ containing 1 mg. cholesterol. The tubes were placed in a thermostat and brought to a temperature of 40°. Acetic anhydride and H₂SO₄ were added as previously described, and when the temperature of the contents had fallen to 40°, two tubes were removed, one being clamped at an angle of 45° to horizontal above a 125 W. Osira H.P. mercury-vapour lamp, and the other 20 cm. above an ordinary 100 W. frosted filament lamp, in such a position as to maintain their temperatures exactly at 40°. The third tube was left in the thermostat (at 40°). All three tubes were allowed to develop for 30 min., after which the extinction value was measured.

A similar set of experiments was conducted using silica test tubes; the results are shown in Table 2. It is clear that only the yellow component absorbing at 430 m μ . is affected, and that under the conditions of the test the white light was more effective than the ultraviolet.

In order to demonstrate that this effect was not due to structural alteration within the cholesterol molecule because of irradiation, a similar series of tests was performed exposing the CHCl₃ solutions alone for 30 min., under the same conditions, then adding the other reagents and developing the colour in the dark thermostat for a further 30 min. The above assumption was shown to be correct since there was no appreciable difference between the readings of these cholesterol solutions and the control solution which had not been exposed. It therefore follows that the effect of white light is upon the yellow-coloured product itself, and although exaggerated in this experiment, it emphasizes the advisability of developing the reaction in the dark, or at least in a uniform subdued light.

Effect of proportions of reagents

Variations in the proportions of reagents materially influenced the final colour. With more than 0.3 ml. H₂SO₄ two layers separated and no measurement could be made. With

The ratio of serum to oxalated plasma cholesterol in the ten samples analysed, and shown in Table 3*b*, gave an average value of 1.11.

On the results of Sperry & Schoenheimer (1935), the differences cannot be attributed to hydrolysis of the esters by lipase, nor can it be explained as being due to absorption of cholesterol on the calcium oxalate precipitate. Since the heparinized sample was initially almost identical with the serum it cannot be related to clot formation. The explanation may lie in a surface phenomenon resulting in the absorption of cholesterol on the red cells as a consequence of the increased potassium ion concentration of the plasma, and investigations are proceeding in this direction to examine this possibility.

METHOD FOR THE DETERMINATION OF CHOLESTEROL IN PLASMA OR SERUM

On the basis of the experimental findings of the earlier sections, the following modification of the method of Bloor (1928) is recommended for the routine clinical estimation of cholesterol in plasma or serum.

Reagents

- (1) Ethanol-ether mixture: 3 vol. 95% ethanol are mixed with 1 vol. ether.
- (2) Anhydrous chloroform.
- (3) Acetic anhydride (Analar).
- (4) Sulphuric acid (Analar).
- (5) Cholesterol standard.
 - (a) Stock solution: dissolve 0.547 g. anhydrous cholesterol in 100 ml. chloroform.
 - (b) Single working standard: prepare a 1 in 50 dilution of (a) in chloroform.
(10 ml. = 250 mg. total cholesterol/100 ml.)

Note on correction for esters. It has been shown in an earlier section that cholesteryl esters give a colour equivalent to 13% more in terms of cholesterol, than does the free sterol itself. If one accepts 28% as the mean value for the free cholesterol fraction (Peters & Man, 1943), then a total cholesterol of say 200 mg./100 ml. would give a reading equivalent to 219 mg. in terms of free cholesterol. An adjustment has been made for this in the preparation of the standard.

Basing a calibration on this assumption it can be shown that variations in the free cholesterol fraction between 20 and 40% of the total would not cause an error of greater than $\pm 2\%$ in the total so determined.

Method

About 15 ml. ethanol-ether mixture are measured into a 25 ml. stoppered volumetric flask, and 1 ml. of the sample is pipetted in, slowly and with constant stirring. The contents of the flask are then carefully brought to boiling point by immersing in a boiling-water bath for a few seconds at a time. The mixture is maintained at the boiling point of the solvent for about 2 min. and is then cooled thoroughly and diluted to 25 ml. with the mixed solvent.

The extract is filtered through a no. 4, 9 cm. Whatman filter paper, the funnel being covered by a watch glass to reduce evaporation. A 5 cm. length of capillary tubing, sealed at its upper end, is placed in a 6 x 1 in. boiling tube and 20 ml. of the filtrate are added and the contents of the tube are taken to dryness in a boiling-water bath.

The last traces of solvent and moisture are removed by heating the tube in a drying oven at 100° for a few minutes. The capillary is broken and the residue in the tube is extracted with 20 ml. chloroform, the walls of the tube being scraped with a glass rod to loosen the particles adhering to the sides and the extraction is completed by heating just to boiling point.

The chloroform extract is filtered hot through a no. 1, 11 cm. Whatman filter paper, with precautions as described above, and cooled. The residue which remains in the tube has been found to give no colour with the reagents. Into a 25 ml. glass-stoppered test tube are pipetted 10 ml. of the final chloroform filtrate and the tube and its contents are immersed in a thermostat at 37°, when 2 ml. acetic anhydride are added, mixed with the contents of the tube, and allowed to stand for 3 min. to attain the temperature of the bath. Then 0.2 ml. of conc. sulphuric acid is added from a microburette and the tube inverted and returned to the thermostat for 30 min., being protected from direct light.

As a blank, 7 ml. of the residual chloroform extract are treated with 1.4 ml. of acetic anhydride and incubated for 30 min. This blank has been found to give a fairly constant reading, even with distinctly pigmented plasmas, and is usually equivalent to about 12 mg. cholesterol/100 ml.

An interval timer is set going when the tubes are placed in the bath, and if a number of tests are to be made sufficient time is allowed between the additions of acid to enable all the tubes to be read at 30 ± 2 min. The extinction value is determined using a filter with a maximum transmission at 430 m μ . and interpolated on a calibration curve. It is suggested that a quantity control chart, such as is used for sodium by Wootton, King & Maclean Smith (1951) should be made in order to establish the reliability of the method.

Distribution curve for normal plasma total cholesterol

For speed of separation and economy in material, plasma cholesterol values have been determined in this laboratory by the above method for a number of years, and the distribution curve shown in Fig. 4 has been obtained.

The results have been drawn from 100 consecutive samples obtained from voluntary donors, and 100 samples from individual patients in this hospital, who, in the course of routine examination showed on an initial determination a basal metabolic rate

falling within the range of -7 to $+14\%$ of standard and were not known to suffer from other conditions associated with abnormal blood cholesterol values.

All the subjects were in the post-absorptive stage. The ages of the individuals, which contained equal numbers of men and women, ranged from 17 to 70 years. The irregularities in the curve must be attributed to the relative smallness of the sample. There is a slight skewness in the distribution which is not altogether unexpected, but for simplicity in calculation a Gaussian distribution has been assumed.

Fig. 4. Distribution curve for normal plasma total cholesterol. 100 males and 100 females, whose ages ranged from 17 to 70 years, are represented. The theoretical curve, $y = 20.7e^{-0.000335x^2}$, which assumes a Gaussian distribution, is indicated. Mean plasma total cholesterol, 197.0 ± 4.1 mg./100 ml.; standard deviation, 38.7 mg.

The mean value of the series was 197 mg./100 ml. and the standard deviation was 38.7 mg. It therefore follows that half the normal plasma cholesterol should fall within the limits 171–223 mg./100 ml., or $\pm 0.67\sigma$, with a 1 in 20 chance of a normal falling outside the limits of 120–274 mg./100 ml., or $\pm 2\sigma$. Twenty replicate determinations on pooled normal plasma gave a result of 217 ± 4.3 mg./100 ml.

DISCUSSION

A comparison of the recovery of cholesterol using various methods of extraction was made by Reinhold (1936), and he concluded that the method of Bloor (1928) gave complete extraction. Methods which involved drying of the sample followed by extraction generally gave lower recoveries. The work of Drecker, Bernhard & Leopold (1935) may give an explanation. They showed that cholesterol was present in two distinct fractions: one part was ether-soluble, and the other appeared to be in combination

with protein and required denaturation by alcohol for its release. The method of Bloor has therefore been used throughout this work.

Gardner & Williams (1921) found that, of the two reagents employed in the development of the final colour, the quantity of sulphuric acid was the more important; considerable latitude could be allowed in the volume of acetic anhydride. Shapiro, Lerner & Posen (1935) suggested the use of a mixed reagent. As stated earlier, the main effect of this scheme of addition is to slow the initial stages of colour development, without greatly influencing the final result. Where small numbers of tests are being carried through, the mixed reagent holds no advantage, and may in fact be an inconvenience.

With the exception of Schube (1932) all authors appear to have recommended the measurement of the green colour, and where a filter is suggested it is one with a maximum transmission in the region of 650 m μ . Schube found that 15 min. development at 20° gave maximum colour for the green component, but he obtained more consistent results by making a second comparison between his standard and unknown after they had stood for 12–24 hr. in a dark cupboard, so that the yellow component was being measured.

The main disadvantage of measuring the yellow component, especially with a blue filter, would appear to be that any yellow pigmentation of the final chloroform extract would result in an increased blank. A correction can be applied by using part of the chloroform filtrate and adding only the acetic anhydride, but it has been found in practice that by far the greater part of any bile pigments present in a sample is absorbed on the protein precipitate during the extraction process. As an example, it was found in one instance where a plasma contained 17.3 mg. bilirubin/100 ml., that the blank, as described in the method, amounted to only 15 mg. cholesterol/100 ml.

The instability of the green colour has been the cause of much criticism being levelled at the Liebermann-Burchard reaction for the determination of cholesterol and has been responsible for the introduction of such artificial standards as naphthol green B by Reinhold (1935), and Carter's ink by Shapiro *et al.* (1935), and also certain schemes of washing such as that of Mueller (1916), in an attempt to eliminate the spurious yellows which are attributed to a fading standard. Sheftel (1944) was able to stabilize the green colour to some extent by the addition of glacial acetic acid.

The work of Hoffman (1940), as extended and discussed here, has clarified this position, and Sperry & Brand (1943), reviewing work on this reaction, stated that in their opinion the green colour was entirely empirical, no doubt implying that it was most variable, and that it was dependent

principally upon the factors of time and temperature, whereas the brown colour (described here as the yellow colour), appeared to be the end product and might be proportional to the amount of cholesterol. The present work has confirmed this view, because it has been shown that a relationship exists between the absorptions in the red and blue regions of the spectrum such that a constant can be obtained which is proportional to the amount of cholesterol present.

Sundeman & Razek (1937), using a Razek-Mulder colour analyser, showed that after 10 min. the reading at 530 m μ . alone remained constant, but the sensitivity was so low as to preclude its use for quantitative work. They found that in the 550–700 m μ . region the absorption increased with temperature, but was thermolabile. In the dark, increasing absorption took place in the blue-violet region, but the component measured at that wavelength was light-sensitive.

A decision has therefore to be taken on whether to measure the green component, making readings within a closely specified time, after developing at a temperature below 30°, or to measure the yellow component which is thermostable after a longer interval of time, and developed at a higher temperature in the dark, or in reasonable subdued light. The latter system has been adopted here.

Noyons (1938) found results 17% lower by the digitonide-precipitation method as compared with colorimetric determination, and Reinhold (1936) found differences averaging 10% in the same direction. These differences can be explained readily by the greater sensitivity shown by cholesterol esters, and an allowance of 9% has been made for this in the preparation of the standard cholesterol solution.

Estimating normal serum cholesterols by their digitonide-precipitation method, Man & Gildea (1933) found a mean value of 208 mg./100 ml. with an experimental error of $\pm 5.5\%$. Later, Peters & Man (1943), using the same technique with 174 cases, found a mean value of 194 mg./100 ml. with a standard deviation of 35.6 mg. Kornerup (1950), using the technique of Sperry (1936), found a mean serum total cholesterol of 218.8 ± 4.4 mg. (s.e.m.)/100 ml., s.d. 47 mg., in a series of adults.

The plasma cholesterols found in the present series of 200 normals, mean 197 ± 4.1 mg. (s.e.m.)/100 ml., s.d. 39 mg., compare favourably with these figures from the point of view of accuracy and agree also in terms of distribution, but, since plasma has been employed, tend relatively to be slightly higher than the first two sets of figures quoted above.

The evidence produced in earlier sections is of general application, and stresses the importance of the strictest control of conditions if reproducible results are to be obtained by any method of cholesterol determination employing the Liebermann-Burchard reaction.

Although plasma cholesterol levels have been estimated in the series quoted here, the author is of the opinion that until a satisfactory explanation is obtained for the differences between serum and plasma values the former should be estimated in preference to the latter, since the serum values have here been found to remain the more constant over a period of 24 hr.

SUMMARY

1. Factors influencing the colour development in the Liebermann-Burchard reaction for the determination of cholesterol have been investigated. The alteration in the densities of the absorption bands with temperature have been studied, and a simple relationship established for the conditions described.

2. A method for the estimation of serum or plasma total cholesterol suitable for routine clinical work has been evolved on the basis of the above findings, measuring the yellow component of the colour and employing a filter having a maximum transmission at 430 m μ .

3. From a series of 200 normals a mean plasma total cholesterol of 197 ± 4 mg. (s.e.m.)/100 ml., s.d. 39 mg., has been found.

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A Micro Glass Electrode

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Glass electrode assemblies as normally supplied by manufacturers require that the glass electrode, as a small bulb, together with either the standard calomel electrode or a potassium chloride-agar bridge, shall dip into the liquid under examination. Under these conditions a reduction in the volume of liquid below 5-10 ml. means that the containing vessel becomes so small that the risk of scratching the thin glass bulb becomes considerable. Rather smaller volumes can safely be used in the Morton (1930) electrode since only the glass electrode dips into the liquid; contact with the calomel electrode being through a stopcock at the base of the electrode vessel.

Most adaptations of the glass electrode to very small volumes have involved a reduction in the area of the glass membrane. Thus Paic (1938) fused a thin septum of glass at a short distance from one end of a narrow U-tube so forming a very small cup in which 0.1 ml. fluid was sufficient to cover the membrane. Chinoy (1947) made use of an eccentrically blown bulb with a depression at its uppermost point in which 0.05 ml. was placed. In both cases a bridge was used to make contact with the calomel electrode. Such electrodes naturally have a much greater electrical resistance than a fully immersed standard type of bulb, and there is again risk of damaging the membrane with the bridge when the depth of fluid is very small.

Other workers, for example Dietz (1948), have used a block of insulating material in which are two

depressions connected by a small channel. The depressions are designed to fit the two electrodes so that each is surrounded by a film of liquid. The smaller the volume of liquid available for such a set up the greater are the chances of damage to the glass bulb as it is lowered into its cavity. By fitting a conventional bulb type electrode concentrically within a wider tube to allow about 1 mm. clearance Hanes (1951) could measure the pH of 1 drop of fluid which was retained in the annular space by capillarity. Such an electrode would be safe from damage as long as it remained rigidly fixed within the outer tube. Britton (1942) has reviewed various types of glass electrode.

Michaelis (1936) designed an electrode in which the glass membrane took the form of a thin-walled tube fused within a wider vertical tube. Such a design ensures complete protection of the glass membrane from mechanical damage. The centre tube held 3-5 ml. of the liquid under examination, while the annular space contained dilute hydrochloric acid into which dipped the silver-silver chloride electrode. At the lower end of the tube was fitted a 5-way stopcock to allow connexion via a potassium chloride bridge to the calomel electrode. Pickford (1937) has described a modification of Michaelis's design, for much smaller volumes (approx. 0.1 ml.). The present design is based upon that of Michaelis but is simpler than the two just mentioned and can be used with 0.3 ml. liquid. Measurements can be made very rapidly