CHANGES IN THE LIPOPROTEINS OF HUMAN BLOOD SERUM DURING PROCESSING

by

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A thesis submitted to the Faculty
of Graduate Studies and Research
in partial fulfilment of the
requirements for the degree
of Master of Science

McGill University,
Montreal

September, 1946

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Dr. O. F. Denstedt, my Director of Research, for stimulating leadership and many acts of kindness.

Special thanks are due to M. J. Tassé and the staff of the Institut de Microbiologie et d'Hygiène at the Université de Montréal, who provided, at considerable inconvenience, the greater part of the materials studied.

I am deeply indebted to Professor D. L. Thomson and to my fellow students in the Department of Biochemistry for valuable discussions.

Thanks are also due to Mrs. Penelope Wilde for careful and patient typing of the thesis, and to my wife for undertaking the burden of the proof-reading.

This work was materially aided by a Bursary granted by the Province of Quebec.

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THEORETICAL PART

PURPOSE OF THE INVESTIGATION

Since 1939 the value of readily available whole blood, plasma and serum has received widespread and deserved recognition. For most purposes whole blood may be considered the ideal material for replacing lost blood, yet certain conditions, such as burns, in which hemoconcentration is to be combatted, call primarily for replacement of circulating fluid. In such cases plasma or serum may be the materials of choice.

The chief advantage of plasma and serum, however, lies in their stability over long periods which makes possible the accumulation of large stores without wastage. The problem of maintaining such plasma or serum in the best possible condition for long periods of time thus assumes considerable importance.

As will be brought out later, plasma stored in the dry state appears to resist change indefinitely, and has been used with notable success during the war. Nevertheless, the frequent necessity of transporting not only the dry plasma or serum, but also sterile water with which to reconstitute it, offsets much of the convenience of the dry preparation. Apart from the obvious advantage of requiring fewer manipulations, it is accordingly frequently desirable to preserve plasma or

serum in the liquid state.

As part of the program (1) of the Canadian Red Cross Society for the preparation of frozen-dried serum, equipment for the purpose was installed in 1943 at the Institut de Microbiologie et d'Hygiene in the University of Montreal. During the first few months of operation several batches of pooled serum were found to be bacterially contaminated, and although all the serum was routinely sterilized by passage through Seitz filters, it was deemed advisable to set aside such previously contaminated batches for observation. After a short time, a white opalescence appeared, followed by the deposition of a precipitate in the bottom of the bottles. The subsequent discovery of similar material in serum which had at no time contained micro-organisms prompted the investigation into the nature and origin of the precipitate which has formed the subject matter of this study.

NATURE OF THE LIPOPROTEINS OF BLOOD SERUM

1. The Lipid Content of Blood Serum

Preliminary analyses served to show that the material deposited from the serum contained large proportions of lipid, indicating serious disruption of the serum lipoproteins. As an introduction to the questions of the mode of occurrence of lipids in blood serum, the interactions between the lipids

and proteins, and the changes which occur during storage, it is important to have a clear conception of the nature and quantities of the various lipids which are found in human blood serum.

So numerous are the conflicting claims which have been published on the subject of blood lipid analysis, however, that it is necessary to undertake a critical survey of the methods which have been employed in order to arrive at reliable values.

a) Extraction of the Lipids

Early methods of extracting lipids from blood were adaptations of the contemporary methods applied to solid organs, and consisted of drying followed by extraction with ether (2). The inconvenience of drying large volumes of a tissue composed principally of water stimulated the development of some rather extravagant procedures, such as drying with large quantities of copper (3) or sodium (4, 5) sulfates.

The inadequacy of direct ether extraction of tissues gradually became apparent as it was compared with methods wherein the tissue, usually muscle, was hydrolyzed by acid (6), alkali (7), or peptic digestion (8), followed by extraction of the acidified digest. This last method was applied to blood by Nerking (9) and by Schulz (10 who showed that

four to five times as much material could be extracted in this way as by ether extraction of the dried blood. Later, after Kumagawa and Suto (11) had established saponification as the most powerful method of determining the total lipid content of muscle, the method was applied to blood by Shimidzu (12).

Hydrolytic procedures, however, can give no information as to the nature, quantities and mode of occurrence of the individual lipids in the intact tissue. Milder methods of the type now almost exclusively employed were introduced in 1866 by Hoppe-Seyler (13) who extracted the blood with large volumes of alcohol and redissolved in ether. It is rather surprising that the method was so slowly adopted, even after successful applications had been reported (14, 15, 16, 17).

In a series of careful experiments, Kumagawa and Suto (11) compared the extractive powers of the common organic solvents for the lipids in muscle. Ethyl and methyl alcohols were equally and most effective, followed in order of decreasing value by ethyl acetate, chloroform, acetone, benzene, diethyl ether and light petroleum. Kumagawa and Suto followed Pfluger (6) in stressing the importance of miscibility with water and ascribed the success of the alcohols to their dehydrating power.

As methods were gradually developed for the

estimation of the individual lipids and as interest in fat metabolism increased, attention became diverted from the macromethods of blood lipid analysis, whose goal was the weighing of the total or partially purified extract, to micromethods suitable for small volumes of blood and making possible the separate estimation of unaltered lipids. For this purpose by far the most widely used method of extraction has been that introduced by Bloor (18). The blood, plasma or serum was run into a large volume of a 3:1 mixture of ethanol and diethyl ether. The mixture was then brought to the boil, cooled immediately, and filtered, aliquots being taken for the estimations. In the words of Bloor, 'the solvent combines the penetrating power of alcohol with the greater solvent power of the ether.'

Considerable variations have been made in the proportions of extractant to blood. Bloor himself has at various times preferred 3 ml. of blood, plasma or serum in 100 ml. of extractant (19, 20), 3 ml. in 50 ml. (21), 5 ml. in 100 ml. (22), 8 ml. in 100 ml. (23), and 10 ml. in 200 ml. (24). The question of the dilution necessary for complete extraction was studied in detail by Boyd (25) who concluded that a ratio of 1:20 was the minimum possible for complete extraction.

Although the alcohol-ether extraction procedure received the early approval of Mueller (26) and of Feigl (27),

Szent-Gyorgyi (28) considered it 10-20% incomplete. Man and Gildea (29) introduced the step of refluxing the extraction mixture for one hour, claiming a greater yield of total fatty acids. Kirk, Page and Van Slyke (30) adopted this procedure, but subsequently abandoned it as unnecessary. Similarly, Boyd (25) found no change with prolonged heating if optimal volumes of extractant were used. Stewart and Hendry (31), using whole blood, demonstrated an increase up to 40% in total fatty acids by titration on refluxing with exposure to air, but no increase when the heating was carried out under hydrogen. After showing that refluxing the extract after separation from the precipitate led to no such change, these authors suggested that iron in the precipitate was responsible for promoting the oxidation of unsaturated fatty acids.

That the alcohol-ether extraction procedure removes all the lipids from blood, plasma or serum has been amply demonstrated. Man and Peters (32) showed that by re-extraction of the washed protein residues only at most 1% more cholesterol, or 2% more lipid phosphorus, could be obtained. Mueller (26) found a mere trace of cholesterol after saponification of the residue. Similarly, Gardner and Gainsborough (33) found the alcohol-ether extraction equivalent to the saponification procedure of Fex (34) in the determination of cholesterol. Perhaps the best evidence for

the completeness of the alcohol-ether extraction lies in the success with which it has been used for many years by the leading workers in the field.

The use of a 3:1 mixture of alcohol and ether nevertheless has not been obligatory. Boyd found no significant differences between 95% alcohol alone, or 3:1, 1:1, and 1:3 alcohol-ether mixtures, although the massive coagulum formed in the last case had to be broken up with a stirring rod. Taking lipid phosphorus as a measure, Stewart and Hendry (31) found alcohol-ether, alcohol-chloroform and alcohol-light petroleum mixtures to be equally effective. Their results incidentally constituted strong evidence of the completeness of extraction (31, 35).

Acetone, although a poor solvent for phospholipids, has been preferred for that very reason when cholesterol alone was to be determined (36), and has been widely used (37) especially mixed with alcohol (38, 39, 40).

Another method which seems promising is that recently introduced by Delsal (41) wherein application was made of the observation by Charonnat (42) that methylal will extract the lipids quantitatively from serum.

The introduction of more complex mixtures, such as acetone-alcohol-trichlorethylene (43), seems to have been rare and unadvantageous. Worthy of mention, however, are the ether-soap (44) and ether-saponoside (45) treatments for

the extraction of cholesterol, novel departures of considerable theoretical interest, to be discussed later.

For the immediate purpose it is sufficient to conclude that the methods of extraction of lipids from blood can be considered reliable, especially those employed since the beginning of the modern period of differential analysis. The necessity for polar, water-miscible solvents and its direct bearing on the question of the mode of occurrence of lipids in blood plasma or serum will be discussed in a later section.

b) Estimation of the Individual Blood Lipids

In the course of the foregoing outline of the methods of extraction of lipids the differences between whole blood, plasma and serum were not emphasized. Since the experimental work to be presented later has been limited almost completely to blood serum, little attention need be paid to lipid analysis of whole blood. Indeed, determinations made on whole blood can scarcely give much information about either intracellular or extracellular lipids, simply because their proportions differ considerably. For instance, it will be shown that at least two-thirds of the extracellular cholesterol is esterified, whereas all (46), or nearly all (47, 48) the intracellular cholesterol is free. Similarly, it will be found that the order of importance of phospholipids in serum or plasma is first lecithin, then cephalin and lastly sphingomyelin; in erythrocytes, on the other hand,

cephalin (47) and sphingomyelin (49, 50) are of primary importance, with lecithin playing a minor role. The choice of whole blood has particularly detracted from the good work of C. P. Stewart (51). Bloor (52, p.126) has stressed that, in view of the relative constancy of the cell lipid levels, plasma and serum are preferable for metabolic studies.

In addition to distinguishing between intracellular and extracellular lipids, note should be taken of the differences between plasma and serum. Plasma and serum differ in the absence of fibrinogen in the latter, and in the presence of an anticoagulant, added before separating the cells, in the former. In 1916, Gettler and Baker (53) reported a series of analyses showing slightly higher protein nitrogen values in serum than in oxalated plasma, despite the loss of protein in the fibrin clot. These findings were later supported by Peters et al. (54). Magistris (55) has reviewed evidence for lower lipid values in oxalated plasma than in serum. In terms of total fatty acids, Man and Gildea (29) found oxalated plasma to give values 3-8% lower than serum, Kirk et al. (30) 13%; for phospholipids, Schmidt (56) found heparinized plasma to give 4-13% higher values than oxalated plasma; and for cholesterol, Sperry and Schoenheimer (57) found a difference of 15.3%. Boyd (25) reported the same trend for all lipids. Boyd and Murray (58), in a more detailed study, showed that potassium oxalate caused

shrinkage of the red cells which reached a maximum almost immediately and was gradually reversed so that the original condition was regained in about 48 hours. Citrate, cyanide and fluoride had similar effects. The shrinkage of red cells with accompanying plasma dilution, apparently due to disturbance of the osmotic equilibrium, was absent in defibrinated, heparinized or hirudinized blood. The variability of the effect with time would seem to introduce a slight uncertainty into most determinations on plasma, and would justify a preference for values obtained on serum or heparinized plasma. Nevertheless, for most purposes the difference is probably not important (59).

General Methods of Separation

The classes of lipid most commonly estimated in blood analysis have been cholesterol, phospholipid and neutral fat. Two general methods for their separate estimation have been used: 1) separation by virtue of solubility properties, followed by determination of the material by weight, colorimetry or oxidation (22, 30); 2) estimation of some component peculiar to the class, such as lipid phosphorus.

Separation by differential solubility has been most widely used. In either case it has been common practice to evaporate the alcohol-ether or other extract and to re-extract the residue with a lipid solvent, usually light petroleum (30). Phospholipids may then be precipitated by acetone and magnesium

chloride (22). The acetone solution containing cholesterol and neutral fat has usually been analyzed for free and esterified cholesterol. Neutral fat has been calculated by difference from the total lipid.

The separation by differential solubility, however, of such similar substances as, for example, the individual phospholipids can hardly be accurately carried out on small samples. Accordingly, recent investigations have been directed more towards the estimation of particular constituents of the various molecular structures. This latter approach will be particularly emphasized during the following survey of methods and analytical values.

Cholesterol In principle at least the determination of cholesterol may be considered both relatively simple and accurate. Two reactions have been most used, the precipitation of the free form by digitonin (60, 61), and the Liebermann-Burchard color reaction (62, 63) applicable to both the free and esterified forms. The precipitate formed with digitonin may be weighed (64, 61, 33, 32), oxidized (65, 66, 67, 30), or redissolved for colorimetric determination of the cholesterol (38, 39, 68). Digitonin has been used almost exclusively for the separation of free and esterified cholesterol. After precipitation of the free form, the esters have usually been saponified and the freed cholesterol estimated by precipitation with digitonin.

The Liebermann-Burchard reaction has the advantage

of making possible the accurate estimation of as little as 0.05 mg.% of cholesterol. Early applications were developed by Grigaut (69) and Autenreith and Funk (70). Bloor (20) applied the latter colorimetric procedure to his alcohol-ether extract. Since that time the Liebermann-Burchard reaction has been widely employed, most notably by Schoenheimer and Sperry (38) and by Kelsey (68). In 1921 Gardner and Williams (71) showed that although both forms of cholesterol gave the same color, that developed by the esters was more intense. This discovery has been the primary reason for introducing hydrolysis prior to color development, although empirical corrections have been employed (72). Ireland (73) has reviewed the effects of temperature and time on the reaction. Recently, the mechanism of the reaction has been partly elucidated (74).

Difficulties encountered in the estimation of cholesterol in a serum extract may be due either to the saponification procedure or to the lack of specificity of the reactions. The saponification has usually been carried out directly on the alcohol-ether extract. Bloor (75) cautioned against heating the mixture to dryness. Okey (66) carried out the saponification under carbon dioxide. Schoenheimer and Sperry (38) and Kelsey (68) obtained complete hydrolysis without destruction using alcoholic KOH at 37-40°.

Neither precipitation with digitonin nor the Liebermann-Burchard reaction is specific for cholesterol.

Digitonin forms insoluble complexes with steroids possessing an hydroxyl group on carbon atom 3 and of the beta configuration. Similarly the color reaction is common to many steroids. Anderson (76) found considerable amounts of material in the unsaponifiable matter of dog plasma lipids which gave the color reaction but not the precipitate. Beef plasma, on the other hand, yielded almost entirely crystalline cholesterol. Graff (77) has found about 80 mg.% of material in human serum which did not precipitate with digitonin and gave a weak Liebermann-Burchard test. Man and Peters (32) reported that decidedly less cholesterol could be precipitated if the serum was first allowed to stand several days in the ice-box. Recently Koehler and Hill (78) have claimed that purified serum cholesterol differed from the form usually obtained commercially and used for standards. These results recall the earlier work of Lifschutz (79) on "Oxycholesterin" in blood and illustrate the uncertainty which persists even in the case of this relatively well known lipid.

Further uncertainty as to the precise condition of cholesterol in a serum lipid extract was introduced by the application (80) of pyridine sulfate as precipitant in place of digitonin (81). It was found that 6-10% of the total cholesterol was directly precipitable with pyridine sulfate, whereas 25-35% was precipitated by digitonin. After saponification precipitation was complete with either reagent. Sobel et al employed the terms 'unbound' and 'loosely bound'.

Separate mention should be made of the recent applications of the Tschugaeff reaction (82) to the determination of cholesterol in serum (83) and plasma (40). The great advantage claimed for this method was the development of the same color and intensity by free cholesterol and all esters (83). The advantages of avoiding saponification seem to recommend this method to study on this continent. Incidentally, Trappe (83) separated free and ester cholesterol chromatographically in the same proportions as were determined by digitonin, and pointed out the apparent disagreement with Sobel's group (80).

Total cholesterol values normally have been found to vary considerably from one individual to another. On the other hand, Boyd (84) showed in a series of eight subjects that the variations for each person of all lipids, and especially cholesterol, during a 24-hour period were rather small and independent of food intake. Bloor and Knudson (85) were among the first to point out the constancy of the ratio of free to total cholesterol. More recently, in a series of 126 samples, Sperry (86) found 24.3-30.1%, average 26.9% of the cholesterol to be free.

Schoenheimer and Sperry (38) reported values ranging between 100 and 350 mg.%, mostly about 150 mg.%, of total cholesterol in human serum. The 118 values collected by Boyd (48) showed total cholesterol, 152 $\pm$ 24 mg.%; ester cholesterol, 106 $\pm$ 25 mg.%; and free cholesterol, 46 $\pm$ 8 mg.%.

Phospholipids The difficulties involved in the analysis of phospholipids are those of separate determination of the three different types, - lecithin, cephalin and sphingomyelin. The determination of total phospholipids on the other hand may be considered a very accurate procedure.

Two general methods of phospholipid estimation have been used; 1) the determination of total lipid phosphorus; i.e., of a constituent peculiar to the class of lipid, a procedure comparable to colorimetry of cholesterol; 2) a separation of the phospholipids due to their insolubility in acetone, analogous to the precipitation of cholesterol with digitonin.

The introduction of the second method, made popular by Bloor and associates (22, 67), was prompted chiefly by doubt as to the specificity of the phosphorus determination (22), whereas its retention in spite of its great complexity has been due mainly to its place in the coherent scheme worked out by Bloor for the application of the Bang oxidative procedure to all plasma lipid fractions. On the other hand, the precipitation procedure would scarcely have survived had it not given excellent agreement with the direct method which has since been amply justified.

The determination of total phospholipid from total lipid phosphorus, also introduced as a routine micro-analytical procedure by Bloor (19), was brought into question by the failure of various workers (30, 87, 88) to recover all the

phosphorus originally present in an alcohol-ether or similar extract after evaporation and re-extraction with light petroleum. The obvious assumption was that some non-lipid phosphorus was present, possibly in the form of inorganic salt or simple ester. Man and Peters (32), however, showed that the sum of inorganic and lipid phosphorus was equal within experimental error to the total phosphorus of a sample of serum, and further, that addition of the Fiske and Subbarow (89) reagents directly to an evaporated aliquot of alcohol-ether gave no colour. Later Wilson and Hansen (90) successfully made use of the inorganic phosphorus liberated by direct saponification of the alcohol-ether extract as a measure of lipid phosphorus. Perhaps the most conclusive evidence came from the success of the procedure introduced by Folch and Van Slyke (91) in which the serum proteins were precipitated and washed free of crystalloids before the extraction of the lipids.

On the other hand, it gradually became apparent that the discrepancies between the phosphorus content of the alcohol-ether extract and that obtained on re-extraction of the alcohol-ether residue with light petroleum were due to changes in the phospholipids themselves. Page et al (87), found that after evaporation of the alcohol-ether, re-extraction with light petroleum gave phosphorus results 20-30 per cent lower. Kirk, Page and Van Slyke (30) in following up this question found that even lower recoveries were obtained if the alcohol-ether was evaporated at 75°C. rather

than 60°C or less. Later, however, Man (92) obtained 95-100.5 per cent recovery when the evaporation was carried out *in vacuo* under nitrogen at a temperature below 37°C. and immediately re-extracted with light petroleum. Man also showed that this yield was reduced by heating the residue in contact with air. Shortly afterwards, Bloor (93) pointed out that he had for some time been employing a light watch-glass as a flutter valve to prevent entrance of air during the evaporation. Air initially present was washed out by the vapours. Incidentally, Bloor's procedure differed from most others in that evaporation was continued till all the alcohol had been removed and only a few drops of water remained. Poor recoveries were obtained when the extract was taken to dryness. In recent work, Chaikoff (94, 95) has obtained perfect recoveries using evaporation under carbon dioxide in a vacuum to a small volume and re-extraction with light petroleum.

The objection might still be raised that complete resolution in light petroleum does not prove that all the phosphorus was present as phospholipid. Bloor (22) quoted the report by LeBreton (96) that up to 20% of the phosphorus redissolved by dry ether was non-lipid. It has seldom been stressed, however, that LeBreton was not concerned with blood but with solid organs wherein active phospholipid metabolism might easily have produced intermediates of uncommon solubility properties. On the contrary, if solubility in light petroleum and insolubility in acetone may be taken as

distinguishing properties of phospholipids, then the fact that at least 97% of the phosphorus soluble in light petroleum has consistently been precipitated by acetone and magnesium chloride (32, 35, 95) would seem to establish that all such phosphorus was present as phospholipid.

The foregoing outline has served to establish the adequacy of total alcohol-ether phosphorus as a measure of lipid phosphorus. If an estimation of the various constituents were desired, however, clearly other methods would be necessary. Nevertheless, all such methods as have been devised have involved calculation by difference, and so have required a knowledge of the total phospholipid.

The three classical types of phospholipid for which analytical methods have been attempted are lecithin, cephalin and sphingomyelin. Lecithin and cephalin both contain one mole of glycerol and phosphate and two moles of fatty acid. They differ only in the presence of choline and ethanolamine as respective bases. Sphingomyelin on the other hand contains no glycerol and but one mole each of choline, phosphate and fatty acid and the peculiar amino alcohol, sphingosine.

In 1930, Schmitz and Koch (97) attempted to determine cephalin from the amino-nitrogen content of a water-washed ether extract of an alcohol-ether residue. Lecithin was calculated by difference from the total phospholipid as derived from the total lipid phosphorus. All workers have

agreed in using a factor in the neighbourhood of 25 for converting lipid phosphorus to phospholipid. The same principle was used four years later by Kirk, Page and Van Slyke (30) for their gasometric oxidative method. These workers discovered that the phospholipids precipitated by acetone and magnesium chloride were not completely soluble in moist ether. The residue was soluble in hot alcohol. A single large sample was prepared and the N:P ratio of the ether-insoluble material was found to be exactly two to one. The material was accordingly assumed to be a diamino phosphatide, presumably sphingomyelin.

Unfortunately, however, the perfect value for the N:P ratio soon became a source of embarrassment when the same group discovered (87) that considerable quantities of nitrogen in the light petroleum extract were not due to phospholipid, and could be only partially removed by washing with acidified water (98). The existence of unquestionably high N:P ratios in both the light petroleum extract and the phospholipid precipitate was rapidly recognized (99, 101, 100) and found to be principally due to urea (100, 101). Since urea was quite insoluble in pure light petroleum (101), it was concluded that the phospholipids were responsible. The phenomenon would seem to be due to salt formation, and may well be similar to the demonstration by Collander (102) of a 35-fold increase in the solubility of urea in olive oil after the addition of 20% of oleic acid. Indeed, the solubility of urea in light

petroleum solutions of lipids is less surprising than the discovery of large quantities of chloride and smaller quantities of sodium in such extracts (103).

A successful method of avoiding contamination by nonlipid extractives was developed by Folch and Van Slyke (91) who made use of "colloidal iron" to precipitate the proteins which were then washed and extracted with alcohol-ether. The interesting observation was made that the presence of water was necessary for complete extraction. This method has been found entirely satisfactory by Artom (104).

In view of the foregoing evidence, it is perhaps surprising that it was not until 1942 that Sinclair and Dolan (105) severely criticised the assumption made by Kirk, Page and Van Slyke (30) as to the nature of the ether-insoluble phospholipid. Previously Kirk (106) had published a scheme wherein the magnesium chloride precipitate was extracted with moist ether and the "ether-insoluble phosphatide" was determined by analysis of the residue for phosphorus. The ether-insoluble material was believed to be primarily sphingomyelin and indeed it was shown that added sphingomyelin was to be found in this fraction.

Sinclair and Dolan (105) during an attempt to apply Kirk's method found that in the absence of magnesium chloride all of the tissue phospholipid and most of the plasma phospholipid that was precipitated was soluble in moist ether. With

the addition of magnesium chloride, the percentage of ether-insoluble material increased in proportion to the amount of salt. In this way a maximum of about 20 per cent of the tissue phospholipid and about 90 per cent of the plasma phospholipid became insoluble in moist ether. Moreover, the N:P ratio of the total phospholipid fractions and of the ether-insoluble fractions were quite similar and did not admit of sphingomyelin as an important constituent. Thannhauser (107) has since admitted that ether-insolubility is inadequate for the separation of mono- and diaminophosphatides.

Even before the ether-insoluble phospholipids had thus been shown to be an artifact, the attempt to fractionate small quantities of such similar substances as phospholipids by differential solubility began to be abandoned. Instead, methods have been devised in which the various constituents esterified to form the phospholipid molecule, phosphate, glycerol, fatty acid, choline and ethanolamine were determined after saponification. Added confusion was recently introduced by the discovery that cephalin contained appreciable quantities of serine in place of ethanolamine (108, 109).

In contrast to the accuracy of the methods which have for some time been available for the determination of small quantities of phosphorus (110, 111, 89, 112, 113), uncertainty still exists in the estimation of the bases. Micromethods for the determination of choline have been mainly dependent on either precipitation as the periodide with

subsequent liberation and titration of the iodine (114), or more often as the reineckate followed by colorimetry of its pink acetone solution (115, 116). A method of determining ethanolamine by distillation was introduced by Blix (117). Artom (118) subsequently estimated both ethanolamine and serine by adsorption of the former on permutite, followed by treatment with periodate. Blix (107) and Ramsay and Stewart (51) have included glycerol determinations in their blood lipid schemes.

Perhaps the greatest uncertainty in the application of the methods of estimation of the individual constituents has been in the preliminary step of hydrolysis, for which conflicting claims have been common. Kirk (106) and Williams et al (119) hydrolyzed the ether-soluble phospholipids with barium hydroxide, then determined the liberated choline by the reineckate method to obtain a value for lecithin, and calculated cephalin by difference. It should be noticed in passing that these procedures, although widely quoted, can be of little value in view of the use of moist ether to extract lecithin and cephalin from the phospholipid precipitate.

Thannhauser, Benotti and Reinstein (120) preferred to hydrolyze the total phospholipid for choline determination. The remaining equivalents of phospholipid were taken as cephalin. Sphingomyelin was determined separately by a modification of an earlier method (50) in which the phosphorus

content of the insoluble sphingomyelin reineckate was determined. The difference between total choline phospholipid and sphingomyelin gave lecithin. These workers found barium hydroxide to be inadequate for the hydrolysis of sphingomyelin and substituted hydrogen chloride in absolute methanol. Unfortunately, Thannhauser preferred to dry the serum in warm air, followed by desiccation over phosphorus pentoxide, then grinding with sand and extraction with a mixture of methanol and chloroform. Oxidation during the drying procedure possibly affected the results.

In contrast to Thannhauser, Brante (121) concluded that alcoholic sodium hydroxide was the best reagent for the hydrolysis of the phospholipids. He did not attempt to determine sphingomyelin, but noted that about 10-15% of the phospholipid was abnormally resistant to hydrolysis. In the same connection it is interesting to note that Wilson and Hansen (90) reported the presence of 'unsaponifiable phospholipid' after prolonged hydrolysis with alcoholic potassium hydroxide.

Of immediate bearing on the results of Wilson and Hansen is the recent note by Schmidt, Benotti and Thannhauser (122), to the effect that lecithin and cephalin were quantitatively transformed into acid-soluble phosphorus compounds during incubation with aqueous potassium hydroxide for 15 hours at 37°C. Under these conditions the phosphorus of sphingomyelin remained insoluble in acids. Recently, too,

Sperry and Brand (123) have criticized the use of methanolic hydrogen chloride and have advocated constant boiling hydrogen iodide as yielding considerably more choline from sphingomyelin than could be obtained with barium hydroxide.

A new approach to the problem of estimation of the individual phospholipids was introduced by Blix (117) who analyzed concurrently for phosphorus, glycerol and fatty acid. The equivalent ratios showed that about 13% of the phospholipids did not contain glycerol, and that that fraction could contain one fatty acid per molecule, in other words could be sphingomyelin. However, two samples of one litre of ox serum were analyzed without detecting sphingosine. It is interesting that in the same paper Blix reported ratios between choline, phosphorus and ethanolamine which showed that part of the non-choline phospholipid was not of the ethanolamine type, thus foreshadowing the discovery of phosphatidyl serine.

The only scheme of analysis published up to the present which has attempted to give values for all the human serum phospholipids, - lecithin, phosphatidyl ethanolamine (the classical cephalin), phosphatidyl serine and sphingomyelin, - was that of Artom (124), based on his earlier scheme (104, 125) with the inclusion of the method for ethanolamine and serine (118). Methanolic hydrogen chloride was used because it was found that barium hydroxide destroyed some serine and considerable ethanolamine. Lecithin alone had to be calcu-

lated by difference between equivalents of choline and of sphingomyelin, the latter determined by a method (125) closely following Thannhauser (120). Ninety-five per cent of the total phospholipids were accounted for with the following distribution: lecithin, 55%; phosphatidyl ethanolamine, 21%; phosphatidyl serine, 7%; sphingomyelin, 12%.

In complete disagreement with Artom's promising work is the finding by Taurog, Entenman and Chaikoff (94) of choline:P ratios of 0.98 for human heparinized plasma and 1.02 for dog plasma, leading to the conclusion that practically all the phospholipids of plasma are of the choline-containing type. Results were the same whether the extraction was carried out directly with alcohol-ether or by the washing method of Folch and Van Slyke (91), and regardless of whether the determinations were made on the whole alcohol-ether extract or acetone-magnesium chloride precipitate. The results were subsequently checked for dog plasma (95). No obvious explanation seems available for the discrepancy between these findings and the general experience, unless it lay in the use of barium hydroxide. But objections to the use of this mild base have been concerned with incomplete hydrolysis of sphingomyelin (120) or with destruction of ethanolamine and serine (118), neither of which has any bearing here. Unless we accept some unsuspected effect of diet (95), there remains no choice but to conclude that the details of phospholipid distribution in blood serum are still very uncertain.

Certain it is, however, that lecithin is the principal species and that a small amount of what appears to be sphingomyelin is also present. The importance of cephalins has usually been considered to lie between the other two, and may prove to be quite variable even in fasting animals.

Cerebrosides The cerebrosides or galactolipids in which sphingosine is esterified on the one hand with a fatty acid and on the other with galactose have rarely received attention in blood lipid analyses. Kirk (126) developed a method in which reducing power was determined after hydrolysis of the lipids, and incorporated it into his general scheme (106). In thirteen determinations on human plasma he found (127) values ranging from 0 to 167 mg.% cerebroside. No such compound was isolated. Sobel (128) has reported that the values obtained by this method decreased with the time taken to work up the lipids, and that nothing could be detected if the analyses were left to the following day. Some such rapid changes might account for the failure of Thannhauser et al (120) to isolate any cerebrosides from 1600 ml. of ox serum. These workers considered the reducing power as measured by Kirk to be due to contaminants. Similarly, Bruckner (129) has expressed doubt as to the presence of cerebrosides in normal serum, although he did detect them in red cells (130).

Triglycerides The glycerol esters of fatty acids contain

no distinguishing constituents by which they may be recognized and estimated in a mixture of other lipids. Consequently until recently only one attempt had been made to determine them at all directly.

As early as 1907, Letsche (131) recognized that very little triglycerides were present in blood.

Ivar Bang in 1918 developed a method (65) wherein a small amount of blood was dried on paper, then extracted first with light petroleum and then separately with alcohol. The first extract was believed to contain triglycerides and free cholesterol only, whereas the second contained cholesterol esters and phospholipids. Triglycerides or 'neutral fat' were determined by chromic acid oxidation of the fatty acids derived from the filtrate obtained after treatment of the first extract with digitonin. Bang's work was corroborated with a similar procedure by Blix who found (132, 133) post-absorptive blood to contain 0-40 mg.% of neutral fat.

Channon and Collinson (134) working with large volumes of ox blood, found very little triglyceride could be present after they had accounted for the other lipids.

On the other hand, the more widely employed methods of microanalysis in which neutral fat was determined by difference have given quite appreciable values. Boyd (48), for example, on a series of 118 samples of human plasma calculated 142±60 mg.% neutral fat or an average of 27% of the total

lipids. It seems probable that poor recoveries of phospholipid in particular, or merely cumulative small negative errors in both phospholipid and cholesterol, may account for the high value for neutral fat. Since such values are undoubtedly incorrect, further consideration is unnecessary beyond pointing out that they have been also widely trusted.

A new method in which triglycerides are hydrolyzed by a lipase from the castor bean was developed by Kelsey (135, 136) in Bloor's laboratory, and first applied by Bloor, Blake and Bullen (24). Further study, however, led Kelsey himself to criticize the method when only about half as much fatty acid was liberated by the lipase as he had expected (136). Nevertheless, Kelsey and Longenecker (137) later applied the method to ox plasma with results similar to those of Channon and Collinson (134).

In 1940 Blix (138) estimated the triglycerides of human plasma by two methods: indirectly by the excess of equivalents of glycerol over lipid phosphorus (corrected for 13% sphingomyelin (117)); and directly from the glycerol soluble in acetone and magnesium chloride. Sixteen determinations by the first method averaged 49 mg.% and six by the second 38 mg.%.

To sum up, triglycerides may be taken to occur at a level of about 40-50 mg.%, or at most 10% of the total plasma lipids.

Free Fatty Acids In 1883, Hoppe-Seyler (139) reported the presence in blood plasma of free fatty acids or soaps. The soaps were separated from the other lipids in an alcohol extract by means of their insoluble lead salts. Hoppe-Seyler found 50-120 mg.% of fatty acid in this form in the blood sera of oxen, horses and dogs.

The determination of small quantities of free fatty acids in blood serum was first attempted by Lemeland (140), who took advantage of the insolubility of the dry soaps in ether. A more extensive study by Szent-Gyorgyi and Tominaga (28) utilized the insolubility of the soaps in dry light petroleum to develop a very complicated procedure leading to the isolation and weighing of the fatty acids. Fifteen determinations on defibrinated blood gave values ranging from 3.1 to 7.5 mg.%, with an average of 5.4 mg.%.

The most recent attempt to determine the free fatty acids of human plasma was made in 1929 by Stewart and White (141). The method was based on the indirect titration of the alkali accompanying the fatty acids in the alcohol-ether extract. Two values of 46.7 and 98.7 mg.% of free fatty acid calculated as oleic acid were given.

It appears to have been generally agreed that the lower values for free fatty acid in fresh serum are more accurate. Sperry (142) considered 20 mg.% as the probable maximum. It is noteworthy that all the methods which have

sought to determine triglycerides by subtracting the fatty acids corresponding to phospholipids and cholesterol esters from the total fatty acids have tacitly assumed the absence of free fatty acids.

In a few cases there has been some evidence of decomposition during analysis leading to the release of free fatty acids. Bloor (143) in 1924 found 10-33% of the total fatty acids of beef plasma to be free. The fatty acids were separated from the other lipids by virtue of the failure of light petroleum to extract them from alkaline 50% alcohol. Similar results have been obtained more recently by Kelsey and Longenecker (137). On the other hand, Channon and Collinson (134, 144) found very little free fatty acid in ox plasma, but obtained considerably higher values for phospholipid, and concluded that the preparation of Bloor had undergone some decomposition of phospholipids to release free fatty acids.

Distribution of Types of Fatty Acid In the outline of the serum lipids which has been presented, the question of differences in unsaturation or chain length among the fatty acids was postponed. It is intended at this point to describe the distribution of these properties among the fatty acids of the various lipid fractions. Since the interesting changes in the fatty acids caused by diet or disease are clearly outside our scope, the discussion will be limited to fasting values in healthy subjects.

The most direct way of determining the total fatty acids of small quantities of blood serum is by virtue of their acidity. Stewart and White (145) in 1925 published a method wherein the lipid extract was saponified with alkali, the exact equivalent of acid added, and then the fatty acids were titrated with sodium hydroxide. This method obviously included acidity due to the phosphate liberated from the phospholipids. More reliable methods have since been developed in which the fatty acids were separated by filtration (146, 147, 29, 31) or extraction with light petroleum (31). Oxidative methods were included in the schemes of Bloor (21) and Kirk, Page and Van Slyke (30).

Man and Gildea (148) found in 1932 that their own method agreed with most of the earlier values in giving an average of 330 mg.% total fatty acids. Boyd (67) and Hansen (90) have agreed on a value about 350 mg.%.

The mean molecular weight of the fatty acids has been obtained by titration of a weighed sample of the isolated acids. Hansen (90, 149, 150) found this value to be about 290. Such a value corresponds roughly to the C_{18} acids which were accordingly assumed to make up the bulk of the mixture. A certain amount of fatty acids of both higher and lower molecular weights nevertheless are probably present (90).

The degree of unsaturation of the fatty acids has

been determined in the usual way in terms of the number of grams of iodine absorbed by 100 grams of lipid. Recent work on blood lipids most frequently has employed Yasuda's (151) application of the pyridine bromide reagent (152) to small quantities of material. Boyd (67) reported an average iodine number of 88.5, whereas Hansen has reported values of 108 (90) and 100 (150). The presence of highly unsaturated fatty acids was reported by Bloor (153) in the plasma of the ox, pig, sheep and dog, and by Channon and Collinson (144) in ox blood.

It is generally agreed that the most unsaturated fatty acids are to be found among those esterified with cholesterol (134, 137). Working with ox plasma, Kelsey and Longenecker (137) found 85% of the fatty acids of the cholesterol ester fraction to be unsaturated, and 62% was identified as linoleic acid. Bloor, Blake and Bullen (24) found iodine numbers of the order of 160 in the fraction of fatty acids derived from the cholesterol esters, whereas phospholipid and triglyceride fatty acids gave values of about 120 and 100 respectively. A similar finding of greater saturation in the phospholipid fatty acids was reported by Channon and Collinson (134) and by Bloor (143). The same workers obtained indirect evidence of an even lower value for the triglycerides, a finding which may explain the otherwise discordant reports that the phospholipids showed a higher iodine number than the total fatty acids.

The difficulty of obtaining accurate iodine numbers and values for mean molecular weight need hardly be stressed. Only recently, MacLachan (154) has overcome some of the obstacles in obtaining reproducible iodine values for whole phospholipid.

Taken as a whole, the values which have been presented show that the fatty acids present in the lipids of fasting serum are composed principally of C_{18} acids such as stearic, oleic, and linoleic, with smaller amounts of the C_{16} palmitic and also of higher, probably more unsaturated, acids.

Collected Values for Lipids in Human Blood Serum In the following table average values have been collected showing the quantities of the principal lipids in human blood serum.

TABLE I

QUANTITIES OF THE LIPIDS OCCURRING IN HUMAN BLOOD SERUM

Lipid	Milligrams per 100 ml.
Cholesterol	
Total	150
Esters	100
Free	50
Phospholipids	
Total	200
Lecithin	125
Cephalin	50
Ethanolamine type	35
Serine type	10
Sphingomyelin	25
Triglycerides	40
Total Fatty Acids	350

The preference for certain values was made clear in the detailed discussions of the individual lipids. The figures chosen for the phospholipids correspond most closely to those of Brante (121) and Artom (124). The inclusion of cephalin is not intended to imply that the work of Chaikoff's group (94, 95) should be questioned.

II. Relation between Lipids and Proteins in Blood Serum

a) Occurrence of Lipids in the Various Protein Fractions

The inadequacy of direct extraction with ether as a means of removing the lipids from blood serum was brought out in the previous section without particular emphasis. As early as 1901, however, Nerking (9) recognized the necessity of assuming some sort of binding between lipids and proteins to explain such behaviour. The correctness of this assumption was demonstrated by Sørensen (155), who also pointed out that the clarity of serum could be explained in no other way.

Early workers using precipitation methods gradually established an association of lipids with the serum globulins. In 1905, Hardy (156) discovered that ox serum globulin prepared by half saturation with ammonium sulfate contained phosphorus which could not be removed by treatment with acid. He noted further that the phosphorus could be concentrated into the fraction insoluble in the absence of salts - the 'euglobulin' as defined by Marcus (157). These observations were confirmed in 1913 by Haslam (158), who went so far as to

employ the presence of phosphorus as a test for the progress of the separation of pseudo- from euglobulin. Soon after, Chick (159) went a step further in making the classical suggestion that euglobulin and pseudoglobulin differed only in the presence of fatty phosphorus-containing material. In 1918, Bang (160) showed that not only phospholipid but also neutral fat and cholesterol were precipitated along with euglobulin.

A few years later, Frankenthal (161) reported that 72% of the total serum lipids were precipitated in the euglobulin fraction. At the same time Handovsky, Lohmann and Bosse (162) found 25% of the ox serum cholesterol firmly bound to globulins, very little to albumins, and the rest was assumed to be combined in some way with phospholipids. Their widely quoted results are perhaps made less convincing by their failure to find any phosphorus in the globulin precipitate. The probable explanation of the low value for protein-bound lipid lies in the use of heat to coagulated the proteins, especially in view of the similar results obtained with the use of heat by Turner and Gibson (163) in 1932.

In 1926, Troensgaard and Koudahl (164) made the important observation that serum albumin contained considerable quantities of cholesterol esters and lecithin, more in fact than they could find in the globulin fraction. In the same year, Theorell (165) prepared from horse plasma the three fractions fibrinogen, globulin and albumin, using as dividing

lines 27% and 50% saturation with ammonium sulfate. He obtained important quantities of lipids in the albumin fraction, and further found that nearly all the lipid could be precipitated and redissolved to give clear solutions. The lecithin/cholesterol ratio increased in the order fibrinogen, globulin and albumin.

Shortly after, Gardner and Gainsborough (166) reported 80-90% of the cholesterol of human plasma to be present in the euglobulin fraction.

In 1929, Macheboeuf (167) acidified a classical horse serum albumin solution half saturated with respect to ammonium sulfate to a pH of 3.8, whereupon almost complete precipitation of the lipids occurred, giving a material composed of 40% of lipid. This material could be redissolved in small volumes of water to give limpid solutions, even to the extent of 50 grams of lipid per litre. Higher concentrations gave clear gels. The lipids comprised almost exclusively phospholipids and cholesterol esters in nearly equal amounts. Since the study of this remarkable lipoprotein fraction has since led to valuable contributions to the understanding of lipid-protein interactions, further consideration will be made in the following section.

Sørensen (155) in 1930 confirmed the precipitation of lipids along with protein fractions, also recognized the necessity of assuming some linkage between the two classes

of substance, but on the basis of his well-known theory concluded that the presence of lipids in any given precipitate did not mean that they were associated with the same proteins in the native serum.

In the same year, Lustig and Katz (168), employing a complicated ammonium sulfate fractionation procedure, arrived at the conclusion that the lipids of ox serum were associated for the most part with albumins and water-insoluble globulins. Somewhat similar results for horse plasma were obtained about the same time by Theorell (169). Here, however, albumin and pseudoglobulin (36-50% saturation with ammonium sulfate) appeared to be chiefly associated with lipids.

Similar conclusions as to the association of lipids with serum proteins were reached by Went and Goreczky (170) and Bendien and Snapper (171), using ultrafiltration through collodion membranes, and also by Sullmann and Verzar (172) employing simple diffusion through semi-permeable membranes. In each case lipids did not penetrate membranes impermeable to proteins, although Went and Goreczky claimed that ether-soluble phosphorus equivalent to 30-40% of the total phospholipid came through. This result almost certainly was due to entrainment of inorganic phosphorus during the direct extraction of the ultrafiltrate with ether. Attempts to fractionate the proteins by the use of graded porosity were inconclusive.

In the same connection,mention should be made of the

experiments of Bruger (173), in which several pathological body fluids, not including blood plasma, were ultrafiltered through membranes or kieselguhr. Some indications were obtained that cholesterol was retained when proteins passed through freely, although the values given are not entirely convincing. Bruger concluded that the cholesterol was dispersed in large micelles independent of protein. It is interesting that Macheboeuf and Fethke (174) previously had described a case of ascites in which the fluid contained crystals of cholesterol and fatty acids. Both lipids and proteins were present in amounts commonly found in clear ascitic fluid.

The first application of the more promising technique of electrophoresis to the problem was made in 1932 by Bennhold (175). The fat-soluble dye Sudan III was first added to the serum which was then subjected to electrophoresis. The color became concentrated in the slower zone, from which it was concluded that the lipids were associated primarily with globulin.

Mellander (176) in 1935 employed a compartmented U-tube which made it possible to analyze the fractions separately. Resolution was on the whole rather poor, probably because the serum was undiluted, although the association of lipids with both albumins and globulins was established. The fastest albumin and globulin fractions seemed to contain

the highest proportions of cholesterol, whereas very little was found in the slowest globulins.

More recent advances in the technique of electrophoresis have contributed greatly to the knowledge of the distribution of the lipids among the serum protein fractions. Tiselius (177) noticed that the opalescence due to the fat droplets or chylomicrons (178) always moved in the beta-globulin (179) fraction and agreed (180) with other suggestions that they were probably coated with that protein (181, 182). Tiselius also pointed out that Bennhold undoubtedly had observed only the beta-globulin in his dye experiments. It should be clearly understood that in the fasting plasma or serum with which we are concerned the chylomicrons are not numerous or large enough to constitute an important part of the total lipid (178).

The most extensive paper on electrophoresis and serum lipids was published in 1941 by Blix, Tiselius and Svensson (183). These workers achieved a good separation of albumin and alpha-, beta and gamma-globulins in sufficient yields to perform separate lipid analyses. The following results were averages from three normal sera, as shown in Table II.

It should be noted that owing to the large amount of albumin and gamma-globulin in serum, at least half of the plasma lipids migrate with them even though their percentage

lipid content is lower. A second electrophoresis served to lower the lipid content of the albumin only very slightly.

TABLE II

THE DISTRIBUTION OF LIPIDS IN SERUM PROTEIN FRACTIONS

Protein Fraction	Lipid content in % of fraction	
	Phospholipid	Cholesterol
Albumin	2.25	1.07
Alpha-globulin	7.75	4.45
Beta-globulin	10.0	8.65
Gamma-globulin	1.0	0.41

Taken from Blix, Tiselius and Svensson (183)

New possibilities have been opened up by the development of the alcohol fractionation procedure of Cohn and coworkers (184). By this method large quantities of relatively pure albumins and alpha-, beta and gamma-globulins have been readily obtained from pools of human plasma. The lipids have been found mainly in Fractions III-0 (beta-globulin) and IV-1 (alpha-globulin). These fractions in turn appear to be capable of further subdivision. It is interesting that considerable quantities of lipid-poor alpha- and beta-globulin were isolated in Fraction IV-4. The lipid-rich proteins were water-insoluble, and might thus correspond to the euglobulin

of Chick (159) and earlier workers. The beta-globulin of Fraction III-0 contained over 20% cholesterol as well as considerable phospholipid.

It is noteworthy that the albumins prepared by Cohn et al. were practically devoid of lipids, in marked contrast to those separated by electrophoresis by Blix et al. (183). The explanation probably lies in the difficulty of separating albumin from alpha-globulin by electrophoresis of whole serum (185). It should be noted also that Svensson (186) and Taylor and Keys (187), in electrophoretic studies of fractions separated by ammonium and sodium sulfates respectively, found considerable further quantities of globulin in the albumin fractions. These results are in agreement with the finding (188) that the albumin/globulin ratio determined by electrophoresis was two-thirds of that determined by salt fractionation.

In the same connection recent electrophoretic studies of Macheboeuf's lipoprotein (189) are of interest. A fairly pure (190) preparation was found to give two sharp boundaries only. The cholesterol/lipid phosphorus ratio was the same throughout the U-tube. Since the absolute values for the lipids in the slower fraction were a little lower, it was concluded that some ordinary albumin was present. It was stressed that the slower lipoprotein could not be separated from the albumin, and this fact was taken to explain the

way in which the lipoprotein had escaped notice during other electrophoretic studies of serum.

Further light on the composition of serum albumin prepared by salt fractionation was provided by the results published in 1938 by Kleczkowski (191), also based on the work of Macheboeuf, in which four horse serum albumin fractions were obtained: 1) the familiar crystalline lipid- and carbohydrate-free albumin; 2) Macheboeuf's fraction, very rich in lipids and poor in carbohydrate; 3) a carbohydrate-rich fraction corresponding to Hewitt's (192); 4) and a fourth fraction containing both lipid and carbohydrate.

Also worthy of note was the finding by Kendall (193) of 2-3% of fatty acid in crystalline serum albumin. This unexpected fact has recently been under study in Cohn's laboratory (194).

In conclusion it is well to draw attention to the fact that the plasma and serum of a considerable number of species have been shown (188, 195) to give rather similar electrophoretic patterns, even in the location of the turbidity (188), and that it has therefore been permissible for our purposes to lump the evidence together without particular attention to species differences.

b) Modes of Interaction or Linkages between Lipids and Proteins

Przylecki (196, 197, 198) has attempted to classify protein-containing complexes according to the possible types of linkage between proteins and other substances. These fall into three classes (198):

1) Electrovalent Symplexes Examples are nucleoproteins, and compounds of proteins with phosphatides, or with acid and basic polysaccharide groups (bacterial antigens). Such compounds are stable only within certain pH ranges.

2) Covalent Symplexes These usually involve esterification, such as N-glycoside linkages in carbohydrate-proteins or between proteins and fatty acids or phospholipids, and are much more stable than the heteropolar types.

3) Co-ordination Symplexes In addition to polyosoproteins and polyproteins, these include lipoproteins which are stable towards water and non-polar organic solvents, but are dissociable under the action of organic acids, bases or alcohols. The lipids involved may be fats, phospholipids, sterols or carotenoids and linkages may be effected through many protein groups. For instance lecithin (197) may be linked electrovalently a) through the $-P-O^-$ to a cationic group, or b) through the quaternary ammonium radical to an anionic group; or by co-ordinate valence through c) the ester linkages, d) an unsaturated bond, or e) by van der Waals cohesion of the paraffin chains with similar groups in the protein.

The term 'symplex', proposed by Willstatter and Rohdewald (199) and adopted by Przylecki, appears to offer no advantages over 'complex' (200). Chargaff (201) has criticized the word 'lipoprotein' as follows: "The indiscriminate use of the term lipoproteins for complexes between proteins and phosphatides, sterols, glycerides and fatty acids respectively is confusing. Besides, one ought to distinguish between compounds, such as the phosphatides, containing functional groups which make possible a combination with proteins by primary valence forces, and the sterols, for example which, if they combine with proteins at all, could do so only by an attraction due to secondary valence forces." Chargaff considered his preparations (201) of cephalin and basic proteins to be truly representative, and has deplored (203) the attention paid to lecithin by other workers. Nevertheless, it will be made evident that the challenge presented by the lipoproteins lies in just that problem of the ill-defined interactions which he has chosen to pass over.

The experimental study of the linkages involved in lipoproteins has been pursued along two principal lines, the study of the extractability of the lipids by various solvents and the preparation of synthetic models. Early failures to extract the lipids from serum (9, 158, 204) or from isolated protein fractions (158) by treatment with ether alone served to establish some sort of binding between the lipids and proteins. The most intensive study of this phenomenon was

undertaken by Macheboeuf and Wahl (205) who, by extracting human serum with frequent changes of ether, showed that in 5 months 35% and in 12 months 48% of the total lipids were removed. On the other hand serum from a case of lipoid nephrosis yielded 87% of its considerably higher lipid content over a period of 5 months.

The significance of the relative effectiveness of direct ether extraction had been studied previously by Eufinger (206), who showed that, in spite of a rise in the total cholesterol of serum during pregnancy, the amount directly extractable with ether progressively declined. Eufinger claimed to have found a lower extractability associated with higher euglobulin values. Similarly, Nekludow (207) in 1931 compared the amounts of cholesterol which could be removed by ether alone or by exhaustive treatment with alcohol. Both 'weakly' and 'strongly' bound cholesterol was found in each of the protein fractions separated by ammonium sulfate. More recently, Lee and Chen (208) demonstrated that phospholipids were confined almost entirely to that fraction of the serum lipids which resisted extraction by ether alone.

Nerking (9) in 1901 found that acidification of serum before shaking with ether increased the yield of lipid very little. This early discovery was followed up in 1930 by Theorell (169) in a study of the effect of pH on the ease of ether extraction of cholesterol from the horse serum fractions prepared by salting-out with ammonium sulfate. The

'fibrinogen' fraction prepared by 27% saturation gave a clear maximum at pH 6.0, leading to practically complete extraction in 12 hours. Euglobulin (27-36% saturation) had a maximum at pH 5.9, but less than half of the total cholesterol was extracted in 24 hours. Pseudoglobulin (36-50% saturation) on the other hand showed no maximum, and was quite readily extracted. Finally, albumin was maximally but still incompletely extracted at a pH a little over 5.5, with a sharp decline on the acid side.

In a similar study, Delage (209) in 1936 found the extraction of total lipid from whole serum by ether to vary little over the pH range from 1.7 to 13.3. A possible maximum about pH 6.5-7.5 agreed with Theorell.

Działoszynski et al. (210) have reported recently that for human plasma the maximal extraction by ether of cholesterol, phospholipid, carotene and vitamin A occurred in the neighbourhood of pH 5. The closeness of this value to the isoelectric point of the proteins was considered significant. These results should be compared to the behaviour of aqueous colloidal solutions of lipids which have been found to give steady increases in extractability with ether as the acidity was increased, reaching completion about pH 2-3 (210).

The serum lipids thus differ markedly from colloidal suspensions. At the same time it should be stressed that the quantity of lipids extractable with ether at any pH is very

small compared to the total content of the serum (210), and hence electrovalent linkages as postulated by Przylecki (198) are not of primary importance. The charges on a protein molecule are known to change so markedly (211, p.444) over the ranges of pH which have been discussed that electrovalent linkages between the lipids and proteins could not conceivably remain almost unchanged. It should also be noted that the extraction of the phospholipids, the very molecules which might be expected to enter into heteropolar combination with proteins, was found by Dzialoszynski et al. (210) to be slightly less influenced by pH than that of either cholesterol or carotene.

The detailed study of the lipid-protein interactions involving close binding was initiated in 1929 by M. A. Macheboeuf (167, 212). The horse serum albumins left in solution in half saturated ammonium sulfate were nearly quantitatively precipitated by acidification to pH 3.8. Nine reprecipitations served to give a material of constant composition, - phospholipid 22.7%, cholesterol esters 17.9%, proteins 59.1%. Macheboeuf later (213) names these lipoprotein complexes 'cénapses' and the fraction came to be known as 'cenapses acidoprecipitables de Macheboeuf' or 'C.A.M.' As mentioned before, this material gave limpid neutral or alkaline solutions. Ether removed no phospholipid, a finding which, in view of the recent (214) demonstration that the phospholipid was composed exclusively of lecithin, would seem to disprove

the previously mentioned assertion of Chargaff (203). Prolonged treatment with boiling alcohol was required to remove the lipids. The presence of salts did not improve the extraction with ether, which was negligible and limited to about 1% of cholesterol esters. When the solution was mixed with an emulsion of lecithin, ether extraction gave 100% recovery of the added lecithin. Similarly, addition of the lipids which had been extracted from the C.A.M. by alcohol to an albumin solution gave unstable emulsions easily extracted with ether.

The material isolated by Macheboeuf contained, therefore, exclusively ether-resistant lipids which could not be returned to their former state after removal from the protein. The effect of alcohol was studied in a second paper (212). Alcohol caused precipitation of the C.A.M. except in alkaline solutions. After a time the precipitate was observed to be composed of amorphous protein and needles of cholesterol esters. With high concentrations of alcohol at pH 9.0, no amorphous precipitate formed at room temperature or below, but cholesterol esters crystallized out. If the material precipitated by alcohol was immediately treated with large volumes of water, clear solutions were obtained.

From the foregoing observations, Macheboeuf concluded that alcohol denaturation was stepwise and led first to a product soluble in strong alcohol which in turn was further altered so as to release cholesterol esters, lecithin which remained

in the alcoholic solution, and an insoluble protein coagulum.

The various studies which have been discussed have shown that some linkages between lipids and proteins appear to be firmer than others, and that strong and weak bindings are widely distributed among the various protein fractions in variable proportions.

It will be remembered that Kumagawa and Suto (11) were the first to compare quantitatively the extractive powers of various solvents for lipids in a protein-containing system, and that they followed Pflüger (6) in ascribing the success of alcohols to their dehydrating power. The question was reopened in 1932 by Macheboeuf and Sandor (215), who sought to study the effect on serum lipids of small concentrations of alcohol, conditions which could hardly be described as grossly dehydrating. To 25 ml. each of horse serum and ether were added increasing amounts of ethanol, after which the mixtures were shaken and left standing for six hours. The ether was then removed, washed, and the residues after evaporation were weighed. The following results were obtained:

ml. ethanol added:	0	0.5	1	1.5	2	2.5	3	3.5	5
mg. lipids extracted (out of a total content of 156 mg.)	1.5	1.6	2.2	3.2	75	74	75	25	42.5

The appearance of the tubes, none of which was

opalescent in the absence of ether, was interesting. The tube containing 1.5 ml. of ethanol was very cloudy, whereas those on either side, including the optimal quantities, were perfectly clear. Heavy opalescence was observed at alcohol concentrations greater than optimal. The opacity of the 1.5 ml. tube corresponded to a viscosity intermediate between that of the tubes on either side. In the higher zone, however, the viscosity was high, increased progressively, and was accompanied by the formation of stable emulsions which undoubtedly interfered with the extraction. The extraction was shown to be dependent upon the alcohol concentration in the aqueous phase.

Macheboeuf and Sandor showed further that subsequent renewals with ether equilibrated against alcohol-water mixtures lead to the extraction of mere traces of lipids such as were extracted by ether alone. The conclusion was drawn that the initially extracted lipids represented a well-defined fraction.

Analysis of the lipids lead to the following conclusions: 1) relatively more cholesterol than phospholipid was extracted; 2) the composition of the lipid mixture was approximately constant within the optimal zone; 3) there was a clear-cut minimum in the extraction of both phospholipids and other lipids at an alcohol concentration of about 14%.

Another interesting discovery was that, even if the

ether was removed at an early stage, the process would continue to completion with the eventual collection of lipid droplets on the surface. The authors concluded: "L'éther dissous dans la phase hydro-alcoolique est donc suffisant pour que le phénomène évolue jusqu'à la libération des lipides."

Strengthening the idea that a definite fraction was involved was the equally important discovery of an induction period (temps préparatoire) which varied from one horse to another. One example showed a rise in extracted lipids from 17.5 mg. at 68 minutes after mixing to 64.5 mg. at 90 minutes. It was significant in the same regard that the quantity of lipid extracted was always independent of the alcohol concentration when this lay between the limits 6-12% by volume, even though the induction period was greatly shortened at the higher concentrations.

In all, the amount of lipid extracted by this procedure averaged about 50% of the total lipids. It was determined that 68% of the lipids accompanying the globulins were extracted in contrast to only 39% of those accompanying the albumins. Similar results were obtained with the serum of men, sheep and rabbits.

Macheboeuf and Sandor developed an interesting theory to explain their findings. They considered that proteins and lipids were associated in complexes with the hydrophobic

groups of each interacting, while the hydrophilic groups of each pointed outwards and were surrounded by a protective layer of water. The alcohol was assumed to modify the affinities of the solvent for the various groups and especially to affect the ionization and hydration of the protein groups. In this way it was assumed that the protective zone could become permeable to ether which would remove the lipids. The process left room for an induction period.

In 1935 Delage (216) undertook to follow up the work of Macheboeuf and Sandor by studying the effect of a series of alcohols and related compounds. It was found that ethanol could be replaced by methanol, propanol, butanol, iso-amyl alcohol, cyclohexanol or acetone. Glycol on the other hand had no effect, and acetaldehyde simply produced an insoluble coagulum. Maxima were the same for ethanol, propanol, iso-amyl alcohol and cyclohexanol. For methanol a slightly higher concentration was required to initiate the process, whereas acetone was needed in twice the concentration of the alcohols. Methanol frequently, and acetone occasionally, gave slightly lower maxima. In each case more than 50% of the total lipids were extractable.

In a companion paper, Delage (217) considered the width of the plateau, and its constancy for a series of compounds and for varying time of contact to be evidence for a definite palier thermodynamique. He also took the ratio

extractable/total lipids to be a measure of an equilibrium between two sets of lipids, and characteristic of a given serum. In order to study the variations in this ratio during disease, he chose the blood serum of heifers used in the production of smallpox vaccine, and was surprised to find that scarcely any lipids were extractable by ether in the presence of dilute ethanol.

In a later paper Delage (218) showed that the ability of various substances to increase the extractive power of ether bore no relation to their influence on the solubility of ether in a dilute salt solution. On the other hand, those substances which did not enhance the extraction, - hydrogen and hydroxyl ions and glycol, - were insoluble in ether. Furthermore, the substances with a positive effect, such as the alcohols and acetone, were soluble in ether, and their effect was regardless of water-solubility. Delage also found some relation between ability to lower the ether/water interfacial tension and to promote extraction. In terms of the Gibbs relation, this would mean that the requisite property was that of ability to concentrate and orient at the ether/water interface and most likely also at the hypothetical lipid/water interface of Macheboeuf and Sandor.

The study of the extractability of lipids from serum by ether in the presence of low concentrations of alcohol has received considerable attention from Italian workers. Marfori repeated (219) the experiments of Macheboeuf and Sandor (215)

and also found (220) that heating to 54° for 30 minutes considerably decreased the amount of lipid extractable in this way. Boscardi (221) claimed to have found a lowered extractability during staphylococcus infections. Similarly, Scotti (222) has attempted to correlate the quantity of ethanol required for maximal extraction with erythroblastosis in chickens.

Mention should also be made of the work of Grigaut (223) in extending the work of Macheboeuf and Sandor to higher concentrations of alcohol. Cholesterol was completely extracted in the presence of 60% ethanol, whereas other lipids required 85%.

The accumulating evidence has suggested that the release of lipids from the serum proteins is primarily dependent on reactions with the proteins. Substantiation for this view is to be found in the report published in 1935 by Drekter, Bernhard and Leopold (224), which for some reason has escaped general notice. These authors showed an inverse relation between the amount of cholesterol extracted by various solvents and the amount of serum protein which could be redissolved in water after the treatment. The correlation between denaturation and extraction was obvious and led to the discovery that serum boiled to dryness with alcohol yielded all its cholesterol readily to ether. Heat denaturation, as had been known for a long time, did not promote ether

extraction. Since denaturation and extraction by chloroform and ether were both greatly increased by the presence of water, Drekter et al. suggested that the effect of ethanol more likely was due to alcoholysis than to dehydration. As was mentioned before, the importance of water in the extraction of lipids from serum by alcohol-ether was recognized in 1939 by Folch and Van Slyke (91). Dzialoszyski et al (210) corroborated Drekter et al. by obtaining a correlation between the denaturation of the proteins and the extractability with ether of the lipids and of bilirubin as a function of pH. Both these authors used solubility as the criterion of denaturation, and in this connection it is perhaps well to keep in mind that the processes leading to the release of the lipids need not correspond exactly to those leading to insolubility (225).

Obviously closely connected with the role of water in removal of lipids from the serum proteins were the experiments published in 1942 by A. S. McFarlane (226). Human serum was shaken with ether and the mixture frozen below -25°C. On thawing considerable quantities of lipid were found in the ether layer. The effect was not obtained when temperatures above -20°C. were used; conversely, temperatures down to - 70°C. did not increase the yield. Successive treatments of 1 litre of serum yielded 3.5, 0.85 and 0.2 gm. of lipid; a further 2.5 gm. could be obtained by treatment with alcohol-ether.

Apart from the fact that a similar proportion of the

lipids were extracted, a point of similarity with the experiments of Macheboeuf and Sandor lies in the fact that McFarlane also found that mere saturation of the serum with ether was sufficient for the process to take place. As before, the fraction of the lipids extracted appeared rather well-defined. Freezing and thawing after the removal of the ether yielded an insoluble gelatinous material which could be removed to leave clear serum.

The only change in the electrophoretic diagram of the serum was a reduction in the concentration of beta-globulin. McFarlane pointed out that serum frozen and thawed or dried from the frozen state and reconstituted in the absence of ether became cloudy. He concluded: "This suggests that the association of lipoid with a stabilizing substance depends on the presence of liquid water and is destroyed by freezing. On subsequent thawing, unprotected lipoid is able to aggregate to visible particles or droplets. These, however, soon reacquire the same stabilizer from solution, because the initial rate of aggregation is not maintained and the formed particles may be observed with the naked eye to migrate with the beta-globulin boundary. The lipoid of these particles is not extractable with ether unless accompanied by freezing." Serum extracted in this way could subsequently be dried from the frozen state and reconstituted clear.

McFarlane gave as the most likely explanation of the process that ether became dissolved in the momentarily freed

fat droplets which then rose to the surface and merged with the ether layer. Apparently he felt that the simple density effect forestalled the coating of the particles with protein. It is also possible that a small amount of protein was carried up to the ether: Hartley (227) remarked on the fact that mixtures containing a large excess of lipid over protein acted as though composed entirely of lipid. In the same connection it should be remembered that milk fat is almost completely extractable with ether, even though the droplets are coated with protein (228). Perhaps the size of the droplets may be influential.

McFarlane's findings should be compared with those of Denstedt (229) who has succeeded in reconstituting small batches of serum after lyophilization without obtaining a cloudy product. Similarly, Tasse has reported (230) obtaining clear reconstitution of horse serum. It is significant that the only difference in treatment between these preparations and those of McFarlane and routine preparations of dried serum and plasma on a large scale was in the ommission of filtration of the serum. Other effects apparently attributable to bacterial filters will be discussed later.

Since the large droplets resulting from freezing without ether were shown by McFarlane to be extractable during a second treatment in the presence of ether, it may be assumed that the chylomicrons were very largely extracted by this method. From the figures previously quoted from Blix,

Tiselius and Svensson (183), it may be seen however, that beta-globulin could not contribute the whole of the lipids which McFarlane was able to extract, and therefore lipids from other protein fractions must have been involved.

Chargaff, Bendich and Cohen (231) found the same effect of freezing with ether on the integrity of the thromboplastic protein. They concluded: "Once the protective water barrier is frozen away, the uniformity of the ostensibly homogeneous complex disappears owing to the removal of lipids by the ether, and separation into discrete components takes place."

Considerable speculation might be made as to the condition of the water which is apparently disrupted during the freezing process. Although much of the earlier confusion (232) about the subject of bound water has disappeared, the nature and location of the water molecules on a protein are not well known. Pauling (233) has offered the attractive suggestion that one water molecule is attached to each polar side chain in the initial process of hydration. Such hydrogen bonds might be susceptible to the attack of alcohols, but the writer is unaware of experimental evidence that freezing would affect them. The entire process described by McFarlane deserves closer study.

It will be recalled that Macheboeuf and Sandor (215)

formulated the theory that lipids are attached to the serum proteins by mutual attraction of polymethylenic chains. Further consideration led Macheboeuf and Tayeau (44, 234) to predict from this theory that compounds containing such polymethylenic chains might be capable of displacing the lipids already present on the protein molecules, provided that the displacing compounds were sufficiently soluble in water to approach the proteins as discrete molecules. Their hypothesis was proved correct when it was found that the addition of neutral aqueous sodium oleate to horse serum resulted in the release of considerable quantities of lipids which were readily collected by shaking with ether. An optimal concentration of soap existed, since excess soap held part of the released lipids in the aqueous phase and prevented their passage into the ether. The interesting observation was made that whereas removal of the lipids from the globulins was practically complete, the lipids of the C.A.M. were quite unaffected.

More details were made available in two papers published by Macheboeuf and Tayeau (235, 236) in 1941. It was found that the same process occurred at a pH of 8.8 as at 7.2, although a minimum in the extraction existed at pH 7.7. It was concluded that the process was due to the action of the fatty acid anion. The procedure served to extract all the free cholesterol from serum, 87% of the ester cholesterol, and 80% of the phospholipids. It is interesting that Charonnat

(237) in 1935 used soaps to improve the extraction of cholesterol from serum by ether, but did not delve into the theoretical implications of his discovery.

Macheboeuf and Tayeau (235), by acidification of the treated serum to pH 4.6, obtained an abundant white precipitate which was denser than serum, insoluble in ether, and rich in proteins and fatty acids. This material could be dissolved by neutralization and reprecipitated without change of composition. Prolonged treatment with ether served to remove almost all the fatty acids from the acidified material.

A study of the optimal quantity of soap required led to the discovery that the same optimum existed for the total lipids, phospholipids or cholesterol considered separately, and hence to the important conclusion that the action of the soap depended on a property of the proteins and not of the lipids. It was also concluded that the optimum corresponded to the capacity of the proteins to bind soap, and not to the quantity of lipid previously bound.

In a study of other factors influencing the removal of lipids by soaps, it was found that the process was progressive with time as far as both cholesterol and phospholipid were concerned, and that the limiting factor was concentration of soap rather than mass of soap. From the latter fact it might be construed that the protein-soap complexes were partly dissociated. As for the nature of the soap, the main

requirement was the ability to obtain a sufficient concentration in the aqueous medium (238). The optimal activity occurred at the same molar concentration for each soap. suggesting a stoichiometric law. The order of decreasing effectiveness of the soaps tested was sodium ricinoleate, linoleate, dibromstearate, oleate, undecylenate, undecylate, and sebacate. Differences in chain length (oleate, undecylenate), although not extensively studied, were less important among the higher fatty acids than was solubility in water (ricinoleate), but the presence of a hydrocarbon end-group was obligatory, as evidenced by the almost complete ineffectiveness of the dicarboxylic sebacic acid. Similar tendencies were found for the sera of man, ox, sheep, pig, dog, rabbit, guinea pig and chicken.

It is interesting to note that in 1938 Peters and Wakelin (239) discovered that the astacin-protein linkage in ovoverdin from lobster eggs was broken by fatty acids with an accompanying change of color from green to red. The effect occurred at physiological pH and was enhanced by increasing chain length. Although no details were given as to the stability of the combination of the fatty acids with the protein, it was stated, "It was not found possible to extract the fatty acid by any means which would leave the protein undenatured."

The same general approach was continued by Tayeau (240), who was anxious to discover the underlying cause for the fact that a part of the cholesterol was resistant to the

action of soaps. He reasoned that the cholesterol which could be displaced by soaps was bound to non-polar side chains of the proteins by virtue of its own side chain or of the esterified fatty acid. On the other hand, the cholesterol which was resistant to soaps might be bound by its nucleus to areas of similar configuration on the proteins. Water-soluble substances possessing a cyclopentenophenanthrene nucleus accordingly might be expected to displace this remaining cholesterol in an exactly analogous manner to that of the soaps in removing the greater part of the cholesterol and phospholipids. In keeping with this hypothesis, Tayeau (240, 241, 242) was able to show that saponosides were capable of releasing cholesterol quantitatively from serum, and even developed a saponoside-ether technique for the clinical determination of total cholesterol (45, 241).

The action of saponosides in making possible the quantitative extraction of serum cholesterol by ether differed from that of the soaps (235) in two important ways: 1) phospholipids remained completely unextracted, and 2) even the peculiarly resistant C.A.M. gave up their cholesterol completely to the saponoside treatment. Otherwise certain similarities were found (241, 242). As with the soaps, excess saponoside caused a diminution in the amount of cholesterol extracted by the ether, due to the formation of water-soluble complexes. The effect of pH in this case was much more marked, in that a sharp optimum at pH 7.5 was found.

Tayeau attributed this effect to the influence of the state of ionization of the protein groups on the film of adsorbed water (c.f. Macheboeuf and Sandor (215)) It is difficult to see how such an explanation could account for a five-fold decrease in extraction at pH 6.7 and 8.4. As with dilute alcohol (215) and soaps (235) the effect did not occur immediately after treatment of the serum with saponoside. An induction period was again evident, giving an S-shaped plot of extraction against the logarithm of the time. Tayeau interpreted these results as showing the existence of weak bindings which were broken immediately and of others which were more gradually decomposed. The presence of ether increased the rate, indicating that the process tended towards equilibrium in its absence. The same behaviour was shown for the sera of man, ox, sheep, guinea pig and rabbit.

Having achieved such success with his hypothesis of the binding between the steroid nucleus and similar arrangements on the serum protein molecules, Tayeau (243, 244, 241) repeated some of the experiments which have just been described, but with bile salts substituted for saponosides. When serum was mixed with a solution of sodium cholate and then shaken with ether, no cholesterol was found in the ether, nor in the proteins separated by salting-out, but it was eventually recovered quantitatively from the mother liquor in the form of a cholesterol-bile salt complex. As

in the case of the saponosides, no phospholipids were removed from the proteins. The bile acids, which unlike the saponosides could be adequately differentiated analytically from cholesterol, were found to be attached to the proteins to form new complexes comparable to the protein-soap complexes of Macheboeuf and Tayeau (235).

Tayeau was led by these discoveries to examine the state of the cholesterol-protein linkages in the serum of patients suffering from obstructive jaundice (241, 245), since such serum would have been treated in vivo by bile salts. The serum was found in each of a numerous series of cases to release abundant quantities of cholesterol and phospholipids when shaken with ether. Tayeau made the interesting suggestion that the loosely-bound lipids, nearly absent in normal serum, could not be utilized by the organism, and accumulated to produce the hyperlipemia and especially hypercholesteremia characteristic of obstructive jaundice. Tayeau and Rolland (246) have recently shown, with the aid of surface tension measurements to determine the point at which excess bile salt was present, that serum from which the lipids had been removed without denaturing the proteins was capable of binding ten to fifty times as much bile salt as untreated serum. This result, taken with the difference in behaviour of normal and jaundiced serum, led them to suggest that in the latter case bile salts became fixed to the proteins prior to the lipids, or in other words

interfered with the normal synthesis of the serum lipoproteins.

Similar to the effect of bile salts was the action of sodium dihydroabietate in dislodging cholesterol from its combination with the serum proteins. Macheboeuf and Rebeyrotte (247) chose this salt for the same reason that Tayeau tested the saponosides and bile salts, namely, similarity with the configuration of the cholesterol nucleus.

Tayeau and Neuzil (248) have recently compared the action of sodium salicylate on the extractability of serum cholesterol <u>in vitro</u> and in living patients. No effect was found in the first instance, but a considerable proportion of the cholesterol of the serum of patients treated with sodium salicylate was extractable with ether. On such meagre evidence it was suggested that the salicylate became attached to the proteins before the cholesterol, in the same way as was suggested by Tayeau and Rolland (246) for bile salts. No attempt to detect the salt in combination with the serum proteins was reported.

As Tayeau himself has pointed out (241), the experiments in which various agents have been shown to displace lipids with considerable specificity from their linkages with proteins are closely allied to the study of the action of metabolic antagonists (249). In fact, the considerable bearing of such investigations on the specificity and mode of

action of enzymes need scarcely be stressed.

The evidence which has been brought forward from extraction studies has shown that the primary linkages between serum lipids and proteins are almost certainly of non-polar co-ordinate valence. The unimportance of purely heteropolar binding was made evident by the failure of pH changes, so marked in their effect on the number and distribution of the charged groups on proteins, to influence directly the extraction of the lipids. Similarly, covalent linkages, the second type listed by Przylecki (198) could scarcely be so readily broken by such mild agents as alcohol, soaps, saponosides or bile salts. On the other hand, Macheboeuf and more recently Tayeau, gave given ample evidence for the importance of van der Waals forces as the primary linkages involved, and have even shown good reason to believe that the effects are quite specific.

Undertaken primarily as a means of studying the reactions of proteins proper, yet perhaps falling more within the realm of synthetic models of lipoproteins, are some recent studies of the reactions between serum proteins and detergents. The Stanford group headed by J. M. Luck have studied in particular the action of caprylate and related compounds in the stabilization of lipid-free serum albumin (250) against denaturation. Experiments employing the cloud-point method (251) have shown that low concentrations of

anions containing a non-polar group had a stabilizing effect against heat denaturation (252). By nephelometry it was shown that caprylate and laurate stabilized when present in a 1:4 mole ratio to the albumin. Viscosimetry showed that the albumin was stabilized in a native state, not partially unfolded as had been assumed at first (251). It was amply shown that both the anionic and non-polar functions were necessary for the stabilization, and to quote Boyer et al. (252): "A tenable conclusion is that this attraction of a single fatty acid molecule for different groups of the protein may aid in preventing the separation of adjacent chains or layers of the albumin molecule, and thus prevent an 'opening out' or extension of the molecule and thereby effect a stabilization against heat denaturation. Since remarkably low ratios of caprylate to albumin will effect a considerable stabilization, it is apparent that certain selected areas or groups play an essential role in the mechanism involved."

The protective action of the fatty acid anions for serum albumin was also manifest in reducing the rate of digestion by papain (253) and in stabilization against urea and guanidine denaturation (254, 255). It is very noteworthy that gamma-globulin not only was not stabilized in a like way but that its thermal stability was even decreased by caprylate. The same structural requirments, namely a charged group and a long hydrocarbon chain, were found for

stabilization against papain and urea and hence a similar process was probably involved.

More information about the mode of binding of the soaps with the serum albumin was afforded by studying the effect of the reaction on electrophoretic mobility (256). At pH 7.7 and ionic strength 0.2, the lower fatty acid anions caused an increase in mobility which was more marked for caprylate than for butyrate, independent evidence of the dependence of affinity on chain length. The concentration of inorganic phosphate in the buffer was found to have a marked influence, presumably indicating an association of this ion with protein cationic groups. The increase in mobility caused by the fatty acid anion could be ascribed either to neutralization of positive charges on the protein or to non-polar combination which would leave the fatty acid carboxyl groups free, either arrangement leading to an increase in net negative charge.

The work of the Stanford group clearly shows that the primary linkage between soaps and serum albumin is through the hydrocarbon chain of the former, yet at the same time establishes that the carboxyl group takes part in the reaction. It is thoroughly reasonable to expect that a charged group brought into close association with a multi-charged molecule would show coulombic interactions with the neighbouring charges.

Dealing more with the quantitative binding of

higher ratios of detergent to protein have been experiments employing sodium dodecyl (lauryl) sulfate. Electrophoretic analysis of detergent-albumin mixtures by Neurath and Putnam (257, 258) in 1944 disclosed three boundaries representing the original albumin, A, a detergent-albumin complex designated AD_n, and a second complex, AD_{2n}, the latter two corresponding to the maximum and minimum ratios for complete precipitation at pH 4.5 (259). Between pH 4.5 and 6.8 combination was found to be independent of pH and was concluded to involve protein groups (presumably cationic) whose ionization did not change over that range. The amount of detergent bound in AD_n (n equal to 55 moles) corresponded to one-half the acid-binding capacity of the albumin, that in AD_{2n} to the whole acid-binding capacity. From the viscosity studies it was suggested that the formation of AD_{2n} from AD_n involved partial unfolding of the lower complex with the liberation of additional positive groups hitherto accessible to protons but not to large organic anions. Ratios of detergent to albumin greater than that corresponding to AD_{2n} were accompanied by the appearance of free detergent in the electrophoretic diagrams, and the composition of the complexes became variable.

Further evidence for the formation of discrete, stoichiometric complexes between serum albumin and sodium dodecyl sulfate was obtained by Neurath and Putnam (260) from studies of the molecular kinetic behaviour of the complexes

AD_n and AD_{2n}. It should be stressed that the transition between the two complexes was sharp, and that apparently no more detergent could be bound by AD_n until it had unfolded. Note should also be taken of the fact that correspondance between the number of cationic groups and the binding of sodium dodecyl sulfate has also been demonstrated for gelatin (261) and beta-lactoglobulin (262).

The work of Neurath and Putnam has thus served to stress the importance which the polar part of a polar-non-polar molecule may assume in linkages with proteins. Just as in the case of the soaps the hydrocarbon chain is the prominent grouping, so the large anionic group of the alkyl sulfate appears to be predominant. The only examples of such clear-cut salt linkages existing in lipoproteins, with the possible exception of salmon roe (263), are the compounds of the type prepared by Chargaff (201, 202) from cephalin and basic proteins. Emulsified brain cephalin was mixed with the protamine salmine (201), with thymus histone or globin (202), to give stoichiometric complexes involving negative groups of the phospholipid with the positive groups of the proteins. Lecithin and sphingomyelin formed complexes only at very high pH. Since the cephalin preparations undoubtedly contained a large proportion of the acidic phosphatidyl serine (203) conclusions as to behaviour should be restricted to it. Indeed, since phosphatidyl ethanolamine as well as lecithin carries one positive and one negative charge (264) at

physiological pH, there is no reason to believe that they behave differently towards proteins.

Also of interest for their own sake, but even less related to serum lipoproteins, were the compounds by Wagner-Jauregg and Arnold (265) and Wagner-Jauregg and Helmert (266). In the first paper, the phosphatidic acid dichaulmoogryl-beta-glycerol-phosphate was shown to combine with clupein and horse albumin and pseudoglobulin to form salts. The second report, published in 1943, apart from corroborating the findings of Chargaff (201, 202), dealt with the formation of salts of clupein with the lysophosphatidic acid monododecanoyl-beta-glycerol-phosphate and the peculiar compound, symmetrical dicholesteryl-pyrophosphoric acid dihydrate.

Before passing on to the discussion of other synthetic model experiments, it is wise to introduce a conception which although obvious has only recently received proper recognition. To quote Schulman (267), "If the lipoid presents an interface, completely different results will be obtained from those obtained if it acts as a dispersant or adsorbent on to the protein." All the experiments which have been considered so far have fallen into the latter category. On the other hand, lipids such as cholesterol, lecithin, phosphatidyl ethanolamine and triglycerides, which are too insoluble in water can only be brought into contact with proteins in the form of aqueous emulsions or monomolecular

layers. In each case the interaction is likely to be controlled by the relatively large lipid interface rather than by the details of the surface of the protein molecules.

Emulsion experiments are likely to be affected by adsorbed impurities on the surface of the lipid droplets in that the surface exposed to the protein may bear no relation to the bulk of the lipid. For instance, even the purest lecithin preparations rapidly become acid due to the splitting off of fatty acids (267, 269, 270, 271). Bull and Frampton (271) found that, in a 1.1% aqueous lecithin-cephalin suspension of isoelectric point pH 3.7, four times as many acid as basic groups were exposed for titration. Bungenberg de Jong and Westerkamp have also shown how widely the properties of lecithin sols may vary according to the mode of preparation (272). Similarly, practically every worker in the field has used cholesterol suspensions stabilized with a fatty acid, either added or present as an impurity, and has thus been testing the interaction of proteins with an oriented film of fatty acid.

Mayer and Terroine (273) in 1907 prepared emulsions of lecithin in cold distilled water, which on mixing with egg or serum albumin formed precipitates from mildly acid solution These precipitates were dissolved by addition of either lecithin or albumin or by dilute salts. The resulting complexes were observed to migrate towards the negative pole, showing a reversal of sign compared to the lecithin alone (the pH

was not closely specified), and were soluble in fat solvents with the exception of acetone.

Substantiating part of the above results was the report by Feinschmidt (274) that the addition of serum to aqueous suspensions of lecithin caused a shift of the flocculation optimum, formerly pH 2-4, to less acid values (not specified).

In 1911 Handovsky and Wagner (275) showed that the lipids extracted with ether from horse serum caused turbidity and precipitation when returned to the same or fresh dialyzed serum in the form of aqueous emulsions. Neutral salt prevented this flocculation. Similarly, lecithin emulsions caused precipitation in dialyzed ox serum. The interesting observation was made that no precipitation of lecithin was obtained with dialyzed albumin. The apparent contradiction of Mayer and Terroine (273) may be explained by differences in pH, which was uncontrolled in these early experiments.

Theorell (169) in 1930 found that when suspensions of cholesterol stabilized with oleic acid were added to serum, part of the cholesterol could be brought into clear solution. Similarly, small quantities of lecithin or serum phospholipids could be brought into clear solution. The interesting observation was made that when the undissolved excess of phospholipid was centrifuged out, part of the cholesterol

originally in the serum accompanied it, and appeared to come from that fraction which was extractable with ether. It seems likely that these interchanges involved only the most loosely bound lipids, which may well have been simply dissolved in more firmly bound fractions.

In 1931, Went and Farago (276), working with horse and pig serum proteins separated by sodium sulfate fractionation, found that the addition of lecithin emulsions tended to facilitate salting-out of the globulins. These results were exactly contrary to those obtained in 1926 by Arnd and Hafner (277).

Bungenberg de Jong and Westerkamp (272) showed in 1931 that 'complex coacervates' between egg lecithin and gelatin were obtained only in the pH range between the isoelectric points of the two components. The same approach was applied to the serum proteins by Elkes, Frazer, Schulman and Stewart (278) in 1945, in a study of the reversible adsorption at charged oil/water interfaces. Emulsions of definite particle size containing 5% olive oil or paraffin were used. For obtaining a negative interface, just sufficient sodium hexadecyl sulfate was added to saturate the interfacial film; hexadecyl trimethyl ammonium bromide was used to obtain a positive interface. The serum proteins were prepared by fractional precipitation with ammonium sulfate and dialysis, mixed with buffer solution, and added to one or

other of the emulsions. Flocculation was seen to take place in the neighbourhood of the isoelectric point of each protein, and extended to the acid side of the isoelectric point if the interface was negative, or to the alkaline side if the interface was positive. Addition of sodium chloride caused some overlapping of the two flocculation ranges, to a maximum overlap of 1 to 1.5 pH units at 5% NaCl. This meant that the proteins, especially albumin and gamma-globulin, were able to adsorb against an overall electrostatic repulsion due to net like charge, in keeping with the concept of localization of charges on the protein molecules.

The experiments of Elkes et al. demonstrate clearly the limitations of the emulsion technique. The simple mixing of lipid emulsions with proteins leads to interactions primarily dependent on the charges borne by the lipid/water interface, which as we have seen are in turn influenced by impurities, pH and salt concentration. Results dependent on specific properties of the proteins have nevertheless been obtained, as witness the behaviour of albumin and gamma-globulin in the experiments of Elkes et al. (278). Indeed, practical application of this fact has been made by Hanger (279) in this cephalin-cholesterol flocculation test for diffuse hepatitis. The protein involved in this case was believed to be a globulin.

In spite of the objections to the emulsion

technique, any modification which might lead to the preparation of emulsions so fine as to be almost molecularly dispersed would make possible the preparation of complexes very similar to natural lipoproteins. Przylecki and Hofer (280) attempted to do so by mixing alcoholic solutions of olive oil, oleic acid, or lecithin with aqueous solutions of egg proteins. The slight opalescence of the resulting solutions suggested that the mixtures were still relatively coarse. Nevertheless, it proved possible to isolate complexes having constant ratios of lipid to protein. Furthermore, the usual vigorous treatment with alcohol-ether was required to remove the lipids completely. Inspection with a dark-field microscope was unfortunately omitted. The authors concluded that secondary valency played the major part in the interaction.

The complexes of serum proteins with fatty acids which were obtained by Macheboeuf and Tayeau (235) after the lipids had been removed by soaps might be referred to as synthetic lipoproteins. More physiological were the preparations of Macheboeuf and Perrimond-Trouchet (281). Oleic or dibromstearic acid was mixed with either sodium taurocholate or glycocholate and the mixture dialyzed (282). Dialyzed horse serum was now added, whereupon the bile salts dialyzed out rapidly, so that in three days only protein and fatty acid were left inside the membrane. The solutions were clear and the fatty acids resisted extraction by ether.

These preparations are interesting in that the concentration of unbound fatty acid in the solution was at all times low. In view of the later discoveries of Tayeau (241), it is equally important that the concentration of free bile salts was also low. Macheboeuf and Perrimond-Trouchet (281) pointed out that their experiments might serve as models for the mode of addition of lipids to serum proteins, but also stressed that the pH of the dialyzing mixture fell progressively farther below that of serum until the isoelectric point of pH 5.5 was reached. It is unfortunate that the dialysis was not carried out against a buffer solution.

Brief mention has already been made of the application of the techniques worked out for the study of monomolecular layers to the interactions of proteins and lipids. The advantage of the monolayer method (283) is that a film of lipid when spread on an aqueous solution will become oriented with the hydrophilic groups directed into the body of the solution and the non-polar parts riding above. If the film is kept closely packed, any protein introduced beneath the film may approach only polar groups in the lipid molecule. Any interaction between protein and lipid must necessarily involve the polar groups of the lipid, although frequently further interaction with the non-polar chains leads to an actual penetration of the film. Interactions can be observed from changes in the lateral pressure, viscosity or surface potential of the film.

In 1935 Hughes (284) found that injection of egg albumin underneath a film of triolein caused a rapid rise in surface potential if the solution were acid to the isoelectric point of the protein. The process was accompanied by gelation of the film which occurred at progressively decreasing rates as the pH was raised. That the protein had not simply displaced the triolein was shown by the fact that the potential was always much greater than that of the egg albumin film alone. Oleic acid and lysolecithin films behaved similarly towards egg albumin, but films of tripalmitin, hexadecanol, cholesterol and lecithin were unaffected by the protein.

In the same year Przylecki, Hofer and Frajberger-Grynberg (196) compared the interfacial tensions at lipid/water interfaces in the presence and absence of proteins. Affinities of peptone-Roche, gelatin, casein, edestin, egg and serum albumins with paraffins, saturated and unsaturated fatty acids, esters and cholesterol were studied. Parallelism between the amino acid distribution of each protein and its affinity for each lipid was established. For instance, egg albumin, serum albumin and edestin, with relatively high contents of non-polar residues such as of leucine, were well adsorbed onto paraffins and cholesterol; the other proteins studied were but poorly adsorbed. Conversely, gelatin and casein interacted strongly with the fatty acids and esters. Similar results were obtained with the emulsion technique.

Again in 1935, Schulman and Hughes (285) found that preparations of pancreatin which had been inactivated as far as reaction in the bulk phase was concerned, were still able to disturb protein monolayers. The explanation was found to be in the presence of fatty acid in the pancreatin, since a film of tripalmitin was replaced by one indistinguishable from palmitic acid. It was concluded that the fatty acid -protein complex was somewhat dissociable. The extent of dissociation need not have been great, since it was found (286) that 0.0005% sodium oleate completely displaced tripalmitin in two hours at pH 7.4.

Schulman and Rideal in 1937 (287) showed that the tendency of films of gliadin to gel was inhibited by low proportions of cholesterol on solutions acid to the isoelectric point of the protein, and by larger amounts over the whole pH range. This was taken to indicate that the cholesterol interacted with the gliadin in different ways on the two sides of the isoelectric point, and was considered in keeping with the interaction previously (288) observed between cholesterol and the carboxyl and amino groups of fatty acids and psychosine respectively.

A different type of experiment was reported in 1938 by Neurath (289). Solutions of potassium myristate and egg albumin were mixed and spread on dilute hydrochloric acid. From changes in the lateral pressure of the films, it was

concluded that interaction was relatively more marked with low concentrations of the soap. This was taken as evidence of limited interaction, and the suggestion was offered that charged groups were involved.

Results of experiments in which serum proteins were injected under various lipid films were reported recently by Schulman (267). Cholesterol interacted strongly with all the protein solutions tested, - fresh and heated horse serum, horse albumins and globulins, human serum, human albumin and human gamma-globulin. Cephalin reacted only at low pH (3.7) and only markedly with fresh horse serum and human albumin. On the other hand, lecithin showed no interaction whatever. C_{16} and C_{20} amines were found to exhibit some rather marked differences in behaviour, indicating very specific effects. Schulman pointed out how these experiments were complicated by the presence of lipids which were already attached to the serum proteins and which might compete with the surface lipids. Owing to its great bulk, the steroid nucleus of the cholesterol undoubtedly jutted down into the aqueous interface along with the primary alcohol group so that the strong interactions observed probably involved both functions.

Considering all the evidence derived from the various methods of extraction and from model experiments, the most important conclusion is that the primary mode of binding between serum proteins and lipids is that of van der

Waals forces between hydrocarbon chains. Indifference to pH, the action of soaps, saponosides and bile salts, and the results of Schulman's experiments with monolayer penetration, all these facts are evidence against the primary involvement of electrostatic forces in the anchoring of the lipids on the proteins. On the other hand, it has already been pointed out that the charged groups of the lipids undoubtedly interact with those of the proteins, as has been especially well established in the case of the detergents, and may even influence the configuration of the proteins.

More details of the association of the lipids and proteins can only be surmised from the extraction experiments. Since the extractability of the lipids has been shown to be related to the condition of the protein, it is tempting to draw a correlation between the unfolding of the polypeptide chains and the availability of the lipids. Particularly difficult at the present stage of our knowledge is the task of accounting for the relative ease of extraction of various proportions of the lipids. The answer may lie in the type of protein side-chain environment in which they find themselves, as pictured by Tayeau to explain the differences between the action of soaps and saponosides on the removal of cholesterol. Or it may be a question of certain small pockets of lipid surrounded by protein groups forming a barrier to non-polar solvents, somewhat after the picture of Macheboeuf and Sandor. The case of the chylomicrons, wherein a

large proportion of the lipids probably do not interact directly with proteins, is less difficult.

The problem of the most firmly bound lipids deserves special attention. The report published in 1926 by the amateur (290) chemists Troensgaard and Koudahl (164), purporting to show that cholesterol was closely bound to serum globulins through the primary alcohol group, has been widely quoted. From the vigorous acetylation of the globulins (291) was isolated a hydrocarbon to which the formula $C_{16}H_{28}$ was assigned. From the products of the same treatment applied to cholesterol alone, the authors obtained a compound whose elementary analysis was taken to agree with the acetyl derivative of the corresponding alcohol. They seemed to ignore the fact that eleven carbon atoms were lost during the process, indicating an extensive disruption of the nucleus.

Perhaps the best example of firm binding is that found in the C.A.M., which have already been shown to be utterly unaffected by ether, dilute alcohol, or soaps. Tayeau (292, 241) put forward the working hypothesis that the linkages might be heteropolar, and thought to test it by determining the influence of the lipids on the titration curve of the protein. In order to avoid the danger of altering the protein during removal of the lipids by prolonged treatment with cold alcohol, he chose to use the lipid-poor horse serum albumins precipitated from half-saturated ammonium

sulfate at pH 5 as examples of the protein moiety of the C.A.M. In this his only justification was in the finding by Macheboeuf and Januszkiewicz (293) that the protein left after the removal of the lipids from the C.A.M. had the same isoionic point (pH 5.2) as the other albumins. Tayeau found (292, 241) by titration that the lipid-rich fraction buffered more strongly than the other on either side of the isoionic point (pH 5.7), and concluded that the effect was due to the ionized groups of the phospholipids, hence that these were not involved in linkages, and therefore that the union of the lipids and proteins was of the van der Waals type.

Unfortunately, every assumption except possibly the last is open to criticism. The identity of the two proteins as claimed by Macheboeuf and Januszkiewicz (293) can not be considered well established. Moreover, the 'lipid-poor' albumins used by Tayeau contained 8% lipid and were undoubtedly a complex mixture. From the titration curves (241) it may be seen that the maximum buffering of the C.A.M. preparation both in absolute terms and relative to that of the 'lipid-poor' albumins came between pH 4.5 and 6.5. Regrettably, the titration was not taken below pH 4.5, and the other points were too widely spaced to allow of close analysis. Nevertheless it is clear that numerous groups of pK in the neighbourhood of 5.5 were present. Since the content of histidine, the only amino acid of pK in that

range, is not large in horse serum albumin (294), the conclusion that buffering is due to a lipid seems justifiable. Without considering the pH values at which the buffering occurred, Tayeau assumed that it was due to phospholipids, and implied that both the positive and negative groups were involved. Since the pK_1 of the secondary phosphate group of all the phospholipids is approximately equal to 1 (264, 269), the pK_2' of lecithin and sphingomyelin approximately 14 (269), and that of cephalin at least 8.9 (264, 295), and the pK of the alpha carboxyl group of phosphatidyl serine can scarcely be greater than the 2.2 of serine (211, p.84), it is plain that no phospholipid could be involved. Fatty acids, on the other hand, have dissociation constants between 1 and 2×10^{-5} (296, p.29), and thus would explain the titration curves admirably. They were presumably present as impurities due to the fact that Tayeau used the less rigorous preparative method of Macheboeuf and Dizerbo (190) rather than that of Macheboeuf (167), because no fatty acids other than of phospholipid or cholesterol ester were found in the earlier preparation.

A final criticism of this fragile proof would be that the ability of hydrogen ions to react with ionized groups is no evidence that such groups were not previously engaged in electrostatic interaction with other charged groups. Even had the phospholipid charges actually been detected by titration, the assumption that van der Waals forces

were involved would not have been proved.

An interesting point which was mentioned earlier is the ability of saponosides to remove the cholesterol esters quantitatively from serum, even from the C.A.M. (241) without disturbing the phospholipids. Tayeau (241) followed up his other titration study by showing that the removal of the cholesterol from the C.A.M. by saponosides did not affect the titration curve. The experiments were undertaken to prove that the cholesterol was not bound to the protein through esterification with glutamic or aspartic acid carboxyl groups, and as such seem to have been misdirected if we are to admit the evidence that all the cholesterol of this fraction was already esterified with fatty acids (167). The results nevertheless are good evidence that the action of the saponosides did not disrupt the protein molecule.

Another approach to the study of the interaction between lipids and proteins in the C.A.M. has been undertaken by Gorter and Hermans (297). Monolayers of C.A.M. showed the characteristics of protein films at pH values above 4, but behaved like lipid films below pH 3.5. The conclusion was reached that the binding of lipids to the proteins was non-polar, but since the original paper has unfortunately not been available, their reasoning cannot be discussed. The same authors have also shown (298) that a mixture of egg albumin and oleic acid spread like a lipid at pH 6 but like a protein at pH values above 7.

In conclusion, it should be stressed that the nature of the difference between strong and weak binding of the serum lipids to proteins is unknown. More information will be required about the shape and dielectric behaviour of more highly purified fractions before and after the various treatments which have been found to release the lipids. Some admittedly inconclusive experiments of this sort will be discussed in the following section.

c) The Influence of the Lipids on the Physical Properties of Serum Proteins

The most direct way of studying the influence of the lipids on the physical properties of proteins is to compare the behaviour of the proteins before and after removal of the lipids. The chief difficulty has been to remove the lipids without altering in any way the configuration of the protein molecules, a difficulty which may actually be insurmountable. The most widely studied property has been solubility, although biological specificity and optical rotatory power have also been considered, and in recent years the methods of electrophoresis and ultracentrifugation have been applied.

Most studies have employed the principle introduced by Hardy and Gardiner (299) in 1910 for the removal of the lipids. Plasma or serum was mixed with ethanol or acetone at -8°C. The precipitate was filtered off and washed with dry ether at 0° until the solvent had been removed, then

extracted with boiling anhydrous ether and finally dried in vacuo. Hardy and Gardiner found the white powder to be soluble in distilled water and capable of clotting if derived from plasma. The material could be fractionated by salts in the usual way, except that the lipid-free globulin was wholly soluble in saturated sodium chloride. A principle of primary importance in the success of this method was the necessity of complete avoidance of moisture when the serum proteins were in contact with either ethanol or ether at temperatures above 0°C.

Numerous workers have applied the method of Hardy and Gardiner in comparing the solubility of natural and lipid-free serum proteins in various concentrations of salt. Impetus for these studies came from the report by Chick (159) that a high phosphorus content was associated with insolubility of globulins in dialyzed solutions. More recently, Cohn et al. (184) have found that the lipid-rich alpha- and beta-globulins were also insoluble in the absence of salts, whereas the lipid-poor were soluble.

Despite Hewitt's (300) previous findings to the contrary, Jukes and Kay (301), using ostensibly the same method of removing the lipids, reported that the insolubility of the euglobulins in distilled water was lost after removal of the lipids. Roche and Derrien (302), on the other hand, found that removal of the lipids by cold alcohol and ether did not affect the solubility of horse serum euglobulins in water.

Macheboeuf and Tayeau, using the soap treatment to remove the greater part of the lipids, found that the euglobulins were rendered soluble in water (303, 236). Tayeau, after his discovery of the effect of saponosides in loosening the cholesterol of blood serum (240), found (242, 241) that such removal of the cholesterol did not affect the solubility of eu- and pseudoglobulin. He concluded that the differences were due to phospholipids, as originally postulated by Chick (159).

The results obtained by Tayeau focus attention on the variability in the degree of extraction of lipids which has been obtained by various workers. As an example of the effectiveness of the cold alcohol-ether method may be taken the results of Greenwald and Levy (304), who reduced the lipid content of the serum to 10% of the original lipid phosphorus, nearly 10% of the cholesterol, and 30% of the total fatty acid. On the other hand, Blix (305), employing cold acetone and ether, obtained complete removal of the cholesterol, but 25% of the phospholipid was retained, which furthermore was found to be chiefly cephalin by the method of Brante (121). Similarly, the method of Macheboeuf and Tayeau (236, 303) left considerable quantities of phospholipid attached to the proteins. These variations, apart from stressing the fact that completely lipid-free serum has never been obtained with these methods without gross denaturation of the proteins, may be taken to explain the conflicting

conclusions which have been reached as to the influence of the lipids on the properties of the serum proteins.

The influence of the lipids on the salting-out of the serum proteins has also received some attention. In keeping with the thesis that the interactions of proteins with emulsified lipids can not be admitted as models of serum lipoproteins, the previously quoted experiments of Went (276) will be ignored.

The available evidence points to a slight increase in the amount of protein which may be precipitated at any given salt concentration, after the proteins have been deprived of lipids. Srensen (306) found that euglobulin was decreasingly soluble in ammonium sulfate as the phosphorus content increased, but that the reverse was true of serum albumin. Boutroux (307) in 1931 treated a sample of human serum with acetone and ether in the cold, and reported that the amount of protein which was precipitated by low concentrations of ammonium sulfate had been increased by the treatment. Similar results were obtained in 1933 by Wu (308) for horse and sheep serum fractionated with sodium and magnesium sulfates. Again, Went and von Kuthy (309) reported the same results for sodium sulfate.

The solubility of lipid-free and natural serum proteins in potassium phosphate solutions at pH 6.5 has yielded similar results. Liu and Wu (310) in 1937 determined the

constants $\mathcal{B}$ and K in the relation of Cohn (311): $\log S = \mathcal{B} - K\mu$, and found that removal of the lipids from horse pseudoglobulin reduced $\mathcal{B}$ from 0.56 to 0.46, but that K remained unchanged at 0.38. These results are to be interpreted as indicating a reduction in the hypothetical solubility at zero ionic strength without any change in the influence of salts on the solubility of the protein. However, in a companion paper Liu and Wu (312) presented other solubility characteristics of their lipid-free serum proteins which strongly suggest that considerable denaturation had taken place during the removal of the lipids.

Roche and Derrien (313) have shown recently that removal of the lipids from horse serum with cold acetone and ether did not change the number of inflections in the curve of salting-out (314) but that the relative proportions of some of the globulin fractions were changed.

Delsal (41) has recently introduced yet another method of removing the lipids from the serum proteins with the use of the acetal methylal. This method has the advantages of effecting complete removal of the lipids without rendering the proteins insoluble in water. Delsal found (315) that the process of removing the lipids with methylal lowered the albumin/globulin ratio, and on repeating the experiment using the cold alcohol-ether method he again found a 10-15% increase in protein insoluble in half-saturated

ammonium sulfate.

Numerous studies on the contribution of lipids to the physical properties of the serum proteins have been primarily immunological. Hardy and Gardiner (299) observed that the antitoxic value of their lipid-free sera was preserved. Hartley (227), in a more detailed study, showed that antigenic properties were unimpaired. Similarly, the agglutination of bacteria and the hemolysis of red cells by the specific sera were independent of the lipids. On the other hand, the precipitin reaction did not occur when both antigen and antiserum had been freed of lipids. The union of diphtheria toxin and antitoxin occurred normally, but flocculation did not take place. Finally, Wassermann positive sera were rendered negative by extraction of the lipids. Went and Lissak (316) and Varteresz (317) have similarly found the immunological specificity of sera to be unchanged by extraction of the lipids, although solubilities underwent various changes.

In 1935, Horsfall and Goodner published (318) the first of a series of reports on the role of the phospholipids in the type-specific reactions of antipneumococcus horse and rabbit sera. The extracted sera were found to give the same *in vivo* protection to mice, but no precipitate was formed *in vitro*. The prozone effect - failure of protection by a large excess of serum - persisted. When lipid-free serum was

injected into a mouse and withdrawn 30 minutes later, it was found to have regained its precipitating power. The extracted serum did not fix complement with homologous pneumococci, nor with specific capsular polysaccharide. It was noteworthy that tenfold dilution of the extracted serum with water caused practically no precipitation.

Addition of aqueous suspensions of the extracted lipids to the same extracted serum did not restore the in vitro reaction. The surprising discovery was made, however, that addition of as little as 0.025 mg. of phospholipid per ml. of extracted serum restored the precipitation effect. If cholesterol were added beforehand, phospholipid was ineffective, which explained the failure of the mixtures. A further specificity was found in that lecithin alone was effective in the restoration of the horse serum, whereas cephalin was required for the restoration of the rabbit antipneumococcus serum. Since it was subsequently shown (319) that pneumococci which had been treated with extracted antiserum were not agglutinated by the normal antiserum, it was concluded that the extracted antibodies did combine with the pneumococci, but that the resulting complexes were soluble.

Horsfall and Goodner found the lipid content of the precipitates composed of antibody and specific polysaccharide to be quite variable (320), and both horse and rabbit precipitates contained (321) no neutral fat and similar quantities of free and ester cholesterol and phospholipids. From the

lipid amino-nitrogen values it was concluded that the phospholipids of the horse and rabbit antibodies were composed chiefly of cephalin and lecithin respectively, in complete contrast to the requirements for restoring precipitating powers.

Polonovski, Faure and Macheboeuf (322) have reported only recently a comparison of the immunological effects of the alcohol-ether and soap methods of removing the lipids from serum. Using egg albumin and the corresponding rabbit antiserum, they found in agreement with Hartley (227) that the alcohol method completely prevented flocculation. The soap method restricted flocculation to a narrower zone of dilutions than usual. It was found that although the antibodies of fresh serum were precipitated by one-third saturation with ammonium sulfate, after treatment with soap they became concentrated in the fraction precipitated between one-third and one-half saturation. There was thus established beyond doubt a relation between the tendency to flocculate with the specific antigen and a solubility characteristic, both of which were changed by treatment with soap. In the same regard, it is interesting that Tayeau and Neuzil (323) were able to show that treatment of horse antidiphtheria serum with saponosides and ether changed neither the zone, quantity nor rate of flocculation with diphtheria toxin. As with the solubility of the euglobulins in water, the conclusion was that cholesterol did not affect the ability to

flocculate the toxin, and it was suggested as before that the phospholipids were primarily involved.

The preceding evidence on the whole has led to the conclusions that the insolubility of euglobulin in distilled water, the ease of salting out of serum proteins, and the tendency of antigen-antibody complexes to flocculate are all influenced by the lipids attached to the proteins. Another interesting contribution of the serum lipids to the properties of the proteins appears to be the protection against proteolytic enzymes. Tayeau and Buffe (324) have shown that pepsin, trypsin and papain preferentially attack the horse serum proteins which are poorest in lipids, without seriously disrupting the lipid-rich fractions. In no case was lipophaneresis observed. Such parallel behaviour by enzymes of definite and separate specificities (325) suggests that the lipids play the determining role in the resistence to proteolysis. Such a probability is strengthened by the well established (253) stabilization of serum albumin by fatty acid anions against digestion by papain. It is interesting that Deutsch, Petermann and Williams (326) have employed peptic digestion to break down gamma-globulin in the presence of the more resistant and lipid-rich beta-globulin.

In 1931 Macheboeuf and Sandor (327) made the interesting suggestion that the lipids bound to proteins would be expected to contribute to the Donnan pressure of the plasma

by virtue of their charged groups. In support of this hypothesis was the discovery that albumin solutions derived from the sera of patients suffering from lipoid nephrosis frequently showed an osmotic pressure over twice that of normal albumins, and generally related to the lipid content. The increased lipid content of the nephrotic serum was concentrated primarily in that fraction of the albumins which were normally poor in lipids, but not in the C.A.M. which were normal in quantity and lipid content (328). The buffering action of the nephrotic albumins was normal on the acid side of the isoelectric point (pH 4.7), but was considerably higher than normal on the alkaline side. Examination of the titration curves does not give a clear-cut answer as to the nature of the increased dissociation. The greatest buffering appears to have occurred in the region between the isoelectric point and neutrality, suggesting the involvement of fatty acids. Yet the curves as drawn (no points marked) show no increased buffering whatsoever on the acid side of pH 4.7, as would be expected to a certain extent if fatty acids were present. Assuming the presence of fatty acids in considerable quantity on the nephrotic albumins, the requirement of an increase in non-diffusing negative charges would be fulfilled, and an increased osmotic pressure should result. These experiments deserve repetition to establish the quantitative relationships.

In 1941, Macheboeuf and Tayeau (329) followed up

the work of Macheboeuf and Sandor (327) by showing that, after removal of the lipids from the nephrotic albumins by the method of Hardy and Gardiner (299), the osmotic pressure of the residual proteins was normal, indicating that the protein part of the albumins was unchanged in the disease. They thereby substantiated the original assumption that the increased osmotic pressure was due to the lipids.

It is interesting that previous to the suggestion of Macheboeuf and Sandor (327), Fishberg (330), from observations on the extensive and repeated bleeding of rabbits, had raised the possibility that an increase in lipids might act as a compensatory mechanism for maintaining the colloid osmotic pressure of plasma.

Another interesting indication of the role of lipids in enhancing the osmotic pressure of plasma proteins is to be found in the report of Callamand (331) that several varities of fish have serum proteins whose osmotic pressure diminishes on removal of the lipids. In the case of the eel the passage from fresh to salt water was correlated with changes in the serum lipoproteins.

In 1943, Popjak and McCarthy (332) compared the osmotic pressure of human serum which had been defatted by freezing with ether by the method of McFarlane (226) with another sample of the same serum which had been frozen without ether. Removal of practically all the cholesterol, two-

thirds of the phospholipid, and two-thirds of the neutral fat was effected. After the results had been corrected for loss of globulins in the extraction process with corresponding increase in albumins, no change in osmotic pressure was found. Once again, the incompleteness of the extraction, and particularly the unlikelihood of removal of free fatty acids, makes this result difficult to assay. Its importance in the preparation of defatted serum for use in therapy is obvious, however.

On the whole, very little attention seems to have been given to the question of the Donnan effect in the colloid osmotic pressure of plasma. For most practical purposes it has been sufficient to consider the Starling pressure as independent of charge, because of the very small changes in net protein charge which may take place over the pH range of normal serum, and hence in the contribution of the Donnan effect. The only reference to the contribution of lipids to the osmotic pressure of plasma to be found in the recent American literature appears to be that of Keys and Butt (333). These workers showed that the concentration of lipids in the veins of the extremities due to standing paralleled that of the proteins. They also calculated the osmotic contribution of the lipids considered as dispersed molecules, and showed it to be ridiculously large. Keys and Butt were apparently unaware of previous discoveries in the field of serum lipoproteins.

As for the contribution of the lipids to the optical activity of the serum proteins, Hardy and Gardiner (299) and Hewitt (300) found small increases in the optical rotation after removal of the lipids. Young (334), on the other hand, found no difference between the optical rotation of serum albumin before and after extraction with cold alcohol-ether. Young's untreated preparations were probably also of low lipid content. It would be useful to repeat these experiments with modern methods of fractionation to obtain purer fractions.

The only report of the effect of removal of the lipids on the dielectric properties of the serum proteins is that of Arrhenius (335) which has been available only in abstract. The serum was lyophilized before treatment with cold alcohol followed by ether. Considerable changes in dielectric behaviour were taken to reflect changes in the internal structure of the molecules. The extracted albumin was found to rotate about its short axis rather than the long axis as with the native molecule. Similarly, the molecules of gamma-pseudoglobulin were found to be split in two. It was also reported that the extracted albumin was more sensitive to proteolytic enzymes, in keeping with the findings of Tayeau and Buffe (324). As is the case with all measures of the effect of removal of the lipids, it is difficult to draw the line between changes due to the absence of the lipids and those due incidentally to the reagents employed.

A study of the electrophoretic patterns of lipid-free horse and human serum was reported in 1941 by Blix (305). As mentioned earlier, the treatment with cold acetone removed the cholesterol completely but left 25% of the phospholipid, mostly cephalin. An alpha-globulin boundary did not appear at pH 6.1 or 7.4, but did at 8.0. Since the alpha-globulin as well as other boundaries showed normal mobility at the latter pH, it was concluded that incipient denaturation did not cause its disappearance at the more acid reactions. When sols of cholesterol, mastic or Sudan III were added, these migrated with the beta-globulin, or occasionally with the alpha-globulin. Blix considered that the beta-globulin had a special function in coating and transporting hydrophobic particles. The unchanged mobilities may be taken as evidence for both the slight influence of the extracted lipids on the net charges on the molecules and for the minimal effect of the process of extraction on the structure and charge of the proteins.

Zeldis, Alling, McCoord and Kulka (336), in a study of the changes in the electrophoretic patterns of dog plasma during chronic protein deficiency, noticed that the total electrophoretic globulin areas increased. Chemical analysis disclosed no such increase in globulins. To test the possibility that the discrepancy was due to the increased lipid content of the plasma in malnutrition, the same group (337) undertook a repetition of the work of Blix (305), this time

with various normal and pathological dog and human plasmas. Extraction of hyperlipemic dog plasma caused mergence of the three or four alpha peaks into one. The extraction caused a loss of as much as 20-30% of the area of the alpha peaks, which in the dog carry the greater part of the lipids. In human plasma, the extraction reduced the beta-globulin area the most, especially in cases of lipoid nephrosis. The surprising discovery had previously been made by Longsworth and coworkers (338, 339) that simple ether extraction markedly reduced the beta-globulins of nephrotic serum. Some correlation with the work of Macheboeuf (327, 329) would be desirable. Zeldis et al. (337) found similar evidence for the contribution of lipids to electrophoretic areas in the plasma of patients suffering from obstructive jaundice, Xanthoma tuberosam and multiple myeloma. Some of the changes caused by the extraction were very complex, however.

In the course of extensive studies on the ultracentrifugation of serum, McFarlane (340) demonstrated the presence of three components: albumins (A), another which he named X, and the most rapidly sedimenting globulins (G). The differentiation of the X-component from the albumins was feasible only in human serum. Extraction with cold alcohol-ether led to the appearance of considerable polydisperse material, and had the principal effect of broadening the peaks.

Pedersen (341) has recently reported extensive

studies on the ultracentrifugation of serum, especially concerned with the X-protein. The components X, A and G were shown to be related to the electrophoretic components beta-globulin, albumin and gamma-globulin respectively. A further heavy (S_{20} equal to 20) component corresponded to alpha-globulin.

Pedersen found that the addition of concentrated magnesium sulfate caused the X-component to sediment more slowly than the others, and it was even possible to concentrate it at the surface of the solution. The explanation for this behaviour may be seen from the following relation for sedimentation velocity (211, p.422):

$$dx/dt = \omega^2 x M (1-V\rho) D/RT$$

where x is the direction of sedimentation, t is time, ω the anglular velocity of the ultracentrifuge, ρ the density of the solvent, D the diffusion constant, M the molecular weight of the particle, V the partial specific volume of the particle, T the absolute temperature, and R the molar gas constant. It may be seen that for proteins of the same partial specific volume the relative rates of sedimentation will be unchanged by variations in the density of the solvent. Conversely, the lowered relative rate of sedimentation of the X-component compared with the other fractions may be explained by a larger value for V. In the presence of sufficient salt to make the product $V\rho$ greater than unity, the protein will rise to the

surface. Pedersen calculated V_{20} to be 0.969 for the X-protein as against 0.749 for the other fractions. Incidentally, the theoretical possibility of the same effect being caused by a change in the partial specific volume (1/density) of the protein itself was demonstrated factually by the addition of 1-2% ether to the serum. The ether was taken up by the X-fraction which accordingly swelled and sedimented more slowly.

Pedersen found that the X-protein was composed not only of beta-globulin but of albumin and gamma-globulin. Whereas dilution of the serum caused the disappearance of the X-component, addition of gamma-globulin to serum without dilution led to an increase in the X-protein. The X-protein is therefore to be considered a reversibly dissociating system. The molecular weight was considered to be about 1,000,000.

Since extraction with alcohol-ether led to the complete disappearance of the X-protein, the role of the lipids in the integrity of the complex was established. The change was accompanied by an increase in heavy inhomogeneous material. The interesting observation was made that removal of the lipids from electrophoretically separated human albumin caused the appearance of globulins as well as albumins in the sedimentation diagram. It was concluded that X-protein was present in the electrophoretic albumins. Similar results were found for calf serum.

It is noteworthy that Wiener (342) believes that the X-protein plays the role of the 'conglutinin' which combines with Rh-positive red cells and blocking antibodies in the conglutination test for Rh sensitivity.

Another serum lipoprotein of low density was recently been reported by Adair and Adair (343). Human serum was treated with 50% saturation of ammonium sulfate, then the supernatant 'albumins' were treated with more salt to bring the saturation to 60%, whereupon a small quantity of material rose to the surface. It was concluded indirectly that the density of the material might be as low as 1.10. The protein was almost homogeneous electrophoretically and contained 8.5% phospholipid, 16.5% cholesterol, and 20.4% fatty acid. By osmotic pressure measurements the molecular weight was estimated at 370,000. It gave a very poor reaction with a rabbit antiserum for total human globulin. The low density was concluded to be due to the high lipid content.

III. Changes Occurring During the Ageing of Blood Serum or Plasma

In recent years the storage of whole blood, plasma or serum has come to be recognized as a military necessity and a great civilian aid. The relatively rapid breakdown of the red cells renders the storage of whole blood uneconomical for periods greater than about one month (344, 345).

The changes occurring in stored plasma or serum are much slower, less dramatic and of less practical importance. Nevertheless, from the point of view of the lipids and their relations with the proteins, the ageing of serum is a far-reaching process, involving changes more subtle than the formation of insoluble material which first inspired this study.

a) Changes in the Proteins

Relatively few studies have been made of the changes occurring during the aseptic storage of serum or plasma. Von Mutzenbecher (346) found that some slowly sedimenting material became detectable with the ultracentrifuge after serum had been stored 41 days. Similar evidence for the formation of split products in serum which had been kept a few weeks at 0°C. was obtained by Roche and Bracco (347) from osmotic pressure measurements on the globulins. At 37°C, however, there was evidence of aggregation of the globulins. On the other hand, Roche and Marquet (348) found that even when serum had been ageing for several years at 0°C. or 37°C., the molecular weight of the albumins remained unchanged at 69,000. Similarly, Mousseron and Huc (349) reported that while the total albumin of horse serum remained unchanged over a period of 8 days, the total globulin as separated by ammonium sulfate decreased. The effect was more marked at 37°C. than at 17°C. or 0°C.

In 1940, with the growing recognition of the need for preserved plasma, Scudder (350) reported electrophoretic patterns of several samples of plasma which had been conserved aseptically for periods of from 12 to 53 days. In plasma which had been stored at 4°C., a decrease in albumin area was accompanied by an increase in gamma-globulin. Unrefrigerated plasma showed a decrease in beta-globulin as well. Clinical results with the preserved plasma were good.

Moore and Mayer (351) in 1944 reported on the changes in crystalline horse serum albumin on ageing. Of a preparation which had been stored in an ice box at 7.4 pH for 46 months, about half had retained its original electrophoretic mobility but the rest migrated more slowly. From the sedimentation velocities and diffusion constants, the molecular weights were calculated as 68,000 and 95,000 respectively. From the frictional ratios, and assuming the shape of oblong ellipsoids, the axial ratios were calculated as 5 and 10 respectively. The rate of formation of the slow component was indicated by the facts that after 20 months in the cold 17% had been altered, and that at room temperature the slow component was discernible within two months. A similar development of a more slowly migrating fraction of horse serum albumin has been found to occur with guanidine denaturation (352).

Lozner et al. (353) have described the changes occurring in human plasma during the second year of storage at

room temperature. In 18 samples preserved between 15 and 24 months the increase in alpha-amino acid nitrogen was 5-10 mg.%, representing 0.5-1.0% of the original protein. A similar increase was noted in the 'polypeptide index' (354). Although proteolysis was thus not very marked, it apparently involved the action of both endo- and exopeptidases.

The same group has reported (355) that in liquid plasma preservation of the anti-hemophilic components was good, but that the prothrombin activity decreased rapidly, so that the ability to coagulate on the addition of calcium was lost in 2-6 months. It should be noted for reference that the prothrombin activity and clotting power were well preserved in frozen-dried plasma. The clinical value of the plasma was unimpaired (356), and in fact fewer untoward reactions were obtained with the older samples.

Krejci, Sweeney and Sanigar (357) have since reported the changes in electrophoretic patterns of pooled plasma which had been stored for three years at room temperature. Large increases in alpha-globulin occurred at the expense of all of the gamma-globulin and fibrinogen, and of part of the beta-globulin. Mobilities were 20-25% greater than in fresh plasma, and the boundaries had broadened. Heat treatment of fresh plasma also caused an increase in alpha-globulins, but at the expense of different plasma constituents and without any increase in the mobilities or broadening of the boundaries. Krejci et al. (357) also showed that glucose

performed some protective action in plasma stored less than one month at room temperature.

The foregoing summary has served to show that the changes which occur in the plasma proteins during storage are slow and have little effect on its practical value as a blood substitute. Nevertheless the electrophoretic studies show marked changes, especially in the increased mobility of all fractions and particularly of the slower beta- and gamma-globulins. It would be extremely valuable to separate such fractions and to perform titrations to discover the nature of the charged groupings contributing to the increased mobility. It would likewise be interesting to determine whether the albumins of the whole serum showed the same loss of mobility (easily masked by the alpha-globulins) as the isolated crystalline albumins of Moore and Mayer (351).

b) Changes in the Linkages between Lipids and Proteins

In 1936, Delage (358) described a study of the effect of ageing on the ether extraction of serum containing low concentrations of alcohol (215). In the case of horse serum stored at 0°C., it was found that at some time usually between one and three months the quantity of lipids so extracted rapidly fell to a very low level. This change was accompanied by a slight shift in the maximum towards higher concentrations of alcohol. The phenomenon was absolutely

general, and was apparently independent of the state of health of the horse. In one case the amount of lipid extracted rose again to the original level, only to fall once more.

Similar changes in the action of soaps (234) were demonstrated three years later by Tayeau (359). The amount of lipid phosphorus and total cholesterol extracted by ether in the presence of various quantities of sodium undecylenate was determined for fresh serum and for serum which had been stored 8 months in the cold. The old serum yielded no phospholipid by this treatment at any concentration of soap, and similarly not only gave up less cholesterol but required a higher concentration of soap before any cholesterol was released.

Perfectly analogous results were obtained by Tayeau (360, 241) in 1944 when he applied his saponoside-ether technique (240) to the determination of total cholesterol in two samples of horse serum which had been preserved aseptically in the cold for 3 months and 6 years respectively. The three month old serum yielded only half of its cholesterol to the saponoside treatment, and barely a tenth was extracted from the serum which had been conserved 6 years. It will be recalled that in fresh serum the extraction was quantitative (241, 45).

More recently, Tayeau (361) has shown that ageing

of serum reduced its capacity to form stable foams on shaking. He considered the effect to be related in some way to the condition of the serum lipoproteins.

Tayeau (362) has also published a discussion of some theoretical interpretations of the reduced extractability of the lipids in stored serum. He favored the hypothesis that heteropolar linkages between lipids and proteins were developed as the serum aged, but offered very little evidence to support it. Indeed it is difficult to see how the objections which have already been given in previous sections to the possibility of primarily electrovalent linkages can be invalidated by any likely changes in the lipids or proteins.

c) Changes in the Lipids

The possibility that esters of cholesterol became hydrolyzed during autolysis of blood received considerable attention from early workers. Cytronberg (363) obtained evidence of an increase in free cholesterol in horse blood, but Kondo (364), Schultz (365), Nemura (366), Mueller (367) and Thannhauser (368) found no change over short periods.

In contrast to the apparent stability of the cholesterol esters in whole blood, increased esterification of the cholesterol of human serum incubated at 37°C. was reported by Sperry in 1935 (369). Employing the method of

Schoenheimer and Sperry (38), he found that in 118 analyses of fresh serum the percentage of cholesterol in the free form was without exception 25-30% of the total. However, in a series of 30 samples which had been incubated for three days, an average of 57% of the free cholesterol originally present had become esterified. Since the changes were prevented by heating the serum for an hour at 55-60°C., it was concluded that the process was enzymatic. The maximum rate of esterification was found to occur at pH 8. The failure to find the same effect in whole blood was explained by the discovery that the addition of a red cell hemolysate inhibited the reaction.

Sperry pointed out that since the free and combined cholesterol were obviously not in equilibrium in fresh serum, the fact of a constant ratio <u>in vivo</u> suggested the influence of factors outside the serum itself. He also made the suggestion that since there are no free fatty acids in blood serum, there must have been a simultaneous hydrolysis of some compound containing fatty acids, such as phospholipids, to furnish the fatty acids required for esterification. On the average only about 20 mg. of fatty acid per 100 ml. of serum were combined in these experiments.

In a further study of the reaction, Sperry and Stoyanoff (370) showed in 1937, that the esterification of free cholesterol in human and dog serum could be inhibited

by bile salts. Glycocholate and taurocholate in particular were able to cause the complete hydrolysis of all the esterified cholesterol in dog serum, but in human serum simply prevented esterification. In a subsequent paper, Sperry and Stoyanoff (371) came to the conclusion that the system in dog serum which led to the hydrolysis of cholesterol esters in the presence of bile salts was quite separate from that present in human and dog serum which caused esterification and which was inhibited by bile salts. The effect of bile salts is especially interesting in the light of Tayeau's (243) discovery of the ability of bile salts to displace cholesterol from linkage with serum proteins.

The original observations of Sperry and Stoyanoff (370) have since been substantiated by European workers. In 1942, Alkalay and Favarger (372) studied the esterification of cholesterol which occurred in sterile human serum maintained at 37°C. for 72 hours. Although likewise employing the method of Schoenheimer and Sperry (38), these workers disagreed with the finding of Sperry that in fresh serum the esterified cholesterol invariably lay between 70 and 75% of the total. The fourteen values of Alkalay and Favarger (372) ranged instead between 55 and 69%, with an average value (recalculated from their table) of 62%. After the incubation, the values ranged between 73 and 86% in the esterified form, with an average of 78%. The free cholesterol which became esterified during the course of the experiments amounted on the average to 42% of that originally present,

in fair agreement with the 57% found by Sperry (369). The other discrepancies with the other results of Sperry were noted but unexplained. Alkalay and Favarger found that the esterification was equally active in serum from patients suffering from gout, but as might be expected was inhibited in cases of obstructive jaundice.

Further evidence for the presence of a cholesterol esterase in human serum was obtained in 1943 by Thoai (373) and by Pantaleon (374). The latter also studied the sera of dogs, rabbits, pigs and oxen. Both workers fully substantiated the inhibitory action of bile salts. Pantaleon reported that 40-75% of the free cholesterol was esterified during 72 hours at 37°C. at the optimal pH of 7.5, and also corroborated Sperry (369) as to the inhibitory effect of red cell hemolysate.

In search of the source of the fatty acids which entered into esterification with the cholesterol, LeBreton and Pantaleon (375) discovered that the quantity of phospholipid determined by the phosphorus or fatty acid content of the acetone-insoluble serum lipids decreased progressively when serum was incubated at 37°C. for 72 hours. Previous heating to 56°C. prevented the decomposition. From the fact that the method of estimation of phospholipid would have included lysolecithin, and also that the incubated serum was not hemolytic, it was concluded that the decomposition did not stop at lysolecithin but proceeded rapidly to lower

products.

In a subsequent paper, LeBreton and Pantaleon (376) described the process occurring during the incubation of serum at 37°C. as involving the coupled action of a lecithinase and cholesterol esterase, as follows:

1) Lecithin + $H_2O \rightleftharpoons$ 2 Fatty acid + glycerophosphorylcholine.

2) 2 Fatty acid + 2 cholesterol $\rightleftharpoons$ 2 cholesterol esters + 2 H_2O

The fate of the glycerophosphorylcholine (GPC) was not discussed.

The following table, taken from LeBreton and Pantaleon (375), shows the quantitative changes which occurred in a sample of horse serum maintained at 37°C.

TABLE III

CHANGES IN CHOLESTEROL AND PHOSPHOLIPIDS DURING INCUBATION OF SERUM AT 37°C.

All values in mg./100 ml.

	0 hrs.	72 hrs.
Total Fatty Acid	278	278
Total Cholesterol	148	147
Free Cholesterol	51.4	20.5
Phospolipid Fatty Acid	75.0	26.0

Taken from LeBreton and Pantaleon (375).

choline and fatty acids. This early evidence for the existence of a phospholipase, in spite of reports to the contrary (381, 382), has since received adequate confirmation (383, 384, 385). King in particular corroborated (386) various scattered studies (387, 388) in showing the presence of enzyme systems which decomposed lecithin to release inorganic phosphate in extracts of kidney, small intestine, spleen, liver, testes, pancreas, and other tissues.

Consideration of the formula for lecithin illustrated in Fig. 1 (a) shows that a variety of bonds must be hydrolyzed to effect complete decomposition of the molecule. Belfanti, Contardi and Ercoli (389) have attempted to classify the enzymes involved (Fig. 1 (b)). The enzyme which catalyzed removal of one fatty acid to yield lysolecithin (390) was designated lecithinase A, whereas that which removed both fatty acids was called lecithinase B. Lecithinase C was assumed to split off choline alone, leaving a phosphatidic acid. Finally, lecithinase D was assumed to split the bond between phosphate and glycerol in lecithin or lysolecithin, yielding choline phosphate and a di- or mono-glyceride.

For complete decomposition of lecithin the following succession of enzymes would be required (391, 389): 1) Lecithinase B or lecithinase A followed by B to yield free fatty acids and glycerophosphorylcholine (GPC). 2) a cholinephosphatase to give choline and glycerophosphate. 3) The familiar alkaline phosphatase (392) to release glycerol and

phosphate. Udagawa (393) has also obtained evidence for the existence in takadiastase of an enzyme which splits GPC at the other bond to yield glycerol and phosphoryl choline, which in turn may be split by a phosphomonoesterase to yield phosphate and choline.

FIGURE 1 (a)

FORMULA OF LECITHIN

For convenience the fatty acids are both represented as stearic, although at least one is usually unsaturated.

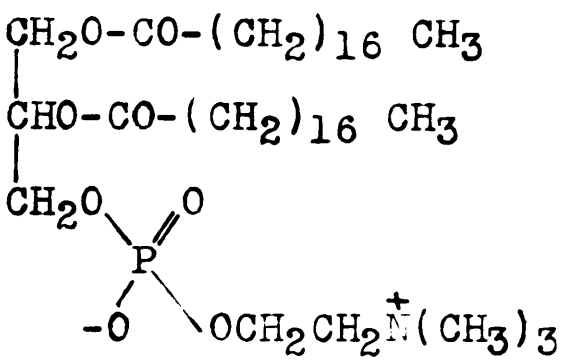
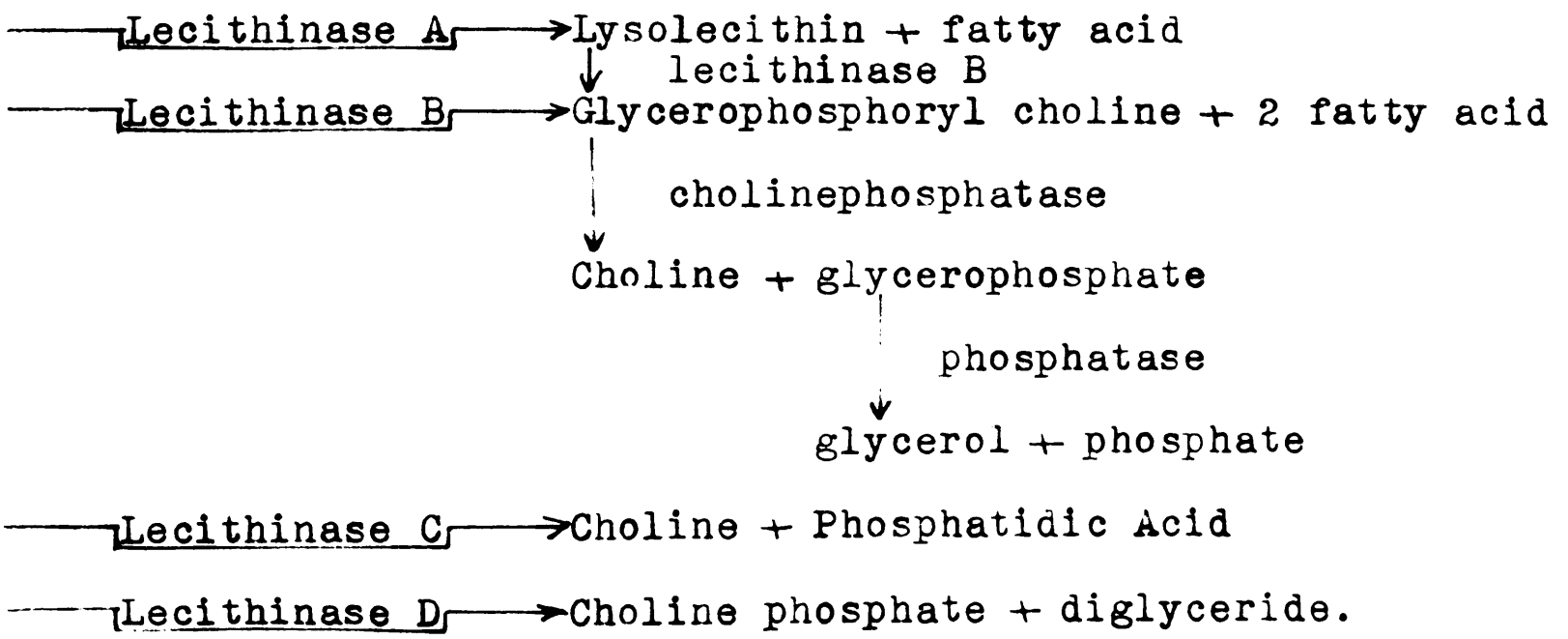


FIGURE 1 (b)

PATHWAYS OF DECOMPOSITION OF LECITHIN



Indirect evidence for the presence of a lecithinase of the B type in blood serum has been known for some time. The hemolytic action of snake venom was shown by Flexner and Noguchi (394) to be dependent on the presence of serum, and the complement present in serum was found by Kyes (395) to be lecithin. Before Delezenne and Fourneau (390) had isolated and characterized the hemolytic agent as lysolecithin, Delezenne and Ledebt (396) were able to show that the hemolytic activity of serum which had been treated with venom reached a maximum and then declined. Eventually the hemolytic properties were completely lost and the serum even acquired the ability to prevent hemolysis. In current terminology, after the original snake venom lecithinase A had manifested itself, lecithinase B activity began to develop. Delezenne and Ledebt found that serum lost the power to destroy the hemolysin after dialysis against saline solution, but that the activity was restored by returning the material which had been removed during the dialysis or by adding cerbrospinal fluid. It is noteworthy that the addition of the serum dialysate or cerebrospinal fluid to a mixture of egg yolk and cobra venom caused destruction of the hemolysin, and indication that the lecithinase B activity may not be a property of a specific serum enzyme.

An interesting suggestion that the decomposition of lecithin in stored serum may involve a temporary accumulation of lysolecithin may be made from the report of Bergenhem and

Fahraeus (397) in 1936. During the incubation of whole blood at 37°C., the red cells tended to assume a spherical shape and to aggregate. The same effect was obtained on addition of a trace of lysolecithin or of cobra venom to fresh blood. A small amount of material having the solubility characteristics of lysolecithin was isolated.

Direct evidence of extensive decomposition of phospholipids in serum was reported in 1939 by Verstraete and Cloetens (398), who thus have priority over LeBreton and Pantaleon (375). Samples of serum from 4 dogs and 8 horses were incubated at 37°C. in the presence of toluene for periods of from 8 to 11 days. The sera were rendered acid or alkaline by the addition of veronal buffers and brought to a 1:1 dilution with water. The increase in inorganic phosphate was found to range between 0.26 and 2.60 mg.% phosphorus. Dog serum showed an optimal pH of 7.6, whereas the horse serum changed most rapidly at pH 9.0. In undiluted dog serum at natural pH increases of 0.9-12.0 mg.% inorganic phosphorus were obtained.

The difference in the pH of optimal activity between horse and dog sera stresses the fact that several pH-dependent processes are involved in the separate steps of decomposition. The overall course of hydrolysis of lecithin to inorganic phosphate by extracts of kidney and intestinal mucosa was found by King (386) to be most rapid at pH 7.5, in agreement with Verstraete and Cloetens (398) for dog

serum. The only well-characterized enzyme involved in the hydrolysis is alkaline phosphatase, whose pH of optimal activity is about 9.0 (392). Verstraete and Cloetens accordingly suggested that in the case of the horse serum the rate-controlling reaction was the splitting of glycerophosphate by the alkaline phosphatase. Whether the value for dog serum of pH 7.6 represented the influence of a single different enzyme or was a composite effect of more than one reaction of similar rate is difficult to decide. King and Dolan (399) found that the hydrolysis of lysolecithin by intestinal extract was most rapid at pH 7.8, which might be taken as the optimum for lecithinase B. Very little appears to be known of the process whereby the phosphate-choline bond is split, although Yosinaga (400) reported that the release of choline from lecithin by takadiastase was most rapid at pH 4.

It should be stressed that the foregoing discussion has made use of information drawn from experiments which employed a wide variety of preparations and enzymes from different sources, and that no definite conclusions may be drawn as to the detailed nature of the hydrolysis of phospholipids in ageing serum. The question of activation and inhibition of the various enzymes will be postponed until the discussion of the experimental results.

Mention should perhaps be made of yet another

lecithin-splitting enzyme which has some connection with serum. Nagler (401) in 1939 discovered that when human serum was inoculated with Type A Clostridium welchii and incubated anaerobically, there developed a reaction in which the serum became opalescent and a layer of fatty material could be centrifuged to the surface. The reaction was prevented by antitoxic serum, and was restricted to the human species with the possible exception of monkeys and chickens. In 1941, MacFarlane and Knight (402) showed that the action was due to a lecithinase which released phosphorylcholine and diglycerides and hence corresponded to the lecithinase D of Contardi and Ercoli (391). The optimum pH was 7.0-7.6. The activity appeared to parallel the lethal power of the alpha-toxin. Macfarlane and Knight and also Crook (403) showed that the active principle of Cl. welchii filtrates was inactivated by heat and did not diffuse through cellophane membranes, and so appeared to be truly enzymatic.

Crook found that not only did the alpha-toxin decompose lecithin but also that the lipids which were released during the reaction represented a cross-section of the serum lipids and were not simply diglycerides, and were mixed with a considerable quantity of protein.

Petermann (404) reported recently the effect of the Cl. welchii lecithinase on the ultracentrifugal and electrophoretic diagrams of human serum, X-protein, and various fractions prepared by the method of Cohn et al. (184). The

most striking result was the destruction of the X-protein which paralleled the increase in turbidity. Inorganic phosphate showed only slight changes, suggesting that the breakdown of phosphoryl choline was slow. It is possible that the lecithin which was split by the enzyme played an important part in the integrity of the X-protein complex.

Since practically all the work which has been discussed was restricted to lecithin, no mention has been made of the other phospholipids. Nevertheless King (405) found that an extract of intestinal mucosa hydrolyzed cephalin nearly as rapidly as lecithin. In this case a broad optimum from pH 5.5 to 7.6 was found. On the other hand Chargaff and Cohen (406) reported that cephalin was unattacked by the lecithinase A of the venom of the cottonmouth moccasin, but Fairbairn (407) presented evidence that both phospholipids were converted to the lyso forms. Detailed work will be required to establish whether or not cephalin and sphingomyelin are hydrolyzed during the autolysis of blood serum.

It appears likely that the only source of the fatty acids which have been observed to esterify with cholesterol during the ageing of serum is phospholipid. True lipases which hydrolyze triglycerides of the higher fatty acids are found in blood serum only in cases of abnormal function of the pancreas (408, 409). The widely distributed esterases which split, for example, ethyl butyrate (409) do not attach higher triglycerides such as triolein (408).

d) Formation of Insoluble Material

The first report of formation of insoluble material in aseptically preserved serum appears to have been made by Petschacher (410) in 1925. Small amounts of precipitate were observed after the serum had stood 14 to 21 days at room temperature, but no attempt at analysis was made.

Roche and Marquet (411) in 1935 reported an analysis of a sediment which appeared in sterile horse serum which had been stored for several months at 0°C. Only one gram was collected from about 20 litres of serum. Prolonged extraction with ethanol dissolved approximately one-fifth of the material. The ratio of P:N in the alcohol was nearly 25:1. Very little cholesterol was present. The residue from the alcohol extraction contained considerable nitrogen and gave strong color tests for protein. Calcium amounted to 12% of the total residue, and phosphorus to 3%. Carbonate was present. The high ratio of lipid phosphorus to nitrogen in the precipitate was reflected by a ratio of 7.5:1 in the supernatant serum, compared with 1.4:1 in fresh horse serum.

Roche and Marquet concluded that the high ratios of lipid phosphorus to lipid nitrogen indicated the hydrolytic removal of the bases from the phospholipids, leaving phosphatidic acids (412, 413). It is interesting that such a process would correspond to the action of lecithinase C according to the terminology of Contardi and Ercoli (391).

In 1936, Khabas (414) reported the analysis of a sediment which was found in antimeningococcus serum after storage for two years with chloroform as preservative. The material contained a large proportion of lipids and inorganic salts, but little organic nitrogen. The cholesterol content was insignificant.

McFarlane (226) in 1942 mentioned that filtered human serum gradually became turbid through the formation of a precipitate "which is mainly lipoid". He did not investigate its composition more closely.

In 1944, Francis, Harrison and Picken (415) reported analyses of a deposit which developed in sterile human serum during storage at room temperature for nine months. The material was taken to be composed primarily of calcium stearate mixed with a small quantity of protein. Since the serum corresponded closely to that which has formed the subject of this investigation, detailed examination of their results will be postponed until the discussion of the experimental results.

EXPERIMENTAL PART

ANALYTICAL METHODS

In the following section are described the methods of analysis which have been employed. Any modifications of the original procedures are presented in full.

Calcium

The method employed was that of Loureiro and Janz (416), with a modification taken from Sendroy (417).

Principle: Ca is precipitated by ammonium oxalate, the washed precipitate is dissolved in nitric acid, and the oxalate treated with a known excess of a special solution of potassium permanganate. The excess permanganate is titrated iodometrically with sodium thiosulfate.

Reagents: 1. Stock Diluent: 50 g. of $MnSO_4.4H_2O$ or 40.5 g. of $MnSO_4.2H_2O$ was dissolved in water in a litre flask, and 350 ml. conc. H_2SO_4 added. After cooling, the volume was made up to 1 litre with water.

2. Standard Permanganate, about N/10: 3.5 g. $KMnO_4$ in 1 litre of stock diluent. 25 ml. were diluted to 250 ml. with stock diluent to prepare a N/10 solution.

3. Standard Thiosulfate Solution, N/100, exactly adjusted against standard iodate. The solution was prepared by dilution of N/10 thiosulfate prepared by dissolving 25 g. $Na_2S_2O_3.5H_2O$ and 0.2 g. Na_2CO_3 in a litre of

freshly boiled water.

4. Potassium Iodide, 5%.

5. Soluble Starch, 1% in saturated NaCl

6. Ammonium Oxalate, saturated

7. Ammonia Solution, 1 volume of concentrated to 3 of water.

8. Nitric Acid, 50% by volume

9. Methyl Red, saturated solution in water

Procedure: To 2 ml. of serum and 10 ml. of water, or an equivalent solution containing about 0.2 mg. Ca, in a 50 ml. centrifuge tube was added 2 ml. of saturated ammonium oxalate followed by two drops of methyl red solution. If necessary, nitric acid was added dropwise to render the solution acid (red), and then ammonia was added until the indicator turned yellow. Following Sendroy (417) no adjustment of pH was made in the case of serum. The tube was carefully swirled to assure complete mixing, stoppered, and left standing at least 16 hours.

The tube was then centrifuged, and the supernatant aspirated off carefully leaving not more than 0.2 ml. in contact with the precipitate. 20 ml. of distilled water were used to wash down the sides of the tube, and the wash-water removed as before after centrifugation.

1 ml. of nitric acid was next added with care to wash down the walls. The tip of the pipette was used to

prevent channeling. As soon as the precipitate had dissolved, 2 ml. of the N/100 $KMnO_4$ solution was added in a similar manner, and the contents of the tube mixed well. After standing at room temperature for 30 minutes, the solution in the tube was transferred with the help of 50 ml. of water to a 125-ml. Erlenmeyer flask. 4 ml. of KI solution was then added, followed by immediate titration of the released iodine with N/100 thiosulfate, using a few drops of the starch solution as the end-point was approached. At the same time 2 ml. of the permanganate was titrated for standardization.

Calculation: (2 x normality $KMnO_4$ - ml. x normality $Na_2S_2O_3$) x 20 = mg. Ca.

Remarks: Loureiro and Janz (416) prescribed precipitation according to the method of E. P. Clark and J. B. Collip (418), but gave as a reference the paper by G. W. Clark (419) on the same subject. The use of 50 ml. centrifuge tubes stems from the latter paper, yet was retained for convenience after the error had been discovered. The precipitation has followed the prescription of Sendroy (417) who found that dilution of serum prior to addition of oxalate gave more reliable results.

Washing once with a large volume of distilled water was a simplification which was justified empirically. As Sendroy (417) has pointed out, the procedure for Ca is

necessarily empirical. Five determinations on a solution of calcium made up from calcium carbonate to contain 12.29 mg./100 ml. yielded an average value of 12.36 mg.% with a mean deviation of 0.10 mg. The method showed a high precision, as exemplified by six determinations on another solution which gave 11.66 mg.% with a mean deviation of only 0.02 mg.%. All values which have been reported, unless specifically stated otherwise, are means of at least 3 determinations whose mean deviation rarely exceeded 1%. As for the stoichiometry of the reaction, an experiment in which a standard solution of sodium oxalate was substituted for the Ca precipitate gave a titration within 0.2% of the theoretical.

Application to Solid Material: The solid material was wet-ashed in a boiling tube with nitric acid, usually for two hours. 30% hydrogen peroxide (superoxol) was used to complete the process. After bringing the residue almost to dryness, the solution was made up to some volume and aliquots taken for calcium and phosphorus determinations, but not for nitrogen.

Phosphate

The method of Fiske and Subbarow (89) modified for use with the Evenlyn colorimeter has been employed.

Principle: Phosphate is treated with ammonium molybdate to form phosphomolybdate which is reduced by 1-amino-2-naph-

ol-4-sulfonic acid. The blue color of the reduced phosphomolybdate is measured photoelectrically.

Reagents: 1. N.HCl.

2. 2N HCl.

3. Ammonium Molybdate, 2.5%.

4. Phosphorus Reagent: 0.125 g. of sodium aminonaphtholsulfonate was weighed into a small beaker and stirred to a paste with a few drops of water, then poured into a 5 ml. vol. flask containing 7.2 g. of sodium bisulphite and a little less than 40 ml. of water. 10 ml. of 5% sodium sulfite was then added, washing in the reducing agent. The flask was brought to volume with water and the solution rapidly became clear on mixing.

Procedure: To 5 ml. of the solution of phosphate in N HCl or its equivalent was added 1 ml. of ammonium molybdate, 0.4 ml. of the reagent and 3.6 ml. of water. After thorough mixing and standing 12 minutes, the tube was read in an Evelyn colorimeter against a blank containing 5 ml. of N HCl in place of the phosphate solution.

Calculation: A standard curve passing through the origin was obtained and plotted. Phosphate values between 4 and 16 micrograms of phosphorus were read off the graph from the determinations of optical density. The relation between phosphorus and optical density was linear over the range used.

Remarks: Since the color development is sensitive to the acidity, special care had to be taken to assure standard conditions, as shown in the following descriptions of applications.

Inorganic Phosphorus in Serum: Most often, 1 ml. of serum was run into 9 ml. of 3% trichloracetic acid in a 15-ml. centrifuge tube, the tube was stoppered, inverted, and left standing for at least 30 minutes. After centrifugation, 1 or 2 ml. were withdrawn and placed in a colorimeter tube. When 1 ml. was taken, 4 ml. of N HCl were added to make up the volume. When 2 ml. of the trichloracetic acid solution were taken, 2 ml. of 2N HCl were added, and the volume finally adjusted by adding 4.6 ml. instead of 3.6 ml. of water. If the supply of serum was small, 0.2 ml. serum were mixed with 2.8 ml. of the trichloracetic acid and 2 ml. of the resulting supernatant were taken for the phosphorus determination. Duplicate determinations were always made.

Total Phosphorus in Serum: 1 ml. of serum was placed in a boiling tube along with 3 ml. of 9N H_2SO_4 (1:3). Addition of a little nitric acid gave a more uniform mixture. The contents of the tube were heated until the water had been driven off, whereupon 30% hydrogen peroxide (superoxol) was added until the mixture was clear. After cooling, 10 ml. of water was added and the mixture was boiled down again to the appearance of dense white fumes to remove traces of H_2O_2. Another 3 ml. of 9N H_2SO_4 was added and the solution made up

to 50 ml. Duplicate samples were taken for the phosphorus determinations. In agreement with the suggestion of Gortner (420), it has been found preferable to allow 20 minutes for color development. If the duplicates differed by more than 1%, the procedure was repeated.

Nitrogen

Nitrogen has been determined by the micro-Kjeldahl method following the directions of Clark (421) and Miller and Houghton (422).

Principle: The material containing nitrogen is ashed in sulfuric acid to convert the organic nitrogen to ammonium ion, and the ammonia is subsequently steam distilled from an alkaline solution and collected in acid. Standard acid is used to titrate the resulting solution to the original pH.

Reagents: 1. Concentrated Sulfuric Acid, reagent grade.

2. Mercuric Oxide, HgO.

3. Potassium Sulfate, K_2SO_4.

4. Boric Acid, 4%.

5. Methyl Red, 0.1% in ethanol.

6. Sodium Hydroxide Solution: 40 g. NaOH, 5 g. $Na_2S_2O_3$, 100 ml. H_2O.

7. Standard Hydrochloric Acid, standardized against carbonate-free NaOH (see section on fatty acids) which in turn was standardized with potassium biphthallate prepared by weight, using phenolphthalein as indicator.

Procedure: Dry samples were weighed onto cigarette paper from which the glued part had been cut; the paper was folded with caution and dropped into a dry boiling tube. Liquid samples were introduced with a pipette. To the unknown and a blank tube were added 40 mg. H_gO, 0.5 g. K_2SO_4 and 1.5 ml. of concentrated sulfuric acid. The contents of the tube were heated cautiously as first to avoid excessive frothing and then boiled strongly with a small funnel placed in the mouth of the tube to act as a condenser. The heating was continued until the mixture was colorless, requiring from 1 to 3 hours depending on the material. One drop of ethanol was now added and heating continued until the mixture was once more clear, then for 30 minutes more.

The digest was then transferred with 8 ml. of water to a round bottom flask of 200 ml. capacity and fitted with a ground glass neck and the boiling tube was washed with 4 small portions of water. The bulb was now placed in position on the apparatus which had been steamed for at least 10 minutes previously. 8 ml. of the NaOH solution was now run in, followed by a few ml. of water. The contents of the bulb were immediately mixed thoroughly by cautious shaking and the steam was turned on and a small electric heater placed under the bulb. 2 ml. of boric acid and 1 drop of methyl red were placed in a 25-ml. Erlenmeyer flask which was arranged under the receiver of the Kjeldahl apparatus with the tip of the tube extending just below the surface. It was found

unnecessary to have the boric acid in position before any liquid came over.

The distillation was continued for 4 minutes after the first condensate reached the acid, the Erlenmeyer was lowered below the tip of the tube and the distillate collected for a further 30 seconds, during which time the tip was washed down with distilled water from a wash bottle. The boric acid solution was then titrated with the N/50 HCl to the original shade of red.

Calculation:

mg. N=(ml. HCl for unknown - ml. HCl for blank)×normality HCl×14

Remarks: The distillation procedure was checked using a solution of ammonium sulfate made up by weight to contain 1.033 mg. N/ml. Recoveries of 98.1, 101.3 and 99.9% were obtained. The entire procedure was tested with a similar solution of urea with a recovery of 99.6%. When an appreciable period had occurred during which the method was not used, the distillation procedure was checked again with a recovery of 99.1% before unknowns were analyzed.

Sulfate

Sulfate was determined by the benzidine method, following directions kindly furnished by Dr. D. E. Douglas.

Principle: Sulfate is isolated as the benzidine salt which is dissolved and titrated with alkali.

Reagents: 1. Ethanol, 95%

2. Benzidine Hydrochloride Solution: 5 g. of benzidine HCl was dissolved in 40 ml. of N HCl and 50% ethanol was added to a volume of 250 ml. The solution was brought to the boil, cooled and filtered.

3. Standard Sodium Hydroxide, N/50 (see procedure for fatty acids).

Procedure: 3 ml. of the solution to be analyzed were placed in a 50-ml. centrifuge tube, followed by 2 ml. of ethanol and 1 ml. of the benzidine solution. The tube was allowed to stand 30 minutes before centrifugation. The supernatant was sucked off and the precipitate washed once with 20 ml. of ethanol. Finally, 5 ml. of water were added, and the tube was placed in a water bath at 70-80°C. and titrated with N/50 NaOH using phenolphthalein as indicator.

Calculation:

mg. Sulfate S = ml. NaOH × normality NaOH × 16.03.

Preparation of Lipid Extracts of Serum

The method of Bloor (18) has been followed.

Reagents: 1. Ethanol, redistilled

2. Diethyl Ether, peroxide free (22): The ether was distilled through a short column, the last third being discarded. It was then shaken with acidified aqueous KI, followed by $Na_2S_2O_3$, and washed with distilled water.

The ether was finally distilled again and stored in a dark bottle over iron wire.

Procedure: About 45 ml. of a freshly prepared 3:1 mixture of alcohol and ether were placed in a 50-ml. volumetric flask. 2 ml. of serum were then run into the flask with constant stirring, after which the contents of the flask were brought to the boil for thirty seconds and allowed to cool. The flask was then brought up to the mark with alcohol-ether, mixed, and filtered through acid- and ether-washed filter paper (Whatman #43). Aliquots were taken for phospholipid and cholesterol determinations.

Alternatively, 1 ml. of serum was extracted in a 25-ml. flask.

Determination of Serum Phospholipid

Direct Method: Phospholipid was estimated by direct determination of the P content of the alcohol-ether extract. The theoretical basis has been amply justified in the Theoretical Part (see p. 17).

Procedure: An aliquot of the alcohol-ether extract, representing usually 0.2 ml. of serum, was pipetted directly into a boiling tube which previously had been marked with a scratch to indicate a volume of 10 ml. A boiling stone was added and the solvent driven off on a water-bath.

The ashing procedure, following Gortner (420), was

similar to that employed for the determination of total serum phosphorus. In this case, however, 1.2 ml. of 9N H_2SO_4 were added and the solution was brought to a final volume of 10 ml. directly in the tube. A 5 ml. aliquot was withdrawn for phosphorus determination.

Calculation: Since the aliquot represented 0.1 ml. of serum, the value obtained could be taken directly to represent mg. of lipid P/100 ml. of serum. Occasionally, such as in cases of very low phospholipid levels in the serum, different aliquots were chosen and required appropriate modifications in the simple calculation.

Acetone-Precipitation Method: On occasion the amount of phosphorus which was insoluble in acetone and magnesium chloride was determined. The procedure followed that of Bloor (22).

Reagents: 1. Light Petroleum (Benzin, Petroleum Ether), reagent grade.

2. Acetone, reagent grade.

3. Magnesium Chloride Solution, 1.2 g. $MgCl_2$ in 25 ml. ethanol.

Procedure: An aliquot of the alcohol-ether extract was evaporated on a steam bath in an Erlenmeyer flask with a light watch-glass over the mouth to act as a flutter-valve. The removal of the organic solvent was considered practically complete after the stage of frothing (never more than one-

quarter the way up the side of the flask) had passed, and the flask was removed from the heat. Light petroleum (L.P.) was immediately added, boiled down to 3-5 ml. and decanted carefully into a 15-ml. centrifuge tube. The extraction was repeated three more times, the L.P. being concentrated in the centrifuge tube by heating in a beaker of hot water. Bumping was prevented by a boiling rod.

The L.P. solution was finally concentrated to 1-2 ml., the boiling rod was removed, and 7 ml. of acetone was added, followed by 2 drops of the $MgCl_2$ solution. The tube was stoppered, rotated, and allowed to stand overnight. The tube was then centrifuged and the supernatant either discarded or saved for the determination of free cholesterol (see below). In the latter case the phospholipid precipitate was washed twice with 5 ml. portions of acetone and the washings were added to the first solution.

Following the procedure of Entenman and Chaikoff (95), the phospholipid precipitate was next suspended in 2 ml. of ether and the phospholipids were brought into solution by the addition of methanol. The solution was transferred quantitatively to a boiling tube with the help of several portions of methanol. The solvent was then driven off on a water bath and the ashing and phosphorus determination made as before.

Cholesterol

The method of Kelsey (68) has been employed, with the substitution of that of Ireland (73) for the final color development.

Principle: The cholesterol is precipitated as the digitonide to separate it from the other lipids and then is freed from digitonin prior to treatment with acetic anhydride and sulfuric acid for the development of color.

Reagents: 1. Alcohol-Ether Mixture, 1:1.

2. Potassium Hydroxide Solution, 35 g. 85% KOH in 100 ml. of water.

3. HCl, 2N

4. Light Petroleum, reagent grade.

5. Digitonin Solution, 0.2% Schuchardt's Digitonin in 95% ethanol.

6. Benzene, redistilled.

7. Chloroform, reagent grade.

8. Acetic Anhydride, reagent grade.

9. Concentrated Sulfuric Acid.

Procedure for Total Cholesterol: An aliquot of the alcohol-ether extract usually representing 0.2 ml. of serum was evaporated as before to a small volume and 10 ml. of 1:1 alcohol-ether and 0.3 ml. of the KOH solution were added. The mixture was brought to the boil and placed in a constant temperature bath at 37°C. for 30 minutes. 1 ml. of 2N HCl

was then added to render the mixture distinctly acid and the solvent was driven off in the usual manner. The residue was extracted 4 times with L.P. and the extracts were concentrated to 0.5 ml. in a 15-ml. centrifuge tube.

4 ml. of digitonin solution were now added and the contents of the tube were evaporated to dryness. Contaminating substances were removed by extraction with 3 portions of boiling L.P., and the extracts were discarded.

The cholesterol-digitonin complex was decomposed by addition of 7 ml. of benzene and boiling slowing and evenly for 45 minutes. The benzene was finally concentrated to 3 ml. and L.P. was added rapidly to a volume of 12 ml. On stirring digitonin precipitated out. After centrifugation, the solution was decanted into a 50-ml. Erlenmeyer flask, followed by three washings of 8 ml. of L.P. each. The solution was evaporated and the last traces of solvent removed with a stream of air.

For the color development the method of Ireland (73) was employed. To the residue in the Erlenmeyer flask 5 ml. of chloroform was added from a burette, after which the flask was tightly corked and the solvent mixed with a swirling motion. 2 or 4 ml., depending on the expected quantity of cholesterol, were withdrawn and placed in a colorimeter tube, also fitted with a tight cork. The volume in the colorimeter tube was then brought to 5 ml. by the addition of chloroform from the burette.

The colorimeter tube was now placed in a water bath at 18°C. along with a tube containing a freshly prepared mixture of 20 ml. of acetic anhydride and 1 ml. of sulfuric acid. After allowing 5-10 minutes for equilibration to take place, 2 ml. of the acetic anhydride-sulfuric acid mixture were pipetted into the chloroform solution containing cholesterol and also into a blank of 5 ml. of chloroform. The contents of the tubes were carefully mixed and they were returned to the bath which was placed in a cupboard to protect the solutions from light. After 60 minutes the tubes were removed, wiped dry with a clean towel, and read in the Evelyn colorimeter using a 635 millimicron filter.

For calibration, six dilutions of cholesterol in duplicate were put through the color development. All except one of the duplicates gave perfect duplication. The slope of the line of regression of optical density on cholesterol content was calculated from the formula (423)

$$m = \Sigma\, xy / \Sigma\, x^2$$

where $x = X - M_X$ and $y = Y - M_y$, M_X and M_y being the arithmetic means of X and Y respectively. Since the line passed through the origin, it was possible to obtain the value for the cholesterol corresponding to a given optical density simply by multiplying the optical density by the value for m.

Calculation: The relation between the cholesterol finally determined and the content of the serum varied according to

the volumes chosen. For example, the choice of 5 ml. of alcohol-ether extract corresponding to 0.2 ml. of serum and of 2 ml. out of 5 ml. of chloroform solution meant a factor of 1/0.2 x 5/2 x 100 = 1250 in converting to mg. cholesterol per 100 ml. of serum.

Remarks: A known sample of cholesterol prepared by Armour was put through the procedure with 95% recovery. This was considered adequate in view of the uncertain relationship between commercial preparations and serum cholesterol (78).

Procedure for Free Cholesterol: An aliquot of the alcohol-ether extract, usually representing 0.4 ml. of serum, was put through the procedure for the precipitation of phospholipid by acetone. The acetone solution and washings were concentrated in a 15 ml. centrifuge tube and 4 ml. of the digitonin solution were added. Henceforth the procedure differed in no way from that for total cholesterol.

Determination of the Iodine Number of Fatty Acids

The method of Yasuda (151) was employed.

Principle: The fatty acids are treated with a known solution of pyridine dibromide. The excess of the latter is determined iodometrically by titration with thiosulfate.

Reagents: 1. Pyridine Sulfate Dibromide: 1.6 ml. of pyridine were run into 5 ml. of glacial acetic acid with

cooling. In a separate flask 1.1 ml. of concentrated sulfuric acid were run into another 5 ml. glacial HOAc. The solutions were combined, and together with 1.7 g. of bromine were made up to 200 ml. with glacial acetic acid. This solution of approximately 0.1 N was diluted 1:1 with HOAc to prepare 0.05 N pyridine sulfate dibromide.

2. Potassium Iodide, 10% solution in water.

3. Soluble Starch Solution, as used in the determination of Ca.

4. Sodium Thiosulfate, 0.01 N, as used in the determination of Ca.

Procedure: Approximately 10 mg. of fatty acid was weighed into a small glass cup which was placed in an iodine number flask fitted with a ground glass stopper and flanged lip. 2 ml. of chloroform were added, followed by 2 ml. of the reagent from a burette. The flask was immediately stoppered and agitated. After standing 15 minutes at room temperature, 0.5 ml. of 10% KI was added and washed in with a little distilled water, and the liberated iodine was titrated with sodium thiosulfate. A blank containing no fatty acid was similarly treated.

Calculation:

$$\text{Iodine Number} = \frac{(B - S) \times \text{normality } Na_2S_2O_3 \times 127 \times 100}{\text{weight of sample}}$$

where B represents $Na_2S_2O_3$ for blank,

and S " " " sample.

Determination of the Mean Molecular Weight of Fatty Acids

Principle: The number of equivalents of alkali which react with a weighed sample of fatty acid is determined, from which is calculated the mean molecular weight of the fatty acids.

Reagents: 1. Ethanol, 95%, redistilled

2. Sodium Hydroxide, N/50, carbonate-free. Prepared by dilution of a saturated solution with water which had been boiled and allowed to cool with a soda-lime tube for communication with the atmosphere. It was stored in a flask which delivered through a siphon, air being admitted to the flask through a trap containing the same solution. In this way the standard solution was protected from carbon dioxide and evaporation. The solution was standardized against the primary standard potassium biphthaliate.

Procedure: To a weighed sample of fatty acids in an Erlenmeyer flask were added 20 ml. of ethanol. The solution was heated to boiling, 2 drops of phenolphthalein were added, and titration with the carbonate-free NaOH followed immediately. A blank on 20 ml. of alcohol was also titrated.

Calculation:

$$\text{Mean Molecular Weight} = \frac{\text{weight of sample}}{(S - B) \times \text{Normality NaOH}}$$

where B represents Ml. NaOH for blank

and S " " " " sample

RESULTS

I. Analysis of the Precipitate

The first two batches of material studied were collected from serum which had been obtained in the dry state from the University of Montreal and reconstituted two years previously by Dr. Denstedt. The first sample was prepared by aspirating off the greater part of the supernatant serum and then concentrating the precipitate in a centrifuge tube, and finally drawing off almost all the remaining serum. The insoluble material was washed twice by suspension in distilled water, recentrifugation, and removal of the wash-water. It was then transferred to a watch-glass and dried in a desiccator. The white material was hard and waxy to the touch.

After a flame test had demonstrated the presence of calcium, the entire sample was ashed with nitric acid, during which process several globules of fatty material were noticed on the surface. A calcium content of 16.1% was found. From a determination kindly carried out by Miss M. I. Currier a phosphorus content of 2.53% was calculated.

The second batch of material was obtained from five bottles each of 250 ml. capacity which had been standing for about two years at room temperature. The wash-water from four successive washings was collected, and appeared to contain in suspension a considerable amount of the precipitate.

Both the precipitate and washings were then shell-frozen and dried under high vacuum (lyophilized). The material from the washings was extremely light and fluffy. The precipitate proper showed an apparently greater density and waxiness at the bottom of the lyophilizing tube, whereas farther up near the mouth of the tube it approximated the material from the washings in density and texture. About 0.35 g. of material were obtained, along with another 0.1 g. from the washings. Calculated on the basis of the total volume of 1250 ml. of serum, the yield was 28 mg. per 100 ml. of serum.

Values for calcium, phosphorus and nitrogen are given in Table IV. Unfortunately the inhomogeneity of the material became apparent only during the actual operation of transferring it to a small bottle, with the result that the chemical evidence of inhomogeneity, though definite, was not very striking. It should be stressed that the variations in Ca and P were far outside experimental error.

TABLE IV

ANALYSIS OF THE PRECIPITATE - SECOND BATCH

Sample	Ca%	P %	N%
1	6.47	0.23	-
2	6.21	0.36	-
3	6.99	0.13	-
4	-	-	0.63
5	-	-	0.61

By ashing in a crucible in the full Bunsen flame the ash was found to constitute 11.1% of the material. The calcium content of the ash was 55.5%, the phosphorus content 2.82%. Calculated on the basis of the total material taken, the contents of Ca and P were 6.17% and 0.31% respectively. The ash was devoid of sulfate.

A sample of the material was then treated with 10 ml. of 10% trichloracetic acid in a centrifuge tube. In a few minutes the coagulum had almost entirely collected on the surface of the solution (sp. gr. 1.044). A few minutes of mild centrifugation served to clear the liquid, after which a pipette was cautiously passed through the coagulum to the bottom of the tube and 1 ml. withdrawn for analysis. On the basis of a similar withdrawal 14 hours later, it was calculated that a total amount of calcium equal to 6.27% of the sample was leached out, 94.5% of which calcium was already in solution at the time the first aliquot was taken, 17 minutes after the initial mixing. A third aliquot gave a negative test for P. The material insoluble in trichloracetic acid was found to contain a trace of cholesterol by the qualitative Liebermann-Burchard test.

Finally, a sample of the material was treated with 6N HCl and extracted several times with light petroleum. A trace of material remained in the interface during the extraction. The light petroleum solution was washed three times with 5% NaCl solution, left standing over anhydrous

sodium sulfate, and finally boiled to dryness together with washings from the sodium sulfate. The residue was dissolved in hot 95% ethanol and titrated with standard carbonate-free NaOH, using phenolphthalein as indicator. 0.1095 milliequivalents of acid were found.

Having thus established the presence of fatty acids, the acids were recovered by diluting the alcohol with an equal volume of water, acidification with HCl, and extraction with light petroleum. The light petroleum solution was washed with 5% NaCl and dried over sodium sulfate. The product obtained on removal of the last traces of solvent melted at 50-52°C., and had a mean molecular weight of 291.

A sample of the untreated material was found to melt at 162-166°C.

Samples of the material which was obtained by drying the wash-water from the frozen state were found to be readily soluble in water, to give a strong biuret reaction with dilute alkaline copper sulfate, and to contain 11.27% nitrogen and 0.13% phosphorus.

Attention was next directed to a relatively large batch of precipitate obtained from the University of Montreal. The serum from which it was collected had been pooled in October 1943, and when found to be bacterially contaminated had been set aside at 0°C. after Seitz filtration. After the appearance of the sediment, the serum was Seitz filtered in

April 1944, and again in September 1945. The material which was taken for study had made its appearance during two months following the last filtration.

The analytical procedure followed the previous pattern. The material was washed by centrifugation in two batches of 0.67 g. and 3 g. respectively, in one case with 3 portions of 30 ml. of distilled water, in the other with 1% NaCl solution to ensure solubility of globulins in the adherent serum. No striking differences in composition appeared to result from this variation in procedure. The two samples melted at 163-176°C. and 168-178°C. respectively. Values for Ca, P and N are shown in Table V.

TABLE V

ANALYSIS OF THE PRECIPITATE - THIRD BATCH

Sample	Ca%	Total P %	Acid-soluble P %	N %
1*	10.00	2.25	-	1.67
2	12.30	1.97	-	-
3	10.50	1.49	-	-
4	8.82	1.69	-	-
5	11.28	-	1.33	-
6	-	-	-	0.89
7	-	-	-	1.24
8	10.27	-	1.61	0.69

* This sample was washed with distilled water; the others were taken from the batch washed with salt solution.

Sample 5 was first extracted with 3:1 alcohol-ether to give a value of 0.013% lipid phosphorus, equivalent to 0.3% phospholipid. Cholesterol determinations on the same extract gave a value of 1.1% for total cholesterol, of which 80% was in the free form. The residue from the alcohol-ether treatment was then treated with N/10 HCl with the results as shown.

The values for Sample 8 were obtained from the aqueous residue after treatment with HCl and light petroleum The aqueous solution was centrifuged and the clear solution taken for the Ca, P and N determinations. A small amount of insoluble material was removed by this operation and after drying was found to amount to approximately 2.5% of the starting material. Examined under the microscope, it was found to be composed partly of needles and partly of smaller fibres. The latter blackened on heating, while the needles showed no sign of melting at 300°C. It was concluded that the material represented traces of the Seitz filter pads (see below) which had broken off during filtration.

In one case the supernatant solution from the precipitation of Ca was tested for magnesium by the method of Hoffman (424), as follows. To 6 ml. of the solution was added 4 drops of 6% 8-hydroxyquinoline prepared by dissolving 3 g. in 50 ml. of 95% ethanol. 8 drops of concentrated ammonia were then added and the mixture was heated to boiling and maintained at 75°C. for 30 minutes. The solution remained

clear, indicating the absence of Mg. To test the unfamiliar procedure, a few drops of very dilute magnesium chloride solution were added and found to produce a heavy precipitate immediately.

Three samples of fatty acid were prepared as before; the properties are presented in Table VI.

TABLE VI

PROPERTIES OF FATTY ACIDS PREPARED FROM THE THIRD BATCH

Sample	Melting Point	Mean Molecular Weight	Iodine Number
1	50-56°C.	286.5	-
2	55-56°C.	289.7	-
3	55-56°C.	295.4	2.42

In view of the similarity of the values, it is instructive to note that Sample 1 was purified by treatment of its alcoholic solution with animal charcoal followed by filtration, whereas the other samples were purified by simply extracting a solution of their soaps in 50% alcohol with light petroleum to remove unsaponified material. In keeping with the conclusion that the fatty acids in the precipitate were in a surprisingly pure state was the finding that the unsaponified material was in each case barely detectable. This conclusion is also in accord with the low yields of cholesterol and phospholipid.

A last batch of serum sediment was obtained from the University of Montreal at the same time as the previous one. The serum had been pooled in December 1944 and re-filtered in September 1945. As before, the material studied had appeared over a two-month period. The material was washed 5 times with 1% NaCl and once with distilled water. The results for Ca, P and N are found in Table VII.

TABLE VII

ANALYSIS OF THE PRECIPITATE - FOURTH BATCH

Sample	Ca%	P%	N%
1	8.71	2.41	-
2	8.44	2.89	-
3	8.53	2.91	-
4	-	-	0.28
5	-	-	0.40
6	-	-	0.36

The precipitate contained 27.37% ash. A sample was stirred with N HCl, centrifuged, and an aliquot taken for phosphorus determination. A value of 0.1% inorganic phosphorus was found. Another sample of the same solution was ashed, giving a value of 0.46% total acid-soluble phosphorus.

Another sample of the material from the fourth

batch was extracted with alcohol-ether, from which a value of 0.018% lipid phosphorus was obtained. Yet another sample was extracted for 100 hours in a Soxhlet apparatus with peroxide-free ether. 87% of the starting material was recovered from the thimble and found to contain 4.00% phosphorus. The total phosphorus content of the ether extract was barely detectable.

Finally, a sample of the material from the fourth batch was saponified by boiling under reflux for 3.5 hours in N/10 alcoholic KOH. The residue was practically entirely soluble in aqueous acid. Fatty acids were isolated from the saponification mixture, and it was found that, unlike the previous preparations of fatty acid, a small amount of liquid material was present. The solid crystals melted (or dissolved) between 42 and 53°C. The iodine number was 14.7.

Samples taken both before and after extraction with ether were observed under a microscope in the melting-point apparatus to melt in part at about 160°C. The remainder of the material contracted slightly above 200°C. but no melting was observed up to 300°C. where the heating was discontinued.

II. Analysis of the Supernatant Serum

Samples of the sera from which the second, third and fourth batches of precipitate were obtained have been designated Serum 2, 3 and 4 respectively. The results of

analyses for calcium and phosphorus are presented in Table VIII.

TABLE VIII

ANALYSIS OF THE SUPERNATANT SERUM

Results are expressed in mg. per 100 ml.

Serum	Ca	Inorganic P	Lipid P	Total P
2	11.04	10.6	-	11.5
3 Nov. 1945	8.84	11.6	-	-
Mar. 1946	7.15	12.2	-	-
4	11.53	11.8	1.8	15.0

Serum 4 was subjected to ultrafiltration as described in Section IV, below. The ultrafiltrate contained 11.2 mg.% inorganic phosphate, 5.57 mg.% Ca and 45.3 mg.% N. The protein nitrogen was calculated by subtracting 49.0 mg.% N in the trichloracetic filtrate (N.P.N.) from 1.163 g.% total N, giving 1.114 g. of protein nitrogen per 100 ml. Using the factor 6.25, 6.96 g.% protein was calculated. Taking the values 11.5 mg.% total calcium and 7 g.% protein, the value of 5.25 mg.% ionized calcium was read off the nomogram published by McLean and Hastings (425).

The pH of Sera 2 and 3 was 7.5 and 7.3 respectively, as measured with the Beckman meter.

III. Analysis of Seitz Filters

Samples of the Seitz filter pads used to sterilize the serum were obtained from the University of Montreal and subjected to analysis. These pads appear to have been prepared from asbestos and paper fibres, pressed to form a sufficiently strong pad to withstand filtration under small pressures. A sample was found to contain 49.2% ash.

Several samples taken from two Seitz filter pads were treated to discover the quantities of Ca and P which could be leached from the material under various conditions. The results are presented in Table IX.

TABLE IX

RESULTS OF LEACHING SEITZ FILTERS WITH VARIOUS SOLUTIONS

Sample	Treatment Solution	Temp.	Time min.	Wt. Sample grams	Ca mg./g.	P mg./g.
1	conc.HCl	25°C.	15	5.12	23.9	-
2	conc.HCl	100	30	1.67	26.6	0.044
3	Veronal, pH 7.5	25	15	5.34	1.53	0.036
4	Veronal, pH 7.5	25	10	5.87	2.14	0.035
5	Boiled dist. water	20	10	6.26	0.27	-
6	Boiled 0.9% NaCl	20	10	5.60	0.70	-

Sample 1 gave a heavy blue precipitate with potassium ferricyanide solution, indicating considerable quantities of iron were present. Even the veronal extract, Sample 3, gave a faint test for iron. Sample 5 was tested with silver nitrate solution, and shown to be free from halides. A white precipitate with barium chloride solution indicated the possible presence of sulfate. A test for sulfide on the same sample using lead acetate in the presence of acetic acid gave a negative result.

A quantitative determination of sulfate on Sample 6 gave the value 0.43 mg./g., equivalent to 0.54 mg. of Ca per gram of filter.

The weight of one pad was about 40 grams.

IV. Analysis of Lipemic Serum

A standard transfusion bottle containing lipemic serum which had been on display for approximately two years was furnished by M. Tasse at the University of Montreal. The serum had been transferred to the bottle without passage through a Seitz filter and thus constituted the only sample of aged serum which had not been so treated. Before being removed from the place where it had been kept at room temperature for several months, the bottle contained clear serum over a layer of sediment. When disturbed, however, the sediment became largely dispersed and could not be cleared completely

even by prolonged centrifugation.

The precipitate was shown to be largely calcium soap by treatment with acid and light petroleum. The presence of Ca was shown by formation of a precipitate with oxalate, and the fatty acids were isolated. A mean molecular weight of 306.8 and iodine number of 43.5 were found. The precipitate, at least in part, took the form of doubly refractive needles about 1-2 mm. long. The milky serum took on a sheen when caused to flow.

A determination of calcium on a centrifuged sample gave the unexpected value of 2.17 mg./100 ml. Similarly, a sample of serum taken directly from the bottle after it had been inverted gave a value of only 3.70 mg.%. When a sample of the latter serum was treated with 5% trichloracetic acid the value jumped to 12.58 mg.%. It was concluded that direct treatment with oxalate had resulted in the surface of the needles becoming coated with calcium oxalate so that further reaction was prevented.

The total phosphorus content of the serum was 17.8 mg.%, of which 2.3 mg.% were due to lipids.

The serum was next subjected to ultrafiltration, using an apparatus which had been prepared by Mr. M. Saffran. The equipment employed was a modified Seitz filtration chamber of about 100 ml. capacity, with the head connected by copper tubing to a cylinder of nitrogen. The gas pressure

was regulated by a needle valve and pressure gauge. About 90 pounds pressure was employed. The cellophane membrane was reinforced in the first experiment with one hard filter paper which had been washed with three changes of N/100 HCl, and twice with water; a Seitz filter which had been leached with 4 changes of HCl until no precipitate was formed with oxalate after neutralization of the washings, and twice with water; these layers were supported in turn on copper mesh. The concentration of calcium in the ultrafiltrate was found to be 28.28 mg.%. Repetition using 6 layers of acid-washed filter paper gave an ultrafiltrate containing no Ca whatsoever. This result was interesting not only for its own sake, but as evidence that even the exhaustively washed Seitz pad still was able to release large quantities of calcium to the serum ultrafiltrate. In both cases the absence of protein was established by testing with sulfosalicylic acid.

The inorganic phosphate in the second ultrafiltrate was 16.8 mg.%. Nitrogen to the extent of 62.4 mg.% was present, and a qualitative test for choline by the method of Booth (426) using KI_3 solution gave needles of the type which Booth reported were formed when the dilution of choline was of the order of 1:20,000, or 5 mg.%.

Cholesterol determinations gave the values of 146 mg.% total cholesterol and 34 mg.% free, from which the percent esterification was calculated as 76.7%.

V. Analysis of Reconstituted Dried Serum

A bottle of dried serum obtained from the University of Montreal was reconstituted by adding sterile distilled water and shaking. The resulting solution was very turbid. It contained 10.52 mg.% Ca and 4.4 mg.% P.

Centrifugation for three hours at 3000 r.p.m. led to the isolation of a very small amount of material which was washed by suspension in 1% NaCl solution. During the latter operation it was observed that the material showed a marked tendency to remain dispersed and required prolonged centrifugation even after separation from the serum.

The material was found to be insoluble in 5 N HCl and 40% NaOH. The biuret test with alkaline copper sulfate was negative, as was likewise the Molisch test with alpha-naphthol and sulfuric acid for carbohydrate. The material burned rapidly with a yellow flame, leaving a minute ash which gave no color in a flame when viewed through a blue glass, an indication that the ash was composed of NaCl from the washing. Under the polarizing microscope the material was non-crystalline and appeared to be utterly unorganized. It was soluble in fat solvents, but an attempt to crystallize it from carbon disulfide was unsuccessful. When burned on a clean silver coin it left a black mark.

A small amount of material was prepared by Dr.

Denstedt by shaking the dry serum with toluene and removing the solvent. Part of it could be recrystallized from acetone and contained fatty acid. The remainder which was soluble in cold acetone was a golden oil, likewise largely soluble in alkaline 50% alcohol and extractable after acidification with light petroleum. By titration, it was calculated that less than 5 mg. of fatty acid were present, derived from the equivalent of 500 ml. of serum.

VI. Incubation Experiments

a) Ox Serum

One gallon of fresh defibrinated ox blood was obtained from a slaughter-house and the serum drawn off after centrifugation and stored in three 500 ml. transfusion bottles under toluene.

The total phosphorus content of the serum was 11.8 mg.%, of which 6.6 mg.% were inorganic. The inorganic phosphorus rose during 5 days in the ice-box to 7.1 mg.%, and in 8 days to 7.8 mg.%. The serum was then removed from the cold and placed in a cupboard. Since too much toluene had been added with the result that considerable emulsification occurred, the bottle was opened to the air and covered with filter paper to allow part of the toluene to evaporate.

Within four more days a precipitate became

noticeable, accompanied by a reduction in inorganic phosphorus in the supernatant serum to 7.0 mg.%. The pH of the serum was 8.8. The precipitate was amorphous and readily soluble in acid. No fatty acid was isolated by treatment with acid and light petroleum. The material was insoluble in ether and gave no biuret reaction. A small amount of the precipitate was found to contain 0.160 mg. Ca and 0.090 mg. P, for a ratio of 1.6:1.

Lipid extracts of the ox serum were prepared by running the serum into 3 volumes of a 4:1 mixture of methylal (dimethoxymethane) and methanol, according to the procedure of Delsal (41). The mixture was centrifuged, the clear solvent mixture decanted off, and the protein residue re-extracted three times by stirring with fresh solvent. The combined extracts were made up to volume and aliquots taken.

Total lipid phosphorus by direct ashing of the extract after removal of the solvent was found to be 5.4 mg.%, in adequate agreement with the 5.2 mg.% calculated as the difference between total and inorganic P.

Re-extraction of the phosphorus-containing compounds from the paste-like residue obtained on evaporation of the methylal-methanol extract gave quite unexpected results. The residue was extracted five times with boiling light petroleum, and both the light petroleum extracts and the aqueous residue were analyzed separately for total phosphorus. The phosphorus

was recovered quantitatively in the aqueous residue, no trace being found in the light petroleum extract. The same result was obtained in one case where the extract was taken to dryness before extraction with light petroleum. Addition of the phosphorus reagents to the residue gave no color, proving that the unextracted phosphorus was in organic combination.

Another aliquot of the methylal-methanol extract was extracted with light petroleum after the addition of enough sulfuric acid to make the residue 2N with respect to the acid. This time, P equivalent to 3.2 mg.% was found in the light petroleum and 2.2 mg.% in the aqueous residue. Similarly, addition of solid sodium chloride before extraction with light petroleum gave 1.3 mg.% in the light petroleum and 4.2 mg.% in the aqueous residue.

An acidified residue from evaporation of the methylal-methanol solution was extracted as before with light petroleum and the latter extract put through the procedure for precipitation of phospholipid from acetone. The phosphorus content of the phospholipid precipitate was equivalent to 2.8 mg.% P.

Duplicate extractions by the Bloor method followed by re-extraction with light petroleum also yielded no phosphorus without acidification.

A sample of fresh human serum containing 11.7 mg.%

total P and 3.3 mg.% inorganic P was treated with methylal-methanol mixture. Two samples were evaporated to dryness under nitrogen and extracted with light petroleum and diethyl ether respectively; a third was concentrated to a small volume and extracted with light petroleum in the usual way. The lipid phosphorus value determined by all three methods was 7.3 mg.%. Direct determination on the original methylal extract gave 8.8 mg.%.

In summary, it may be concluded that the difficulty encountered in re-extracting the phospholipids from the methylal-methanol extracts of ox serum was due to a peculiarity of the serum rather than of the methylal extraction procedure. It has been shown that true phospholipid was present in these extracts but could not be re-extracted with light petroleum unless the residue was acidified. The effect was of a magnitude hitherto undescribed. It should be stressed that the effect of acid was by no means quantitatively reproducible. Several experiments in which increasing quantities of acid were added to identical residues from evaporation of the methylal-methanol showed only an irregular trend towards increased extraction of the phosphorus by light petroleum.

The addition of acid or salt was suggested by the hypothesis that the phospholipid in the aqueous emulsion obtained on evaporation of the methylal-methanol extract was in a colloidal state which prevented intimate contact with

the light petroleum. The finding by Bloor (143) and by Kelsey and Longenecker (137) of large amounts of free fatty acid in ox plasma might have some bearing. Kelsey (68) has pointed out that if complete extraction of cholesterol from the mixture of cholesterol and fatty acids obtained after saponification of an alcohol-ether extract is to be obtained, the mixture must first be acidified.

b) Human Serum

Experiment No. 1. 90 ml. of human blood were collected in a sterile 100 ml. centrifuge tube and allowed to clot. After standing for three hours, the tube was centrifuged and the perfectly clear serum aspirated off. Half the serum was transferred to a sterile flask containing 160 mg. of sodium citrate. The untreated serum was designated Sample 1, and that containing citrate Sample 2. At the same time a little less than 40 ml. of blood was collected in a tube containing 8 ml. of 3.2% sodium citrate. The plasma was separated and designated Sample 3. A little water had remained after the autoclaving in the flask which was to receive Sample 2, with the result that this serum was slightly more dilute than Sample 1. The plasma was still more dilute not only because of the added citrate solution but because of cell shrinkage (see page 9). The three samples were kept in a constant temperature bath at 37-38°C.

Determinations of inorganic and lipid phosphorus

total and free cholesterol, and, in a few cases, the phosphorus content of the phospholipid precipitate were carried out immediately after the samples were set up and at intervals over the following 10 weeks. The results are presented in Table X.

TABLE X

CHANGES IN THE SERUM LIPIDS DURING INCUBATION AT 37°C.

Experiment No. 1.

Days	Sample No.	Phosphorus: Inorganic	Phosphorus: Total Lipid	Phosphorus: Acetone-Insoluble	Cholesterol: Free	Cholesterol: Total	Cholesterol: % Ester
0	1	4.3	9.3	-	48	195	75.4
	2	4.0	8.9	-	47	180	73.9
	3	2.6	6.6	-	29	139	79.1
3	1	4.6	-	-	-	-	-
	2	4.2	-	-	-	-	-
11	1	5.6	8.0	6.1	37	166	77.7
	2	5.6	8.3	5.6	40	-	-
	3	3.7	5.8	4.5	25	-	-
17	1	7.6	6.0	5.4	33	110	70.0
	2	8.4	5.6	5.3	34	-	-
76	1	11.9	-	-	-	-	-
	2	8.2	5.4	-	19	89	78.7
	3	5.6	4.5	-	18	69	73.9

With the exception of the percentage values, all values are expressed in mg./100 ml.

Fine precipitates began to appear about the 17th day. Unfortunately, the supply of Sample 1 was insufficient to complete the analysis.

After the experiment had been completed, sufficient Sample 2 was left to perform another cholesterol determination a week later. This time, however, the alcohol-ether extract was saponified and acidified as usual but the light petroleum extracts, which were colorless, were poured directly into the colorimeter tube. After removal of the solvent, chloroform was added and the color developed in the usual way. A value of 109 mg.% was obtained. It should be stressed that the determination was carried out on saponified material and that no correction for esterification was necessary.

Experiment No. 2. Blood from a second donor was drawn and allowed to clot as before. After centrifugation, the clear serum was transferred with sterile precautions to 4 tubes. Tubes 1, 2 and 3 were empty. Tube 4 contained 1-2 mg. of potassium oxalate. The volume of serum in each tube was about 3 ml. Tube 1 was taken immediately for analysis, whereas the other tubes were placed in the constant temperature bath at 37-38°C. for 26 days. Tube 3 was heated to 56°C. for 30 minutes before being placed in the bath. The results are presented in Table XI.

TABLE XI

CHANGES IN THE SERUM LIPIDS DURING INCUBATION AT 37°C. FOR 26 DAYS

Experiment No. 2

Values are expressed in mg./100 ml.

	Sample 1	Sample 2	Sample 3	Sample 4
Age	0 days	26 days	26 days	26 days
Inorganic P	3.4	6.7	5.0	6.2
Lipid P	7.2	4.3	5.1	5.3
Cholesterol Free	37	17	31	13
Total	80	61	60	64
% Ester	53.8	73.1	48.3	79.7

Concurrently with the final determinations, an aliquot of the alcohol-ether extract of Sample 2 was evaporated directly in a colorimeter tube and the color developed as usual. A value corresponding to 217 mg.% cholesterol was obtained. In this case a correction for the 20% greater color developed by the esters was necessary, and assuming 75% esterification of the material giving the color, a corrected value of approximately 190 mg.% was calculated.

DISCUSSION

The most striking fact which is brought out by the analyses of the precipitate is the variability of its composition, both from one batch to another and in different samples of the same batch. Nevertheless, with the possible exception of the fourth batch, the differences can be ascribed to variations in the relative proportions of three main constituents, - calcium soap, calcium phosphate and protein.

As shown by the absence of inorganic phosphorus, the proportion of calcium phosphate in the second batch was negligible. From the number of milliequivalents of the fatty acid isolated, and taking the mean molecular weight of 291 as found, a weight of fatty acid equal to 86.1% of the total material may be calculated. As the calcium salt, this would constitute over 92% of the material. The remaining 8% may easily be accounted for as protein, phospholipid and cholesterol. Since no inorganic phosphorus was found in this batch, the phospholipid phosphorus may be taken to lie between 0.13% and 0.36%, which represents, using the factor 25, 3 - 9% phospholipid. Similarly, from the nitrogen content of 0.62% and the customary factor 6.25, the possible protein content would be 3.9%. These two values are partly mutually exclusive, since the same nitrogen atoms cannot be present in both phospholipid and protein.

The chief difference between the second and third

batches lies in the presence in the latter of large quantities of phosphorus which were extracted in inorganic form by acid. The process of precipitation of calcium phosphate as described by Logan and Taylor (427) involves an initial aggregation of $CaHPO_4$ which subsequently loses phosphate and approaches the 3:2 mole ratio of tertiary calcium phosphate, $Ca_3(PO_4)_2$. The growth of this latter salt, which is amorphous, depends on the adsorption on the lattice of Ca salts present in amounts near their solubility product, a process very favorable to the occlusion of carbonate and hydroxide. The tertiary salt is very insoluble, but its direct precipitation is impossible since it would involve a reaction of the fifth order (428).

The explanation of the presence of calcium phosphate in the third but not the second batch of precipitate clearly lies in the solubility of the more soluble $CaHPO_4$. Shear, Washburn and Kramer (429) have shown empirically that solutions containing calcium and phosphate at the pH of serum will deposit $CaHPO_4$ only when the solubility product expressed in mg. per 100 ml. exceeds 50. Normal serum contains approximately 10 mg.% Ca but only 5 mg.% of the Ca is in ionized form (425), so that with an inorganic phosphate concentration of 3.5 mg.% the product is only 17.5. The possibility of precipitation of calcium phosphate becomes real in the sera investigated when the inorganic phosphorus reaches 10 mg.%. From Table VIII it may be seen that Serum 2 and

Serum 4 were on the verge of precipitation. Serum 3 had already reached the stage of precipitation of the phosphate, and it is interesting to note a considerable fall in calcium concentration over a period of four months. Once the presence of $CaHPO_4$ is established and conversion to the tertiary form has begun, it is no longer necessary to have a Ca x P product of 50.

The effect of high pH, by increasing the concentration of the monohydrogen phosphate, is to allow the precipitation of $CaHPO_4$ to occur at lower values of the empirical product. This is illustrated by the experiments on ox serum which became alkaline through loss of carbon dioxide and deposited calcium phosphate but no calcium soap. Even in the newly-formed precipitate the Ca:P ratio was approaching the theoretical 1.87:1 by weight.

The composition of the third batch of precipitate (Table V) may be calculated in the same way as before. In this case however, the minimum content of calcium soap would be between 75% and 80% of the material. If a content of 1.5% inorganic phosphorus be taken, 3% of calcium would be accounted for, and the whole molecule of tertiary calcium phosphate would amount to 8% of the precipitate. Any calcium unaccounted for would probably be present as co-precipitated carbonate and hydroxide. Protein would amount to as much as 10% of this batch, and cholesterol a further 1%. Finally, the insoluble material which appeared to come from

the Seitz filters accounts for 2.5%.

Conclusions as to the nature of the fourth batch of precipitate are less easily drawn. From the nature of the melting, the presence of calcium soap is again evident. However, since the phosphorus is not acid-soluble, it is presumably present as a constituent of some lipid, a suggestion supported by the saponification experiment. Yet the insolubility of the phosphorus in alcohol-ether shows that ordinary phospholipids are not present. The attractive hypothesis that part of the precipitate, like that of Roche and Marquet (411), is composed of calcium phosphatidate appears to have been ruled out by the insolubility of the phosphorus in ether (412, 413). Unlike the other samples, the material before saponification could be fractionated to only a limited extent between aqueous acid and ether. It is unfortunate that the analysis of this batch was undertaken too late to permit more extensive investigation.

The reason for the presence of the protein in the precipitate even after thorough washing out of adherent serum can not be established with certainty. The most likely explanation would be adsorption of layers of protein on the solid particles, with subsequent occlusion and probably some degree of denaturation.

The low iodine numbers show the fatty acids to be almost completely saturated. Even though the mean molecular

weight determinations would be expected to give slightly high values, it appears certain that the bulk of the fatty acid in each case was stearic acid, molecular weight 284. This assumption gains support from the melting points of the precipitate which approached that of 179-180°C. for calcium stearate (430). As has been mentioned previously, Francis, Harrison and Picken (415) reported without analytical values that a similar deposit in Seitz-filtered serum was composed mainly of calcium stearate with a trace of protein. These authors also explained the presence of inorganic phosphorus in the same way as has been followed here.

The presence of a precipitate containing large quantities of fatty acids suggested at an early point in the investigation that some decomposition of lipids had occurred. The hypothesis was immediately supported by the discovery of very high values for inorganic phosphorus in the supernatant serum, as shown in Table VIII. Soon afterwards, the paper by Verstaete and Cloetens (398), describing the same process in dog and horse serum, was found.

The rather high values for calcium which were found in Serum 2 seemed to be at variance with the presence of large quantities of calcium in the precipitate. Accordingly, the analysis of the Seitz filters was undertaken, with the results as shown in Table IX. It will be seen that important quantities of calcium were leached out even at neutral pH. From the very high yield of calcium inadvertently obtained

during the first ultrafiltration of the lipemic serum, it is clear that prolonged contact will yield large quantities of calcium. An accurate estimate of the amount of calcium which would be released during the processing of the serum can hardly be made, but obviously it would be sufficient to explain the findings. Calcium added in this way probably influences the rate of formation and quantity of the precipitate, and indeed it has been observed (431) that pools of serum which had already deposited some precipitate rapidly formed more after filtration through the Seitz pads.

Hunter (432) has reported a similar study of bacteriological filter pads which grew out of the discovery that acid-fast saprophytes of the genus Mycobacterium showed active growth in a presumably inadequate medium which had been sterilized by filtration. Positive tests for calcium, ferric iron and copper were obtained. The presence of calcium in Seitz filters has also made filtration of citrated plasma difficult (433, 434).

The fate of the various products of decomposition of the phospholipids merits attention. It is obvious from the values for the iodine number of the phospholipids of serum (see p. 32) that the decomposition of the greater part of the phospholipids must release large proportions of unsaturated as well as saturated fatty acids. On the other hand, the fatty acids of the precipitate have been shown to be almost exclusively saturated, so that the conclusion is

inescapable that unsaturated fatty acids were free in the serum. A few experiments in which serum extracts were taken up in light petroleum, washed with dilute sulfuric acid followed by water, and titrated with sodium hydroxide yielded acid values corresponding to 100-150 mg.% fatty acid. No attempt was made, however, to isolate sufficient quantities for determination of the iodine number. The results nevertheless show that free fatty acids were still present even in sera such as 2 and 4 where the calcium content was high, and incidentally show that secondary esterification had not taken place to any important extent.

The condition of the free fatty acids which are not precipitated as calcium salts is an important point of speculation. It has already been mentioned in the Theoretical Part that fatty acids when added to serum are not quantitatively bound to the proteins but that a small proportion remains in equilibrium in what might be termed the 'aqueous phase' surrounding the protein molecules or micelles. That the dissociation of the protein-fatty acid complexes is small has long been known from experiments in which the addition of serum to a suspension of red cells prevented hemolysis by fatty acids (435, 436, 437, 438). Some experiments which did not warrant full description in which samples of Serum 2 and Serum 4 were shaken with ether showed that fatty acids were not extractable in this way. The results were unchanged after treatment of the serum with citrate, oxalate or acid.

It is probably a safe assumption that the fatty acids released by decomposition of phospholipids immediately become attached to the serum proteins. Jarisch (439) in 1922 showed that addition of soap to serum (in quantities less than those employed by Macheboeuf and Tayeau (44)) did not cause cloudiness, even when calcium chloride was added subsequently. The process of precipitation of the calcium soaps in the present case may be explained as a gradual removal of the trace of saturated fatty acids from the 'aqueous phase' with the concurrent release of more from the proteins to maintain the equilibrium. The selective precipitation of saturated fatty acids in spite of the likelihood that their concentration in the aqueous phase is lower than that of the unsaturated acids may be explained by the lower solubility of their calcium salts. Even with the higher concentration of the unsaturated acids, the solubility product of their calcium salts apparently is not reached.

After the release of the fatty acids from the phospholipids, the rate-controlling process is almost certainly the diffusion of the fatty acids away from the proteins to a region where they may aggregate with calcium ions and precipitate. The activation energy of the diffusion (440) of the fatty acid anion out of the field of the protein is certainly quite high and might be calculated from measurements on such a system.

Another aspect of the release of free fatty acids in

serum is the effect on the ionized calcium. The effect of the precipitation of calcium stearate is obvious and well illustrated by the complete absence of calcium in the ultrafiltrate from the lipemic serum. It is interesting that in 1922 McBain and Jenkins (441) used ultrafiltration to determine the extent of dissociation of soap micelles. In the case of Serum 4, however, where added calcium from the several filtrations was present, and very little calcium soap was in suspension, the ultrafiltrable calcium was close to the theoretical value for ionized calcium in fresh serum, showing that the binding of calcium by the proteins was undiminished. In view of the decomposition of the phospholipids, it is interesting to compare such undiminished calcium binding with the estimate of Drinker and Zinsser (442) that 30 to 40% of the bound calcium in serum is bound to cephalin which in turn is fastened to the proteins. It is of course conceivable that the fraction of the phospholipids which had not decomposed contained the cephalin. On the other hand, it is likely that free fatty acids, whether saturated or unsaturated, which were still attached to the serum proteins would bind calcium, as pointed out by Jarisch (439).

The fate of other remnants of the phospholipid molecule deserves attention. Glycerophosphorylcholine is apparently broken down completely, in view of the consistent failure to find significantly greater quantities of total acid-soluble phosphorus than of inorganic phosphorus. The

same might be said of phosphoglycerol and phosphorylcholine. The free choline was detected, and presumably would show some pharmacological activity if the serum were used for transfusion.

The incubation experiments with human serum were undertaken with a double purpose: to verify the changes in esterification of cholesterol which had been found by other workers, and to test whether calcium ions were required for the decomposition of the phospholipids.

The results of the cholesterol analyses (Tables X and XI) show most strikingly a diminution in both free and total cholesterol as the serum aged. The two experiments in which the lipids as a whole were put through the color reaction, in one case after saponification and in the other without saponification, show that both the property of precipitation with digitonin and the development of color were involved in the decrease. A similar decrease in precipitation with digitonin was noted by Man and Peters (32) in 1933. The low value obtained even from the first for the total cholesterol in Experiment 2 is surprising, especially in view of the value of 190 mg.% calculated from the sample which was subjected directly to color development. In no case, however, was there reason to suspect manipulative error.

Equally striking is the failure, with the exception of Sample 1, Table XI, of any marked changes in percentage esterification to appear. Whatever such changes may have

occurred, they were overshadowed by the more profound changes in the cholesterol molecule. The lipemic serum, which may have escaped such extensive changes in the cholesterol, showed a normal percentage esterification.

As for the effect of depression of calcium ions, a comparison of Samples 1 and 2 in Table X shows that the addition of citrate to the serum did not markedly retard the decomposition of the phospholipids or the appearance of inorganic phosphate. The slower changes in Sample 3 are almost certainly due to dilution, shown by Verstraete and Cloetens (398) to be a factor of great importance. The differences between total alcohol-ether phosphorus and that insoluble in acetone might be taken as evidence for accumulation of intermediates, but their nature is quite unknown. It is noteworthy that these differences were less on the 17th day than on the 11th day.

In Table XI are shown the results of adding potassium oxalate to remove the calcium quantitatively and of repeating the heat 'inactivation' experiment of LeBreton and Pantaleon (375). It is seen that removal of calcium had scarcely any effect, and that the heat treatment considerably reduced the decomposition of the phospholipids but did not eliminate it.

Although it is unwise to draw conclusions from only two experiments, it seems clear that during the ageing of the serum, 1) cholesterol became fundamentally altered; 2) the decomposition of phospholipids proceeded without dependence on calcium ions; 3) heating the serum to 56°C. did not prevent these changes.

The study of the importance of calcium ions was prompted by the fact that calcium is known to be an activator of other lecithinases (389, 402). Removal of ionized calcium does not appear to inhibit the reaction in this case, however. It would be desirable to test the effects of the inhibitors reported by LeBreton and Pantaleon (375), monobromoacetate, phloridzin, or most simply heated red cell hemolysate, with the view to preventing such changes in stored serum. Unfortunately, the series of papers by the French workers became available too late to influence the planning of the experiments.

In view of the report by Marfori (220) that heating reduced the extraction of lipids from serum by ether in the presence of low concentrations of ethanol, it is interesting to speculate on whether the same process was involved in this case. The evidence for enzyme activity in the slow process with which we are concerned is not striking, and the underlying changes may be initiated by some configurational arrangement which is altered by heating.

Finally, it is important to note that the turbidity

in freshly reconstituted dried serum is not identical with the precipitate deposited during ageing in the wet state. The former material is very finely divided and appears to offer little practical difficulty in transfusion. It is particularly noteworthy that the turbidity in reconstituted frozen-dried serum does not appear to be related to the chylomicron-like structures described by McFarlane (see p. 56), but rather seems to be composed of elemental sulfur.

In conclusion, it is well to point out that the precipitation of calcium soap from serum does not appear to detract seriously from the therapeutic value of the serum. The presence of traces of the material in the serum when administered is undoubtedly harmless, as testified by the lack of reports to the contrary and by the fact that calcium oleate actually enjoyed a brief popularity in the treatment of tuberculosis (443) and of malnutrition, chronic sepsis and emotional disturbances (444).

The chief objection to the formation of the deposit in stored serum is that it entails filtration of the serum before administration, with the accompanying inconvenience and danger of contamination. The substitution of calcium-free filter pads cannot be expected to prevent the changes, and there may even be some advantages in maintaining the calcium distribution in the serum near normal. The development of practical methods of preventing the formation of the deposit will require more study of inhibitors, following the

pattern of the incubation experiments. For the present, it may be concluded that the only way of avoiding such lipolytic changes is to preserve the serum in the dry state.

The meagre information on the formation of insoluble material was collected.

The experimental study involved adaptations of various techniques which were fully described. The results bring out the following points:

1) The insoluble material was composed principally of calcium salts of higher saturated fatty acids, chiefly stearic acid.

2) The calcium soaps were contaminated with small and variable quantities of calcium phosphate and protein. One sample was found to contain phosphorus in an unidentified lipoid form. A theory of the precipitation of the phosphate was described, and another of the precipitation of the calcium soap was developed qualitatively.

3) The fatty acids which deposited in the precipitate were shown to be derived from the decomposition of phospholipid. The breakdown of the phospholipids in serum stored for one to two years in the cold was shown to be qualitatively complete, and nearly so quantitatively.

4) Serum which had been sterilized by filtration was found to contain slightly abnormally high concentrations of calcium and the cause was traced to the release of calcium by the Seitz filter pads. The discovery that the proportion of ultrafiltrable calcium in such serum was normal was discussed.

5) A sample of lipemic serum which had been preserved without filtration was found to contain no ultrafiltrable calcium.

6) Freshly reconstituted frozen-dried serum showed no decomposition of phospholipid. The turbidity in this case was found to be due to very finely divided material which was taken to be composed of elemental sulfur.

7) Samples of freshly drawn sterile human serum were shown to undergo decomposition of the phospholipids at a considerable rate when maintained at 37°C. The rate was not markedly affected by addition of sodium citrate or potassium oxalate. Contrary to some recent reports, heating to 56°C. for 30 minutes did not check the decomposition completely.

8) No consistent changes in the proportion of cholesterol in the esterified state were found. A marked reduction in the values for both total and free cholesterol did occur, however, and was shown to be due to changes which modified the properties both of precipitation with digitonin and of color development in the Liebermann-Burchard reaction.

The practical importance of the changes described and the preferable approach to their prevention were discussed briefly.

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