Recent Advances in the Chemistry of the *Plasma Proteins* and their Complexes.*

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CONTENTS.

INTRODUCTION.

THERE is no department of physiological chemistry of more vital importance to the physiologist, the pathologist and the clinician than that of blood chemistry, consequently research upon the constituents of the blood is continuous and progressive year after year. New substances are continually being detected as normal or pathological constituents of cells or plasma, methods improved, various inter-relationships discovered, and the structure of well recognized constituents is being gradually more completely elucidated.

In any review of blood chemistry which is to be kept within reasonable proportions, some restriction of subject is imperative. In the following pages attention will be directed towards one particular department of blood chemistry in which our outlook has, in the past ten or fifteen years, suffered the most profound changes. It will not be possible to deal with fibrinogen and the mechanism of blood clotting, but an attempt will be made to summarize the modern conception of the individual identities and interrelationships of the remaining serum proteins and to discuss recent advances in the chemistry of the complexes which these proteins are known to form with lipoid and carbohydrate materials.

Any attempt at the inclusion of pathological data would open up so wide a field that it would be impossible in the present review to deal adequately with this phase of the subject. It is felt that by confining attention to normal materials and normal processes a more useful purpose will be served. For similar reasons no attempt will be made here to deal with recent advances in methods applied to blood chemistry.

THE PROTEINS OF THE SERUM.

The earlier work at the beginning of the present century revealed the fact that normal serum contained about 7 per cent. of protein material amongst which could be distinguished an albumin and a globulin fraction. For a comprehensive review of the earlier work, reference may be made to Petschacher (1930). Hammarsten's (1902) article is a good presentation of the subject from the point of view prevailing at the close of the century before the classical work of Hardy (1905) upon the serum globulins. It is really Hardy's work which ushered in the new era culminating in the researches of Sørensen from the Carlsberg Laboratory and his theory of the constitution of proteins as reversibly dissociable compound systems (Sørensen 1930).

At the time that Panum (1851) showed that a precipitate of protein material was invariably formed when normal serum was diluted with water and slightly acidified or a stream of carbon dioxide gas passed through the diluted liquid, this was considered to be the only protein body of the serum. It was orginally called "casein" or "serum casein" by Panum (1851), but later designated "globulin" by Schmidt (1862), who considered it to be a single substance. Kühne (1868), however, was of the opinion that two different substances were produced according to the mode of pre-peration, by acidification with acetic acid or by carbon dioxide, and accordingly proposed the terms "paraglobulin" and "sodium

albuminate ". A return to the earlier views of Panum was, however, made by Weyl (1877), in whose opinion the precipitate consisted of a single substance which he named " serum globulin ".

The quantity of protein which separates from serum by the use of any of the above methods is only a little over 1 per cent. Actually much larger quantities are present. Hammarsten (1878) found that by saturation of the serum with magnesium chloride, no less than 63 per cent. of the total protein was precipitated, although this procedure was calculated to remove only globulins, leaving any albumin in solution. He therefore came to the conclusion that serum contained much greater quantities of " globulin " than had hitherto been supposed. Burckhardt, some years later, challenged this view on the grounds that when the precipitate obtained according to Hammarsten was redissolved in water and dialysed until salt free, only a fraction of it separated out, neither could further quantities be precipitated by acidification or treatment with carbon dioxide gas. It appeared to Burckhardt that Hammarsten's precipitate must consist of globulin admixed with albumin. It is of interest, in view of our later experience regarding the behaviour of globulin fractions on dialysis, to note that Hammarsten (1884) in reply argued that the presence in serum of other substances might greatly affect the solubilities of the protein constitutents and that by treatment of such a globulin fraction, not precipitable by dialysis, with sodium chloride after the manner of fractional precipitation, such interfering substances could be largely removed and a conversion of non-precipitable into precipitable globulin be brought about. He pointed out that only in solubility and temperature of coagulation did these substances appear to differ, but added, however, " weder die Indentität beider bewiesen, noch die Möglichkeit, dass der Niederschlag ein Gemenge von zwei oder mehereren Globulinen sei, in abrede gestellt sein soll ".

With the introduction of the technique, due to Hofmeister of salting out the different proteins by means of ammonium sulphate solutions of varying degrees of saturation, a big advance took place. Kauder (1886) was able to show that by precipitation of serum with ammonium sulphate up to half saturation, substantially the same material was precipitated as was thrown out by full saturation with magnesium chloride, as in Hammarsten's technique. As emphasized by Pick (1902), there is an interval of no precipitation from serum by ammonium sulphate, between the upper precipitation limits of the globulins and the lower limits of the albumins. From this time onwards, the separate individuality has been generally conceded of two main fractions of the serum proteins—albumins and globulins. With reference to the numerous attempts to prepare artificial globulins from albumin, compare the recent work of Hooker and Boyd (1933). Of course, chemical data, based upon analyses of the various fractions, furnish much more reliable evidence of similarity or dissimilarity than qualitative physical characteristics, such as those of solubility. Elementary analysis of protein materials is of limited help, however, on account of the small differences encountered in carbon, hydrogen and nitrogen content of various individuals. The content of sulphur or phosphorus is nevertheless frequently a valuable guide.

The following figures from Kestner (1925) illustrate the fact that the sulphur content of serum albumin is distinctly higher than that of the globulin fraction:

	C	H	N	S
Serum albumin cryst. ...	51·92 – 52·08	6·96 – 7·11	15·89 – 16·03	1·73 – 1·9
Serum albumin amorph. ...	53·04 – 53·5	6·75 – 7·1	15·71 – 16·04	1·77 – 2·31
Serum globulin	52·71	7·01	15·82 – 15·85	0·97 – 1·38

The difference in phosphorus content is even more pronounced. Sørensen (1930) has obtained purified serum albumins containing no phosphorus, whilst the globulin fractions all contained appreciable quantities of phosphorus, the proportion of which to nitrogen increased the less soluble the particular fraction was. Some pseudoglobulin preparations contained only minimal quantities of this element.

The difference in composition between (total) serum albumin and (total) serum globulin is still more clearly brought out by a comparison of the figures for the nitrogen distribution due to Hartley (1914) (see table 1), and those of amino-acid content cited from Kestner (see table 2). It will be noticed that glycocol is absent from serum albumin.

TABLE I.

	Total globulin. %	Total albumin. %
Ammonia N	7·7	5·8
Melanin N	2·0	1·1
Cystine N	2·0	3·5
Arginine N	10·9	10·4
Histidine N	6·3	6·7
Lysine N	9·0	16·3
Total basic N	28·2	36·9
Total filtrate N	62·0	56·5
Filtrate amino N	59·9	54·2
Filtrate non-amino N ...	2·2	2·3

TABLE II.

	Globulin.	Albumin.
Glycocol	3·5	absent
Alanine	2·2	2·7–4·19
Valine	2·0	present
Leucine	15·0–18·7	20·0–30·0
Aspartic acid	2·5	3·1–4·43
Glutamic acid	8·5	7·7
Proline	2·5–2·8	1·0–2·34
Tryptophane	4·0	1·4
Oxyproline		1·04
Phenylalanine	2·7–3·8	3·1–4·24
Tyrosine	2·5–6·6	2·1–5·8
Serine		0·56–0·6
Cystine	0·7–4·1	2·3–7·1
Histidine	0·8–1·7	2·2–3·72
Arginine	3·4–4·5	4·38–4·75
Lysine	4·6–6·8	7·48–11·29
Ammonia	1·75	0·95

A comparison of the ratio of total nitrogen to amino nitrogen titratable by formol was made by Obermeyer and Wilhelm (1912-1913) for serum albumin and globulin. Their figures were 14-10 for the former and 23-18 for the latter, which is in accordance with the fact that serum albumin contains the greater proportion of lysine.

Differences in specific refraction were found by Robertson (1912), who gave the figures for serum globulin 0·00229 and for serum albumin 0·00177.

Characteristic differences in the ultra-violet absorption spectrum have also been described by Dhéré and by Smith (1929), who proposes to determine the ratio of albumin to globulin in a mixture of the two by an analysis of the ultra-violet absorption spectrum.

Further confirmation of the individuality of the albumin and globulin fractions of serum is afforded by the immunological experiments of Dale and Hartley (1916), and of Doerr and Berger (1922). The former demonstrated the anaphylactogenic properties of these proteins and showed that the response of the sensitised animal was specific for the individual fraction with which it had been sensitised. Doerr and Berger further showed that, in the case of globulin sensitisation, the latent period is shorter and the resultant shock more intense than in the case of albumin. Berger has also pointed out that the increase of the one serum fraction in the organism and decrease of the other, are in no way related.

It would appear from an impartial appraisal of the various lines of evidence that there is good reason to believe that the albumin and globulin fractions produced in the ordinary way from serum represent chemically distinct proteins or associations of chemically similar proteins. The strongest argument in favour of this view is the demonstrable difference in composition between the materials. So little is known concerning the factors exciting biological sensitivity and formation of immune bodies, that the immunological evidence, at first sight so dramatically convincing, must be considered with great caution. This is all the more imperative, since Dale and Hartley (1916) found the euglobulin and pseudoglobulin fractions to exhibit a well-marked biological difference from which one would be tempted to draw the conclusion that here at least two distinct individual proteins were under consideration. The recent work of Sørensen (1925) however, makes it abundantly clear that the globulin complex consists not of two proteins but of a group of associated complexes whose individual components may be gradually and progressively dissociated by suitable treatment, a subject which will be more fully discussed under the heading of the serum globulins.

Association with other materials, differences in degree of dispersion, etc., may so affect the properties of an unstable colloid system such as a protein dispersion, that it is difficult to draw rigid conclusions from observed differences in precipitability by electrolytes, etc. The theory of salt precipitation advanced by Hafner and Kürthy (1924) and based upon relative polarisability may here be mentioned. The experiments of Adolf and Pauli (1924) should also receive attention, since these authors showed that, by electrodialysis, the serum proteins could be separated into an albumin

fraction, soluble in water and not precipitated by half saturation with ammonium sulphate and a globulin fraction which was completely insoluble in water.
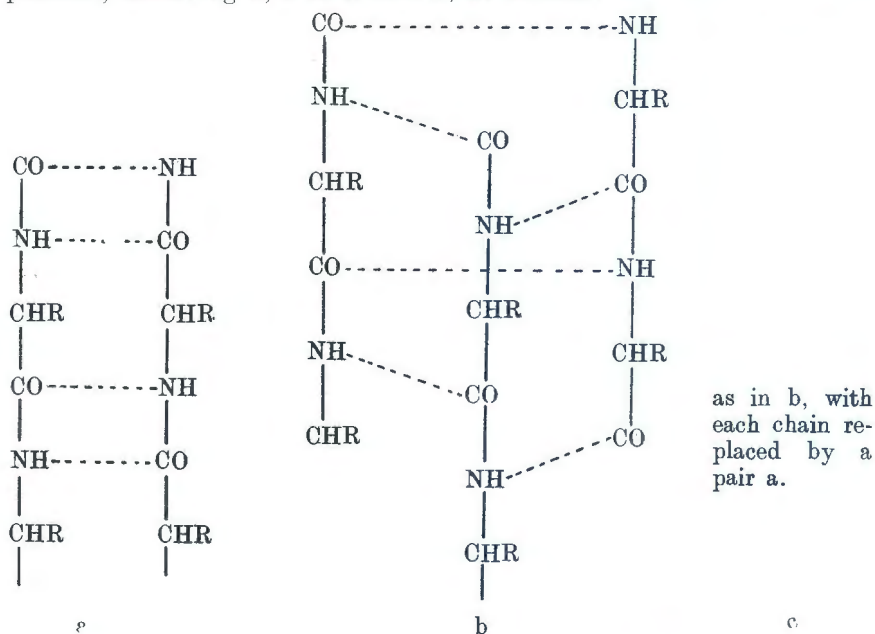
That there is a large difference in the degree of dispersion between the albumin and globulins in native serum seems to be certain from ultra-filtration and other experiments, e.g. Mutzenbecher (1931), also from Svedberg and Sjögren's (1928) researches upon the isolated proteins, but this difference in physical state with its consequent reflections upon chemical behaviour is just one of the reasons why great diffidence should be shown in coming to the conclusion that albumins and globulins are different in the sense, for example, that glucose and galactose are different. Is it not possible that in native serum we have a number of polypeptide associations (hauptvalenzketten) capable of grouping themselves into larger units by virtue of residual or secondary valencies, as is suggested by Sørensen in his theory of soluble proteins as reversibly dissociable compound systems, and that what really happens when the equilibrium is disturbed by the introduction of a quantity of an electrolyte, such as ammonium sulphate, is that those components with similar electro-chemical properties are forced into association and precipitate as a fraction to which we give the name albumin or globulin, as the case may be?

If such a protein precipitate represented a single, chemically pure substance, its physical properties should remain constant, such as, for example, its solubility in salt solutions of given concentration and hydrogen ion activity. That this is not so in the case of crystallized serum albumin, has been shown by Sørensen (1930), so we are forced to the conclusion that even crystallinity, in the case of these complex systems, is no criterion of purity. Sørensen's experimental data will be considered later on.

It may be as well to digress somewhat at this point in order to consider Svedberg's results, more particularly as they affect the serum proteins and decide what weight should be given to such evidence in coming to a decision regarding protein individuality.

Svedberg and his associates (1930) have applied the ultra-centrifuge to measure the molecular complexity of proteins in solution. In general, it was found that all proteins exhibited a molecular weight which was a multiple of a common factor 34,500. A small group possessed much larger molecular weights, but the others fell regularly into classes of 1, 2, 3 or 6 times 34,500. In many cases the values so obtained checked well with those derived from such direct measurements as those of osmotic pressure, as in the case of egg albumin, investigated by Sørensen. Svedberg also showed that each protein possessed a comparatively small range of pH on each side of its iso-electric point within which its molecular weight, as measured by the ultra-centrifuge, remained unchanged. In the case of the 2, 3 and 6 multiple groups, however, cautious regulation of pH resulted in a dissociation of the protein into components of the lower orders. Outside this range again irreversible dissociations or decompositions took place. Svedberg's brilliant results at once call for two explanations, why the basic figure of 34,500 for all proteins and why only the 1, 2, 3 and 6 multiples, never 4 and 5?

A solution to these two problems has been suggested by Astbury and Woods (1931). In the first place it is assumed that the figure 34,500 is a statistical average, for which there is some evidence, and that actually polypeptide chains could be produced of indefinite length and weight were it not for the instability caused by atomic vibrations. It is this disruptive vibration which is assumed to limit the length of the chain to a unit having the approximate molecular weight 34,500. One would like to see quantitative evidence advanced in support of this hypothesis, against which, however, nothing definite will be said at the moment. The second question, once the answer to the first is conceded, can, according to Astbury and Woods, be solved by application of the elementary principles of crystalography. A polypeptide chain can form an association with another by virtue of the residual attractions resident in its $-NH_2$ and $>CO$ groups. Three combinations, and three only, are then possible, involving 2, 3 or 6 chains, as follows:

```
                              CO- - - - - - - - - - - - - - -NH
                              NH·.                          CHR
                                 ·.
                              CHR   ·. CO
                                       |
                              CO· · · · CO
                                     NH·
                              CO- - - -|- - - - - - - - -NH
       CO- - - - - - -NH       CHR                        CHR
                               NH·.
       NH· · · · · · ·CO           ·. CO
                               CHR   ·. CO
       CHR          CHR             NH·
                               CHR
       CO- - - - - - -NH            NH
                                    |
       NH· · · · · · ·CO            CHR                as in b, with
                                                      each chain re-
       CHR          CHR                               placed by a
                                                      pair a.
```
                ϱ   b   c

The four classes of proteins having complexities represented by 1, 2, 3 and 6 times 34,500 can thus be explained.

It is very important to note that in some cases Svedberg found that a certain minimal concentration of protein was necessary before homogeneity of particle size was obtained. This is significant in view of the fact that Sørensen has demonstrated that in systems such as gliadin, dissociation of the components occurs only when the protein concentration is very low. As previously pointed out (Rimington, 1931), the dominance of the figure 34,500 throughout all Svedberg's results must point to some uniformity of pattern common to all natural proteins, but it is not certain that this represents the lowest unit out of which the more complex structures are built. A protein which under Svedberg's ultra-centrifugal

conditions would appear to be homogeneuous can, by Sørensen's comparatively simple technique of applying the elementary physico-chemical principles of solubility, be shown to be divisible into dissimilar portions.

THE ALBUMIN FRACTION.

Doubts as to the homogeneity of the serum alubumin fraction have not been wanting. Thus Halliburton (1884) on the grounds of differences in coagulation temperature distinguished at least three albumins in serum, α-, β- and γ-albumin, which he considered to be separate proteins. The first, α-serum albumin, coagulated at 70-73°, β-serum albumin at 76-78°, and the γ-albumin at 82-85°. It is, however, very questionable whether these temperature differences are sufficient to warrant the existence of three distinct proteins. Even Halliburton's (1886) own results would seem to indicate that other factors may influence the behaviour of the proteins in a given serum. Thus in many cold-blooded animals the coagulation temperature indicated that only γ-serum albumin was present, whilst in eel blood α- and β- and in avian and mammalian bloods all three α-, β- and γ-albumins appeared to be present. These anomalies are somewhat difficult to understand. Even more forceful arguments have been put forward based upon the finding that in no case can the entire albumin of serum be obtained in the crystalline condition, a non-crystallizable portion invariably remains in greater or lesser proportion. In fact, it is a matter of the greatest difficulty to get any albumin crystals at all from the serum of such animals as guinea pigs, cats and bovines (Gruzewska, 1899). As previously pointed out, however, crystallisability is no guarantee of the purity of a particular protein. Wichmann (1898) drew attention to the fact that when a mixture of serum albumin and egg albumin or of these two with lactalbumin is treated with ammonium sulphate in the usual way, the crystals which form convey the impression of perfect homogeneity

Sørensen's (1930) more recent researches have established beyond doubt that apparently homogeneous crystalline serum albumin is in reality an association of mutually interacting component systems having different solubilities, etc. His evidence will now be considered in detail.

Experiments were conducted with crystalline serum albumin made by the method of Hopkins and Pinkus (1898) both from native serum and containing about 0·3 to 1·5 mg. P per gm. N and also with practically phosphorus-free preparations obtained from serum protein powder according to the Hardy-Young (1910, 1922) alcohol technique, or the Hewitt (1927) alcohol-ether method. No appreciable difference in behaviour was found between these preparations, thus indicating that the attachment of lipoids cannot be alone responsible for the irregularities in solubility observed. The crude Hopkins-Pinkus preparation, when subjected to the first recrystallization, deposited a lipoid-containing, greenish, amorphous substance.

In every series of experiments all factors except those intentionally varied were kept strictly under control.

A well-marked dependence of solubility upon the total quantity of protein present in the system was demonstrated, employing three fractions T_1, T_3 and M_5 of a crystalline serum albumin. These designations were arrived at in the following way: To the protein solution sufficient ammonium sulphate solution was added to throw down a slightly soluble fraction T_1. As the salt concentration was increased the main bulk of the albumin separated in a middle fraction M, the filtrate containing easily soluble L_1. T_1 and L_1 were recrystallized and the mother liquor from the first and precipitate from the second added jointly to M_1, which was now subjected afresh to the entire scheme of fractionation yielding T_2, M_2 and L_2. This process was repeated five times.

Fig. 1 represents the results graphically, S being the protein hydrate in gm. per 100 gm. of water found dissolved at ammonium sulphate concentration S where Z was the quantity of protein hydrate present in the experimental mixture. [For experimental details, etc., see Sørensen C.R. Lab. Carlsberg 16, No. 12, p. 17 (1927).]
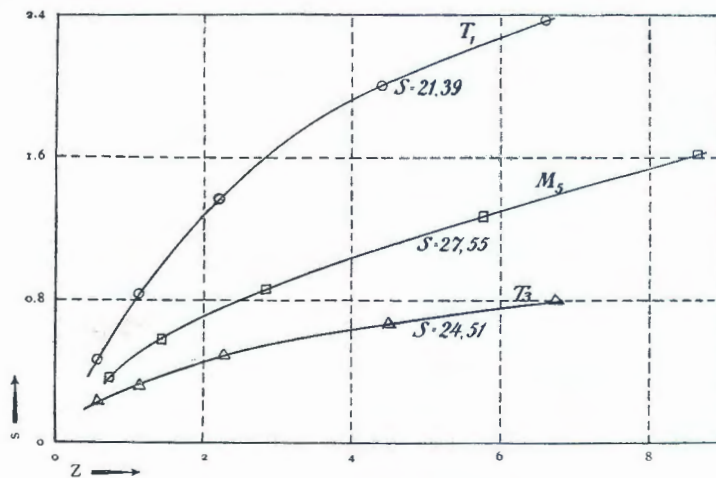

Fig 1. (from Sørensen, 1930).

Again it was shown by selecting particular fractions and determining their solubility under strictly controlled conditions that under identical circumstances their individual solubilities varied widely, also the solubilities at varying pH as is shown by Fig. 2. The fractions designated A_1, A_{11} and A_{111} were the sparingly, moderately and easily soluble fractions of a phosphorus-free albumin preparation and were obtained by crystallization to ammonium sulphate concentrations of (40), (47) and (60) respectively in Sørensen's notation. T_1C was gained by recrystallizing T_1 three times; T_3b and M_5c are to be similarly interpreted.

Such striking differences in solubility behaviour would suggest that there might be found underlying differences of a chemical nature in the various fractions, indicating the separation of a heterogenous mixture of proteins or, possibly, actual decomposition, carried to a greater or lesser degree, of the original albumin material.

The results of Sørensen's analyses did show that quite marked differences in composition existed between the various fractions, but only certain easily determined amino-acids were estimated and the experimental evidence was insufficient to reveal any connection between solubility and composition.

The figures quoted below illustrate this fact:—

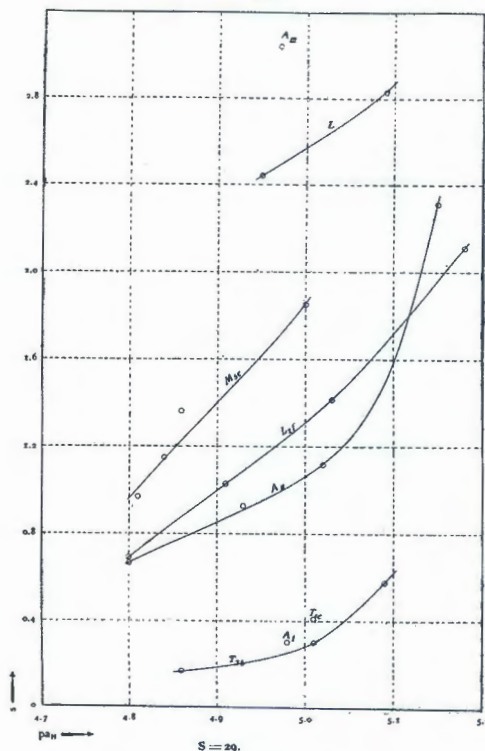|	T_3b	L_2f	L
Humin N	0·02	0·39	0·04
Ammonia N	5·80	7·16	5·97
Cystine N	4·38	3·65	4·57
Tryptophane N	0·66	2·10	0·91

Fig. 6.

Fig. 3 (from Sørensen, 1930).

As was earlier pointed out, T_3b represents a twice recrystallized sparingly-soluble fraction, whilst L and L_2f represent fractions at the other extreme, characterized by great solubility. It is evident that T_3b and L do not differ markedly in composition. L_2f on the other hand shows that by successive recrystallizations of the more soluble L fractions, a product pronouncedly richer in tryptophane N and in humin N accumulates.

Determinations of the acid- and base-binding capacities showed, on the other hand, that under equal conditions all fractions were alike, as were their optical rotations. Previous work (Sørensen,

1924) had shown that crystalline fractions of widely differing solubilities all exhibited the same osmotic pressure. These facts would seem to disprove any suggestion that processes of decomposition or denaturation had taken place.

As Sørensen himself remarks, the chemical analysis of the various fractions was not detailed enough to reveal the connection which must undoubtedly exist between solubility and chemical composition. In view of the significant figure for humin N in the fraction L_2f quoted in the table and the demonstration by Rimington (1929, 1931) that both serum albumin and serum globulin contain quite considerable quantities (about 1 per cent. in the former and 3·8 per cent. in the latter) of a complex polysaccharide as an integral part of their molecules, also the work of Lustig and his associates (1931) upon the carbohydrate content of various serum globulin fractions, it would be of the greatest interest to examine whether solubility of the various serum albumin fractions can be related directly to their carbohydrate contents. The experiments of M. Sørensen and Haugaard (1933) would seem to suggest that this may be so (see section on bound carbohydrate of blood).

THE GLOBULIN FRACTION.

As has already been pointed out, the addition of acid or CO_2 gas to diluted serum results in the precipitation of only about one-sixth of the total quantity of protein; by salting out with magnesium chloride or half saturation with ammonium sulphate, a further quantity precipitates, representing about another 62 per cent. of the total. Hammarsten regarded both fractions as globulin, a view which was strongly contested by Burckhardt, who showed that reprecipitation was incomplete after the fraction was subjected to dialysis. Marcus (1899) confirmed these findings, but pointed out that in other respects the materials—the water soluble and water insoluble—were closely similar. Hammarsten (1884), whilst not denying the possible association of two or more globulins in the precipitate, demonstrated that their solubilities were largely affected by the presence of impurities which he succeeded in removing by sodium chloride precipitation.

The duality of the globulins was clearly shown by Fuld and Spiro (1901-2), who found that the rennetic ferment and anti-rennetic substance of serum could be separated by fractional precipitation of the globulin fraction with ammonium sulphate. The former appeared in the 28-33 per cent. fraction, the latter in that precipitated between 43 and 46 per cent. saturation. Full and Spiro first applied the terms euglobulin and pseudoglobulin, which are still in use to-day.

Pick (1902) considers that there are no sharp limits of precipitation for these two substances, but that they precipitate reciprocally. That some such phenomenon occurs appears also to be borne out from experience with antitoxins and immune bodies in different animal species (Pick, 1902), Belfanti and Carboné, Freund and Joachim (1902), Marcus (1899), Dieudonné (1897), etc., also by the fact that by often-repeated dialysis and resolution, it is impossible to obtain a pseudoglobulin which is completely soluble in water.

Adolf and Pauli's (1924) electrodialysis method does seem capable, however, of effecting a division into completely soluble and completely insoluble globulin.

For an explanation of this peculiar behaviour it is natural to turn at first to the possibility of associated substances, such as lipoids, affecting the solubilities of the priteins. An association of the globulins with lipoids was early recognized and many studies have been made with the object of elucidating the nature of the combination. Thus Chick (1914) thought it not improbable "that euglobulin in serum is a complex material formed from pseudoglobulin by association with some serum lipoid to the presence of which it owes its phosphorus content"; the process could even be pictured as a gradual and progressive one.

With reference to the nature of protein-lipoid associations, more will be said in a later section, but from Sørensen's (1930) results it would appear that a part at least of the phosphorus of eu- and possibly also of pseudoglobulin is an integral part of the protein and independent of lipoid material.

As in the case of serum albumin, Sørensen's (1925, 1926, 1930) researches from the Carlsberg Laboratory have done much to clear our views concerning the individuality of the two serum globulins. In brief, his conclusion is that one is here dealing with an association of mutually interacting complexes the conditions of whose solubility, association or dissociation are governed by factors such as the concentration of the solution, the presence or absence of electrolytes, etc. In the first series of experiments upon serum globulin, Sørensen (1925) used preparations made by fractionation of the diluted serum with ammonium sulphate, subsequent dialysis, etc. The proteins so obtained contained fairly large amounts of phosphorus, whereas the later preparations which he employed and which were made from "serum powder", the total protein obtained from serum by the use of alcohol or ether-alcohol at low temperature, contained considerably less phosphorus as the following figures show. There was no significant difference, however, in behaviour between the proteins of the former or latter series:

	mg. coagulable P/gm. protein N	
	From crude serum.	From serum powder.
Pseudoglobulin	0·5–3·0	0·1–0·4
Euglobulin	2 – 40	0·1–0·4

An euglobulin preparation, four times reprecipitated by ammonium sulphate, collecting only the material thrown out at concentrations of the salt up to (30), was subjected to dialysis and the precipitate and residual solution worked up separately. The precipitate, after washing, was dissolved in 0·6 N KC1 and the solution then diluted to 0·03 N whereby a part of the protein was precipitated. This was again dissolved in 0·6 N KC1 and the mixture diluted to 0·1 N. A further separation into soluble and insoluble fractions occurred. The quantities of protein remaining in the first and second KC1 mother liquors were dialysed until salt-free but only a portion was precipitated. These processes were shown to be capable of indefinite repetition. There is thus a continuous and progressive dissociation or "scaling off" of pseudoglobulin

particles from the complex originally present. Similarly it was shown that, by suitable treatment, the more easily soluble pseudoglobulin fraction could be made to yield certain quantities of euglobulin. Sørensen denotes the relationship as follows:—

$$E_p \; P_q \rightleftharpoons a \; Px + \; \ldots\ldots\ldots + b \; E_n \; P_m + \; \ldots\ldots\ldots + E_r \; P_s$$

where E denotes an euglobulin and P a pseudoglobulin complex or molecule and

$$p = bn + \; \ldots\ldots\ldots\ldots \; r$$
$$q = ax + \; \ldots\ldots\ldots\ldots \; bm + \; \ldots\ldots\ldots + s$$

Turning now to the solubility relationships at different salt concentations, it was again found, as in the case of serum albumin, that the solubility depended upon the total quantity of protein added to the system, the exact behaviour being also related to the degree of previous fractionation to which the samples had been subjected. Thus, only by very prolonged fractionation and washing could an euglobulin be obtained which exhibited a fairly constant solubility in $0 \cdot 02N$ NaCl. When stronger salt solutions were used as solvent for this material, dissociation of pseudoglobulin complexes again took place and the solubility of the material was, consequently, once more dependent upon the total amount of protein in the system. The stronger salt solutions have a more potent effect in bringing about dissociation.
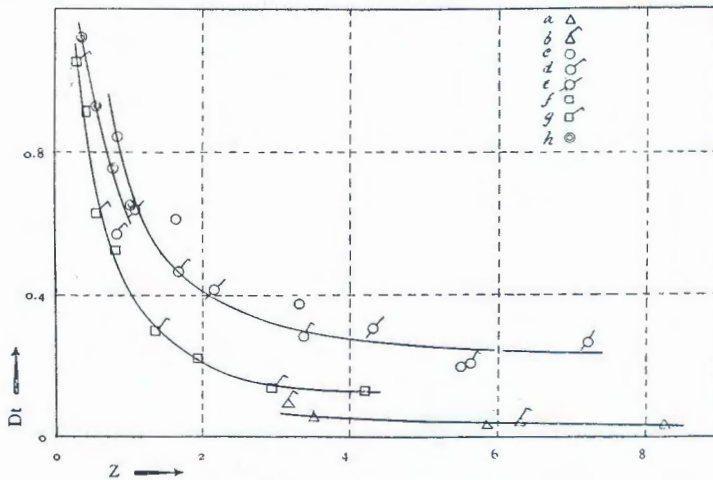

Fig. 3 (from Sørensen, 1930).

a and b : egg albumin; c d, and e : serum albumin; f and g : pseudoglobulin; h : euglobulin.

Reciprocal action between precipitate and dissolved complexes was clearly shown by Sørensen to occur in these systems.

It must be concluded that by these means it is impossible to obtain either pure euglobulin or pure pseudoglobulin, all preparations being complexes whose solubility rises in proportion to the quantity of pseudoglobulin they contain. It is of interest as a close to this section to reproduce a figure from Sørensen's work illustrating the dissociation tendency (Dt) of the various proteins he has studied as a function of their concentration (Z).

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BEHAVIOUR OF THE SERUM LIPOIDS.

As has been mentioned earlier, the precipitate produced by adding an equal volume of saturated ammonium sulphate to serum, contains quite considerable quantities of lipoid material as does also the first crude deposit of crystalline albumin. Upon recrystallization of the latter, most of the accompanying lipoids are left behind in the form of a greenish insoluble residue (cf. Troensegaard and Koudahl, 1926). It is probable that one is dealing with a mechanical admixture or loose association between protein and lipoid rather than true chemical combination, since there seems to be no regularity in the relative proportions of the two constituents. However, the fact that serum is normally clear and translucent requires an explanation. By shaking serum with ether only minimal quantities of lipoid material are removed which would suggest that some mutual interaction must exist between these substances. Addition of ammonium sulphate or other electrolytes suffices to break down this equilibrium so that the two materials are easily separated. Treatment with alcohol or ether-alcohol in the cold gives rise to protein preparations which are practically lipoid-free, so that here again conditions must have been disturbed.

It is noteworthy that in Sørensen's (1930) experience, all attempts to restore the combination of protein and lipoid have failed to reproduce the original clear serum. Fairly stable emulsions could be formed but these were always opalescent.

A few years ago, Mâcheboeuf (1927, 1929) subjected the problem of the serum lipoids to a fresh investigation and claimed to have isolated a true protein-lipoid combination in a yield of about 2·5 gm. per litre from horse blood. The globulins were first precipitated by ammonium sulphate and the filtrate acidified to pH 3·8 which caused a voluminous precipitate to settle. This was dissolved in water to which sufficient ammonia was added to bring the pH to 7. Solution and reprecipitation at pH 3·8 was repeated 10 times, by which time the composition of the substance was said to become constant.

It was an association of 50 per cent. protein, 23 per cent. lecithin and about 18 per cent of cholesterol esters, and dissolved in water at neutral or alkaline reaction to produce perfectly clear solutions from which the lipoids could not be removed by shaking with ether.

On extracting crude serum with cold alcohol or ether-alcohol as in the preparation of " serum powder ", one would expect to find Mâcheboeuf's substance in the mother liquors, but as Sørensen (1930) shows, its detection in these is not realisable in practice. One must conclude that on evaporation of the solvent cleavage of the compound takes place.

More recently Theorell (1930) has subjected the whole question of the serum lipoids and their relationship to the proteins to one of the most searching investigations of the subject so far performed. In addition, his paper may be referred to for a critical review of the previous literature on the subject. As he justly states, far too little attention has been paid to the purity of the materials used in

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attempts to reconstitute stable lipoid-protein emulsions resembling serum. Thus, ordinary cholesterol is fairly readily emulsified in water by suitable technique, but this property is lost on repeated recrystallization. Theorell describes in detail the special procedure by which he was able to obtain $0 \cdot 7$ to $0 \cdot 85$ per cent. emulsions of pure cholesterol.

When such an emulsion is added to fresh serum a portion of the cholesterol is taken up in clear solution and the sedimentation velocity of erythrocytes is found to be markedly retarded thereby. At the same time the resistance to hypotonic salt solutions (fragility test) is increased. It appears that a part of the dissolved cholesterol is taken up—possibly adsorbed by the cells.

Examination of such a choloesterized serum shows that the precipitation limits of the proteins towards ammonium sulphate have not altered, but that the main quantity of the added cholesterol falls with the globulin fraction. Fibrinogen, in the case of cholesterised plasma, carries down little. In a similar way it was shown that when lecithin sols are added to serum or plasma and the excess centrifuged off, a certain quantity has gone into clear solution in the liquid, whilst the excess has removed with it a fraction of the cholesterol originally present. Lecithin, as does cholesterol, causes a diminution of sedimentation velocity but acts antagonistically to cholesterol in that it decreases the resistance to hypotonic sodium chloride solutions. A part of the added lecithin appears to be adsorbed by the corpuscles, although no alteration in electric charge of the latter can be detected.

On salting out with ammonium sulphate, the precipitation limits of the proteins are found to be unaffected. A part of the excess lecithin is carried down by the fibrinogen, the remainder by the globulin fraction.

For the sake of comparison with the above findings Theorell (1930) prepared an euglobulin fraction from horse serum by electrodialysis in a Pauli apparatus and also the total globulin and albumin fractions from the same serum by ammonium sulphate precipitation. All these protein preparations were analysed for cholesterol and lecithin (i.e. Phosphorus x 25) with the following results, from which it will be seen that the quotient cholesterol/lecithin falls progressively from euglobulin to albumin :—

Protein fraction.	Cholesterin lecithin.	Cholesterin protein.	Lecithin protein.
Total protein	$0 \cdot 51$	$0 \cdot 013$	$0 \cdot 0255$
Euglobulin	$0 \cdot 79$	$0 \cdot 023$	$0 \cdot 0292$
Pseudoglobulin plus albumin (=dialysate filtrate) ...	$0 \cdot 45$	$0 \cdot 011$	$0 \cdot 0246$
Total globulin	$0 \cdot 75$	$0 \cdot 011$	$0 \cdot 0153$
Albumin	$0 \cdot 41$	$0 \cdot 015$	$0 \cdot 0366$

The effect of the pH upon the distribution of the plasma lipoids among the various protein fractions was also studied. The results may be presented summarily in the following table, although in attempting to interpret these figures it is important to note that

altering the pH towards the acid side causes a progressive co-precipitation of the less highly dispersed fractions at the usual salt concentrations, and in all probability also an alteration in the equilibrium between free and protein-bound lipoid:—

			$\dfrac{\text{Cholesterin}}{\text{Lecithin}}$ for		
Protein fraction		pH	" Fibrinogen."	" Globulin."	" Albumin."
I	... ...	7·46–7·41	∞	1·08	0·50
II	... ...	6·54–6·42	∞	1·05	0·50
III	... ...	5·99–6·12	∞	1·20	0·52
IV	... ...	5·57–5·59	1·44	0·71	0·59
V	... ...	5·06–5·02	1·00	0·66	0·73
VI	... ...	4·70–4·77	0·54	0·66	0·80

A general comparison of the data for sedimentation velocity with total cholesterol content fails to reveal any correlation, although it would appear to be certain that this phenomenon is largely influenced by the lipoids of the plasma. The existence of both free and protein-bound cholesterol in serum was made very probable by the work of Handovsky, Lohmann and Bosse (1925), who also found a correlation, slight in degree, between the euglobulin content and the ether-extractable cholesterol. Theorell, in the work under discussion, by
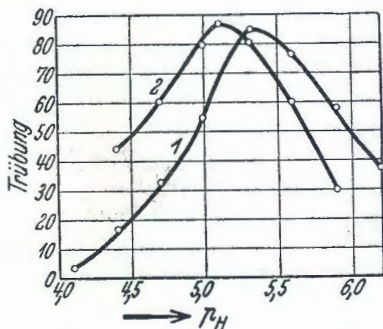

Fig. 4   Fig. 5

(from Schmitz and Fischer, 1933, A).
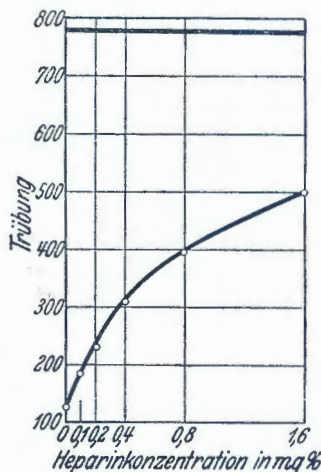
means of ether extraction experiments at different pH values of the serum and of varying duration, establishes quite clearly the fact that a part of the total cholesterol is easily extracted at any pH and is probably, therefore, free cholesterol in simple, colloidal solution, whilst the remainder is only extracted with difficulty and shows a well-marked pH effect, being most easily extractable by ether at pH 5·5–6·0, a reaction suggestively close to the iso-electric points of fibrinogen and the globulins. A reversible association between cholesterol and protein was successfully demonstrated with isolated protein fractions. Only at still more acid reactions than pH 5·5 do albumin and cholesterol form associations.

As a result of a large number of extraction and sedimentation velocity experiments upon a large number of sera, the hypothesis was substantiated that only the cholesterol not in combination with fibrinogen or globulin exerts an inhibitory effect upon sedimentation. A correlation coefficient of $-0\cdot63 \pm 0\cdot10$ was found between the free cholesterol and sedimentation velocity.

The effect upon sedimentation of heating plasma is to be associated with alterations in the colloidal conditions and is independent of the lipoids.

Schmitz and Fischer (1933, A, B) distinguish between the effect of H ions upon the serum proteins (acid reaction) and that of salts, particularly ammonium sulphate (salt reaction), and show how these two factors operating together may be made to indicate the lability of the colloidal protein system.

Lipoids such as heparin were shown by Fischer (1932) to increase the acid reaction, the flocculation optimum being displaced towards the acid side, they act, therefore, in the sense of stabilisers of the serum proteins. This effect with a mixture in their original proportions of albumin and globulin isolated from serum, is well brought out in Fig. 4.

pH 5 was chosen as a suitable value at which to make comparative determinations of turbidity and the effect of the progressive addition of heparin to such a protein system upon the turbidity at pH 5 is illustrated in fig. 5. It will be noticed that the salt reaction remains uninfluenced.

When now the results are so expressed as in Fig. 6 plotting the difference between the salt reaction and acid reaction D as ordinate against the logarithm of the concentration of stabilisor (heparin), log S, a linear relationship is found to hold, i.e. the stability decreases regularly as the logarithm of the stabilisor increases.
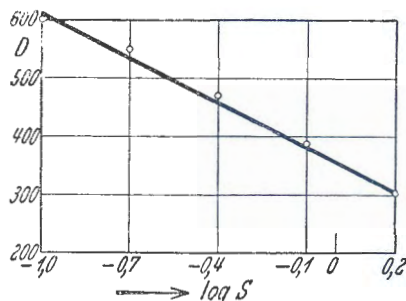

Fig. 6 (from Schmitz and Fischer, 1933, A).

For the sake of obtaining a characteristic for any serum the " degree of lability " may be calculated by means of the expression $L = 100 - \dfrac{100 \times J_1}{J_2}$ where J_1 and J_2 are the values for the acid and salt reaction respectively. Log L then gives the " degree of lability " of the serum.

No reference has as yet been made to the work of Brinkman and Van Dam (1920) and Brinkman and Wastl (1921), to whom many of the early results in this field were due. In particular, attention will here be called to their demonstration of the effect of diet upon red cell fragility, haemolysis and regeneration. The feeding of lecithin caused a well-marked intravital haemolysis in the rabbit with increased red cell fragility. Lecithin in the diet was also found to be essential for normal blood cell regeneration.

Adopting a procedure of progressive precipitation by ammonium sulphate, Lustig and Katz (1930) were able to show that the protein fractions brought down from normal serum at concentrations of 33 per cent. ("euglobulin"), 50 per cent. ("pseudoglobulin"), 50-60 per cent. ("albumin I"), 60-66 per cent. ("albumin II"), 66-75 per cent. ("albumin III") and then separated according to solubilities in water, sodium chloride, sodium carbonate and sodium hydroxide solutions (Freund and Joachim, 1902), differed from each other in such respects as total nitrogen content, number of amino and of carboxyl groups, etc. In a further communication the same authors (1931) have investigated the lipoid contents of the various ox serum protein sub-fractions. Lustig and Botstiber (1930) had already shown that the lipoids of human euglobulin, pseudoglobulin and albumin fractions were characteristic for each protein, and Lustig and Katz extend this specificity to the sub-fractions also. The highest lecithin content was found in the sodium chloride-soluble sub-fractions of the eu- and pseudoglobulins, the lowest in the water soluble sub-fractions. Their table of results is reproduced below:—

Distribution of lipoids in the sub-fractions of serum proteins.

Proteins	Lipoids in 100 g. protein %	Lecithin		Cholesterin	
		in 100 gm. protein	in % of total lipoid.	in 100 gm. protein.	in % of total lipoid.
Euglobulin water sol. ...	0·56	0·11	19·7	0·12	21·4
Eug. NaCl sol.	2·33	0·41	17·6	0·48	20·6
Eug. Na$_2$CO$_3$ sol.	1·66	0·46	28·3	0·41	24·7
Euglobulin NaOH sol.	9·70	1·98	20·4	2·95	30·4
Pseudoglobulin water sol. ...	3·32	1·08	33·4	0·92	27·7
Pseudoglobulin NaCl sol. ...	3·29	1·35	41·0	0·92	28·0
Albumin I ...	3·15	1·18	37·8	1·02	32·3
Albumin II ...	6·26	2·82	45·0	2·10	33·5
Albumin III...	4·86	1·53	31·6	2·13	44·0
Total proteins of ox serum ...	4·75	1·44	30·3	1·61	33·9

The immunological behaviour of the various serum sub-fractions has been the subject of additional communications by Freund and Lustig (1932) and Lustig and Katz (1932). The relative proportions and chemical characteristics, NH_2- and COOH groups, total N and carbohydrate contents of the sub-fractions in such pathological conditions as cirrhosis, sarcoma and carcinoma, have been investigated by Lustig (1931) with interesting results.

"BOUND SUGAR" OF THE BLOOD AND PROTEIN-CARBOHYDRATE COMPLEXES.

In a recent review of 250 pages supplemented by over 500 references, Grevenstuk (1929) subjected to a detailed, critical examination the experimental results and claims of those who have investigated the so-called " bound sugar " or " protein sugar " of the blood. No attempt will be made, therefore, to traverse this field again from its historical aspect, since Grevenstuk's paper is readily available; the issue will be narrowed down, rather, to a discussion of the occurrence of carbohydrate groups as an integral part of the serum proteins.

The fact that the reducing power of whole blood is increased by acid hydrolysis is an incontestable fact, but to assume, as have most workers in this field, that the increase in reducing power is due to the liberation of *glucose*, previously held in combination with the *protein*, is quite unwarrantable. In order to avoid the prejudice of terms, Grevenstuk in the monograph referred to alludes to the precursor of the extra reduction as the " hydrolysable substance ", " Hy–S ", thus avoiding any implication as to its nature. His discussion suffers, however, in another respect, that of engendering in the mind of the reader, unintentionally, the idea that the same Hy–S may be found in the plasma, corpuscles and possibly also in the serum proteins. In any approach to the subject, the very reverse should be uppermost in the mind. The methods employed by different investigators should be critically compared before endeavouring to appraise the value of their respective results, which so often stand in mutual contradiction. Grevenstuk has performed a signal service in reproducing, in all essential detail, the procedures adopted by the workers of the French and Italian schools, especially are the methods of deproteinization employed of the greatest significance.

Let us now clarify the problem. As stated above, the fact is undisputed that, when whole blood is subjected to acid hydrolysis, the reducing power of the deproteinized filtrate is found to be greater than that prior to hydrolysis. The general concensus of opinion is also to the effect that plasma behaves similarly. With regard to the proteins, we have conflicting results.

Now, plasma or serum contains substances, some of which are known to liberate reducing bodies on boiling with acids or to increase in reducing power such as, for example, hexose phosphates, glycuronates, creatin, etc. It is of interest that with regard to the latter, Benedict found that by heating a solution with $n - HCl$ at $117°$ for 30 minutes, the change from creatin to creatinine, with higher reducing power, was practically complete. The conditions are almost identical with those used by the French workers in their " bound-sugar " determinations (Bierry and Fandard, 1918).

Turning next to the deproteinizing agent, it is well known that some mixtures precipitate protein fission products less completely than others and differences in final reducing power have in fact been found according to whether, for example, mercuric nitrate (Patein-Dufau) or sodium tungstate (Folin) mixture was employed for the

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removal of the protein cleavage products formed as a result of the treatment with acid. The use of the Hagedorn-Jensen method where zinc hydroxide is the deproteinizing agent, as in the experiments of Bigwood and Wuillot (1927, A, B, C) is quite unpermissable!

Not only is there a danger of incomplete removal of interfering protein bodies, however, there is also the chance that particular deproteinizing agents might remove one or other of the actual carbohydrate substances it is desired to estimate, a point which has been most persistently and unaccountably overlooked. In 1929 Dische pointed out that mercuric nitrate and alkali, as used by Bierry and his collaborators, removed some constituent from the hydrolysate which in view of the writer's own, later experiments, was almost certainly glucosamine. Thus the figures obtained by the French school of workers are, probably, without exception, too low.

Fontès and Thivolle (1927, A, B) found that deproteinization of blood with mercuric nitrate led to lower reducing values than when sodium tungstate was used, but also pointed out that if such a tungstate-deproteinized blood filtrate was hydrolyzed with acid for half an hour and then treated with mercuric nitrate and the reducing power again determined on this filtrate, it was found to be unchanged. Grevenstuk comments upon this finding as follows: '' Blut enhält eine Substanz, die nach Hydrolyse ebenso stark reduziert wie zuvor (also nicht zur Hy—S gehört), aber vor Hydrolyse wohl, danach aber nicht mehr von Mercurinitrat gefällt wird! ''

The facts allow of another interpretation. Suppose there is present in the tungstate filtrate a carbohydrate complex represented by G–M where M possesses a free reducing group. G–M is supposed to be removed from solution by mercuric nitrate and alkali by virtue of groupings in the component G, but to remain unprecipitated by sodium tungstate. The higher reducing power of a tungstate filtrate follows at once. Now suppose such a filtrate is hydrolyzed, G–M being split into its components and the mercuric nitrate reagent added. G will in all probability be precipitated and M remain behind in solution. The filtrate from this last precipitation would then exhibit a total reduction equal to that of the tungstate blood filtrate but greater, by M, than that of an original mercuric nitrate blood filtrate.

Apart from the points afore mentioned, the method of direct acid hydrolysis possesses other serious drawbacks which may be enumerated below as follows:—

(a) If the period of heating be too short, or the temperature not sufficiently high, hydrolysis may be incomplete and the reducing value found, therefore, too low.

(b) Even boiling pure sugar solutions with acids leads to a certain amount of destruction the degree of which varies both with the particular carbohydrate, the kind of acid, its concentration and the temperature and duration of heating, so that it is quite impossible to make any quantitative allowance for this effect [Pavy (1896), Krok (1918), Glassmann (1926), and unpublished results of the writer].

(c) In the presence of certain amino-acids or protein break-down products and hot, strong acids, as in the usual condition of hydrolysis, carbohydrates readily condense with the nitrogenous substances to form " humin ", a dark amorphous material devoid of reducing properties. Gortner (1916) and his associates have shown that tryptophane is the amino-acid chiefly responsible and in unpublished experiments the writer has found that when tryptophane and glucose in the molecular proportions $2:1$ are refluxed in 20 per cent. hydrochloric acid for 24 hours, the formation of humin is nearly quantitative. Glucosamine reacts to only a very slight extent under these conditions.

Clearly, it must be accepted as a foregone conclusion that when whole blood, or even the isolated blood proteins, are autoclaved with acids and reducing power determinations carried out, the quantity of sugar found will be considerably less, to a variable extent, than that which was actually formed. Had any of the methods in use been tested by a simple recovery experiment, this would at once have been obvious.

Considering the unsatisfactory state of the whole problem of " bound " or " protein-sugar ", the writer some years ago endeavoured to find a solution to one aspect at least, whether purified serum proteins contained any carbohydrate material as an integral part of their molecule—by the only method possible, namely, that of isolating the carbohydrate constituent in a state of purity and determining its chemical structure.

It is surprising that previous investigators have given so little forethought to the efficient purification of their starting material and to the proper choice of hydrolytic agent. Mineral acids have been almost exclusively employed in spite of the obvious objections to their use outlined above. Thus both Krawkow (1896) and Langstein (1902) claimed to have isolated an osazone from acid-hydrolyzed serum albumin and the latter author identified glucosamine among the reaction products by the method of benzoylation.

Somewhat similar results were obtained in the case of serum globulin by Krawkow (1896), Eichholz (1898), Abderhalden et al. (1904), Langstein (1903, 1905, 1906), Condorelli (1924, A ; 1926) and others. Their individual findings were, however, far from being in good agreement, and no clear insight was obtained into the nature of the " bound sugar ".

In the writer's own work (Rimington, 1929; 1931) attention was especially paid to the purification of the serum protein fractions used and an alkaline hydrolyzing agent, barium hydroxide was employed, this having the advantage that the carbohydrate complex was fairly readily liberated from its attachment to the protein without in itself suffering much resolution into its components. The greater stability of glucosides towards alkalis than towards acids is well known. The same complex was also obtained after digesting the protein with trypsin. Its isolation was achieved as follows (Rimington, 1931):—

The purified protein, serum albumin or globulin, was refluxed with 10 per cent. baryta for 36 hours, barium removed as sulphate and the carbohydrate precipitated by basic lead acetate and baryta,

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the process being controlled by Molisch tests upon the filtrate. After washing, reprecipitation, etc., carbon dioxide was passed through a suspension of the precipitate in water until all carbohydrate material was liberated. Protein decomposition products were then removed by acid mercuric sulphate, keeping the H_2SO_4 concentration below 5 per cent., and after removal of Hg, Ba and SO_4 ions, the solution concentrated in vacuo to a syrup. This was mixed with dry methyl alcohol and the carbohydrate precipitated by addition of ether. It was purified by frequent reprecipitation and then dried in vacuo over sulphuric acid.

The substance so obtained contained nitrogen, and it was at first thought that the percentage of this element became constant at 4·1 per cent. However, such preparations always contained a little chlorine and it was subsequently found that this was due to the carbohydrate complex being incompletely freed from histidine, to which it appears to be bound in the protein molecule. More prolonged hydrolysis with baryta gave a substance, no longer giving a positive diazo reaction (Pauli's test) and free from chlorine, with a constant nitrogen content of 2·78 per cent.
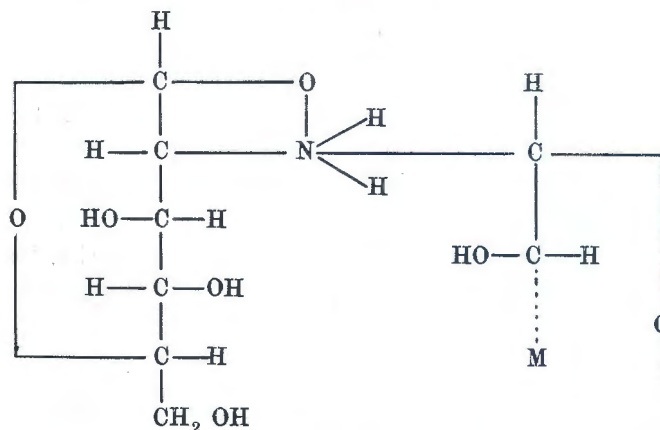
This carbohydrate was found to be a complex of glucosamine (1 mol.) and mannose (2 mols.), most probably polymerized. No other sugar could be identified. It does not exhibit reducing properties until after acid hydrolysis, which liberates the individual components, and was optically inactive. It was not attacked by enzyme preparations. In order to account satisfactorily for its chief properties, the following structural formula was tentatively suggested for this carbohydrate; detailed evidence is presented in the writer's first paper. M represents the second molecule of mannose.

The same complex was present in both albumin and globulin, but the quantity was much greater in the latter.

In the light of these findings, many of the observations of former investigators fell into their true perspective. Thus the isolation of glucosamine from acid hydrolysates is readily understandable; also the fact that Dische (1929) found mannose to be present in an acid hydrolysate of whole plasma.

Many of the claims of Bierry, it is true, are at variance with the above results. Thus he (1928) failed to find any mannose in one careful experiment with an acid hydrolysate but isolated glucosazone and consequently attributed the whole of the extra reducing power to glucose, although, in a later publication he (1931) considered the presence of mannose, galactose and glucose to be probable.

As before mentioned, the chances of isolating, with certainty, any of the carbohydrate constituents from an *acid* hydrolysate of blood or plasma are remote except in the case of the comparatively stable glucosamine, but this, of course, yields the same osazone as glucose.

It is of interest to note, in passing, that Fränkel and Jellinek (1927) obtained a nitrogen-containing polysaccharide from egg-albumin and Levense and Mori (1929) later obtained this substance in pure form and showed it to be glucosamino-dimannose. They consider, however, that it was probably derived from ovomucoid adhering to their protein preparations since with recrystallization the carbohydrate-content of egg albumin decreases.

This interpretation was strongly criticized by the writer, who pointed out that, by repeated recrystallization a fractionation of the protein, in the Sørensen sense, was really achieved. It was suggested that the carbohydrate content of the individual components of the blood serum proteins might vary quite considerably, thus, perhaps, conferring upon them their respective solubility characteristics (see previous discussion).

A fractionation of this sort has quite recently been carried out in Sørensen's laboratory by M. Sørensen and Haugaard (1933), who actually found a very marked difference between the carbohydrate content of a readily soluble, crystalline serum albumin fraction (0·47 per cent.) and a sparingly soluble (also crystalline) fraction which contained not more than a trace (0·009 to 0·017 per cent.). These values were obtained by a colorimetric method.

Various methods have been proposed for the colorimetric determination of sugars in complex mixtures, glucosides or proteins. These are reviewed by Dische (1931). The writer, in his study of the serum proteins (1931) used both the methods proposed by Dische and Popper (1926), and by Tillmans and Philippi (1929) based upon colour reactions with indole and with orcinol respectively. Neither gives any colour with glucosamine but mannose, according to the indole method, develops only 65-70 per cent. of the colour of an equivalent quantity of glucose, whereas with orcinol it reacts quantitatively. The polysaccharide isolated from the serum proteins gave, as would be expected from a consideration of the above facts, 66·6 per cent. of the colour of an equal weight of glucose by the orcinol method and only 40 per cent. according to the indole method.

Serum globulin gave 1·468 per cent. of carbohydrate reckoned as glucose by the Dische-Popper method, and 2·279 per cent. by that of Tillmans and Philippi. A very carefully purified preparation afforded a figure of 1·3 per cent. as glucose (Dische-Popper) corresponding, therefore, to 3·7 per centfl of total carbohcdrate-glucose aminodimannose.

When examined in the same way, serum albumin was found to be much less rich in carbohydrate groups. Sørensen and Haugaard (1933) made a thorough study of the conditions under which the Tillmans-Philippi method could be carried out to best advantage and found that, by suitable adjustments, characteristic differences in the behaviour of the individual hexoses were observed such as to make it possible to identify them in mixtures, or in combination in more complex polysaccharides. In this they had recourse to measurements of colour absorption by the " step-photometer " and by selecting the two spectral filters 43 and 53 and plotting the ratio of the extinctions as measured by these, $\frac{E\ 43}{E\ 53}$, against the time of heating of the reaction mixture were able to obtain their basic reference curves for each sugar. Since the individual components of a polysaccharide contributed their effects additatively, the constitution of the latter could be " deduced " by finding the combination best superposed upon the experimental $\frac{E\ 43}{E\ 53}$ time curve yielded by the substance in question.

From the results of their experiments, they conclude that not only mannose but also galactose is present in serum albumin and globulin preparations, glucosamine, of course, gives no colour with the reagent and would not be detected by this method.

Whilst these findings must undoubtedly be looked upon as suggestive and significant, no certain conclusion can be drawn until galactose has actually been isolated from these proteins and fully identified.

The quantity of carbohydrate found by Sørensen and Haugaard in serum globulin was 1·82 per cent., as glucose, which as they show is equivalent to 3·41 per cent. of a glucosaminodimannose or glocusamino-galacto-mannose in good agreement with the figure of 3·7 per cent. obtained by the writer. Attention must also be drawn to the work of Lustig and Haas (1931) who, using the Tillmans-Philippi method as originally described, showed that the carbohydrate content of their serum proteins was not altered by prolonged dialysis or repeated reprecipitation from alkaline solutions, after denaturation, by the addition of acid. Further, they investigated the carbohydrate content of the various sub-fractions of ox serum protein and found very marked differences. The following table is taken from their work:—

Protein sub-fraction.	Carbohydrate content %.
Euglobulin water—soluble	0·84
Euglobulin NaCl—soluble	0·98
Euglobulin Na$_2$CO$_3$—soluble	2·38
Euglobulin NaOH—soluble	8·50
Pseudoglobulin water—soluble	0·98
Pseudoglobulin NaCl—soluble	0·64
Pseudoglobulin Na$_2$CO$_3$—soluble	6·40
Pseudoglobulin NaOH—soluble	7·03
Albumin I	0·47
Albumin II	0·55
Albumin III	0·65

It is clear from the small difference in carbohydrate content between albumins I, II and III compared with the large difference found by Sørensen and Haugaard (1933) between that of their easily soluble and sparingly soluble albumin fractions, that the different procedures adopted do not effect the same type of separation.

Some space has been devoted to the consideration of " bound " or " protein " sugar in blood since it is felt that there are already sufficient indications in the literature to show that it may prove to be of distinct importance not only in the normal physiology of carbohydrate metabolism but also in pathological conditions. Much of the existing work, it is true, is marred by the uncertainty as to what substances were actually included in the determination and by the unreliability of the methods employed. The proper basis for future investigations has now been supplied, however, with the recognition of the nature of the carbohydrate and methods for its accurate determination.

No attempt will be made to discuss the various contributions to the physiology of bound sugar as most are referred to in Grevenstuk's (1929) article. Attention will only be drawn to the papers of Glassmann (1926), Freund (1885) and the Italian workers, especially Condorelli (1924), B; 1924, C), and to point out that Bordet's (1922) argument is not necessarily sound. He claimed that the bound carbohydrate of blood could not be regarded as attached to the proteins since Bierry's quotient " $\dfrac{\text{Protein N}}{\text{Protein sugar}}$ ", is by no means constant, at least not in pathological cases. It was not appreciated, apparently, that different serum protein fractions might have widely differing carbohydrate contents [compare the table from Lustig and Haas (1931), quoted above] and thus an increase in the relative quantity of any one, considerably alter the " $\dfrac{\text{Protein N}}{\text{Protein sugar}}$ ", quotient. Especially is it likely that such conditions might arise in pathological sera.

As the writer has previously pointed out (Rimington, 1929), the question whether the carbohydrate complex of the serum proteins takes a part in immunological phenomena is a pertinent one and should be explored by experimental investigation. Heidelberger (1927) and his associates have demonstrated the peculiar rôle played in this connection by the polysaccharides, some nitrogen-containing, of various strains of pneumococcus. Other examples have since been brought forward and the name " haptene " has been proposed for this class of specifically active substance. The demonstration that the serum protein polysaccharide is incapable in itself, of provoking antibody formation, does not exclude its possible function in a manner analogous with that of the haptenes. What grouping is responsible for the biological specificity of proteins is still unsolved. Possibly the phenomenon of specificity and antigenicity resembles that of enzymic action in requiring a specifically active centre situated on a colloidal carrier, the dependence for the manifestation being mutual.

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It is significant that Avery, Goebel and Barbers (1932) have demonstrated that the synthetic gluco-proteins formed by coupling globulin with $\alpha-$ and $\beta-p-$aminophenol glucosides (by diazotization and mixing in alkaline solution) are not only antigenic but exhibit a specificity very similar to that encountered in the case of pneumococcus type II and Friedländer Bacillus type B.

Thus, whilst addition of the homologous glucoside to its anti-serum completely inhibits the precipitins for both homologous and heterologous test antigens, the heterologous glucoside inhibits only for heterologous antigen having but slight effect upon the antibodies reactive with the homologous antigen.

In the previous study, made by Avery and Goebel (1929), where a gluco-globulin and galacto-globulin were employed, that is to say, antigens made by coupling globulin with the p-aminophenol glycosides of glucose and of galactose respectively and differing therefore only by the relative positions of one H and OH group in the sugar residue, a much stricter degree of specificity was found to obtain, each developing an antibody specific for the gluco-protein which had induced its formation. Moreover, the specific rôle of the sugar radical was demonstrated by the fact that the glycosides alone, unattached to protein, were capable of inhibiting specifically the precipitin reaction between the corresponding antiserum and homologous antigen.

Before leaving the subject of " bound sugar ", mention must be made of the third protein of the serum, the so-called serum mucoid. Surprisingly little attention has been paid to this substance, the most complete study being that of Bywaters (1909), who also proposed a method for its determination (1906-7). Eichholz (1898) reported the isolation of a considerable quantity of an osozone melting at 204° from the products of its hydrolysis and Zanetti (1903) the identification of glucosamine as (tetra) benzoylglucosamine.

Bywaters, as a result of his study came to the conclusion that serum mucoid was a true protein, closely resembling ovomucoid, and not merely a proteose or protein split-product, as some had supposed on account of its not being coagulated by heating its solutions to boiling. He also determined its carbohydrate content by the yield of reducing substances after acid hydrolysis to be of the order of 24 per cent. Glucosamine was identified by the isolation of penta-benzoylglucosamine from such hydrolysates. Bywaters considered that the quantity of serum mucoid, normally about 0·3 to 0·9 gm. per litre of blood, varied markedly in response to various physiological conditions such as hunger, starvation, etc.

As a result of unpublished experiments carried out some years ago, the writer is able to substantiate Bywater's claims on the chemical side.

The mucoid was prepared from ox or sheep serum after first removing the albumin and globulin by heating, by evaporating in vacuo to a small volume, dialyzing thoroughly and then precipitating by addition of alcohol. The crude material was several times re-dissolved in warm water, the solution filtered and poured into about four volumes of alcohol. A second dialysis was also usually included.

It was obtained as a white powder, soluble in warm water and giving most of the usual protein colour reactions, with the exception, however, of the labile sulphur test, although analysis showed it to contain about 1·7 per cent. S. Fehlings solution was not reduced on boiling with a solution of the mucoid, but the Molisch reaction was strongly positive. Analyses of different preparations gave figures similar to those quoted by Bywaters for his own and Zanetti's preparations, thus:—

	C	H	N	S
Ox serum mucoid	47·43	7·23	12·31	
Sheep serum mucoid	47·78	7·13	12·75	0·91
Mean of Bywaters preparations	47·62	6·85	11·59	1·8
Zanetti's preparations	47·6	7·1	12·9	2·2

The carbohydrate content was determined by the method of Tillmanns and Philippi (1929). Expressing the results as glucosaminodimannose it approached 22 per cent., but some variation was noticed, possibly due to irregularities of the method (see Sørensen and Haugaard, 1933). The isolation of the carbohydrate complex has not yet been undertaken, but it is hoped to do this and to complete the study of this interesting protein in the near future when it will be seen whether the same carbohydrate grouping is present in the mucoid as in the other serum proteins.

That serum mucoid does exist in blood and is a distinct protein seems to be incontestable. It would be of interest to examine its fluctuations in various pathological conditions. Lewis and Wells (1926) have examined the immunological behaviour of both serum- and ovo-mucoids of different species and their results still further support the view that these proteins are distinct individuals. Thus serum mucoid was found to be antigenic, that of the sheep being more active (anaphylactic shock method) than that of the ox. No protection was afforded by the heterologous protein. Compliment fixation tests also showed a fairly high degree of specificity. Dog and sheep serum mucoids were shown by the uterine strip method to be distinct from each other and from the ovomucoids.

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