CCCXII. SEPARATION OF SERUM ALBUMIN INTO TWO FRACTIONS. I.

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THE differentiation of proteins is made uncertain by their complexity and instability, by the difficulties of separation and by the inapplicability of many of the usual criteria of chemical purity. For example, the position with regard to the existence and interrelation of different serum globulins is still highly controversial [Hardy & Mellanby, 1905; Chick, 1914; Sørensen, 1925; Hewitt, 1927; Mutzenbecher & Svedberg, 1933]. It is generally agreed that serum albumin and globulin are separate and distinct fractions and, since serum albumin can be obtained in a crystalline condition, there should be less difficulty than with most proteins of isolating it in a reasonably pure state. Grave doubts have, however, been expressed from time to time as to whether serum albumin is a single chemical compound or a mixture. Differences in heat coagulability [Halliburton, 1884], solubility [Sørensen, 1930], and carbohydrate content [Hewitt, 1934] have been observed, but despite the indications given in experiments of this kind, definite and conclusive proofs of the existence of different serum albumins have not been easy to obtain. The starting point of the present investigation was the observation recorded in a previous communication [Hewitt, 1934] that by fractionation with ammonium sulphate it was possible to separate albumin into fractions of widely different carbohydrate content. The present communication describes the separation of horse serum albumin into two fractions which can be distinguished readily by chemical or physical methods.

Methods of fractionation.

The aim of the experiments was to separate albumin into two main fractions, the crystalline fraction which separates first from ammonium sulphate solutions and the most soluble fraction remaining in the mother-liquors.

The general method of obtaining crystalline albumin has not been altered fundamentally for over 30 years [Hopkins & Pinkus, 1898]. Frequent alterations in *p*H tend to have deleterious effects on serum proteins, so the procedure of Adair & Robinson [1930] of dissolving the crystals in ammonium acetate solution and reprecipitating with acid was avoided. Prolonged dialysis is a further source of possible change in the proteins and this was also obviated. Although alcohol-ether extraction at low temperature is an efficient method of obtaining stable preparations of proteins in the dry state [Hardy & Gardiner, 1910; Hartley, 1925; Hewitt, 1927], this procedure was rejected on the ground of possible criticism [McFarlane, 1935].

The main points in the present method are that oxalate plasma was used instead of serum, that the plasma was diluted with an equal volume of water before addition of saturated ammonium sulphate solution, that the globulins were removed as usual by 50 % saturation with ammonium sulphate, and that crystallization in the 50 % saturated ammonium sulphate filtrate was effected by addition of acetic acid and not sulphuric acid.

In order to save space details will be given of only one fractionation process, in which fractions A_3, B, C and D were isolated.

The plasma from oxalated horse blood was diluted with an equal volume of water, saturated ammonium sulphate solution was added to make the mixture 50 % saturated with ammonium sulphate and the globulins were removed by filtration. To 100 l. of the clear filtrate (corresponding to 25 l. of plasma) were added 160 ml. of glacial acetic acid. After a short time crystallization commenced and this was allowed to continue overnight. The crystals were filtered off and redissolved in 10 l. of water and insoluble green-coloured material was filtered off, 9·5 l. of clear filtrate being obtained. To this was added saturated ammonium sulphate solution (5·7 l.). The crystals which separated were filtered off and redissolved in 3 l. of water, the solution being clarified by passing it through a Sharples centrifuge. 1650 ml. of saturated ammonium sulphate solution were added to the clear solution and the fine needles which separated out were filtered off, dried in towels and dialysed in cellophane bags against running water for 48 hours. In this way fraction A_3 (yield about 73 g., calculated as dry weight of protein) was obtained.

To the filtrate of the first crystallization (97 l.) was added more saturated ammonium sulphate solution (20 l.) and glacial acetic acid (100 ml.). The precipitate produced (fraction B) was dried and dialysed as described in the case of fraction A_3, the yield being about 76 g. To the filtrate of the second crystallization 5 l. of saturated ammonium sulphate solution and 50 ml. of glacial acetic acid were added and the precipitate was collected as described previously. In this way fraction C, yield about 73 g., was obtained. The filtrate from the third crystallization (4 l.) was also treated with saturated ammonium sulphate solution (2·5 l.) and fraction D was obtained (yield 4·8 g.).

As a result of other fractionations the following fractions were obtained: E_8 was a crystalline albumin obtained from 100 l. of the 50 % saturated ammonium sulphate filtrate (corresponding to 25 l. of horse plasma). The fraction had been recrystallized eight successive times and was obtained in a yield of about 117 g. One quarter of this preparation was subjected to four further recrystallizations making twelve recrystallizations in all, this yielding fraction E_{12}.

Fraction H_1 (50 g.) was a soluble fraction obtained from the mother-liquors of 100 l. of 50 % saturated ammonium sulphate filtrate after two less soluble fractions F_4 and G had been removed. F_4 appeared to contain globulins and was not further investigated, whilst the intermediate fraction G was obtained in a final yield of about 147 g.

Nitrogen content and protein determinations.

The discrepancies between the figures given for the nitrogen content of proteins by different investigators are very remarkable. Two comparatively recent investigations in which this matter received detailed consideration yielded very different results. For serum albumin Adair & Robinson [1930] found a nitrogen content of 15·6 %, whilst Smith *et al.* [1932] give a figure of only 13·8 % of nitrogen. It appears that there must be some difference in experimental conditions to account for the divergence.

For nitrogen determinations in the present investigation a modification of the micro-Kjeldahl method using the Parnas and Wagner apparatus was employed. The dry weights of the proteins were determined in some cases by drying *in vacuo* over sulphuric acid and in others by coagulating the protein by heating at the isoelectric point and drying with alcohol and ether. The nitrogen content

observed in the case of crystalline fractions was from 14·1 to 14·4 % (mean 14·2 %). In the case of the very soluble fractions however the nitrogen content is much lower (about 12·9 %). The low nitrogen content of the soluble fractions is explained, as will be shown later, by the very high carbohydrate content of these fractions but it is surprising that the crystalline fractions have so much lower nitrogen contents than those of Adair and Robinson. Since the nitrogen content has been an incidental determination in the present investigation, whilst Adair and Robinson directed special attention to the point, it is not advisable unduly to stress the present figures. The specific refractive increment was determined roughly with an Abbé refractometer and gave a mean value of 0·00177. This value is lower than Adair & Robinson's figure of 0·00183 but again these investigators devoted much greater attention to the point.

Carbohydrate contents of fractions.

The carbohydrate content was found to be a valuable index to the course of the fractionation. As described previously [Hewitt, 1934] the method used for determining carbohydrates was that of Sørensen & Haugaard [1933]. It may be reiterated here that the solutions to be used for photometric examination must be kept shielded from light and that the values for the protein blank determinations are unreliable and may be ignored. As the result of the combined work of the investigators just mentioned and of Fränkel & Jellinek [1927], Levene & Mori [1929], Rimington [1931] and Bierry [1934], it seems probable that the carbohydrate group present in serum proteins is a polysaccharide containing equimolecular amounts of galactose, mannose and glucosamine. No results obtained during the present work presented any serious evidence to contradict the conclusions reached by other workers. Hence all results quoted will be based on the assumption that the carbohydrate present is galactose-mannose-glucosamine (for brevity referred to as g.-m.-g.). The protein content was calculated with the use of the protein:nitrogen factor 7·0. The carbohydrate content of the total albumin fraction of serum is usually about 2·8 % (g. of g.-m.-g. per 100 g. of protein). The fractions now described have the following carbohydrate contents:

	E_{12}	E_8	A_3	G	C	D	B	H
Carbohydrate	0·05	0·08	1·18	1·80	2·46	3·96	7·3	8·5 %

The fractions may be divided roughly into three classes: (i) the highly purified crystalline fractions E_{12} and E_8 with so small a carbohydrate content that it cannot be determined accurately, and may be ignored; (ii) the intermediate fractions; and (iii) the soluble fractions B and H from the mother-liquors containing up to 8·5 % of carbohydrate. Our chief attention will be devoted to the two end fractions since the intermediate fractions behave as mixtures of the high- and low-carbohydrate content fractions. In each case the crystalline, sparingly soluble fractions have the low carbohydrate content (in the most highly purified fractions no appreciable amount) and the soluble fractions from the mother-liquors have high carbohydrate contents. Fraction B was diluted, and heated in a boiling water-bath at the isoëlectric point to coagulate the protein and the precipitate was centrifuged down and washed with alcohol and ether. When redissolved the coagulum was found to contain 7·8 g. of g.-m.-g. per 100 g. of protein, so that the polysaccharide had not been removed by boiling water, alcohol or ether. When hydrolysed with boiling hydrochloric acid for a

few minutes no glucosamine could be detected in E_8 using the method of Elson & Morgan [1933]. With B, glucosamine was detected but could not be determined accurately owing to the large amount of humin formation during the hydrolysis.

Humin formation.

It has been suggested [Gortner, 1916] that humin formation is due to the interaction of carbohydrates and certain amino-compounds. It is not surprising therefore that E_8 and E_{12}, which are free from carbohydrates, remained colourless when hydrolysed with hydrochloric acid whilst B and H, with their high carbohydrate content, gave rise to large quantities of humin under the same conditions.

Optical rotations.

The value of optical rotatory powers in differentiating optically active compounds suggested its use in the investigation of the albumin fractions. A Bellingham and Stanley polarimeter fitted with direct-vision spectroscopic eyepiece was used, and a mercury vapour lamp provided the light source. The mercury green line ($\lambda = 5461$ Å.) was used throughout the measurements.

It will be seen in Table I that the rotations measured varied from $[\alpha]_{5461}$ $-57 \cdot 1°$ in the case of H up to $-70 \cdot 8°$ for E_{12}. The most soluble proteins had the lowest rotatory powers and the crystalline specimens the highest rotations, there being a graded variation amongst the intermediate fractions.

Table I. *Some properties of the different fractions.*

Fraction	Carbohydrate content. g. of g.-m.-g. per 100 g. protein	Optical rotation. $[\alpha]_{5461}$	Van Slyke amino-nitrogen. g. per 100 g. protein
E_{12}	0·05	−70·8°	1·07
E_8	0·08	−70·2	0·90
A_3	1·18	−66·5	0·87
G	1·80	−66·8	0·85
C	2·46	−61·0	0·82
D	3·96	−60·3	0·79
B	7·3	−57·2	0·66
H	8·5	−57·1	0·65

Two observations on these figures should be made here. First, the proteins were not treated with alcohol and ether as described in a previous communication [Hewitt, 1927] for reasons stated in a previous section and hence the optical rotations are not directly comparable with those in the former paper. Second, the calculation of specific rotatory power is based on the nitrogen content of the proteins, using a constant protein : nitrogen factor of 7·0. If the dry weight of the proteins be used the difference between the crystalline albumin and the soluble fractions is even greater, since the specific rotation of fraction H, for example, is reduced to $-50 \cdot 1°$ whilst the figures for the purest crystalline specimens remain at $-70 \cdot 8°$.

Titration curves.

Titration curves indicated a higher base binding capacity by the purest crystalline fractions than by the more soluble mother-liquor fractions. This difference was greater when formaldehyde was added before the titration. Typical curves for the fractions E_8 and H in the presence of about 7 % formaldehyde are given in Fig. 1. The glass electrode was used for these measurements.

Van Slyke amino-nitrogen.

Since the electrometric formaldehyde titration curves indicated a difference between the different fractions, amino-nitrogen determinations were performed using the Van Slyke micro-method. The nitrous acid was allowed to react for 4 min. at 20°. It will be seen in Table I that there is a gradation in the free amino-nitrogen figures of the different fractions, the purest crystalline albumins having the highest amino-nitrogen content (E_{12} = 1·07 % amino-nitrogen) whilst the most soluble fractions had the lowest figures (H = 0·65 %). The results are quoted in terms of g. of amino-nitrogen per 100 g. of protein.

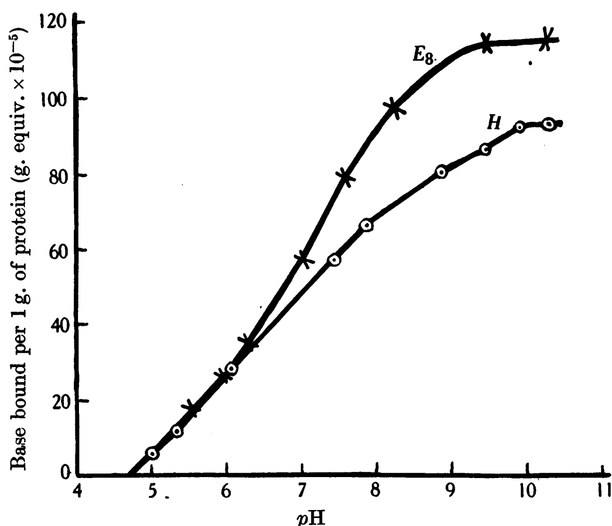

Fig. 1. Titration curves of fractions in presence of formaldehyde.

Heat coagulation.

Protein solutions were diluted to contain about 1 % of protein in 0·85 % sodium chloride solution, the *p*H was adjusted to a value of 4·7 to 4·8 and the solutions were slowly warmed in a water-bath. The crystalline albumin fractions began to coagulate at 60° but the most soluble fractions B and H behaved very differently. It was particularly difficult to coagulate H; very careful adjustment to the isoelectric point had to be made. Some coagulation began in these fractions at about 80° but the supernatant fluid was opalescent and not clear, and the coagulum was not granular but of soft consistency.

The fact that the crystalline low-carbohydrate fractions coagulate at 60° whilst the soluble high-carbohydrate fractions did not coagulate below 80° suggested the interesting possibility that separation of the fractions might be effected by coagulating a mixture at a temperature a little above 60°.

Hence a sample of albumin containing the total albumin fraction of horse serum (carbohydrate content 2·6 g. of g.-m.-g. per 100 g. of albumin) was subjected to a fractional coagulation process. The first coagulation was effected by heating at 62–64°, the coagulum I was centrifuged down, the mother-liquor was reheated and coagulum II collected by heating at 64–66° and so on. The precipitates obtained were dissolved in dilute alkali and analysed for nitrogen and carbohydrate content. The results are given in Table II.

Table II. *Fractional coagulation of sample of serum albumin.*

Fraction	Coagulation temperature	Volume of solution ml.	Nitrogen content %	Carbohydrate g. of g.-m.-g. per 100 g. protein
I	61–62°	5	0·196	3·8
II	64–66°	2	0·038	4·8
III	72–74°	2	0·030	2·1
IV	77–81°	2	0·016	2·7
V	93–100°	2·2	0·008	4·5
M. L.	Not coagulated	12	0·006	8·2

The results of the experiment were not those that would be expected from the low coagulation temperature of the low-carbohydrate content albumin. Evidently even the first coagulum at the lowest temperature carries down appreciable amounts of the high-carbohydrate fraction.

When fraction H was partially coagulated by heating at 80° there was a fairly even distribution of carbohydrate between the precipitate and the supernatant fluid. The precipitated protein contained 9·14 % of carbohydrate and the protein in the supernatant fluid contained 7·74 % of carbohydrate (calculated as g. of g.-m.-g. per 100 g. of protein).

It appears not to be possible, therefore, to utilize the differing coagulation temperatures of the separated fractions to effect a separation of the two.

Tryptophan and tyrosine determinations.

The Folin & Marenzi [1929] method was used for determining tryptophan and tyrosine, the only departures from the published method being that in place of one sample of 20 ml. of the hydrolysate, duplicate samples of 5 ml. were used, the final volume for colorimetric comparison being 25 ml. in place of 100 ml., and the colorimetric comparisons were made in a Stufenphotometer instead of a colorimeter. Duplicate analyses agreed extremely well and the photometer was most useful when the hydrolysates were coloured. With care the method yields very consistent results. In a previous paper [Hewitt, 1934] the tryptophan content of serum albumin was found to be from 0·44 to 0·52 %. Folin & Marenzi's figures were 0·52 and 0·53 % of tryptophan in serum albumin. In the literature the figures are generally higher than these.

It is now found that the tryptophan contents of the purest fractions (E_{12} and E_8) of crystalline albumin are 0·26 and 0·30 % respectively. These extremely low values were readily explained when it was found that the most soluble fractions (B and H) from the mother-liquors of the albumin had tryptophan contents of over three times as much, namely 1·0 %. The tryptophan content of a specimen of serum albumin evidently depends upon the proportions present in it of the different albumin fractions.

The tyrosine contents of the different fractions do not vary so much. The purest crystalline specimens had tyrosine contents of 4·74 and 4·79 % respectively compared with Folin & Marenzi's figures of 4·66 and 4·67 % and the author's previous figure of 4·7 %. The soluble fractions H and B had tyrosine contents of 5·38 and 6·06 % respectively.

Discussion.

Before the theoretical implications of some of the conclusions reached are discussed it will perhaps be well to state clearly in outline what results have been obtained. In Table III are summarized the contrasting properties of the two

Table III. *Contrasting properties of two horse serum albumin fractions.*

Description	Crystalline serum albumin	Mother-liquor fraction
Solubility in ammonium sulphate solution	Least soluble fraction	Most soluble fraction
Preparation	Twelve times crystallized	Remaining in mother-liquors after removal of two less soluble fractions
Carbohydrate content (as g.-m.-g.)	0·05 %	8·5 %
Nitrogen content	14·4 %	13·0 %
Van Slyke amino-nitrogen content	1·0 %	0·65 %
Rotatory power $[\alpha]_{5461}$	− 70·8°	− 57·1°
Coagulation temperature	60°	80°
Tryptophan content	0·26 %	1·0 %
Tyrosine content	4·79 %	5·38 %
Hydrolysis with hydrochloric acid	Remained colourless	Much humin formation

"end" fractions separated from serum albumin, the most sparingly soluble fraction and the most easily soluble fraction being selected. In this connexion the solubility referred to is in ammonium sulphate solution and not in water.

It is clearly proved that horse serum albumin may, by simple methods not involving any rigorous treatment, be separated into two fractions of quite different chemical and physical properties. The crystalline fraction free from carbohydrate is not difficult to identify; it is probably true serum albumin in a higher state of purity than is generally encountered. It seems that serum albumin is usually contaminated with varying amounts of some other protein which is entirely removed only after a quite extensive crystallization process. The outstanding characteristics of "pure" serum albumin apparently are: (1) its solutions are almost colourless, (2) it contains no carbohydrate, (3) humin formation does not occur during acid hydrolysis and (4) it has a low tryptophan content, namely between 0·26 and 0·30 %. This last point is of some interest, for if it be assumed that serum albumin has a molecular weight of 69,000 [Mutzenbecher & Svedberg, 1933], then the presence of one molecule of tryptophan in each albumin molecule would require a tryptophan content of

$$\frac{204}{69{,}000} \times 100 = 0{\cdot}298\,\%,$$

a figure in remarkable agreement with the experimental determination.

The identity of the second protein is the next point of interest. It is not suggested that the easily soluble fraction B or H represents a pure protein but it seems probable that they are each a mixture of albumin with another protein It is hoped in the future to obtain further evidence as to the nature of this other protein material, which may be serum mucoid. Serum mucoid has not been studied extensively but its marked characteristics appear to be that it is not coagulated on heating, that it has a high carbohydrate content of about 25 %, and that it has a low nitrogen content, 11·9 % [Zanetti, 1897; Bywaters, 1909; Rimington, 1931]. The soluble fraction from serum albumin has a fairly high carbohydrate content (8·5 %) and is not easily coagulated on heating. Apparently, however, when large quantities of serum albumin are present the coagulum of serum albumin produced on heating carries down with it a considerable portion of this almost non-coagulable protein. It is perhaps not surprising that

mixtures of proteins, with their large number of reactive groups, behave somewhat differently from the individual components of the mixture and are difficult to separate.

Serum mucoid is prepared by coagulating serum or even whole blood by heating and precipitating the filtrate with alcohol after dialysis. A yield of 0·1–0·5 g. per litre of blood is obtained by this method. If, as seems possible, the carbohydrate content of serum albumin (about 2·5 % g.-m.-g.) is due to admixture with serum mucoid (containing about 25 % g.-m.-g.), there must be in each litre of serum say 25 g. of albumin and 2·2 g. of serum mucoid, 90 % of which is carried down by the albumin when it is coagulated by heating. It is worthy of note that Levene & Mori [1929] conclude that the carbohydrate content of egg albumin is due to admixture with ovomucoid.

It is certain that the second fraction present in serum albumin presents certain similarities to the protein known as serum mucoid but it is immaterial for the moment whether the two are identical. The important fact established is that there is present in the albumin fraction of serum considerable quantities of some other protein, perhaps amounting to as much as one-tenth of the total albumin fraction.

Summary.

1. Serum albumin, as ordinarily prepared, is apparently not a single protein but is contaminated with varying amounts of a more soluble fraction.

2. By careful fractionation horse serum albumin has been separated into two widely different fractions:

(*a*) Crystalline albumin free from carbohydrate and with a tryptophan content corresponding roughly to one molecule of this amino-acid in each molecule of protein;

(*b*) A freely soluble fraction which is not coagulated readily on heating. This fraction contains considerable amounts of carbohydrate, has a higher tryptophan content than the albumin fraction, has a lower rotatory power and a lower nitrogen content and has fewer free amino-groups.

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