LXXII. OBSERVATIONS ON THE CRYSTALLIZABLE ALBUMIN FRACTION OF HORSE SERUM

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THE advantage of sodium sulphate as a reagent for use in the precipitation and crystallization of proteins has been pointed out in a previous paper [Kekwick & Cannan, 1936] and it was considered of value to attempt to extend this procedure to the serum proteins, and in particular to the crystallization of the albumin fraction. Extensive investigations on the fractionation of serum proteins with sodium sulphate have been made by Howe [1921], but in no case was a crystalline product prepared.

It soon appeared that the crystalline albumin fraction of horse serum was probably not homogeneous, a fact which has been observed a number of times, and most extensively investigated by Sørensen [1930].

Two crystalline fractions have been prepared and evidence with regard to their chemical individualities is presented, together with a survey of some of their physico-chemical characteristics.

The fractionation of horse serum proteins with sodium sulphate

Globulin precipitation.

In Howe's [1921] investigations diluted serum was always used and Homer [1919] stated that in undiluted serum sharp separation of the protein fractions did not occur either with sodium sulphate or other precipitating salts.

The avoidance of large dilutions is an obvious advantage for large scale preparations and consequently the precipitation curve of undiluted serum with sodium sulphate was reinvestigated.

Horse serum for this purpose, and for all subsequent preparations described in the paper, was obtained from healthy horses by collecting blood in a receptacle in the bottom of which had been poured sufficient 3 % sodium oxalate solution to make the final concentration 0.1 % when the jar was filled with blood. This concentration of sodium oxalate did not inhibit clotting, but a very loose clot was formed which shrank to about one-third of its initial volume in less than 12 hr. in the cold. Practically no haemolysis occurred during this period. After siphoning off the supernatant serum and centrifuging lightly to remove suspended particles a water-clear pale yellow serum was always obtained. Such serum occasionally contained minimal quantities of fibrinogen.

The precipitation curve was investigated at 30°, 50 ml. samples of a batch of serum being added to weighed quantities of anhydrous sodium sulphate, which was dissolved by the aid of mechanical stirring. After 2 days' standing the precipitates were filtered off and washed with 25 ml. sodium sulphate solution of the concentration used for the precipitation. The filtrate and washings were combined and made up to volume and the N content determined directly by the micro-Kjeldahl method.

In Fig. 1 the filtrate N per 100 ml. serum is plotted against sodium sulphate concentration. A distinct plateau appears in the curve extending from 19 to 21 g. Na_2SO_4 per 100 ml. The use of 20 g. Na_2SO_4 per 100 ml. serum for the precipitation of the globulins was therefore adopted for undiluted serum.

Fig. 1. The precipitation curve of horse serum with sodium sulphate. $T=30^\circ$.

Howe [1921], using serum diluted 1:10, found breaks in the precipitation curve from 13.5 to 14.5, 16.4 to 17.4 and 21 to 22 g. Na_2SO_4 per 100 ml. He recommended the use of 21.5 g. Na_2SO_4 per 100 ml. for globulin precipitation under these conditions. For undiluted serum a second discontinuity is suggested by the present data around 16 g. Na_2SO_4 per 100 ml. but this was not adequately investigated.

Crystallization and fractionation of albumin fraction

As a general rule the serum was worked up in 4 l. batches, all the procedures being carried out in a warm room at 30° unless otherwise stated.

The globulins were precipitated by addition of 20 g. Na_2SO_4 per 100 ml. serum and allowed to stand overnight before filtration. The albumin filtrate was then acidified with 0.2 *N* H_2SO_4 , the usual precautions being observed (continuous mechanical stirring; delivery of the acid from a small orifice below the surface of the liquid) until an apparent pH of 4.8 to 4.9 was attained.

This usually required 230 ml. acid per l. of filtrate. The pH was controlled externally by the use of suitable phthalate buffers [Clark, 1928] with bromocresol green as an indicator, allowance being made for the colour of the serum and for the effect of the Na_2SO_4 present. The pH is advisedly referred to as "apparent", since the protein error of the indicator is considerable, and larger with serum albumin than figures in the literature indicate.

Crystallization usually commenced as the final amounts of acid were stirred in; then for every 100 ml. of acid added 20 g. of anhydrous Na_2SO_4 were stirred in and crystallization was allowed to come to completion by standing for 24 hr.

After standing, the crystals were filtered off and allowed to drain thoroughly. In order to ascertain that crystallization had been complete the filtrate was on many occasions tested out for further crystallization; it was found that either a slight amorphous precipitate formed, or if there were any crystals the amount was negligible.

The carefully drained crystals were then extracted with a volume of distilled water at 30° equal to one-tenth that of the initial serum, the extraction being continued for 16 hr. with occasional stirring. The undissolved material, mostly

crystalline but showing some amorphous substance was then filtered off, drained and extracted for several days at 2° with a quantity of distilled water equal to that used in the first extraction, after which the green amorphous residue was filtered off and discarded.

The filtrate from the first extraction will be referred to as fraction A, that from the second as fraction B, and the green material as "insoluble residue". The data given in Table I show the average quantities of these materials obtained by such a fractionation procedure. The actual amounts vary with the different samples of serum, and the yield of crystalline material tends to fall if the serum is kept.

Table I

	g. N ₂ /l. serum
Total albumin fraction	3.7
Total crystalline albumin	2.5
Fraction A	0.6
Fraction B	0.9
"Insoluble residue"	1.0

Recrystallization and the carbohydrate-nitrogen ratio of the fractions

The fact that crystalline serum albumin can be separated into fractions of different carbohydrate contents was first shown by Sørensen [1930] and Sørensen & Haugaard [1933]. Recently Hewitt [1936; 1937] has reinvestigated this and other aspects of the problem.

It was considered that an investigation of the carbohydrate-nitrogen ratio of the fractions A and B, particularly from the point of view of its variation during subsequent recrystallizations, would give some evidence as to the chemical homogeneity of the fractions.

The recrystallizations were carried out at 30° and conditions were sought such that at each recrystallization between 85 and 95 % of the fractions was recovered. For fraction A this was achieved by recrystallization from solutions containing 3.0 g. protein per 100 ml., the sodium sulphate concentration being brought up to 20 g. per 100 ml. For fraction B the corresponding conditions were 4.5 g. protein per 100 ml. and 15 g. Na₂SO₄ per 100 ml. The pH was carefully maintained at 4.8–4.9 as in the initial crystallization procedure. The protein concentrations were determined by micro-Kjeldahl, an arbitrary factor of 6.5 being adopted for the conversion of nitrogen to protein for both fractions.

Samples of crystallized material and mother liquors were removed at each recrystallization and dialysed in cellophane against toluene-saturated distilled water at 0° until the dialysate showed no SO₄²⁻ test after 16 hr. contact with the solution. In some cases the mother liquors were concentrated by pressure dialysis to obtain a suitable protein concentration. The carbohydrate-nitrogen ratio was then determined for each sample, the nitrogen by the micro-Kjeldahl method and the carbohydrate by the Sørensen & Haugaard [1933] method. Details of the procedures are given below.

Table II. *Carbohydrate-nitrogen ratios for crystallizations of fractions A and B*

Crystallization no.	Fraction A		Fraction B	
	Crystals	Mother liquor	Crystals	Mother liquor
1	0.204	—	0.022	0.204
2	0.123	0.25	0.019	0.179
3	0.141	0.111	0.0069	0.048
4	0.123	0.104	0.0052	0.023
5	0.136	0.038	0.0055	0.0079

In Table II the results of one investigation of this type are given for both fractions A and B, the ratios having been calculated as mg. carbohydrate per mg. nitrogen. Similar data, not included here, have been obtained from further experiments which confirm the results in Table II.

Considering fraction A it will be observed that its ratio remains substantially constant from crystallization 2 onwards after a material very rich in carbohydrate was eliminated by crystallization 2. The mother liquors from crystallizations 3 and 4 approximate in ratio to the crystals though that from crystallization 5 shows an unusual discrepancy.

For fraction B the ratio is of a different order of magnitude and becomes sensibly constant after the second crystallization. The data for the mother liquors shows the elimination of a substance considerably richer in carbohydrate than the crystals, but when the fifth crystallization is reached this material has been practically completely removed.

On the basis of these data it is suggested that the method of fractionation described results in the separation of two chemically distinct crystallizable substances.

It may be emphasized here that to adduce evidence proving conclusively that a protein is chemically homogeneous is a matter of the utmost difficulty.

The recrystallizations were also controlled by sedimentation velocity measurements (for methods see p. 556). These showed the presence in fraction A, during early crystallizations, of a very slight amount of heavily sedimenting material, probably globulin, which was however completely removed after four crystallizations. A twice crystallized sample of fraction B showed no contamination.

In general appearance the two fractions are rather alike, the crystals being of similar needle-shaped form, but those of fraction A were usually smaller than those of fraction B. Fraction A was however pigmented, a 2% solution being a bright yellow colour, whereas a corresponding solution of fraction B was almost colourless.

The crystal precipitate removed by filtration can be rapidly reduced to a dry powder by circulating air at 25° over it. The product can easily be ground to a fine powder and is completely redissolved on treatment with water, from which solution it can be recrystallized. A sample of such material which had been kept at room temperature for 3 years retained its ability to be crystallized. Ultra-centrifugal investigation showed that this treatment caused the protein to become slightly polydisperse, but for many purposes this is not a very serious disadvantage. None of the preparations used in the investigations in this paper had been dried.

The nitrogen and carbohydrate content of the fractions

A determination of the percentage of nitrogen in each of the fractionated materials, in addition to being of interest itself, enables an estimation of the percentage of carbohydrate to be made when taken in conjunction with the ratios of Table II.

Samples of five times crystallized material were dialysed as before. On aliquots of the solution nitrogen was determined by micro-Kjeldahl, and the amount of protein by drying to constant weight at 105° followed by ashing in an electric muffle at 450°.

The nitrogen values found, together with carbohydrate contents calculated by combination with the data of Table II for the five times crystallized material, are given in Table III, on an ash-free basis.

Table III. *Nitrogen and carbohydrate content of fractions A and B (five times crystallized)*

	% N	% carbohydrate	Ash (% dry wt.)
Fraction A	14.35	1.95	0.38
Fraction B	15.16	0.083	0.23

EXPERIMENTAL

(1) *Determination of nitrogen.*

Estimations were carried out in triplicate by the micro-Kjeldahl method, suitable blank determinations being made concurrently. Selenium oxychloride was used as catalyst.

The method was tested on a sample of crystallized egg albumin in parallel with the ordinary copper sulphate method and identical results were obtained. With the selenium catalyst, however, the digestion time was considerably shortened.

(2) *Estimation of carbohydrate.*

Estimations of carbohydrate were carried out by the method of Sørensen & Haugaard [1933]. Into 15 ml. 60 % H_2SO_4 were pipetted 1 ml. protein solution and 2 ml. 2 % orcinol in 60 % H_2SO_4 , a blank omitting the orcinol being set up for each protein solution. After thorough mixing the solutions were heated in a water bath at $80 \pm 1^\circ$ for 20 min., plunged into ice water and cooled in the dark. The extinctions were measured in a Zeiss step photometer¹ using the filters S 43 and S 53.

Calibration curves were made using a mixture of equal quantities of glucose and mannose, the carbohydrate content of the protein solutions being read off from the curve.

More weight was given to the extinctions with the S 43 filter, those from the S 53 filter being used for corroboration of the general magnitude of the carbohydrate content of the protein solutions.

The use of a glucose-mannose solution for reference was arbitrary, comparative data being the chief objective.

Some physico-chemical characteristics of the fractions

Having established a chemical differentiation, it was considered of interest to determine whether the fractions showed any divergence in some of the more common physico-chemical characteristics usually determined for proteins. For this purpose measurements of sedimentation velocity, electrophoretic velocity and diffusion were made on both fractions. For these measurements a six times crystallized fraction A and a three times crystallized fraction B were used.

Sedimentation velocity measurements.

Sedimentation velocity measurements were made using the light adsorption method, a mercury vapour lamp in conjunction with chlorine and bromine filters ($\lambda = 250\text{--}270\text{m}\mu$) being used as a light source [Svedberg, 1925; Svedberg & Eriksson, 1932]. The centrifugal field was approximately 200,000 times gravity in all experiments.

The sedimentation constants corrected to pure water at 20° together with other relevant data are given in Table IV. Sufficient electrolyte was present in the solutions to suppress any charge effect on the sedimentation.

¹ I am indebted to Prof. C. R. Harington for allowing me to use this apparatus.

Table IV. *Sedimentation constants of fractions A and B*

Buffer	Total salt conc. (<i>M</i>)	<i>pH</i>	$S_{20} \times 10^{13}$	
			Fraction A	Fraction B
HOAc, NaOAc, NaCl	0.2800	3.74	(4.10)	(4.31)
HOAc, NaOAc, NaCl	0.3000	5.04	4.34	4.52
KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl	0.2800	6.63	4.52	4.55
KH ₂ PO ₄ , Na ₂ B ₄ O ₇ , NaCl	0.2500	8.54	4.46	4.36
Mean			4.44	4.48

The experiments cover almost completely the stability range given for serum albumin, viz. *pH* 4–9 [Svedberg & Sjögren, 1930]; in fact the first pair are just outside this range. The mean values for each fraction, excluding the first pair of results, are in excellent agreement with the established value for the sedimentation constant for horse serum albumin, viz. $S_{20} = 4.5 \times 10^{-13}$ [v. Mutzenbecher, 1933], and the difference between them is not significant. No sign of heterogeneity was shown by the absorption curves of either fraction.

Measurements of electrophoretic mobility.

Using the method of Tiselius [1930] a series of measurements of the electrophoretic mobilities of the fractions was made, with the same recording conditions as for the sedimentation velocity experiments. Acetate buffers (0.02 *M* NaOAc + *xM* HOAc) were used throughout, the region studied being in the vicinity of the isoelectric point alone. All experiments were conducted at 20°. The data obtained are given in Table V and Fig. 2.

Table V. *Electrophoretic mobilities of fractions A and B*

<i>pH</i>	Mobility cm./sec. 10^{-5}		Direction
	Fraction A	Fraction B	
4.19	9.35	8.47	Cathodic
4.36	5.56	5.88	"
4.66	1.81	—	"
4.67	—	1.68	"
4.97	1.51	2.25	Anodic
5.27	4.19	—	"
5.28	—	4.39	"
5.48	5.80	—	"
5.49	—	5.95	"

Fig. 2. The electrophoretic mobilities of crystallized horse serum albumin fractions. $T = 20^\circ$. Fraction A: $\circ$. Fraction B: $\square$.

An examination of the figure reveals that no significant differences occur in the electrophoretic mobilities of the two fractions over the pH range studied. It remains possible, however, that at pH farther removed from the isoelectric point differences might be found in the mobilities of the fractions although this is not apparent at pH 8.03 [Tiselius, 1937].

The curves obtained during the investigations again showed no sign of heterogeneity in either fraction.

The value obtained for the isoelectric point, pH 4.80, is lower than that found by Tiselius [1930] (pH 4.88) for horse serum albumin, but agrees excellently with an extensive series of results obtained by Pedersen (unpublished) using the same method, and which also indicate an isoelectric point pH 4.80 for horse serum albumin. For a product isolated electrophoretically, Tiselius [1937] reports a value pH 4.64 for the isoelectric point of horse serum albumin, using a modified method. This latter value is not directly comparable as it was determined at $T = 0^\circ$ and at an ionic strength $\mu = 0.1$, whereas for the value reported in this paper $T = 20.0^\circ$ and $\mu = 0.02$, as in Pedersen's measurements.

Diffusion constant measurements¹ and molecular weights.

Determinations of the diffusion constant for each fraction were made by the method of Lamm & Polson [1936]. Two concentrations of protein were used in each case, and the data were analysed by equations (2) and (4a) of Lamm & Polson's paper. The mean values corrected to pure water at 20° are given in Table VI.

Both fractions were shown to be homogeneous, as the normal dispersion curve coincided exactly with experimental curves for each fraction.

No significant differences appear in the diffusion constants for the two fractions. The actual values found are rather lower than those of Lamm & Polson for horse serum albumin, but the preparation used by them was not completely homogeneous. They agree however with the value quoted by Svedberg [1937].

Table VI. *Diffusion constants and molecular weights of fractions A and B*

Fraction	Protein g./100 ml.	D corr. cm. ² /sec. $\times 10^7$	$S_{20} \times 10^{13}$ Mean	M
A	0.425	6.12	4.44	70,400
A	0.850	6.01		
B	0.720	6.28	4.48	70,200
B	1.44	6.01		

$T = 20.0^\circ$.

In Table VI are included also the molecular weights (M) of the two fractions calculated from the equation:

$$M = \frac{RTs}{(1 - V\rho)} \quad [\text{Svedberg, 1925}].$$

The mean values for the sedimentation constants and diffusion constants reported for the fractions in this paper were used, and the partial specific volume was taken as 0.748 in each case.

The values obtained for M agree with those quoted by Svedberg [1937].

¹ I am indebted to Mr A. G. Polson for making these determinations.

DISCUSSION

The two main points arising for discussion would appear to be the relation of the results presented here to the recent findings of Hewitt [1936; 1937], and the significance of the physico-chemical data.

Hewitt has isolated a crystallizable albumin of low carbohydrate content from horse serum and also an amorphous material of high carbohydrate content. These he calls crystalbumin and seroglycoid respectively.

There seems little doubt that the fraction B of this paper and Hewitt's crystalbumin are identical, despite the fact that the nitrogen content of crystalbumin (14.4%) is considerably lower than that found here (15.16%). The latter figure is in much better agreement with the determination of Adair & Robinson [1930] on "crystalline serum albumin", viz. 15.6%. With a molecular weight of 70,200 for this material, a carbohydrate content of 0.083% leads to a value of 58 g. carbohydrate per mol. protein, or about one-third of a hexose residue per mol. protein. The conclusion seems justified that this material contains no carbohydrate residue, but that the orcinol reacts with some grouping other than carbohydrate in this particular protein, under the conditions of the experiment. Glucosamine gives no colour with the orcinol reagent and its presence is not excluded.

The data obtained during the recrystallization of fraction A suggest strongly that it too is a chemical individual. Its carbohydrate content, 1.95% about 8 hexose residues per mol. protein, is considerably lower than that of Hewitt's amorphous seroglycoid.

No quantitative significance is claimed for these carbohydrate values in view of the arbitrary nature of the reference solution.

It has already been pointed out that the mother liquors from crystallization 2 of fraction A have a higher carbohydrate:nitrogen ratio than the crystals. Further, the value of this ratio is greater than that of serum globulin, of which a small amount was shown to be present by ultracentrifugal analysis. Consequently if one accepts the chemical individuality of fractions A and B, at least one more non-dialysable material of high carbohydrate content must be assumed present to account for the facts.

It has been shown [Pedersen & Waldenström, 1937] that bilirubin migrates with serum albumin both in the ultracentrifuge and electrophoretically. In the experiments described here only fraction A was associated with a yellow pigment, probably bilirubin, fraction B being colourless.

It is of considerable interest that no significant differences were observed in the sedimentation constants, diffusion constants and electrophoretic mobilities of the two fractions, which are manifestly chemically differentiated. It would appear that the problem here is beyond the present analytical capabilities of these physico-chemical methods.

A further differentiation between easily soluble and less soluble fractions of crystalline serum albumin is shown by the dielectric constant increment [Oncley & Ferry, quoted by Cohn, 1937], values of 30,000 and 11,000 having been found for such fractions respectively.

Some preliminary measurements on the solubility of fraction B in aqueous sodium sulphate indicate that the quantity of protein dissolved is dependent on the amount of solid phase present. This agrees with the findings of Sørensen [1930], but is surprising in view of the variety of criteria of purity to which the substance has been shown to conform in this paper.

SUMMARY

1. The crystallization and fractionation of horse serum albumin is described, using sodium sulphate as a precipitating agent.
2. Evidence regarding the chemical individualities of two crystalline fractions of horse serum albumin is presented.
3. A survey of some physico-chemical characteristics of the fractions is given and certain limitations of the methods pointed out.

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APPENDIX

ON THE SEROLOGICAL PROPERTIES OF THE ALBUMIN FRACTIONS

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The purpose of the following experiments was to distinguish serologically between the two albumin fractions described above.

Antisera to fraction A (carbohydrate-rich), fraction B (carbohydrate-poor) and, as a control, to a specimen of thrice recrystallized horse serum albumin (from a different animal), were prepared as follows. Three rabbits were used for

each antigen; for 3 weeks the animals received tri-weekly intravenous doses of antigen, starting with a 10 mg. dose and increasing by 10 mg. each week, thus receiving 180 mg. in all. Serum was collected on the 8th day after the last injection, and stored at 2° without preservative.

Ring tests. Tenfold dilutions of antigen were layered on the surface of neat antiserum, and the appearance of a disk of precipitate at the serum-antigen interface noted after about 30 min. at 20°. With all three antisera, the reactions of the heterologous antigens were nearly as marked, as regards degree and end-point, as those of the homologous antigens. In addition, two zones of maximum precipitation occurred in some heterologous and homologous systems. The production of strong antisera to fraction B (carbohydrate-poor) does not confirm the findings of Hewitt [1937].

Bulk tests. The crude ring test results were checked by bulk tests, in which equal volumes of antiserum (1/5) were incubated at 37° with each of a threefold dilution-series of antigen.

Table I shows the optimal flocculating concentration of the antigen: the optima recorded appeared at widely different times, sometimes after 15 min. and sometimes only after 24 hr. incubation.

Table I. *Bulk precipitin tests. Optimal reacting concentrations of three antigens with the three antisera*

Antigens: Recrystallized albumin ... R.		Anti-R ... Antisera		
Carbohydrate-rich fraction ... A.		Anti-A		
Carbohydrate-poor fraction... B.		Anti-B		
Optimal flocculating concentrations of antigen				
Antisera 1/5	Antigen R		Antigen A	Antigen B
	1	1	1 *	1
	320'	38400	5120	80' 40960
	1	1	1	1
Anti-R	200'	25600	540	20' 14580
Anti-A	1	1	1 *	1
Anti-B	100'	6400	3840	20' 4860

* Indicates wide zone of flocculation.

Thick type indicates optima with rapid and copious precipitation.

It will be seen that several of the systems show two clearly marked optimal reacting concentrations of antigen; this may be taken as evidence that, in these at least, more than one antigen-antibody reaction is taking place [cp. Dean *et al.* 1935; Taylor & Adair, 1935; Goldsworthy & Rudd, 1935].

Precipitin absorption tests were then attempted, by absorbing the three antisera with heterologous antigen in a concentration corresponding to the optimum, or, in cases in which two zones appeared, to the optimum occurring with the more concentrated antigen. The mixtures were held at 37° for 20 hr., the precipitates centrifuged down, and the supernatants tested against antigen solutions as in Table II.

It will be seen that as far as these absorption tests go, R and A behave as though they were identical, and B appears to be lacking in an antigenic component possessed by R and A. This result suggests that the carbohydrate partly determines the antigenic specificity of horse serum-albumin (R) and of the

Table II. *Precipitin absorption tests. Supernatants of optimal reactions tested in ring test for specific antibodies*

Antiserum	Absorbed by antigen	Supernatant tested with antigen		
		R	A	B
Anti-R ...	A	-	-	-
Anti-R ...	B	+	+	-
Anti-A ...	R	-	-	-
Anti-A ...	B	+	+	-
Anti-B ...	R	-	-	-
Anti-B ...	A	-	-	-

carbohydrate-rich fraction (A), and though the capacity to stimulate anti-carbohydrate antibodies has not necessarily been wholly lost by the carbohydrate-poor fraction (B), yet it has little power to absorb such antibodies from anti-R and anti-A sera.

Such an interpretation must be accepted with caution, for it fails to account fully for the distribution of double-zones recorded in Table I. The evidence of double zones, and of other tests now in progress, suggests an antigenic pattern of greater complexity.

We desire to thank Prof. A. A. Miles for his help in analysing the results of the serological tests.

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