LII. SEPARATION OF SERUM ALBUMIN INTO TWO FRACTIONS

II. OBSERVATIONS ON THE NATURE OF THE GLYCOPROTEIN FRACTION

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Experiments recently reported led to the conclusion that serum albumin, even when obtained in the crystalline condition, contains considerable amounts of another protein which is removable only with difficulty [Hewitt, 1936]. It would thus appear that material regarded as being reasonably pure and used as the starting point of many investigations was a mixture of different proteins.

A clear-cut separation of the albumin fraction of horse serum into two distinct fractions of widely different chemical and physical properties was described in the former paper. One of the fractions was crystalline serum albumin in a purer state than that usually attained and was virtually free from polysaccharide. Since the total albumin fraction of serum contains considerable amounts of polysaccharide it is not surprising that the second fraction isolated contained a glycoprotein, the identity of which was not established.

In view of the possible importance of these observations the experiments now described were carried out in order to obtain further evidence in support of the conclusions reached and to collect more information about the glycoprotein present in the second fraction of serum albumin.

Nomenclature

Lack of a clearly defined nomenclature leads to difficulties in discussing these proteins. The term albumin, derived originally from egg white, has been applied to any protein material but its use is now generally restricted to proteins soluble in 50% but insoluble in 100% saturated ammonium sulphate solution. There is, however, no name available except albumin to apply to the crystallizable carbohydrate-free protein present in the albumin fraction of serum. The description crystalline serum albumin, besides being cumbrous is not necessarily accurate since the protein can be precipitated in a non-crystalline condition and it is indeed generally encountered in solution. It would appear therefore that there is need for a new word to describe this protein. The name should suggest the crystallizability of the protein and its occurrence in the albumin fraction. It is suggested that *crystalbumin* might be appropriate, and for convenience this term will be used throughout this paper.

In a later section the identity of the second protein present in the albumin fraction is discussed but in the earlier sections it is referred to in general terms as a glycoprotein.

Experimental

The general methods used were similar to those previously described [Hewitt, 1936]. Horse plasma was the source of the proteins employed. Protein determinations were carried out by the micro-Kjeldahl method and in view of the

varying N contents of different fractions a conversion factor of 7·0, corresponding to a protein-N content of 14·3 %, was used throughout.

Carbohydrate determinations were based on the methods of Tillmans & Philippi [1929] and Sørensen & Haugaard [1933] with modifications that were found to be improvements. The reagents used were 60 % (by volume) H_2SO_4 and 1·6 % orcinol in 30 % H_2SO_4; and a solution of equal parts of galactose and mannose was used for comparison. 1 ml. of solution, corresponding to 0·02–0·2 mg. of carbohydrate, was heated with 2·5 ml. of 1·6 % orcinol solution and 15 ml. of 60 % H_2SO_4 in a 7 × 1 in. boiling-tube in a water-bath at 80° for 20 min. At the end of this time the tube was plunged into cold water. Colorimetric comparison was carried out by measuring the extinction coefficients of 20 mm. layers in a Stufen-photometer using the blue (470 mμ) and the green (530 mμ) filters. It is necessary to take certain precautions to obtain reproducible results. A curve must be constructed relating the extinction coefficients to the carbohydrate content, and the solutions must be shielded from light in order to avoid photochemical changes. The use of "blank" corrections by heating the protein and sulphuric acid alone cannot be justified, since humin formation proceeds differently in the presence and absence of orcinol. On the basis of the work of Fränkel & Jellinek [1927], Levene & Mori [1929], Rimington [1929; 1931], Sørensen & Haugaard [1933] and Bierry [1934] it is assumed that the polysaccharide present in the proteins is galactose-mannose-glucosamine (g.m.g.) and the calculations are based on this assumption.

Since the work previously reported five batches of albumin, each obtained from 25 litres of horse plasma, have been fractionated. Details are omitted in order to save space but it may be stated that the previous results have been confirmed.

By repeated recrystallization crystalbumin is obtained containing only traces of carbohydrate (less than 0·1 % in the twelve times recrystallized specimens). The crystalbumin fractions are sharply differentiated from the glycoprotein fractions which contain upwards of 4 % of polysaccharide. It now remained to obtain the glycoprotein as pure as the nature of the material would allow, and a series of fractionations with this object in view was started, the polysaccharide content being taken as the main index to the course of the fractionation. It is more difficult, in general, to purify the more soluble constituent of a mixture than the less soluble and the tenacity with which the fractions appeared to adhere made their separation more difficult than is usual even with proteins.

Fractionation methods

Fraction S had the highest polysaccharide content (9·5 %) of the fractions mentioned above, and it may be stated at once that it was found difficult to raise the polysaccharide content of any fraction much above this figure. This fraction had remained in solution after removal of the crystalline fraction and was reprecipitated with $\frac{2}{3}$ saturated $(NH_4)_2SO_4$. It is not necessary to detail the many fractionations which failed to yield a fraction of appreciably higher polysaccharide content than S, but mention may be made of the highest content reached in any salting-out refractionation process. The original protein (B) had 7·3 % polysaccharide and precipitation with $\frac{2}{3}$ saturated $(NH_4)_2SO_4$ separated it into one fraction (63 mg.) containing 10 % polysaccharide and a second fraction (107 mg.) containing only 5·7 % of g.m.g. No fractionations with $(NH_4)_2SO_4$ were successful in raising the polysaccharide content above 10 %.

Fractionation with organic solvents. The glycoprotein was precipitated fairly readily by ethyl alcohol and this suggested a possible method of effecting

fractionation. Chilled protein solution was added to an equal volume of chilled alcohol. The precipitate was centrifuged, redissolved in water and reprecipitated with alcohol. In this way the polysaccharide content of the fraction (H) was raised from 8·5 to 9·6 %. Attempts to obtain a more considerable separation of fractions were unsuccessful. Precipitation with methyl alcohol and acetone failed to provide a satisfactory method of fractionation.

Alum precipitation. Albumin is precipitable by potassium alum and attempts were made to utilize this fact in a fractionation process. 1·5 ml. of 5 % potassium alum were added to 9 ml. of 0·4 % protein solution (fraction H) and maximum precipitation was obtained by adding 3·3 ml. of 0·1 *N* NaOH. The precipitated protein contained 8·6 % of polysaccharide and the unprecipitated protein 7·1 %. When the precipitation zone was approached from the alkaline side, by adding the alkali to the protein before the alum solution, the fractionation was unsuccessful, the precipitated and unprecipitated proteins having closely similar polysaccharide contents.

Trichloroacetic and tungstic acids. A fairly high concentration of trichloroacetic acid is necessary to precipitate the glycoprotein from dilute solutions, but in 1 % protein solutions the addition of 2·2 % trichloroacetic acid did not give any consistent fractionation. The use of tungstic acid was equally unsuccessful.

Heat-coagulation. It was reported in the previous communication that whilst crystalbumin coagulates quickly on heating at the isoelectric point at about 60°, the glycoprotein fractions, on the other hand, coagulate only with difficulty and at temperatures above 80°. Attempts to separate the fractions by heating at an intermediate temperature were unsuccessful, the coagulable protein carrying down with it the glycoprotein. The heat-coagulation phenomena in the glycoprotein fractions have now been studied under a variety of experimental conditions. Solutions of the glycoprotein fractions B and H containing approximately 1 % of protein were adjusted to *p*H 4·8 and heated in a boiling water-bath. As only very slight coagulation was observed, dilute alkali and acid were added to the hot solution and a considerable coagulation occurred. In neither case were the polysaccharide contents of the precipitated and unprecipitated proteins appreciably different. In another experiment the experimental conditions were the same but a protein solution of less than 0·1 % was employed. In this case the polysaccharide content of the uncoagulated protein reached nearly 11·5 %.

The conditions for obtaining satisfactory coagulation were now studied. Fraction S was diluted with physiological saline until it contained about 0·15 % of protein. 10 ml. portions were transferred to eight tubes and each was adjusted to a different *p*H by addition of dilute acid or alkali. No buffer salts were used owing to the confusing effects of different buffer solutions [Hewitt, 1929]. The *p*H values varied from 4·0 to 8·1. The tubes were immersed in a boiling water-bath for 10 min. but only one of the tubes showed any coagulation and this was very slight. The sample in which coagulation had occurred had been adjusted to *p*H 4·6 before heating and was at *p*H 5·0 after heating. The other tubes showed varying degrees of opalescence, this being greatest in those tubes adjacent to *p*H 4·8 and least in those furthest removed from this *p*H. The samples were cooled and each was adjusted to *p*H 4·7 or 4·8. No precipitation occurred and the tubes were immersed in a boiling water-bath for a second time for 5 min. Coagulation now occurred in varying degree in every tube except the one previously heated at *p*H 4·0, which merely developed a slight opalescence. The amount of precipitation was greatest in the tube originally heated at *p*H 6·1 and least in the tubes furthest removed from this *p*H. The polysaccharide con-

tents of the proteins remaining uncoagulated were determined and the results of the experiment are summarized in Table I.

Table I. *Effect of heating glycoprotein solutions containing 1·38 mg. of protein per 1 ml., first at different* p*H levels and then at* p*H 4·8*

Sample no.	*p*H of first heating	Opalescence after first heating	Protein coagulated after second heating mg./ml.	Polysaccharide content of uncoagulated protein %
1	8·1	–	0·03	9·3
2	7·1	–	0·12	10·2
3	6·6	–	0·45	11·7
4	6·1	–	0·48	10·8
5	5·9	∓	0·39	11·4
6	5·6	±	0·09	10·1
7	5·0	+	0·05	9·8
8	4·1	–	0	9·3

It will be seen that the highest polysaccharide contents (about 11 %) are reached in the samples originally heated at *p*H values between 5·8 and 6·6, and the lowest polysaccharide contents in the samples heated at *p*H values furthest removed from this range. The proteins remaining uncoagulated in samples 3, 4 and 5 are, judging from their high polysaccharide content, as nearly pure specimens of the glycoprotein as any so far obtained and repetition with other samples of glycoprotein gave similar results, but there are several further points of interest in the experiment.

In order to obtain maximum coagulation in these glycoprotein solutions it was necessary to heat at two different *p*H levels, both quite definitely fixed. The coagulable protein and the non-coagulable glycoprotein seem to be combined to some extent and the first heating (at *p*H 6·0) is necessary to effect dissociation of the complex, whilst the second heating (at *p*H 4·8) coagulates the protein dissociated during the first heating. In more concentrated solutions dissociation of the complex does not occur and the glycoprotein is carried down in the coagulum, as described previously.

Cystine determinations

The cystine contents of the fractions have proved of great value in considering their identity. The method used for determining cystine was based on Tompsett's [1931] useful modification of Folin & Marenzi's [1929] method and can be applied to quantities of protein as small as 50 mg. From 10 to 20 ml. of pure concentrated HCl are placed in a 100 ml. Kjeldahl flask and heated to boiling point. The protein solution (1–5 ml. containing 40–120 mg. of protein) is pipetted directly into the boiling acid. The flask is heated on an electric sand-bath, the contents being kept just boiling for 18 hours. The hydrolysate is then evaporated to dryness *in vacuo*, the residue is dissolved in water and the volume is made up to 25 ml. in a graduated flask. Humin is filtered off and an aliquot part of the filtrate is taken for analysis. In the present experiments 1–6 ml. of filtrate were used for each determination, the final volume of the colorimetric solutions being 25 ml., and 0·2–0·5 mg. of cystine was used for comparison. To each flask 0·8 ml. of 20 % Na_2SO_3 was added and after 2 min. 5 ml. of 8 % $NaHCO_3$ and then 2 ml. of the uric acid reagent (free from phenol reagent) of Folin & Marenzi [1929]. The colour develops rapidly and after

10 min. the extinction coefficients of the solutions are measured in a Stufen-photometer, 20 mm. layers of solution being used. When the red filter S 72 (approximate wave-length 720 mμ) is used the extinction coefficients bear an almost linear relationship to the amount of cystine used for analysis. The brown colour of the glycoprotein hydrolysates tends to make ordinary colorimetric comparison of the blue colour developed very difficult, as will be seen from Table II which gives the extinction coefficients of the colorimetric solutions used during the analysis of the hydrolysates of a glycoprotein (S) and of a crystalbumin. The figures given by 0·4 mg. of cystine are included for comparison.

Table II. *Extinction coefficients (2 k.) of 20 mm. layers of solutions used for cystine determinations*

Filter	Colour	0·4 mg. cystine	Glycoprotein	Crystalbumin
S 43	Blue	0·16	0·22	0·23
S 47	Blue	0·19	0·22	0·29
S 50	Green	0·25	0·24	0·36
S 53	Green	0·36	0·35	0·53
S 57	Yellow	0·51	0·44	0·72
S 61	Red	0·66	0·55	0·93
S 72	Red	1·08	0·83	1·40
S 75	Red	1·10	0·84	1·45

In the case of the glycoprotein, comparison of the coefficients for the red filter S 72 gives a cystine equivalent of 0·31 mg. but using blue light (filter S 43) a figure of 0·55 mg. is obtained owing to the yellow colour of the hydrolysate itself. In the case of the crystalbumin the discrepancy using the different coloured lights is much less, the figures being 0·52 and 0·57 mg. respectively. This last fact illustrates the great difference in behaviour of the glycoprotein and crystalbumin when heated with concentrated HCl. The glycoprotein developed a deep brown colour and deposited a black precipitate of humin whilst the crystalbumin remained almost colourless.

The results of the cystine determinations of various fractions are given in Table III, in which are included also the polysaccharide contents for comparison.

Table III

Fraction	Polysaccharide content %	Cystine content %
E	0·05	5·8
M	0·09	5·4
R	4·3	4·1
N	5·6	2·4
K	6·6	2·3
H	8·5	2·1
B	7·3	1·9
S	9·5	1·8

In each case the crystalbumins with low polysaccharide contents have a high cystine content whilst the glycoproteins have low cystine contents. Not only is this of interest in demonstrating once more the contrasting properties of the two proteins but it enables certain conclusions to be drawn concerning the nature and composition of the glycoprotein fraction. This last point is discussed in the next section.

Discussion

Repeated experiments confirm the conclusion previously reached that by simple recrystallization serum albumin may be obtained practically free from polysaccharide. The importance of this lies in the fact that nearly every specimen of crystalbumin regarded by investigators as materially pure must have contained appreciable amounts of another protein and many of the properties described are those of a mixture of proteins.

In the literature the polysaccharide content of the albumin used has not in many cases been reported but where it is given it is frequently considerable. Dische & Popper [1926] describe serum albumin as containing 1·08% of carbohydrate, Rimington [1929; 1931] gives a carbohydrate content of about 2%, the figures of Lustig & Haas [1931] are between 0·47 and 0·65%, Sørensen & Haugaard [1933] give 0·47% for one specimen and a very low figure for another, and the present author [1934] found contents between 0·29 and 0·78% for crystalline albumins and up to 4·4% for other fractions. Since it has been shown in the two papers of this series that the polysaccharide content of purified crystalbumin can be reduced to values below 0·1%, it is evident that appreciable amounts of glycoprotein must be present in specimens of albumin not subjected to careful repurification processes.

Nature of glycoprotein

The identity of this glycoprotein must now be considered. The only known serum protein which it resembles is serum mucoid which has a high carbohydrate content and is not coagulated on heating [Zanetti, 1897; Bywaters, 1909; Rimington, 1931]. The resemblance is, however, only superficial and the fact that the glycoprotein is different from mucoid becomes clear on closer inspection.

Although, when nearly pure the glycoprotein now investigated may be heated in dilute solution without being coagulated, when it is heated in the presence of appreciable amounts of coagulable proteins it is carried down with the coagulum. Serum mucoid, on the other hand, remains in solution when serum is heated and it is, in fact, prepared by this means.

Serum mucoid contains 25% of polysaccharide [Rimington, 1931], but, despite the use of a wide variety of fractionation methods it has not been possible to obtain the present glycoprotein with a content of more than 10 or 11% of polysaccharide. Consideration of the cystine content of the glycoprotein proves that it cannot be serum mucoid. As an example we may consider fraction S which had a carbohydrate content of 9·5%. If the carbohydrate present were due to serum mucoid (containing 25% carbohydrate) then there is present 38% of serum mucoid and 62% of crystalbumin. The cystine content of crystalbumin was found to be 5·8%. The cystine content of the glycoprotein must be the sum of the cystine contents of its constituents. Therefore, even assuming that the serum mucoid contributes no cystine at all, the crystalbumin present necessitates a cystine content of at least $\frac{62}{100} \times 5{\cdot}8 = 3{\cdot}6\%$ in fraction S. Actually, however, this fraction contains less than 1·8% of cystine, so that it is impossible for the glycoprotein to be serum mucoid. In fact no protein so far described has the properties of this glycoprotein, and one is forced to the conclusion, supported by all the available evidence, that this is a new serum protein, to which perhaps the name seroglycoid may be provisionally applied.

The properties of seroglycoid are different in almost every respect from those of crystalbumin, since it has a high polysaccharide content (at least 10% calculated as g.m.g.), a lower N content, a lower Van Slyke amino-N figure, different titration curves, a lower rotatory power, difficult heat-coagulability, higher tryptophan content and lower cystine content. In its amino-acid distribution seroglycoid resembles globulin rather than albumin but it is not, of course, precipitable by 50% saturated $(NH_4)_2SO_4$. As far as the quantity of the protein in serum is concerned the following rough figures may be taken as an indication of the composition of horse serum: 3·6% globulin, 2·8% crystalbumin, 0·3% seroglycoid, 0·05% serum mucoid.

Summary

1. The observation is confirmed that crystalline serum albumin (crystalbumin) when pure is free from polysaccharide but as usually prepared it contains varying amounts of a glycoprotein.

2. The identity of this glycoprotein is discussed and evidence is adduced that it is a new serum protein for which the name seroglycoid is provisionally suggested.

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