VII. PREPARATION AND PROPERTIES OF A GLOBULIN PRESENT IN THE ALBUMIN FRACTION OF SERUM

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(*Received 30 November 1937*)

It is now generally accepted that the albumin fraction of blood serum consists of a mixture of proteins and recent chemical and immunological work [Hewitt, 1934; 1936; 1937] has led to the recognition of the presence of crystalbumin, seroglycoid and a globulin in addition to traces of a mucoid. Some characteristic properties of crystalbumin and seroglycoid have been described in previous papers and it is proposed to deal in this communication with the separation of the globulin. This globulin is of interest not only from the point of view of the purification of crystalbumin but also in view of its bearing on the question of the existence of different serum globulins.

Many workers have advanced the view that all globulins have fundamentally the same structure with minor modifications of a physical or extraneous character accounting for the observed divergencies in the behaviour of the different globulin fractions that have been isolated. The diverse properties of euglobulin and pseudoglobulin, for example, have been ascribed to different amounts of lipin adsorbed by the proteins [Chick, 1914], it being assumed that the globulins are otherwise structurally identical. This view is in conformity with the similar amino-acid analysis recorded for the two globulins [Hartley, 1914]. On the other hand it has been found that even after the removal of the lipins the solubilities and optical rotatory powers of euglobulin and pseudoglobulin remained different [Hewitt, 1927]. Various analytical differences between various globulin fractions are described by Lustig & Haas [1931] and differing carbohydrate contents have also been observed [Hewitt, 1934]. Sørensen [1925] regards the globulin fraction as a labile association of euglobulin and pseudoglobulin which dissociates during fractionation processes. The ultracentrifuge has enabled Mutzenbecher & Svedberg [1933] to postulate the existence of different globulins and this has recently been confirmed by Tiselius [1937] using an electrophoretic method. As an example of an opposing point of view serum has been regarded as containing a single protein called "orosin" [Block, 1934].

When a question is open to such divergent views further experimental evidence is obviously desirable and it is possible that eventually different viewpoints may be united. Differences between globulins may in some cases be due to minor modifications such as denaturation, etc., whilst other divergencies may be traced to definite variations in chemical structure. Various globulin fractions have been examined with these objects in view.

Experimental

Horse serum or plasma was the source of the proteins investigated, except in a few experiments referred to later in which ox serum was used. Nitrogen determinations were carried out by the micro-Kjeldahl method and carbohydrate

determinations by the Sørensen & Haugaard [1933] modification of the Tillmans & Philippi [1929] method. For the purposes of calculation it is assumed that the polysaccharide present in serum proteins contains equimolecular amounts of galactose and mannose, and results are quoted in terms of galactose-mannose (g.-m.).

Globulin from the albumin fraction

Serum or plasma was diluted with an equal volume of water, treated with sufficient saturated $(NH_4)_2SO_4$ was added to render the mixture 52% saturated and filtered. Crystalbumin crystallized out when the filtrate was adjusted to *p*H 4·7 by the cautious addition of acetic acid. The amount of precipitate was in some cases increased by the addition of a further small amount of $(NH_4)_2SO_4$. After being kept overnight the precipitate was filtered off, leaving the bulk of the seroglycoid in the filtrate which was discarded. A further small amount of seroglycoid was removed by dissolving the precipitate in water and reprecipitating by cautiously adding $(NH_4)_2SO_4$ until crystallization commenced. The precipitate was filtered off after a short time and the filtrate was again discarded. The precipitate was now dissolved in water and the solution adjusted to *p*H 7·0 by addition of *N* NaOH. To the neutral solution an equal volume of saturated $(NH_4)_2SO_4$ was added. This yielded a copious precipitate, although, before the removal of seroglycoid, all the fraction had been soluble in half-saturated $(NH_4)_2SO_4$. The globulin precipitate was filtered off, leaving the bulk of the crystalbumin in solution. Incidentally it may be mentioned that this process provides an excellent method of preparing pure crystalbumin since one or two crystallizations of the protein in the filtrate yield a very pure specimen.

The globulin precipitate was dissolved in water, reprecipitated by half-saturation with $(NH_4)_2SO_4$, filtered off and the process repeated once or twice. Dialysis to remove salts completed the preparation of the protein. The immediately characteristic property of this fraction is its high carbohydrate content. This is about 5% calculated as galactose-mannose, corresponding to some 7·5% of polysaccharide assuming that this has the constitution galactose-mannose-glucosamine commonly assigned to the serum protein polysaccharides. As will be seen in a later section this polysaccharide content is much higher than has been observed with any other globulin and it differs in other respects. In order to distinguish it therefore it is necessary to find a term which, without prejudice to any consideration of its structure, will suggest briefly its behaviour as a globulin and its high carbohydrate content; it is tentatively suggested that "*globoglycoid*" may be a suitable term.

Globoglycoid has been obtained from normal horse serum, from the blood serum of horses immunized against diphtheria and scarlet fever toxins and from normal ox serum. The amount isolated has been of the order of 1 g. from 1 litre of serum. This amount was of course obtained from the albumin fraction and there is probably a further quantity present in the main globulin fraction. It seems probable that globoglycoid is of general occurrence. Other globulin fractions have been prepared for comparison; these are described in the following section.

Serum globulin fractions

The total globulin precipitate obtained by half-saturation of serum with $(NH_4)_2SO_4$ was purified by dissolving in water and reprecipitating with 50% saturated $(NH_4)_2SO_4$. After several repetitions of this process the globulins were fractionated in various ways, including precipitation with varying concentrations of $(NH_4)_2SO_4$ or NaCl and by isoelectric precipitation. Three main types

of fraction may be distinguished, the pseudoglobulin fractions soluble in distilled water at all *p*H values between 4 and 8, the euglobulin I fractions insoluble in water at *p*H 7 and euglobulin II which is soluble in water at *p*H 7 but insoluble at *p*H 6.

The variations in carbohydrate content of the different fractions are quite considerable as shown in the following table.

Table I. *Carbohydrate contents of various globulin fractions*

	g. of galactose-mannose per 100 g. of protein			
	Euglobulin I	Euglobulin II	Pseudoglobulin	Globoglycoid
Least soluble	1·8	2·2	1·4	4·6
Intermediate	2·4	2·8	1·7	
Most soluble	2·9	3·6	2·3	

The more soluble fractions tend to have the higher carbohydrate content but in no case is the high value of globoglycoid reached. Apart from differences in precipitability and carbohydrate content the main characteristics of the fractions were as follows. Euglobulin I required a salt concentration of well over 1% before it was dissolved and even then gave a turbid solution; euglobulin II dissolved readily in 0·9% NaCl giving a clear greenish-blue solution; the pseudoglobulin was, of course, soluble in water, whilst globoglycoid although soluble gave a slightly opalescent solution in water.

The antitoxic properties in the sera of horses immunized with diphtheria toxin are carried almost exclusively by the water-soluble globulins and the distribution in the case of a batch of antitoxic sera is shown in Table II.

Table II. *Diphtheria antitoxin distribution*

	Total antitoxin content A.U. $\times 10^4$	Purity of antitoxin A.U. per 1 mg. protein	Flocculation time under standard conditions min.
Pseudoglobulin, sparingly soluble fraction	3·2	5·4	25
Pseudoglobulin, main bulk	5000	20·0	68
Globoglycoid	27·4	5·5	175

It will be seen that only a very small amount of antitoxin is carried by globoglycoid and its purity, in terms of the number of units of antitoxin in a given weight of protein, is little more than a quarter that of the main pseudoglobulin fraction. The rapid flocculation of the sparingly soluble fraction and the slower flocculation of the more soluble fractions have been described in a previous communication [Hewitt, 1934].

Amino-acids

Cystine and tyrosine determinations, carried out as described previously [Hewitt, 1934; 1936; 1937], revealed no great difference between globoglycoid and the other globulin fractions but the tryptophan figures are very characteristic. The tryptophan contents of the globulin fractions ranged from 2 to 3% in conformity with previous figures [Hewitt, 1934; Holiday, 1936] but the value for globoglycoid was much lower, being in the neighbourhood of 0·8%.

This low tryptophan content, less than half that of other globulins, may account for the behaviour of globoglycoid when hydrolysed with acids. The amount of humin formed is much less with globoglycoid than with the other

globulins when heated with mineral acids. It will be recalled that crystalbumin gives no humin when hydrolysed with acids, owing, it was suggested, to the absence of carbohydrate, and it is interesting that globoglycoid despite its high carbohydrate content should yield so little humin. This strengthens the view that tryptophan is of importance in this respect and that tyrosine and cystine play little part.

Discussion

The presence of a globulin in the albumin fraction of serum has been detected immunologically [Hewitt, 1937] and by an electrophoretic method [Tiselius, 1937], and this globulin fraction has now been separated. When examined it is found to exhibit characteristic properties differentiating it from other globulin fractions. It has an extremely high polysaccharide content, probably about 7·5% calculated as galactose-mannose-glucosamine, and to distinguish it from other globulins the name globoglycoid is tentatively suggested. As observed in the case of seroglycoid and ovomucoid the high polysaccharide content tends to confer increased solubility and decreased precipitability compared with other proteins. Globoglycoid requires a higher concentration of $(NH_4)_2SO_4$ to precipitate it than other globulins but it is precipitated by 2% trichloroacetic acid and is coagulated when heated in boiling water, unlike pure seroglycoid or ovomucoid.

A further striking characteristic of globoglycoid is its low tryptophan content which is less than half that of the other globulins. This low tryptophan content is reflected in a decreased tendency to humin formation when heated in acid solution.

In serum from horses immunized with diphtheria toxin only some 0·5% of the antitoxin is found in the globoglycoid and the potency measured in antitoxic units per g. of protein is much less than in the main pseudoglobulin fraction. The phenomenon previously observed [1934] of the rapid flocculation of the antitoxin present in the sparingly soluble pseudoglobulin fractions and the slower flocculation in the more soluble fractions is confirmed but no explanation is forthcoming.

An immediate practical application of the observation of the properties of globoglycoid is the facilitation of the preparation of pure crystalbumin which is frequently required for physico-chemical or immunological experiments.

Incidentally to these observations on globoglycoid some evidence becomes available as to the essential uniformity or multiplicity of the serum globulins. The carbohydrate content of serum globulin was found by Rimington [1931] to be about 2%, by Sørensen & Haugaard [1933] to be 1·8% and by the present author [1934] to range from 1·5 to 3·0% in different fractions (all calculated as galactose-mannose). It is of interest also to note that globulins of different carbohydrate contents are separated by electrophoresis, but Tiselius [1937] states that his figures are of comparative value only. These are 2·2, 0·7 and 0·4% respectively for the β-, γ- and α-globulins. In the present series of observations the carbohydrate contents ranged in the case of pseudoglobulin from 1·4 to 2·3%, the low values being associated with the sparingly soluble fractions, and for the euglobulins from 1·8 to 3·6%.

If it be conceded that the polysaccharide present is an intrinsic part of the protein molecule, and its firm attachment and difficult removal make this conclusion appear probable, then the evidence offered by the different carbohydrate contents seems to show quite definitely that chemically distinct proteins occur in the globulin fraction of serum. The low tryptophan and high carbo-

hydrate contents of globoglycoid suggest that it is different from the main bulk of the globulin fraction.

Doladilhe [1936] has commented on the different physical properties of the euglobulins insoluble in distilled water at *p*H 7 and at *p*H 6 respectively. Difficulties arise in considering all such evidence owing to the susceptibility of proteins to denaturation with consequent modification of properties. Considerable differences in chemical composition, however, can hardly be due to minor denaturation changes, and it is difficult to envisage the range of denaturation effects extending to profound hydrolyses of the protein molecule in the absence of any drastic reagent or *p*H change or any considerable rise in temperature. Nevertheless it is necessary to be wary in accepting evidence of differences between different proteins unless denaturation effects can be definitely excluded, especially when mixtures of proteins are being considered.

It should be emphasized that the term globulin in this paper is used in its customary sense of any protein fraction precipitable by half-saturated $(NH_4)_2SO_4$. This is, of course, an arbitrary classification and may lead to difficulties but its use in this sense is retained in view of its general acceptance.

Summary

1. A globulin fraction tentatively named globoglycoid has been separated from the albumin fraction of blood serum.

2. Removal of globoglycoid facilitates the preparation of pure crystalbumin.

3. Globoglycoid has a high carbohydrate and a low tryptophan content.

4. Both euglobulin and pseudoglobulin are separable into fractions of different carbohydrate content.

5. The evidence available is discussed in relation to the question of the existence of different serum globulins.

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