CCI. SERUM PROTEINS IN NORMAL AND PATHOLOGICAL CONDITIONS

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Although Loeb [1922] has shown that the properties of proteins need not be described vaguely as complex colloidal phenomena but that ordinary physico-chemical principles provide a basis for adequate explanation, and although chemical methods have been successfully applied in demonstrating that other biologically important substances are compounds of definite chemical constitution, nevertheless, in the case of proteins their complexity has led to the tendency to place them in a class apart. Blood serum, for example, is sometimes regarded as a single complex protein incapable of being separated into fractions of definite chemical composition, and any fractions that are obtained are regarded as artefacts [Block, 1934]. An alternative suggestion is that serum consists of innumerable fractions differing only slightly from one another or which are even interconvertible.

These views, which would render the chemical investigation of proteins of very limited value, have not been accepted in this or preceding communications but, as a working hypothesis, it has been assumed that the serum proteins consist of a limited number of chemical individuals of reproducible properties. The objects of the investigation, therefore, have been to prepare fractions as pure as possible, to seek means of identifying individual proteins and to obtain criteria of purity of each.

Although the albumin fraction of serum has been generally regarded as consisting essentially of a single protein, nevertheless as early as 1884 Halliburton, on the basis of coagulation temperatures, suggested that albumin did not consist of a single homogeneous fraction. The same conclusion was reached by Sørensen [1930] on the basis of solubility measurements and by Hewitt [1934] as the result of carbohydrate determinations and it was eventually possible [1936; 1937; 1938] to separate from the albumin fraction of serum three distinct proteins, crystalbumin, seroglycoid and globoglycoid, which are distinguishable by biological, chemical and physical methods.

Chemical and physical evidence suggests that serum globulin is a mixture of different proteins [Hewitt, 1927; 1934; 1938; Lustig & Haas, 1931; Tiselius, 1937; Stenhagen, 1938] and this communication describes attempts to carry further the identification of chemically individual proteins in the blood serum of various animals and in various body fluids of human patients.

I. THE BLOOD SERUM OF NORMAL ANIMALS

The isolation and properties of crystalbumin and seroglycoid, the main constituents of the albumin fraction, have been described previously and attention in this section is directed mainly to the globulin fractions.

In a former communication [1938] two euglobulins, designated I and II respectively, were described in the case of horse serum. Euglobulins of similar characteristics have now been obtained from human, ox and rabbit sera. Euglobulin-I separates out first when globulin fractions are dialysed in neutral solution; it is obtained in the form of a white precipitate requiring a fairly high concentration of salt to render it soluble. Euglobulin-II is precipitated when the solution from which euglobulin-I had been precipitated is either brought to *p*H 6 or else is subjected to prolonged dialysis against distilled water to remove traces of salt. Euglobulin-II is obtained in the form of a semi-liquid precipitate frequently greenish-blue in colour and it is soluble in the presence of traces of salts. In many respects it resembles the "viscous protein" of Doladilhe [1936] and its composition will be dealt with in later sections of this paper.

The water-soluble globulins or pseudoglobulins may be separated into fractions of varying properties, the fraction most readily precipitated by $(NH_4)_2SO_4$, Na_2SO_4 or NaCl having the lowest carbohydrate content [Hewitt, 1934; 1938]. The precipitin reaction of this fraction has been studied by Kendall [1937] who calls it water-soluble euglobulin or α-globulin. Since euglobulins are insoluble in water and since the latter name may be confused with α-globulin of Tiselius [1937], it is considered preferable to call the fraction *pseudoglobulin-A*.

The main bulk of the pseudoglobulin-A fraction is precipitated by one-third saturation with $(NH_4)_2SO_4$, or by saturation with NaCl, but considerable amounts remain unprecipitated and nearly all the globulin fractions so far examined contain a certain amount of this protein. It is precipitated by comparatively low concentrations (about 20 %) of alcohol in the absence of salt but contrary to common expectation higher concentrations of alcohol are necessary in order to effect precipitation in the presence of small amounts of salt.

The main pseudoglobulin fraction is not precipitable by one-third saturated $(NH_4)_2SO_4$ but is precipitated when the $(NH_4)_2SO_4$ concentration is raised to 50 % saturation. After dialysis to remove salts and euglobulins, the pseudoglobulin remaining in solution, as will be shown later, appears to consist of at least three different proteins, including pseudoglobulin-A and globoglycoid.

Globoglycoid is a globulin fraction first obtained from the albumin fraction of horse serum [Hewitt, 1938]. After removal of the main bulk of the seroglycoid the crystalbumin frequently contains considerable amounts of globoglycoid which may be separated by neutralizing the solution and precipitating with 55 % saturated $(NH_4)_2SO_4$ solution. It is now found that the serum globulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ is rich in globoglycoid, and a similar fraction was obtained by precipitation between the limits 20 and 22 % Na_2SO_4 [Hewitt, 1934]. Removal of other albumin and globulin fractions from globoglycoid is difficult and the difficulties are enhanced by the accumulation of lipins in the fraction. In the case of human, ox and rabbit serum globoglycoids the solutions are frequently milky in appearance whilst even with horse serum globoglycoid the protein is difficult to centrifuge down owing to the presence of fatty matter. As will be shown later globoglycoid is of importance from the point of view of euglobulin formation.

It is not easy to make quantitative determinations of the distribution of the various proteins in serum by fractionation processes, since the repeated reprecipitations necessary to effect purification result in large and unpredictable losses. In addition, as will be shown later, figures for euglobulin depend upon a variety of experimental conditions so that figures in the following table must be regarded as approximate only, but they serve to show the differences between the serum protein distributions of different animal species.

Table I. *Distribution of serum proteins in blood of different animals*

Results are given in g. per 100 g. total protein.

	Horse	Ox	Rabbit	Human
Euglobulin	12	17	8	17
Pseudoglobulin	46	28	16	22
Crystalbumin	33	47	66	53
Seroglycoid	9	8	10	8

In the serum of a horse immunized with diphtheria toxoid the crystalbumin content fell from 33 to 13 % of the total with a corresponding increase in the globulin fractions. Pathological changes in human serum are dealt with in a later section.

The value of the carbohydrate content in distinguishing protein fractions has been indicated in previous papers [1934; 1936; 1937; 1938]. Crystalbumin has been shown to be carbohydrate-free and seroglycoid to contain about 7 % of galactose-mannose [1936; 1937]. In the case of rabbit serum as mentioned previously [1937] the solubility relations of the constituents of the albumin fraction are such that separation is sometimes difficult as mixtures of proteins tend to be precipitated together. Some specimens of seroglycoid have a carbohydrate content appreciably greater than 7 % owing to the presence of mucoid which is readily separated by reprecipitation processes.

It is, however, the carbohydrate contents of the globulin fractions with which we are chiefly concerned at present and some of the results obtained are summarized in Table II. The modification of the Sørensen & Haugaard [1933] method previously described was used except that for colorimetric comparison, in place of the step-photometer, a photoelectric Spekker absorptiometer with filter 5 was used for some of the measurements.

Table II. *Carbohydrate contents of globulin fractions*

g. of galactose-mannose per 100 g. of protein.

Fraction	Horse	Ox	Rabbit	Human
Pseudoglobulin-A	1·4	1·4	1·5	1·5
Main pseudoglobulins	2·4	3·6	2·5	3·4
Globoglycoid	5·6	4·9	4·1	5·4
Euglobulin-I	1·8	2·1	2·6	2·8
Euglobulin-II	3·6	3·4	3·2	4·0

Just as in the cases of crystalbumin and seroglycoid the carbohydrate contents of the corresponding globulin fractions of the different animal sera resemble one another but there are several points of additional interest.

In each case pseudoglobulin-A has the lowest carbohydrate content, about 1·4 %, and preliminary experiments indicate that further purification processes, although leading to considerable losses of the material, may reduce the carbohydrate content even further. The main pseudoglobulin fractions of human and

ox serum have a higher carbohydrate content than those of horse serum probably owing to the smaller proportion present of pseudoglobulin-A with its low carbohydrate content.

Composition of euglobulin-II

Some specimens of pseudoglobulin-A and the main pseudoglobulin fraction when mixed together produce a precipitate and Kendall [1937] suggests that euglobulin is a mixture of pseudoglobulins. The matter is, however, a rather complicated one.

As mentioned above and in a previous communication [1938] there appear to be two distinct euglobulins and it is euglobulin-II of which the properties and behaviour suggest that it may be composed of mixed water-soluble pseudoglobulins. Furthermore, experiments have so far failed to produce euglobulin precipitates when horse, ox or rabbit serum protein fractions were mixed and there has been success only when human serum fractions participate in the reaction, although as will be seen later there is evidence that horse serum euglobulin-II contains pseudoglobulin-A.

The next observation was that pseudoglobulin-A gave the heaviest precipitation of euglobulin when it was mixed not with the main pseudoglobulin fraction but with globoglycoid.

Proteins from the sera of different animals were mixed and it was found possible to obtain a euglobulin-II of mixed origin, the components being derived from different animal species. The results are summarized in Table III.

Table III. *Euglobulin-II precipitates from serum globulin fractions of different animal species*

+ indicates precipitation, 0 no precipitation.

Globoglycoid from	Pseudoglobulin-A from			
	Human	Horse	Ox	Rabbit
Human	+	0	0	0
Horse	+	0	0	0
Ox	+	0	0	0
Rabbit	+	0	0	0

It will be seen that in every case the pseudoglobulin-A constituent must be of human origin, whilst the globoglycoid may be derived from human, horse, ox or rabbit serum and yet produce a precipitate. The globoglycoid fraction is rich in lipins which have been considered to be concerned in euglobulin formation and it was decided to investigate the precipitating powers of globoglycoid freed from lipins. For the removal of lipins a more rapid modification of a method previously described [Hewitt, 1927] was used.

60 ml. of globoglycoid solution cooled to 0° were added gradually to 360 ml. of alcohol-ether (7 : 3) mixture kept at −15°. After being allowed to stand for an hour the precipitate was centrifuged down in chilled centrifuge buckets. The supernatant fluid was decanted off and the solid was suspended in 400 ml. of alcohol-ether mixture at −15°. After centrifuging the solid was washed twice in a similar manner with chilled ether which had been distilled over sodium metal. The whole process up to this stage had occupied less than 2 hr. The solid was now transferred to a Soxhlet apparatus and extracted with ether, sodium metal being present in the extraction flask to remove any water or alcohol present. After 2 hr. extraction, fresh ether and sodium were added and the extraction was continued for a further period of 3 hr. The solid was then dried

in vacuo over sulphuric acid and was finally obtained in the form of a fine white powder readily soluble in water.

It was found that this lipin-free globoglycoid still precipitated with human pseudoglobulin-A indicating that the lipin content of the globoglycoid was not an essential factor for the precipitation.

Some quantitative experiments on the amount of precipitate obtained under different experimental conditions are summarized in Table IV.

Table IV. *Amount of euglobulin precipitated by mixing human pseudoglobulin-A with various proteins*

Precipitate in mg. obtained from 10 mg. of mixed proteins.

Proportion of pseudoglobulin-A present	Human serum pseudoglobulin (main fraction)		Serum globoglycoid from			
%	*p*H 6	*p*H 7	Human	Horse	Rabbit	Ox
85	—	—	4·0	—	1·2	—
75	—	—	5·0	3·4	1·9	—
67	—	2·6	6·0	—	2·4	4·1
60	3·1	2·0	6·0	2·4	2·9	—
50	2·7	1·1	—	2·0	—	—
40	2·0	—	—	—	—	—

Precipitation is greater at *p*H 6 than at *p*H 7 as would be expected from the properties of euglobulin II already mentioned. The precipitate obtained with globoglycoid was greater than with the main pseudoglobulin fraction, suggesting that the globoglycoid present in the main pseudoglobulin fraction was the active precipitant.

Further discussion of the data described is postponed until consideration of the evidence given in later sections.

II. HUMAN BLOOD SERUM AND PATHOLOGICAL BODY FLUIDS

Human sera, urine, ascitic fluids and pleural effusions have been subjected to fractionation processes by the method already outlined with a view to detecting any considerable abnormality in the distribution of the various protein fractions.

The results of some of the fractionations are summarized in Table V. Dr Alston and Dr S. H. Robinson of the Archway Hospital Group Laboratory very kindly sent the specimens investigated.

Table V. *Protein fractions obtained from various human body fluids*

g. per 1 l. fluid.

Fraction	Normal serum	Heart failure ascitic fluid	Liver Cirrhosis ascitic fluid	Chronic parenchymatous nephritis					
				Serum	Urine	Ascitic fluid	Ascitic fluid	Pleural fluid	Pleural fluid
Pseudoglobulin-A	5·4	0·4	1·9	2·6	—	0·5	0·2	1·6	0·4
Pseudoglobulin (main fraction)	5·1	0·8	1·7	5·7	1·2	0·9	0·6	1·7	1·5
Euglobulins	8·4	1·1	1·3	5·0	1·2	1·2	0·8	2·6	1·7
Crystalbumin	25·3	8·0	3·4	3·8	1·7	1·1	1·0	3·2	2·0
Seroglycoid	3·6	2·3	2·2	5·4	1·8	0·8	0·7	1·2	0·4

The number of specimens examined is limited and it is inevitable that material is lost during fractionation processes, but despite its limitations the information obtained presents several points of interest, especially with regard to the various body fluids obtained from the case of chronic parenchymatous nephritis. All these specimens were obtained from the same patient, a man aged 51 with albuminuria and generalized oedema of a recurrent type; the blood urea concentration varied from 0·06 to 0·11 %.

The low albumin : globulin ratio in such cases of nephritis is well-known but we now know that the albumin fraction includes seroglycoid and globoglycoid in addition to crystalbumin. It is interesting, therefore, to consider the crystalbumin concentration apart from the other components of the albumin fraction. Thus the albumin : globulin ratios of the normal and nephritic sera in the table were 1·4 and 0·8 respectively, whilst the ratios of crystalbumin : "other proteins" were 0·8 and 0·2 respectively. The difference is, therefore, much more striking when the crystalbumin alone is considered instead of the whole albumin fraction. The explanation of this is discussed later.

There is some evidence of a decreased proportion of pseudoglobulin-A in the globulin fraction of the nephritic specimens as has been indicated by the precipitin tests of Kendall [1937].

The properties so far examined of the various fractions were similar irrespective of whether their origin was serum, urine, ascitic fluid or pleural effusion. The carbohydrate content of the pseudoglobulin-A fractions approximated to 1·5 % and that of the globoglycoids to 4 or 5 % although the quantity of material available precluded careful purification. The carbohydrate contents of the main pseudoglobulin fractions varied from 2·3 to 4·4 % according to the proportions present of the various proteins comprising the fraction. Pseudoglobulin-A and globoglycoid derived from different body fluids, when mixed, yielded a precipitate of euglobulin-II just as in the case of proteins obtained from serum.

The high carbohydrate content (16·4 %) of a crude specimen of seroglycoid from nephritic ascitic fluid suggested the presence of the mucoid investigated by Zanetti [1897] and Bywaters [1909], and fractionation with $(NH_4)_2SO_4$ resulted in the separation of two fractions, one mainly seroglycoid containing 8·5 % of galactose-mannose and the other, probably mucoid containing 20 % of carbohydrate.

III. HORSE SERUM STUDIED BY MEANS OF THE PRECIPITIN REACTION

The difficulty of obtaining pure antigens and the corresponding specific antisera and the ease with which biological tests are influenced by slight variations in experimental conditions, must necessarily set limits to the value of the precipitin reaction, and the accuracy attainable in chemical analysis is not reached. Nevertheless, precipitin tests are of great value for the qualitative examination of the homogeneity of protein fractions. Results of some quantitative significance may be obtained by observation of the first tube in a graded series of dilutions to exhibit flocculation [Dean & Webb, 1926], and, in conjunction with this method, by determining the nitrogen content of the floccules precipitated under appropriate conditions [Heidelberger & Kendall, 1932].

The importance of the route and mode of inoculation in the preparation of antisera is apparently greater than is generally appreciated and this will be discussed in a later section.

The antisera used were prepared by twice-weekly intravenous injections into rabbits of suspensions of alum-precipitated proteins over a period of 3 or 4 weeks, the course of injections being repeated if necessary. Antisera to four different fractions were investigated and they are described below.

Antipseudoglobulin-A serum reacted only with pseudoglobulin-A and on other zone of precipitation was observed.

Antigloboglycoid serum reacted principally with globoglycoid but a subsidiary zone of precipitation was observed and this was traced to the presence of antibody to crystalbumin, which was removed by absorption in the optimal proportion with pure crystalbumin.

Anticrystalbumin and *antiseroglycoid sera* reacted only with the homologous antigens.

Titrations of the antigens with the homologous antisera are summarized in Table VI.

Table VI. *Precipitin reactions of antigens with their homologous antisera*

Antisera were diluted 1 in 20 and mixed with equal volumes of antigen of dilutions given in table.

Antigen	100	300	1000	3000	10,000	30,000	100,000
Pseudoglobulin-A	–	–	–	–	+ + +	+ +	+
Globoglycoid	–	+	+ + +	+ +	+	–	–
Crystalbumin	–	–	–	–	+ + +	+ +	+
Seroglycoid	–	–	–	–	+ + +	+ +	+

Having thus obtained four specific sera each reacting with one of the protein constituents of serum, it became possible to investigate the composition of various sera and serum protein fractions. The method of carrying out the precipitin tests was to mix equal volumes of antiserum, diluted 1 in 20, with graded dilutions of the protein solution ranging from 1 % down to one-hundred thousandth or less of this concentration, and to observe the tubes at intervals for the first appearance of opalescence and flocculation.

Tested in this way good specimens of pseudoglobulin-A and crystalbumin behaved as homogeneous proteins, seroglycoid contained small amounts of crystalbumin whilst globoglycoid was found to contain small amounts of pseudoglobulin-A and crystalbumin. The main pseudoglobulin fraction also contained pseudoglobulin-A and it appears that nearly all globulin fractions contain this protein in greater or less amount.

Suitable proportions were found by preliminary tests in graded dilutions, and quantitative determinations were then carried out [Heidelberger & Kendall, 1932] by mixing the antisera with various fractions, keeping the proportions present in the region of antibody excess. After standing overnight in the ice-chest the precipitates were centrifuged down, washed with saline and the nitrogen contents determined by the micro-Kjeldahl method, the protein : nitrogen factor of 6·45 being used throughout in the calculations.

The antisera used were first standardized by determining the amount of precipitate produced by known amounts of the appropriate protein and plotting the curve connecting these quantities (Fig. 1). The amount of any of the four

proteins present in a fraction may be ascertained by determining the amount of precipitate obtained with the appropriate antiserum and reading off from the curve the corresponding quantity of antigen.

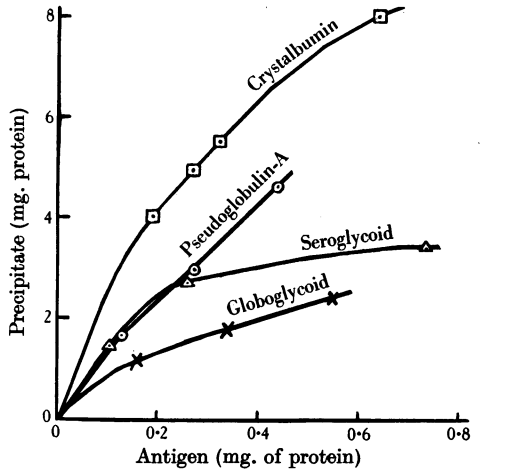

Fig. 1. Amount of protein precipitated on adding various horse serum proteins to the corresponding rabbit antisera.

The results are summarized in Tables VII, VIII, IX and X.

Table VII. *Pseudoglobulin-A antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Pseudoglobulin-A present (calculated) mg.
Pseudoglobulin-A	0·13 mg.	1·68	—
,,	0·27 mg.	2·99	—
,,	0·44 mg.	4·61	—
Horse serum 471	0·02 ml.	5·02	0·490
489	0·02 ml.	5·68	0·565
478	0·0033 ml.	3·37	0·310
Globoglycoid	2·2 mg.	2·95	0·270
Total globulins	0·91 mg.	5·85	0·585
Euglobulin-II	0·45 mg.	3·08	0·283
Pseudoglobulin (main fraction)	0·41 mg.	3·16	0·290

Table VIII. *Globoglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Globoglycoid present (calculated) mg.
Globoglycoid	0·16 mg.	1·17	—
,,	0·34 mg.	1·79	—
,,	0·55 mg.	2·39	—
Horse serum 471	0·10 ml.	2·07	0·46
489	0·10 ml.	2·08	0·47
478	0·10 ml.	2·36	0·59
Pseudoglobulin (main fraction)	3·50 mg.	1·68	0·34

Table IX. *Crystalbumin antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Crystalbumin present (calculated) mg.
Crystalbumin	0·19 mg.	4·02	—
,,	0·27 mg.	4·96	—
,,	0·32 mg.	5·52	—
,,	0·64 mg.	8·05	—
Horse serum 471	0·02 ml.	5·78	0·345
489	0·02 ml.	6·30	0·400
478	0·07 ml.	8·05	0·640
Globoglycoid	4·40 mg.	5·24	0·290

Table X. *Seroglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Seroglycoid present (calculated) mg.
Seroglycoid	0·11 mg.	1·45	—
,,	0·26 mg.	2·71	—
,,	0·74 mg.	3·43	—
Horse serum 471	0·10 ml.	3·03	0·35
489	0·02 ml.	1·45	0·11
478	0·10 ml.	2·71	0·26

Some interesting conclusions can be drawn from the tables. The main pseudoglobulin fraction of the horse serum examined was found to consist of 70 % of pseudoglobulin-A and 10 % of globoglycoid, leaving 20 % to be accounted for in the form of some other pseudoglobulin. Human serum pseudoglobulin contains only 30 % of pseudoglobulin-A according to Kendall [1937]. The higher proportion of pseudoglobulin-A in horse serum pseudoglobulin probably accounts for the lower carbohydrate content of horse serum fractions compared with human serum fractions, as mentioned previously.

Globoglycoid was first separated from the albumin fraction [Hewitt, 1938], but precipitin tests now show that the main pseudoglobulin fraction also contains about 10 % of globoglycoid. This is supported by the fact that the pseudoglobulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ has the properties of globoglycoid.

Euglobulin-II from horse serum was found to contain 63 % of pseudoglobulin-A; this may be compared with the figures 48–65 % obtained for human euglobulin [Kendall, 1937].

The difficulties of purifying globoglycoid have already been discussed and they are illustrated by precipitin reaction results which indicated that the specimen of globoglycoid examined contained 6 % of crystalbumin and 12 % of pseudoglobulin-A.

Results of the precipitin analysis of whole horse serum are of particular interest and are summarized in Table XI.

Table XI. *Analysis of horse serum by precipitin reactions*

Results are given in g. of protein per 100 ml. of serum.

Fraction	Normal horse No. 471	Normal horse No. 489	Diphtheria antitoxin horse No. 478
Pseudoglobulin-A	2·45	2·83	9·30
Globoglycoid	0·46	0·47	0·59
Crystalbumin	1·73	2·00	0·91
Seroglycoid	0·35	0·55	0·26
Other proteins	2·02	1·82	1·34

The diphtheria antitoxin horse (No. 478) had received a series of injections of formalized diphtheria toxin, the antitoxin content of the serum had risen to 500 A.U. per 1 ml. and was investigated because the protein content had increased considerably. It will be seen that the pseudoglobulin-A content had increased threefold, whilst the crystalbumin content had fallen to less than half the normal value. It is possible that the fall in crystalbumin content might be a compensatory mechanism to balance the increased osmotic pressure of the serum caused by the increase in globulin concentration.

The figures given for "other proteins" present in serum are obtained by difference and are approximate only. These other proteins probably include euglobulin-I, and a pseudoglobulin. This pseudoglobulin is difficult to isolate owing to the difficulties of removing pseudoglobulin-A, etc.

The antisera were used to study the absorption of proteins from the peritoneal cavity. A rabbit was injected intraperitoneally with 10 ml. of horse serum. At intervals blood was withdrawn from the ear vein and the amounts of the various horse serum proteins in the rabbit's blood serum were followed by carrying out precipitin tests, using the optimal proportions method [Dean & Webb, 1926]; the considerable quantities of antisera required for the Heidelberger & Kendall [1932] method were not available. By comparison of these results with those obtained using purified antigens of known protein content it was possible to calculate the approximate quantity of each protein present.

Table XII. *Amount of horse serum proteins present in the blood serum of a rabbit injected intraperitoneally with 10 ml. of horse serum*

	mg. protein per 100 ml. rabbit serum.			
Time hours	Pseudoglobulin-A	Globoglycoid	Crystalbumin	Seroglycoid
0·25	25	10	20	6
1	80	—	140	—
4	100	100	210	60
24	80	—	180	—
96	80	—	140	—
336	25	—	7	—

The highest concentration of the foreign proteins was reached after 1–4 hr. and diminished quite slowly.

The serum and cerebrospinal fluid of several patients who had received intravenous injections of horse serum containing meningococcus antitoxin were also examined by the optimal proportions technique. Dr Burtenshaw of the Southern Group Laboratory kindly sent these specimens. The horse serum proteins were readily detectable in the presence of human serum proteins, and the highest concentrations recorded were: in the serum, 800 mg. of pseudoglobulin-A and 150 mg. of crystalbumin; and in the cerebrospinal fluid, 9 mg. of pseudoglobulin-A and 4 mg. of crystalbumin. The results refer to the quantity present in 100 ml.

Discussion

It is becoming increasingly clear that serum cannot be regarded as a mixture of only two proteins, globulin and albumin. The albumin fraction has been separated into three different proteins, crystalbumin, seroglycoid, and globoglycoid, of distinctive properties [Hewitt, 1934; 1936; 1937; 1938]. Dr Philpot kindly examined a specimen of seroglycoid in the ultracentrifuge and reported that the sedimentation constant was not appreciably different from that of crystalbumin. Albumin prepared by electrophoresis [Tiselius, 1937] still contains

large amounts of seroglycoid as shown by its high carbohydrate content. Thus although both by chemical and precipitin tests crystalbumin and seroglycoid have been shown to be distinct and different proteins, their properties are such that certain physical methods may fail to differentiate them. Kekwick [1938] obtained an albumin fraction which by ultracentrifugal and electrophoretic methods appeared to be homogeneous, yet which by precipitin tests [Gell & Yuill, 1938] was found to be a mixture.

Previous work has revealed the presence in serum of globulins of different properties [Hewitt, 1927; 1934; 1938]. Pseudoglobulins may be obtained by fractionation with $(NH_4)_2SO_4$, Na_2SO_4 or NaCl, with carbohydrate contents ranging from 1·4 to 6·0 % (calculated as galactose-mannose). The pseudoglobulin of lowest carbohydrate content, about 1·4 %, is obtained by several reprecipitations with one-third saturated $(NH_4)_2SO_4$ solution. As the result of a study of precipitin reactions of a similar fraction obtained from human serum Kendall [1937] concluded that it was a single protein. The alternative names suggested for this protein both have disadvantanges and without making any assumption as to its identity the protein is therefore described as *pseudoglobulin-A*. Closely similar pseudoglobulin-A fractions have been obtained from human, horse, ox and rabbit sera. There is, however, a difference between pseudoglobulin-A obtained from human sera and that from the sera of other animals, and this difference will be described in the next few paragraphs.

Under certain conditions, when pseudoglobulin-A and the main pseudoglobulin fraction are mixed a precipitate is obtained [Kendall, 1937]. The semi-fluid consistency of this precipitate resembles that of euglobulin-II, and precipitin tests also indicate that pseudoglobulin-A is a constituent of euglobulin-II. It has been found that pseudoglobulin-A gives a heavier precipitate of euglobulin with globoglycoid than with the main pseudoglobulin fraction. This, and the fact that the main pseudoglobulin fraction has been found to contain globoglycoid makes it seem likely that this latter protein is the active precipitant. Since much lipin accumulates in the globoglycoid fraction it was thought possible that this was an essential factor but it was found that globoglycoid freed from lipin still acted as a precipitant.

Mixing pseudoglobulin-A and globoglycoid from human sera (or other body fluids) resulted in precipitation of euglobulin but this did not occur when the appropriate fractions of the sera of other animal species were mixed. Furthermore, mixture of human serum globoglycoid with pseudoglobulin-A from horse, ox and rabbit sera gave no precipitate. On the other hand pseudoglobulin-A from human serum gave a precipitate when it was mixed with human, horse, ox or rabbit serum globoglycoid. It is of some interest that it is possible to prepare a euglobulin of mixed origin derived from two different animal species.

Antisera have been prepared in rabbits reacting specifically with pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid from horse serum. With these antisera it has been possible, using a quantitative precipitin technique, to obtain analytical figures for the composition of various fractions and for horse serum itself. It is now possible to quote figures for the bulk of the serum protein in terms, not of arbitrary fractions, but of proteins which although difficult to purify nevertheless may be regarded as chemical entities.

The tentative figures obtained for normal horse serum were: pseudoglobulin-A, 2·6 %; globoglycoid, 0·5 %; crystalbumin, 1·9 %; seroglycoid, 0·5 %; other proteins, 1·9 %. The figure for "other proteins" is arrived at by difference and is not accurately determined but it probably includes a new pseudoglobulin fraction and euglobulin-I as well as traces of mucoid.

Precipitin analysis of the serum of a horse after a series of injections of formalized diphtheria toxin showed that the pseudoglobulin-A fraction had increased threefold to 9·3 % whilst the crystalbumin content had fallen to 0·9 %, less than half the normal value. The reason for the increase in pseudoglobulin-A is obscure since diphtheria antitoxin appears not to be associated with this fraction [Hewitt, 1934; 1938].

Fractionation of the proteins present in human serum and pathological body fluids revealed that, as in the case of urinary and serum albumin [1927; 1929], the proteins from different sources could not be distinguished from each other. Thus pseudoglobulin-A and globoglycoid when mixed gave a precipitate of euglobulin regardless of whether the proteins originated in serum, urine, ascitic fluid or pleural effusions. The carbohydrate contents of the proteins from different sources also were similar.

It was found that there was a very marked fall in the crystalbumin content of the serum of a case of chronic parenchymatous nephritis but the seroglycoid constituent of the albumin fraction was not diminished in amount. Seroglycoid was present in considerable amounts in nephritic urine, ascitic fluid and pleural effusions but yet the concentration in the serum had not fallen as it had in the case of crystalbumin. It would seem, therefore, that mechanical loss through the kidney did not of itself account for the diminished amount of crystalbumin in the blood stream, and it appears that regeneration of crystalbumin must proceed more slowly than that of seroglycoid or the globulins. Perhaps it is significant in this connexion that crystalbumin, alone among the serum proteins so far isolated, is chemically different in not containing polysaccharide in the molecule. The slow regeneration of albumin compared with globulins after plasmaphoresis was observed by Kerr *et al.* [1918]. Liver damage appears to delay the resynthesis of albumin [Foley *et al.* 1937]. As mentioned previously the explanation is lacking of the large increase in pseudoglobulin-A content of the serum of horses injected with diphtheria toxin and indeed the whole problem of serum synthesis is obscure.

The species specificity of the various serum proteins made it possible to study the appearance of foreign proteins in the blood stream or cerebrospinal fluid after intraperitoneal or intravenous injection of foreign protein. A rabbit received an intraperitoneal injection of horse serum. Within a quarter of an hour after the injection horse serum pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid could be detected in the rabbit's blood by means of precipitin tests. Disappearance of the foreign proteins from the rabbit's blood was slow, and traces were still detectable after a fortnight. On the other hand, it is reported that after intravenous injection of egg albumin the foreign protein disappeared from the blood stream very rapidly, 94 % having gone after 2 hr. and 99·6 % after 24 hr. [Kenton, 1938].

After intravenous injection of horse serum containing meningococcus antitoxin into patients, traces of pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid.

The question of the relative antigenicities of different proteins is complicated; there is, for example, considerable divergence in the literature concerning the antigenicity of serum albumin. That albumin is non-antigenic is maintained by some workers [Nolf, 1900; Landsteiner & Calvo, 1902; Ruppel *et al.* 1923] whilst others find it yields a specific antibody [Michaelis, 1902; Hektoen & Welker, 1924; Kabat & Heidelberger, 1937; Gell & Yuill, 1938]. The confusion was in some measure due to the fact that the serum albumin fraction contains at least three different antigenic proteins, crystalbumin, seroglycoid and globoglycoid,

each giving rise to a separate antibody, but there appears to be an additional source of confusion. It was reported previously that purified crystalbumin was only feebly antigenic when injected intraperitoneally even in large doses, whilst serum globulins, under these conditions are potent antigens [1937]. When injected intravenously crystalbumin is antigenic [Kabat & Heidelberger, 1937; Gell & Yuill, 1938] especially when the injected material is in the form of an alum suspension. The use of alum in enhancing antigenic activity was reported by Glenny *et al.* [1926]. Not only does the antigenic activity increase in the presence of alum but the risks of anaphylaxis after intravenous injection, previously reported [Hewitt, 1937], appear to be minimized. It is important, therefore, in attempting to evaluate the relative antigenicities of different proteins to take into account the route and method of injecting the antigens.

Summary

1. Attempts have been continued to separate serum into chemically distinct homogeneous individual proteins.

2. Pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid have been detected in human, horse, ox and rabbit sera.

3. Conditions have been studied under which mixing of pseudoglobulin-A and globoglycoid produces a precipitate of euglobulin-II.

4. In a nephritic patient the protein fractions were similar whether derived from serum, urine, ascitic fluid or pleural effusion and they could not be distinguished from normal serum proteins.

5. Both seroglycoid and crystalbumin were excreted in the urine of a nephritic patient in considerable amounts and the crystalbumin content of the serum fell to a low value, but the seroglycoid content did not fall appreciably. A different mechanism of regeneration of different serum proteins is indicated.

6. Specific precipitating sera were prepared for the four horse serum proteins, pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid. The route and method of injection of crystalbumin have marked effects on the antigenicity.

7. Approximate figures for the composition of normal horse serum in terms of four of the individual protein constituents have been obtained by means of quantitative precipitin tests. The results are: pseudoglobulin-A 2·6%; globoglycoid 0·5%; crystalbumin 1·9%; seroglycoid 0·5%; other proteins 1·9%.

8. In the serum of a horse immunized against diphtheria toxin the pseudoglobulin-A content had increased threefold and the crystalbumin content had fallen to half its normal value.

9. A rabbit was injected intraperitoneally with horse serum and the various horse serum protein fractions were detected by precipitin tests in the rabbit's blood stream within 15 min. of the injection. The maximum concentration of foreign protein was reached after 1 to 4 hr. and fell only slowly, traces being still detectable after a fortnight's interval.

10. Traces of horse serum pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid of patients injected intravenously with horse serum.

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