THE IMMUNOLOGICAL SPECIFICITY OF THE EUGLOBULIN AND PSEUDOGLOBULIN FRACTIONS OF HORSE AND HUMAN SERUM

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Serum is generally considered to contain at least two proteins, albumin and globulin, differing both in their isoelectric points, (literature summarized by Freundlich) and molecular weights (Svedberg and Sjögren), and readily separable by half-saturation with ammonium sulfate, which precipitates the globulin. There has been considerable investigation tending to show that the globulin fraction itself is not homogeneous, but consists of two distinct entities. Thus we find many early investigators dividing the total globulin into water-soluble and water-insoluble fractions, which they called pseudoglobulin and euglobulin respectively, whereas others have applied the term euglobulin to that fraction of the serum protein precipitated by 33 per cent saturation with ammonium sulfate, and the term pseudoglobulin to the fraction precipitated between 33 and 50 per cent saturation (literature summarized by Kato).

Such differences in solubility do not, however, necessarily mean that the fractions are actually different proteins. The fact that only part of the serum globulin is precipitated on 33 per cent saturation with ammonium sulfate hardly suffices to distinguish two fractions, as even proteins known to be homogeneous can be partially precipitated by arbitrarily chosen precipitating agents. With respect to water-solubility as a distinguishing criterion between protein fractions, there are reports that the water-soluble fraction undergoes a progressive loss in solubility as it ages, presumably going over to euglobulin (Chick), and that simple dialysis does not suffice to effect complete separation, which is achieved only by electrodialysis (Ruppel, Stern). Finally, Svedberg and Sjögren report that globulin

rapidly separated from serum is a homogeneous protein of uniform molecular size as determined by ultracentrifugation, but that globulin "purified" or fractionated by repeated precipitation with ammonium sulfate and dialysis has been profoundly altered, insofar as the solutions now contain particles of widely varying size.

It would therefore appear that some clear-cut difference other than solubility must be found between fractions of globulin in order to establish them as distinct entities. Gay and Adler have reported that the fraction of globulin precipitated by 33 per cent saturation with ammonium sulfate sensitizes guinea pigs more readily, but causes anaphylaxis less readily, than the remainder of the globulin. Kato found the water-soluble fraction more active in both respects. Chemical differences have been reported by several investigators (Chick, Block). Hunter tested the immunological specificity of the water-soluble and water-insoluble fractions of globulin by means of the precipitin reaction. Although he was able to produce heavier precipitates in every case by adding the homologous antigen to an antiserum, there were always some demonstrable antibodies to the other fraction as well. Dale and Hartley by sensitizing guinea pigs to the globulin fractions and testing the reactivity of the excised uterus, were apparently able to demonstrate the absolute specificity of the water-insoluble fraction, but not of the water-soluble. However, numerous other investigators (Kato, Györffy, Otto and Iwanoff, Ruppel *et al.*), have found that guinea pigs sensitized to either euglobulin or pseudoglobulin often go into anaphylactic shock on the subsequent injection of either fraction: and Otto and Iwanoff have been unable to demonstrate any clear-cut difference between the two fractions using the passive anaphylaxis technique with rabbit antisera.

Not only are these results inconclusive, but even if it were possible to establish a definite immunological or chemical difference between globulin fractions, such a difference might conceivably be due to changes in the proteins induced by the physical and chemical manipulations involved in their separation, a possibility which cannot be disregarded in view of the findings of Svedberg and Sjögren already cited, and confirmed by von Mutzenbecher.

The present experiments were therefore undertaken to determine (1) whether the immunological specificity of fractions of serum globulin indicated by the anaphylaxis technique could be confirmed by a

quantitative precipitin technique; (2) if any difference which might be demonstrated was an artifact induced by the purification of the fractions; (3) to what extent lipoids associated with the several globulin fractions were responsible for their immunological specificity, as suggested by Dale and Hartley.

Methods and Materials

An obvious difficulty in the work just reviewed is that even if globulin contained two or more distinct fractions, the methods of fractionation used could hardly be expected to separate them quantitatively. Each preparation would probably be a mixture of the fractions in varying proportions. In the second place, two different methods have been used to separate so called euglobulin and pseudoglobulin, and these do not yield the same products. Water-soluble and water-insoluble fractions each contain some material precipitable at 33 per cent saturation with ammonium sulfate and some precipitated between 33 and 50 per cent saturation; conversely, the fractions obtained at 33 and 50 per cent saturation each contain both water-soluble and water-insoluble protein. The serum globulin can thus actually be divided into four parts,[1] as indicated in Table I; and which of these four parts should be called euglobulin and which pseudoglobulin is a matter of arbitrary definition.

In order to minimize this confusion the two portions studied in these experiments were those satisfying both definitions: the 33 per cent precipitable water-insoluble protein being arbitrarily taken as euglobulin, and the 33 to 50 per cent precipitable, water-soluble protein being termed pseudoglobulin.

The experiments to be described were carried out on both human and horse serum.

Preparation of the Protein Fractions

To 150 cc. of inactivated human serum were added 75 cc. of a saturated solution of ammonium sulfate at room temperature. The resulting precipitate

[1] Strictly speaking, there are eight fractions rather than four. The four fractions obtained by first salting out with ammonium sulfate and then dialyzing (Table I) are not necessarily the same, either qualitatively or quantitatively, as those obtained by first dialyzing the serum and then further fractionating the water-soluble and water-insoluble proteins so obtained with ammonium sulfate.

was centrifuged, washed twice with 50 cc. of 35 per cent saturated ammonium sulfate, redissolved by adding a measured minimum volume of distilled water, reprecipitated by raising the saturation with salt to 33 per cent, washed with 35 per cent salt, and finally redissolved by adding distilled water.

To the supernatant from the original 33 per cent precipitate (164 cc.) were added 55 cc. of saturated ammonium sulfate, which made the solution 50 per cent saturated. This precipitate was now washed twice with 30 cc. of a 52 per cent solution, dissolved in a measured volume of water, reprecipitated at 50 per cent, washed with a 52 per cent solution, and redissolved.

The two solutions, representing the serum globulin precipitable by 33 and by 33 to 50 per cent saturation with ammonium sulfate, were dialyzed in cellophane bags against running tap water overnight, then against running distilled water overnight. The contents of each bag were centrifuged to separate the water-soluble from the water-insoluble fractions. The washed water-insoluble precipitates were dissolved in 0.85 per cent salt solution.

Portions of the 33 per cent precipitable water-insoluble (euglobulin) and of the 50 per cent precipitable water-soluble (pseudoglobulin) fractions were dehydrated from the frozen state in the Flosdorf-Mudd apparatus in order to insure the preservation of the protein, and the remainder was used for rabbit immunization.

Essentially the same method, on a somewhat larger scale, was used for the preparation of similar fractions of horse serum globulin.

Preparation of the Antisera

Nine rabbits the sera of which contained no demonstrable antibodies to human globulin were selected. Three of these received 0.5 cc. of whole human serum, three received 20 mg. of euglobulin, in solution, and the remaining three an equal quantity of pseudoglobulin. The injections were made into the marginal ear vein three times a week for 3 weeks. At the beginning of the 4th week 30–40 cc. of blood were withdrawn from the heart of each rabbit under sterile precautions, and centrifuged soon thereafter in individual bottles. The injections were continued another week and the seven surviving rabbits were again bled. There were thus obtained three different antisera against each of the three antigens in amounts varying between 30 and 70 cc. Three antisera each to whole horse serum, horse euglobulin, and pseudoglobulin—nine in all—were similarly obtained.

Nitrogen Analyses

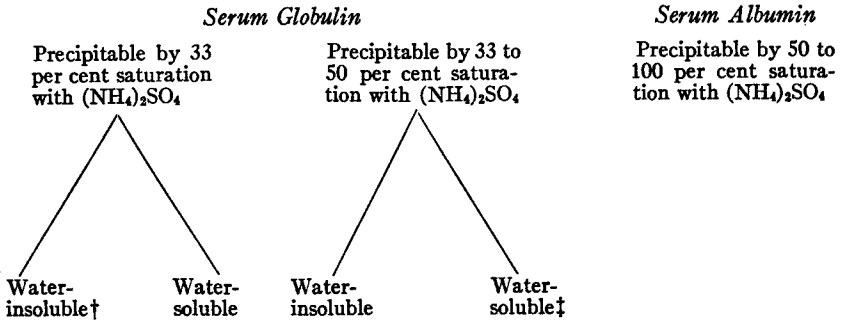
The precipitates obtained by adding antigen to antiserum as described in the text were washed in several changes of 0.85 per cent salt solution to remove excess serum and then analyzed for N content by a micro-Kjeldahl analysis, using the Meeker-Wagner technique. The results on samples containing > 1 mg. N are believed to be accurate to less than 1 per cent.

Method of Extracting the Lipoids from the Globulin Fractions (Experiment of Page 393)

In lieu of a soxhlet extraction flask, a sample of each of the proteins was placed in an alundum cup, which was suspended in a 150 cc. flask just above 50 cc. of the extracting agent (ether, petroleum ether, alcohol). A reflux condenser was fitted into the flask, so arranged that fluid dropping from the condenser would fall into the alundum cup. A water bath surrounding the flask was then heated until the rate at which the solvent dropped into the cup from the condenser just equaled the rate at which it seeped through the cup and its contents to drop into the bottom of the flask.

TABLE I

The Four Fractions of Serum Globulin Which Can Be Separated on the Basis of Salt-Precipitability and Water Solubility*



* See footnote 1, page 385.

† This fraction is arbitrarily taken as euglobulin throughout this paper.

‡ This fraction is arbitrarily taken as pseudoglobulin throughout this paper.

The extractions were maintained for 2 to 3 hours. The fluid in the bottom of the flask was then centrifuged clear of any particulate matter and evaporated. The lipoid residue was taken up in a small quantity of ether, transferred to a small weighed beaker, dried, and weighed. Each sample of protein was extracted twice with anhydrous ether, and the residue then extracted twice with petroleum ether. A second sample was extracted with absolute alcohol.

The Immunological Specificity of the Fractions of Serum Globulin

If the two fractions of serum globulin chosen for study, which differ in water solubility and salt precipitability, were also entirely distinct as regards immunological properties, we would find that the antisera produced by injecting each antigen reacted specifically with

that antigen, and that only the antisera to whole serum reacted with both. Instead, it was found that every one of the three groups of antisera gave precipitation with each of the two globulin fractions, generally more marked with the homologous antigen, as Hunter has observed, but not invariably so. At first sight, this would seem to imply that the fractions were devoid of immunological specificity. However, the possibility suggested itself that there actually were two distinct globulins, but that the euglobulin and pseudoglobulin fractions as obtained by us contained both antigens in varying proportion. The following experiment was undertaken to test this possibility.

5 cc. of each of the nine antisera to human serum and its fractions were placed in Wassermann tubes. Dissolved euglobulin (0.1 cc. of a solution containing 19 mg./cc.) was added to each of the tubes. The tubes were kept at 37°C. for 3 hours (or overnight in the ice box), centrifuged, and a second portion of antigen was added. After the second or third addition excess antigen was demonstrated in the supernatant fluids by adding 0.1 cc. of these to 0.2 cc. of fresh rabbit anti-human serum. In each case, two further additions were made to the tubes after excess antigen in the supernatant had been thus demonstrated to assure complete precipitation of the antibody. (See Table II.)[2]

The combined precipitates from each serum were centrifuged, washed twice in 7 to 8 cc. of 0.85 per cent NaCl to remove the serum, and reserved for nitrogen analysis. 4.50 cc. of each of the nine supernatant fluids which had been absorbed with euglobulin in the manner described were now treated with 1.9 mg. of pseudoglobulin. In every case, a heavy precipitate formed, indicating that all the antisera contained antibodies not absorbed by euglobulin, and reacting specifically with pseudoglobulin. This residual antibody was now completely precipitated by successive additions of 1.9 mg. pseudoglobulin, and the combined precipitates also washed and analyzed for nitrogen (Table II).

There were thus obtained for each serum two figures representing the amount of precipitate obtained on primary absorption with euglobulin, and the amount found when the supernatant fluid was subsequently precipitated with pseudoglobulin.

The entire procedure was now repeated in reverse order, absorbing the sera first with pseudoglobulin and then with euglobulin, with

[2] In spite of the excess of antigen, a slight precipitate continued to form on the addition of more antigen, insignificant in quantity as compared with the total precipitate, or that obtained on the subsequent addition of pseudoglobulin, but interesting as regards its implications (*cf.* page 391).

similar results. All the sera, after complete[2] absorption with pseudoglobulin, were found to contain residual antibodies specific for euglobulin. The results of this experiment are summarized in Table II. A similar experiment carried out with antisera to horse serum and to the globulin fractions of horse serum is summarized in Table III. The

TABLE II

The Demonstration in Antisera to Human Euglobulin, Pseudoglobulin, and Whole Serum of Antibodies Specific for the Two Globulin Fractions

Type of antiserum used	(A) Precipitate from 5 cc. antiserum on addition of euglobulin*	(B) Precipitate from supernatant of (A) on addition of pseudoglobulin†	(C) Precipitate from 5 cc. antiserum on addition of pseudoglobulin	(D) Precipitate from supernatant of (C) on addition of euglobulin†	Total precipitate on absorbing 5 cc. serum	
					First with euglobulin and then with pseudoglobulin (A + B)	First with pseudoglobulin and then with euglobulin (C + D)
	mg.	*mg.*	*mg.*	*mg.*		
Euglobulin.....	12.7‡	5.0	8.3	9.8	17.7	18.1
	28.5	2.5	17.6	8.1	31.0	25.7
	25.5	3.9	11.7	11.1	29.4	22.8
Pseudoglobulin....	8.1	1.4	4.2	3.0	9.5	7.2
	3.8	0.9	1.9	2.8	4.7	4.7
	18.9	12.6	21.6	8.0	31.5	29.6
Whole serum.....	9.0	3.4	5.8	6.6	12.4	12.4
	14.0	8.2	11.9	7.7	22.2	19.6
	19.8	7.1	12.6	10.1	26.9	22.7

* See text for method of addition.

† In Columns B and D the actual experimental readings were multiplied by 5.5/4.5 to correct for the fact that only 4.5 cc. of the supernatant were used out of a total volume of 5 + 0.5 cc. = 5.5 cc.

‡ These figures were obtained by multiplying the mg. of nitrogen in the precipitate by 6.25, assuming the protein to be 16 per cent nitrogen.

results were qualitatively similar, and the following discussion applies equally to both experiments.

As is seen in the tables, every antiserum contained fraction-specific antibodies for euglobulin and pseudoglobulin. After absorbing any serum with either one of the two antigens there was always residual antibody for the other fraction as well. This result is adequately

explained on the basis that serum contains two immunologically distinct proteins which we may term globulin I and globulin II, but that the "euglobulin" and "pseudoglobulin" fractions as obtained by a combination of salting out and dialysis each contain a preponderance of one antigen and a trace of the second. On this hypothesis, euglobulin would consist largely of globulin I, but would contain a trace of globulin II; and conversely for pseudoglobulin. Since even a trace of contaminating protein would suffice to cause the formation of

TABLE III

The Demonstration in Antisera to Horse Euglobulin, Pseudoglobulin, and Whole Serum of Antibodies Specific for Two Globulin Fractions

Type of antiserum used	(A) Precipitate from 5 cc. of serum on adding euglobulin	(B) Precipitate from supernatant of (A) on adding pseudo-globulin	(C) Precipitate from 5 cc. serum on adding pseudo-globulin	(D) Precipitate from supernatant of (C) on adding euglobulin	Total precipitate on absorbing 5 cc. serum	
					First with euglobulin and then with pseudo-globulin (A + B)	First with psuedo-globulin and then with euglobulin (C + D)
	mg.	*mg.*	*mg.*	*mg.*		
Euglobulin.....	29.2	1.5	9.6	20.6	30.7	30.2
	11.0	2.1	3.1	10.6	13.1	13.7
	7.4	4.0	3.3	8.9	11.4	12.2
Pseudoglobulin....	3.5	6.4	9.2	2.4	9.9	11.6
	1.1	3.5	4.1	0.1	4.6	4.2
	9.3	10.0	17.0	2.6	19.3	19.6
Whole serum.....	14.2	14.3	28.4	3.7	28.5	32.1
	10.3	11.8	15.7	6.6	22.1	22.3
	11.4	14.2	18.0	7.0	25.6	25.0

antibodies on injection into the experimental animals, each preparation would cause the formation of antibodies to both immunologically specific fractions. As is well known, the amounts of antibody produced to two different antigens bear no necessary relationship to the amount of antigen injected; it is nevertheless to be noted that with the exception of some of the antisera to human protein, the antisera generally contained more antibody for the homologous antigen than they did for the heterologous, corresponding to the large discrepancy in the amounts of antigen injected.

Confirmatory evidence that the foregoing is the correct explanation is furnished by two additional observations. When *e.g.* an anti-euglobulin serum was absorbed with successive small amounts of either euglobulin or pseudoglobulin, there was at first a heavy precipitate which continued to form until excess antigen was demonstrable in the supernatant fluid. Even after this excess was present, however, further additions of the antigen solution continued to cause faint precipitation. Comparatively large amounts of euglobulin failed to exhaust the antibody responsible for this slight precipitation. This phenomenon was observed with every one of the eighteen antisera, and is explained on the basis that the first additions of the euglobulin preparation removed all the antibodies for globulin I as such, but that the traces of globulin II impurity removed only a small part of the specific antibodies to that fraction. Accordingly, on further addition of euglobulin, the globulin II impurity continued to produce slight clouds of precipitate. When a solution of pseudoglobulin was now added, there was a rapid and heavy precipitation of the residual antibody to globulin II, and the supernatant then gave no precipitation with either antigen.

Another observation which bears out the thesis of incomplete separation of two immunologically distinct proteins is the fact that in every case the residual antibody to *e.g.* euglobulin after absorption of the serum with pseudoglobulin is less than the antibody precipitable by euglobulin from fresh serum. Thus, the figures in Column B are in every case less than the corresponding figures in Column C, and Column D is likewise consistently less than A. We would explain this on the basis that Column C, for example, represents the total antibody to globulin II *plus* that portion of the antibody to globulin I precipitated by the traces of the latter in pseudoglobulin; while Column B represents the total antibody to globulin II *minus* that portion precipitated by the traces of globulin II in euglobulin; and conversely for Columns A and D.

To summarize, our experimental data are adequately explained on the thesis that serum globulin consists of at least two immunologically distinct proteins, only incompletely separated even by a combination of the methods ordinarily used in separating euglobulin and pseudoglobulin.

The observation (Svedberg and Sjögren, von Mutzenbecher) that globulin repeatedly precipitated differs in such an essential property as size from the globulin in whole serum suggests that the immunological specificity of these fractions might be a similar artifact. However, the presence of these fraction-specific antibodies in the antisera to whole serum strongly suggests that this immunological difference is not an artifact due to changes in the protein induced by its physical or chemical manipulation, but is an inherent difference between two globulin fractions present as such in whole serum. It is, however, possible that in serum these two proteins are associated as a single large molecule, somehow broken up during the salting out and dialysis into its component parts.

After complete absorption of the antisera with the euglobulin and pseudoglobulin preparations, they gave no further precipitation on the addition of the "anomalous" fractions indicated in Table I: the fraction precipitated by 33 per cent saturation with ammonium sulfate, but water-soluble on subsequent dialysis, and the fraction not precipitated by the salt, but water-insoluble. It would therefore appear that the latter two globulin preparations do not contain any antigenic factor which is not also present in the euglobulin and pseudoglobulin fractions as defined in this paper.

The experimental data do not exclude the possibility that, in addition to the two globulins already demonstrated, there may be other immunologically distinct globulins which are uniformly distributed among the several fractions as obtained by salting out and dialysis, and which could therefore not be differentiated by the technique here used.

The Rôle of Lipoids Associated with the Euglobulin and Pseudoglobulin Fractions on Their Immunological Specificity

In view of the reports by Chick to the effect that euglobulin (water-insoluble globulin) can be artificially produced from pseudoglobulin (water-soluble) by the addition of a lecithin suspension to the latter, and in view also of the suggestion by Dale and Hartley that lipoid associated with euglobulin is of importance in determining its immunological properties, it became of interest to ascertain to what extent lipoids associated with the two fractions were responsible for

the specific immunological reactivity just demonstrated. Accordingly, lipoids extracted from the euglobulin and pseudoglobulin fractions were added to highly reactive antisera and the precipitate analyzed for nitrogen to determine whether the lipoid particles had combined with antibody protein. As a further check, the sera so "absorbed" were tested for residual antibody to globulin, to determine whether removal of such antibodies to lipoids as might be present had in any manner affected the reactivity of the antisera to the globulin fractions.

TABLE IV

The Extraction of the Euglobulin and Pseudoglobulin Fractions of Human and Horse Serum with Ether, Petroleum Ether, and Alcohol

Extraction with	Human euglobulin			Human pseudoglobulin			Horse euglobulin			Horse pseudoglobulin		
	Sample	Lipoid extracted	Lipoid in sample	Sample	Lipoid extracted	Lipoid in sample	Sample	Lipoid extracted	Lipoid in sample	Sample	Lipoid extracted	Lipoid in sample
	gm.	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*
Ether	0.82	72.5	8.85	0.33	3.3	1.00	1.07	51.5	4.81	2.10	11.4	0.54
Petroleum ether		2.4	0.29		0.4	0.12		9.0	0.84		2.7	0.13
Alcohol 95 per cent	0.55	50.2	9.09	0.15	12.0	8.00	0.50	21.0	4.20	0.60	10.0	1.67
Total lipoids obtained, *mg.*		125.1			15.7			81.5			24.1	

The amounts of lipoid obtained from weighed dry samples of the globulins on extraction with ether followed by petroleum ether on one sample and with 95 per cent alcohol on another are summarized in Table IV, the method of extraction being that described on page 387. These lipoid fractions were pooled, brought to a concentration of 1 per cent in 95 per cent alcohol, and to this solution were added cholesterol and corn-germ sterol to a concentration of 0.6 per cent of each. The alcoholic solution was now diluted by rapidly blowing in 1.3 volumes of 4 per cent NaCl, producing a microscopically crystalline suspension. The sterols were added in order to facilitate

aggregation on the addition of antisera, and to allow quantitative recovery of the lipoid particles on centrifuging (Eagle). The suspensions were now analyzed for nitrogen content, and added to the antisera in the quantities indicated in Table V. The lipoid-antiserum mixtures were centrifuged, the precipitates washed twice to remove excess serum protein, and analyzed for nitrogen.

TABLE V

Showing That Lipoids Extracted from the Globulin Fractions Do not Combine with Demonstrable Amounts of Antibodies to Those Fractions

Source of lipoids	Antiserum to original globulin	Lipoid suspension*	Lipoid added*	Protein from which lipoid was extracted	N content of the lipoid suspension	N content of sediment after centrifuging the serum-lipoid mixture	Antibody absorbed by the lipoid	Antibody precipitable by a corresponding amount of globulin
	cc.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Human euglobulin.....	10	5.0	21.8	143	0.05	0.03	0†	3.8
Human pseudoglobulin.....	10	1.0	4.4	92		Not recoverable		3.4
Horse euglobulin.....	10	5.0	21.8	284	0.08‡	0.11‡	0.19 (?)‡	11.2
Horse pseudoglobulin.....	10	1.0	4.4	380	0.06	0.04	0†	6.6

* Added in five equal parts.

† The amount recovered was actually less than that added, due either to incomplete recovery of the lipoid particles on centrifugation or to the presence of water-soluble nitrogenous material in the lipoid suspension.

‡ Probably not a significant difference.

In no case was there visible flocculation of the lipoid particles by the antiserum, and, as seen in Table V, there was no demonstrable combination with antibody protein. In view of the fact that the antibodies in the serum used would have been completely precipitated by much less than 10 mg. of globulin, while the lipoid extracted from 92 to 380 mg. of globulin failed to combine with any demonstrable antibody we conclude that lipoids extracted from the globulin frac-

tions of human and horse serum cannot combine with antibodies to these proteins.

As corollaries to this finding, antisera absorbed with this large excess of lipoid were not impaired either qualitatively or quantitatively in their reactivity with the globulin itself (Table VI); and globulin extracted at room temperature with ether and petroleum ether was unaffected as regards its reactivity with the antisera.

TABLE VI

Showing That Antisera Absorbed with Lipoids Extracted from the Corresponding Serum Globulin Are Unaffected As to Reactivity with the Original Globulin

Antigen used for precipitation	Precipitate obtained from 5 cc. of antiserum		
	Orginal antiserum	Antiserum absorbed with euglobulin lipoid	Antiserum absorbed with pseudoglobulin lipoid
	mg.	*mg.*	*mg.*
Human euglobulin	11.9	10.6	13.1
Human pseudoglobulin	10.6	10.0	8.1
Horse euglobulin	35.0	33.2	33.8
Horse pseudoglobulin	20.6	21.2	20.3

SUMMARY

That portion of horse and human serum globulin precipitated by 33 per cent saturation with ammonium sulfate and precipitated on subsequent dialysis was taken as euglobulin; and the fraction precipitated between 33 and 50 per cent saturation and remaining in solution on subsequent dialysis was taken as pseudoglobulin.

The sera of rabbits injected with either of these antigens gave precipitation with both. However, two distinct and fraction-specific antibodies could be demonstrated by absorbing the sera with the one antigen, and testing the supernatant fluid with the other. The experimental results are adequately explained on the basis that there are at least two antigenically distinct globulins in serum which we may term globulin I and globulin II and which are largely associated with so called euglobulin and pseudoglobulin respectively. The or-

dinary methods of salting out and dialysis do not effect complete separation and each globulin preparation contains a trace of the other antigen. The antisera to these euglobulin and pseudoglobulin preparations therefore contain antibodies to both antigens. Each protein solution precipitates all the antibody specific for the one antigen and in addition, by virtue of the trace of contaminating protein, precipitates a *portion*, and only a portion of the antibody specific for the other antigen.

The fact that antisera to whole serum contain these same fraction-specific antibodies suggests that this immunological specificity is an inherent property of two globulins present as such in serum and is not an artifact induced by their precipitation and purification.

Lipoids extracted from the globulins by ether, petroleum ether, and alcohol give no demonstrable reaction with antisera to these globulins; antisera absorbed with a large excess of lipoid are not affected as regards their reactivity with the original protein; and globulins extracted with ether and petroleum ether at room temperature are not affected as regards their reactivity with antisera. It is concluded that the immunological specificity of the globulin fractions as evidenced by the precipitation reaction is not determined by lipoids associated with the protein.

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