THE FRACTIONAL PRECIPITATION OF THE GLOBULIN AND ALBUMIN OF NORMAL HORSE'S SERUM AND DIPHTHERIA ANTITOXIC SERUM, AND THE ANTITOXIC STRENGTH OF THE PRECIPITATES.

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In December, 1899, I published in this Journal[1] a preliminary note on the fractional precipitation of the globulin and albumin of normal horse's serum and diphtheric antitoxic serum. I now present the completed work, though there is still much which I cannot explain and which I have not as yet been able to work out.

The proteids of diphtheria antitoxic serum do not show any determinable differences chemically from those of the normal serum. The globulin precipitate[2]—which possesses the antitoxic power—increases in quantity but not necessarily proportionately to the antitoxic increase (see the preceding article, p. 47).

The method employed in the precipitation is as follows: 10 cc. of serum are pipetted into a beaker glass of suitable size and diluted up to a volume of about 50 cc. with water; the globulin is then removed by saturating the diluted serum with magnesium sulphate and filtering the precipitated globulin. The globulin precipitate is dissolved in water and again precipitated by magnesium sulphate, filtered off, and washed with saturated magnesium sulphate solution. It is thus freed from the albumin. The filtrates, which contain the albumin, are combined. The globulin derived from the 10 cc. of serum, is dissolved in about 200 cc.

[1] *Journal of Experimental Medicine*, 1899, iv, p. 649.

[2] The article of Brieger and Boer entitled "Ueber Antitoxine und Toxine" (*Zeitschr. f. Hygiene u. Infectionskrankh.*, 1896, xxi, p. 259), and the more recent work of Freund and Sternberg, entitled "Ueber Darstellung des Heilkörpers aus dem Diphtherieheilserum" (ibid., 1899, xxxi, p. 429) show that the antitoxin is not carried down mechanically by the globulin, but that there is a true precipitation of the antitoxic substance.

of distilled water and the solution is saturated with sodium chloride. The albuminous filtrate is also saturated with sodium chloride and set aside for the fractional precipitation of the albumin.

The globulin solution saturated with sodium chloride is allowed to stand over night at the room temperature (15-20° C.) before filtering off the precipitate which forms upon saturating. A shorter time may do quite as well but I have in all my experiments allowed it to stand over night in order to be sure of saturation. The precipitate after filtering is washed with the saturated sodium chloride solution, dissolved in distilled water and coagulated. 350-400 cc. of water is a convenient volume from which the coagulation of the R.° (room temperature) precipitate may be made by bringing the solution to a complete state of boiling and then adding weak acetic acid drop by drop until the liquid is faintly acid. The coagulated R.° globulin-precipitate is allowed to settle and is then filtered on a Gooch crucible, washed with hot distilled water, then with absolute alcohol, dried in the air-bath to constant weight, cooled in the desiccator over calcium chloride and weighed.

The beaker containing the filtrate from the R.° precipitate is placed in a water-bath, the temperature of which can be regulated easily, and the temperature is raised to 40° C., sodium chloride having been added to ensure saturation. At this temperature a turbidity makes its appearance and increases until 45° C. is reached. The solution is constantly stirred while the temperature is rising. The 40-45° C. precipitate can now be filtered off and the filtrate should run through the filter perfectly clear after 20 or 25 cc. have come through and have been poured back on the filter. If it continues to run through cloudy, the precipitation is not complete and the beaker containing the solution from which the precipitation is being made should be put back in the water-bath and the temperature run up to 45° C. again with constant stirring.

This precipitate also dissolves readily in water, and is coagulated, filtered, washed, dried and weighed as described. In the same manner one obtains a third turbidity at 49° C. and complete precipitation at 54° C.; a fourth turbidity at 57° C. and complete precipitation at 62° C. These four precipitates are soluble in water. Finally a fifth turbidity appears at 67° C. and there is complete precipitation at 72° C. The final precipitate is not entirely soluble in water but the few insoluble flocks are easily soluble in a little sodium hydroxide, and after neutralization with acetic acid their solution can be added to the general solution of this precipitate. The dissolved precipitates are coagulated, filtered, washed, dried and weighed as described.

TABLE I.

COMPARISON OF THE FRACTIONAL PRECIPITATES OF GLOBULIN OF NORMAL AND ANTITOXIC SERA, SHOWING ALSO THE LOSS OF GLOBULIN IN WEIGHT DURING THE PROCESS OF PRECIPITATION. 10 CC. OF SERUM WERE USED IN EACH CASE.

Temps. of Precip.	R° ppc.	40° ppc.	49° ppc.	57° ppc.	67° ppc.	Sum of Frac.	Total Glob.	Loss of Glob.
Horse No. 137 Normal Serum	.1457	Trace	.0482	.0746	.0071	.2756	.3235	.0479 gms.
137, bled 10/20 '99, 1200 units per cc.	.0648	.1026	.1247	.1899	.3038	.7856	.8292	.0436 gms.
137, bled 10/20 '99, 1200 units per cc.	.4419	.0397	.2970	.0362	Trace	.8144	.8292	.0148 gms.
137, bled 11/9 '99, 650 units per cc.	.1729	.1086	.1253	.0950	.0124	.5142	.5934	.0792 gms.
137, bled 11/14 '99, 600 units per cc.	.0613	.0992	.1007	.1486	.0218	.4316	.5116	.0800 gms.
137, bled 11/18 '99, 400 units per cc.	.2200	.0733	.0842	.0679	Trace	.4454	.4743	.0289 gms.
" "	.2225	.0724	.1308	.0227	Trace	.4448	.4743	.0295 gms.
" "	.0853	.0637	.0846	.2038	.0188	.4562	.4743	.0181 gms.
" "	.1691	.0446	.0713	.1386	Trace	.4236	.4743	.0507 gms.
Horse No. 135, Normal Serum.	.1154	Trace	.0373	.0749	.0399	.2375	.3864	.1489 gms.
135, bled 10/26 '99 500 units per cc.	.2209	.1408	.1944	.0714	.0149	.6424	.8987	.2563 gms.
Horse No. 136, Normal Serum.	.1005	.0056	.0597	.1450	.0058	.3166	.3536	.0370 gms.
136, bled 10/20 '99, 600 units per cc.	.1277	.0530	.1249	.1919	.1118	.6093	.7389	.1296 gms.
Horse No. 133, Normal Serum.	.0485	.0124	.0378	.0731	.1473	.3191	.3727	.0536 gms.
133, bled 9/18 '99. 400 units per cc.	.1376	.0604	.0995	.1304	.2600	.6879	.7782	.0903 gms.

The final filtrate from the 67-72° C. precipitate fails to give the biuret reaction, and on boiling and subsequent addition of a little acetic acid shows no turbidity. These reactions are common to the globulin of normal and diphtheric antitoxic globulin. Duplicate analyses give frequently different results for corresponding temperatures (see Table I). These differences cannot be accounted for by incomplete precipitation, for if, for any temperature the precipitation was found to be incomplete, the solution was again raised in temperature to the proper degree and the globulin was filtered out and added in its proper place. They could be accounted for by considering each precipitate as caused by the formation of a globulin salt. The amount of the precipitate at any given precipitation tempera-

ture would then depend upon the amount of the compound formed. At the close of the paper I shall consider more fully this hypothesis.

The globulin suffers a loss during the separation (see Table I), and I have found nitrogen in the 67-72° C. filtrates in varying quantities. In order to determine whether the action of sodium chloride had a destructive action on the globulin, three determinations were made by coagulating the globulin from 10 cc. of serum for each experiment.

1. From the watery solution containing some magnesium sulphate which remained with the globulin when it was separated from the albumin.
2. From a one-half saturated sodium chloride solution.
3. From a saturated sodium chloride solution.

Table II gives the results, which show that sodium chloride does not exercise a destructive action on the globulin.[3]

TABLE II.

COMPARISON OF COAGULATION WEIGHTS OF GLOBULIN, EACH FROM 10 cc. OF SERUM, FROM A MAGNESIUM SULPHATE SOLUTION AND FROM SODIUM CHLORIDE SOLUTIONS.

Horse 133.
Normal serum.
Weak $MgSO_4$ solution = .3727 grms. of coagulated globulin.
One-half saturated NaCl solution = .3656 grms. of coagulated globulin.
Saturated NaCl solution = .3648 grms. of coagulated globulin.

The globulin fractions from antitoxic serum each contain antitoxin and the final filtrate is free from antitoxin. Some of the antitoxin is always destroyed by the process of precipitation. In this reaction the relation of "diphtheria antitoxin" to globulin is possibly still further shown, the loss in antitoxic power and the loss in weight of the globulin being at the same time and by the same cause.

Table III gives the analyses of a serum containing 400 units per each cubic centimetre which during the process of precipitation lost about 46% of its antitoxic strength.

[3] Solutions 2 and 3 also contained a little magnesium sulphate which remained with the globulin when it was separated from the albumin.

TABLE III.

THE RELATION OF ANTITOXIC STRENGTH TO WEIGHT OF FRACTIONAL GLOBULIN PRECIPITATES PER CUBIC CENTIMETRE.

Serum of Horse 133, bled September 18, 1899—400 units per cc.

Temperature of precipitation.	R° ppc.	40° ppc.	49° ppc.	57° ppc.	57° filt.	67° ppc.	Sum of frac.	Original am't of globulin per cc.	Loss of globulin.
Weight of globulin precipitates in grms.	.0457	.0036	.0132	.0078	.0103	.0011	.0714	.0778	.0064
Antitoxic strength of precipitates in units.	100 to 115	10 to 15	50 to 75	25 to 50	40	10 to 15	195 to 237	Original strength 400 units per cc.	Mean loss of antitoxic strength 184 units = 46%.

This table also shows that antitoxic power is lost during the heating process, while the proteid may be scarcely affected in weight, for if the globulin from the 57° filtrate is substituted for the globulin obtained from this filtrate, *i. e.* the 67° ppc., we have a sum of the fractions .0028 grms. greater in weight than the total globulin. That is, the sum of the fractions and the total globulin are practically the same, and we have a loss of 40% of antitoxic power. We may explain this by considering the antitoxic molecule rearranged by the lower temperature during the process of precipitation without the destruction of the coagulable properties of the proteid.

It will be seen by Table III that there is only a general relationship between the number of antitoxin units and the corresponding weight of globulin.

The destruction of the globulin and antitoxin by the process of precipitation is directly shown by the difference in globulin and antitoxin found in the 57° C. filtrate and the amount found in the precipitate obtained from it.

These reactions of normal and antitoxic globulins toward the salt precipitants, coupled with the fact that normal horse's serum is found to possess decided antitoxic properties against diphtheria toxin and that this antitoxin in normal horse's serum is also found in the globulins, compels us, I believe, to place diphtheria antitoxin among the globulins. The non-correspondence of change in globulin weight to antitoxic increase and decrease, except in a very general way, indi-

cates in immunized as well as in normal serum the presence of other forms of globulin besides the antitoxic, all of which are affected by the group reagents and coagulated by heat in the same way.

The fractional separation of the albumin (Table IV) is carried out as described for the globulin.

The albuminous filtrate which has been saturated with sodium chloride is poured off from the mass of salts[4] at the bottom of the beaker. The salts are washed with a saturated sodium chloride solution and the washings are added to the main portion of the solution. Some albumin may be held back in the crystals, and in order not to have too large a volume to work with, I have not washed very carefully but have dissolved the crystals and coagulated the proteid which they held. This is afterwards distributed proportionately among the various precipitates. The first precipitate of the albumin makes its appearance at 56° C. and may be filtered off at 61° C. This precipitate is soluble in water. The second precipitate appears at 67° C. and may be filtered off at 72° C. This precipitate is partially soluble, a portion being coagulated and needing weak sodium hydroxide for its solution. The third and fourth precipitates are not soluble, but are coagulated. They are thrown out of solution at 73-76° C. and 77-81° C. respectively. They are at best only slight and in some cases only represented by traces. The final filtrate is free from coagulable proteids and fails to give the biuret reaction. As in the globulin so in the albumin, some of the proteid is lost during the separation.

Dr. Thomas B. Osborne of the Connecticut State Agricultural Station has shown that certain vegetable proteids form definite compounds with

[4] In the preliminary communication, I stated that a double salt was formed by saturating the magnesium sulphate filtrate, containing the albumin, with sodium chloride. The statement is incorrect. Almost every analysis showed the salt to be sodium sulphate. The following are 3 selected analyses of the salt:

No. 1.	1st crystallization.	No. 2. 1st crystallization.
Mg =	9.45%	Mg = 4.54%
SO_4 =	38.26%	No other constituent quantitatively determined. Cl present.
H_2O =	51.89% determined by loss in weight.	
	99.60%	
No. 3.	Recrystallized four times.	
H_2O =	56.29%	Impurity of Cl and Mg in 1st crystallization. Cl persisted until fourth crystallization.
SO_4 =	29.81%	
Na =	13.9% by difference	
	100.00	

mineral acids. Thus, he has separated edestin[5] mono- and bihydrochlorate. The nitrate, phosphate, sulphate and acetate of edestin are indicated by the reaction. The behavior of the animal globulins and albumins may find their explanation in the same way. The individuals of the globulin group may form sulphates insoluble in saturated magnesium sulphate solution or may form double salts (globulin magnesium sulphate) insoluble in saturated magnesium sulphate solutions. If we saturate the "globulin solution" with sodium chloride at room temperature a precipitate separates out. The precipitate may be accounted for by considering the chlorine or sodium chloride as uniting with or effecting a double decomposition with the globulin sulphate or globulin magnesium sulphate, and the formation of either a simple globulin chloride or double salt of globulin sulphate and chloride, or, perhaps, a complex salt containing globulin magnesium sodium chloride and sulphate. The precipitations at the other temperatures could be accounted for by the same reactions.

TABLE IV.

COMPARISON OF THE FRACTIONAL PRECIPITATES OF ALBUMIN OF NORMAL AND ANTITOXIC SERA; SHOWING ALSO THE LOSS OF ALBUMIN IN WEIGHT DURING THE PROCESS OF PRECIPITATION.

10 cc. of serum were used in each case.

Temperature of precipitation.	56° ppc.	67° ppc.	73° ppc.	78° ppc.	Sum of fractions.	Total albumin.	Loss of albumin.
Horse No. 137, Normal Serum	.2259	Traces for 67°, 73° + 78°			.2259	.3127	.0868 grms.
137, bled 10/20 '99, 1200 units per cc.	.1558	.0180	Traces for 73° + 78°		.1738	.2046	.0308 grms.
137, bled 11/9 '99, 650 units per cc.	.1987	.0204	Traces for 73° + 78°		.2191	.2727	.0536 grms.
137, bled 11/14 '99, 600 units per cc.	.1791	.0351	Traces for 73° + 78°		.2142	.2938	.0796 grms.
137, bled 11/18 '99, 400 units per cc.	.1253	.0457	73° + 78° = .0080		.1790	.3133	.1343 grms.
Horse No. 135, Normal Serum	.0304	.0672	.0074	.0061	.1111	.3680	.2569
135, bled 10/26 '99, 500 units per cc.	.1520	.0188	73° + 78° = .0133		.1841	.2341	.0500
Horse No. 136, Normal Serum	.2149	.0428	73° + 78° = .0078		.2655	.3618	.0963
136, bled 10/20 '99, 600 units per cc.	.0290	.1193	73° + 78° = .0612		.2095	.2715	.0620
Horse No. 133, Normal Serum	.2438	.0986	73° + 78° = .0362		.3786	.4146	.0360
133, bled 9/18 '99, 400 units per cc.	.0877	.0407	73° + 78° = .0266		.1550	.2101	.0551

[5] *Jour. of the American Chem. Soc.*, 1899, xxi, No. 6, June.

The precipitation of the albumin by means of sodium chloride could be accounted for in the same manner. At 73° C. and 77° C. we have coagulation and these portions of albumin are therefore beyond this hypothesis.

The presence of the SO_4 is necessary for these fractional precipitations of the globulin and albumin. According to standard text-books some of the globulin can be precipitated from the serum by saturating the serum with sodium chloride at room temperature. I have obtained another precipitate from the room temperature filtrate by saturating with sodium chloride at 56° C. These precipitates are slight however. In a serum containing 600 units of antitoxin per cc. I obtained a precipitate containing only 10 units per cc. by saturating with sodium chloride at room temperature. Halliburton[6] states that serum albumin is soluble in saturated sodium chloride and magnesium sulphate solutions, yet when the serum has been treated with magnesium sulphate, one can precipitate fractionally the globulin from its watery solution, and the albumin from its magnesium sulphate solution by saturating these solutions with sodium chloride and raising the temperature.

If it be true that we have a series of compounds of globulin and albumin in the described reactions it would account for the differences in the quantities of globulin obtained at corresponding temperatures from duplicate samples, the amount of the precipitate depending upon quantities of the compound formed.

I hope to be able to show later in what form these precipitates of globulin and albumin occur, whether as pure proteids, simple halogen salts, double salts or complex salts.

CONCLUSIONS.

1. The globulins of both normal and diphtheria antitoxic serum exhibit chemically toward reagents the same reactions, being precipitated by magnesium sulphate and split up into fractions in precisely the same way.

2. All of the diphtheric antitoxic power of both normal and immunized serum is always carried by the globulin and its fractional precipitates.

3. During the fractional precipitation of the serum globulin of horses immunized from diphtheria toxin and horses not immunized

[6] Halliburton, *Text-book of Chemical Physiology and Pathology*, pp. 127 and 236–237.

from diphtheria toxin, some of the globulin is lost, likewise at the same time some of the antitoxic power of the globulin of the immunized serum is lost.[1]

4. These reactions, considered in connection with the fact that different observers as well as we ourselves have found diphtheric antitoxic power in normal horse's serum and that this antitoxin separates with the globulin, strongly incline us to consider "diphtheria antitoxin" a form of globulin.

5. The reactions of globulin, previously separated from the serum by magnesium sulphate, with sodium chloride lead one to think that there is a formation of globulin salts.

6. Since serum albumin in a magnesium sulphate solution gives fractional precipitates at definite temperatures, it seems not improbable that the albumin is precipitated in the form of albumin salts.

I take this opportunity of expressing to Dr. William H. Park, Assistant Director to this laboratory and Dr. Alexander Lambert, Assistant Bacteriologist, my appreciation of their suggestions and kind criticisms during my work.

[1] On the addition of the fractions of antitoxin tested in guinea pigs, and the addition of the fractions of globulin determined by weighing the coagulated globulin, we find that both have lost, but that the antitoxin has been affected especially by the higher temperature.