SERUM-GLOBULIN AND DIPHTHERIC ANTITOXIN.—A COMPARATIVE STUDY OF THE AMOUNT OF GLOBULIN IN NORMAL AND ANTITOXIC SERA, AND THE RELATION OF THE GLOBULINS TO THE ANTITOXIC BODIES.[1]

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The varying statements concerning the amount of globulin in serum from immunized and non-immunized horses, and concerning the relation of the antitoxic principle to this constituent and to other constituents of serum, leave no doubt as to the confusion existing in regard to these questions, and indicate a promising field of research.

The propositions upon which our work was primarily based were the following: To analyze normal sera and antitoxic sera of different values for the determination and comparison of the amounts of globulin and albumin, and of the antitoxic values of these globulins and albumins, and also of the various filtrates accompanying their preparation.

In the analysis of blood-serum various methods have been devised and recommended for obtaining the different ingredients of the serum in a pure state. Of these methods, those recommended for the precipitation of the globulins are of chief interest to us and will be briefly considered. The principal methods are the following: (*a*)

[1] Received for publication February, 1900. The experiments recorded in this paper were begun in May, 1897, and have extended over the intervening years. They have been performed at the Research Laboratory of the Department of Health of New York City, where exceptional opportunities for such investigations exist, owing to the large number of horses constantly undergoing immunization for the production of diphtheric antitoxin.

precipitation of the globulins by super-saturation of the serum with magnesium sulphate or semi-saturation with ammonium sulphate; (*b*) precipitation by means of CO_2 gas, by passing it through solutions containing the serum or globulins; and, (*c*) finally, precipitation by dialysis, this method depending for its success upon the removal of the natural or added salts, the globulins being looked upon as insoluble in pure water and hence precipitated by removal of the salts.

Dieudonné [2] has shown that marked differences exist in the action of the globulins prepared by different methods, when tested for their antitoxic power. Globulins obtained by precipitation with CO_2, according to him, display the least antitoxic value; those obtained by precipitation with magnesium sulphate the greatest; while globulins, the product of dialysis, hold an intermediate place in the antitoxic scale.

In his experiments, Dieudonné used a normal horse's serum which, he says, showed marked antitoxic properties. One cubic centimetre of this serum mixed with twice the fatal dose of diphtheric toxin, in vitro, protected the animal even from marked local symptoms. Globulin prepared from this serum by CO_2 gas was found to have practically no antitoxic value. This result differed so widely from the results claimed by Smirnow,[3] that another experiment was undertaken in which the globulins were precipitated by the addition to the serum of an excess of magnesium sulphate. This was the method that Smirnow had employed, and Dieudonné found "the preparation obtained by the magnesium sulphate acted very differently from that prepared by carbonic acid; it showed toxin-neutralizing properties like those obtained by Smirnow in his experiments with globulin." Further experiments brought to light the fact that globulins won by means of dialysis were markedly less active than the precipitate obtained by magnesium sulphate, but that the filtrate had a noticeable antitoxic value.

Dieudonné concludes from his experiments "that the antitoxic property does not belong to the globulin, but to some unknown body contained in the serum which in the preparation of the globulin is carried down mechanically in the precipitate, or becomes closely bound up with it; and that by the rapid precipitation with magnesium sulphate this

[2] *Arb. a. d. k. Gesundheitsamte*, 1897, xiii, p. 297.
[3] *Arch. d. sciences biol.*, St. Pétersb., 1895, iv, p. 328.

active substance is much more easily seized, than in the slower separating out of the globulins by carbonic acid or by dialysis, in which only a small portion is taken with the precipitate." This active body contained in serum must therefore, he concludes, be least precipitable by carbonic acid, somewhat more precipitable by dialysis, and most precipitable by magnesium sulphate; and hence the action of the various filtrates obtained in the different methods of globulin preparation is also different. After precipitation by magnesium sulphate these fluids are poorest in antitoxic bodies; after carbonic acid preparation, richest. This becomes especially apparent if the following experiments of Dieudonné are considered.

The precipitate previously obtained by magnesium sulphate was redissolved and subjected to dialysis in flowing and distilled water. The precipitate thus obtained by dialysis was redissolved in 2% salt solution. The original magnesium sulphate globulin precipitate had a high protective value, while even 2 cc. of the fluid remaining after the abstraction of the globulin was totally inactive. These proportions were very different after dialysis. The globulin precipitated by dialysis was only weakly active, while of the fluid freed from the globulin, 0.5 ccm. still displayed marked neutralizing powers. These differences are more apparent still in the second experiment, *i. e.*, the purification of the magnesium sulphate globulin by means of carbonic acid gas. One gramme of the dried magnesium sulphate globulin was dissolved in 50 cc. of water. In passing CO_2 gas directly into this solution not a trace of precipitate was to be noted. The solution was therefore subjected to dialysis, first in running water, then in distilled water, until no trace of sulphuric acid could be determined. In the parchment tube an abundant precipitate appeared, which was redissolved in 2% sodium chloride solution. Into this solution carbonic acid gas was run for two hours and a fine flocculent precipitate obtained. In testing the antitoxic value of this precipitate it was found to have lost its value and that the antitoxin was for the most part in the filtrate instead of with the globulin as in the original magnesium sulphate precipitation.

These experiments have seemed worth detailing in this connection since an explanation of the results obtained has been advanced by Seng[4] in his recent paper. Seng mentions that Brieger's[5] observation that the antitoxins from zinc chloride precipitation go over into the filtrate from reprecipitation with carbon dioxide, while those from zinc sulphate

[4] *Zeitschr. f. Hygiene*, 1899, xxxi, p. 513.

[5] Ibid., 1896, xxi, p. 267.

precipitation remain with the precipitate when reprecipitation by carbon dioxide is practiced, demonstrates that the chemical nature of the resulting precipitate or of the precipitating method decides whether the antitoxin falls with the precipitate, but shows that it is in no wise carried down by the precipitate.

He cites experiments in support of this view and against Dieudonné's conclusion that the antitoxins are mechanically carried down with the precipitates, and says: "That a further weighty support to this view was brought by the researches of Dr. Sternberg," who, finding that "it was inconvenient to work with such richly albuminous antitoxin solutions as those composing the native diphtheric serum," was able to show "that the albumin could be removed by other than the Brieger method from the antitoxin, and that by the addition of $\frac{1}{3}$ vol. of a 5% (concentrated) solution of potash alum to the serum, a great part of the albuminous bodies would be precipitated as an abundant, voluminous, precipitate, the antitoxins, however, remaining in solution."

Here, then, the antitoxins remained in solution in spite of a voluminous precipitate, and by further experiments it was shown that no matter whether the albumins were previously removed or whether the globulins were precipitated by magnesium sulphate or ammonium sulphate, nevertheless, the entire antitoxin always remained with the globulins.

The vital point in Seng's experiments was reached in removing the excess of the precipitating salts from the globulins. The removal of the salts was accomplished by dialysis, and it was to be expected, according to previous conceptions, that, by a dialysis which was carried on until all traces of chlorine, ammonia, and sulphuric acid reaction had disappeared, all globulins would be precipitated in the salt-free solution, but it appeared that only a very small part, 1:23 to 1:11, of all the globulin separated itself out as insoluble, the majority of the globulin and with it the entire antitoxin remaining in solution. It was determined by experiment that the antitoxins clung to these "soluble globulins."

This may be the explanation of the discrepancies noted when globulins obtained by different methods are tested for their antitoxic value, the antitoxin remaining apparently with the "soluble globulins" only. Both the soluble and the insoluble globulins are precipitated by magnesium sulphate, but only the insoluble by dialysis.

Early in our own work it became apparent that the magnesium sulphate method was the most trustworthy known to us for obtaining

globulins and the antitoxic principles of the serum. Throughout the paper, therefore, the results of precipitation by magnesium sulphate will alone be given.

In obtaining the globulin content of the various sera, both normal and antitoxic, the following method has been followed:

Separation of the globulins from blood-serum.—10 cc. or 20 cc. of the serum was diluted to 50 cc. or 60 cc. with a saturated solution of magnesium sulphate. Crystals of magnesium sulphate were then added and the mixture stirred until completely saturated. When precipitation was complete, the precipitate was filtered out and redissolved in water together with the crystals of magnesium sulphate still remaining in the original beaker glass, the quantity of solution thus resulting amounting generally to 350 cc. to 400 cc. From this solution the globulin was again precipitated with magnesium sulphate and filtered out, the beaker being washed with a saturated solution of magnesium sulphate, which fluid was then poured onto the filter. The globulin thus obtained was washed on the filter paper with a saturated solution of magnesium sulphate, and finally redissolved in distilled water.

These precipitates were then tested upon guinea-pigs in order to determine their antitoxic values and to compare these with the values of the sera from which they were derived.

All tests of serum for antitoxic value were made by mixing the toxin and serum and then injecting the mixture into the test animal.

Determination of the antitoxic value of the globulins.—In making the tests for antitoxin in globulin solutions, exactly the same method was followed as that employed in testing serum itself. The globulin from the 10 cc. or 20 cc. of the serum was dissolved in a known quantity of sterile distilled water. Of this solution a quantity corresponding in globulin content to 1 cc. of the serum, from which it was derived, was diluted to the required strength and mixed as usual with the 10-times fatal dose of toxin.

Table I shows the results of the tests with globulins. Some tests of albumins from the sera are recorded in Table II.

Toxin control animals for all these tests died in the usual time, 4 to 7 days.

From Tables I and II it can be readily seen that the tests of the globulins obtained by magnesium sulphate precipitation show that practically the total antitoxin content of the various sera is associated

with the globulins, these globulins showing an antitoxic value equal to the strength of the antitoxin contained in the serum from which they were derived.

TABLE I.

DETERMINATIONS OF ANTITOXIC VALUE OF GLOBULINS FROM ANTITOXIC SERA.

Date.	No. of Horse.	Units of Antitoxin in 1 ccm. of serum.	Units of Antitoxin tested for in glob. from 1 ccm. of serum.	Weight of test Guinea Pig.	Result of Test.
26-III-'97 ..	83	500	500	278	Animal not affected.
30-XII-'97..	105	500	500	270	" " "
1-XII-'97..	112	550	550	230	" " "
1 " '97..	112	550	550	228	" " "
1 " '97..	112[6]	550	550	260	" " "
1 " '97..	112[7]	550	550	237	" " "
3-VI-'97 ..	128	500	500	218	" " "

TABLE II.

DETERMINATIONS OF ANTITOXIC VALUE OF ALBUMINS.

Date.	Horse.	Units of Antitoxin in 1 ccm. of serum.	Units of Antitoxin tested for in Albumin from 1 ccm. of serum.	Weight of test Guinea Pig.	Result of Test.
26-III-'97 ..	83	500	5	222	Animal died in 5½ days.
3-VI-'97 ..	128	500	5	272	Animal died in 1½ days.

To determine the action of magnesium sulphate by itself upon the toxins, and thus rule out any error that might arise from such action, if present, the following experiment was undertaken:

A solution of magnesium sulphate calculated to be the same in strength as would be found with solutions of the globulins was mixed with toxin as follows and injected into guinea-pigs:

TABLE III.

TO DETERMINE ACTION OF MAGNESIUM SULPHATE ON TOXIN.

Weight of test Guinea Pig.	ccm. of $MgSO_4$ sol.	Fatal doses of toxin.	Result of test.
225 gm.	2 ccm.	2	Died in 3 days.
" "	2 "	4	" " 2½ days.
" "	2 "	6	" " 2½ days.

[6] Same globulin solution as 112, but kept exposed to light at room temperature in stoppered blue-glass bottle for 2½ months.

[7] Same globulin solution as 112, but kept in bottle in ice-chest three months before testing.

As is evident from Table III, no neutralizing action upon the toxin was determined, the test animals dying in about the usual time; we may also conclude from these experiments that the Mg SO_4 had no stimulating effect upon the animal.

To determine, on the other hand, the evil effects of injecting Mg SO_4 into guinea-pigs the tests recorded in Table IV were made:

TABLE IV.

TO DETERMINE INJURIOUS EFFECTS OF INJECTION OF MAGNESIUM SULPHATE.

Weight of test Guinea Pig.	ccm. of sat Mg SO_4 sol.	Result of test.
300 gm.	0.5 ccm.	Animal not affected.
335 "	0.25 ccm.	" " "
307 "	0.1 ccm.	" " "
250 "	1 ccm.	" died in 10 minutes.

As the globulin from 1 cc. of an antitoxic serum of 500 units strength, when tested for this strength, is diluted 5000 times, it is plain from the above results that the amount of magnesium sulphate injected into an animal with the globulin solution must be too small in quantity to have any appreciable effect on the animal.

Having, by the foregoing experiments, demonstrated to our satisfaction that the magnesium sulphate precipitates contained practically all the antitoxic bodies—be these globulins or bodies precipitated with them in the presence of magnesium sulphate—we were then in a position to carry on the major part of our work, *i. e.* the comparison of the weights of these precipitates as obtained from the serum of non-immunized and of immunized horses at various stages of resistance. These determinations were made in the manner detailed below, the greatest care being taken to avoid error, several determinations often being made from the same serum.

Coagulation and weighing of the total coagulable albuminous bodies (proteids); globulins, and albumins.—The globulin and albumin were coagulated together from the horse serum in the following manner: 10 cc. or 20 cc. of the serum, as the case might be, were diluted to 350 cc. or 400 cc. with water, and the temperature gently raised over the Bunsen burner until coagulation began. The temperature was then quickly raised to boiling point, the solution being constantly stirred. When

the solution was boiling well, dilute acetic acid was added drop by drop until a faint acidity was reached. After a few moments more of boiling, the coagulation was complete and the flocculent mass quickly settled to the bottom of the beaker glass. The coagulated proteids were filtered onto a weighed filter paper, washed with boiling water, and, finally, slowly dried in the air bath to constant weight.

The globulin obtained by precipitation with Mg SO_4 was coagulated and filtered from its watery solution in precisely the same manner. The difference between the weight of the globulin and the total albuminous (proteid) precipitate was considered as the weight of the albumin in the serum taken. This method is open to some objections, but with careful technique very accurate results for comparison may be obtained.

Table V gives the results of these experiments. The determinations are given in grammes.

A consideration of the figures of Table V convinces us that although there is no absolute conformity in the amount of the precipitates from sera of the same antitoxic value, still even in the comparison of these sera from different horses it is apparent that the lower the antitoxic value of the serum the lower is the globulin content, *i. e.* if normal serum, according to our determination for 20 ccm., gives 0.8 grm. of precipitate, then 200-units serum will average much higher, 300 higher still, and so on, if a long series is taken. But there were such marked exceptions to this rule that it was evident that a high antitoxic value is possible in a serum of comparatively low globulin content, as low often as a serum of practically no antitoxic value. The absolute amount was then no index of antitoxic value. This dispelled our hope of determining before undertaking immunization the value of a horse for the production of antitoxin by globulin determinations.

It early became evident, except for the interest of general statistics and comparison, that determinations based upon the sera of different horses were of little value, and that the only safe data upon which to found conclusions would be those obtained from the serum of the same animal, from the normal state on to a high degree of immunity. In several cases it has been possible to make such determinations.

From Table VI it is obvious that, no matter what the initial globulin content, this amount was always increased as the immunization

TABLE V.

DETERMINATION OF TOTAL ALBUMINOUS BODIES (PROTEIDS)—GLOBULINS AND ALBUMINS—FROM SERA OF DIFFERENT ANTITOXIC VALUES.

Date.	Horse.	ccm. of serum used.	Antitoxic units per ccm.	Weight of globulin.	Estimated wt. of albumin.	Weight of albuminous bodies (glob. and alb.) precipitated by heat.
16-XI-'97.....	7	20	300	1.4077	.4928	1.9005
18-XII-'97....	"	"	400	1.5548	.5194	2.0742
11-V-'97	83	"	200	.9834		
29-VI-'97.....	"	"	400	1.3344	.2354	1.5698
?	"	"	500	1.4683	.3959	1.9642
6-XII-'97	"	"	850	1.5298	.4695	1.9993
?	89	"	Normal.	.5710	.4707	1.0417
21-V-'97	91	"	200	.7358	.3263	1.0621
13-IV-'97.....	"	"	300	1.1720	.3928	1.5648
?	95	"	Normal.	.7265	.6465	1.3730
8-XII-'97	96	"	300	1.3540	.4123	1.7663
29-XI-'97	"	"	400	1.5949	.5143	2.0806
27-IX-'97	98	"	Normal.	.6171	.8057	1.4228
?	99	"	"	.7593		
?	100	"	"	.8150	.5610	1.3760
?	101	"	"	.7602		
?	101	"	200	1.4193		
?	103	"	Normal.	.8685	.6535	1.5220
21-IV-'98.....	108	"	"	.6743	.6871	1.3614
	"	"	200	.8436		
?	109	"	Normal.	.5818	.8376	1.4194
10-XII-'98.....	110	"	"	.7559	.6834	1.4393
	110	"	300	1.2101	.5773	1.7874
27-XI-'98.....	111	"	Normal.	.6377	.8221	1.4598
	"	"	500	1.5447	.2455	1.8902
	112	"	450	.7569	.7281	1.4850
	"	"	500	1.0591	.5918	1.6509
	116	"	Normal.	.7977		
	117	"	"	.9943	.6826	1.6819
	118	"	"	.7773	.7373	1.5146
	119	"	"	.7673	.6532	1.5205
	"	"	150	.8056	.5908	1.4964
	121	"	Normal.	1.1429	.5944	1.7373

proceeded, although no fixed amount of increase seemed to represent a given number of units of antitoxic value. The same thing is apparent in Table VII, where lists are given of determinations from horses in whose case it was impossible to obtain data concerning the normal serum.

TABLE VI.

GLOBULIN DETERMINATIONS FROM NORMAL AND ANTITOXIC SERA.[8]

		Units of Antitoxic Strength.				
	Normal.	150	200	300	400	500
Horse 89.....	.5710					
" 95.....	.7265					
" 98.....	.6171					
" 99.....	.7593					
" 100.....	.8150					
" 101.....	.7602		1.4193			
" 103.....	.8685					
" 108.....	.6743		.8436			
" 109.....	.5818					
" 110.....	.7559			1.2101		
" 111.....	.6377					1.5447
" 116.....	.7977					
" 118.....	.7773					
" 119.....	.7673	.8056				
" 121.....	1.1429					

We again find demonstrated the excess of the magnesium sulphate precipitates from the sera of horses which have reached high degrees of immunization over the sera of the same animals at a less advanced stage.

TABLE VII.

GLOBULIN DETERMINATIONS FROM ANTITOXIC SERA.[9]

	200	300	400	450	500	550	600	650	700	750	800	850
Horse 7		1.4077	1.5548									
" 83			1.3344		1.4683							1.5297
" 91	.7358	1.1720										
" 96		1.3540	1.5949									
" 112				.7569	1.0591							

In Table VIII are given some globulin and albumin determinations made from 10 cc. amounts of various sera. The globulins were pre-

[8] Determinations made from 20 cc. of serum, and results given in grammes.

[9] Determinations made from 20 cc. of serum, and results given in grammes.

cipitated with $MgSO_4$ in the regular manner; and the albumins were then precipitated from the filtrate by heat. The precipitates were collected, however, on Gooch crucibles instead of on filter-papers, and the weighings thus made with probably less error than in the methods used by us earlier in making these determinations. Nevertheless, remembering that we are dealing with 10 cc. instead of 20 cc. amounts of serum, we find the results practically unaltered. It is interesting to note the decrease shown by the albumins as the globulins increase. Whether this is a coincidence, or a regular gain of the one at the expense of the other, it would be difficult to determine from our few tests. If it is a constant occurrence, it may be that a transmutation takes place, or simply that the process which regulates the albumin content is depressed during the period of exaltation of globulin production.

TABLE VIII.

GLOBULINS.

Horse.	Normal.	400	500	600	650	1200
133	.3727	.7782				
135	.3864	....	.8987			
136	.3566	....	....	.7389		
137	.3235	.4743	....	.5116	.5934	.8987

N. B.—Horse No. 137 went from normal to 1200 units at first bleeding; subsequent bleedings with no intervening toxin inoculation showed constant decrease in the number of units, likewise in globulin.

ALBUMINS.

133	.4146	.2102				
135	.3680	....	.2341			
136	.3618	....	....	.2715		
137	.3127	.3133	....	.2938	.2727	.2341

TABLE IX.

GLOBULIN DETERMINATIONS FROM SERUM OF HORSE 83.

	200	300	400	500	850
Horse 83.....	.9834				
" ".....	.9666	....	This serum dropped from 700 units.		
" ".....	....	1.0218	" " " " 500 "		
" ".....	....	....	1.3344		
" ".....	....	....	1.1478	This serum dropped from 600 units.	
" ".....	....	....	....	1.4683	
" ".....	....	....	....	....	1.5297
Average.	.9750	1.0218	1.2411	1.4683	1.5297

The comparison given in Table IX is exceedingly interesting. The table shows the determinations from one of the horses that has long been a source of high grade antitoxin. Figures are given for the magnesium sulphate precipitates from 200 unit serum to that containing 850 units, with the difference of 0.5 grm. between the determination of 200 unit serum and the 850 unit specimen.

The most interesting point in this connection is that some of these specimens when first drawn and tested gave a high number of units, and that, after having been kept, they dropped very considerably in their antitoxic value and globulin content, this being due possibly to some obscure chemical change or more probably to contamination with microörganisms. Comparing two 200-unit specimens, one of which had dropped from 700 units, and the other having been freshly drawn, we find their globulin content practically the same. A 400-unit specimen, which had dropped from 600 units, showed a like decrease in the amount of globulin; likewise a 500-unit serum, which had fallen to 300 units, gave a precipitate in weight between that of the 200 and 400-unit specimens.

Although we do not feel justified in positively concluding from the foregoing facts that the antitoxin, or antitoxic bodies as we prefer to call them, are globulins, yet we cannot help feeling that such facts point very strongly to their being globulins, or at least bodies which have in all points, in the light of our present knowledge, the same reactions as the globulins. It seems a remarkable coincidence that the antitoxic bodies and the globulins should have been destroyed in such exact proportion in those sera which had lost in antitoxic value, if they were not the same bodies.

Undoubtedly included under the general term "serum globulin" there are several or many bodies of slightly varying chemical characters that eventually we may be able to distinguish one from the other, and determine their absolute amounts. Some of these globulins, it seems not unlikely, may, at times, be "cell globulins" in contradistinction to the true "serum globulins," and be found in the serum after some unusual destruction of the formed elements of the blood.

The variations in the initial amounts of globulin present in the serum of different animals may be due to normal variations in the physiological processes, but many of them are possibly to be explained as pathological conditions, and among these may be increases due to various past infections through which the animal has successfully passed, or to minor infections, less obvious, but existing before and at the time of the taking of the specimen for analysis. All such complicating conditions should be taken into consideration, and an attempt made to reduce results to uniformity by excluding such complications or allowing for their existence when making comparisons.

In considering initial amounts of globulin the antitoxic value of so-called "normal serum" in each case should be carefully determined, as a partial explanation of variations in initial amounts of globulin may be found in the variations in the protective value of these sera. In our work this has not been done in all cases, but Table X gives the results of some tests undertaken to determine the neutralizing power of serum from normal horses, and Table XI the results in determining the neutralizing power of the globulin from this normal serum. It will be seen that many among these display a marked neutralizing action when tested against one or more fatal doses of diphtheric toxin. The accepted unit of antitoxin—the amount of antitoxin which will protect the test animal against 100 minimal fatal doses of the diphtheric toxin—represents in reality a fair neutralizing action, for 1 cc. of a serum possessing only a strength of one unit has the power of rendering harmless 100 minimal fatal doses of toxin. Remembering this in considering these tables, it will be seen that, although these sera show only very exceptionally as high a power as one antitoxic unit, yet some of them in amounts of one ccm. protect the test animal (guinea-pig, 250 grm.) against many minimal fatal doses of the toxin—in the case of Horse 137, against 300 minimal fatal doses. Reference to Table XI will also show that the globulins from these sera act in a like manner against the toxins of diphtheria. These sera, however, in many instances do not contain more globulin than others of less neutralizing power, hence the variations in diphtheric antitoxin or neutralizing substances are again demon-

strated to be only one of the factors causing increase in the globulin precipitates, and can but partially explain the variations in these precipitates.

TABLE X.

DETERMINATIONS OF THE NEUTRALIZING POWER OF NORMAL SERA.

Horse.	Weight of Guinea Pig.	Serum + Toxin.	No. of fatal doses protected against.	Result.
126	225	10 cc. + 1.5 f.d.t.[10]		Loss in wt.; no induration. Recovered.
"	315	5 " + " "		Died in 3 1/2 days.
127	280	5 " + " "		No loss in wt.; no induration.
"	335	3 " + " "	0.5	Loss in wt.; induration. Recovered.
"	320	1 " + " "		Died in 3 1/2 days.
135	365	1/2 " + " "		No loss in wt.; no induration.
"	270	1/10 " + " "	15	Induration; loss in weight.
"	248	1/20 " + " "		Died in 4 1/2 days.
"	243	1/2 " + 5 f.d.t.		?
136	374	" " + 1.5 "		Much induration.
136	240	1/4 " + " "		Much induration; loss in weight.
137	255	1/20 " + 1.5 "	30	Gain in weight; no induration.
"	260	1/5 " + 5 f.d.t.	25	No induration; no loss in weight.
137	270	1/10 " + 10 "	100	" " "
"	225	1/100 " + 1.5 "	150	" " "
"	250	1/30 " + 10 "	300	" " "
"	254	1/35 " + 10 "		Died in 3 1/2 days.
139	355	3 " + 1.5 "		Much induration; slight loss in wt.
"	325	1 " + " "		Died in 48 hours.
140	270	10 " + " "	0.15	Induration; slight loss in weight.
"	340	5 " + " "		Died in 3 1/2 days.
141	270	1/4 " + " "	6	No induration; gain in weight.
"	275	1/10 " + " "		Loss in wt.; induration.
"	255	1/20 " + " "		Died in 3 1/2 days.
142	313	10 " + " "		No induration.
"	365	5 " + " "	0.3	Induration; no loss in weight.
"	325	3 " + " "	0.5	Loss in weight; induration.
"	325	1 " + " "		Died in 3 1/2 days.

All controls of 1 1/2 f.d.t. died in 2 1/2 to 3 1/2 days. Toxic dose corrected for weight in all tests.

It is then not at all surprising that the amount of so-called normal globulin should be a very varying one if we keep in mind all the various factors which must certainly influence its increase and decrease; nor is it curious that no definite amount of increase can be determined for a given increase in antitoxic value, since many of

[10] f. d. t. = minimal fatal dose of toxin.

these same causes are constantly at work masking results. More refined methods of analysis and separation may bring out differences between the substances increased and show us that there is a constant factor, but at present we cannot say more than that the globulins and antitoxin increase and decrease under the same conditions, and respond in the same manner to all tests.

Although the determination of the protective value of sera from normal horses was undertaken by us principally to establish some relation between these values and the amount of globulin precipitate obtained from the sera, still we hoped also that such determinations might in themselves serve as indications of the value of horses for the production of diphtheric antitoxin.

TABLE XI.

DETERMINATIONS OF THE NEUTRALIZING POWER OF THE GLOBULINS FROM NORMAL SERA.

Horse.	Weight of Guinea Pig.	Globulin sol.[11] and Toxin.	No. of fatal doses protected against.	Result.
135	270	1/2 cc. + 1.5 f.d.t.	3	Gain in weight; no induration.
"	245	1/2 " + 5 "		Died in 7 1/2 days.
137	244	1/5 " + 5 "	25	No induration; no loss in weight.
"	260	1/30 " + 10 "	300	" " " " "

An analysis of the results obtained is so dependent upon one's conception of the relation of the normal protective or neutralizing substances to the true antitoxin, and also upon one's conception of the origin of antitoxins, that some reference to these questions is necessary, although it must be remembered that any discussion of these subjects is at present necessarily largely hypothetical.

It may be generally true that normal horses, the sera of which possess marked neutralizing powers against the diphtheric toxin, are more promising subjects for immunization than are those from whose sera such power is practically absent or only poorly developed; yet this is not the constant experience of ourselves or of others,[12] and

[11] Globulin solution contains the same amount of globulin in 1 cc. as was contained in 1 cc. of the serum from which it was derived.

[12] See Bolton, *Journal of Experimental Medicine*, 1896, I, p. 543.

directly opposed to it has been our experience especially with one of our horses. In this case the normal serum showed quite a marked neutralizing action, while, even after long treatment with toxins, only a very weak antitoxic serum was obtained, and the animal, as a source of antitoxin, was abandoned. Such occurrences make us feel that it is possible for the animal body to take care of a poison by other methods than the increased production of anti-substances, for instance by an unusual power of excretion of the poison, or by rendering it inert by destruction rather than by antagonistic action or neutralization. It seems well at least not to allow our conceptions to become too narrow when dealing with these special problems, lest we overlook some side-reactions which may be going on hand in hand with the main process, and may at times even play a major part, and thus account for the behavior of some animals which show neither great systemic disturbances when inoculated, nor produce antitoxins in quantity in response to such inoculations.

Reference to Horse 136 shows on the other hand that an animal possessing only a very weak protective serum primarily, may be perfectly capable of producing very high grade antitoxin. The experience of Meade Bolton confirms our own, for he writes[13] "that it would seem that the presence or absence of more or less antitoxin normally has no effect upon the ultimate production of artificial antitoxin by inoculation, but its presence enables the inoculation to be made with less risk to the animal." Bolton refers to these substances occurring in apparently normal sera as antitoxin, but we have spoken of them advisedly simply as protective or neutralizing substances, since their identity with the true diphtheric antitoxin is considered by some observers as at least doubtful.

Cobbett[14] has recently published a paper on the question whether the normal horse's serum contains diphtheric antitoxin. To solve the problem of the identity or non-identity of the protective substance with antitoxin he attacks it experimentally, basing his work and conclusions upon an acceptance of the toxin-toxoid composition of diph-

[13] Op. cit., p. 545.

[14] *Lancet*, 1899, ii, p. 332. Also, *Centralbl. f. Bakt.*, 1899, xxvi, p. 548.

theria-culture filtrates as set forth by Ehrlich. Testing the protective normal sera against the toxins in the manner indicated by Ehrlich, he finds that they act in all respects as typical antitoxins. He concludes that these experiments afford a proof of the identity of these substances. He does not, however, think that it follows that the antitoxin is a normal constituent of the serum of the normal horse, but rather that the diphtheric antitoxin present in the blood of certain men and horses is probably acquired, or, in the case of the young, inherited from the mother, by whom immunity has been acquired. "The presence of antitoxin in these animals cannot therefore," he claims, "be held to throw any light on the origin of antitoxin."

To enter into the question as to whether these protective substances are, in the accepted sense of the term, antitoxins or not, would lead us far afield into a discussion of the mechanism of and bases for the production of immunity in general, and of antitoxins in particular; yet we cannot but feel that, so far, the weight of evidence is on the side of those who hold that these substances are the same in composition as the so-called specific antitoxin, and further, that they are either normally present in the blood or capable of being produced under stress by the cells. By the term "specific" in describing antitoxin we mean derived from a personal infection, or an ancestral infection, near or remote, with the specific organism of diphtheria, or from an inoculation with its poisons.

Considering this subject briefly, we find in some horses, with no individual or ancestral history of infection with the diphtheria bacillus, a substance or substances which will protect test animals from multiple fatal doses of diphtheric poison; we find, also, we believe, both from our own experiments and from those of others, that the normal protective or neutralizing substances are globulins (or associated substances that cannot be differentiated from them) just as are the true antitoxic substances, which are formed during an infection or artificial immunization; and, furthermore, whether we accept or do not accept the views of Ehrlich[15] upon which Cobbett has based

[15] For a consideration of these views, based upon experimental work, see Park and Atkinson, *Journal of Experimental Medicine*, 1898, iii, p. 513.

his conclusions, still the fact remains that his experiments have shown that these protective substances in normal serum act toward the diphtheric toxins in precisely the same manner as do the true antitoxins.

If we look upon these facts as in any way proving the identity of the normal protective substances and specific antitoxin, then a disbelief in the antitoxins and the normal protective substances being normal physiological products or potential products of the cells of antitoxin-producing animals finds for its chief foundation a hypothetical past infection or past or present non-pathogenic association with the germs of diphtheria. Even if such infections or associations could be proven, the problem would simply be referred back in the process of evolution, but in nowise elucidated, since there must have been a point in history where no such infection or association had yet taken place, hence the fundamental question at issue is in regard to the bacterial (toxic) or cellular origin of the antitoxins.

Much weighty and convincing experimental evidence[16] has already been brought forward against such views as those of Fraser[17] "that the antitoxic or immunizing substances originate not from vital reactions upon constituents of the body, but from the toxins themselves, being produced by chemical changes in them, or being actually among their normal ingredients"; so that even assuming the presence of toxins, these would not account for the production of antitoxins, unless the latter were products of the cells, and simply increased in response to toxic action or stimulation.

Since we have, then, as far as we can determine, normal animals producing under normal conditions protective substances, which are, according to our tests, identical with the specific antitoxin produced in animals and man by disease or inoculation; and since, unless we assume in all cases a recent past infection or some constant source of toxin supply, neither of which can in most cases be shown to have a basis in fact, we are forced to turn to the animal economy for the supply of antitoxic substance. Hence we are inclined to look upon these substances as products of certain cells of the bodies of the animals in whose sera they are found, or as products which may be

[16] See Salomonsen and Madsen, *Annales de l'Institut Pasteur*, 1898, xii, p. 763.

[17] *Lancet*, 1898, ii, p. 247.

regarded as potential in the cells of these animals which show no normally present antitoxic bodies, but develop them in the presence of toxins. That this last supposition is not purely hypothetical is indicated, as shown above, by horses with practically no normally present protective bodies, but which develop antitoxic sera in response to inoculations.

That these protective substances subserve some other purpose in the normal physiological economy may or may not be true—but that they must have existed as normal physiological products or potentialities even in the first surviving animal attacked by the specific disease, providing cure resulted from the formation of an antitoxin, seems at least probable, if not undeniable.

It appears to us, therefore, that these substances or antitoxins, have in all likelihood had no absolute dependence upon or relation to toxin even remotely in the history of the race or individual, except as they may have been increased and adapted to the protection of the organism in the presence of an infection;[18] and hence that the normal protective substance and specific diphtheric antitoxin are one and the same substance or substances, which fluctuate within certain limits as do all other physiological products, according to the present need and condition and past history of the animal.

Conclusions.

The results of the foregoing experiments may be briefly summarized as follows:

The amount of antitoxic substance obtained by precipitation with magnesium sulphate from the blood-serum of the horse corresponds, as nearly as can be determined by the use of test guinea-pigs, in full to the protective power of the serum from which it is obtained, *i. e.* the precipitate from 1 cc. of serum will protect against the same amount of toxin as 1 cc. of the serum itself.

Equal amounts of the precipitates by magnesium sulphate from immunized and non-immunized horses act differently toward toxin; *i. e.* the proportion of protective substance to the precipitate from

[18] Cobbett has recently reported a conclusive observation of the natural occurrence in a horse of nasal and laryngeal diphtheria caused by Bacillus diphtheriæ (*Lancet*, Aug. 25, 1900, p. 573).—Editor.

non-immunized serum is exceedingly small as compared with the proportion of antitoxin to the precipitate from sera of immunized horses.

The average precipitate from the sera of immunized horses, as obtained by magnesium sulphate, is more abundant than the average precipitate from sera of non-immunized horses.

In the case of the same animal before and after immunization, the serum before immunization gives a less abundant precipitate with magnesium sulphate than the serum tested after immunization.

The proportion of increase per unit of antitoxic strength for the same or different horses is not constant. This may be due to an increase of inactive substances (in their relation to diphtheric toxin) or to imperfect methods of determination.

The precipitates obtained by magnesium sulphate give all the reactions recognized as characteristic of globulins, and as distinguishing them from other albuminous bodies. We are not warranted, then, in the present state of our knowledge, in considering any part of these precipitates as other than globulin. But it does seem warrantable to conclude, from the fact that the globulins of normal serum do not protect, or only in comparatively large amounts, against diphtheric toxin, that new globulins are formed, or rather greatly increased in the serum of immunized horses, and that these globulins protect against the toxin. These increased globulins and the inert globulins (which from obvious causes are a very variable factor) are both precipitated by magnesium sulphate.

Every animal has a physiological and pathological history more or less widely diverging from the normal, hence absolute conformity in the results obtained is not to be expected, at least with our present methods of differentiation.

We desire to express our sincere thanks to Dr. William H. Park, Assistant Director of the Research Laboratory, for the many courtesies shown us throughout the continuance of this work; and also to Dr. George P. Biggs for his valuable assistance in aiding us to procure suitable material for our experiments.

We are especially indebted to Prof. T. Mitchell Prudden of Columbia University for helpful suggestions during the preparation of this article.