ON THE RELATION OF THE ANTITOXIN TO THE GLOBULIN-CONTENT OF THE BLOOD SERUM DURING DIPHTHERIA IMMUNISATION.

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Within recent years a considerable amount of attention has been directed to the subject of the relationships subsisting between the proteids of the blood serum and the antibodies elaborated therein by various methods of immunisation.

One result of such study has been to give an impetus to the more accurate differentiation of the several proteid constituents regarded as bodies possessing not only definite physical and chemical properties, but also what we, with our present knowledge, may call functional affinities.

The investigations recorded in the present paper, dealing with the quantitative relations of the serum globulins during the process of immunisation with diphtheria-toxin, were instigated by the fact that, hitherto, with the exception of the work of Hiss and Atkinson (1901) to be later referred to, only isolated observations have been made of the globulin variations in relation to potency.

It was felt that the only satisfactory method of procuring reliable data on the globulin-antitoxin question was to institute frequent quantitative estimations throughout the whole period of immunisation of individual horses or goats and not at random intervals in their history as antitoxin-producers.

For some considerable time it has been a well-established fact that antitoxin is precipitable from serum by any precipitants which throw down the globulins, but more recent research has shown that the term "globulin" comprises two or more bodies having different salt precipitation limits as well as different antitoxin-contents.

The history of this subject, dealing with the differentiation of the globulins and their relation to the antitoxic substance, is so important in connexion with the observations to be presently recorded, that brief reference must be made to the literature. The work of Marcus (1899), following at long interval after the preliminary observations of Burckhardt (1883), went to show that the hitherto accepted characteristic of serum-globulin in contradistinction to serum-albumin, viz. its insolubility in salt-free water was untenable because, after precipitation by dialysis of the typical insoluble globulin, there remained in solution a relatively much larger quantity of a proteid body, presenting no essential differences from the first either in chemical reaction, coagulation temperature, or elemental constitution.

Consequently, according to Marcus, one must either give up insolubility in water as a characteristic property of globulin or consider the soluble globulin as constituting a new proteid group.

So far, it was impossible by fractional salt precipitation to isolate bodies corresponding to these soluble and insoluble globulins.

Before Marcus' work, attempts had been made to determine the chemical nature of diphtheria-antitoxin and its relation to the serum-proteids.

Thus Aronson (1893) found that the globulin precipitated by dialysis was potent as well as the globulin-free filtrate, while Dieudonné (1897), by magnesium sulphate precipitation, obtained the greater part of the antitoxic substance in the precipitate and only a small part in the globulin-free filtrate.

Brodie (1897) was the first to show that diphtheria-antitoxin was completely precipitated from a solution by any means which removed the globulins. He also attempted a fractional precipitation of serum with ammonium sulphate up to half-saturation, thus separating four successive portions, all of which were found to contain antitoxin in equal amount. That the mode of preparation of the globulin was of importance was shown by Belfanti and Carbone (1898), who found that the antitoxin was carried down along with the globulins from ammonium or magnesium sulphate precipitation but not with the precipitate obtained by acetic acid.

In the course of an attempt to follow up Brieger and Boer's work on the preparation of a proteid-free antitoxin, Freund and Sternberg (1899) discovered that by addition of a solution of potash-alum to serum (to one volume serum, one-third volume of a 5 % solution of potash-alum) all the antitoxin remained in the filtrate. In fact, by this

precipitant the albumin was removed first and a filtrate left which, according to Freund and Sternberg, contained practically all the globulin and consequently all the antitoxin. Further, by precipitating this globulin filtrate with ammonium sulphate, it was possible to obtain in small bulk the globulin-content of a large quantity of serum. No further light, however, could be thrown by these investigators on the chemical nature of antitoxin. All that could be said was that the antitoxic substance was precipitated along with the globulins.

The method of treating serum with potash-alum in order to get rid of albumin was employed by Seng (1899), who dealt with the question whether the sera of normal and immune animals presented differences in the amount and nature of their proteid constituents. The "soluble" and the "insoluble" globulin fractions were separately estimated, and the total proteid determined by Kjeldahl.

From the few isolated estimations on horses immune to diphtheria-toxin, no conclusive facts could be elicited as to the relative quantities of total globulin, albumin, and insoluble globulin. Though no quantitative differences could be found between the "soluble" globulin-content of normal and antitoxic serum, certain slight differences in coagulation temperature and specific rotation were made out. It was further determined that prolonged dialysis of the potash-alum filtrate until no reaction to chlorine, ammonia, or sulphuric acid remained, rendered only a very small fraction of the globulin insoluble, viz. 1:23 to 1:11. All the antitoxin was recoverable from the solution. These latter experiments of Seng threw light on the rather discordant results of earlier writers. Thus the result obtained by Belfanti and Carbone that the antitoxin falls out with the globulins when precipitation is effected by magnesium or ammonium sulphate, but not by acetic acid or carbon dioxide, is explained by the fact that simple neutralization of the serum by acetic acid or carbon dioxide precipitates only the "insoluble" globulin which contains none of the antitoxin. So also the statement by Dieudonné that "reines globulin" precipitated by carbon dioxide or by dialysis contained no antitoxin is explained on similar grounds.

A notable advance was made by Hiss and Atkinson (1901), who estimated the globulin-content of the serum of a large number of horses at different stages of immunisation against diphtheria-toxin. The globulin was precipitated by the magnesium sulphate method. The total proteid was obtained by heat coagulation and the albumin estimated by difference. As a rule not more than two globulin estimations were made on each horse, one before the commencement

of immunisation and the other at some stage in the process. When the potency had reached a level of 300 units or over, the globulin was invariably found to have risen, sometimes to double its original value. In one horse five globulin estimations were made during immunisation, the figures (obtained from 10 c.c. serum) being as follows:

Normal	400 units	600 units	650 units	1200 units
·3235	·4743	·5116	·5934	·8987

A progressive increase in globulin was thus evident as the potency rose. While the globulin rose the albumin fraction progressively diminished. It appeared generally that a low potency coincided with a low globulin-content, but it was impossible to take the absolute amount of globulin as an index of the antitoxin-content of the serum.

Within the last few years important contributions to our knowledge of the globulins have emanated from the Hofmeister school. Fuld and Spiro (1900) determined that by fractional precipitation of serum with ammonium sulphate two globulin fractions could be obtained, one precipitable by 28–33 % saturation and the other only by 34–46 % saturation. To the former fraction precipitable by one-third saturation, Hofmeister gave the name "euglobulin," and to the latter precipitable only by half-saturation, the name "pseudoglobulin." Included in the euglobulin fraction is the fibrinoglobulin, which Reye (1898) had already found to be precipitated by 21·5 % saturation with ammonium sulphate. The interesting fact was recorded that these three fractions differed with regard to their influence on milk coagulation. The fibrinoglobulin had no constant action either in producing or preventing coagulation. The euglobulin had decidedly the power of coagulating milk, while the pseudoglobulin had a more or less pronounced inhibitory action on milk coagulation.

The relation of diphtheria-antitoxin to the three globulin fractions precipitable by ammonium sulphate up to half-saturation was investigated at great length by Pick (1902), who found that neither the fibrinoglobulin nor the euglobulin of horse serum contained the antitoxin. The pseudoglobulin, on the other hand, contained all the antitoxin. On the other hand, when goat serum was salted out in the same way and the fractions tested as to their antitoxin-content, it was found that the euglobulin contained the antitoxic substance.

Freund and Joachim (1902) repeated much of Spiro's work, and were unable to identify completely the euglobulin and pseudoglobulin with the "insoluble" and "soluble" globulin obtained by dialysis,

acetic acid or sodium chloride. It was determined that both the euglobulin and the pseudoglobulin could again be split up into two portions, one of which was soluble and the other insoluble in water. They accordingly recommended the following as a more accurate classification of the globulins:

Fraction obtained by one-third saturation	*a.*	Insoluble in H_2O.	Paraeuglobulin.
	β.	Soluble in H_2O.	Euglobulin.
Fraction obtained by half-saturation	*a.*	Insoluble in H_2O.	Parapseudoglobulin.
	β.	Soluble in H_2O.	Pseudoglobulin.

There is no doubt that the division into euglobulin and pseudoglobulin is a more or less artificial one from the purely chemical point of view as Hammarsten maintains, but the remarkable differences exhibited by them in their capacity as antibody-carriers proclaim a real duality and render it highly essential that we should retain this somewhat arbitrary mode of separation.

Porges and Spiro (1903) admitted the correctness of Freund and Joachim's statement that the eu- and pseudoglobulin fractions did not represent exactly the sum total of the insoluble and soluble globulins respectively. In the same year Joachim (1903) recorded a few quantitative estimations of the globulin fractions in a horse immune to diphtheria-toxin. The estimations were made on two occasions, one before the commencement of immunisation and the other three months later, when the serum had reached a potency of 500 units. He found no essential increase of the total proteid contrary to what had previously been noted by Butjagin (1902) and Szontagh and Wellman (1898). The total globulin was, however, markedly increased at the expense of the albumin, but contrary to expectation the rise affected solely the euglobulin fraction which contained none of the antitoxin. His figures were:

Before immunisation,

Eugl. : Pseudogl. : Alb. :: 12·74 : 37·04 : 50·21.

After immunisation,

Eugl. : Pseudogl. : Alb. :: 26·21 : 36·74 : 37·05.

This remarkable result we shall discuss later.

The question of globulin variations in other experimental infections has been approached by Langstein and Mayer (1904), who showed that in rabbits immunised against typhoid, pneumococcus, sheptococcus, dysentery, cholera, and swine erysipelas the serum-globulin rose while

the albumin diminished. It was determined also by Mayer (1905) that the serum of dogs infected with *Trypanosoma* showed an increase of globulin and a diminution of albumin, although the total proteid was not markedly affected.

The objections which several authors have recently brought forward against the view that globulin-change is a necessary concomitant of the elaboration of antibodies will be discussed in the course of this paper in the light of our own observations.

Description of Technique and Mode of Investigation.

Though the main object of the present investigation was to ascertain by examination at frequent intervals whether any definite relationship could be traced during immunisation between the rise in potency and the globulin variations, it appeared desirable also to determine how far Pick's statements as to the antitoxin-contents of the various globulin fractions held good in the horse and goat.

For the first two series of investigations a horse (Plug) and a goat (Mephistopheles) were employed. The former was treated by the usual method which obtains at this institute, and previous to the commencement of immunisation estimations were made of the normal globulin and normal antitoxin present in the blood serum.

Half-saturation with ammonium sulphate was always employed to separate the total globulin from the albumin. The precipitate was redissolved in water and again treated with ammonium sulphate. After filtration the precipitate was again dissolved and coagulated by heat. The coagulated globulin was collected on a weighed filter paper, thoroughly washed with hot water and then dried in vacuo over sulphuric acid for several days. After a final dessication in an air-bath at 80° it was weighed.

The filtrates containing the albumin were also coagulated and weighed in a similar manner. In estimating the total proteid a fixed quantity of serum, 10 c.c., was diluted by addition of 190 c.c. of distilled water, and carefully acidified with acetic acid. It was then coagulated by heat and the precipitate dried and weighed. Where complete estimations of the euglobulin and pseudoglobulin were made, as well as of the total globulin and total proteid, three quantities of serum (each 10 c.c.) were used. With one quantity the total coagulable proteids were estimated, with another the total globulin and total albumin, and with the third quantity the euglobulin was obtained by third-saturation

with ammonium sulphate, the filtrates being employed for the estimation of the combined pseudoglobulin and albumin. The difference between this last and the albumin previously estimated, gave the amount of pseudoglobulin.

The potency of the serum was estimated in Ehrlich-Behring units by the usual methods practised in this Institute.

Immunisation of Horse (Plug).

Immunisation was commenced on 17 Nov. 1905, with an inoculation of ·01 c.c. toxin. Inoculations were made thereafter every third or fourth day, provided all local swelling had disappeared, each successive dose being double the preceding one.

In the following table (p. 72) are indicated the toxin-doses and the potency of the serum on the different dates.

According to the practice at this Institute the horses are bled nine days after they have received the final dose of 1000 c.c. toxin. The potency of the serum when tested at this time, *i.e.* after the first bleeding varies as is well known in different horses quite apart from the methods of immunisation employed. In our experience here the great majority of the horses attain a potency of 600 units and over, at the first bleeding, but there is always a small percentage of horses which do not reach 300 units. These latter are of course discarded so far as their employment for the production of diphtheria-antitoxin is concerned.

From the following table it will be seen that unfortunately for the object of our experiment, this horse proved unsuitable for the production of high grade antitoxin. Our hopes that the serum might attain a high antitoxin-content so that a suitable comparison might be made between this and the globulin-content were disappointed.

From the purely theoretical point of view, however, the facts brought by the immunisation of this refractory horse, present numerous points of interest and enable one to institute some comparison between the reaction to diphtheria-toxin of such horses and the more responsive animals.

On the 10th day after the first dose of 1000 c.c. toxin (2nd Feb. '06), (see Table I. and Chart I.), the serum had an antitoxin-content of only 180—200 units per c.c. One litre of blood was drawn and a further dose of 1000 c.c. given on 5th Feb. On the 9th day thereafter (14 Feb.) the potency had risen slightly to 240—250 units. On the 19th Feb., 22nd Feb., and 26th Feb. further doses of 500 c.c., 700 c.c., and 1000 c.c.

respectively, were administered with little appreciable effect on the potency.

TABLE I.

Date	Toxin-dose	Potency (in units)
Nov. 10 '05	—	$\frac{1}{20}$—$\frac{1}{15}$ per c.c.
17	·01	—
20	·02	$\frac{1}{16}$—$\frac{1}{4}$
23	·04	$<\frac{1}{8}$
27	·1	1—2
30	·2	2—4
Dec. 4	·5	2—4
7	1·0	<6
11	2	4—6
15	4	—
19	8	8—12
22	15	8—12
26	30	12—16
29	60	12—20
Jan. 1 '06	120	<20
4	250	20—28
8	500	40—50
12	500 (new tox.)	60—80
16	800	80—100
19	—	110
23	1000	110—120
26	—	140—160
31	—	160—180
Feb. 2	—	180—200
5	1000	—
9	—	<200
14	—	240—250
19	500	—
22	700	—
26	1000	<240
Mar. 7	—	250—300
12	1000 intraven.	—
21	—	<200

Thus on 7th Mar. the serum had only between 250 and 300 units per c.c. A final attempt to raise the potency was made on 12th Mar. when 1000 c.c. toxin were injected intravenously by the jugular vein. The animal suffered no bad effects from the injection, but after a lapse of 10 days the potency instead of being raised had actually fallen below 200 units per c.c. In fact this latter experiment is in complete accord with a previous one made by Dr Dean and which goes to show that the intravenous injection of diphtheria-toxin has little or no effect in raising the potency in a horse whose antitoxin-content is falling.

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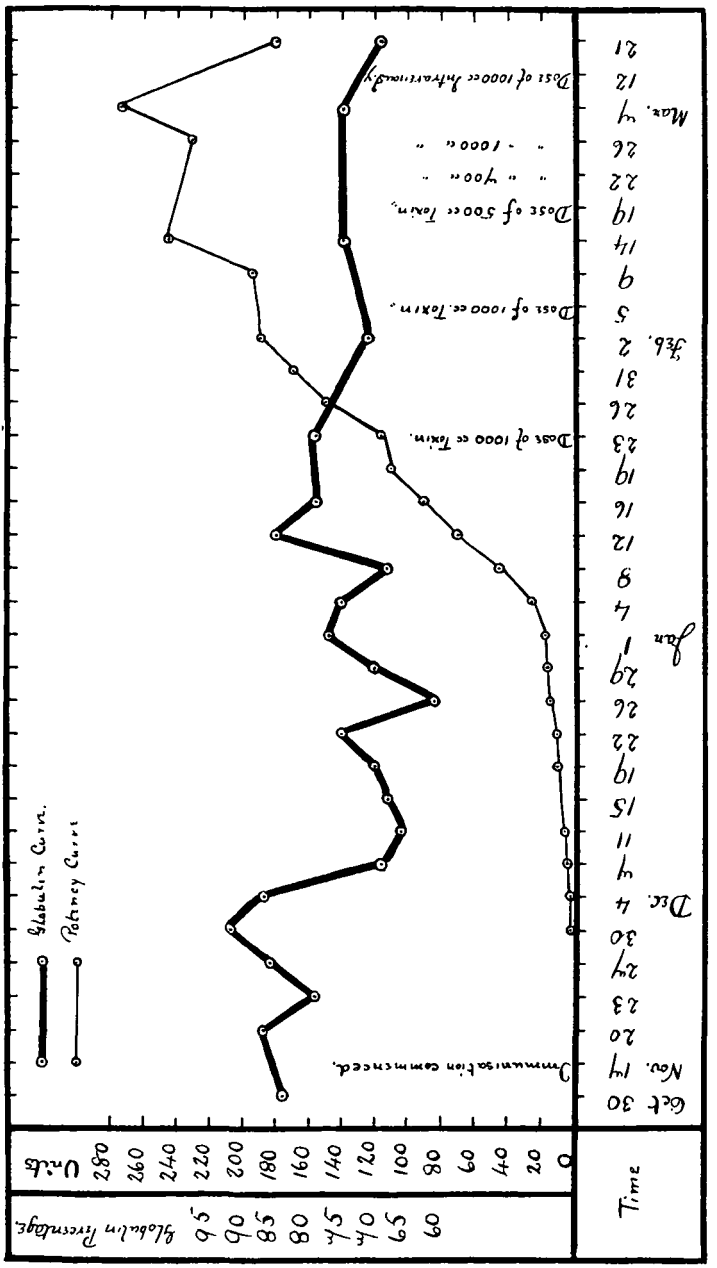

CHART I.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid during immunisation of horse (Plug).

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TABLE II.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
Oct. 30 '05	·8532	·7191	84	·1341
Nov. 20	·8816	·7608	87	—
23	·8330	·6618	79	—
27	·8702	·7529	86	—
30	·8034	·7400	92	—
Dec. 4	·8220	·7182	87	—
7	·8987	·6247	69	—
11	·8201	·5945	66	—
15	·8304	·5710	68	—
19	·8006	·5648	70	—
22	·8334	·6278	75	—
26	1·0622	·6532	61	—
29	·9382	·6582	70	—
Jan. 1 '06	·9273	·7213	77	·2060
4	·9372	·7064	75	·1696
8	·8906	·6074	68	—
12	·7460	·6653	89	—
16	·9294	·7390	79	—
23	·9711	·7727	79	·1478
Feb. 2	1·0272	·7357	71	·2370
14	·9561	·7204	75	—
Mar. 7	·9083	·6811	75	·1804
21	·8468	·5890	69	·1728

Horse (Plug).

In the above table (Table II.) are recorded estimations of the total coagulable proteid, and total globulin with a few albumin estimations. In all cases the amounts given are those derived from 10 c.c. serum. Starting with an initial value of ·8532 gm. on Oct. 30 before immunisation, the total proteid exhibits very slight fluctuations throughout the period ending Dec. 22 the average value being ·8406 gm. During the next period ending Jan. 23 the total proteid showed a slight though quite appreciable rise, the average for the period being ·9252 gm. From Jan. 23 onwards this higher level was maintained.

Regarding the globulin variations it will be seen in the first place that the initial value was much higher than that recorded by Hammarsten for the normal horse. In fact the value ·7191 gm. is nearly double the normal value. Thus three normal horses described by Hiss and Atkinson had initial values of ·3085 gm., ·4342 gm., ·3988 gm. in 10 c.c. serum while the horse "Wright," presently to be described, had an initial globulin value of ·4365 gm.

The fluctuations in the percentage globulin-content of the total proteid will be more readily followed from Chart I.

No progressive rise in the globulin-content occurred during the process of immunisation. While the total proteid remained stationary during the period Nov. 17 to Dec. 22 the globulin-content maintained its initial level only for a short time. It then fell persistently attaining its lowest level on Dec. 26.

During the second period when the total proteid was at a higher level, the globulin-content slowly rose to its initial value beyond which it never mounted. The values recorded in the period Jan. 23 to Mar. 21, show that a fall to a lower level had again occurred.

The rise in the total proteid from Dec. 22 onwards was due not to an increase of the globulin but of the albumin fraction.

The important question, whether our failure to obtain a high-grade antitoxic serum in this horse, is in any way related to the peculiar behaviour of the globulin, will be discussed later.

Immunisation of Goat (Mephistopheles).

In the immunisation of the goat, almost exactly the same procedure was adopted as in the case of the horse except that the initial dose of toxin was only ·001 c.c.

The inoculations were made not intramuscularly but subcutaneously in the dorsal region over the erector spinae mass. Before immunisation the normal serum was tested for the presence of normal antitoxin and quantitative estimations of the proteids made. Further in view of the possibility of globulin-variations due to lowered condition etc. the animal was carefully weighed every week. The annexed table (Table III.) shows the scheme of immunisation and the potency of the serum on the different dates.

It was found that the toxin doses were well tolerated up to the middle of February when the animal began to show signs of weakness. After the dose of 100 c.c. the goat developed slight paresis in the hind limbs but appetite remained good and a further injection of 150 c.c. was made on the 19th February. The paresis however became worse and latterly affected the fore limbs so that the animal had to lie on its side. Though thus prostrated appetite remained good but death took place suddenly on 3rd March probably from heart failure.

At the post mortem the stomach was enormously dilated with food-stuffs. Apart from haemorrhagic oedema of the lower pulmonary lobes there were no gross changes in the organs. The serum obtained post mortem had between 20 and 30 units per c.c.

TABLE III.

Date	Toxin-dose	Weight	Potency (in units)
Nov. 6 '05	—	—	$<\frac{1}{5}$
9	—	—	$<\frac{1}{20}$
20	—	—	$\frac{1}{40}$
Dec. 1	·001 c.c.	90 lbs.	—
5	·002	—	—
9	·004	—	—
13	·01	89	—
18	·02	—	—
23	·04	—	$<\frac{1}{3}$
27	·1	80	—
30	·2	—	—
Jan. 3 '06	·5	88	—
8	1	—	—
12	2	87	—
16	3	—	4
19	6	80	—
23	12	—	5—10
29	25	—	—
Feb. 3	50	—	<5
7	50 (new tox.)	—	—
12	100	—	5—10
19	150	—	15—20
Mar. 3	Death	—	20—30

From the potency curve (see Chart II.) it will be seen that it required six weeks' immunisation to raise the antitoxin-content to one unit per c.c. Thereafter progress was more rapid, but for a period of three weeks, Jan. 23 to Feb. 12, the potency appears to have remained more or less stationary with an interval of depression.

After the 12th Feb. the antitoxin-content rose markedly and continued to do so up to the fatal conclusion in spite of the diphtheritic paralysis. Records of immunisation of goats against diphtheria-toxin are not numerous and it does not appear that these animals are capable of producing serum of high potency.

In one goat previously immunised at this Institute a potency of 40 units was reached. The goat serum with which Pick worked had a very low antitoxic value inasmuch as ·1 c.c. was required to neutralize 10 lethal doses of a toxin whose M.L.D. was only about ·01 c.c. (The toxin employed here in the immunisation of the horses and goat had a M.L.D. of ·002 c.c.)

In Table IV. are recorded the values of the total proteid, total globulin and albumin, during the immunisation of the goat. The figures for the normal serum of Nov. 6 show, as in the horse, a marked

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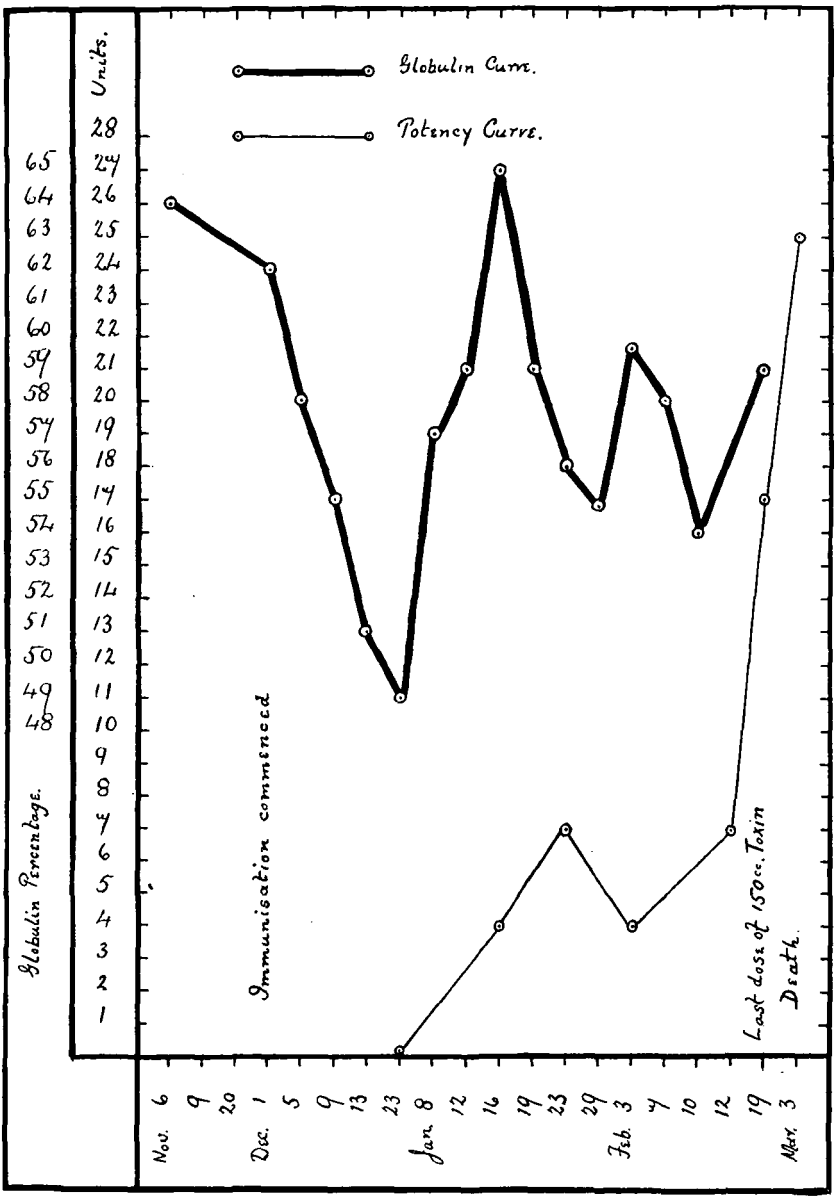

CHART II.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid, during the immunisation of goat (Mephistopheles).

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preponderance of the globulin—over the albumin—fraction. The total proteid shows a pronounced and fairly progressive rise from ·5974 gm. on Dec. 1 to ·8555 gm. on Jan. 29. This increase which was equivalent to one of 43 % on the initial value greatly exceeded in amount the corresponding increase in the horse "Plug," but when we consider the tables we find that the very slight increase of total globulin during immunisation, is quite insufficient to account for the greatly increased total proteid. Indeed, as will be apparent from Chart II. the percentage globulin-content of the total proteid fell markedly during the first three

TABLE IV.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
Nov. 6 '05	·5867	·3775	64	·2092
Dec. 1	·5974	·3739	62	—
5	·6668	·3868	58	—
9	·6908	·3820	55	·2261
13	·6161	·3156	51	—
18	·6388	—	—	—
23	·6428	·3204	—	—
27	—	—	—	—
30	—	—	—	—
Jan. 3 '06	—	—	—	—
8	·7430	·4252	57	—
12	·7410	·4399	59	—
16	·7150	·4644	65	—
19	·7608	·4548	59	—
23	·8128	·4554	56	—
29	·8555	·4702	54·9	—
Feb. 3	·7615	·4547	59·7	·2873
7	·7669	·4476	58	—
10	·7581	·4098	54	—
19	·6852	·4047	59	·2597

Goat (Mephistopheles).

weeks. It then rose gradually till it attained a value slightly above the initial figure of 64 %. The rise, however, was only temporary and was succeeded by a second fall which lasted with slight fluctuations during the period of maximum potency. It must be said, therefore, that the great increase in the total proteid was due in far greater measure to the albumin than to the globulin fraction

Such a result, in view of the fact that the globulin is the antitoxin carrier, seems rather surprising and will demand our consideration later.

Immunisation of Horse (Wright).

As the horse "Plug" proved unsatisfactory for the production of high grade antitoxin and consequently did not permit of our drawing any very definite conclusions regarding the relationship of the serum-globulins to the potency, it was decided to immunise another horse.

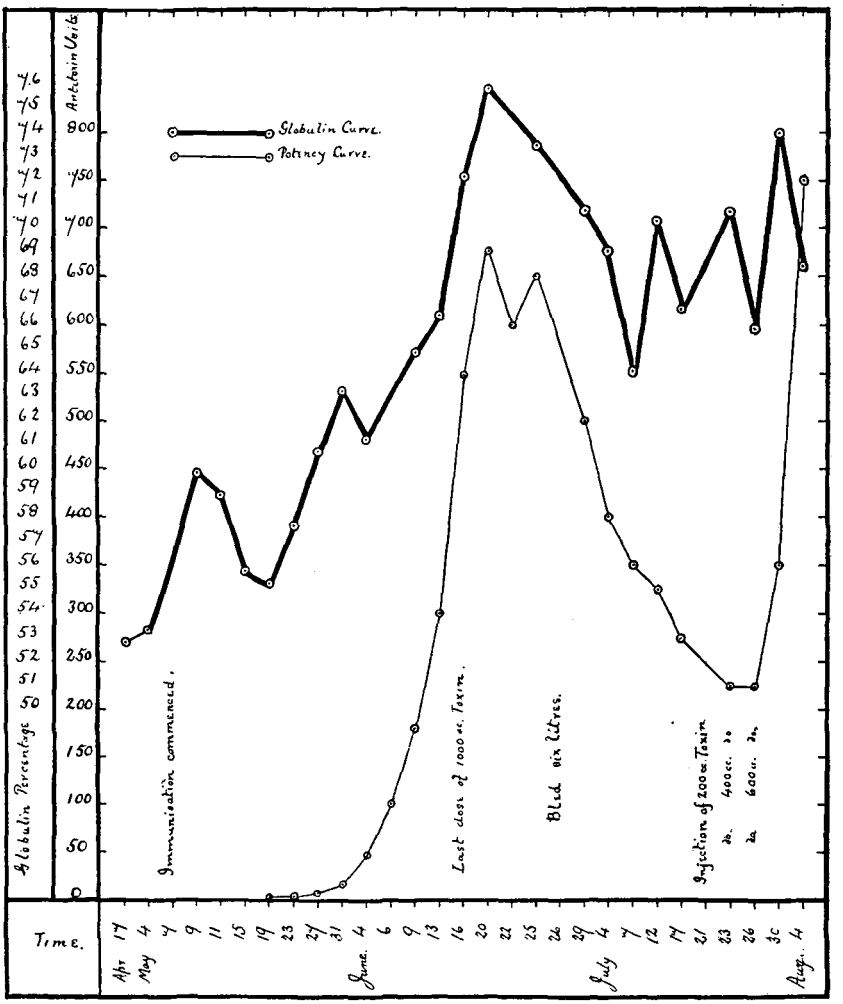

CHART III.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid during the immunisation of horse (Wright).

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The procedure adopted was in all respects similar to that in the case of "Plug" except that the inoculations were made, where possible every second day. No delay was experienced until doses of 200, 400, and 600 c.c. were administered when the inoculations were postponed for a day or two to allow local swelling to subside.

In the following table (Table V.) are recorded the toxin-doses and potency of the serum during the course of immunisation.

It will be observed that "Wright" reacted in a much more characteristic way to diphtheria-toxin than "Plug," and the potency curve (see Chart III.) may be considered a fairly typical one.

TABLE V.

Date	Toxin	Potency (in units)
April 17 '06	—	$<\frac{1}{4}$
May 7	·01	—
9	·02	—
11	·04	$<\frac{1}{3}$
13	·1	—
15	·2	—
17	·5	—
19	1	2—4
21	2	—
23	4	2—4
25	8	
27	15	8
29	30	—
31	50	16
June 2	100	—
4	200	>40
6	400	100
9	600	180
13	—	300
16	1000	550
20	—	675
22	—	600
25	—	650
26	Bled 6 litres	—
29	—	500
July 4	—	400
7	—	350
12	—	325
17	—	250—300
21	200	—
23	400	225
26	600	200—250
30	—	>300
Aug. 4	Bled 4 litres	750

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After the first bleeding, the potency gradually fell to 300 units and under, but a second series of inoculations caused it to mount upwards again to a level higher than that attained at the end of the first series. Such a phenomenon is not unfrequent.

In Table VI. are recorded the values of the total proteid, total globulin and albumin during immunisation. A series of euglobulin and pseudoglobulin estimation are also entered.

TABLE VI.

Date	Total proteid	Total globulin	Albumin	Euglobulin	Pseudo-globulin	Globulin percentage	Albumin percentage
Apr. 17 '06	·8266	·4365	—	—	—	52·8	—
May 4	·7954	·4241	—	—	—	53·3	—
9	·8440	·5050	·2734	·1600	·3333	59·8	32·3
11	·7964	·4692	—	—	—	58·9	—
15	·8289	·4622	·2636	·0952	·3544	55·7	31·8
19	·7278	·4021	·2826	—	—	55·2	38·8
23	·7884	·4546	·2545	—	—	57·6	32·2
27	·8226	·4998	·3027	·0916	·3893	60·7	36·7
31	·8043	·5094	·3005	·1495	·3131	63·3	37·3
June 4	·8065	·4941	·3046	·1358	·3072	61·2	37·7
9	·8346	·5419	·2456	—	—	64·9	29·4
13	·9452	·6278	·1930	·2178	·4295	66·4	20·4
16	1·0234	·7379	·2363	·1146	·6040	72·1	23·0
20	1·0600	·8046	·2004	·2934	·4080	75·9	18·9
25	·9387	·6893	·2043	·2795	—	73·4	21·7
29	·8519	·6026	·1916	·1320	·4286	70·7	22·5
July 4	·8190	·5655	·2151	—	—	69·0	26·2
7	·7963	·5102	·2333	—	—	64·0	29·3
12	·8225	·5785	·2596	—	—	70·3	31·5
17	·7853	·5238	·2874	—	—	66·7	36·6
23	·8398	·5940	—	—	—	70·7	—
26	·9342	·6159	—	—	—	65·9	—
30	·9429	·6986	—	—	—	74·0	—
Aug. 4	·9276	·6343	—	—	—	68·3	—

Horse (Wright).

During the first month the total proteid remains fairly constant with slight fluctuations, but a rapid rise takes place between the 9th and 20th June, a period corresponding to the maximum antitoxin development. This rise is succeeded by an appreciable fall during the resting period following the bleeding. A second rise, however, makes its appearance after the second series of inoculations. Roughly the total proteid rose from 8 % before immunisation to 10 % at the period of maximum antitoxin development.

Apart from slight fluctuations, the absolute amount of globulin rises very slowly during the first three weeks so that the percentage globulin-content of the total proteid also rises (see Chart III.). The increase is much more rapid in the second month when the potency is rising the figure for 20 June being almost double the initial figure before immunisation. At the period of maximum globulin-content the albumin percentage is reduced to 18·9 %.

We have, therefore, to record in this horse a very marked rise in the globulin-content coincident with the development of a high potency and with a reduction of the albumin fraction. Regarding the euglobulin and pseudoglobulin estimations the results are somewhat indefinite but would at least indicate that in the globulin increase, both fractions are implicated and the euglobulin more so than the pseudoglobulin. The result is in more or less agreement with that recorded by Joachim and to which we have already referred.

After the bleeding and during the resting stage the total proteid and total globulin fall while the albumin remains more or less stationary or even tends to rise. When the inoculations were renewed, the total proteid and total globulin again rose but more slowly and even at the period when the potency had reached its second maximum the total globulin had not quite reached a value equal to its previous maximum at the end of the first series of inoculations. The globulin values, however, never attain the low limits prevailing before the commencement of immunisation and during the earlier part of the process.

The question of the influence of inanition or repeated bleedings, on the constitution of the blood plasma has undoubtedly to be considered in all experimental work designed to bring out a causal connexion between rise in potency and rise in the globulin fraction of the blood serum. In this connexion we may cite the work of Githens (1904) who found that in hungering dogs a rise in the globulin fraction occurred especially affecting the fibrinoglobulin and euglobulin, while after repeated bleedings the albumin-content rose markedly.

This peculiar relationship of the albumin and globulin fractions inclined the author to believe that the albumin behaves as if it came nearest to the "Nahrungseiweiss" and suggested the possibility that the globulins under these conditions might be derived from the albumins either inside or outside the circulating blood.

Glässner (1905) investigated the changes in the blood plasma before and after the immunisation of rabbits with bacterial emulsions, bacterial

toxins and proteid bodies as horse or ox sera. He came to the conclusion that a rise in the globulin fraction was only marked in those animals which had lost weight during immunisation, and that consequently a rise in the globulin fraction could not be considered an essential concomitant of the formation of antibodies but only as a secondary symptom depending on inanition. Provided immunisation is cautiously proceeded with and the animals not allowed to get into an enfeebled condition, a rise in globulin need not necessarily follow. Glässner thinks his views are supported by the results obtained by Githens already quoted but as has also been pointed out recently by Moll (1906) the globulin values obtained by Githens after inanition are not to be compared to the high values obtained by immunisation.

In Moll's own experiments on fasting dogs and rabbits no marked rise in the globulin fraction was noted. In two cases the globulin even fell. By immunising with horse serum however a great rise in globulin was observed and was accompanied by the development of marked precipitin formation. He does not believe that the rise in globulin is an inanition effect though it is possible that pure inanition may influence this phenomenon during immunisation.

Before commencing our work we had in view the possibility of inanition or loss of weight as possible disturbing factors. Unfortunately the horses were not weighed during immunisation but it is the general experience in this Institute that, so far from losing weight the horses appear actually to put on flesh. They receive as much nourishment as they will take and enjoy regular exercise. It is questionable, however, how far we are to consider this apparent well-being as a criterion of an undisturbed metabolism. The occasional occurrence of waxy disease, hepatic haemorrhage etc. causing sudden death in apparently healthy horses undergoing immunisation for long periods, suggests that metabolism may be profoundly altered without much visible sign being afforded of its effects.

Determinations of the antitoxin-contents of the proteid fractions in the horse and goat.

We have already referred to Pick's work showing that in the horse the pseudoglobulin is the antitoxin carrier, while in the goat the euglobulin performs that function. As no confirmatory evidence on this important question is yet available, and as the possibility was always present that the sera of different horses might comport themselves

differently in regard to the antitoxin-contents of their respective fractions, it seemed desirable to test the potency of a number of proteid solutions isolated from horse and goat serum at different stages in immunisation.

The separations were all performed by the ammonium sulphate method and the various precipitates were dissolved in distilled water. Where the final fluid exceeded in bulk that of the original serum quantity taken, the necessary allowance was made in testing the potency.

For each test three or four guinea-pigs of standard weight were employed.

Experiments on Horse serum.

Exp. I. Total globulin.

Serum employed: Plug's serum of March 7, 1906 containing 250—300 units per c.c.

Mode of separation: A single precipitation with $(NH_4)_2SO_4$ to half saturation.

Dissolved precipitate tested for 100, 200 and 250 units in a series of three pigs.

Result: Pig No. (3) died on 4th day while others lived.

Remarks: The globulin contained practically all the antitoxin.

Exp. II. Total albumin.

Mode of separation: Filtrate from Exp. I.

Tested for 10, 20 and 40 units in three pigs.

Result: All three pigs died on 2nd day.

Remarks: Apparently the albumin contains none of the antitoxin.

Exp. III. Euglobulin.

Serum employed: Plug's serum of March 21, 1906 containing 200 units per c.c.

Mode of separation: A single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 10, 20 and 40 units in three pigs.

Result: All three pigs lived.

Remarks: No conclusion can be drawn as a certain amount of the pseudo-globulin may have been mechanically carried down in the first precipitate.

Exp. IV. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. III.

Tested for 50, 100 and 150 units.

Result: Pig No. (1) lived, pig No. (2) died on 4th day, and pig No. (3) died on 2nd day.

Remarks: The combined pseudoglobulin and albumin contained more than half the antitoxin after a single separation from euglobulin by one-third saturation.

Exp. V. Euglobulin.

Same serum as in Exp. III.

Mode of separation: Complete separation of euglobulin by repeated precipitation with $(NH_4)_2SO_4$.

Tested for 10, 20 and 40 units.

Result: Pig No. (3) died on 2nd day while the others lived.

Remarks: The euglobulin fraction even after complete separation from the pseudoglobulin and albumin contained 20 units per c.c.

Exp. VI. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. V.

Tested for 50, 100 and 150 units.

Result: Pig No. (3) died on 3rd day while others lived.

Remarks: The pseudoglobulin-albumin therefore contained three-fourths of the antitoxin.

Exp. VII. Euglobulin.

Serum employed: Serum of "Togo" containing 600 units per c.c.

Mode of separation: Single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 25, 50 and 100 units.

Result: All three pigs lived.

Remarks: The remarks on Exp. III. apply here also.

Exp. VIII. Euglobulin.

Same serum as in Exp. VII.

Mode of separation: Complete separation from pseudoglobulin by repeated precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 25, 50 and 100 units.

Result: All three pigs died on 2nd day.

Remarks: The euglobulin apparently contains none of the antitoxin.

Exp. IX. Pseudoglobulin-albumin.

Mode of separation: Combined filtrates from Exp. VIII.

Tested for 400, 500 and 600 units.

Result: Pig No. (1) lived while pig (2) and (3) died on 2nd day.

Remarks: The pseudoglobulin-albumin certainly contained over two-thirds of the antitoxin but some loss of antitoxin has apparently occurred in the preparation of the different fractions.

Experiments on Goat serum.

Exp. I. Total globulin.

Serum employed: Mephistopheles' serum of 19 Feb. 1906 containing 15—20 units per c.c.

Mode of separation: Complete separation from albumin by repeated precipitation with $(NH_4)_2SO_4$ to half saturation.

Tested for 5, 10 and 15 units.

Result: Pig No. (3) died on 3rd day while the others lived.

Remarks: The globulin appears to contain all the antitoxin.

Exp. II. Euglobulin.

Serum employed: Meph. serum of Feb. 7, 1906 containing about 5 units per c.c.

Mode of separation: Single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 3, 4 and 5 units.

Result: Pig No. (3) died on 3rd day while the others lived.

Remarks: The euglobulin appears to contain all the antitoxin but the experiment is not conclusive, as some of the pseudoglobulin may have been carried down in the first precipitation.

Exp. III. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. II.

Tested for 2, 3 and 4 units.

Result: All three pigs died on 2nd day.

Remarks: Evidently a single precipitation with $(NH_4)_2SO_4$ to one-third saturation removed all the antitoxin, leaving none in the pseudoglobulin-albumin filtrate.

Exp. IV. Euglobulin.

Serum employed: Meph. serum of Jan. 29, 1906 containing about 5 units per c.c.

Mode of separation: Complete separation from pseudoglobulin-albumin by repeated one-third saturation with $(NH_4)_2SO_4$.

Tested for 2, 3, 4 and 5 units.

Result: All pigs died on 2nd day.

Remarks: It would appear that on this occasion none of the antitoxin was contained in the euglobulin fraction.

Exp. V. Euglobulin.

Serum employed: Meph. serum of 19 Feb. 1906, containing 15—20 units per c.c. (same serum as in Exp. I.).

Mode of separation: Complete separation from pseudoglobulin-albumin by repeated one-third saturation with $(NH_4)_2SO_4$.

Tested for 5, 10 and 15 units.

Result: All three pigs died on 1st day from toxaemia.

Remarks: Taken in conjunction with Exp. I. it would seem that on this occasion all the antitoxin must have been contained in the pseudoglobulin fraction.

General Summary of Experimental results.

There appears to be no doubt that the greater part, if not the whole of the antitoxin of horse serum is contained in the pseudoglobulin fraction. In the horse "Togo" which was yielding serum of high potency not more than 2—3 % of the antitoxin could have been present in the euglobulin when completely separated from the pseudoglobulin.

In the horse "Plug," however, the euglobulin when completely separated from the pseudoglobulin still contained fully 10 % of the antitoxin. It will be remembered that from Feb. 14, 1906 onwards

little change took place in the potency in spite of repeated stimulation. The serum of Mar. 21 (see Exp. V.) was drawn at a time when the potency was evidently falling or at least stationary so that the conclusion is forced upon us that in the serum of a refractory horse which responds sluggishly or not at all to stimulation there may be no such sharp delimitation of antitoxin to the pseudoglobulin as in the serum horses more susceptible to stimulation.

In order to obtain reliable data on this point, however, it would be necessary to make a larger series of experiments on the above lines, at different stages in the immunisation of refractory horses.

In the case of the goat I am unable to confirm Pick's statement that the antitoxin is invariably linked on to the euglobulin fraction. The analysis of sera on 29th Jan. and 19th Feb. showed that the euglobulin contained none of the antitoxin, while on the intervening date, 7th Feb., the euglobulin (though here incompletely separated) contained all the antitoxin. It is interesting to observe that between the 23rd Jan. and 12th Feb. the potency remained stationary with an intervening depression so that again we have the possibility suggested to us that during a refractory period which corresponds in all probability to an abnormal metabolic activity, the distribution of the antitoxin-molecules may be altered.

The fact too that in the globulin-increase during immunisation the euglobulin appears to take a larger share than the pseudoglobulin which acts as the antitoxin-carrier seems to show that some compensatory mechanism is at work. In the horse whose reaction to stimulation is prompt and is evidenced by increased potency, we may presume that a more or less constant number of pseudoglobulin molecules has to combine with or hold together a very largely increased number of antitoxin molecules. In order to prevent any diversion of these pseudoglobulin molecules to simple functions of nutrition the euglobulin undergoes what one may call a "compensatory hypertrophy."

In the refractory animal, however, or during a period of stationary potency such a mechanism may not exist, or once established may readily be upset in which case a redistribution of the antitoxin molecules would not be an unexpected phenomenon. Hitherto no explanation has been afforded of the different reactions displayed by different horses to diphtheria-toxin. From the data furnished by the immunisation of "Plug" and "Wright" there would seem to some intimate relation between the amount of antitoxin developed and the quantity and quality of the globulins. In both cases (as also in the goat whose power

of elaborating antitoxin is very limited) the total proteid was increased, but in the first case the increase affected largely the albumin, and in the second case, the globulin solely. In the goat the albumin was mostly implicated in the increase of total proteid. Now both horses had practically the same amount of total proteid initially but in the case of "Plug" the globulin fraction preponderated enormously over the albumin fraction. It was only at the height of antitoxin-production that the globulin of "Wright" attained a value equal to that of "Plug" initially. Had the globulin fraction of "Plug's" serum increased in the same proportion as that of "Wright," quite apart from increase of potency, the total proteid would have consisted practically entirely of globulin.

But there is no doubt a physiological limit to the amount of globulin in serum and that limit was attained in "Plug," for some unexplained reason, before immunisation began. Hence the increase in the total proteid fell largely on the albumin fraction which however does not functionate as an antitoxin carrier. If in view of Hiss and Atkinson's work and the data supplied by "Wright" we are to consider increase of globulin as a necessary concomitant of high grade antitoxin-development then the failure of "Plug" to yield such high grade serum is not surprising.

We use the term "high grade serum" advisedly because there is no doubt that a certain degree of potency is attainable without any very marked disturbance in the constitution of the serum-proteids. In this connexion it seemed advisable to exclude any possible globulin variation that might occur from the injections of large quantities of bouillon alone, apart from the toxin they contain. To make certain on this point a horse "Lister" was inoculated with increasing quantities of alkaline bouillon according to the following scheme.

The bouillon employed was the same as that used in the production of diphtheria-toxin. It was kept in the incubator for ten days and finally filtered through a Berkefeld in order to approximate as closely as possible the conditions of the toxin-containing bouillon.

The initial dose which was inoculated intramuscularly was 50 c.c. Thereafter doses of 100, 200, 400, and 800 were given at intervals of two or three days.

After each injection a well marked pyrexia was evident but otherwise no untoward symptoms appeared.

Before the commencement of the inoculations estimations were made of the total proteid, total globulin and albumin.

In Table VII. are recorded the various proteid estimations throughout

the period of inoculation. It will be seen that no appreciable change took place in the amount of total proteid. The globulin fluctuations were also very slight as will also be evident from Chart IV. which shows the fluctuations in the percentage globulin-content of the total proteid.

TABLE VII.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
July 31 '06	·7493	·4880	65·1	·2702
Aug. 8	·7898	·4654	58·9	—
13	·8063	·5246	65	·3201
16	·8478	·5006	59	·2074
20	·7590	·4639	61·1	·2070

Horse (Lister).

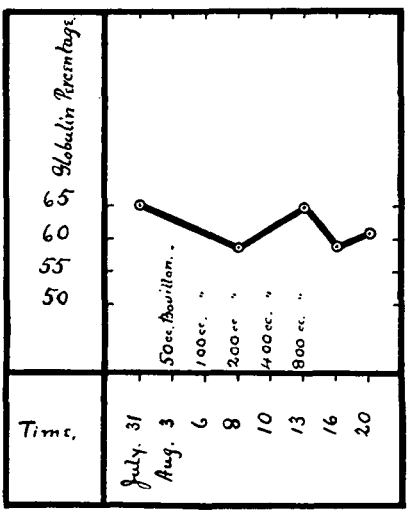

CHART IV.

Chart showing the variations in the globulin-content of the total proteid during the course of the Bouillon-injections (Lister).

We may therefore exclude the inoculation of large quantities of bouillon as a possible factor in the production of the marked globulin-increase which occurs during the immunisation of susceptible horses with diphtheria-toxin.

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Summary and Conclusions.

1. During the immunisation of a horse which ultimately failed to yield high grade antitoxic serum, the globulin-content of the total proteid showed no tendency to increase. The slight rise in total proteid which occurred was due to an increase in the albumin fraction. It is probable that the failure of this horse to yield high grade antitoxin was in some way connected with the initial high globulin-content of the serum.

2. During the immunisation of a goat, the rise in total proteid affected mainly the albumin fraction and the globulin fraction in lesser degree.

3. During the immunisation of a horse which ultimately yielded high grade antitoxic serum, the percentage globulin-content of the total proteid, progressively increased. This increase affected the euglobulin fraction more than the pseudoglobulin fraction.

4. In the horse the pseudoglobulin contains the greater part if not all the antitoxin but it seems probable that this relationship holds good only when the antitoxin-content of the serum is steadily rising.

5. In the goat the antitoxin-content of the euglobulin and pseudoglobulin fractions may vary at different periods in the course of immunisation.

In conclusion I have to express my thanks to Dr Dean the Bacteriologist-in-charge for kindly help and suggestions in the course of this work.

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ADDENDUM.

In the *Proceedings of the Society for Experimental Biology and Medicine*, vol. IV, No. 1, New York, Dec. 1, 1906, is contained the abstract of a paper by Gibson and Collins on the fractionation of agglutinins and antitoxin. These authors report:— "Precipitation of anti-diphtheria goat serum showed that about half the antitoxin remained in the pseudoglobulin; practically none was found in the euglobulin while the $\frac{1}{3}$rd saturated $(NH_4)_2SO_4$ solution washings contained the balance." The results of their experiments, so far as they had gone, appeared to indicate the unreliability of Pick's differentiation of the antibodies by fractional precipitation of the globulins.