CONCENTRATION OF THE PROTECTIVE BODIES IN ANTIPNEUMOCOCCUS SERUM BY MEANS OF SPECIFIC PRECIPITATION.*

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Previous work has shown that the occurrence of a specific precipitin reaction between a serum of one animal species and its antiserum may result in a very definite action on normal or immune antibodies contained in the first (precipitinogenic) serum.

Such studies were inspired by the work of Camus and Gley (1), Kossel (2), and Bordet (3), who had shown that antihemolysins were formed by immunization against a hemolytic serum, and were designed to determine whether similar antibodies could be produced to antitoxins, agglutinins, precipitins, and the like.

Kraus and Eisenberg (4) succeeded in producing an antilactoserum, the action of which was apparently due to the carrying down of the precipitin of the lactoserum with its precipitinogen which had been allowed to interact with the antilactoserum. In other words, the serum of a rabbit immunized against milk when precipitated by the serum of a goat immunized against rabbit serum removes the precipitin for milk from the lactoserum. The first combination was for some reason not effective when a dog-antirabbit serum was employed. These same authors failed, however, to demonstrate fixation of tetanus antitoxin or of typhoid agglutinin in horse serum after precipitating the horse serum by means of rabbit-antihorse serum. Their failure to remove the antitoxin was, however, due to the excess of precipitinogen (horse antitoxin) employed, an inhibiting factor to precipitate formation that was then unknown. This difficulty was overcome by Dehne and Hamburger (5), who found that diluted (1:500) horse antitoxin was entirely removed from the supernatant fluid on producing a precipitate with rabbit-antihorse serum. These experiments were fully corroborated by Kraus and Pribram (6), Hamburger (7), and von Eisler and Tsuru (8), and analogous facts were produced in respect to the fixation of diphtheria antitoxin in horse serum by Weill-Hallé and Lemaire (9) and by Atkinson and Banzhaf (10). Experiments which indicate that a similar removal of antitoxin takes place in the body of immunized animals were performed with diphtheria antitoxin by Sacharoff (11) and with tetanus antitoxin by Dehne and Hamburger (5).

Some difference of opinion exists as to the carrying down of agglutinins with a precipitinogen when antiserum is added, the results obtained doubtless depending on technical differences. Thus, Kraus and Eisenberg (4) at first failed to remove immune agglutinins, but later Kraus and Pribram (6) succeeded. Von Eisler and Tsuru (8) and Landsteiner and Prašek (12) brought down normal

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agglutinins, but the latter at times failed with immune agglutinins. Wassermann and Bruck (13) failed to remove antityphoid protective bodies by precipitation with rabbit-antigoat serum, whereas some such removal is indicated by the experiments of Walker (14) performed *in vivo*. Zebrowski (15) failed to remove the hemolytic sensitizer (amboceptor) by similar treatment.

These facts are of significance in considering the mode of action of antisensitizers (antiamboceptors) as bearing on the relations of lysins to "Bordet's fixation bodies," and particularly as bearing on the relation of the fixation reaction to the formation of specific precipitates (16). We do not propose, however, to discuss any of these interesting and somewhat puzzling questions at this time, but to direct attention to the one fact that an antiserum (antitoxin, agglutinating serum) when employed as a precipitinogen by the addition of an homologous antiserum (precipitin) may yield to the precipitate its entire content of antibody.

It is the reverse of this reaction which we wish to describe, employing antipneumococcus serum as precipitin and producing a precipitate by the addition of an extract of the pneumococcus. We find that such a precipitate brings down more or less completely the antibodies which protect animals against pneumococcus infection. Owing to a corresponding reversal of the relative dosage of the reacting substances, this reaction becomes at once of practical as well as of theoretical interest. It enables us not only to concentrate the specific antibodies of the antipneumococcus serum in small volume, but, as we shall see, in a fluid of extremely low protein content.

Although bacterial precipitins were the first to be described (Kraus, 1897 (17)), interest in them has for practical reasons been far exceeded by that taken in the serum and protein precipitins later described by Tchistovitch (18) and by Bordet (19). Neufeld (20) first described a precipitin for pneumococcus extract in the serum of specifically immunized animals. His observations were extended by Wadsworth (21), by Panichi (22), by Heyrovský (23), and by Norris (24).

In our own work we have employed potent antipneumococcus sera produced in horses by prolonged treatment with dead and living cultures of either type I or type II (25) strains of pneumococcus. Such sera agglutinate specifically the type of pneumococcus employed for

immunization in low dilutions, but have no effect on other types. They further protect mice in a dose of 0.2 of a cubic centimeter against 0.1, or rarely 0.2, of a cubic centimeter of a twenty-four hour bouillon passage culture of pneumococcus of which 0.000,001 of a cubic centimeter suffices to kill controls.

As antigen (precipitinogen) we have used water-clear extracts of dried and ground pneumococci obtained by precipitating considerable amounts of washed concentrated bouillon cultures by the addition of equal parts of 95 per cent. alcohol, or, in later experiments, by adding them to ten volumes of acetone (26) (Kahlbaum's C. P.).[1] These extracts were made in the manner that Gay and Claypole (27) have described for producing plain typhoid vaccines, and are made by suspending weighed amounts of ground culture in carbolated (0.5 per cent.) saline solution in the proportion of one milligram to one cubic centimeter, shaking at intervals at a temperature of 37° C. for from three to six hours, and then allowing them to remain in the ice box over night. On the following day the suspended mixture is vigorously centrifugalized until the supernatant fluid is cloudless and water-clear, or at most slightly opalescent. Such supernatant fluids from ground pneumococci are very toxic for mice, distinctly more so than the bacterial sediment from which they have been separated.

This clear antigen gives a distinct precipitin reaction with one cubic centimeter of strong immune serum in a dose of 0.01 of a cubic centimeter or less, when read from the sediment after twenty-four hours at room temperature. The maximal precipitate and rapidity of reaction are produced by the addition of from 0.3 to one cubic centimeter of the antigen when added in a single dose. We have as yet no information at hand as to the zone of inhibition or resolution of the precipitate in an excess of antigen. In producing precipitates to test for protective purposes our method has been to endeavor to produce the maximum precipitate from a given amount of immune serum. For this purpose we have added the antigen in divided doses, allowing the cloudy or flocculent precipitate to form for from a few minutes to several hours, centrifugalizing, and then

[1] This was done at the suggestion of Dr. Van Slyke.

adding more precipitinogen to the supernatant serum until no further cloud is produced.

We have by no means fully determined the ideal conditions for producing the maximal precipitate, or perhaps, more correctly speaking, the optimal protein complex in a precipitate, as is indicated by the considerable variations in the total nitrogen determinations of our washed precipitates. Nor have we in all instances succeeded in bringing down all the protective antibodies in our precipitates. The fact, however, that we have repeatedly found such precipitates, washed free of serum, to contain as much protective value in aliquot parts as the immune serum, seems to us to justify this communication, pending a greater uniformity of results.

EXPERIMENTAL.

Experiment 1.—To 20 c.c. of antipneumococcus serum (type I, lot 14, Dec. 6, 1913) were added 6 c.c. of clear carbolated extract of dried pneumococci. An immediate voluminous precipitate occurred. This was centrifugalized five minutes later and another 6 c.c. of antigen were added to the supernatant fluid. A slow cloudiness appeared which was allowed to form for two hours at 37° C. The second supernatant fluid in a dose of 1 c.c. gave no further precipitate with doses of from 0.1 to 1 c.c. of antigen. The combined precipitates were shaken in 20 c.c. of carbolated saline, centrifugalized, and resuspended. 20 c.c. of the same specimen of antipneumococcus serum were diluted *pari passu* with the above by addition of two doses of 6 c.c. each of carbolated saline solution instead of antigen.

One-half the above washed precipitate was dissolved by 2 c.c. of $\frac{N}{10}$ sodium hydrate. It was further diluted with saline until 0.5 c.c. of the solution corresponded to the precipitate derived from 0.2 c.c. of original serum. The undissolved precipitate, the original diluted serum, and the exhausted (precipitin-free) serum were also made up to corresponding volume (0.5 c.c. equals 0.2 c.c. of original serum).

A Kjeldahl determination by Mr. Cullen gave a total nitrogen in the undissolved precipitate, indicating a protein content of 0.34 per cent.

With these mixtures the following experiments were performed on mice (tables I, II, and III).

TABLE I.

A. Protective Experiment with Serum and Derivatives Mixed in a Fixed Amount with Decreasing Amounts of Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococcus, Type I, 96–1,[2] in a Total Volume of 0.5 C.C. The Mixtures Were Injected Intraperitoneally in Mice.

Mouse.	Protective fluid.	Culture.	Result.
1	Control	0.0001 c.c.	Died, 45 hrs.
2	Control	0.00001 c.c.	Died, 45 hrs.
3	Control	0.000001 c.c.	Died, 45 hrs.
4	Original serum 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
5	Original serum 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
6	Original serum 0.2 in 0.5 c.c.	0.01 c.c.	Died, 34 hrs.
7	Original serum 0.2 in 0.5 c.c.	0.001 c.c.	Survived.
8	Exhausted serum 0.2 in 0.5 c.c.	0.5 c.c.	Died, 20 hrs.
9	Exhausted serum 0.2 in 0.5 c.c.	0.1 c.c.	Died, 45 hrs.
10	Exhausted serum 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
11	Exhausted serum 0.2 in 0.5 c.c.	0.001 c.c.	Survived.
12	Whole washed precipitate 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
13	Whole washed precipitate 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
14	Whole washed precipitate 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
15	Whole washed precipitate 0.2 in 0.5 c.c.	0.001 c.c.	Died, 45 hrs.
16	Dissolved precipitate 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
17	Dissolved precipitate 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
18	Dissolved precipitate 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
19	Dissolved precipitate 0.2 in 0.5 c.c.	0.001 c.c.	Died, 4 dys.

TABLE II.

B. Protective Experiment with Fixed Dose of Culture and Decreasing Doses of Serum and Serum Derivatives.

Mouse.	Protective fluid. Total volume 0.5 c.c.	Culture. Pneumococcus, type I, 98–1.	Result.
1	Original serum 0.1 c.c.	0.05 c.c.	Survived.
2	Original serum 0.05 c.c.	0.05 c.c.	Survived.
3	Original serum 0.025 c.c.	0.05 c.c.	Survived.
4	Original serum 0.0125 c.c.	0.05 c.c.	Survived.
5	Original serum 0.00625 c.c.	0.05 c.c.	Died, 14 dys.
6	Dissolved precipitate 0.1 c.c.	0.05 c.c.	Survived.
7	Dissolved precipitate 0.05 c.c.	0.05 c.c.	Survived.
8	Dissolved precipitate 0.025 c.c.	0.05 c.c.	Survived.
9	Dissolved precipitate 0.0125 c.c.	0.05 c.c.	Survived.
10	Dissolved precipitate 0.00625 c.c.	0.05 c.c.	Survived.
11	Exhausted serum 0.1 c.c.	0.05 c.c.	Survived.
12	Exhausted serum 0.05 c.c.	0.05 c.c.	Died, 36 hrs.
13	Exhausted serum 0.025 c.c.	0.05 c.c.	Died, 36 hrs.
14	Control	0.05 c.c.	Died, 20 hrs.
15	Control	0.0001 c.c.	Died, 18 hrs.
16	Control	0.00001 c.c.	Died, 40 hrs.
17	Control	0.000001 c.c.	Died, 48 hrs.

[2] This indicates the ninety-sixth passage through animals with a single passage on culture media.

TABLE III.

C. Duration of Protection Afforded by Antipneumococcus Serum and Its Precipitate.

In this experiment two series of mice were given, respectively, whole serum and dissolved precipitate in a dose of 0.2 c.c. subcutaneously, and inoculated subsequently at intervals with suitable controls.

Series *A*, on Dec. 15, 1914, was given 0.2 c.c. of diluted serum in saline (volume 0.5 c.c.) subcutaneously.

Series *B*, on the same date, was given 0.2 c.c. of dissolved precipitate from serum (0.2 in 0.5 c.c.) subcutaneously.

Mouse.	Interval.	Dose of culture.	Result.
A–1	4 dys.	0.1 c.c.	Died, 14 hrs.
A–2	4 dys.	0.01 c.c.	Died, 18 hrs.
A–3	4 dys.	0.001 c.c.[3]	Survived.
A–4	8 dys.	0.01 c.c.	Died, 20 hrs.
A–5	8 dys.	0.001 c.c.	Died, 10 hrs.
A–6	8 dys.	0.0001 c.c.[4]	Survived.
A–7	10 dys.	0.0001 c.c.	Survived.
A–8	10 dys.	0.00001 c.c.[5]	Survived.
B–1	4 dys.	0.1 c.c.	Died, 18 hrs.
B–2	4 dys.	0.01 c.c.	Died, 18 hrs.
B–3	4 dys.	0.001 c.c.[3]	Survived.
B–4	8 dys.	0.01 c.c.	Died, 10 hrs.
B–5	8 dys.	0.001 c.c.	Died, 10 hrs.
B–6	8 dys.	0.0001 c.c.[4]	Died, 4 dys.
B–7	10 dys.	0.001 c.c.	Died, 24 hrs.
B–8	10 dys.	0.0001 c.c.	Died, 30 hrs.
B–9	10 dys.	0.00001 c.c.[5]	Died, 42 hrs.

The first two sections of this experiment (*A* and *B*) show that the precipitate derived by adding an extract of pneumococcus to antipneumococcus serum may contain as much protective power against pneumococcus infection in mice as the original serum from which it is derived. It is active whether employed in its original precipitated condition or dissolved in a small amount of alkali. In this experiment the duration of the immunity passively conferred by precipitate was not so great as when serum was used. This failure in durability, however, may well have been due to the fact that the solution of

[3] Controls with doses of 0.00001 and 0.000001 c.c. died in 40 hrs.

[4] Controls with doses of 0.00001 and 0.000001 c.c. died in 20 and 48 hrs.

[5] Controls with doses of 0.00001 c.c. died in 42 hrs.

precipitate in an alkali is by no means without harmful effect on its protective power. The amount of alkali needed to dissolve the precipitate has varied not only with the apparent opacity of the precipitate but also with its age, freshly formed precipitates being more readily soluble than older ones. At all events the addition of the alkali has frequently robbed the precipitate of its protective properties, and it has likewise a harmful effect on the original serum. A solution of the precipitate seemed desirable in view of its possible application by intravenous injection in human cases of pneumonia. It is questionable, however, if the precipitate itself would be dangerous to employ; we have given considerable amounts to rabbits intravenously without ill effect.

It seemed to be of importance, when employing the original precipitate in protection against intraperitoneal infection in mice, to show that the protective effect is not in some measure due to a possible mechanical effect of the precipitate rather than to specific antibodies. This would seem to be ruled out in the following experiment, in which a precipitate from a serum of type I was employed mixed with cultures of pneumococci of both type I and type II.

Experiment 2.—Protective value of precipitate I.[6] The dried culture in this experiment differs from the preceding antigen in that it was precipitated by alcohol directly from the bouillon culture without washing. An extract was made in carbolated saline in dilution of 1 mgm. to 1 c.c. This antigen solution was added in doses of 50 and 40 c.c., with 12 hours' interval, to 100 c.c. of serum of type I, lot 3. Precipitates were produced on each addition, which were then washed and suspended in one-fifth the original volume. Not all the precipitate was removed from the treated serum, as could be shown by further addition of antigen. A Kjeldahl determination of the precipitate diluted to original volume gave a protein content of 0.108 per cent.

The protective values of the precipitate concentrated five times, of the precipitate at original volume, and of the concentrated precipitate dissolved in sodium hydrate,[6] are compared with the original serum. Mixtures of the various fluids were made with the culture dilutions, each in a volume of 0.5 c.c., and injected intraperitoneally in mice (table IV).

[6] 0.3 c.c. of $\frac{N}{1}$ sodium hydrate was used to dissolve 5 c.c. of five times concentrated precipitate.

TABLE IV.

Mouse.	Protective fluid.	Culture.	Result.
		Pneumococcus, type I, 100–4	
1	Original serum 0.2 c.c.	0.1 c.c.	Survived.
2	Original serum 0.2 c.c.	0.01 c.c.	Survived.
3	Original serum 0.2 c.c.	0.001 c.c.	Survived.
4	Precipitate emulsion, original volume 0.2 c.c.	0.1 c.c.	Died, 36 hrs.
5	Precipitate emulsion, original volume 0.2 c.c.	0.01 c.c.	Survived.
6	Precipitate emulsion, original volume 0.2 c.c.	0.001 c.c.	Survived.
7	Precipitate emulsion concentrated 5 times	0.1 c.c.	Survived.
8	Precipitate emulsion concentrated 5 times	0.01 c.c.	Survived.
9	Precipitate emulsion concentrated 5 times	0.001 c.c.	Survived.
10	Precipitate concentrated 5 times dissolved in sodium hydrate	0.1 c.c.	Died, 4 dys.
11	Precipitate concentrated 5 times dissolved in sodium hydrate	0.01 c.c.	Died, 40 hrs.
12	Precipitate concentrated 5 times dissolved in sodium hydrate	0.001 c.c.	Died, 13 hrs.
		Pneumococcus, type II, 34–7	
13	Precipitate emulsion concentrated 5 times	0.1 c.c.	Died, 15 hrs.
14	Precipitate emulsion concentrated 5 times	0.01 c.c.	Died, 15 hrs.
15	Precipitate emulsion concentrated 5 times	0.001 c.c.	Died, 18 hrs.
16	Precipitate emulsion concentrated 5 times	0.0001 c.c.	Died, 15 hrs.
		Pneumococcus, type I, 100–4	
17	Control	0.00001 c.c.	Died, 32 hrs.
18	Control	0.000001 c.c.	Died, 24 hrs.
		Pneumococcus, type II, 34–7	
19	Control	0.00001 c.c.	Died, 18 hrs.
20	Control	0.000001 c.c.	Died, 14 hrs.

This experiment indicates the protective value of undissolved precipitate emulsion, shows its specificity, and that concentration increases its potency. The deleterious effect of solution in sodium hydrate is also shown.

We are at present engaged in determining other methods of dissolving the precipitate which may have no effect on the protective bodies. Our experiments show further that the agglutinins as well as the protective bodies are brought down with the precipitate. Preliminary experiments indicate that a comparison of the protective value of precipitates with that of the original serum may be made more convincing by employing rabbits instead of mice. It is at least evident that a simultaneous injection of serum or of the precipitate will protect rabbits from an intravenous dose of the pneumococcus that is many times the fatal dose. The precipitates have shown themselves to be as protective as the original serum in these animals, although the end-point of the protective value has not been reached in our experiments to the present time.

TABLE V.

Protein Content of Antipneumococcus Serum before and after Precipitation, and of Its Precipitates with Indication of Protective Value of Precipitates.

Precipitate lot.	Protein content.		Exhausted serum.	Protective value of precipitate.
	Original serum.	Antigen.		
I^1				Protects well.
I^2 0.34%				Protects well.
I^3 0.18%	5.76%		7.0 %	Partial protection.[7]
I^4				Partial protection.[7]
I^5 0.09%	6.49%	0.015%	7.19%	Partial protection.[7]
I^6 0.108%				Protects well.
I^7 0.101%	5.9 %	0.022%	5.07%	Protects well.
II^1 0.21%	5.9 %		5.90%	Does not protect.
II^4 0.157%	5.56%	0.022%	5.72%	Protects well.

It remains to discuss the relative protein contents of serum and precipitate. Our results here are somewhat varying and fragmentary, but show that the protein content of the precipitate as compared with serum is surprisingly low. There would, moreover, seem to be no necessarily direct relation between protein content and protective value. We are indebted to Mr. Cullen for the Kjeldahl determina-

[7] The failure to demonstrate good protection in this serum is probably due to the fact that only the dissolved sodium hydrate precipitate was tested.

tions of total nitrogen content on which these figures are based. The estimates were made in duplicate and usually from five cubic centimeters each of a dilution of each fluid.

It will be noted from table V that in all instances of precipitates from antipneumococcus sera of type I, where only partial protection was obtained by the precipitates, the original precipitate emulsions were not tested, but only the precipitates after solution in sodium hydrate which, as has been evidenced, may destroy the protective power in precipitate or in serum.

The average protein content of more or less perfectly protective precipitates is, as will be noted, very low, ranging from 0.09 to 0.34 per cent., or from one-seventieth to one-thirty-second of the amount of protein in the original serum in those instances in which full computation was made. The obvious value of the possible employment of concentrated immune bodies in a solution of such low protein concentration for intravenous injection in human beings is apparent. The avoidance of serum sickness and of the possible formation of antagonistic bodies seems possible by this means. The advantage of this method of concentration of the protective bodies over concentration by chemical precipitation lies not only in the low protein content of the precipitate, but also in the fact that it may be produced rapidly and under conditions of absolute asepsis.

CONCLUSIONS.

The addition of a water-clear extract of pneumococci to homologous antiserum produces a voluminous precipitate which carries down with it the agglutinins and practically the totality of the protective bodies against pneumococcus infection in animals. This precipitate when washed and resuspended in saline solution to the original volume of serum protects as well as the whole serum. The protein content of such solutions has varied from 0.09 to 0.34 per cent., as contrasted with about 6 per cent. in the original serum. The solution of this precipitate is not necessary to insure protection, and when produced by dilute alkali (sodium hydrate) frequently destroys the immune bodies.

In conclusion the senior author wishes to express his appreciation of the courtesy which Dr. Cole has shown in placing the facilities of the hospital at his disposal for this work.

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