THE CONCENTRATION OF THE PROTECTIVE BODIES IN ANTIPNEUMOCOCCUS SERUM. SPECIFIC PRECIPITATE EXTRACTS.

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(Received for publication, May 28, 1915.)

It has been noted in a previous communication (1) that the immune substances in antipneumococcus serum may be removed by specific precipitation with extracts of the pneumococcus. It was further shown that the precipitates, when suspended in normal salt solution, and to a less extent when dissolved in a weak solution of sodic hydrate, protect susceptible animals against many times the lethal dose of pneumococcus. The present work is a further study of the action of the precipitates as well as of extracts of precipitates made by various methods.

An attempt was made to render soluble the specific precipitates by the use of weak alkaline salts. As in the preceding experiments (1), it appeared that the amount of sodic hydrate necessary to dissolve the precipitates was so great that in many cases the solution proved either toxic for animals or had suffered diminution in protective properties. Such weak alkalis as sodium phosphate, sodium biphosphate, and sodium carbonate were not strong enough to cause a solution of the precipitate. However, it was noted that when sodium carbonate was added to an emulsion of the whole precipitate in salt solution, a definite flocculation of the suspended particles occurred, and the flocculated particles quickly settled down, leaving an opalescent supernatant fluid which contained protective bodies, agglutinins, and precipitins. This observation suggested the possibility of bringing about a dissociation of the antigen and antibody of the specific precipitate.

It has been shown by Pfeiffer and Friedberger, and Bail and his pupils that the formation of the antigen-antibody complex that takes place in the precipitate

which forms when the appropriate bacterial precipitinogen is added to an immune serum, is relatively a loose one, and can be dissociated subsequently by extraction in salt solution. In 1903 Pfeiffer and Friedberger (2) found that when thoroughly washed sensitized cholera vibrios were injected into the peritoneal cavity of a guinea pig, the guinea pig was able to survive a second lethal dose of fresh live cholera vibrios, when injected one or two hours later. This work has been subsequently confirmed and elaborated by Landsteiner and Jagić (3), Hoke (4), Bail and Rotky (5), Bail (6), and Bail and Tsuda (7); and more recently Krauss (8) and Matsui (9) have demonstrated this dissociation of antigen and antibody, both *in vivo* and *in vitro*. They precipitated the immune substances in normal beef serum with live cholera vibrios and with *Bacillus typhosus*, and determined the optimum relation of culture to serum and the effects of varying degrees of temperature on the formation of the precipitate and on the potency of the extracts obtained from such precipitates. In many plate experiments the bactericidal or inhibiting action of these extracts on the growth of cholera vibrios has been demonstrated.

In the present work it will be shown that extracts of the precipitates formed in antipneumococcus serum by the addition of bacterial precipitinogen not only exert an inhibiting influence on the growth of virulent pneumococci *in vitro*, but the extracts contain agglutinins and precipitins and protect susceptible animals, such as the mouse and rabbit, as efficiently as does the original antipneumococcus serum. On the other hand, these extracts contain only a minimal amount of protein as compared with the whole serum. Moreover, considerable experimental evidence suggests that the extracts, and more especially the whole precipitates, produce active as well as passive immunity to pneumococcus infection in mice.

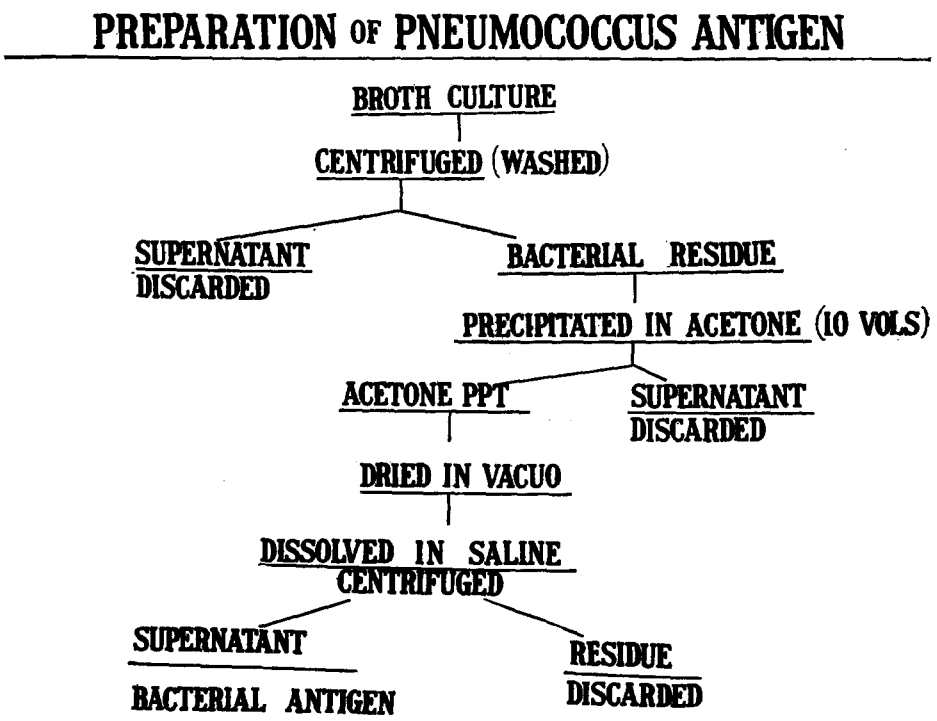
Methods.

As certain modifications have been made in the methods described in the first communication, the preparation of the bacterial precipitinogen and the method of precipitating the serum are reviewed in detail. For instance, acetone instead of alcohol has been used exclusively to kill the bacteria used in the preparation of the extract. Methods have also been employed for the more complete exhaustion of the immune serum.

Preparation of Pneumococcus Extracts (Precipitinogen).

An outline of the method is shown in Text-fig. 1. A twenty-four hour plain broth culture of pneumococcus of Type I or Type

II (10) is centrifuged and the bacterial residue washed twice in normal salt solution. The washed bacterial residue of a liter of broth culture is then emulsified in 5 cc. of normal salt solution and is added slowly to 10 volumes of acetone. There is an immediate flocculation of the bacteria. The mixture is then quickly centrifuged within five minutes, and the supernatant acetone decanted. The bacterial residue is then quickly dried *in vacuo*. The average



TEXT-FIG. 1.

yield of dried bacteria from a liter of broth is about 100 to 150 mg. The acetone-killed bacteria may be stored in dry form until ready for use in precipitating the immune serum.

The dried bacteria are dissolved in normal salt solution, usually 1 to 2 mg. per cc. On shaking thoroughly, the solution of the bacteria is rapid and almost complete. After the bacteria have been dissolved as completely as possible, the solution is centrifuged at high

speed for one-half hour. Usually a very small amount of sediment collects at the bottom of the tube. The opalescent supernatant fluid is used as the bacterial extract in precipitating the immune substances from antipneumococcus serum.

Since acetone does not kill the ferments (Van Slyke and Cullen[1] (11)) these bacterial extracts may undergo autolysis. Hence it is advisable to store the bacterial precipitinogen in dry form from which fresh extracts can be readily prepared when needed. However, saline extracts of the bacteria have been used after storage on ice for two months and have shown no appreciable decrease in their ability to precipitate immune sera.

Acetone kills the pneumococcus probably by very rapid dehydration. Cultures of these bacterial extracts are always sterile when the procedure is carried out with aseptic precautions. Smears of the bacterial extracts show a Gram-negative staining amorphous material. Kjeldahl determinations made under Mr. Cullen's direction show that the bacterial extract is practically pure protein, as it contains 16.2 per cent nitrogen.

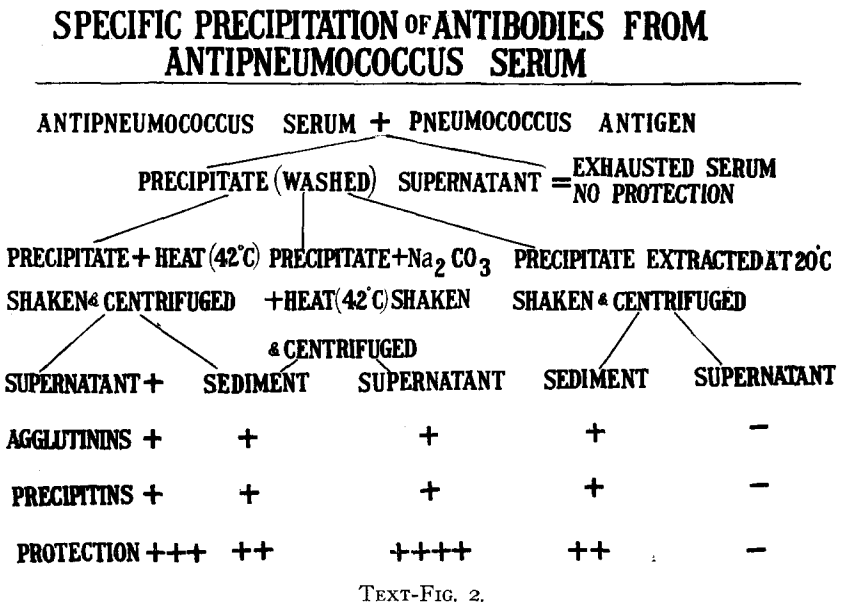
Method of Specific Precipitation of Antipneumococcus Serum.

An outline of the methods employed, showing the method of obtaining the various fractions, is shown in Text-fig. 2. The presence or absence of immune bodies in the various fractions is also indicated. These facts are shown in detail later.

To an antipneumococcus serum, prepared by actively immunizing a horse to live cultures of pneumococcus of Type I or Type II, a bacterial extract of pneumococcus of the corresponding group is added until the immune serum is exhausted of its antibodies. Testing the absence of agglutinins in the serum from which the precipitate has been removed is a more accurate criterion of complete exhaustion of the serum than is the repeated addition of bacterial precipitinogen until no further precipitate occurs. By this method it is possible to exhaust completely the serum of its protective properties. To exhaust antipneumococcus serum it is necessary to add

[1] Buchner was the first to note that acetone did not kill the oxidases. Van Slyke, having prepared an active form of urease by precipitation with acetone, suggested its use in the preparation of pneumococcus extracts.

on the average the bacterial extract of 50 mg. dry weight of bacteria per 100 cc. of serum. It makes little difference, as regards the total amount of bacterial extract necessary to exhaust a given quantity of serum, whether the precipitinogen be added at once or repeatedly in small amounts. The precipitate formed by the addition of the bacterial extract as precipitinogen is apparent at once and is very volu-



TEXT-FIG. 2.

minous. The precipitated serum is incubated at 38° C. for two hours to render the reaction complete and is then stored on ice over night, whereupon it is centrifuged. The supernatant serum fluid is pipetted off and is called exhausted serum. The precipitate is washed three times in normal salt solution, in order to free it completely of serum, and is then ready for extraction.

Methods of Extracting the Protective Substances from Specific Precipitates of Antipneumococcus Serum.

Extraction in Normal Salt Solution at 20° C.—The washed specific serum precipitate is emulsified in normal salt solution in one-half to one-fifth the volume of the original serum. It is allowed to

stand at room temperature for twenty-four hours, being shaken occasionally. In the following experiments this extract is designated saline extract. This preparation is unsatisfactory, as the extract does not protect animals as highly as do extracts prepared by the methods described below.

Extraction in Normal Salt Solution at 42° C.—The washed serum precipitate is suspended in normal salt solution as described above. It is then heated for one hour at 42° C., being shaken gently at intervals, after which it is centrifuged at low speed. The supernatant fluid from this heated saline emulsion is called heat extract. This extract contains agglutinins and precipitins, and protects mice against many times the lethal dose of pneumococcus.

Extraction in Normal Salt Solution at 42° C. with Sodium Carbonate.—The washed serum precipitate is suspended in salt solution as described above, and about 1 cc. of a 1 per cent sodium carbonate solution is added to the precipitate from 100 cc. of serum, or an amount sufficient to flocculate the suspended particles of precipitate. The flocculated emulsion is then heated for one hour at 42° C. and is gently shaken at intervals, after which it is centrifuged. This supernatant fluid is designated as carbonate extract. With this extract the best results have been obtained, both as regards protection of animals against pneumococcus infection and content of agglutinins and precipitins. In the preparation of the extracts, it is important not to subject the precipitates to prolonged shaking, as this procedure diminishes or destroys their antibody content. Shaklee and Meltzer (12) have previously shown that prolonged shaking destroys the ferments trypsin and pepsin.

EXPERIMENTAL.

Protective Properties of Specific Precipitates and Extracts of Precipitates from Antipneumococcus Serum.

The whole precipitates, and more especially the extracts of the precipitates, protect susceptible animals, rabbits and mice, as well, or almost as well, as the whole antipneumococcus serum.

In Table I, which is a protocol of one of the many protection experiments which have been done upon mice with various specific

TABLE I.*

Protective Experiment with a Fixed Amount of Serum of Type II or Serum Derivatives Formed by Various Methods and Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococcus of Type II, in Total Volume of 0.5 Cc. The Mixtures Were Injected Intraperitoneally into Mice.

Mouse No.	Protective fluid.	Culture.	Result.
		cc.	
1	Control	0.00001	D., 30 hrs.
2	"	0.000001	" 35 "
3	Original serum, 0.2 cc. in 0.5 cc.	0.2	" 3 days.
4	" " " " " " "	0.1	" 24 hrs.
5	" " " " " " "	0.01	S. 5 days.
6	" " " " " " "	0.001	D., 4½ "
7	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.	0.2	" 10 hrs.
8	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.	0.1	" " "
9	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.	0.01	" 40 "
10	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.	0.001	" " "
11	Whole washed precipitate, 0.2 cc. in 0.5 cc.	0.2	" 4 days.
12	Whole washed precipitate, 0.2 cc. in 0.5 cc.	0.1	" 56 hrs.
13	Whole washed precipitate, 0.2 cc. in 0.5 cc.	0.01	S. 5 days.
14	Whole washed precipitate, 0.2 cc. in 0.5 cc.	0.001	" " "
15	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.	0.2	D., 5 "
16	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.	0.1	" 24 hrs.
17	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.	0.01	S. 5 days.
18	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.	0.001	D., 3 "
19	Carbonate extract, 0.2 cc. in 0.5 cc.	0.2	" 4 "
20	" " " " " " "	0.1	S. 5 "
21	" " " " " " "	0.01	" " "
22	" " " " " " "	0.001	" " "
23	Heat extract, 0.2 cc. in 0.5 cc.	0.2	D., 56 hrs.
24	" " " " " " "	0.1	" 24 "
25	" " " " " " "	0.01	S. 5 days.
26	" " " " " " "	0.001	" " "

* In the tables D. stands for "died," S. for "survived."

precipitates and extracts of precipitates of both Type I and Type II immune serum, it will be noted that the whole precipitate dissolved in sodic hydrate failed to protect. This result with alkaline solutions of the precipitates has been noted several times. As the injection of the dissolved precipitate itself does not harm the mice, it is presumable that the sodic hydrate has a deleterious effect on the immune substances in the precipitate. Of the mice receiving whole

TABLE II.

Protective Experiment with a Fixed Dose of Culture Plus Decreasing Doses of Serum or Serum Derivatives (Same Lot as Experiment I).

Mouse No.	Protective fluid, total volume 0.5 cc.	Culture, pneumococcus Type II.	Result.
		cc.	
1	Control.....	0.00001	D., 44 hrs.
2	"	0.000001	" " "
3	Original serum 0.2 cc.....	0.01	S. 5 days.
4	" " 0.15 "	0.01	" " "
5	" " 0.1 "	0.01	" " "
6	" " 0.05 "	0.01	" " "
7	" " 0.01 "	0.01	" " "
8	Whole washed precipitate 0.2 cc.	0.01	" " "
9	" " " 0.15 "	0.01	" " "
10	" " " 0.1 "	0.01	" " "
11	" " " 0.05 "	0.01	" " "
12	" " " 0.01 "	0.01	D., 4 "
13	Carbonate extract 0.2 cc.....	0.01	S. 5 "
14	" " 0.15 "	0.01	" " "
15	" " 0.1 "	0.01	" " "
16	" " 0.05 "	0.01	" " "
17	" " 0.01 "	0.01	D., 60 hrs.

serum or whole precipitate, those receiving 0.01 cc. of culture survived, while of those receiving the carbonate extract, those receiving as much as 0.1 cc. of culture survived. In other words, the carbonate extract possessed a higher protective power than the original serum, or the whole precipitate. The group of animals receiving heated extract was not as well protected as the group receiving carbonate extract. This is not a sporadic result of the protective action of carbonate extracts, for many experiments with different lots of serum and various samples of extracts of specific precipitates have yielded approximately the same results. Increasing the dose of whole precipitate by concentration does not increase its potency.

This same result has been noted when using whole serum or concentrated globulin fractions of serum as prepared by the method described by Avery (13). Apparently there is a maximum dose of culture against which it is possible to protect a mouse, and increasing the size of the dose of the protective agent does not increase the protection.

On the other hand, if one infects a series of mice with equal doses of culture and attempts to protect them with decreasing doses of serum or serum derivatives, the results have shown that the protective agent is in excess of the needed amount when 0.2 cc. is used as the standard protective dose. Table II illustrates this point. When mice were infected with doses of 0.01 cc. of culture, the mouse that received 0.01 cc. of original serum was protected as well as the one that received 0.2 cc. of serum. In titrating in this manner the protective value of the serum derivatives made up to original volume of serum, it will be seen that there is some loss in potency of the serum derivatives as compared with the original serum. But this can be explained as due to the inevitable slight loss in material during the manipulations incident to the preparation of the specific precipitates and their extracts.

In these experiments the carbonate extract has consistently shown greater protective qualities than the extracts prepared by simple extraction at room temperature or by heating at 42° C.

Further experiments were carried on to determine whether removal of the precipitate from the serum completely exhausted its protective power, and also whether the precipitate still retained protective power after extraction with sodium carbonate.

From the protocol (Table III) it is evident that the exhausted serum and the salt solution used in washing the precipitate free from serum afforded no protection to mice. Apparently the process of washing the precipitate free from the exhausted serum did not diminish appreciably its protective substances. The first carbonate extract protected well; subsequent extractions of the precipitate showed but little potency. While a single extraction removed most of the protective substances that are dissociable from the whole precipitate, some protective power still existed in the residue precipitate which could not be removed by repeated extractions.

TABLE III.

Experiment to Show the Comparative Protective Value of Serum, Exhausted Serum, Wash Water, and Repeated Extraction. The Mixtures Were Made Up in All Cases to a Constant Volume, 0.5 Cc., and Injected Intraperitoneally into Mice.

Mouse No.	Protective fluid.	Culture II 40.	Result.
		cc.	
1	Control	0.00001	D., 42 hrs.
2	"	0.000001	" " "
		Culture I 107	
3	"	0.000001	" 30 "
		Culture II 40	
4	Original serum, 0.2 cc. in 0.5 cc	0.1	" 18 "
5	" " " " " " "	0.01	" " "
6	" " " " " " "	0.001	S. 5 days.
7	" " " " " " "	0.0001	" " "
8	Exhausted " " " " " "	0.1	D., 18 hrs.
9	" " " " " " "	0.01	" 20 "
10	" " " " " " "	0.001	" 18 "
11	" " " " " " "	0.0001	" 42 "
12	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc	0.1	" 18 "
13	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc	0.01	" " "
14	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc	0.001	" 29 "
15	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc	0.0001	" 42 "
16	Carbonate extract 1st extraction, 0.2 cc. in 0.5 cc	0.1	" 18 "
17	" " " " " " "	0.01	S. 5 days.
18	" " " " " " "	0.001	" " "
19	" " " " " " "	0.0001	" " "
20	Carbonate extract 2d extraction, 0.2 cc. in 0.5 cc	0.1	D., 18 hrs.
21	" " " " " " "	0.01	" 4 days.
22	" " " " " " "	0.001	" 3 "
23	" " " " " " "	0.0001	S. 5 "
24	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc	0.1	D., 18 hrs.
25	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc	0.01	" " "
26	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc	0.001	S. 5 days.
27	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc	0.0001	" " "
		Culture I 107	
28	1st carbonate extract, 0.2 cc. in 0.5 cc	0.1	D., 18 hrs.
29	" " " " " " " "	0.01	" " "
30	" " " " " " " "	0.001	" 42 "
31	" " " " " " " "	0.0001	" 72 "

The carbonate extract and heat extracts contained agglutinins and precipitins, but the titer was lower than that of the original serum. A fresh live culture of pneumococcus was agglutinated by the extracts when made up to the original volume of the serum, but not in dilutions of 1 to 5 or 1 to 10. The whole serum itself, on the other hand, agglutinated in dilutions of 1 to 20 or 1 to 30.

The amount of protein in the carbonate extracts is, of course, much less than in the original serum. The whole serum used in this experiment contained 4.98 per cent protein. After removal of the extract the carbonate extract and the residue precipitate each contained 0.08 per cent protein, or about one-sixtieth of the amount in the original serum. It will be further noted that the protection conferred by the use of such extracts is specific, there being no protection against a pneumococcus infection of Group I by an extract of a precipitate from an antipneumococcus serum of Group II, and *vice versa.*

Experiments like the following have been done to ascertain whether the protective substances in antipneumococcus serum can be removed by live washed cultures of pneumococcus. To 25 cc. of antipneumococcus serum, Type I, were added live washed pneumococci of Type I from 150 cc. of a twenty-four hour broth culture. An immediate precipitation occurred. After twenty-four hours the mixture was centrifuged, the precipitate washed in normal saline and emulsified in 12 cc. of normal salt solution. To this emulsion 0.5 cc. of 1 per cent sodium carbonate was added, and flocculation occurred. The mixture was then heated at 42° C. for one hour, being shaken gently at intervals, and then it was heated to 56° C. for one-half hour. After centrifuging, the supernatant fluid was pipetted off and diluted to the original volume of the serum, and is called carbonate extract (Table IV). The sediment was reemulsified and made up to the original volume of the serum with salt solution and is called sediment of precipitate.

This and similar experiments show that antipneumococcus serum can be exhausted of its antibody content by live cultures. To accomplish this, a relatively much larger quantity of live bacteria must be used to exhaust the serum than when a bacterial extract is employed. The washed precipitate of live agglutinated bacteria, after

TABLE IV.

Protective fluid.	Culture I 10⁶.	Result.
	cc.	
Original serum I, 0.2 cc. in 0.5 cc.	0.1	S. 6 days.
" " " " " " " "	0.01	" " "
" " " " " " " "	0.001	" " "
" " " " " " " "	0.0001	" " "
Exhausted " " " " " " "	0.001	D., 18 hrs.
" " " " " " " "	0.0001	" 30 "
" " " " " " " "	0.00001	" 48 "
Carbonate extract, " " " " "	0.1	" 18 "
" " " " " " "	0.01	S. 6 days.
" " " " " " "	0.001	D., 4 "
" " " " " " "	0.0001	S. 6 "
Sediment of precipitate, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.
" " " " " " " " "	0.01	" " "
" " " " " " " " "	0.001	S. 6 days.
" " " " " " " " "	0.0001	" " "
Control	0.00001	D., 30 "
"	0.000001	" " "

being killed by heating to 56° C. for one-half hour, as well as carbonate extracts therefrom, protects susceptible animals against many times the lethal dose of pneumococcus. They are not as potent as precipitates and extracts of precipitates formed from antipneumococcus serum by acetone-killed bacterial extracts. It will be seen from Table IV that mice are protected fairly well by both the carbonate extract of the precipitate and the residue of the precipitate after removal of the carbonate extract. In no case did the precipitates and extracts of precipitates of antipneumococcus serum, treated with live cultures, protect as well as the original serum. The carbonate extracts from these precipitates contain agglutinins and precipitins as well as protective substances.

The Development of Active and Passive Immunity to Pneumococcus Infection by the Use of Antipneumococcus Serum Derivatives.

The injection of specific serum precipitates and extracts of such precipitates from antipneumococcus serum into mice produces a certain amount of active, as well as passive immunity to pneumo-

coccus infection. This is to be expected, as the specific precipitate itself contains bacterial antigen, and it is presumable that in the extracts of these precipitates a certain amount of antigen as well as antibody goes into solution.

It is well known that the passive immunity conferred on mice by the injection of antipneumococcus serum is of short duration and

TABLE V.

Protective fluid.	Mouse No.	Culture, pneumococcus Type I.*	Result.	Mouse No.	Culture, pneumococcus Type I.†	Result.
		cc.			*cc.*	
Whole serum, 0.2 cc. in 0.5 cc.	1	0.1	D., 20 hrs.	4	0.1	D., 18 hrs.
" " " " " " "	2	0.01	S.	5	0.01	" 24 "
" " " " " " "	3	0.001	"	6	0.001	" 48 "
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	7	0.1	D., 20 "	11	0.1	" 18 "
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	8	0.01	" 24 "	12	0.01	S.
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	9	0.001	" 44 "	13	0.001	"
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	10	0.0001	" " "			
Carbonate extract, 0.2 cc. in 0.5 cc.	14	0.1	" 20 "	18	0.1	D., 18 "
Carbonate extract, 0.2 cc. in 0.5 cc.	15	0.01	" " "	19	0.01	" 24 "
Carbonate extract, 0.2 cc. in 0.5 cc.	16	0.001	S.	20	0.001	S.
Carbonate extract, 0.2 cc. in 0.5 cc.	17	0.0001	"	21	0.0001	"
Exhausted serum, 0.2 cc. in 0.5 cc.	22	0.1	D., 20 "	25	0.1	D., 18 "
Exhausted serum, 0.2 cc. in 0.5 cc.	23	0.01	" 26 "	26	0.01	" " "
Exhausted serum, 0.2 cc. in 0.5 cc.	24	0.001	" 44 "	27	0.001	" 2 days.
Exhausted serum, 0.2 cc. in 0.5 cc.				28	0.0001	" " "
Control	29	0.00001	" 26 "	31	0.00001	" 26 hrs.
"	30	0.000001	" 44 "	32	0.000001	" 48 "

* These cultures were given 5 days later.

† These cultures were given 10 days later.

usually disappears after a period of seven days. On the other hand, the active immunity response of an animal to the injection of a bacterial antigen does not usually appear until the period of passive

immunity has passed. To determine the presence or absence of passive and active immunity in mice in response to the injection of antipneumococcus serum and serum derivatives, a series of mice were injected intraperitoneally with 0.2 cc. each of (1) antipneumococcus serum, (2) whole precipitate derived from antipneumococcus serum, (3) a carbonate extract of the whole precipitate, and (4) exhausted serum. After intervals of five and ten days, varying dilutions of live virulent cultures were injected intraperitoneally.

Table V shows that considerable passive immunity was conferred on mice by the intraperitoneal injection of antipneumococcus serum, which immunity persisted for at least five days but had disappeared after ten days. On the other hand, the immunity conferred by the carbonate extract persisted up to ten days at least, when the period of passive immunity had presumably passed. This would seem to indicate that some degree of active immunity had been produced by the carbonate extract. The mice treated with exhausted serum showed, as was to be expected, no evidence of either passive or active immunity.

Of the mice treated with whole specific precipitate, all died when infected with pneumococci five days later. It is possible that the early disappearance of passive immunity in this case might be due to a state of lowered resistance caused by the injection of the large amount of bacterial antigen contained in the whole precipitate. After ten days other mice in this same group showed a considerable resistance which must be interpreted as due to active immunity. Experiments have been carried on to learn whether the early loss of passive immunity after the injection of whole precipitate, as mentioned above, might be prevented by preliminary heating of the bacterial extract used for precipitation. Heating the bacterial extract for one hour at 56° C. does not injure its precipitative power. The precipitates formed with such heated extracts, however, showed no differences from those formed from unheated extracts. The passive immunity produced by the injection of such precipitates lasts no longer than that after the injection of precipitates formed with unheated extracts.

TABLE VI.

Protective Experiment with a Fixed Amount of Polyvalent Serum or Serum Derivatives and Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococci of Type I and Type II in Total Volume of 0.5 Cc. The Mixtures Were Injected Intraperitoneally in Mice.

Polyvalent serum of Types I and II + bacterial extract of Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + bacterial extract of Type II = Precipitate II.

Polyvalent serum + bacterial extract of Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + bacterial extract of Type I = Precipitate IV.

Bacterial extract was added in all cases until the serum was exhausted of the corresponding agglutinins.

All precipitates were suspended in saline with sodium carbonate and heated at 42° C. for one hour, shaking gently. Extracts of each were used for protection tests, diluted to the original volume of serum.

Protective fluid.	Culture I 108.	Result.	Culture II 41.	Result.
	cc.		*cc.*	
Original polyvalent serum, 0.2 cc. in 0.5 cc.	0.1	S. 6 days	0.1	S. 6 days.
	0.01	" " "	0.01	D., 18 hrs.
	0.001	" " "	0.001	S. 6 days.
	0.0001	" " "	0.0001	" " "
Extract of Precipitate I, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.	0.1	D., 18 hrs.
	0.01	" 3 days	0.01	" 30 "
	0.001	S. 6 "	0.001	" 18 "
	0.0001	" " "	0.0001	" 30 "
Extract of Precipitate II, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.	0.1	" 18 "
	0.01	" " "	0.01	" 30 "
	0.001	" 30 "	0.001	" 30 "
	0.0001	" 42 "	0.0001	S. 6 days.
Extract of Precipitate III, 0.2 cc. in 0.5 cc.	0.1	" 18 "	0.1	D., 18 hrs.
	0.01	" " "	0.01	" 56 "
	0.001	" " "	0.001	S. 6 days.
	0.0001	S. 6 days	0.0001	" " "
Extract of Precipitate IV, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.	0.1	D., 18 hrs.
	0.01	" 24 "	0.01	" " "
	0.001	S. 6 days	0.001	" " "
	0.0001	D., 3 "	0.0001	" 24 "
Controls	0.00001	" 24 hrs.	0.00001	" 18 "
	0.000001	" " "	0.000001	" " "

TABLE VII.

The Specificity of Agglutinins Contained in Extracts of Specific Precipitates from Polyvalent Antipneumococcus Serum. The Extracts Are Obtained by Extracting the Specific Precipitates (Experiment VI) in Saline with Weak Sodium Carbonate + Heat.

Polyvalent serum + bacterial extract of Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + bacterial extract of Type II = Precipitate II.

Polyvalent serum + bacterial extract of Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + bacterial extract of Type I = Precipitate IV.

Original polyvalent serum dilution.	+ Culture, pneumococcus Type I.		Original polyvalent serum dilution.	+ Culture, pneumococcus Type II.	
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
1 : 1	++	++	1 : 1	++	++
1 : 5	++	++	1 : 5	++	++
1 : 10	+	+	1 : 10	+	+
1 : 20	−	−	1 : 20	−	−
Extract of Precipitate I + Culture I			Extract of Precipitate I + Culture II		
5 : 1	++	++	1 : 1	−	−
1 : 1	−	−	1 : 5	−	−
1 : 2.5	−	−	1 : 10	−	−
1 : 5	−	−	1 : 20	−	−
Extract of Precipitate II + Culture I			Extract of Precipitate II + Culture II		
5 : 1	−	−	5 : 1	++	++
1 : 1	−	−	1 : 1	−	−
1 : 2.5	−	−	1 : 2.5	−	−
1 : 5	−	−	1 : 5	−	−
Extract of Precipitate III + Culture I			Extract of Precipitate III + Culture II		
5 : 1	−	−	5 : 1	++	++
1 : 1	−	−	1 : 1	−	−
1 : 2.5	−	−	1 : 2.5	−	−
1 : 5	−	−	1 : 5	−	−
Extract of Precipitate IV + Culture I			Extract of Precipitate IV + Culture II		
5 : 1	++	++	5 : 1	−	−
1 : 1	−	−	1 : 1	−	−
1 : 2.5	−	−	1 : 2.5	−	−
1 : 5	−	−	1 : 5		

All agglutination tests are done by the macroscopic method, 0.3 cc. of twenty-four hour broth culture + 0.3 cc. of the serum or extract dilution being used. Readings are made after 2 hours in the water bath at 38° C. and after 24 hours on ice. If agglutination occurs it is usually apparent in 5 minutes.

TABLE VIII.

The Specific Adsorption of Agglutinins from a Polyvalent Pneumococcus Immune Serum by Use of Live Washed Cultures of the Pneumococcus and the Dissociation of These Agglutinins from the Specific Precipitates. Live Washed Cultures of Pneumococci of Type I and Type II Were Used in Obtaining the Precipitates and Extracts from a Polyvalent Antipneumococcus Serum.

Polyvalent serum + live culture of pneumococcus Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + live culture of pneumococcus Type II = Precipitate II.

Polyvalent serum + live culture of pneumococcus Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + live culture of pneumococcus Type I = Precipitate IV.

All precipitates were heated for one-half hour at 56° C., and extracted with saline + carbonate.

The extracts were used for agglutination tests.

Dilution of serum.	Culture I.		Dilution of serum.	Culture II.	
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
Polyvalent serum after removal of Precipitate I by live culture of pneumococcus Type I.					
1 : 1	−	−	1 : 1	+	+
1 : 5	−	−	1 : 5	−	−
1 : 10	−	−	1 : 10	−	−
1 : 20	−	−	1 : 20	−	−
Polyvalent serum after removal of Precipitate III by live culture of pneumococcus Type II.					
1 : 1	+	+	1 : 1	−	−
1 : 5	−	+	1 : 5	−	−
1 : 10	−	−	1 : 10	−	−
1 : 20	−	−	1 : 20	−	−

Extract I.			Extract II.		
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
Culture I.	+	+	Culture I.	−	−
" II.	−	−	" II.	−	+
Extract III.			**Extract IV.**		
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
Culture I.	−	−	Culture I.	+	+
" II.	+	+	" II.	−	−

The Specific Adsorption of Immune Substances from Polyvalent Serum by Bacterial Extracts and Live Bacteria.

A polyvalent antipneumococcus serum may be specifically exhausted of its immune bodies for one of the types of pneumococcus by the addition of a bacterial extract of the corresponding type. The immune substances of the other type remain intact and can be removed subsequently by the addition of the appropriate antigen (Tables VI and VII). During the process of fractional precipitation the titer of the immune bodies is somewhat diminished. This diminution may be accounted for partly by the loss of precipitate incident to the manipulation in washing, and partly by the supposition that the antigen-antibody combination is only partially dissociated. Table VIII shows that the specific agglutinins may be removed in like manner from the polyvalent serum by the use of living pneumococci in place of bacterial extracts.

Nature of the Union between Precipitin and Precipitinogen.

In the above detailed experiments it has been shown that the protective substances in antipneumococcus serum can be removed specifically by precipitation with an extract of the homologous type of pneumococcus, and that this precipitate, when suspended in salt solution and injected into susceptible animals, will protect them against many times the lethal dose of pneumococcus.

It has been further shown that the union between the bacterial extract (precipitinogen) and the immune substances in the serum can be to some extent disunited by suitable chemical and physical agents.

The following observations which we have made suggest strongly that in the formation of the precipitate an actual union occurs between the precipitin of the serum and precipitinogen of the bacterial extract. If, before adding the precipitinogen to the serum, the latter be heated at 60° C. for an hour, no precipitation takes place. Nor can the precipitin be reactivated by the addition of unheated normal serum. This fact, that precipitins are inactivated by heat, and then cannot be reactivated by fresh serum, has already been demonstrated by Pick (14) and Kraus and von Pirquet (15). That

a union, however, has occurred between the precipitin and precipitinogen, even in the absence of precipitate, is made evident by the fact that if to a mixture of bacterial extract and heated immune serum fresh unheated immune serum be added, no precipitate occurs. The most likely explanation is that the precipitable substance is already saturated with the inactivated precipitins.

By suitable extraction methods as shown above, an apparent dissociation of antigen and antibody occurs. It seems likely, however, that, although a dissociation has occurred, and the agglutinins and protective substances have been set free, a permanent change has occurred in the precipitinogen, for when fresh immune serum is added to the extracts of the specific precipitates, even if the latter be concentrated, no fresh precipitate forms.

DISCUSSION.

These studies suggest that the use of precipitate extracts, prepared as described, may offer certain advantages over the use of whole serum in the treatment of lobar pneumonia, since the extracts possess practically the entire immunizing and protective power of immune serum, and yet contain a very small fraction of the serum proteins. The patient may thus be relieved of the strain incident to the metabolism of the large amounts of protein contained in the large quantities of serum which it is now necessary to employ. It is altogether probable, moreover, that the symptoms of serum sickness, which so frequently follow the use of large amounts of serum, may be lessened, if not prevented entirely, by the use of precipitate extracts. The precipitates and extracts of precipitates are still able to produce the phenomena of anaphylaxis and serum sickness, however, as is shown by the fact that in guinea pigs sensitized to horse serum, acute anaphylactic death may be induced by the injection of precipitates or precipitate extracts. To produce this phenomenon, however, fairly large amounts of these substances are required.

The extracts of precipitates, moreover, may have an additional advantage over serum alone in treatment, in that, in addition to conferring passive immunity, they also induce the production of

some degree of active immunity. While the active immunity becomes evident at so late a period that in acute lobar pneumonia it could not be of much therapeutic importance, yet in other, more chronic infections, a similar active reaction induced by suitable extracts might prove of value.

CONCLUSIONS.

1. The protective substances contained in specific precipitates from antipneumococcus serum can be extracted by suitable chemical and physical agents, dilute sodium carbonate at 42° C. being especially advantageous as an extractive agent.

2. The resulting water-clear extracts, when made up to the original volume of the serum used for precipitation, protect animals almost as well as does the whole serum.

3. The bacterial extracts used in precipitating the protective substances from the serum act specifically; that is, a bacterial extract of pneumococcus of Type I removes the protective substances from a Type I immune serum only.

4. In a polyvalent serum of Type I and Type II, the protective substances of each type may be removed independently of each other by the successive addition of the homologous antigens.

5. Extracts of specific serum precipitates contain only one-fiftieth to one-sixtieth of the protein in the original serum, and about one-half the protein of the whole precipitate.

6. Extracts contain not only protective substances but agglutinins and precipitins.

7. Extracts and whole precipitates not only confer passive immunity but stimulate the production of active immunity to pneumococcus infection in rabbits and mice.

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