

ANTIPNEUMOCOCCUS PROTECTIVE SUBSTANCES IN NORMAL CHICKEN SERUM.*

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(Received for publication, February 23d, 1921.)

The natural immunity of birds, particularly the pigeon and the domestic fowl, to infection by the pneumococcus has been a subject of research on the part of experimental immunologists since the earliest days of bacteriology. The solution of the mechanism of this phenomenon has been the chief objective, and the desire of the investigators has been to acquire a knowledge of the fundamental laws of immunity phenomena which might serve to guide them in the production and use of artificial immunity as a prophylactic and therapeutic agent.

While studying his "maladie nouvelle" (septicemia produced in rabbits by inoculations of sputum) Pasteur (1) discovered a new organism which, on microscopic examination, reminded him of the bacillus of chicken cholera. He inoculated chickens with the new organism and found them to be quite refractory to it. After the identity of Pasteur's sputum-septicemia organism and the pneumococcus of Fränkel was established, other workers became interested in Pasteur's original observation and it was soon found that not only chickens but pigeons and other birds are highly resistant to infection by the pneumococcus. Fränkel (2), Gamaléia (3), Tchistovitch (4) and Welch (5) were among the earliest workers who confirmed and extended Pasteur's experiments. More recently Strouse (6), Keyes (7) and Berry and Melick (8) have done interesting and important work on this subject, especially on the mechanism of the phenomenon.

Tchistovitch (4) concluded that wandering phagocytic cells play a decisive part in the pigeon's resistance to the pneumococcus. This author injected pneumococcus cultures into the anterior eye chamber of pigeons and found that the organisms did not multiply and that phagocytes soon appeared and engulfed the cocci which he had injected. Similar experiments performed on the rabbit showed that in

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this animal the cocci rapidly increased in number, few phagocytes appeared and a general infection developed causing death within a short time. Strouse's (6) experiments seemed to show that the normal high temperature of the pigeon is an important, if not decisive, factor in its immunity to the pneumococcus. Keyes (7) questions Strouse's conclusion and presents experiments which show that certain phagocytic cells (the hemophages) of the liver and spleen are very active in the destruction of pneumococci following intraperitoneal or intravenous injections of the organism. Keyes admits the possibility of a high temperature influence but the results of his experiments on this point tend to exclude the temperature of the pigeon as an important factor.

That phagocytic cells finally take up and destroy pneumococci which have been injected into refractory animals (including both naturally immune and artificially immunized animals) seems to be well established. It is also well established that the sera of immunized animals contain immune bodies which can be demonstrated in vitro, and also confer passive immunity upon susceptible animals. Whether normal humoral factors play a significant or essential part in the mechanism of the resistance of naturally immune animals is, however, still debatable. Neither Strouse (6) nor Keyes (7, 9) found any evidence of antibodies in the body fluids of either the chicken or the pigeon. In testing the serum of chickens super-immunized with pneumococcus, Keyes (9) used the serum of normal chickens as a control and found that the normal serum did not protect mice against infection with the pneumococcus, while the serum of the artificially immunized chickens was very potent in this respect. Bull and Bartual (10) published experiments which showed that pneumococci are phagocytized in the fresh, undiluted blood of both chickens and pigeons. This furnished at least presumptive evidence that the blood contained opsonins for the pneumococcus since it is a general rule that washed cells of the most resistant animal are not capable of phagocytizing virulent bacteria (pneumococci particularly) if they have not been previously sensitized (opsonized) with the serum of an immune animal. In a further investigation of the humoral phase of the natural immunity of the domestic fowl to pneumococci, we have made the observations presented in this paper.

EXPERIMENTAL.

Our first experimental objective was to prove the presence or absence of protective substances in normal chicken serum for any or all

of the types of pneumococci. Experiments were planned with the view of affording optimal conditions for protection. Experiment A will serve to illustrate the type of protocol used for this purpose.

Experiment A.—(a) The serum was obtained by taking blood (20 to 40 cc.) from each of four or five large hens or roosters. The blood was collected aseptically, allowed to clot at room temperature, put in an ice box over night and the clear serum removed the next morning.

(b) The strains of pneumococci used in this and subsequent experiments had been passed through mice and possessed a high degree of virulence for these animals, the lethal dose being not less than 0.0000001 cc. of an eighteen-hour culture. The cultures were grown in veal infusion broth to which defibrinated rabbit blood (5 per cent. by volume) had been added.

(c) Mice of about 25 grams weight were given, intraperitoneally, 1 cc. of fresh chicken serum and four hours later inoculated, also intraperitoneally, with from 10 to 100,000 lethal doses of pneumococcus culture. Control mice which had received no serum were infected at the time of inoculating the test animals.

The experiment is tabulated in Protocols 1, 2, 3, 4, 5 and 6 which represent respectively infection with pneumococci of Types I, II, II A, II B, III and Group IV.

PROTOCOL 1.

Mouse protection against Type I pneumococcus with normal chicken serum.

Mice.	Serum.	Type II pneumococcus.	Results.
1.....	1 cc.	0.01 cc.	D. 48 hrs.
2.....	1 cc.	0.001 cc.	S. 5 days
3.....	1 cc.	0.0001 cc.	S. 5 days
4.....	1 cc.	0.00001 cc.	S. 5 days
5.....	1 cc.	0.000001 cc.	S. 5 days
6.....	—	0.0000001 cc.	D. 36 hrs.

D., dead; S., survived 5 days and discarded.

PROTOCOL 2.

Mouse protection against Type II pneumococcus with normal chicken serum.

Mice.	Serum.	Type I pneumococcus.	Results.
1.....	1 cc.	0.01 cc.	D. 48 hrs.
2.....	1 cc.	0.001 cc.	S. 5 days
3.....	1 cc.	0.0001 cc.	S. 5 days
4.....	1 cc.	0.00001 cc.	S. 5 days
5.....	1 cc.	0.000001 cc.	S. 5 days
6.....	—	0.0000001 cc.	D. 24 hrs.

PROTOCOL 3.

Mouse protection against Type II A pneumococcus with normal chicken serum.

Mice.	Serum.	Type II A pneumococcus.	Results.
1.	1 cc.	0.01 cc.	D. 48 hrs.
2.	1 cc.	0.001 cc.	S. 5 days
3.	1 cc.	0.0001 cc.	S. 5 days
4.	1 cc.	0.00001 cc.	S. 5 days
5.	1 cc.	0.000001 cc.	S. 5 days
6.	—	0.0000001 cc.	D. 36 hrs.

PROTOCOL 4.

Mouse protection against Type II B pneumococcus with normal chicken serum.

Mice.	Serum.	Type II B pneumococcus.	Results.
1.	1 cc.	0.01 cc.	D. 48 hrs.
2.	1 cc.	0.001 cc.	S. 5 days
3.	1 cc.	0.0001 cc.	S. 5 days
4.	1 cc.	0.00001 cc.	S. 5 days
5.	1 cc.	0.000001 cc.	S. 5 days
6.	—	0.0000001 cc.	D. 36 hrs.

PROTOCOL 5.

Mouse protection against Type III pneumococcus with normal chicken serum.

Mice.	Serum.	Type III pneumococcus.	Results.
1.	1 cc.	0.0001 cc.	D. 48 hrs.
2.	1 cc.	0.00001 cc.	S. 5 days
3.	1 cc.	0.000001 cc.	S. 5 days
4.	1 cc.	0.0000001 cc.	S. 5 days
5.	—	0.0000001 cc.	D. 36 hrs.

PROTOCOL 6.

Mouse protection against Group IV pneumococcus with normal chicken serum.

Mice.	Serum.	Group IV pneumococcus.	Results.
1.	1 cc.	0.01 cc.	D. 48 hrs.
2.	1 cc.	0.001 cc.	S. 5 days
3.	1 cc.	0.0001 cc.	S. 5 days
4.	1 cc.	0.00001 cc.	S. 5 days
5.	1 cc.	0.000001 cc.	S. 5 days
6.	—	0.0000001 cc.	D. 36 hrs.

The protocols above, which were repeated a number of times, show very decisively that the serum of normal chickens contains protective substances for every type of the pneumococcus. The serum gave less protection against Type III than against the other types. In no instance did mice survive which had received more than 0.000001 cc. of culture. The 24-hour lethal dose of this culture was 0.0000001 cc.

Protection against the other types was quite regular up to 0.001 cc. of culture. Occasionally mice which had received 0.001 cc. died of infection from two to five days after being inoculated and there were sometimes irregular deaths, i.e., a mouse which had received 0.0001 cc. of culture would die while the mouse having received ten times this amount of culture survived. The most regular results were obtained in protection against our Type II culture. Time was saved by always running duplicate experiments and thus eliminating the disturbance of irregular deaths from specific infection and accident.

Experiments planned to determine the optimum conditions for protection and the nature of the protective substance gave the following results: (1) Mice manifest the highest degree of resistance when infected from eight to twelve hours after the serum has been given; there is much less protection when the serum and culture are mixed and immediately injected; twenty-four hours after the administration of the serum the mice are only slightly more resistant than normal controls. (2) If the serum is given subcutaneously, the mice do not manifest an appreciable increased resistance at any time. (3) Less than 0.5 cc. of the serum affords little protection; while 3 cc. of serum is injurious to the mice. Two cc. of serum gives more protection than 1 cc. but the difference is not great. (4) Serum from 2 to 3 weeks old is as potent as fresh serum and it is less toxic. (5) Heating the serum for thirty minutes at 56° C. does not affect its potency, while serum heated for thirty minutes at 65° C. has lost its protective power. (6) The serum of old chickens is more potent than that of young ones.

Two other points that have been determined are of sufficient interest to be given in detail: (1) The fraction of the serum in which the protective substances reside, and (2) the type specificity of the protective substances.

FRACTIONATION OF THE SERUM.

For this work the serum was obtained by collecting blood from a large number (100 to 200) of chickens, allowing it to clot, and centrifugalizing the clot within two or three hours after collecting the blood. The serum was not sterile but there was no opportunity for bacterial growth. A type experiment is given below (Experiment B).

Experiment B.—Two liters of serum were diluted with four liters of tap water. Two liters of a saturated solution of ammonium sulphate were slowly added to the diluted serum, the mixture being constantly stirred. This stood at room temperature until the following

day. A light, flocculent precipitate (precipitate A) had formed. The precipitate was collected on hardened filter paper. 3.75 liters of saturated sulphate solution were added to 7.5 liters of the filtrate obtained above. This caused a heavy precipitate to form which was filtered off the next day. This precipitate will be designated precipitate B. The filtrate obtained here was saturated with ammonium sulphate crystals and allowed to stand for 24 hours when the precipitate, which was now rising and leaving a clear and colorless fluid below, was collected on hardened filter paper. This last precipitate will be called precipitate C. The three precipitates were thoroughly pressed and then put into collodion bags and dialyzed in running tap water until they were free of the sulphate. The contents of the bags containing precipitates A and C were removed and rendered isotonic with sodium chloride crystals. Precipitate C went into solution during the dialysis and did not reprecipitate as the sulphate disappeared. Precipitate A did reprecipitate and dissolved only slightly on the addition of the sodium chloride, but almost complete solution was effected by rendering it alkaline with sodium bicarbonate. A heavy precipitate had formed in the bag containing precipitate B. A portion of the supernatant fluid was removed and rendered isotonic with sodium chloride. The precipitate and the remainder of the supernatant fluid were removed together and made isotonic. The precipitate dissolved immediately on the addition of the salt. The four solutions obtained from the dialysates were passed through Mandler filters and then tested for antipneumococcus protective substances. The first solution ("fibrinoglobulin"), it will be remembered, came from precipitate A which was formed when one fourth volume of saturated ammonium sulphate was added to the diluted serum. The second solution ("pseudoglobulin") is the supernatant fluid from the dialysate of precipitate B (one half saturation precipitate) and is therefore largely the water soluble globulin. The third solution ("euglobulin") is from the precipitate from dialysate B and consists principally of the water insoluble globulin. The last solution (albumin) is the dialysate from precipitate C or the precipitate which formed between 50 and 100 per cent. saturation of the serum with ammonium sulphate and consists, therefore, of the albumin fraction of the serum.

Guinea pigs weighing between 180 and 200 grams were used in testing the potency of the serum fractions which we have just described. A strain of Type II pneumococcus was used to infect the pigs. This strain had been passed through a number of guinea pigs

and was very virulent for these animals, 0.000001 cc. of an 18-hour blood broth culture being the 24-hour lethal dose. Mice were also used in a few experiments and the results corresponded with those obtained with the guinea pigs. Protocols 7, 8, 9, and 10 will serve to illustrate the type of experiment employed in these tests and the results obtained. The guinea pigs were infected three hours after the serum fractions had been given, both being given intraperitoneally.

PROTOCOL 7.

Guinea pig protection against Type II pneumococcus with the "Fibrinoglobulin" of chicken serum.

Guinea pig.	Globulin.	Type II pneumococcus.	Results.
1.....	2 cc.	0.01 cc.	D. 40 hrs.
2.....	2 cc.	0.001 cc.	D. 4 days
3.....	2 cc.	0.0001 cc.	D. 3 days
4.....	2 cc.	0.00001 cc.	D. 4 days
5.....	—	0.000001 cc.	D. 40 hrs.
6.....	2 cc.	—	S. 7 days

D., dead; S., survived 7 days and discarded.

PROTOCOL 8.

Guinea pig protection against Type II pneumococcus with the "Pseudoglobulin" of chicken serum.

Guinea pig.	Globulin.	Type II pneumococcus.	Results.
1.....	2 cc.	0.01 cc.	S. 7 days
2.....	1 cc.	0.01 cc.	D. 6 days
3.....	1 cc.	0.001 cc.	S. 7 days
4.....	1 cc.	0.0001 cc.	S. 7 days
5.....	—	0.000001 cc.	D. 40 hrs.
6.....	2 cc.	—	S. 7 days

PROTOCOL 9.

Guinea pig protection against Type II pneumococcus with the "Euglobulin" of chicken serum.

Guinea pig.	Globulin.	Type II pneumococcus.	Results.
1.....	2 cc.	0.1 cc.	S. 7 days
2.....	1 cc.	0.1 cc.	D. 6 days
3.....	1 cc.	0.01 cc.	S. 7 days
4.....	1 cc.	0.001 cc.	S. 7 days
5.....	—	0.000001 cc.	D. 40 hrs.
6.....	2 cc.	—	S. 7 days

PROTOCOL 10.

Guinea pig protection against Type II pneumococcus with the albumin of chicken serum.

Guinea pig.	Albumin.	Type II pneumococcus.	Results.
1.....	2 cc.	0.1 cc.	D. 21 hrs.
2.....	2 cc.	0.01 cc.	D. 24 hrs.
3.....	2 cc.	0.001 cc.	D. 28 hrs.
4.....	2 cc.	0.0001 cc.	D. 40 hrs.
5.....	—	0.000001 cc.	D. 40 hrs.
6.....	2 cc.	—	S. 7 days

From Protocol 7 it is seen that the precipitate formed on adding one fourth volume of saturated sulphate solution to the diluted serum contained very little of the protective substances. Two cc. of the dialysate from this precipitate did not give permanent protection. Other tests showed that the precipitate formed by 23 per cent. saturation did not contain even a trace of the protective substances. The albumin fraction did not give protection (Protocol 10). Both the water-soluble and the water-insoluble fractions gave permanent protection (Protocols 8 and 9). The water-insoluble fraction was, however, definitely more potent than the water-soluble fraction, 2 cc. of the former protecting against 0.1 cc. of culture and 1 cc. against 0.01 cc. of culture, while it took 2 cc. of the water-soluble fraction to protect against 0.01 cc. of culture. These results might be taken to indicate that the protective substances are associated with both globulin fractions but the methods used in this experiment did not insure complete separation of the two fractions. A further experiment (Experiment C) was undertaken in which a complete separation of the globulin on the basis of water solubility was attempted.

Experiment C.—The globulin was removed from the serum by salting and consisted of the precipitate which formed on one half saturation with ammonium sulphate, the one fourth saturation precipitate having been previously removed. The precipitate was dialyzed against running tap water until it was free of sulphate. The fluid portion of the dialysate was removed and dialyzed against distilled water for 24 hours. A precipitate had formed. This was removed by filtering through hardened paper and the filtrate was put into a new bag and redialyzed. This process was repeated until a 24-hours' dialysis against distilled water caused no precipitate to form. The dialysate was now rendered isotonic filtered and tested for anti-pneumococcus protective potency. The solid portion of the origi-

nal dialysate was put into two liters of distilled water, thoroughly shaken and recollected by centrifugalization. The washing process was repeated and the sediment from the centrifugalization was dissolved in the smallest possible quantity of physiological salt solution. This solution was filtered and tested for antipneumococcus protective power. The protection tests are given in Protocols 11 and 12.

PROTOCOL 11.

Guinea pig protection against Type II pneumococcus with water-insoluble globulin.

Guinea pig.	Globulin.	Type II pneumococcus.	Results.
1.....	0.1 cc.	0.1 cc.	D. 40 hrs.
2.....	0.1 cc.	0.01 cc.	S. 7 days
3.....	0.1 cc.	0.001 cc.	S. 7 days
4.....	0.2 cc.	0.1 cc.	S. 7 days
5.....	0.2 cc.	0.01 cc.	S. 7 days
6.....	0.2 cc.	0.001 cc.	S. 7 days
7.....	—	0.000001 cc.	D. 40 hrs.

PROTOCOL 12.

Guinea pig protection against Type II pneumococcus with water-soluble globulin.

Guinea pig.	Globulin.	Type II pneumococcus.	Results.
1.....	2.4 cc.	0.1 cc.	D. 40 hrs.
2.....	2.4 cc.	0.01 cc.	D. 40 hrs.
3.....	2.4 cc.	0.001 cc.	D. 40 hrs.
4.....	—	0.000001 cc.	D. 40 hrs.
5.....	2.4 cc.	—	S. 7 days

Protocols 11 and 12 show that only the water-insoluble fraction of the globulin contains the protective substances. Two tenth of this fraction of the serum protected against 0.1 cc. of culture or 100,000 lethal doses, while 2.4 cc. of the water-soluble fraction did not protect against 0.001 cc. of culture.

TYPE SPECIFICITY OF THE PROTECTIVE SUBSTANCES.

It has been shown above that the serum of chickens protects mice and guinea pigs against any of the various types of pneumococci. Since in artificially produced immune sera the protective substances are type specific (a distinct substance for each type) and can be selectively removed by bacterial adsorption (11), it becomes of interest to know whether the chicken serum contains a distinct protective substance for each type or only one substance which has

the property of protecting against any and all of the different types. The principle of selective antibody adsorption was here taken advantage of. The details of the work are given under Experiment D.

Experiment D.—(General technique.) About 150 cc. of a 24-hour broth culture of pneumococci was centrifugalized at high speed until the cocci were packed firmly on the bottom of the tubes. The supernatant culture fluid was carefully and completely removed, an effort being made to get the bacteria as dry as possible. Fresh chicken serum (30 cc. for each 150 cc. of culture) was put into the tubes containing the sedimented culture and agitated until an even suspension of the cocci had been effected. The suspension was placed in the incubator (37° C.) for two hours and then sedimented by centrifugalization. The supernatant serum was removed and passed through a Mandler filter to remove the remaining bacteria. A series of three mice for each type of pneumococcus to be used for infection was given, intraperitoneally, 1 cc. each of the adsorbed serum, and an equal number of mice was given 1 cc. of an unadsorbed portion of the same lot of serum. Four hours later all the mice were infected as indicated in the following representative protocols.

PROTOCOL 13a.

Mouse protection against Types I and II pneumococcus with chicken serum adsorbed with Type I pneumococcus.

Mice.	Ad. S.	N. S.	Pn. I.	Pn. II.	Results.
1....	1 cc.		0.0001 cc.		D. 24 hrs.
2....	1 cc.		0.00001 cc.		D. 24 hrs.
3....	1 cc.		0.000001 cc.		D. 30 hrs.
4....		1 cc.	0.0001 cc.		S. 5 days
5....		1 cc.	0.00001 cc.		S. 5 days
6....		1 cc.	0.000001 cc.		S. 5 days
7....	1 cc.			0.0001 cc.	S. 5 days
8....	1 cc.			0.00001 cc.	S. 5 days
9....	1 cc.			0.000001 cc.	S. 5 days
10....		1 cc.		0.0001 cc.	S. 5 days
11....		1 cc.		0.00001 cc.	S. 5 days
12....		1 cc.		0.000001 cc.	S. 5 days
13....			0.0000001 cc.		D. 30 hrs.
14....				0.0000001 cc.	D. 24 hrs.

Ad. S., Adsorbed serum.

N. S., Normal serum.

Pn. I, 18-hour blood broth culture of Type I pneumococcus.

Pn. II, 18-hour blood broth culture of Type II pneumococcus.

PROTOCOL 13b.

Mouse protection against Types I and II pneumococcus with chicken serum adsorbed with Type II pneumococcus.

Mice.	Ad. S.	N. S.	Pn. I.	Pn. II.	Results.
1.	1 cc.		0.0001 cc.		S. 5 days
2.	1 cc.		0.00001 cc.		S. 5 days
3.	1 cc.		0.000001 cc.		S. 5 days
4.		1 cc.	0.0001 cc.		S. 5 days
5.		1 cc.	0.00001 cc.		S. 5 days
6.		1 cc.	0.000001 cc.		S. 5 days
7.	1 cc.			0.0001 cc.	D. 24 hrs.
8.	1 cc.			0.00001 cc.	D. 24 hrs.
9.	1 cc.			0.000001 cc.	D. 24 hrs.
10.		1 cc.		0.0001 cc.	S. 5 days
11.		1 cc.		0.00001 cc.	S. 5 days
12.		1 cc.		0.000001 cc.	S. 5 days
13.			0.0000001 cc.		D. 36 hrs.
14.				0.0000001 cc.	D. 24 hrs.

From Protocols 13 *a* and 13 *b* it is seen that serum adsorbed with Type I pneumococci no longer protects mice against this type but still protects against Type II as well as the unadsorbed serum, and that adsorption with Type II removes the protective substance for this type without removing the Type I protective substance. It appears, therefore, that normal chicken serum contains distinct protective substances for these two types of pneumococci.

Other experiments, similar to Experiment D, showed that chicken serum contains a specific protective substance for each type of pneumococcus. Only one virulent strain of Group IV was available but serum adsorbed with this strain no longer protected against itself but still protected against Types I, II and III, and adsorption with any of these types did not remove the protective substance for the Group IV strain.

It now became of interest to know whether the type specificity of chicken serum extended to the subtypes of Type II strains. These subtypes, it will be remembered, consist of atypical Type II organisms or strains of pneumococci which are agglutinated only slowly and incompletely with Type II serum. In a study of these atypical Type II strains Avery (11) discovered two new serological types which he designated Type II A and Type II B. The relation between these new types and Type II was as follows: A Type II immune serum contains agglutinins and protective substances for all three types but an im-

immune serum produced with a strain of either of the other types (II A or II B) contains antibodies only active against the homologous type. A Type II serum adsorbed with a Type II culture no longer protects against or agglutinates Type II, II A or II B strains, all the antibodies being removed. If a Type II serum is adsorbed with either II A or II B organisms only the homologous antibodies are removed, and the antibodies are not removed from a Type II A immune by adsorption with a II B culture and vice versa. It is seen, therefore, that a Type II immune serum contains one group of major antibodies and two groups of minor antibodies, the latter groups being distinct from each other or, in other words, Types II A and II B are subgroups of Type II.

Avery's experiments were repeated, using chicken serum instead of the sera of immunized animals. Our work is given in detail under Experiment E.

*Experiment E.**—Three series of mice were given, intraperitoneally, 1 cc. of chicken serum which had been adsorbed (see Experiment D) with either a Type II, II A or II B culture and were infected four hours later—one series with Type II culture, one with Type II A and one with Type II B. The three following protocols give the different experiments in tabular form.

PROTOCOL 14.

Mouse protection against Types II, II A and II B pneumococcus with chicken serum adsorbed with Type II pneumococcus.

Mice.	Ad. S.*	Pn. II.	Pn. II A.	Pn. II B.	Results.
1....	1 cc.	0.0001 cc.			D. 24 hrs.
2....	1 cc.	0.00001 cc.			D. 24 hrs.
3....	1 cc.	0.000001 cc.			D. 24 hrs.
4....	1 cc.		0.0001 cc.		D. 3 days
5....	1 cc.		0.00001 cc.		S. 5 days
6....	1 cc.		0.000001 cc.		S. 5 days
7....	1 cc.			0.0001 cc.	D. 2 days
8....	1 cc.			0.00001 cc.	S. 5 days
9....	1 cc.			0.000001 cc.	S. 5 days
10....		0.0000001 cc.			D. 24 hrs.
11....			0.0000001 cc.		D. 48 hrs.
12....				0.0000001 cc.	D. 48 hrs.

* The strains of Types II A and II B pneumococci used in this experiment were kindly furnished us by Dr. O. T. Avery, of the Hospital of the Rockefeller Institute.

* The same number of mice was given 1 cc. each of an unadsorbed portion of the same lot of serum and infected in the same way and at the same time as the

PROTOCOL 15.

Mouse protection against Types II, II A and II B pneumococcus with chicken serum adsorbed with Type II A pneumococcus.

Mice.	Ad. S.	Pn. II.	Pn. II A.	Pn. II B.	Results.
1....	1 cc.	0.0001 cc.			D. 24 hrs.
2....	1 cc.	0.00001 cc.			D. 24 hrs.
3....	1 cc.	0.000001 cc.			D. 24 hrs.
4....	1 cc.		0.0001 cc.		D. 24 hrs.
5....	1 cc.		0.00001 cc.		D. 24 hrs.
6....	1 cc.		0.000001 cc.		D. 48 hrs.
7....	1 cc.			0.0001 cc.	S. 5 days
8....	1 cc.			0.00001 cc.	S. 5 days
9....	1 cc.			0.000001 cc.	S. 5 days
10....		0.0000001 cc.			D. 24 hrs.
11....			0.0000001 cc.		D. 48 hrs.
12....				0.0000001 cc.	D. 48 hrs.

PROTOCOL 16.

Mouse protection against Types II, II A and II B pneumococcus with chicken serum adsorbed with Type II B pneumococcus.

Mice.	Ad. S.	Pn. II.	Pn. II A.	Pn. II B.	Results.
1....	1 cc.	0.0001 cc.			D. 24 hrs.
2....	1 cc.	0.00001 cc.			D. 24 hrs.
3....	1 cc.	0.000001 cc.			D. 24 hrs.
4....	1 cc.		0.0001 cc.		S. 5 days
5....	1 cc.		0.00001 cc.		S. 5 days
6....	1 cc.		0.000001 cc.		S. 5 days
7....	1 cc.			0.0001 cc.	D. 24 hrs.
8....	1 cc.			0.00001 cc.	D. 24 hrs.
9....	1 cc.			0.000001 cc.	D. 48 hrs.
10....		0.0000001 cc.			D. 24 hrs.
11....			0.0000001 cc.		D. 48 hrs.
12....				0.0000001 cc.	D. 48 hrs.

The protocols above show, (1) that chicken serum adsorbed with Type II pneumococci loses only the Type II protective substances, still protecting against Type II A and II B strains; (2) that serum adsorbed with Type II A culture loses the Type II A and Type II antibodies but not the Type II B antibodies; and (3) that serum adsorbed with Type II B culture loses the Type II B and Type II protective substances but not those for Type II A.

mice which received the adsorbed serum in order to make sure that the serum contained protective substances for all three types before it was adsorbed. The same was done in Protocols 15 and 16.

In terms of the protective substances of chicken serum, therefore, Types II A and II B pneumococci seem to form two distinct main groups with Type II a subgroup to both of them. This relationship is just the reverse of that which Avery found in terms of sera from specifically immunized animals.

DISCUSSION.

The observations recorded above, in conjunction with previous observations, afford ground for concluding that specific humoral elements are essential factors of the natural immunity of the chicken to infection by the pneumococcus. The exact nature of these humoral elements or the manner in which they operate to effect resistance has not been determined. It is probable, however, that they are of the nature of opsonins and prepare the pneumococci for phagocytic destruction. The basis of these conclusions is briefly as follows: (1) The serum of chickens has the power of conferring upon other animals an immunity to the pneumococcus, (2) virulent pneumococci are phagocyted in fresh chicken blood *in vitro*, (3) pneumococci grow readily in fresh chicken serum or plasma, and (4) opsonization and phagocytosis are known to be essential factors in other instances of acquired and artificial immunity to the pneumococcus.

It may be objected that the passive immunity conferred upon other animals with chicken serum is not comparable in degree to that of the chicken and that the methods employed fail to demonstrate pneumococcus opsonins in chicken serum. It is to be remembered, on the one hand, that it is not necessary to have a high concentration of antibodies in the body tissues and fluids of an animal in order to have protection against infection, provided the antibodies are constantly present and are inexhaustible. On the other hand, the usual methods of studying antibodies *in vitro* entail a dilution of the serum and a one-fold dilution may suffice to inhibit their action. These conditions seem to obtain in the present case. The production of antipneumococcus protective substances is, no doubt, a natural function of the tissues of the chicken and whenever the normal concentration of these substances in the blood is reduced the tissues immediately liberate more of them and reestablish the equilibrium, the resistance of the animal being thus maintained. As already stated, pneumococci are phagocyted in the undiluted fresh blood of the chicken while in a mixture of one part serum, one part bacteria and one part leucocytes no phagocytosis occurs. The demonstration by Keyes (7) that phago-

cytosis plays a prominent rôle in the destruction of pneumococci in the pigeon is another point in favor of this explanation of the chicken's immunity to the pneumococcus. In all probability similar mechanisms operate in both instances, but as yet the humoral phase in the former and phagocytic in the latter have not been satisfactorily demonstrated.

Why chicken serum protects mice against infection with the pneumococcus when it is given intraperitoneally and fails to protect when it is given subcutaneously is not entirely clear. There are two facts, however, which afford a possible explanation for this anomaly. First, animals are more resistant to infection several hours after they have received antibacterial sera than they are if they are infected at the time of administering the serum. This is true notwithstanding the fact that the infecting organisms may be exposed to the action of the concentrated serum when the culture and the serum are given at the same time and not when the serum is given several hours before the animals are infected. It appears, therefore, that some kind of reaction between the immune serum and the tissues of the host must take place before the protective serum becomes effective. This reaction may be nothing more than the adsorption of the protective bodies by the cells of the host or it may be a more specific reaction between factors from the host and from the immune serum resulting in the formation of a new substance to which protection is due. In any case, time elapses between the administration of the serum and the development of the maximum resistance on the part of the host. The second fact is that the chicken serum is rapidly destroyed or eliminated by mice for, on intraperitoneal injection, the mice are not protected for more than twenty-four hours. Horse serum is not so quickly eliminated since antipneumococcus horse serum protects mice for at least six days. It is possible, therefore, that on subcutaneous injection the chicken serum is destroyed before the reaction necessary for protection occurs and, for this reason, a resistant state never develops.

The discovery of antibodies in the serum of a normal animal which are specific for the different serological types of the pneumococcus shows that the classification of these organisms, although done with artificially prepared sera, has a fundamental biological significance. If this classification had first been done by means of the protective substances of chicken serum, it would have been just as it is with the exception of the relation of the group II subtypes to the main type. This difference is due, doubtless, to differences in the antigenic origin of the two sets of antibodies. The observation is of interest in con-

nection with existing theories of the origin and relationships of major and minor antibodies.

How the chicken came to possess these antipneumococcic substances is quite a puzzle to the immunologist. Existing theories concerning the origin of natural bacterial antibodies do not offer a satisfactory explanation in this instance. Is it possible that these substances owe their origin to a specific antigenic stimulation which occurred in some earlier period of the life history of the species, the germplasm being so fundamentally impressed that an acquired property became inheritable? If specific antigenic stimulation is eliminated, it gives reason for questioning the soundness of this theory with reference to other natural antibodies found in both man and the lower animals.

SUMMARY.

1. It has been shown that the serum of normal chickens is capable of protecting mice and guinea pigs against infection with pneumococci.

2. The protective substances are found in the water-insoluble fraction of the serum globulin.

3. There are, in chicken serum, particular protective substances for each serological type of pneumococcus. These substances are selectively removed from the serum by the process of bacterial adsorption.

4. In terms of the protective substances in chicken serum, Types II A and II B pneumococci constitute two distinct main groups and Type II strains form a subgroup to both of them.

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