THE IMMUNIZING ANTIGEN OF THE PNEUMOCOCCUS.

III. The Purification of the Water-Soluble Antigen.

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It has been shown by Perlzweig and Steffen (1) that the immunizing antigen of the pneumococcus is easily soluble in and extracted by water and salt solutions. This observation was later corroborated by Ferry and Fisher (2). It appeared desirable, therefore, to attempt to extract the antigen from the filtrates of broth cultures of pneumococcus.

Source of the Material.

The material used was obtained in the following manner. Massive cultures of Pneumococcus Type I were grown in the medium recently described by Huntoon (3) consisting of aminoids, peptone, sodium chloride, phosphates, and lactose. At the end of 18 hours of growth the culture was killed by heating at 56–60°C. for 1 hour, and centrifugalized through a Sharples machine. The supernatant fluid was passed through Mandler filters and then concentrated under reduced pressure at 40–44°C. to about one-fourteenth of the original volume.[1] This concentrated material was preserved with 0.5 per cent chloroform and kept in the refrigerator. On cooling, a large amount of lactose and the salts of the culture medium sedimented out. In the experiments described below the supernatant fluid only was used, as it was found that the sediment contained no appreciable quantity of the active material.

Purification by Pressure Filtration.

Since the original solution contained large amounts of salts, amino acids, lactose, peptones, and peptides it was first attempted to separate these by dialysis. The

[1] The growth of the cultures and the carrying out of the above preliminary treatment was done for us at the H. K. Mulford Laboratories at Glenolden Farms, Pa., under the supervision of Dr. Huntoon. The authors wish to express their appreciation to the Mulford Company and to Dr. Huntoon for their generous gift and cooperation.

osmotic pressure of the solution, however, proved to be too great, resulting in the passage of much water into the dialyzing bags, and thus materially retarding dialysis. Consequently it was found more advantageous to make use of ultrafiltration

TABLE I.

Experiment No	Fraction of antigen.	Total nitrogen per 100 cc.	Dose of antigen administered to mice.		Culture.	No. of mice.	
			1st dose.	2nd dose.		Dead.	Survived.
		mg.	*cc.*	*cc.*			
1 A	Original material including sediment.	1025	0.1	0.5	10^{-3}	3	1
					10^{-4}	1	3
					10^{-6}	1	2
1 B	Supernatant fluid of No. 1 A.	1005	0.1	0.5	10^{-3}	3	1
					10^{-6}	1	3
1 C	Ultrafiltration residue within 6 bags, obtained from No. 1 B.	32	0.1	0.5	10^{-3}	3	1
					10^{-6}	0	4
1 D	Ultrafiltrate of No. 1 C.	950	0.1	0.5	10^{-3}	4	0
					10^{-6}	4	0
2 A	Precipitate at pH 4.1 obtained from No. 1 C.	16	0.5		10^{-5}	0	8
2 B	Filtrate from No. 2 A.	16	0.5		10^{-5}	3	4
2 C	Hydrochloride of picrate precipitate from No. 1 C.	17	0.5		10^{-5}	1	7
					10^{-3}	1	0
Control (accompanying each of above experiments).					10^{-7}	2	0
					10^{-8}	2	0

under pressure. Stout collodion bags made from 8 to 12 per cent collodion in 1:1 alcohol-ether solution were used and found capable of withstanding pressures of 300 to 400 mm. of mercury. This method of ultrafiltration has been previously used with success by Glenny and Walpole (4) for the purification of diphtheria

antitoxin and of other similar substances. We did not, however, use water on the outside of the bags, but the filtrate was collected in glass jars without dilution. The material in the bags was washed for several days by continuous feeding into the bags of distilled water under pressure from a reservoir included in the system.

In this way it was possible to filter out through the membranes at least 97 per cent of the original content of nitrogen of our material (see Table I) and practically all of the inorganic salts. The total nitrogen content of the solution remaining within the bags was 32 mg. per 100 cc., whereas the original solution contained 1005 mg. per 100 cc. The dialysate gave positive protein color tests. It may be seen from the table that the content of immunizing antigen in this dialyzed fraction remaining in the bag compares well with that of the original solution.

Further Purification by Precipitation.

Further purification of the above dialyzed fraction was attempted by two methods: by precipitation of an active fraction at the isoelectric point and by the separation of a soluble picrate fraction. The first of these methods was proposed by Michaelis and Davidsohn (5) for the purification of diphtheria toxin, tuberculin, trypsin, etc., while the second was developed for the purification of insulin by Dudley (6) and applied by Baker, Dickens, and Dodds (7).

In a series of preliminary test-tube experiments it was found that a maximum sharply defined precipitation occurred in the solution of dialyzed material when a small amount of it was added to an excess of N/10 acetic acid-sodium acetate buffer mixture at pH 4.1.

Accordingly, to 100 cc. of the dialyzed solution 20 cc. of a N/2 acetate buffer mixture were added to pH 4.1. The precipitate was removed by decantation and centrifugalization and washed repeatedly in the centrifuge tube with diluted acetate mixture (pH 4.1). Finally, the precipitate was dissolved in water with the aid of a few drops of N/10 NaOH solution. This isoelectric fraction contained 20 mg. total nitrogen per 100 cc. calculated on the basis of the volume of the original solution. It protected mice against 1000 fatal doses of the homologous pneumococcus culture.

The picrate procedure was carried out according to the micro method of Baker, Dickens, and Dodds (7). 200 cc. of dialyzed solution were ground up in a mortar with 2.5 gm. of picric acid. The resulting precipitate was separated by centrifugalization and decantation, and washed several times in the centrifuge tube with saturated picric acid solution. The precipitate was then stirred in 70 per cent

acetone, whereupon much of it dissolved, yielding a dark brown-red solution and an insoluble bright yellow portion. The latter was separated in the centrifuge tube, and after decantation of the supernatant solution, the residue was extracted with two further portions of 70 per cent acetone. The supernatant acetone solutions were combined and evaporated to a small volume before a fan until a precipitate began to appear. On the addition of an equal volume, 10 cc. of water, a bulky, dark, gummy precipitate appeared. This was again separated in the centrifuge and washed repeatedly with ether to remove the excess of picric acid. The picrate was then changed into a hydrochloride by decomposing it with alcoholic HCl solution (25 cc. 3 N HCl diluted with 95 per cent alcohol to 100 cc.). All but a small portion dissolved. To the supernatant solution obtained on centrifuging 10 volumes of absolute acetone were added. A light flocculent precipitate separated out which darkened and became gummy on standing. This precipitate, representing the hydrochloride, was then removed by centrifugalization, washed with ether, and dried in a current of air. It was dissolved easily in very dilute $NaHCO_3$ solution. Before use in the protection experiments the pH of this solution was adjusted to 7.4. The hydrochloride fraction derived from the soluble picrate contained but 17 mg. of the 1005 mg. of total nitrogen in the original solution, and it also protected immunized mice against 1000 fatal doses of virulent Pneumococcus Type I culture.

The table given above shows the total nitrogen content of the various fractions calculated on the basis of the original solution. It also shows the results of the protection tests on mice. These were carried out according to the technique employed in the first paper of the series (8).

In some of the earlier experiments two doses of the protecting material were administered at 7 day intervals to the mice subcutaneously, while later it was found that one dose sufficed to produce immunity. For the purposes of infection a virulent 16 to 18 hour Pneumococcus Type I culture was given intraperitoneally 10 days after the last dose of antigen. Every experiment was controlled by at least two normal untreated mice which received intraperitoneally 0.5 cc. of 10^{-8} dilution of the same culture. This latter dose was adopted as the standard minimal fatal dose. Unless the control mice succumbed within 48 hours and pneumococci were recovered from a peritoneal smear or a subculture from the heart's blood, the experiment was discarded. All mice reported as dead succumbed within 60 hours and pneumococci were found in a stained peritoneal fluid smear. All mice reported as living or protected, survived at least 4 days and were in good condition at that time. In these experiments no effort was made to determine the maximal protecting power of the fractions.

DISCUSSION OF RESULTS AND SUMMARY.

It may be seen from the table that the soluble immunizing antigen which is present in the filtrate from cultures of the pneumococcus can be separated from non-antigenic material by means of ultrafiltration through collodion under pressure. It can be further purified by means of precipitation at a definite hydrion concentration and by the application of the picrate method used in the purification of insulin and of the antineuritic vitamine (9).

Whether the substance dealt with above corresponds to the protein obtained from bile solutions of pneumococci by Avery and Heidelberger (10) has not yet been established. This question is being studied along with others dealing with the chemical nature of the active antigen. Sufficient material for this purpose has been lacking until the present. The problem of species and type specificity of the various fractions is also to be investigated.

The immunologic experiments presented are to be regarded purely as qualitative tests to show relatively the absence or presence of some of the immunizing antigen in the fraction tested. No attempt has been made thus far to ascertain quantitatively the distribution of the antigen.

These methods obviously furnish a ready means for at least the initial separation and purification of the immunizing antigen from the complex mixtures present in culture media and in the bacterial cells themselves. Further chemical and immunologic studies will be made upon the fractions obtained by these methods.

SUMMARY.

Actively immunizing fractions of protein nature have been isolated from broth culture filtrates of Pneumococcus Type I by ultrafiltration, precipitation at a definite hydrion concentration, and the separation of a soluble picrate fraction. The method appears to be suitable for the initial purification of this antigen.

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