ANTIGENIC ACTIVITY OF EXTRACTS OF PNEUMOCOCCI

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Antigenic substances have recently been extracted in soluble form from many bacteria, and the work of Boivin & Mesrobeanu (1934), Raistrick & Topley (1934) and Topley *et al.* (1937) has shown that polysaccharides in combination with other non-protein substances extracted from certain Gram-negative bacilli stimulate an effective immunity in mice and specific antibody formation in rabbits. The antigenicity of similar extracts of pneumococci has not been so readily demonstrated. Berkefeld filtrates of pneumococci lysed by bile or by alternate freezing and thawing were found to be non-antigenic in rabbits (Avery & Morgan, 1925; Avery & Neill, 1925), although it was possible to immunize mice effectively with bacteria-free solutions obtained from cultures of pneumococci (Perlzweig & Steffen, 1923; Perlzweig & Keefer, 1925; Meyer, 1927). The essential antigen for the production of immunity in mice has been shown to be the type-specific polysaccharide which is effective only in small doses, usually between 0·01 and 0·00001 mg. (Schiemann & Caspar, 1927; Schiemann, 1929; Enders, 1930; Wadsworth & Brown, 1931; Avery & Goebel, 1933). The type-specific polysaccharide has, however, been found to be without antigenic activity in rabbits (Avery & Goebel, 1933; Downie, 1937).

Barach (1928) has recorded that the injection into rabbits of filtrates of broth cultures of pneumococci produced sera which would protect mice against infection with the homologous type of *Pneumococcus*. Day (1930, 1933) and Harley (1934) described experiments with type-specific antigen solutions, believed to be free from formed bacteria, which produced active immunity in mice and stimulated the formation of type-specific agglutinins and mouse-protective antibodies in rabbits. It seemed reasonable to suppose that by their methods a soluble antigen might be obtained which was fully antigenic for rabbits, and it was in view of these findings that the present work was undertaken. It was hoped to determine, if possible, the relation of the type-specific antigen of Day to the specific polysaccharide.

Experimental

Material and methods. The cultures of types I, II and III pneumococci used in the course of this work were of such virulence that one to ten pneumococci injected intraperitoneally usually produced a fatal infection in mice. The

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cultures were maintained in broth containing 5–10 % of fresh rabbit blood and were passed through mice once every 2 or 3 weeks. All extracts used in immunization experiments were prepared from the type I strains, and immunizing injections were made in rabbits by the intravenous route and in mice by the intraperitoneal route. Tests for active immunity in mice were made by the intraperitoneal injection of 0·5 c.c. amounts of tenfold dilutions made in broth from cultures grown in rabbit blood broth 8–18 hr. at 37° C. The number of bacteria in the broth dilutions was estimated by preparing pour-plates in blood agar from the last two dilutions used. Tests for mouse-protective antibodies in sera were made by the intraperitoneal injection into normal mice of 0·5 c.c. serum, undiluted or diluted 2 in 5 or 1 in 5 with saline, together with 0·5 c.c. of tenfold dilutions of culture. The culture dilution and serum were mixed in the syringe immediately before injection.

The polysaccharide preparations were kindly supplied by Dr Goebel and had been prepared by the method of Avery & Goebel (1933). The type I material was therefore an acetylated product which had been shown to be antigenic for mice when the proper dosage was used.

In the preparation of their specific antigen extracts Day & Harley collected the organisms from 19 hr. cultures and suspended them in saline. An equal volume of $N/10$ HCl was added, and after heating at 60° C. for 50 min. the suspension was "thoroughly centrifuged" and the supernatant after neutralization used as type-specific antigen. While it was not claimed that this supernatant was completely free from bacterial debris, it was believed that the fluids did not contain sufficient formed bacteria to produce immunity in mice or rabbits.

Since the inoculation of relatively small numbers of heat-killed pneumococci may produce immunity in mice (Killian, 1924) experiments were made to determine the antigenicity of small doses of extracted pneumococci such as might be present in the supernatant fluid after centrifugation.

The number of pneumococci remaining in the supernatant fluid after centrifugation

In experiments made with living broth cultures the number of organisms in the supernatant was determined by pour-plate cultures prepared from dilutions made in broth, and in the case of suspensions which had been extracted with acid, enumeration by direct observation in a Helber slide under dark-ground illumination was carried out.

In the experiment using 5·0 c.c. quantities of broth culture the number of pneumococci was reduced from 264 millions per c.c. to 1·5 millions in 30 min. at a speed of 3000 r.p.m. and to 10,000 in 15 min. at a speed of 15,000 r.p.m. When 200 c.c. quantities of a suspension of pneumococci which had been extracted with acid and heat were spun for 1 hr. at approximately 2500 r.p.m. the number of bacteria in the supernatant was found to be in the neighbour-

hood of 50 millions per c.c. That such pneumococcal cells after extraction with acid and heat may still retain their full antigenic activity is demonstrated in the experiments which follow.

EXPERIMENTS WITH EXTRACTS AND VACCINES

Mode of preparation. To 400 c.c. of culture of type I *Pneumococcus*, grown in broth containing 2% sterile rabbit serum for 12 hr., 200 c.c. of 1·0% glucose broth was added and incubation at 37° C. continued for 2 hr. longer. The culture was centrifuged for 40 min. and the deposit obtained was suspended in 200 c.c. of saline. From this saline suspension four preparations were obtained:

A. Heat-killed vaccine containing approximately the same number of bacteria per c.c. as the original culture, to be used as control material. Type-specific polysaccharide in the supernatant was equivalent to a dilution of 1 in 400,000.

B. Acid heat extract (unfiltered). This faintly opalescent extract contained many millions of cocci per c.c., and most of the organisms were Gram-positive. Type-specific polysaccharide in the supernatant was equivalent to a dilution of 1 in 800,000.

C. Berkefeld filtrate of "B". No cocci present, polysaccharide content as in "B".

D. Deposit obtained by prolonged centrifugation from "B" and washed twice with saline to remove free polysaccharide. Contained 48,500,000 cocci per c.c., mostly Gram-positive. No polysaccharide detectable by precipitation tests with immune serum.

Material "B" was prepared by adding to 100 c.c. of the original saline suspension 100 c.c. of $N/10$ HCl and heating to 60° C. for 50 min. The heated suspension was centrifuged for 40 min. at a speed of 2500 r.p.m. The supernatant which was turbid was twice spun for 1 hr., the final supernatant was neutralized to pH 7·0 and the volume made up to 300 c.c.

Preparation "C" was obtained by passing 150 c.c. of "B" through a Berkefeld N candle.

The deposit of bacteria obtained from the final centrifugation used in preparing material "B" was twice washed in a high-speed centrifuge at 15,000 r.p.m. and suspended in 200 c.c. of saline as preparation "D".

The concentration of free polysaccharide was determined by precipitation tests made on the supernatants obtained by high-speed centrifugation of "A", "B" and "D", using a type I immune horse serum. Dilutions of a purified preparation of type I polysaccharide were put up in parallel tests. Observations on the morphology of the organisms were made by examination of the deposits.

Experiments in mice with fractions A, B, C and D

Active immunization. Two intraperitoneal injections of 0·5 c.c. amounts were made with an interval of 3 days between injections. Six mice from each

group were bled out for serum 8 days after the second injection, and the remainder were tested for resistance to infection with type I and type II pneumococci. The results are shown in Table I.

Table I. *Active immunity produced in mice by type I antigens A, B, C and D*

Dilution of infecting culture		Mice immunized with A	B	C	D	Control normal mice	Colonies from 0·5 c.c.
Type I:	10^{-1}	D D	D D	—	D S	—	—
	10^{-2}	D S	D D	—	S S	—	—
	10^{-3}	S S	D D	D S	S S	—	—
	10^{-4}	S S	S S	D S	S S	—	—
	10^{-5}	S S	S S	S S	S S	—	—
	10^{-6}	S S	S S	S S	S S	D D	—
	10^{-7}	—	—	S S	—	D D	23
	10^{-8}	—	—	—	—	D S	4
Type II:	10^{-6}	D D	D D	D D	D D	D D	—
	10^{-7}	D D	D D	D D	D D	D D	28
	10^{-8}	D D	D S	D D	D D	D D	2

D = died within 4 days of pneumococcal infection.
S = survived 7 days.

The numbers of mice used are small, but in view of the virulence of the cultures and the results with the mice tested with type II culture it would appear that all four preparations induced resistance against infection with the homologous organisms only. Preparation C, which was free from formed cells, was not so effective as the other three but apparently did contain an antigenic substance which was type specific.

Examination of the sera of immunized mice. The sera from mice of the various groups failed to show agglutination in a dilution of 1 : 5 (the lowest tested) with type I or type II organisms, nor did they precipitate with solutions of polysaccharide prepared from type I or type III pneumococci. The sera were tested for protective antibodies by injecting 0·2 c.c. of the sera diluted to 0·5 c.c. with saline together with 0·5 c.c. of culture dilutions into normal mice. The results are shown in Table II.

Table II. *Passive protection with the sera of mice immunized with antigens A, B, C and D*

Dilutions of culture, type I	Sera from mice immunized with A	B	C	D	Control normal mice	Colonies from 0·5 c.c.
10^{-4}	S S	S S	S S	S S	—	—
10^{-5}	S S	S S	S S	S S	—	—
10^{-6}	S S	S S	S S	S S	D D	—
10^{-7}	S S	S S	S S	S S	D D	53
10^{-8}	—	—	—	—	D D	6

No controls with cultures of heterologous type and serum were used in this experiment owing to the limited amount of serum available, but experiments to be described later showed that the protection afforded to normal mice by such sera was effective only against the homologous organisms.

These findings indicate that filtrate C contained in solution a substance antigenic for mice, while the results with preparation D show that preparation B contained, in addition, pneumococcal cells which retained their specific immunizing power apparently unimpaired.

An attempt was made to determine the minimum effective antigenic dose of extracted cells by injecting tenfold dilutions of suspension D into groups of mice. The results of these experiments indicated that approximately 500,000 cocci injected in two doses gave a high degree of immunity, while one-tenth of this amount gave protection to some animals only and smaller doses had no effect.

Experiments in rabbits with fractions A, B, C and D

Immunization. Sample bleedings were taken from rabbits before treatment. Immunizing injections were made intravenously at weekly intervals in doses of 2·0, 4·0, 6·0, 8·0 and 10·0 c.c. Vaccine A was injected into two rabbits and three animals were used for each of the other three preparations. At the same time similar amounts of preparation D diluted 1 : 10 with saline were injected into one rabbit. Seven days after the last injection all animals were bled from an ear vein and the serum obtained was tested for agglutinins and precipitins against type I and type III polysaccharide, and mouse-protective antibodies.

Test of active immunity. Four days after bleeding the rabbits were tested for active resistance to infection with type I *Pneumococcus*, using the intradermal method described by Goodner (1928). The strain of *Pneumococcus* used had been shown to produce fatal infections in the majority of normal rabbits injected with 0·01 c.c. of a young culture. In the present experiment 0·2 c.c. of a 14 hr. culture was injected into the skin of the flank of the treated animals and two normal controls.

Results. The two normal rabbits 459 and 464 died 44 and 29 hr. after injection. They showed at the site of injection very extensive areas of inflammation with marked oedema and purplish discoloration extending down the flank to the midline of the abdomen and beyond it. Pneumococci were recovered from the heart blood at death.

The two rabbits immunized with vaccine A, 431 and 432, showed the type of immune reaction described by Goodner. The temperature did not rise above 102·5° F., and the lesion at the site of injection was raised and sharply circumscribed. It attained its maximum in 24 or 48 hr., but at the end of a week was still present as a small firm nodule.

The three animals immunized with the unfiltered extract B also showed the immune type of response, although the local lesions were somewhat more extensive than in the rabbits which had been immunized with vaccine A.

The three animals which had received injections of filtrate C, 436, 437 and 438, all showed extensive oedematous lesions with redness and purplish discoloration extending to the middle line. The temperatures rose to over 106° F.

on the first or second day, and the animals died of generalized infection 118, 142 and 54 hr. after injection.

The three animals injected with preparation D, and the animal injected with the same preparation diluted tenfold, showed an immune reaction similar to that observed in rabbits 431 and 432.

The infecting dose used in this experiment was many times the amount required to kill normal rabbits, and the duration of life in rabbits 436 and 437 suggests that, while they were not resistant to the same degree as those immunized with the other three preparations, they were rather more resistant than the controls.

Examination of the serum of rabbits injected with preparations A, B, C and D

The agglutination tests were carried out with serum dilutions of 1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 40, etc., and heat-killed saline suspensions of pneumococci which had been centrifuged out from 16 hr. broth cultures. The results were read after the tubes had been kept 2 hr. at 37° C. in a water-bath. The precipitation tests were carried out with sera undiluted, and type I and type III polysaccharides in dilutions of 1 : 10,000 and 1 : 100,000; the results were read after 2 hr. at room temperature and again after the tubes had been kept overnight in the refrigerator.

Results. The results of the tests are summarized in Table III.

Table III. *Antibodies in the sera of rabbits immunized with antigens A, B, C and D*

Rabbit no.	Antigen preparation	Agglutinins	Precipitins for type I polysaccharide	Protective titre in mice	Test for active resistance
431	A	1 : 40	+ + +	0	S
432		1 : 320	+ + +	10^{-1}	S
433	B	1 : 5	+		S
434		1 : 5	+	10^{-2}	S
435		1 : 5	+		S
436	C	–	–	–	D 118 hr.
437		–	–	10^{-4}	D 142 hr.
438		–	–	–	D 54 hr.
439	D	1 : 80	+ + +	0	S
440		1 : 40	+ +	0	S
441		1 : 10	+ +	0	S
442	D/10	1 : 80	+ + +	10^{-1}	S
459	Normal	.		.	D 44 hr.
464	controls	.	.	.	D 29 hr.

– = negative. 0 = not tested.

The figures in column 5 indicate the largest amount of culture against which 0·2 c.c. of rabbit serum protected normal mice.

The findings with rabbit 442 show that immunization with a suspension containing approximately 5 million cocci per c.c. which had been extracted with $N/20$ HCl at 60° C. gave a satisfactory immunological response which was

type-specific in character. The bacteria apparently still retained the specific polysaccharide antigen.

The animals injected with preparation C showed no agglutinins or precipitins but, while the sera of rabbits 436 and 438 showed no protective antibody, the serum from rabbit 437 protected mice against many lethal doses of type I pneumococci. The combined results of two tests with this serum are shown in Table IV. The serum of this rabbit obtained before immunization failed to show any protective action.

Table IV. *Mouse protection tests with the serum of rabbit* 437 *which had been immunized with filtrate C*

Culture	Serum 437	Controls	Colonies in 0·5 c.c.
Type I: 10^{-3}	D S	—	—
10^{-4}	D S S S	—	—
10^{-5}	S S S S	—	—
10^{-6}	S S S S	D D	—
10^{-7}	S S S S	D D	57
10^{-8}	—	D D	6
Type II: 10^{-5}	D D	—	—
10^{-6}	D D	D D	—
10^{-7}	D D	D D	31
10^{-8}	—	D D	2

In view of these findings serum 437 was examined further in attempts to demonstrate antibodies by *in vitro* tests. When tested for antibodies to type I polysaccharide by precipitation and complement-fixation tests the serum gave negative results, but positive reactions were obtained by these methods when filtrate C was used in place of polysaccharide solutions. Agglutination tests using suspensions of type I and type II pneumococci which had been washed six times with saline to remove as much polysaccharide as possible were negative. Mixtures of serum 437 and type I and type III polysaccharide after 2 hr. incubation at 37° C. and standing overnight in the refrigerator still gave visible precipitates on the subsequent addition of filtrate C. These findings suggest that the reaction with filtrate C was not due to the presence of antibodies to the specific polysaccharide. It seems possible therefore that the protective power of serum 437 was due to antibodies to *Pneumococcus* protein which may have been present in small amount in the filtered extract. Avery & Morgan (1925) have noted the presence of specific mouse-protective power of slight degree in the serum of one rabbit of a number which had been immunized by repeated injections of *Pneumococcus* protein. The sera of these rabbits had shown precipitins for the protein but no detectable antibodies to the specific polysaccharide.

EXPERIMENTS WITH EXTRACTS FROM PNEUMOCOCCI GROWN IN BROTH AND ON AGAR

Further extracts were prepared by the same method as before except that in the case of the culture from broth the 100 c.c. of filtrate obtained was derived from 230 c.c. of broth culture. In preparing extracts from type I

pneumococci grown on agar, a saline suspension of organisms obtained from 19 hr. growth in Roux bottles containing agar with 2% rabbit serum and 0·1% glucose was extracted in the same way as the broth culture. In each case in addition to the filtrate of the extract (broth C and agar C), a little of the deposit after extraction was washed several times in saline and finally suspended in saline to contain approximately 50 million cocci per c.c. (broth D and agar D).

Injections of 1·0 c.c. amounts were made intravenously into rabbits on three successive days each week for 6 weeks. All animals were bled and tested for active resistance to infection as before. Table V shows the results of these tests and the examination of the sera of the rabbits for antibodies. The results are essentially the same as those in the previous experiment. The rabbits treated with filtered extracts did not show active resistance to infection, nor did their sera contain any demonstrable antibodies.

Table V. *Results of examination of the sera and test for active immunity in rabbits treated with extracts from type I pneumococci grown in broth and on agar*

Rabbit no.	Antigen preparation	Agglutinins	Test for antibodies to type I polysaccharide: Precipitins	Test for antibodies to type I polysaccharide: Complement fixation	Protective titre in mice	Test for active resistance
331	Agar C	–	–	–	–	D 70 hr.
351		–	–	–	–	D 70 hr.
352		–	–	–	–	D 22 hr.
333	Agar D	1 : 80	+ + +	+ + +	0	S
335	Broth C	–	–	–	–	D 118 hr.
336		–	–	–	–	D 118 hr.
353		–	–	–	–	D 70 hr.
350	Broth D	1 : 640	+ + +	+ + +	10^{-1}	S
423	Normal rabbit	.	.	.	.	D 70 hr.

0 = not tested. – = negative.

Experiments with a concentrated extract

Although the results of the last experiment were entirely negative, it seemed possible that the findings with rabbit 437 in the first experiment might have been due to the presence of an immunizing substance too small in amount to give consistent results. A more concentrated extract was therefore prepared.

Three litres of broth containing 2% rabbit serum were inoculated with an actively growing culture of type I *Pneumococcus*. After 16 hr. incubation 1·5 l. of broth containing 1% glucose were added and incubation continued for 2½ hr. more. The cultures were then centrifuged and the deposit of pneumococci was twice extracted with *N*/20 HCl as before. The reaction of the pooled extracts obtained after centrifugation, approximately 1 l. in volume, was adjusted to approximately *p*H 5·0 with NaOH. The fluid was evaporated down under reduced pressure at a temperature of 40–50° C. to approximately 200 c.c. This was filtered through a Berkefeld N candle and the reaction adjusted to *p*H 7·0 before use.

The concentration of specific polysaccharide in the filtrate was determined by precipitation tests with immune sera using the optimum proportions method and following the technique described by Smith (1932). The results of these tests showed that the extract contained specific polysaccharide in a concentration of approximately 1 in 3000. The extract gave positive results when examined by complement fixation and precipitation tests with an immune rabbit serum which had been completely absorbed by a preparation of type I polysaccharide prepared by the older method of Heidelberger *et al.* (1925). It seemed likely therefore that the polysaccharide in the extract was in the acetylated form.

Experiments with rabbits

Three rabbits were injected intravenously at weekly intervals with amounts of extract increasing from 2·0 to 10·0 c.c. as in the first experiment. Two rabbits were given series of three injections of 1·0 c.c. on successive days each week for 4 weeks, so that while each of the first three rabbits received a total volume of 30 c.c. the other two received 12 c.c. The animals were bled one week after the last injection.

Test for active resistance. Twelve days after the animals had been bled 0·02 c.c. of 16 hr. broth culture of type I *Pneumococcus* was injected intradermally into each animal and into two normal controls. The two normal animals died 23 and 51 hr. after injection. Two of the five treated rabbits died after 30 and 47 hr., and the remaining three were found dead 70 hr. after injection. The temperature reactions and the skin lesions were similar in the control and in the treated rabbits.

Examination of the sera of the treated rabbits. No agglutinins for type I or type II pneumococci were present, nor did the sera precipitate in tests made with type I polysaccharide or the extracts used for the previous intravenous injections. All sera gave completely negative results in mouse-protection tests. The sera in 0·5 c.c. amounts failed to protect mice against 0·5 c.c. of 10^{-7} dilution of culture (66 colonies) when injected together with the culture or 18 hr. before by the intraperitoneal route. The serum of all five rabbits when tested with type I immune rabbit serum gave positive complement fixation and precipitation reactions showing that the polysaccharide contained in the extract injected was still present in the blood stream 1 week after the last injection.

The results show that the extracts failed to induce active immunity in rabbits or to give rise to demonstrable antibodies in their sera.

Experiments with mice

For the injection of mice the extract was used undiluted and in a dilution of 1 : 500. This dilution was chosen because it appeared likely from the results of various workers that this would contain a concentration of polysaccharide which would produce effective immunity in these animals. Three injections of

0·5 c.c. each were made at 3 day intervals into two groups of mice. Six mice from each group were bled out from the heart 7 days after the last injection to obtain serum for examination. The results of tests for active immunity on the remaining mice are shown in Table VI.

Table VI. *Active immunity produced in mice with a concentrated extract from type I pneumococci*

	Mice immunized with			
Culture dilutions	Extract diluted 1 : 500	Extract undiluted	Control normal mice	Colonies from 0·5 c.c.
Type I: 10^{-2}	D D D	—	—	—
10^{-3}	D D D	—	—	—
10^{-4}	D D D	—	—	—
10^{-5}	S S S	D D D	—	—
10^{-6}	S S S	D D S	D D D	—
10^{-7}	S S S	D D S	D D D	78
10^{-8}	—	—	D D D	6
Type III: 10^{-5}	D D	D D	—	—
10^{-6}	D D	D D	D D	—
10^{-7}	D D	D D	D D	46
10^{-8}	—	—	D D	4

The serum of both groups of mice failed to agglutinate type I pneumococci even when equal volumes of serum were mixed with heat-killed suspensions of the organism.

The serum from the mice immunized with the diluted extract gave a slight but distinct precipitate with a 1 : 100,000 dilution of type I polysaccharide, while the serum from the other animals was negative. Precipitation tests with a type I immune rabbit serum were negative with the first mouse serum but positive with the second, indicating that antigenic material, presumably the specific polysaccharide, was still present in the circulating blood of the mice 7 days after the last injection of undiluted extract.

In protection tests each normal mouse received 0·1 c.c. of serum to be tested together with the culture dilution. The results are shown in Table VII.

Table VII. *Passive protection tests with the sera of mice immunized with concentrated extract*

	Sera of mice immunized with		
Culture dilutions	Extract diluted 1 : 500	Extract undiluted	Colonies in 0·5 c.c.
Type I: 10^{-4}	D S S	—	—
10^{-5}	S S S	—	—
10^{-6}	S S S	D D D	—
10^{-7}	S S S	D D D	83
10^{-8}	—	D D S	9
Type III: 10^{-6}	D D	—	—
10^{-7}	D D	—	51
10^{-8}	D D	—	5

It has been pointed out previously that successful immunity in mice is only produced by the purified type-specific polysaccharide of pneumococci within a certain range of dosage. The difference in the results obtained with the two groups of mice in the above experiment and the negative results in rabbits,

when considered in relation to the concentration of polysaccharide in the extract used, strongly suggest that the mouse-immunizing antigen present in filtered acid extracts of pneumococci was the specific polysaccharide.

EXPERIMENTS WITH ALCOHOLIC EXTRACTS OF TYPE I PNEUMOCOCCI

It has been observed by various workers that alcoholic extracts of pneumococci may contain a substance which on injection into mice produces active immunity to infection with the homologous type of pneumococcus (Perlzweig & Steffen 1923; Schiemann *et al.* 1931; Felton, 1932). Although such extracts may fail to give specific precipitation with immune sera, it was pointed out by Schiemann and his associates that active preparations of pneumococcus carbohydrate may be most effective in mice at a concentration which is too dilute to yield a positive precipitation reaction. It has been suggested by these workers and by Felton (1934) that the immunizing activity of alcohol-soluble preparations may be due to traces of polysaccharide which may be soluble in alcohol under certain conditions. Most of these experiments had been carried out in mice, so it was decided to test the antigenic activity of alcoholic extracts of type I *Pneumococcus* in rabbits.

Preparation of the extracts. The bacteria obtained by centrifugation of 4½ l. of culture of type I *Pneumococcus* grown for 19 hr. in 2% rabbit serum broth were extracted three times with a mixture of 66% ethyl alcohol and 33% isotonic salt solution. Extraction was carried out at room temperature over a period of 24 hr. After removal of the alcohol by evaporation under reduced pressure at a temperature of 40° C. the pooled extracts had a total volume of 250 c.c. A slight opalescence developed when the extract was placed overnight in the refrigerator at a temperature of 4° C. but became clear on warming to 40° C. At this temperature the material was filtered through a Berkefeld N candle. The extract gave weak positive reactions in precipitation and complement-fixation tests with type I immune rabbit serum.

Immunization of rabbits. Four rabbits were injected into the ear veins with the extract as in the third experiment, two rabbits being treated by each method. Seven days after the last injection the animals were bled and tested for active immunity by intradermal injection of virulent type I organisms as in previous experiments. The results showed that the animals were no more resistant than normal controls and all four died within 40 hr. The serum of the animals failed to react with type I polysaccharide or to agglutinate homologous or heterologous pneumococci, and afforded no protection to mice against infection with type I or type III organisms. These extracts apparently contained no antigen which was effective in immunizing rabbits. Jungeblut (1927) has recorded a similar failure to immunize a rabbit with an extract prepared from type I pneumococci with 95% alcohol, although his material under suitable conditions gave flocculation reactions with some type I immune rabbit and horse sera.

Discussion

The experiments in the present paper have shown that pneumococci after extraction with acid and heat retain their immunizing properties for rabbits. A suspension containing approximately 5 million such organisms in each cubic centimetre was quite clear and yet sufficed to produce active immunity in a treated rabbit and gave rise to the production of type-specific antibodies in its serum. When the extracts were filtered so as to remove the bacterial cells the filtrates were no longer antigenic in the rabbit although still effective in producing type-specific immunity in mice. The activity of these filtrates was probably due to the type-specific polysaccharide which was present in them in an active form. This conclusion is supported particularly by the experiments with the concentrated extract recorded in this paper. It is a common experience that vaccines, prepared from cultures of pneumococci in which autolysis has set in and in which the majority of the organisms are Gram-negative, are poor antigens for the preparation of antisera in the laboratory. The recent work of Dubos (1937) has shown that pneumococci subjected to the action of bacteriolytic enzyme lose their antigenicity for rabbits when the cells are merely rendered Gram-negative without undergoing actual autolysis. Boivin & Mesrobeanu (1935) were unable to obtain a complete antigen by extraction of pneumococci with trichloracetic acid, a procedure which serves to extract soluble antigens from many other bacteria. It would appear that the substance in the young Gram-positive *Pneumococcus* which determines antigenicity for the rabbit and perhaps some other animal species is extremely labile, and attempts to extract it in soluble form have so far been unsuccessful.

Conclusions

1. Unfiltered extracts of *Pneumococcus* type I prepared by treating the bacterial cells with $N/20$ HCl produced active immunity in mice and rabbits and gave rise to the production of specific agglutinating, precipitating and mouse protective antibodies in their sera.

2. Filtrates of such extracts were fully antigenic in mice and this activity was almost certainly due to the presence of type-specific polysaccharide.

3. The filtrates were without immunizing action in rabbits and the antigenicity of the unfiltered extracts in these animals was shown to be due to the presence of pneumococcal cells.

4. Extraction of type I *Pneumococcus* with 66 % alcohol also failed to yield preparations antigenic in the rabbit.

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