AN IMMUNOLOGICAL STUDY OF THE A SUBSTANCE OR ACETYL POLYSACCHARIDE OF PNEUMOCOCCUS TYPE I

By JOHN F. ENDERS, Ph.D., and CHAO-JEN WU,* M.D.

(*From the Department of Bacteriology and Immunology, The Harvard Medical School, Boston*)

In 1930 (1) one of us described a type-specific substance in the autolysates of Pneumococcus Type I which caused precipitation in homologous antiserum after all precipitating antibody reacting with the specific carbohydrate of Heidelberger, Goebel and Avery (2) had been removed by absorption. This antigen which appeared to be non-protein was provisionally designated the A substance. Because of its instability on heating in the presence of dilute alkali, which was in marked contrast to the stability of the specific carbohydrate under the same conditions, and because of its precipitating and anaphylactic reaction with specific antiserum deprived of antibody capable of reacting with the specific carbohydrate, we at first regarded the A substance as a second and distinct antigenic entity, probably belonging to the group of partial antigens or haptenes. However, the analysis of Pappenheimer and Enders (3) on the chemical composition of the substance obtained by preserving an acid reaction during all steps in the purification of the specific carbohydrate, indicated a close relationship between the A substance and the specific carbohydrate. These authors found that the elementary analysis of the product derived under the constant condition of a low pH was practically identical with that found for the specific carbohydrate isolated according to the procedure of Heidelberger, Goebel and Avery, during which the material is exposed to the alkaline action of concentrated barium hydroxide. Moreover, no significant difference was noted in the

---

capacity of the two substances to change the plane of polarized light. The carbohydrate obtained under the condition of acidity differed, nevertheless, in two respects from the specific carbohydrate: in aqueous solution it failed to precipitate at pH 3.4, the isoelectric point of the specific carbohydrate; it reacted by precipitating with antiserum after absorption with the specific carbohydrate. Moreover, the capacity to cause precipitation in such an antiserum was removed by boiling in dilute alkali. It was observed that the material thus treated with alkali precipitated at pH 3.4 and was able to react to titre with antiserum which contained antibody for the specific carbohydrate. Absorption of a specific antiserum with the A carbohydrate removed all antibodies precipitating either with itself or the specific carbohydrate. These facts led to the suggestion by Pappenheimer and Enders that the specific carbohydrate of Heidelberger, Goebel and Avery represented a hydrolytic product of the A substance. They believed that possibly the A substance more closely approximated the specific antigen of Type I pneumococcus as it exists in the living cell.

During the course of the experiments which are recorded in this paper, Avery and Goebel (4) reported that by maintaining an acid reaction during the preparation, they obtained a specific substance from Type I pneumococcus, the precipitating power of which, in the presence of an antiserum absorbed with specific carbohydrate, was removed by treatment with alkali. On elementary analysis, the material appeared to be identical with the specific carbohydrate. In addition they made the significant observation that this carbohydrate contained the acetyl group which was not found in the specific carbohydrate and which was apparently split off upon treatment with alkali. In their study of the immunological properties of this material, these authors showed that injection of small quantities of the acetyl polysaccharide actively immunized mice against an otherwise fatal dose of virulent pneumococci, while absorption of an antiserum with this carbohydrate appeared to eliminate its protective action in those animals—a result not obtained with comparable amounts of the substance after treatment with alkali. In the present paper a study of certain immunological aspects of the A substance purified according to the procedure described by Pappenheimer and Enders (3) is presented.

In particular the action of the substance was investigated in phago-cytic systems containing normal opsonins, in bactericidal systems of leucocytes and normal and immune sera. Its capacity to absorb the mouse-protective immune body from antiserum as well as its ability to induce active immunity in mice, was also examined. The quantities necessary to induce such immunity and the time of its appearance and defervescence have been subjected to study. The sera of mice actively immunized with the material has been tested for specific antibody and the effect of injection of the A substance in A-immunized mice on their resistance to pneumococcus infection has been observed.

The material has been analyzed chemically to determine whether or not it contains the acetyl group. This has been found to be present.

The results serve to identify the A substance as prepared by Pappenheimer and Enders with the acetyl polysaccharide described by Avery and Goebel and, in general, to confirm and extend the observations of these authors and our own in respect to its immunological effects.

Materials

Preparation of the A Substance.—The A substance used in these experiments was prepared according to the procedure given by Pappenheimer and Enders (3). The data obtained on elementary analysis and determination of specific rotation have been presented in their report. Subsequent to the appearance of the paper of Avery and Goebel on the acetyl polysaccharide, this material has been subjected to analysis for the presence of the acetyl group according to the method of Friedrich and Rapoport (5). It has been found to contain 5.8 per cent acetyl. This finding, taken together with the data of the elementary analysis, appears to offer sufficient reason for concluding that the acetyl polysaccharide and the A carbohydrate are to be regarded as identical.

In the majority of experiments, the effect of the A carbohydrate was compared with the effect of the substance after boiling in alkaline solution. As Avery and Goebel (4) have shown, such treatment removes the acetyl group. Deacetylization was effected in $N/50$ and $N/10$ concentrations of alkali. After addition of the volume of either $N/5$ or 1 N sodium hydroxide required to give these final concentrations to a measured quantity of 1:100 A carbohydrate, the solution was immersed in boiling water for 1 hour and then neutralized with the required amount of $N/5$ or 1 N hydrochloric acid.

Preparation of Type I Specific Soluble Substance.—The specific carbohydrates designated as SSS I, SSS II and SSS III were derived from 0.1 per cent dextrose phosphate buffer broth cultures of the three types of pneumococcus, according to the procedures described by Heidelberger, Goebel and Avery.

Preparation of Antisera.—Three Type I antipneumococcus rabbit sera were used. Of these one was prepared by repeated intravenous injection of pneumococcus suspension in 0.3 per cent formolized saline. Antigens employed in the production of the remaining two were suspensions of pneumococcus killed by heating at 56° and 60°C. The route of administration was intravenous.

Strains of Pneumococci.—The strain of Type I pneumococcus used in all the experiments was of such virulence that 0.00000005 cc. of a 6 hour 0.1 per cent dextrose infusion broth culture was uniformly fatal for white mice within 48 hours. Virulence was maintained by weekly passage through mice. In a few cases in which control of type specificity was required, stock strains of Type II and III pneumococcus were utilized. The virulence of these for mice was of the same order as that of the Type I pneumococcus.

Mice.—White mice varying in weight from 15 to 20 gm. were used.

Technique

Technique Employed in Phagocytic Experiments.—Ward and Enders (6) have described in detail this technique.

Technique Employed in Bactericidal Experiments.—In the experiments in which human defibrinated blood was used as a base for testing the antibactericidal action of the A carbohydrate against the normal antibody of the serum, the technique described by Ward (7) was followed, except that 0.25 cc. of defibrinated blood was used instead of 0.5 cc. For the study of the effect of the substance on the bactericidal power of immune antibody, a modification of the technique introduced by Robertson and Sia (8) was developed, in which polymorphonuclear leucocytes of the rabbit obtained by intrapleural injection of aleuronat, are added to the defibrinated blood of the same animal. A normal rabbit of about 2 kilos weight is injected into the right pleural cavity with 5 cc. of aleuronat suspension, prepared according to the direction of Robertson and Sia. After about 24 hours, 5 cc. of 1 per cent citrate in saline is injected into the same pleural space and withdrawn in the same syringe, together with the exudate of leucocytic cells. The suspension of cells is then slowly centrifuged for 7 minutes and then washed twice with about 20 cc. of physiological salt solution. A count of the total white blood cells present is made after resuspension in the second volume of saline, before centrifuging. A quantity of this suspension is removed which, when mixed with the desired amount of rabbit's defibrinated blood, will give a count of 10,000 per c. mm. This measured volume of suspension is then slowly centrifuged, the supernatant removed and the predetermined quantity of defibrinated blood added to the deposit of cells and well mixed with them. The number of leucocytes in the blood-leucocyte mixture is then checked. It is usually greater than that found for the saline suspension by 1,000 to 2,000 cells per c. mm. due to the presence of the leucocytes in the defibrinated blood. For satisfactory results, the final white blood count of the mixture should not be below 10,000 per c. mm. Counts from 15,000 to 17,000 do not appear to influence significantly the effectiveness of the

system. 0.25 cc. of blood-leucocyte mixture is then placed in small pyrex glass tubes (10 cm. long, 7 mm. inside diameter). 1 drop each of antiserum, carbohydrate and 18 hour blood broth pneumococcus culture, diluted in infusion broth, is added in the order named. After addition of each substance, the tube is gently shaken. The tubes are then sealed and placed in a rotating box (fifteen revolutions per hour) at 37°C. Rotation is maintained for 48 hours when the results are read by the darkening of the blood caused by bacterial growth. These results are checked by plating the contents of the last tube in a series showing color change and the first tube remaining unaffected.

Technique Employed in Mouse Protection Tests

A. *Passive Immunization with Antipneumococcus Rabbit Serum.*—Equal volumes of 6 hour 0.1 per cent dextrose infusion broth culture, diluted 1:20 in broth, and the various dilutions of antiserum are well mixed and injected intraperitoneally into mice in a total volume of 0.4 cc. Three animals were usually employed for each dilution of the serum tested. Mice serving as controls for virulence received 0.5 cc. of the culture, diluted to 1:10,000,000 and 1:10,000,000 in infusion broth. 0.1 cc. of these dilutions was plated to indicate the number of pneumococci injected. The animals were considered to have been protected if they survived 8 days.

B. *Active Immunization with the A Carbohydrate.*—The A carbohydrate and its derivative after treatment with alkali, were diluted in saline. 0.5 cc. of the saline dilutions was injected intraperitoneally in mice. After varying intervals the presence of active immunity was determined by injecting 0.5 cc. of 6 hour 0.1 per cent dextrose infusion broth culture of pneumococcus, suitably diluted in sterile broth. The animals were observed for a period of 8 days after injection.

Technique Employed in Absorbing Antisera with the A Carbohydrate, Its Deacetylated Derivative and with Pneumococcus Boiled on the Acid and Alkaline Sides of Neutrality.—Antibody precipitation with the A carbohydrate, or its derivative after boiling in alkali, was removed from Pneumococcus Type I antiserum by repeated addition of these antigens and removal of the resulting precipitate. Preliminary titrations were done to determine the quantity of the carbohydrate which would remove the maximum amount of antibody from a definite volume of antiserum. This is most conveniently determined by placing 2 drops of antiserum in each of a series of small tubes used for precipitin ring tests. One drop of the carbohydrate in serial dilutions, ranging from 1:200 to 1:1,000, is added to each tube. The contents of the tubes are well mixed and allowed to stand at room temperature for 2 hours. The precipitates are thrown down in the centrifuge and on the supernatant fluid is layered the carbohydrate, diluted 1:2,000.

The original mixture of highest dilution of carbohydrate and antiserum which fails to give a positive ring test is taken to indicate optimum proportion of antigen and antiserum. On this basis, the amount of carbohydrate required to remove the antibody from any desired volume of antiserum is calculated and added in

the smallest possible volume of diluent. After standing at 37°C. for 1 hour and overnight in the ice box, the precipitate is removed by centrifuging and the supernatant tested with the carbohydrate diluted 1:200 and 1:2,000. If any precipitate is observed the absorption is repeated according to the procedure described above.

18 hour 0.1 per cent dextrose infusion broth cultures of Type I pneumococcus were centrifuged and the deposit resuspended in a volume of saline equivalent to that of the broth removed. The saline suspension was divided into two equal portions. To one, sufficient acetic acid was added to give a final concentration of N/100. To the other, sodium hydroxide was added to give the same concentration of alkali. The suspensions were then allowed to stand in the Arnold sterilizer for an hour at 100°C. After neutralizing they were centrifuged. Absorption of Pneumococcus Type I antiserum, diluted 1:4, was twice carried out with the deposit from 100 cc. of the saline suspension. The mixture of serum and organisms stood for 1 hour at 37°C. following each absorption.

EXPERIMENTAL

A. The Effect of the A Carbohydrate on the Phagocytosis of Type I Pneumococcus in Normal Human Blood

In this experiment, the results of which are presented in Table I, the effect of the A carbohydrate and its derivatives on the phagocytic power of normal human defibrinated blood was determined. It was found that the A carbohydrate in a concentration of 1:2,000 practically eliminated the opsonic capacity of the blood, whereas the substance after boiling in N/10 and N/50 sodium hydroxide, or the soluble specific substance (SSS I) prepared according to the procedure of Heidelberger and his associates in the same concentration, reduced the count only about two-thirds. It will also be noted that, in the case of the A carbohydrate, there is a definite reduction in the count in a concentration of 1:512,000 while the action of its derivatives is not definite in concentrations above 1:8,000. By this method of comparative testing, it appears that the A carbohydrate is at least 64 times as effective in removing opsonins from normal serum as the deacetylated polysaccharides. The effect of the A carbohydrate is type-specific since it has no action on the opsonic titre of the same blood in the presence of Type II pneumococcus. These results possess an additional interest in that they elucidate the finding of Ward and Enders (6) concerning the failure of SSS I to remove completely the phagocytic power of normal human blood against Pneumococcus

Type I, even when that substance was used in as high a concentration as 1:400.

TABLE I

Type of pneumococcus	Carbohydrate	Concentration of carbohydrate*	No. of organisms phagocyted by 50 cells	Percentage of cells taking part
I	0	0	279	98
	A	1:2,000	12	20
		1:8,000	52	46
		1:32,000	85	62
		1:128,000	63	58
		1:512,000	120	76
		1:2,048,000	259	96
	A boiled N/50 NaOH†	1:2,000	112	84
		1:8,000	169	90
		1:32,000	290	100
		1:128,000	258	98
		1:512,000	302	100
		1:2,048,000	295	100
	SSS I	1:2,000	132	90
		1:8,000	182	90
		1:32,000	218	92
		1:128,000	223	96
		1:512,000	306	98
		1:2,048,000	298	100
II	0	0	616	100
	A	1:2,000	790	100

* Concentration of carbohydrate present in the phagocytic system.

† Titration of A boiled in N/10 NaOH gave counts not significantly different from those for A boiled in N/50 NaOH.

B. The Effect of the A Carbohydrate on the Opsonin in Anti-Type I Pneumococcus Serum

Anti-Type I pneumococcus serum, absorbed with the A carbohydrate and with that substance boiled in N/50 sodium hydroxide, was added in suitable dilutions to normal defibrinated human blood which,

by itself, exhibited only a feeble phagocytic action against Type I pneumococcus. The results are summarized in Table II. In the same experiment these absorbed sera were titrated for opsonic power in a system containing the exudative leucocytes and defibrinated blood of a normal rabbit prepared according to the method described for preparing this system for use in bactericidal tests. The results were entirely comparable to those recorded in Table II. From the data recorded therein, it was calculated that the immune opsonin was reduced in the serum absorbed with A carbohydrate to about 1.6 per cent of its original titre. The deacetylated polysaccharide was less effective in removing the opsonin of the serum, since after absorption from 6.3 to 26 per cent of the antibody remained. This difference in the percentages of residual antibody after absorption with deacetylated polysaccharide depends upon whether the counts denoting the end-point of the action of the immune serum are taken as a basis for calculation or those which indicate no diminution in the maximum opsonic effect, as compared with the count obtained with the unabsorbed antiserum. It is necessary to point out here that the antibody, although greatly reduced by absorption with the A carbohydrate, was not completely removed. This fact, we believe, need not be explained by assuming the presence in the serum of any qualitatively distinct antibody, but most probably may be attributed to the conditions upon which the reaction between antibody and the antigen in solution depend. In favor of this view is the fact that suspensions of pneumococcus, boiled in N/100 acetic acid, have been found capable of removing even more opsonic antibody than the A carbohydrate. That this substance is the antigen involved, but obviously in a somewhat different physical state, is indicated by the fact that absorption of the serum with organisms boiled in N/100 sodium hydroxide only slightly reduces its opsonic titre. Such absorbed serum fails to precipitate with the deacetylated polysaccharide, but reacts to titre with the A carbohydrate.

On the basis of these experiments, it is apparent that the A carbohydrate is decidedly more effective in reducing both the normal and immune opsonin involved in the phagocytosis of Type I pneumococcus.

TABLE II

	Anti-Type I pneumococcus serum	Dilution of antiserum	No. organisms phagocyted by 50 cells	Percentage of cells taking part
	0	—	66	50
	Unabsorbed	1:256	502	100
		1:1,024	396	100
		1:4,096	162	96
		1:16,384	100	74
Normal human defibri-nated blood + sus-pension of Type I pneumococcus	Absorbed with A	1:1	460	96
		1:4	482	100
		1:16	333	98
		1:64	161	88
		1:256	80	62
	Absorbed with A boiled in N/50 NaOH	1:4	506	100
		1:16	770	100
		1:64	524	100
		1:256	216	94
		1:1,024	97	76
		1:4,096	69	60

C. The Effect of the A Carbohydrate on the Bactericidal Action of Normal Human Blood

Employing the defibrinated blood of the same normal individual which formed the base for the opsonic experiment, the antibactericidal action of the A carbohydrate was compared with that of SSS I, prepared according to the method of Heidelberger, Goebel and Avery. The results recorded in Table III parallel those obtained using the opsonic technique. It is evident that the bactericidal action of blood is significantly depressed by as small a concentration of the A carbohydrate as 1:512,000. The concentration of SSS I required to produce the same inhibition of bactericidal capacity is 1:32,000 which represents at least a sixteenfold decrease in activity. It will be noted that the A carbohydrate does not significantly influence the capacity of the same blood to kill Type II pneumococcus. Essentially similar findings have been recorded in a comparison of the antibactericidal effect of the A carbohydrate and that substance after boiling in N/10 sodium hydroxide.

TABLE III

Type of pneumococcus	Carbohydrate	Concentration of carbohydrate	Approximate No. pneumococci killed by 0.25 cc. blood
I	0	—	40,000
	SSS I	1:2,000	40
		1:8,000	400
		1:32,000	400
		1:128,000	40,000
	A	1:2,000	0
		1:8,000	4
		1:32,000	4
		1:128,000	40
		1:512,000	400
II	0	—	400,000
	A	1:250	40,000

D. The Effect of the A Carbohydrate on the Bactericidal Action of Anti-Type I Pneumococcus Rabbit Serum

The determination of the effect of the A carbohydrate on the bactericidal action of anti-Type I pneumococcus rabbit serum was carried out in two different ways. In one, a suitable dilution of the antiserum was chosen and held constant while the quantity of A carbohydrate or its deacetylated derivative was varied. In the other type of experiment, the antiserum was absorbed with the A carbohydrate or its derivative and serial dilutions of these absorbed sera were tested for bactericidal power. In both cases a mixture of exudative leucocytes and defibrinated blood of the normal rabbit was used as the basis of the system. Both methods of testing showed that the A carbohydrate was several times more efficient in impairing the ability of the antiserum to promote the destruction of the pneumococcus in the presence of fresh normal serum and polymorphonuclear leucocytes. It is, however, to be emphasized that by the method of absorption it was found that in every case a not inconsiderable amount of the antibody involved in the destruction of the organism remained. This failure to absorb completely all antibody active in the bactericidal

TABLE IV

Experiment I

Effect of Carbohydrates on Constant Dilution of Anti-Type I Pneumococcus Rabbit Serum

Concentration of antiserum	Carbohydrate	Concentration of carbohydrate	Approximate No. pneumococci killed
—	0	—	0
1:2,000	0	—	70,000
	A	1:2,700	0
		1:8,000	7
		1:16,000	700
		1:32,000	700
		1:64,000	7,000
		1:128,000	70,000
	A boiled in N/50 NaOH	1:2,700	7,000
		1:8,000	70,000

Experiment II

Bactericidal Action of Anti-Type I Pneumococcus Rabbit Serum Absorbed with Carbohydrates

Antiserum absorbed with carbohydrate	Concentration of antiserum	Approximate No. pneumococci killed
0	—	0
Unabsorbed	1:8,000	7,000
	1:32,000	700
	1:128,000	0
A	1:32	70,000
	1:128	700
	1:500	70
	1:2,000	0
A boiled in N/50 NaOH	1:500	70,000
	1:2,000	70
	1:8,000	7
	1:32,000	0

system was expected from the results obtained when similarly absorbed serum was tested for residual opsonin and, we are inclined to believe, is due to the same cause that we have previously postulated. In discussing these experiments, it is important to note the fact that it proved impossible—using the constant dilutions of antiserum 1:250 and 1:500—to show any significant effect of the deacetylated carbohydrate in the highest concentration used (*i.e.*, 1:2,700) on the bactericidal property of these quantities of antiserum which, however, were definitely impaired by dilution of the A carbohydrate in concentrations ranging from 1:16,000 and 1:32,000. In Table IV the results of two experiments among several carried out are summarized. They support the observations already described in showing that the A carbohydrate is about sixteenfold more active in removing antibody than is the deacetylated polysaccharide. Nevertheless, it requires approximately equal concentrations of antiserum and A carbohydrate before complete inhibition of the bactericidal action is obtained. The type specificity of the action of the highest concentration of A carbohydrate was assured by adding it to Type III pneumococcus, together with diluted anti-Type III pneumococcus serum. It exerted no antibactericidal effect in this system.

E. The Effect on the Mouse-Protective Action of Anti-Type I Pneumococcus Rabbit Serum Absorbed with the A Carbohydrate

Although repeated efforts were made to show that the A carbohydrate would remove all protective action for mice from anti-Type I pneumococcus rabbit serum, none were completely successful. From the data recorded in Table V, it is clear that absorption with the A carbohydrate markedly reduces the protective titre of the antiserum. It is difficult, because of the small number of animals involved, to assess precisely the extent of this reduction, but if one selects the highest dilution of serum which protects three out of three mice as a basis for calculation, it will be found that absorption with A has lowered the titre to 1.5 per cent of its original value, compared with a reduction to 6.2 per cent after absorption with the deacetylated compound. These figures indicate that the A carbohydrate is about four times more effective in removing the protective titre of the antiserum. Experiments of the same type in which the A carbohydrate used for

absorption was boiled in N/10 NaOH yield comparable results. Thus, the protection test carried out with serum absorbed with A and its derivatives shows less difference in activity between them than is revealed either by the bactericidal or opsonic techniques. However, a greater discrepancy in absorptive capacity is found if portions of the serum previously saturated with Type I pneumococcus boiled in N/100

TABLE V

Effect on the Mouse-Protective Titre of Anti-Type I Pneumococcus Rabbit Serum Absorbed with the A Carbohydrate

Dilution of antiserum....................	1:4	1:16	1:64	1:256	1:1,024
Unabsorbed serum	Not done	Not done	Not done	S S S	D D D
Serum absorbed with A	S S S	S S D	S D D	D D D	Not done
Serum absorbed with A boiled in N/50 NaOH	S S S	S S S	S S D	S D D	D D D
Serum absorbed with Pneumo-coccus I boiled in N/100 HAc	S S D	S S D	D D D	Not done	Not done
Serum absorbed with Pneumo-coccus I boiled in N/100 NaOH	S S S	S S S	S S S	S S S	S D D

Three mice received the same dilution of antiserum.

D = died. S = survived.

acetic acid and N/100 sodium hydroxide are tested for their protective titre in mice. The results of an experiment of this kind are included in Table V. It will be seen that absorption with pneumococci boiled in alkali does not definitely affect the protective action of the serum, although this treatment had removed all precipitating antibody reacting with the deacetylated carbohydrate. In contrast, absorption with organisms boiled in N/100 acetic acid removes a large portion of the

protective action. There can be little doubt that it is the A carbohydrate present in the pneumococcus upon which the removal of the antibody depends.

This failure to eliminate completely the mouse-protective action of an antiserum by addition of the A carbohydrate is not in entire agreement with the findings of Avery and Goebel (4). In addition to the possibility which has already been discussed concerning the physical state of the antigen, the discrepancy may be either in the fact that these authors did not test serum less dilute than 1:10, or that they employed antipneumococcus horse serum, while we used antipneumococcus rabbit serum. Powell and his coworkers (9) have shown that heterophile antibody of Forssman, together with very small quantities of specific pneumococcus antibody, greatly increases the therapeutic properties of the serum in rabbits. Since the Forssman antibody may be present in antipneumococcus rabbit serum, it is possible that its presence in the antiserum employed might account for this difference in results.

F. The Antigenic Action of the A Carbohydrate

It has been found that when comparatively large amounts of the A carbohydrate are injected into mice no immunity against virulent Type I pneumococcus develops. If the quantity of carbohydrate is decreased, definite, though limited resistance to infection develops. Boiling the A carbohydrate in N/10 sodium hydroxide removes completely its capacity to function as a complete antigen. Similar treatment in N/50 sodium hydroxide appears to only partially destroy the antigenic property of the substance. Briefly summarized, the experimental findings were as follows: Nine mice were vaccinated by three intraperitoneal injections of 0.00025 mg. of the A carbohydrate, administered every 3rd day, and infected with 0.5 cc. doses of Pneumococcus Type I, increasing by tenfold from 1:1,000,000 to 1:10,000 dilution of broth culture, on the 6th day after the last dose of the carbohydrate. Two only, of the nine mice, died with pneumococci in the heart's blood. These animals had received the largest number of organisms. Out of four mice vaccinated in the same manner, with equivalent quantities of A boiled in N/50 sodium hydroxide, two survived. Of eight animals vaccinated with A boiled in N/10 sodium

hydroxide and infected with doses of pneumococci ranging from 1:100,000,000 to 1:100,000, all died.

Quantities of A as large as 8 mg. given at 3 day intervals, in doses of 2 mg. each, failed to confer any protection on the 11th day after the last injection. It was thought that if a longer period was allowed to elapse, subsequent to the course of immunization with such large doses, before the animals were infected, immunity might develop. Accordingly, an interval of 6 weeks was allowed before infecting two mice, one of which had received 1 mg. and the other 2 mg. of the A carbohydrate. No indication of resistance was observed. Additional confirmation of the fact that large doses of the A carbohydrate fail to immunize is given in Table IV.

It was believed unnecessary to present these experiments in greater detail, since they fully confirm those of Avery and Goebel (4).

We have noted that extremely small quantities of the A carbo-hydrate may actively immunize mice: 0.5 cc. of 1:10,000,000 dilution of the material (*i.e.*, 0.00005 mg.), administered intraperitoneally, at a single dose, will protect a certain percentage of the animals under test, against at least 10 M. L. D.'s of virulent pneumococci. Increasing the immunizing dose tenfold appears to improve slightly the immunity obtained. This dose of 0.0005 mg. proved to be about the optimum, since a further increase to 0.005 mg. is definitely less effective. Larger amounts of the A carbohydrate produce practically no resistance to infection. There is a suggestion that with a dose of 0.005 mg. some immunity may develop within 24 hours. After 3 days 50 per cent of the mice vaccinated with 0.00005 and 0.0005 mg. showed resistance. Maximum immunity exists from 6 to 25 days after vaccination, but apparently is in a state of defervescence by the 49th day. These statements are based on the experimental results included in Table VI. The manner of procedure was as follows: 60 normal mice were divided into five lots. The twelve mice in each lot were injected intraperi-toneally with the same dose of A carbohydrate. The quantities received by the various lots of mice ranged from 0.00005 mg. to 0.5 mg. At varying intervals after vaccination, two mice were selected from each lot and injected intraperitoneally with the same dose of Type I pneumococci, which was equivalent to at least 10 M. L. D. In every case the heart's blood of the mice which died was cultured.

TABLE VI

Lot No. of mice	Amount of A used for vaccination	Two mice from each lot injected intraperitoneally with 0.5 cc. culture Pneumococcus I diluted 1:1,000,000					
		Days after vaccination on which mice were infected					
		1	3	6	12	25	49
	mg.						
1	0.00005	D 24	D 40	D 98 HB—	D 60	S	D 36
		D 48	S	S	S	S	D 60
2	0.0005	D 48	D 40	D 48	S	D 144	D 36
		S	D 70 HB—	S	S	S	S
3	0.005	D 48	D 40	D 48	D 60	D 60	D 36
		D 60	D 48	S	S	S	S
4	0.05	D 48	D 40	D 48	D 36	S	D 36
		D 48	D 48	D 60	D 36	D 36	D 36
5	0.5	D 24	D 40	D 24	D 36	D 36	D 44
		D 48	D 36	D 48	D 36	D 36	D 44
Normal mice injected intraperitoneally with 0.5 cc. 1:10,000,000 culture Pneumococcus I		D 48	D 36	D 48	D 36	D 60	D 60

D = died; numerals following indicate number of hours after infection in which death occurred.   S = survived.   HB— = heart's blood culture negative.   In all cases not indicated, culture of heart's blood was positive for pneumococcus.

G. Elimination of Immunity Induced by Vaccination with the A Carbohydrate

It became of interest to ascertain whether the immunity resulting from vaccination with the A carbohydrate could be annulled by injecting that substance just prior to infection with the pneumococcus. Five mice were given three injections of 0.00025 mg. intraperitoneally, every 3rd day.   9 days after the last injection, three of the mice were injected intraperitoneally with 0.5 mg. of the A carbohydrate.   After an interval of $4\frac{1}{2}$ hours, these animals, together with the two which had been vaccinated but had received no immediate preliminary

treatment with A, were infected with 0.5 cc. of Pneumococcus Type I culture, diluted 1:1,000,000. A normal mouse was injected at the same time with 0.5 cc. of a 1:10,000,000 dilution of the same culture. The three mice which received 0.5 mg. of A before infection all died within 3 days, together with the normal control. Of the two vaccinated mice which had not received 0.5 mg. of A before infection, one survived 6 days and the other was killed after 7 days. Preliminary injection, then, of a comparatively large quantity of the A substance shortly before infection appears to remove the protective mechanism developed in response to small doses of the same material previously administered.

H. Passive Transfer of Immunity Developed in Response to Injection of the A Carbohydrate

The question arose as to whether demonstrable antibody appeared in response to vaccination with the A carbohydrate in the serum of the mouse. Eight mice were immunized at the same time, and according to the procedure in the preparation of animals used in the previous experiment. 10 days after the last immunizing dose of the A carbohydrate was given, the mice were bled from the heart under ether anesthesia, employing a tuberculin syringe and a 27 gauge hypodermic needle. The blood was pooled and allowed to clot. About 4 cc. of serum were obtained. Four normal mice were each injected intravenously with 0.75 cc. of this pooled serum. At the same time, two normal mice were injected, by the same route, with 0.75 cc. of the pooled serum of four normal mice. After an interval of 20 hours, these six animals were infected intraperitoneally with 0.5 cc. of Type I pneumococcus culture, diluted 1:1,000,000. A normal mouse was injected with 0.5 cc. of 1:10,000,000 dilution of the same culture. The results which are presented in Table VII show clearly that the serum of mice immunized with the A carbohydrate contains substances protective against Type I pneumococcus.

With the remainder of the pooled serum obtained from the mice vaccinated with the A carbohydrate, tests for the presence of agglutinins for Pneumococcus Type I and precipitins for the A carbohydrate were carried out. In both cases the results, entirely negative, indicated that the amount of antibody present was very small.

TABLE VII

Passive Transfer of Protective Substance in Serum of Mice Vaccinated with the A Carbohydrate

Mouse	Pooled serum A-vaccinated mice	Pooled serum normal mice	Dilution of infecting dose Pneumococcus I (0.5 cc.)	Result
	cc.	*cc.*		
1	0.75	0	1:10,000,000	S
2	0.75	0	1:1,000,000	S
3	0.75	0	1:1,000,000	S
4	0.75	0	1:1,000,000	S
5	0	0.75	1:1,000,000	D
6	0	0.75	1:1,000,000	D
7	0	0	1:10,000,000	D

Serum administered intravenously, pneumococcus culture intraperitoneally 20 hours after serum was given.

DISCUSSION

In general the experimental evidence presented shows that the A carbohydrate, which, on the basis of chemical analysis and biological action, is to be identified with the acetyl polysaccharide of Avery and Goebel, is distinctly more effective than the deacetylated derivative in reducing the efficiency of the several immunological systems which have been studied. The A carbohydrate, then, closely approaches, if indeed it be not identical with the substance developed by Type I pneumococcus which enables the organisms to resist the defensive mechanisms of the host, in so far as these are type-specific.

Capable, like the organism itself, of inducing resistance to subsequent infection, as Avery and Goebel have shown for the acetyl polysaccharide, the A carbohydrate, introduced parenterally into mice, arouses a state of active immunity which may appear as early as the 3rd day after vaccination. This immunity, closely correlated with the quantity of carbohydrate employed—since doses of over 0.005 mg. fail to protect—is not of great magnitude nor of marked duration. It may be completely eliminated by introducing into the body of the vaccinated mouse an appropriate amount of A carbohydrate shortly before infection. That such resistance is dependent upon substances which may appear in the serum has been demonstrated by the passive transfer of the immune state to normal mice from animals vaccinated with the A carbohydrate.

Perhaps the chief interest and value of these experiments with the A carbohydrate and those of Goebel and Avery with the acetyl polysaccharide lie in their application to our ideas on the nature of antigens. It is extremely improbable that the acetylated carbohydrate contains even a minute quantity of protein, yet it functions as a complete antigen, in that it not only actively immunizes the animal body against subsequent invasion by the homologous organism, but also gives rise to demonstrable protective substances in the serum. Tentatively, it would seem that the conception of all complete antigens as being protein in nature should be modified.

There is a question which has been in our minds during the course of this work and which still remains unanswered: Is there one antibody which reacts in a quantitatively different manner with the A carbohydrate and the deacetylated compound, or are there two antibodies corresponding to the two forms of the antigen? Against the acceptance of the first alternative is the fact that a serum containing a high titre of precipitin reacting with the A carbohydrate may fail entirely to precipitate with the deacetylated material. On the other hand, there is ample evidence to show that the introduction into an antigen of chemical groupings, no more complex than the acetyl group, may definitely change the specificity. However, if two antibodies are postulated, it would be necessary, in view of experimental fact, to assume that the A carbohydrate could unite with both, whereas the deacetylated carbohydrate could react with only that antibody specifically produced in response to its antigenic stimulus.

In this connection, the experiments of Avery, Goebel and Babers (10) on the serology of artificially prepared antigens containing substituted glucosides present an interesting analogy to the behavior of the A carbohydrate and the deacetylated substance. These authors showed that an antiserum prepared by injecting p-aminophenol α-glucoside linked to globulin was precipitated by both p-aminophenol α-glucoside-globulin and p-aminophenol β-glucoside-globulin. If p-aminophenol α-glucoside was added to this antiserum, precipitation was inhibited in the presence of both α- and β- glucosides linked to globulin. However, addition of β-glucoside inhibited precipitation only against p-aminophenol β-glucoside linked to globulin. Since in this case apparently only one antibody is concerned, their findings

indicate that the difference in reaction capacity of the A carbohydrate and the deacetylated product could be explained on the assumption that only a single antibody was involved.

SUMMARY

1. The A carbohydrate isolated from Type I pneumococcus by Pappenheimer and Enders, on the basis of elementary analysis, the presence of the acetyl group and its immunological properties, appears to be identical with the acetyl polysaccharide described by Avery and Goebel.

2. The A carbohydrate possesses a greater anti-opsonic action than either the deacetylated substance obtained by boiling in alkali or the soluble specific substance of Type I pneumococcus prepared according to the procedure of Heidelberger, Goebel and Avery. The opsonic titre of normal human serum is practically eliminated upon the addition of the A carbohydrate—an effect not observed with equivalent amounts of either the deacetylated material or the specific soluble substance. In immune serum, the A carbohydrate brings about a quantitatively greater reduction in opsonic activity than its derivatives, but it has not been possible to demonstrate complete inhibition of phagocytic action by the method of absorption of antibody.

3. In a system of normal human serum and leucocytes capable of destroying Type I pneumococcus, the bactericidal effect may be entirely removed upon the addition of the A carbohydrate. It proved impossible to inactivate the bactericidal action with the deacetylated substance in equivalent proportions. In this system, the A carbohydrate was about 64 times more effective as an antibactericidal agent than the deacetylated compound. Essentially similar results were obtained in a study of the antibactericidal properties of the A carbohydrate and the deacetylated derivative in the presence of anti-Type I pneumococcus rabbit serum added to a mixture of exudative leucocytes and the defibrinated blood of the rabbit.

4. The mouse-protective titre of anti-Type I pneumococcus rabbit serum is lowered to a greater degree after absorption with the A carbohydrate than it is by similar treatment with the deacetylated compound. Absorption with the A carbohydrate does not, however, completely remove the protective antibody.

5. As Avery and Goebel have shown in the case of the acetyl polysaccharide, so the A carbohydrate, when administered in very small quantities, protects mice against an otherwise fatal dose of Type I pneumococcus. Active immunity in mice has been obtained with as little as 0.00005 mg. of the A carbohydrate administered in a single dose. Doses larger than 0.005 mg. confer no protection on these animals. Deacetylization of the A carbohydrate after boiling in N/10 sodium hydroxide destroys its protective capacity while similar treatment in N/50 alkali does not completely remove its immunizing property. Active immunity may arise within 3 days following a single injection of the A substance. It appears to be at its height from 6 to 25 days thereafter, and is retrogressive by the 49th day following vaccination. Injection of the A carbohydrate into immunized mice immediately before infection deletes the state of resistance.

6. The immunity actively induced as a result of injection of the A carbohydrate may be passively transferred to normal mice with the serum of vaccinated animals.

7. Since the evidence obtained in the course of this study indicates that the A carbohydrate of Type I pneumococcus and the acetyl polysaccharide of Avery and Goebel represent the same chemical substance, it is suggested that the designation "A carbohydrate" or "A substance" be relinquished in favor of the more exactly descriptive term "acetyl polysaccharide."

BIBLIOGRAPHY

1. Enders, J. F., *J. Exp. Med.*, 1930, **52**, 235.
2. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 727.
3. Pappenheimer, A. W., and Enders, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 37.
4. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1933, **58**, 731.
5. Friedrich, A., and Rapoport, S., *Biochem. Z.*, 1932, **251**, 432.
6. Ward, H. K., and Enders, J. F., *J. Exp. Med.*, 1933, **57**, 527.
7. Ward, H. K., *J. Exp. Med.*, 1930, **51**, 675.
8. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, **39**, 219.
9. Powell, H. M., Jamieson, W. A., Bailey, H. G., and Hyde, R. R., *Am. J. Hyg.*, 1933, **27**, 102.
10. Avery, O. T., Goebel, W. F., and Babers, F. H., *J. Exp. Med.*, 1932, **55**, 759.