CHEMOIMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

I. The Isolation and Properties of the Acetyl Polysaccharide of Pneumococcus Type I

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During the past several years it has become increasingly evident that Pneumococcus Type I possesses type-specific immunological properties not completely accounted for by those of the capsular polysaccharide in the form in which it was first chemically isolated and identified in this laboratory as the soluble specific substance (1–3). This fact was foreshadowed in the earlier studies of Perlzweig and his coworkers (4, 5), and has since been amply demonstrated by a number of European and American investigators, notably by Schiemann and his collaborators (6, 7) abroad, and by Enders (8, 9), Sabin (10), Wadsworth and Brown (11, 12), Ward (13) and Felton (14) in this country.

In view of this evidence, a further study has been made of the chemical and immunological properties of the soluble specific substance of Pneumococcus Type I, with the hope of acquiring a fuller knowledge of the nature of the relationship existing between the specifically reacting derivatives studied by other investigators and the type-specific polysaccharide previously described in this laboratory. The results of this study form the subject matter of the present report.

Evidence will be presented that the soluble specific substance has now been isolated in a chemical form more closely approximating that in which it exists as a natural constituent of the cell capsule. It will be shown that the type-specific carbohydrate present in the intact bacterial cells, and in filtrates of autolyzed broth cultures has been chemically identified as an acetyl polysaccharide. This naturally occurring acetyl polysaccharide differs chemically from the spe-

cific carbohydrate as originally isolated, principally in respect to the presence of acetyl groups which, as will be pointed out, endow the native substance with additional specific properties not possessed by the polysaccharide after removal of these labile groups by alkaline hydrolysis.

Owing to the marked instability of the acetyl groups and the ease with which they are removed by treatment with alkali, the soluble specific substance as originally isolated will be shown to represent the deacetylated polysaccharide which, although still retaining the dominant type specificity of the native substance, has through the loss of its acetyl groups suffered a corresponding loss of certain specific properties possessed only by the acetyl polysaccharide itself. Thus, specific differences between the properties of the cell fractions studied by other investigators and those of the soluble specific substance as originally defined, now appear to be due to the presence or absence of acetyl groups in the polysaccharide molecule. Indeed, so distinctive are the immunological reactions of the acetyl polysaccharide and those of its deacetylated derivative, that it is now possible to clarify many of the apparently conflicting views still current concerning the nature and properties of the specific carbohydrate of Pneumococcus Type I.

I. Isolation of the Acetyl Polysaccharide of Pneumococcus Type I

Methods for the isolation of the acetyl polysaccharide differ from those previously described for isolation of the soluble specific substance, principally in the avoidance of the use of an excess of alkali in the chemical procedures employed. The following methods have been used in the recovery and purification of the acetyl polysaccharide from the bacterial cells and concentrates of autolyzed cultures.

1. Bacterial Cells.—The bacterial cells from 18 hour broth cultures of Pneumococcus Type I, grown in 3 liter lots, were collected by centrifugation. The unheated cells from each lot were taken up in 50 cc. of sterile saline and stored in the ice box until the bacteria from 50 liters had been collected. The suspension of partially autolyzed cells was then treated with 15 cc. of N/1 acetic acid and heated in an Arnold sterilizer for 30 minutes. The precipitate of coagulated protein and bacterial debris was removed by centrifugation, and washed several times. The washings together with the original supernatant were concentrated to 150 cc. *in vacuo*. 150 cc. of alcohol were added to the concentrated liquid. The copious precipitate, containing the specific polysaccharide, was separated

by centrifugation, and the alcoholic supernatant liquid was discarded. The carbohydrate was dissolved in 75 cc. of water, 1 cc. of N/1 acetic acid was added and the small amount of insoluble precipitate which formed was separated and discarded. The polysaccharide was precipitated from the solution by the addition of an equal volume of alcohol, the precipitate was recovered and again dissolved in 50 cc. of water. The solution of polysaccharide at this point was clear and colorless. It gave none of the usual protein tests. The solution was acidified by the addition of 2 cc. of 5 N HCl and dialyzed until no chlorine ion was detectable in the dialysate. At this point, a small amount of degraded (deacetylated) polysaccharide which had separated from the solution was removed by centrifugation. The viscous, clear and colorless solution of the specific acetyl polysaccharide was precipitated by pouring it into 10 volumes of acidulated acetone. The polysaccharide was separated by filtration on a porous, sintered glass funnel, and was washed with absolute alcohol and ether. 0.5 gm. of dry substance was recovered.

2. *Autolyzed Broth Cultures.*—50 liters of 0.5 per cent dextrose broth were seeded with an actively growing culture of Pneumococcus Type I, and incubated at 37°C. for 5 days during which time the bacterial cells had undergone marked autolysis. The reaction of the culture fluid after growth and autolysis had occurred was distinctly acid. The 50 liters of autolyzed broth culture were autoclaved and concentrated to one-tenth of the original volume in a steam kettle. The concentrated material was cooled to 0° and neutralized, while stirring, with solid sodium bicarbonate, 6 liters of alcohol were added to the concentrate and after standing for 18 hours at room temperature, the clear, dark supernatant liquid was syphoned off. After removal of the excess fluid by centrifugation, the precipitate was dissolved as completely as possible in 800 cc. of water. The deep brown and turbid aqueous solution containing the pneumococcus polysaccharide was clarified by centrifugation at high speed. The precipitated bacterial debris and coagulated protein was next washed with slightly acidulated water, until the washings gave only a faint Molisch test. Four to five washings were found necessary. To the combined solution of polysaccharide and wash waters, now at a volume of about 1 to 1.2 liters, were added 5 cc. of N/1 acetic acid and 50 gm. of sodium acetate. A slight precipitate separated which was removed by centrifugation and discarded. 1.2 volumes of alcohol were now added to the supernatant and the precipitate was recovered by centrifugation. The precipitate was dissolved in about 250 cc. of water and 25 gm. of solid trichloracetic acid were dissolved directly in the turbid solution. After standing 2 hours at 0°, a heavy deposit of coagulated protein precipitated from solution and was separated by centrifugation. The clear, pale yellow and strongly acid supernatant liquid, containing most of the polysaccharide, was placed in the ice box. The precipitate of coagulated protein, still containing a considerable amount of adsorbed specific polysaccharide, was suspended in 100 cc. of water, cooled to 0° and brought as completely as possible into solution by the *cautious* addition of ice-cold 2 N NaOH. The solution was not allowed to become definitely alkaline at any time. A solu-

tion of 50 per cent trichloracetic acid was then carefully added until the point of maximum precipitation was reached. After standing 10 minutes, the coagulated protein was centrifuged and the supernatant liquid was added to that in the ice box. Solution and reprecipitation of the coagulated protein were repeated until the supernatant fluid gave only a faint or a negative Molisch test. A total of two or three reprecipitations sufficed. To the combined supernatant liquids containing the specific polysaccharide in a volume of about 500 cc., 20 gm. of sodium acetate were added and the solution was cooled to 0°. On the addition of 1.2 volumes of alcohol to the chilled solution, the polysaccharide precipitated as a white flocculent mass. The substance was collected in the usual way and dissolved in 100 cc. of water. After adjusting the reaction to approximately pH 4 and allowing the solution to stand for several hours, a small amount of insoluble residue was separated by centrifugation. 5 gm. of sodium acetate were added and the polysaccharide was precipitated by adding an equal volume of cold alcohol. The precipitate was separated, and dissolved in 75 cc. of water, yielding a clear and almost colorless solution which gave none of the usual protein tests. The material was made definitely acid to Congo red with HCl, and was dialyzed until no chlorine ions were detectable in the dialysate. A small quantity of deacetylated specific polysaccharide which had precipitated from the solution was separated by centrifugation. The viscous solution of acetyl polysaccharide was poured into 10 volumes of acidulated acetone. The precipitated carbohydrate was separated by filtration and washed with alcohol and ether. 2.1 gm. were recovered. The product thus obtained was nearly white in color. A 1 per cent solution gave no test for protein.

3. *Analytical Methods.*—The acetyl polysaccharide was analyzed for total nitrogen by a modification of the Pregl (16) method. Amino nitrogen was determined by the method of Van Slyke (17). Acetyl determinations were made on samples of 15 mg. by Pregl's method (18). The specific optical rotation was observed in a 2 dm. tube on aqueous solutions of known concentration. The acid equivalent of the specific acetyl polysaccharide was determined by titrating an aqueous solution of known concentration at 0° to pH 7 with N/50 NaOH. The acid equivalent of the deacetylated carbohydrate was determined by dissolving a weighed sample in a known quantity of N/10 HCl, then neutralizing the measured HCl with an equivalent quantity of N/10 NaOH, and finally titrating the finely suspended isoelectric polysaccharide to pH 7 with N/50 NaOH. Reducing sugars were determined by the Hagedorn-Jensen method (19) after hydrolyzing solutions of known concentration with 1.5 N HCl in sealed tubes at 100°.

II. Chemical Properties of the Acetyl Polysaccharide of Pneumococcus Type I

By the methods described above, in which treatment with alkali was purposely avoided, the soluble specific substance of Pneumococcus Type I has been isolated in the form of an ash-free acetyl polysaccha-

ride possessing marked acidic properties. The specific carbohydrate in this form is very soluble in water, and gives solutions of high viscosity. Aqueous solutions show a specific optical rotation of about +270°. The naturally acetylated polysaccharide contains 4.85 per cent of nitrogen, approximately one-half (45 per cent) of which is liberated in the amino form when the substance is treated with nitrous acid in the cold. It does not reduce Fehling's solution until after hydrolysis with dilute mineral acids. At the same time that reducing sugars appear in the solution, the serological specificity of the acetyl polysaccharide is destroyed. The behavior in this respect is identical with that of the deacetylated polysaccharide.

On the addition of hydrochloric acid-napthoresorcinol, both forms of the specific carbohydrate give a marked color reaction indicating the presence of uronic acids. In addition to being soluble in water, the acetyl polysaccharide is soluble in 80 per cent acetic acid. Aqueous solutions of 0.5 per cent concentration are precipitated by the following reagents: phosphotungstic acid, silver nitrate, neutral and basic lead acetate; and are incompletely precipitated by barium hydroxide. Unlike the deacetylated product, the acetyl polysaccharide is precipitated by tannic acid but not by uranyl nitrate. It gives no color reaction with iodine-potassium iodide solution. Weak solutions of potassium permanganate are not immediately decolorized by the acetyl polysaccharide. The biuret, ninhydrin, sulfosalicylic and picric acid tests are negative. No traces of phosphorus or sulfur were detectable in the most highly purified preparations of the specific acetyl polysaccharide.

The following experiments were carried out, in order to identify the organic acid liberated from the naturally acetylated polysaccharide by alkaline hydrolysis, and to determine whether the chemical properties of the carbohydrate after deacetylation are identical with those of the specific polysaccharide formerly obtained by methods involving the use of alkali in the process of isolation.

1. Conversion of the Acetyl Polysaccharide to Its Deacetylated Derivative by Alkaline Hydrolysis

0.0693 gm. of acetyl polysaccharide (Preparation 2, Table I) was dissolved in 7 cc. of water and neutralized to phenolphthalein with 1.42 cc. of N/10 NaOH. 7 cc. more of N/10 alkali were added and the mixture was heated in a boiling water

bath for 35 minutes. The reaction mixture was then neutralized and finally 1.5 cc. of N/10 HCl were added in excess. A precipitate appeared in the solution. The flask containing the mixture was placed in the ice chamber for 24 hours, after which time the precipitate was separated by centrifugation and washed several times with small quantities of ice water. 0.052 gm. of material was recovered. The substance was dried at 100° in high vacuum.

TABLE I

Analyses of the Acetyl Polysaccharide of Pneumococcus Type I

Preparation No.	Source	Acid equivalent	Specific rotation	Ash	C	H	Total nitrogen	Amino nitrogen	Acetyl	Phosphorus	Reducing sugars after hydrolysis	Highest dilution of polysaccharide reacting with antipneumococcus serum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	Bacterial cells	—	+270°	0.0	—	—	4.89	2.30	5.9	0.0	—	1:5,000,000*
2	" "	—	+265°	0.0	—	—	4.86	2.21	6.9	—	—	1:5,000,000*
3	Autolyzed broth cultures	576	+277°	0.0	42.55	6.58	4.85	2.22	6.0	0.0	32.0	1:5,000,000*
2 A, deacetylated	†	535	+297°	0.0	‡	‡	5.05	2.50	0.0	—	27.6	1:5,000,000§

* Type I antipneumococcus serum previously absorbed with Preparation 2 A (deacetylated).

† This sample of deacetylated polysaccharide was obtained by alkaline hydrolysis of Preparation 2. This material is identical with the carbohydrate formerly known as the soluble specific substance of Type I Pneumococcus.

‡ An analysis of carbon and hydrogen was made on a sample of deacetylated carbohydrate which had been reprecipitated five times at its isoelectric point. The material contained no ash, and had a carbon content of 40.33 per cent and a hydrogen content of 6.23 per cent.

§ Unabsorbed Type I antipneumococcus serum.

The analytical data presented in Table I show that the deacetylated product (Preparation 2 A) obtained by alkaline hydrolysis of the acetyl polysaccharide (Preparation 2) contains no acetyl groups[1] and is in all respects chemically identical with the polysaccharide which has hitherto been known as the soluble specific substance.

[1] Heidelberger and Kendall have previously found that the Type I polysaccharide (deacetylated) contains no acetyl groups. (See *J. Exp. Med.*, 1931, **53**, Table III on page 636.)

2. Identification of Acetic Acid in the Acetyl Polysaccharide of Pneumococcus Type I

0.80 gm. of Preparation 3 (Table I) was dissolved in 25 cc. of water and neutralized. 25 cc. of N/1 NaOH were added, and the solution was heated in a boiling water bath for 40 minutes. The mixture was cooled and 6 cc. of 5 N H_2SO_4 were added. The solution was placed in a Claissen flask and distilled *in vacuo*. The delivery tube of the Claissen flask was so bent that it extended to the bottom of a receiving flask. The latter contained a suspension of freshly prepared and carefully washed silver carbonate, and the entire flask was packed in ice. The contents of the Claissen flask was distilled nearly to dryness, then 50 cc. of water were added and the distillation repeated. This was done three times in all. The receiving flask was then disconnected, and the excess silver carbonate was removed by filtration. The filtrate from the silver carbonate, which contained the silver salt of a volatile organic acid, was concentrated to 20 cc. *in vacuo*. The solution was warmed slightly and filtered. The filtrate was cooled to 0°, and 50 cc. of neutral, freshly distilled ethyl alcohol were slowly added. A snow-white product crystallized promptly from the solution. In crystalline form this material was identical with an authentic sample of silver acetate. The unknown silver salt was filtered, washed with a little dilute alcohol and dried *in vacuo* at 56° to constant weight; 0.080 gm. was recovered. The product was analyzed for silver by the usual method. The silver was weighed as silver iodide.

Analysis: 0.0483 gm. substance gave 0.0677 gm. AgI.

CH_3COOAg	Calculated:	Ag 64.7 per cent.
	Found:	Ag 64.4 per cent.

From the above analysis it is seen that the volatile organic acid liberated from the polysaccharide by alkaline hydrolysis is acetic acid, bringing chemical proof that the specific carbohydrate exists as an acetyl polysaccharide. The exact mode of linkage of the acetic acid is not as yet definitely known. Judging from the ease with which the acetyl groups are removed by dilute alkali, even at room temperature, it seems certain that the nitrogenous groups of the polysaccharide are not acetylated. It appears more likely that in the native polysaccharide the acetic acid is bound directly to the hydroxyl groups attached to a carbon atom.

The possibility remains, of course, that in addition to the acetyl groups, the specific carbohydrate, in the state in which it exists as a natural constituent of the capsular substance, may also have other labile groups attached to it. However, there is certain evidence which lends support to the view that the only chemical difference between

the naturally acetylated and the artificially deacetylated polysaccharide lies in the presence of acetyl groups in the former substance. For it has been found that the acid equivalent of the acetyl polysaccharide is 576, while that of the deacetylated substance is 535, when each is titrated to pH 7 with N/50 NaOH. The difference in the observed values of the acid equivalent is 41, which is in close agreement with the theoretical value of 43, representing the difference required if the native polysaccharide contains one acetyl group per 576 of formular weight. The specific polysaccharide may therefore be regarded, tentatively at least, as an acetyl ester.

The chemical evidence thus far available indicates that the soluble specific substance of Pneumococcus Type I exists in its native state as an acetyl polysaccharide, and that the specific substance recovered by the earlier methods must now be regarded as the deacetylated derivative of the native carbohydrate from which the labile acetyl groups have been removed by treatment with alkali during the chemical manipulations incident to its isolation.

In the following experiments the immunological properties of the two forms of the specific polysaccharide and their serological relationships to each other will be considered in terms of these chemical findings.[2]

III. Immunological Properties of the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

The preceding experiments have revealed the chemical relationship existing between the naturally acetylated and artificially deacetylated form of the specific capsular polysaccharide of Pneumococcus Type I. In the following experiments these chemical differences are shown to be reflected in the immunological reactions of the two forms of the specific carbohydrate. The acetyl polysaccharide is found not only to fulfil all the immunological criteria of type specificity, but also to possess certain additional specific properties which the deacetylated polysaccharide lacks through the loss of the extremely labile acetyl groups.

[2] The authors wish to express their appreciation to Mr. Frank H. Babers for his kindly cooperation in much of the analytical work and to Dr. Michael Heidelberger and Dr. P. A. Levene for their interest and helpful suggestions.

1. Precipitation of the Acetyl and Deacetylated Polysaccharide in Absorbed and Unabsorbed Type I Antipneumococcus Serum

Immune horse serum[3] was absorbed by the fractional addition of a 1:2000 solution of the acetyl polysaccharide, until the supernatant serum after removal of the successive precipitates no longer reacted on the further addition of the specific substance. An equal portion of the same lot of serum was similarly absorbed with the deacetylated polysaccharide.

10 cc. of antipneumococcus horse serum (Type I) were diluted with 8 cc. of salt solution. To the diluted serum, 1 cc. of 1:2000 solution of the specific polysaccharide was added. The mixture was incubated for 2 hours at 37° and then placed in the ice box for the same length of time. The precipitate was thrown down by centrifugation in the cold and the clear supernatant serum was pipetted off. This procedure was repeated three times in all. Finally, 0.5 cc. of the solution of polysaccharide was added to the serum and the mixture was incubated for 2 hours at 37° and then allowed to stand in the ice box 24 to 48 hours. After removal of the final traces of precipitate by centrifugation in the cold, the clear supernatant serum was pipetted off and made up to a volume of 25 cc. with salt solution, so that each 0.5 cc. of absorbed serum used in the tests equalled 0.2 cc. of original serum.

The original serum and the two separately absorbed portions of the same serum were tested for the presence or absence of precipitins for both forms of the capsular polysaccharide. The results of the precipitin tests are given in Table II.

From the data recorded in Table II it is seen that both the acetyl and the deacetylated polysaccharide were precipitated by the original, unabsorbed serum in the highest dilution tested, representing a final concentration of 1 part in 3 million. The serum absorbed with the deacetylated polysaccharide, after removal of all precipitins for this form of the specific carbohydrate, still reacted with the acetyl polysaccharide in equally high dilution. On the other hand, after absorption with the acetyl polysaccharide, the serum was completely exhausted of all precipitins for both forms of the carbohydrate, as shown by the lack of reaction when tested with each substance in dilutions ranging from 1:20,000 to 1:3,000,000. It is a significant fact that the

[3] The antipneumococcus horse serum used in these experiments was provided through the courtesy of Dr. Augustus Wadsworth, Director of the Division of Laboratories and Research, New York State Department of Health, Albany.

deacetylated polysaccharide selectively takes out from the serum only the precipitins for itself, whereas the acetyl polysaccharide completely removes all the precipitating antibodies for both forms of the specific substance.

Enders (8) (1930) demonstrated that there exists in the autolytic products of Pneumococcus Type I a substance which is specifically precipitable in immune serum devoid of all antibodies for the soluble specific substance (deacetylated). He further showed that this material after being heated in a weakly alkaline solu-

TABLE II

Precipitation of the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I in Homologous Antiserum before and after Absorption of the Anti-carbohydrate Precipitins

Antipneumococcus Serum Type I	Acetyl polysaccharide					Deacetylated polysaccharide				
	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000
Unabsorbed	++++	++++	+++±	+++	++	++++	++++	++++	+++	++
Absorbed with deacetylated polysaccharide	++++	++++	++±	++	+	−	−	−	−	−
Absorbed with acetyl polysaccharide	−	−	−	−	−	−	−	−	−	−

++++ = complete precipitation, compact sediment with clear supernatant.
− = no precipitate formed, fluid clear.

The final readings were made after incubating the reacting mixtures 2 hours at 37° and overnight in the ice box.

tion lost its capacity to react in the same serum. The substance therefore appeared to be so sharply differentiated by its immunological reactions and its instability to alkali, that Enders considered it to be quite distinct, and provisionally called it "the A substance" to distinguish it from the specific carbohydrate. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a substance which, like the A substance of Enders, precipitated with Type I antipneumococcus serum that had previously been absorbed with the soluble specific substance (deacetylated). They also found that the substance designated by them "the cellular carbohydrate," when boiled for 2 minutes in N/100 NaOH no longer reacted in the absorbed serum, indicating, as the authors suggest, that boiling in alkaline solution had so altered their original material that its activity under these conditions approximated that of the soluble specific substance (deacetylated).

The specific precipitation of the acetyl polysaccharide in serum previously absorbed with the deacetylated carbohydrate, and the readiness with which the former substance is converted into the latter by alkali and heat, are similar to the relationships observed by Enders (8), and by Wadsworth and Brown (12), between the substances isolated by them and the soluble specific substance which they prepared according to methods previously described in this laboratory. Since the specific substance thus prepared is now known to be the deacetylated polysaccharide, it seems not improbable that the differences they observed, like those noted in Table II, represent the reactions not of two different carbohydrates but of a single substance in two chemically different forms; namely, the naturally acetylated and the artificially deacetylated polysaccharide.

2. Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and Deacetylated Polysaccharide

Type I antipneumococcus serum was separately absorbed with the acetyl and the deacetylated polysaccharide as previously described. The results of the agglutination tests of Type I pneumococci in homologous antiserum before and after specific absorption are given in Table III.

The experimental data presented in Table III show that absorption of Type I antiserum with the acetyl polysaccharide completely removed all the type-specific agglutinins, as evidenced by the fact that, after absorption, the serum no longer agglutinated the homologous organisms. On the other hand, the serum similarly absorbed with the deacetylated polysaccharide still agglutinated the bacteria although the titer of agglutinins was considerably reduced. The fact that after absorption with the deacetylated polysaccharide the precipitin titer appeared undiminished for the acetyl polysaccharide, while the titer of agglutinins for the bacterial cells was reduced, may be attributed not to essential differences in the antibodies involved in the two forms of immune reactions, but to differences in the technical procedures of diluting the antiserum in the agglutination test, and of maintaining an excess of serum throughout the range of the precipitin titration. Of special significance in the present study is the fact

that the acetyl polysaccharide by itself completely exhausted the serum of all demonstrable type-specific precipitins and agglutinins.

Sabin (10) and Enders (9) previously demonstrated that Type I antiserum after absorption with the specific carbohydrate (deacetylated) still agglutinated pneumococci of the homologous type. Enders further showed that when the bacterial cells were heated in a weakly alkaline medium, they lost the capacity to react in the absorbed serum although in this altered state they were still specifically agglutinated by the original, unabsorbed serum. The fact that the bacteria were agglutinated by immune serum containing no antibodies reactive with the specific carbohydrate (deacetylated), and the further observation that after boiling for

TABLE III

Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide

Antipneumococcus Serum Type I	Agglutination of Pneumococcus Type I in serum dilutions						
	1:10	1:20	1:30	1:40	1:60	1:80	1:100
Unabsorbed	++++	++++	++++	++++	+++	++	+
Absorbed with deacetylated polysaccharide	++++	++	+±	±	−	−	−
Absorbed with acetyl polysaccharide	−	−	−	−	−	−	−

++++ = complete agglutination, compact sediment with clear supernatant.
− = no agglutination.

10 minutes at pH 8.8 the cells no longer reacted in this same serum, led Enders to the conclusion that there exists a type-specific substance distinct from the specific carbohydrate in Pneumococcus Type I.

As pointed out earlier in this paper, the acetyl polysaccharide is readily converted into the deacetylated substance by treatment with alkali. This fact, together with the observations just cited on the serological reactions of the two forms of the specific polysaccharide, not only substantiates the findings of the former investigators but also furnishes evidence of the immunological significance of this hitherto unrecognized relationship. Thus, on the basis of the present evidence, it appears that the acetyl polysaccharide represents the soluble specific substance in a form that fulfils all the serological requirements

of type specificity without the necessity of predicating a second substance distinct from the specific carbohydrate itself. These observations are further confirmed by the results of the following protection experiments.

3. *Protective Action of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and Deacetylated Polysaccharide*

Protection tests in mice were carried out according to the method described by Felton (15).

Dilutions of the unabsorbed and absorbed serum, calculated on the basis of original serum volume, were made, ranging from 1:10 to 1:500. 0.5 cc. of each dilution of serum together with 0.5 cc. of 1:200 dilution of a 12 hour plain broth culture of Pneumococcus Type I was injected intraperitoneally into white mice weighing from 18 to 21 gm. The virulence of the organisms was such that 10^{-8} cc. of culture caused the death of normal control mice in 48 hours.

All mice alive and well 7 days after inoculation were considered effectively protected and were recorded as survivals.

The results of experiments to determine the protective action of Type I antipneumococcus serum before and after absorption with the acetyl and deacetylated polysaccharide are given in Table IV.

The outcome of the protection tests (Table IV) shows that after absorption with the deacetylated polysaccharide, the serum still possessed protective action, although the titer of protective antibodies was considerably reduced. In an earlier study of the neutralizing effect of the soluble specific substance, Sabin (10) showed that this substance, which in the light of the present results was presumably in the form of the deacetylated polysaccharide, only partially neutralized the protective power of Type I antipneumococcus serum He attributed the residual protective action of the absorbed serum to the presence of type-specific antibodies not neutralized by the homologous soluble specific substance and distinct from the anticarbohydrate precipitins. The comparative data presented in Table IV show that the soluble specific substance in the form in which it naturally occurs as the acetyl polysaccharide completely removed the protective antibodies in Type I antiserum. This neutralizing effect is shown by the fact that after removal of all the anticarbohydrate precipitins by absorption with the acetyl polysaccharide, the immune serum was de-

void of protective action when titrated by the method employed in the present experiments.

These results again emphasize the relationship existing between the natural acetyl polysaccharide and its deacetylated derivative. It now becomes apparent why the specific carbohydrate in the form in which

TABLE IV

Protective Action in Mice of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

Dilution of serum	Antipneumococcus Serum Type I					
	Unabsorbed		Absorbed with			
			Deacetylated polysaccharide		Acetylpolysaccharide	
1:500	S	S	D 21	D 28	D 22	D 25
1:250	S	S	D 26	D 51	D 20	D 23
1:100	S	S	S	S	D 17	D 18
1:50	S	S	S	S	D 17	D 21
1:10	S	S	S	S	D 19	D 23

All mice were injected intraperitoneally with 0.5 cc. of diluted serum together with 0.5 cc. of 1:200 dilution of broth culture of Pneumococcus Type I.

Controls

Pneumococcus Type I	Mice receiving no serum
cc.	
10^{-6}	D 45
10^{-7}	D 45
10^{-8}	D 48

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

it was first isolated was later found to be deficient in certain specific properties, notably in its failure to absorb completely the type-specific antibodies from immune serum. By the methods employed in the original isolation, the specific carbohydrate is now known to have been artificially deacetylated, and its immunological deficiencies have been found to be associated with the loss of the highly reactive but ex-

tremely labile acetyl groups. The significance of this fact is made evident by the results of the preceding experiments, in which it has been shown that the acetyl polysaccharide with these chemical groups intact specifically bound and completely removed from the serum all the type-specific antibodies.

4. Antigenic Action of the Acetyl Polysaccharide in Mice

In order to determine whether the acetyl polysaccharide is capable of inducing active immunity against infection with pneumococci of the homologous type, the antigenicity of this form of the specific carbohydrate was tested in mice and its action compared with that of the deacetylated polysaccharide

Six mice were given three intraperitoneal injections, at 3 day intervals, of 0.5 cc. of 1:2 million dilution of Type I acetyl polysaccharide; another group of six mice was similarly treated with identical amounts of the deacetylated carbohydrate prepared by heating the original material in N/20 alkali for 30 minutes at 100°. 6 days after the last immunizing injection both groups of mice were infected by the intraperitoneal injection of a virulent culture of Pneumococcus Type I in amounts ranging from 10^{-5} to 10^{-7} cc., the maximum number of infecting organisms being 1000 times greater than that which proved fatal in the normal control mice.

The results of the experiments on the active immunization of mice with both forms of the specific carbohydrate are given in Table V.

As shown in Table V, the mice which had received in divided doses an amount of acetyl polysaccharide totaling only 0.00075 mg. of specific substance survived the injection of an amount of virulent culture of Pneumococcus Type I greatly in excess of that causing fatal infection in the untreated control animals. Repetitions of this test in mice have shown that the active immunity induced by the acetyl polysaccharide is strictly type-specific, affording no protection against infection with pneumococci of the heterologous Types II and III. It is equally clear from the results of this and other similar experiments that the deacetylated polysaccharide is wholly devoid of antigenic action. This total lack of immunizing effect is all the more striking in this particular instance, since the deacetylated substance was derived from the originally active acetyl polysaccharide by

merely heating the latter in alkaline solution—a procedure previously shown to deprive the native carbohydrate of its acetyl groups.

During the past ten years a number of investigators using various methods have recovered from Pneumococcus, substances which have been shown to possess the property of inducing active immunity in mice against infection with organisms of the homologous type. In many instances, the antigenic and serological behavior of these substances was so distinct that the authors designated them by special terms in order to distinguish them from the soluble specific substance (deacetylated). Thus, there are now current in the literature

TABLE V

Active Immunity Induced in Mice by the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I

Amount of culture Pneumococcus Type I	Normal mice controls (untreated)	Mice receiving 3 injections of 0.5 cc. of 1:2 million solution of			
		Acetyl polysaccharide Type I		Deacetylated polysaccharide Type I	
cc.					
10^{-5}	—	S	S	D 68	D 68
10^{-6}	D 44	S	S	D 34	D 44
10^{-7}	D 52	S	S	D 58	D 76
10^{-8}	D 93	—	—	—	—

The treated mice were infected 6 days after the last immunizing injection.

— = not done.

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

descriptive terms such as the following: "the water-soluble fraction" of Perlzweig and his coworkers; "the A substance" of Enders; "the cellular carbohydrate fraction" of Wadsworth and Brown, and "the non-polysaccharide and probably non-protein derivative" of Felton. With the possible exception of the A substance, which Enders did not test for antigenicity in mice, these various cell derivatives have been found to produce type-specific immunity in this particular species of animal.

Since it is obviously impossible within the scope of this paper to review the individual contributions in detail, brief reference will be made only to those studies concerned with the antigenicity of specific fractions derived from Pneumococcus Type I.

Perlzweig and Steffen (4) (1923) extracted from the bacterial cells a water-soluble fraction which induced specific immunity in mice. This observation was later confirmed by Ferry and Fisher (20) (1924, 1925) who obtained from washings of the organisms an aqueous extract which had similar antigenic properties in mice. The water-soluble antigen of Perlzweig and Steffen proved resistant to the prolonged action of autolysis and tryptic digestion. They further pointed out the suggestive fact that boiling the antigenic material for 5 minutes in alkaline solution (pH 9) destroyed its immunizing action in mice, while similar exposure to heat in a slightly acid medium (pH 6) did not impair its antigenicity. Perlzweig and Keefer (5) (1925) recovered from the filtrate of broth cultures a substance which, like that derived from the cells, produced active immunity in mice. Although Perlzweig and his coworkers regarded the immunizing material as protein in character they pointed out evidence suggestive of its non-protein nature.

Schiemann and his collaborators (6, 7) (1927, 1931) first brought convincing evidence that the type-specific polysaccharide of Pneumococcus Type I, in the form isolated by them, produced specific immunity when injected in relatively minute amounts into mice. They also found that if administered to mice in larger doses, this form of the specific carbohydrate not only failed to incite immunity but on the contrary was often toxic and caused purpura. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a specific fraction designated by them "the cellular carbohydrate." This substance corresponded in its antigenic and purpura-producing action to the carbohydrate of Schiemann and Casper (6), and was similar in its immunological reactions to the A substance of Enders (8). Felton (14) (1932) isolated from Pneumococcus Type I a substance inducing type-specific immunity in mice which from its chemical properties he concluded was "a non-polysaccharide and probably non-protein derivative" of the bacterial cells.

The consistently negative results of all former attempts in this laboratory to induce active immunity in mice with the specific carbohydrate are now known to have been due to the fact that the polysaccharide was then used only in its deacetylated form. As shown in Table V, the change from the antigenic to the non-antigenic form of the carbohydrate is brought about whenever the originally active acetyl polysaccharide is converted by alkali into its deacetylated derivative. This difference in antigenic action, like that already noted in the serological behavior of the two forms of the polysaccharide, is referable to known differences in chemical constitution.

An analysis of the specific reactions of the acetyl polysaccharide discloses a previously unsuspected similarity between this form of the specific carbohydrate and the antigenically active fractions described

by other investigators. From the chemical and immunological properties of the acetyl polysaccharide it seems highly probable that this substance in the purified state accounts for the antigenic action of the carbohydrate of Schiemann and Casper (6) and of Wadsworth and Brown (11, 12). As in the case of these substances, the acetyl polysaccharide is antigenically effective in mice only when administered in extremely minute quantities. Although an extensive study of the purpura-producing action of the acetyl polysaccharide has not been made, in several instances purpura has been noted in mice injected with amounts of this substance ranging from 0.4 to 4.0 mg.

That the antigenic action of the water-soluble fraction of Perlzweig and his coworkers (4, 5) may have been due to the presence of traces of unhydrolyzed acetyl polysaccharide seems not unlikely from the readiness with which it lost its immunizing capacity when heated in alkaline solution.

As pointed out earlier, the A substance of Enders (8) and the cellular carbohydrate of Wadsworth and Brown (11, 12) correspond in their serological reactions to those of the acetyl polysaccharide. In addition, both of these substances were shown to be equally sensitive to the destructive action of alkali. While it cannot be stated with certainty that these substances are identical, their properties parallel those of the acetyl polysaccharide so closely that it seems probable that their biological activity is due to this substance.

5. Antigenic Action of the Acetyl Polysaccharide in Rabbits

Despite the number of observations on the antigenicity in mice of specific fractions derived from Pneumococcus Type I, comparatively little work has been done to determine the capacity of these substances to incite antibody formation in rabbits. The following experiments, therefore, were carried out to ascertain whether the acetyl polysaccharide possesses the property of stimulating the production of type-specific antibodies in rabbits.

Six rabbits were given intravenous injections of acetyl polysaccharide daily for 6 days, followed by a rest period of 1 week. Three courses of injections were given in all. Two rabbits received 1 cc. of 1:1000 solution of the substance; two others were injected with 1 cc. of 1:10,000 solution; the remaining two were given 1 cc. of 1:100,000 solution. At the end of the third course of injections, each of

the three groups of rabbits had received a total quantity of acetyl polysaccharide amounting to 18 mg., 1.8 mg. and 0.18 mg. respectively. 7 days after the second and third series of injections, test bleedings were made and the sera were tested for the presence of type-specific agglutinins, precipitins and protective antibodies.

The detailed protocols of the serological tests are omitted, since in no instance were type-specific agglutinins, precipitins or protective antibodies demonstrable in the serum of the rabbits which had previously received repeated injections of Type I acetyl polysaccharide.

Ten days after the last course of injections, each rabbit was infected by the intradermal injection of 0.2 cc. of undiluted blood broth culture of Pneumococcus Type I according to the method described by Goodner (21). The infected rabbits developed at the site of inoculation typical lesions characterized by areas of massive edema and hemorrhagic necrosis. All six of the animals died within 48 to 96 hours after the onset of infection. In no instance, therefore, was there any evidence of increased resistance brought about by the prolonged series of injections of Type I acetyl polysaccharide in amounts totaling 0.18 to 18 mg. In view of the fact that only minute amounts of the acetyl polysaccharide were effective in evoking an immune response in mice, it is conceivable that these rabbits were given too large doses; however, in terms of body weight, the dosage in those rabbits that had received a total of 0.18 mg. was presumably within the range of the amounts found to be effective in mice.

In view of these results, it is significant that the presence of the acetyl polysaccharide was demonstrated by the precipitin reaction in the serum of these rabbits 7 days after the second and third course of injections. This observation indicates that the acetyl polysaccharide is only very slowly excreted, and indirectly suggests that no antibodies were formed, otherwise the substance would in all probability have disappeared more rapidly from the circulation. That the acetyl polysaccharide is actually excreted as such by the kidney and appears in the urine in this specifically reactive form was shown in the case of two other rabbits. One of these animals was given a single large dose of 17 mg. of active substance intravenously and the other a similar amount intraperitoneally. Samples of urine from these animals were collected and tested for the presence of the acetyl polysaccharide. Specific precipitation occurred in the urine of both rabbits

on the addition of Type I antipneumococcus serum from which all antibodies reactive with the deacetylated polysaccharide had been previously removed by specific absorption. The specificity of this test conclusively demonstrates that the polysaccharide was excreted in the acetylated form. The urine of both rabbits still showed the presence of the acetyl polysaccharide, as demonstrated by the specific precipitin reaction, 7 days after injection, at which time the observations were discontinued.

Under the conditions of this experiment, the acetyl polysaccharide failed to induce any immune response in rabbits. The serum of the treated animals contained no demonstrable antibodies, and the animals themselves were not protected against subsequent infection with organisms of the homologous type. Moreover, it has been shown that the acetyl polysaccharide persisted in the circulation of the treated rabbits for considerable periods of time, was slowly excreted by the kidney and appeared in the urine in its naturally acetylated form.

It is of course possible that in the present instance the failure of the acetyl polysaccharide to induce antibody formation or to incite active immunity may be attributed to the inadequate number of rabbits tested or to the use of improper amounts of the substance. No assumption is made as to the difference in the antigenic action of the acetyl polysaccharide in mice and in rabbits. The explanation must await further study of this interesting and significant phase of the problem.

DISCUSSION

So far as is known, the only chemical difference between the acetyl polysaccharide and its deacetylated derivative lies in the presence or absence of the acetyl groups. Evidence in support of this view is the difference observed in the acid equivalents of the two forms of the specific carbohydrate. On alkaline hydrolysis there is liberated from the acetyl polysaccharide approximately 6 per cent of acetic acid which is organically bound in the intact molecule in the form of an acetyl ester. From solutions of the acetyl polysaccharide that have been treated with alkali, the deacetylated carbohydrate has been recovered and the substance thus derived has been found to correspond in chemical and serological properties to the polysaccharide formerly known as the "soluble specific substance."

The acetyl polysaccharide possesses all the specific immunological characteristics of the deacetylated derivative and in addition exhibits other distinctive properties. In highly purified form the acetyl polysaccharide, in contradistinction to the deacetylated substance, completely absorbs all demonstrable type-specific antibodies from antiserum of the homologous type; it induces active immunity and incites purpura in mice; it is specifically precipitable in immune serum from which the type-specific anticarbohydrate precipitins reactive with the deacetylated polysaccharide have been removed by specific absorption; it is extremely unstable to the action of alkali.

The results of the present study offer an explanation of many of the perplexing problems that have arisen concerning the nature and specific properties of the soluble specific substance. One of these is the question of the antigenicity of the specific carbohydrate. In the form in which it was originally isolated the polysaccharide was found to be devoid of antigenic action in mice and in rabbits, and considerable evidence was presented that this substance functioned only as a hapten. However, a number of investigators (4, 5, 14, 20) have isolated substances, in some instances of undoubted carbohydrate nature (6, 12), which were antigenic, inducing type-specific immunity in mice. The present experiments show that minute quantities of the purified acetyl polysaccharide give rise to active immunity in mice. While it is impossible to state that the antigenic activity of the specific fractions isolated by others is due to the presence of the acetyl polysaccharide in the preparations employed, this possibility seems not unlikely. The differences between the antigenic and nonantigenic forms of the specific carbohydrate are thus related to known differences in chemical constitution. The antigenicity of the acetyl polysaccharide, in mice at least, is intimately associated with the presence of the acetyl groups in the polysaccharide molecule.

The writers have never maintained that complex carbohydrates may not function as antigens, but until the present experiments with the highly purified acetyl polysaccharide they had obtained no evidence in experimental animals that this was the case. Many years ago, Ulenhuth (22) (1905) presented evidence of the antigenic action of gum arabic, pointing out that this was the first time that specific antibodies had been demonstrated in the serum of animals immunized

with a carbohydrate. Recently in collaboration with Remy, Ulenhuth (23) (1933) has confirmed his early observations, showing that after prolonged immunization with gum arabic, the serum of the treated rabbits contained specific precipitins and complement-fixing antibodies. Ford (24, 25) (1906–07) found that the serum of rabbits immunized with extracts of *Amanita phalloides* and *Rhus toxicodendron*, possessed marked antihemolytic and antitoxic properties. The active principle of these extracts was isolated and identified in each instance as a glucoside.

A question that has been difficult of interpretation is that relating to the purpura-producing activity of the specific carbohydrate. Specific substances of carbohydrate nature isolated by other workers have been found to incite purpura in mice, while the polysaccharide in the form originally isolated does not possess this activity. However, the fact that the mere presence of acetyl groups in a physiologically active substance may greatly modify its activity is well known in the case of acetyl choline which has at least one thousand times the activity of the parent base (26). It is not certain that the purpura-producing activity of the acetyl polysaccharide is solely related to the presence of these groups in the molecule. However, it is known that with loss of acetyl groups, the polysaccharide also loses the capacity to induce purpura.

Another of the perplexing problems that have arisen has been the possibility, indicated by the work of several investigators, that the specific carbohydrate is not the only substance concerned in the type specificity of Pneumococcus Type I. The concept of two type-specific substances has its origin in the observation that the polysaccharide in the form first isolated does not absorb all the type-specific antibodies from immune serum (10). This fact suggested the presence in the serum of antibodies distinct from the anticarbohydrate precipitins, and the coexistence in the cell of another substance unrelated to the polysaccharide. Support for this view was found in the demonstration and subsequent isolation by others (8, 12) of a substance that was specifically precipitable in antiserum from which all precipitins for the specific carbohydrate had been removed. Several explanations were proposed, chiefly that there exist in the cell two specific sub-

stances giving rise to two distinct antibodies, both type-specific, but each reactive only with the corresponding antigen. On the other hand, in support of the view that only a single substance is responsible for type specificity Wadsworth and Brown (11) suggested that the specific carbohydrate as first isolated may be only a radical of a more complex substance. Ward (13) suggested "the possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate—perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate itself."

From the work of Landsteiner and others it is known that the mere presence of relatively small chemical groups in an immunologically active substance exerts a determining influence on its specificity. The present study brings evidence that in Pneumococcus Type I the specific carbohydrate with the acetyl groups intact fulfils all the serological requirements of type specificity. On the other hand, when the acetyl groups are removed the resultant product retains the polysaccharide structure and the dominant type specificity of the original carbohydrate, but loses many of the specific characteristics that distinguish the naturally acetylated polysaccharide.

SUMMARY

The soluble specific substance of Pneumococcus Type I has been chemically isolated from the bacterial cells and from autolyzed cultures as an acetyl polysaccharide.

So far as could be determined by the methods employed, the acetyl polysaccharide in highly purified form absorbs from Type I antipneumococcus serum all demonstrable type-specific precipitins, agglutinins and protective antibodies.

Mice injected intraperitoneally with minute quantities of the acetyl polysaccharide develop active immunity to subsequent infection with Pneumococcus Type I. The immunity thus induced is type-specific. In several instances purpura has been observed in mice following the injection of larger amounts of the acetyl polysaccharide.

Under the experimental conditions of this study, no type-specific precipitins, agglutinins or protective antibodies were demonstrable

in the serum of rabbits following repeated intravenous injections of the Type I acetyl polysaccharide. The treated rabbits were not immune to subsequent infection with Pneumococcus Type I.

The acetyl polysaccharide is readily converted into its deacetylated derivative by treatment with dilute alkali.

The chemical and immunological properties of the deacetylated polysaccharide are identical with those of the soluble specific substance in the chemical form in which it was originally isolated; the deacetylated form of the specific carbohydrate is non-antigenic, does not produce purpura in mice, and only incompletely absorbs the type-specific antibodies from Type I antipneumococcus serum.

The immunological significance of the acetyl polysaccharide and its possible relationship to the specific substances isolated from Pneumococcus Type I by other workers are discussed.

CONCLUSIONS

The soluble specific substance of Pneumococcus Type I is now regarded, tentatively at least, as an acetyl polysaccharide. In this form it accounts adequately for all the serological phenomena of type specificity of Pneumococcus Type I.

Addendum.—During the course of publication of the present work there has appeared a paper by Pappenheimer and Enders[4] on the specific carbohydrate of Type I Pneumococcus. On the basis of elementary analysis, amino nitrogen content and specific rotation, these authors conclude that the A substance of Enders and the specific polysaccharide previously isolated in this laboratory are closely related and that the latter is possibly a hydrolytic product of the former substance.

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