IMMUNOLOGICALLY SPECIFIC POLYSACCHARIDES

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In the present state of knowledge of the field a review of polysaccharides which react specifically with immune sera may appear somewhat premature. The possibilities and limitations of the subject are barely understood, few of the substances in question have been investigated in detail, and indeed, a rigorous proof has never been given that the polysaccharides isolated are the actual specific substances and not merely adsorbents carrying them. However, the evidence on the last point seems so overwhelming that a new and unsuspected field of sugar chemistry would appear to have been opened up. Moreover, the subject is of such importance to immunology in general and to the understanding of bacterial specificity in particular, that the writer will, perhaps, be pardoned if he attempts to call attention to the data already collected and to such advances as they may have occasioned, leaving final conclusions and final judgment to some future time.

It has long been known that polysaccharides with the properties of gums could be produced through the agency of microörganisms. As early as 1861 Pasteur (1) observed the formation of gum from saccharose solutions when seeded with certain "petits globules reunis en chapelet." The subsequent literature has been reviewed by Buchanan (2), who described a gum which he considered the capsular material of *B. radicicola.* The polysaccharide material from the encapsulated, pathogenic Friedländer bacillus was isolated by Toenniessen (3) and later by Kramár (4). None of these investigators ascribed any immunological significance to the gums which they isolated and it is only in the light of the work now to be discussed that their classification with the specific polysaccharides would seem justified.

Following the demonstration (5) by serological methods of the existence of three fixed antigenic type of Pneumococcus, Dochez and Avery (6) showed in 1917 that whenever pneumococci of Type I, II, or III are grown in fluid media, there is present in the cultural fluid a substance which precipitates specifically in antipneumococcus serum prepared by injection of animals with a strain of the homologous type. This soluble substance was demonstrated in culture filtrates even during the initial period of rapid multiplication when little or no cell death or disintegration is occurring. Examination of the blood and urine of experimentally infected animals gave proof of the presence of this substance in considerable quantities in the body fluids, showing that this soluble material, elaborated at the focus of the disease, readily diffuses throughout the body, is taken up in the blood, passes the kidney, and appears in the urine unchanged in specificity. Similarly, a study of the serum of patients suffering from lobar pneumonia revealed a substance of like nature in the circulating blood during the course of the disease in man. Furthermore, examination of the urine of patients having pneumonia due to pneumococci of Types I, II, and III, showed the presence of this substance in some stage of the disease in approximately two-thirds of the cases.

In 1923, Zinsser and Parker (7) prepared from filtered alkaline extracts of bacteria of several varieties, including pneumococci, products which they called "residue antigens," which appeared free from coagulable protein and were specifically precipitable by homologous antisera. These observers considered their acid- and heat-resistant antigenic materials analogous to the soluble specific substance of Pneumococcus described by Dochez and Avery.

In their earlier studies Dochez and Avery found that the specific substance is not destroyed by boiling; that it is readily soluble in water and precipitable by acetone, alcohol, and ether; that it is precipitated by colloidal iron, and does not dialyze through parchment; and that the serological reactions of the substance are not affected by proteolytic digestion by trypsin. Since the substance is easily soluble, thermostable, and type-

specific in the highest degree, it seemed an ideal basis for the beginning of a study of the relation between bacterial specificity and chemical constitution, and this was accordingly undertaken (8).

The Type II pneumococcus was first selected for study, and the fractionation and purification of the specific substance were followed at each step by means of the precipitin test with sera prepared by immunization of horses against Type II pneumococcus. Eight-day cultures of the organism in meat-infusion phosphate broth were concentrated on the water-bath to about one-fifteenth volume, precipitated with 1.2 volumes of alcohol, and centrifuged. Of the three layers formed, the middle gummy layer contained most of the specific substance, and a large proportion of the accompanying impurities could thus be eliminated in a single step. Further purification depended upon repeated precipitation with alcohol under varying conditions, also by means of saturation with ammonium sulfate and final precipitation by pouring into 10 to 15 volumes of acetone. The yield from 300 liters of culture was 2.5 to 3.5 grams.

It has generally been supposed that substances with immunologically specific properties are derived from proteins. It was therefore an entirely unexpected occurrence that as the precipitin titer of the recovered soluble specific substance rose during the process of purification, the nitrogen content dropped until, after the final acid precipitation, this element remained present only in undeterminable traces. On the other hand, the material isolated took on more and more the properties of a polysaccharide, so that it became evident that a sugar derivative was at least the carrier of whatever might be the true specific substance itself.

Attempts were made to separate this hypothetical specific substance from the polysaccharide, but no significant change in the properties of the material could be effected by precipitation with basic lead acetate, uranyl nitrate, or safranine, or by adsorption on alumina and recovery from this, or even by specific precipitation with a large quantity of immune serum and recovery of the specific substance from the immune precipitate. Furthermore a substance with the properties of a polysaccharide was obtained whether the starting material was the entire broth

culture, the centrifuged pneumococci themselves, or the urine of pneumonia patients. When exposed to the action of 1:1 hydrochloric acid in the cold the specific activity did not diminish appreciably until reducing sugars appeared, so that the specific substance and the polysaccharide, if not identical, appeared at least to be very closely associated. Attempts at a separation by means of polysaccharide-splitting enzymes also failed, as the sugar derivative proved very resistant to this type of hydrolysis.

On acid hydrolysis the soluble specific substance of Type II pneumococcus, in the state of purity outlined above, yielded about 70 per cent of reducing sugars, calculated as glucose. These sugars actually consisted mainly of glucose, as shown by the isolation and identification of glucosazone, and the formation of saccharic acid on oxidation, isolated as the acid potassium salt. Other possible constituents have not yet been identified.

Thus the soluble specific substance of Type II pneumococcus is apparently identified with a weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its acid equivalent is about 1250 and the specific optical rotation is about $+74°$. It is not precipitated by barium hydroxide or heavy metal salts with the exception of basic lead acetate and uranyl compounds. It reacts at a dilution of 1:5,000,000 with Type II antipneumococcus serum but does not precipitate Type I and Type III antisera at a concentration of 1:400. The substance is converted by acetic anhydride and pyridine into a very sparingly soluble triacetyl derivative.

Little work was necessary on the soluble specific substance of Type III pneumococcus to show that marked chemical differences existed between it and the corresponding substance of Type II. The method of concentration and purification was at first the same, but it soon developed that not only is the Type III specific substance precipitated by heavy metal salts such as those of silver, mercury, and copper, which do not precipitate the Type II substance, but that, in conformity with this, the Type III substance is the soluble alkali or alkaline earth salt of an *insoluble* strong acid which is thrown out of solution in the presence of an

excess of strong hydrochloric acid. This property was most useful in separating the specifically reacting polysaccharide acid from accompanying glycogen or erythrodextrin, derived either from the meat broth or from the bacteria themselves. By repeated purification as the insoluble free acid the Type III specific substance was easily obtained in a state of relative purity without the aid of precipitation by ammonium sulfate and with fewer fractionations from alcohol than proved necessary with the Type II substance. Successive batches also agreed very closely in their chemical and physical properties, indicating that one is apparently dealing here with a much more definite chemical entity than in the case of the Type II substance, in which, in the absence of so characteristic a property which can be made use of in the process of purification, the variation in the findings is over a much wider range.

The Type III soluble specific substance is thus also isolated as a nitrogen-free polysaccharide. It is, however, not a dextrorotatory, weak acid like the Type II substance, but a levorotatory, strong acid, with an equivalent value of about 340 and $[\alpha]_D$ of about $-33°$. It is precipitated by barium hydroxide in excess and by heavy metal salts, and is also rendered insoluble by the addition of strong hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum. On hydrolysis the substance yields glucose and a disaccharide acid which will be discussed below. No further purification was effected by precipitation with barium hydroxide or by adsorption on highly active alumina prepared according to Willstätter and Kraut (9).

The chief stumbling-block to chemical progress with the bacterial polysaccharides has been the small amount of material available. It has been found possible in the case of the Type III pneumococcus to overcome this objection to some extent. In the first place this microbe produces far more specific substance than do the other two antigenic types of Pneumococcus, and the substance is the easiest of the three to isolate and purify. Furthermore, once the properties of the Type III specific substance had

been established it was considered proper to add glucose to the usual Pneumococcus broth. In this way a greatly increased growth was obtained, and whereas the yields of specific substance were originally from 6 to 9 grams per 300 liters of broth, they now rose to 35 to 40 grams. With these increased amounts of material it became possible to throw new light on the structure of the polysaccharide and its hydrolysis products (8f).

When the Type III specific substance is partially hydrolyzed in the cold with 75 per cent (by weight) sulfuric acid until it no longer precipitates on dilution, and is then further hydrolyzed with boiling normal sulfuric acid until the maximum reducing power is attained, a small amount of glucose is formed and may be identified through the analysis, melting point, and mutarotation of the osazone, and through oxidation to saccharic acid. By far the largest portion of the hydrolytic products, is, however, precipitated by basic lead acetate and yields a crystalline morphine salt which melts at 153–6° and shows an initial $[\alpha]_D$ of $-47.9°$, changing to $-54°$. This salt, on decomposition, gives rise to an hitherto uncrystallizable sugar acid, differing little from the crude material before purification. Its reducing power is 50 per cent that of glucose; its acid equivalent is found to be 363, while the value calculated for a disaccharide acid, $C_{11}H_{19}O_{10}$. COOH is 356; on prolonged hydrolysis only a small amount of glucose, in addition to unhydrolyzed material, can be isolated, the acid half of the portion hydrolyzed apparently decomposing similarly to glucuronic acid and yielding approximately the expected amount of furfural; the reducing group of this bionic acid is aldehydic, as shown by its quantitative determination by the Willstätter-Schudel method (10); the acid also gives the color reaction with naphthoresorcinol characteristic of the glucuronic acid type, but since the easily isolable mucic acid is not found on hydrolysis and oxidation with nitric acid, the acid portion of the molecule can scarcely be galacturonic acid. Whether the saccharic acid actually isolated from the oxidation mixture arises only from the glucose half of the molecule, or from the acid portion as well, can not be stated at present.

As to the position of the union of the glucose to the sugar acid the evidence at hand does not permit any conclusion. The linkage may be either through the reducing group of the glucose or else the reducing group of the sugar acid is involved in the glucosidic union. This type of union is indicated by the fact that on further hydrolysis the reducing power of the aldobionic acid increases to about 65 per cent before dropping owing to destruction of the hexose-uronic acid liberated.

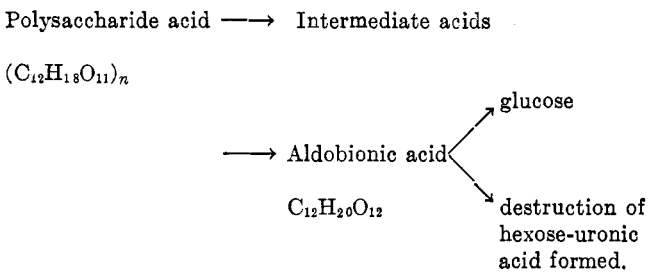
It is evident from quantitative data obtained on the hydrolysis of the original polysaccharide (8f, p. 412) that the aldobionic acid accounts for about 85 per cent of the total products of hydrolysis, while only 9.5 per cent is glucose, and 5.5 per cent unaccounted for. Now it has also been found (8f. p. 412) that the aldobionic acid itself slowly hydrolyzes, with liberation of glucose to the extent of about 1 per cent per hour on boiling with dilute mineral acid. It is therefore not illogical to assume that the glucose set free during the hydrolysis of the polysaccharide owes its origin, not to a separate part of the carbohydrate molecule, but chiefly to a secondary reaction involving the disaccharide acid. This assumption is all the more justified by the fact that no glucose is split off during the preliminary hydrolysis by 75 per cent sulfuric acid in the cold. Since, also, these partial hydrolysis products show, by their acid equivalents, one carboxyl group for every two sugar nuclei, it would seem that the polysaccharide as a whole is built up of units of the disaccharide acid.

The condensed, or polysaccharide form of a hexose-hexose-uronic acid should have the formula

$$(C_{12}H_{20}O_{12})_n - (n - 1)H_2O, \text{ or } (C_{12}H_{18}O_{11})_n.$$

A substance of this composition should have an acid equivalent of 338 and a carbon and hydrogen content of 42.6 per cent and 5.4 per cent respectively. These figures are practically identical with actual analytical values obtained (cf. Table 1). Thus one may justifiably conceive of the Type III soluble specific substance as a condensation product of the aldobionic acid, $C_{12}H_{20}O_{12}$,

built up in such a way that the reducing groups disappear and carboxyl groups remain free. Hydrolysis by means of acid follows the course:



In view of the evidence collected it is believed that the soluble specific substance of Type III pneumococcus is a definite chemical individual, polysaccharide in nature, and that it is composed of units of a difficultly hydrolyzable aldobionic acid in which glucose and a hexose-uronic acid are combined in such a way that one aldehyde group and the carboxyl remain free. The polysaccharide is thus unusual not only in its possession of the property of immunological specificity, but in its chemical constitution as well.

In the case of the soluble specific substance of Type I pneumococcus (8e) it was again found necessary to modify somewhat the method of isolation, owing to its presence in the culture fluid in relatively smaller amount than are the Type II and Type III specific substances, and because of the insolubility of the product at its isoelectric point, which lies at about p_H.[4] It was also found advisable to wash the alcoholic precipitates with 0.5 normal acetic acid, in which the isoelectric Type I specific substance is insoluble, while the accompanying glycogen or erythrodextrin dissolves. Finally, the substance was precipitated at as low a temperature as possible with strong barium hydroxide solution, reprecipitated by alcohol in the presence of hydrochloric acid, and dialyzed free from chloride ion. The Type I substance is a weak base and precipitates as the excess of hydrochloric acid is removed. The yield from 300 liters was 2 to 3 grams.

The Type I soluble specific substance also appears to be a sugar

derivative, but differs from the other two substances in its lower percentage of sugar liberated on hydrolysis and in containing nitrogen as an apparently essential component. It rotates the plane of polarized light about 300° to the right, is a strong acid and a weak base, and is very sparingly soluble at its isoelectric point, which lies at about pH 4. When in concentrated solution it also separates from hydrochloric acid at an acidity of 1.3 normal, redissolving as more strong acid is added. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated on treatment with nitrous acid and reducing sugars appear at the same time, while the specific reaction vanishes. Under the same conditions the Type II and Type III substances are unaffected by nitrous acid. It would thus appear that this portion of the nitrogen, at least, is an integral part of the specific substance, and is possibly linked to the reducing group of a sugar derivative, as Karrer believes is the case in the polyglucosamines (11). The substance gives the color reaction for glucuronic acid with naphthoresorcinol, but yields mucic acid on oxidation, indicating a relationship to galactose. Since the carbon and hydrogen contents of the substance are close to the theoretical values for polysaccharides it appears possible that in it a nitrogenous sugar derivative is linked to galacturonic acid through the reducing group of the latter. The Type I substance is precipitated by barium hydroxide in excess, by heavy metal salts, and by phosphotungstic acid. In the specific precipitin reaction with Type I antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000, while at a concentration of 1:400 it gives a faint cloud with Type III antiserum.

A preparation derived from Type I pneumococci which had been separated from the accompanying broth by centrifugation and one subjected to additional purification by adsorption on alumina failed to show differences from those isolated as above.

The properties of the soluble specific substances of the three antigenic types of Pneumococcus, in the state of purity attained up to the present are summarized in Table 1.

The three polysaccharides contain no sulfur or phosphorus

and differ from the starch-glycogen group of carbohydrates in their acid properties, in giving no color with iodine, and in their resistance to the ordinary carbohydrate-splitting enzymes. Each substance breaks down on hydrolysis into reducing sugars, a part of which, at least, is peculiar to itself. The Type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acidic properties, while of the other substances, the Type II is a dextrorotatory weak acid and the Type III a levorotatory strong acid. Especially striking is the

TABLE 1

Soluble specific substances of the three fixed antigenic types of pneumococcus and of Friedländer bacillus (Type B)

TYPE	$[\alpha]_D$	ACID EQUIVALENT	C	H	N	REDUCING SUGARS ON HYDROLYSIS		HIGHEST DILUTION GIVING PRECIPITATE WITH HOMOLOGOUS IMMUNE SERUM
						calculated as glucose		
I	+300°		43.3*	5.8	5.0†	28	(Galacturonic acid) (Amino sugar derivative)	1:6,000,000
II	+74°	1250	45.8	6.4	0.0	70	Glucose	1:5,000,000
III	−33°	340	42.7	5.3	0.0	75	Aldobionic acid, glucose	1:6,000,000
Frdl. bac.	+100°	685	44.6	6.1	0.0	73	Glucose	1:2,000,000‡

* Theory for $(C_6H_{10}O_5)_n$: C, 44.4 per cent; H, 6.2 per cent.

† Amino N, 2.5 per cent.

‡ Rabbit antiserum.

occurrence of specific substances of such widely differing properties in microörganisms as closely related as are the three fixed types of Pneumococcus.

With the possible exception of the Type III substance it is doubtful whether in their present state of purity, each of the specific substances represents a definite chemical compound. However, in the case of the three fixed types of Pneumococcus three totally distinct polysaccharides have been isolated from cultures grown in the same medium. Successive preparations

of each specific substance have been quite uniform regardless of the widely different methods employed in the process of purification. Moreover, substances reactive to the same degree with homologous antisera have been derived both from the microorganisms themselves and from autolyzed broth cultures. Furthermore, the only one of these substances hitherto investigated in detail, namely that of Type III pneumococcus, appears to differ in its structure from that of any other known nonnitrogenous polysaccharide.

It is thought that these and other considerations based on the data presented warrant the belief that the three polysaccharides isolated represent the actual specific substances, stripped of at least a large portion of accompanying impurities, and that they do not merely represent inert material carrying an extremely minute amount of the true specific compounds. If this be admitted, it affords a chemical corroboration of the fact of type specificity in Pneumococcus, in the discovery of which only biological methods had been used (5).

An extension of the methods of isolation used above to the Friedländer bacillus resulted in the preparation from agar cultures of the so-called "E" strain of this bacillus (now known as Type B (12)) of another polysaccharide with specific properties of the order possessed by the soluble specific substances of the three fixed types of Pneumococcus (13). After repeated fractionation by precipitation with alcohol, then with barium hydroxide, and finally with alcohol in the presence of hydrochloric acid, it was obtained as a nitrogen-free strong acid with an equivalent value of about 685, sparingly soluble in water after drying, but yielding soluble alkali salts. The specific optical rotation is $+100°$.

A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, or phosphotungstic acid, but yields precipitates with barium hydroxide and with both neutral and basic lead acetates. It gives no color with iodine. The substance thus differs markedly from the material isolated from the Friedländer bacillus by Toenniessen (3) and Kramár (4), as this gave a red color with iodine and yielded galactose on hydrolysis.

The polysaccharide itself is non-reducing, but on hydrolysis with mineral acid yields reducing sugars, among which glucose was shown to be present by isolation of its osazone and by oxidation to saccharic acid.

From Table 1 it will be seen that this substance greatly resembles that of Type II pneumococcus, and the similarity was found to extend even to precipitation of Type II antipneumococcus serum. The Type II substance in its present state of purity, however, is a weaker acid and is not precipitated by barium hydroxide or neutral lead acetate. It is possible that these differences depend only on the presence of different impurities in the two cases, but opposed to this view are the findings that different preparations of each substance, purified by widely different methods, showed great constancy in each case, and that the absorption of agglutinins and precipitins is not reciprocal with the two organisms. That is, Type II antipneumococcus serum, absorbed with Type B Friedländer bacillus, lost its power to precipitate the Friedländer soluble substance, but still precipitated the Type II pneumococcus substance. Similarly, the Type B Friedländer antiserum, absorbed with Type II pneumococcus, precipitated the Friedländer specific substance, but not that of Type II pneumococcus. If the fact that bacteria possess mutual absorptive capacity be accepted as the criterion of their antigenic identity then the failure of the organisms in question to exhibit this property may be taken as further evidence of the lack of identity of the substances involved.

Granted a chemical difference between the two specific substances, it becomes necessary to account for their marked immunological similarity. In the absence of further evidence as to the structural relations of the two substances, which can only be obtained when large amounts of material become available, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.

A striking and probably analogous example of common anti-

genic properties in substances of remote biological origin is furnished by the phenomenon of heterogenetic specificity originally described by Forssman (14), who showed that following the injection of animal tissues of unrelated species common hemolytic antibodies for sheep corpuscles appear. Landsteiner (15) and Taniguchi (16) have shown that such heterogenetic antigens consist of two component parts, one a protein, the other probably a lipoidal substance. Landsteiner and Simms (17) have found that the lipoid constituent, although itself practically devoid of antigenic properties, acquires true antigenicity when combined with protein, and that the antibodies thus induced react with the isolated lipoid fraction.

The fact that two biologically unrelated organisms, Pneumococcus Type II and Friedländer's bacillus (Type B), possess certain similar serological and antigenic properties suggests that examples of heterogenetic specificity likewise occur among bacteria. In the case of the bacteria hitherto studied, however, the specific substance appears to be a polysaccharide, instead of a lipoid, and it further appears probable that when the analogous specific polysaccharides of otherwise totally unrelated microorganisms correspond sufficiently in chemical constitution an immunological correspondence also results.

From another strain of Friedländer's bacillus, the type of which has not yet been identified, Mueller, Smith, and Litarczek (18) have isolated carbohydrate-containing material with a nitrogen content of 1.3 per cent, and showed that this product at high dilutions caused precipitation of homologous immune serum.

A polysaccharide with specific properties has also been isolated from the tubercle bacillus and somewhat similar products have been obtained by widely differing methods, on the one hand by Laidlaw and Dudley (19) and on the other by Mueller (20). In the former case the defatted bacilli were treated with weak alkali, and the portion not precipitated by acetic acid or copper sulfate was adsorbed on uranium hydroxide and freed from accompanying glycogen by repeated fractionation with alcohol, while in the latter case the culture concentrate, freed from nucleic acid derivatives, was treated with "antiformin" and fractionated

under varying conditions from alcohol. In both instances a product giving the reactions of a pentose-containing polysaccharide was obtained, dextrorotatory and practically nitrogen-free, and reacting at high dilutions with the sera of animals immunized to the tubercle bacillus. While the material obtained by Mueller showed $[\alpha]_D$ $+24°$ in acid solution and yielded 99 per cent of reducing sugars on hydrolysis, that of the British workers showed $[\alpha]_{Hg\ green}$ $+79°$ and 50 per cent of reducing sugars on hydrolysis. In neither case were the actual sugars identified. The carbohydrate itself appears responsible for the precipitin reaction given by the complex mixture known as tuberculin.

Specifically reacting material, also apparently carbohydrate in nature, has been isolated from yeast by fractionation with alcohol. The product, closely associated with or identical with the yeast gum, precipitates the sera of rabbits immunized against the yeast (21).

The specific polysaccharides discussed up to the present point were elaborated by microörganisms of the most diverse types. The wide distribution of these substances made it seem not improbable that there might occur among higher plant forms other carbohydrates with specific properties. In a series of tests on water-soluble plant gums samples of gum arabic (gum acacia) were actually found which showed some degree of specific activity (22). By fractional hydrolysis with 1:1 hydrochloric acid it was found possible to remove about one-half of the pentose in combination. The recovered gum showed $[\alpha]_D$ about $-10°$ and a specific activity of 100 to 150 times that of the original material. The interpretation of these findings is deferred until further data are available.

It has thus been shown that microörganisms of the most diverse types, and even a vegetable gum yield individually characteristic polysaccharides which behave not merely as the inert carriers of specific substances of undetermined chemical nature, but react as though themselves actually endowed with immunologically specific properties. Whether or not one accept this view it should be of some interest to consider the immuno-

logical significance of the soluble specific substances and their relationship to other cell constituents of microörganisms.

Initial studies of this phase of the subject have been made in the case of Pneumococcus (8b, 23, 24, 8d). When pneumococci are dissolved, either with the aid of bile or by repeated freezing and thawing, the resulting solution yields a precipitate of so-called "nucleoprotein" on acidification with acetic acid. While probably a mixture consisting largely of nucleoprotein and mucoid, it still possesses definite immunological properties which differ sharply from those of the soluble specific substance.

In the first place the protein is antigenic, since it gives rise to antibodies on injection into animals, while the soluble specific substance, though reacting specifically with antibodies to the highest degree, in non-antigenic, that is, unable by itself to stimulate the production of antibodies when injected into animals.

Moreover, the protein isolated from any of the three fixed types of Pneumococcus, or from a strain of the heterogeneous Group IV, appears serologically the same as that from any of the other types, for not only does it precipitate antisera to the protein of the other types as well as its homologous antiserum, but its homologous antiserum also reacts equally well with the protein isolated from the other types of Pneumococcus. Thus this portion of the pneumococcus protein is not type-specific, like the soluble specific substance, but is rather, species-specific.

Before considering further the immunological characters of these two cellular constituents of Pneumococcus it may add to the clearness of the discussion to picture the form or pattern of the cell as it relates to the disposition of these substances. Many of the immune reactions participated in by microbes are presumably surface phenomena, and the nature of the reactive material at the periphery of the cell may determine the readiness of response and even the specificity of reaction. Pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic, or outer, layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type-specific substance. On the other hand,

the endoplasm, or somatic substance, consists largely of protein which, as previously pointed out, is species- and not type-specific. This protein is possessed in common by all pneumococci while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends. Thus the structure and, as will be pointed out, the morphological integrity of the cell are determinative factors in bacterial specificity.

Immunization with dissolved pneumococci results in the formation of antibodies which precipitate the pneumococcus protein, but not the soluble specific substance. The fact that this specifically reactive carbohydrate is non-antigenic when separated from the other cellular constituents and is capable of inciting antibody formation only in the form in which it is present in the intact cell, forces the conclusion that in the latter instance it exists not merely as free carbohydrate but also in combination with some other substance which confers upon it specific antigenic properties. Immunization with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells but precipitate solutions of the carbohydrate isolated from pneumococci of the homologous type. How the specific polysaccharide is combined in the cell, whether with protein or some other constituent is not yet clear, but it is evident that the compound thus formed is the dominant and essential antigen of the cell, and the one responsible for type specificity. It would also appear to be readily dissociated into its components when the cell is dissolved.

The immunological relationships of the protein and carbohydrate fractions of the cell are graphically presented in Table 2, in which S represents the soluble specific substance (carbohydrate) and P the protein of Pneumococcus.

It is thus evident that morphological dissolution of pneumococci is accompanied by antigenic dissociation, for sera prepared from filtered solutions of disintegrated cells free of formed elements fail to exhibit any of the dominant type-specific prop-

erties which characterize sera obtained by immunization with whole bacteria. Morphological integrity of the bacterial cell, therefore, is requisite for the expression of its full antigenic power, and the character of the antibody response is determined by the nature of the cell material used for immunization. The injection of suspensions of pneumococci into animals induces the formation of antibodies against S alone or against both S and P separately, depending upon whether or not these suspensions contain only intact cells or a mixture of both intact and dissolved

TABLE 2

PNEUMOCOCCUS AND CELL CONSTITUENTS		ANTIBODIES DEMONSTRABLE IN SERUM						
Material used for immunization	Effective antigen	Agglutinins	Precipitins		Complement fixation		Specificity	
			S	P	S*	P	Type	Species
Intact cells (SP)†	(SP)	+	+	−	+	−	+	−
Carbohydrate S‡	None	−	−	−	−	−	−	−
Protein P§	P	−	−	+	−	+	−	+
Solutions, extracts containing free S and free P	P	−	−	+	−	+	−	+
Suspension of intact cells and dissociated cell constituents (SP), free S, free P	(SP),P	+	+	+	+	+	+	+

* = Free S, as antigen, does not fix complement with immune horse serum; is active with immune rabbit serum (unpublished data obtained by E. Vollmond).

† (SP) = Carbohydrate and protein combined, antigen of cell.

‡ S = Free carbohydrate, the soluble specific substance of cell.

§ P = Free protein of cell.

cell bodies. Since pneumococci readily undergo autolysis and dissolution, suspensions and indeed cultures of these organisms almost invariably contain not only formed elements, but also more or less of dissociated cell constituents in solution. Therefore, use of suspensions of pneumococci containing both intact cells and the soluble products of cell disintegration yields on immunization not only type-specific antibodies but antibodies reacting with the protein substance which is common to all pneumococci. While the former generally predominate it is the

presence of this protein antibody with its broader zone of activity which is responsible for the confusing cross-immunity reactions occasionally encountered in supposedly type-specific sera, and which has in some instances led workers even to deny the existence of distinct antigenic types of pneumococci. That the two sets of antibodies involved are separate and distinct is shown by absorption tests: the antiprotein reacting bodies in such sera can be removed by absorption with the protein of a heterologous type without diminishing either the titer of specific agglutinins for the homologous culture or the precipitins for the specific polysaccharide of the corresponding type.

A practical result of the above analysis is the clarity with which the conditions are defined for the production of the most sharply differentiated type-diagnostic sera so important in the study and recognition of the etiological agents of pneumonia in human beings. By the application of the knowledge gained from this study it has also been possible for the first time to classify serologically the bacilli of the Friedländer group and to separate them into three main antigenic types and a heterogeneous group, much as in the case of Pneumococcus (12). An understanding of the immunological behavior of the non-hemolytic streptococci has also been facilitated by the same type of analysis of the specific factors involved (25).

The elaboration of the type-specific carbohydrate is a specialized function most active in pneumococci rapidly multiplying in the animal body or in suitable culture media. Under these conditions pneumococci exhibit maximal capsular development, exalted virulence, and distinct type specificity. Without further supporting evidence it would of course be hazardous to venture the assertion that all three of these characters are necessarily and causally related to the S-producing function of the cell. However, it is now known (26) that under certain cultural environments pneumococci lose their type specificity and become avirulent, and that these changes are accompanied by a loss of the capacity to elaborate this soluble specific substance.

In 1917 Cole (27) observed that infected exudates and sera containing soluble reactive substances of pneumococci possessed

the property of neutralizing pneumococcus antibodies, and pointed out the significance of this fact in relation to the therapeutic administration of immune serum. Moreover it has recently been reported that the addition of the soluble specific substance of pneumococcus in very small amounts exerts a definite and specific effect in annulling the inhibitory action of the sera of naturally resistant animals on the growth of pneumococci in serum-leucocyte mixtures (28). It has also been reported (29) that injections of the soluble specific substance lower the resistance of mice to infection with partially attenuated pneumococci.

If then, final proof be brought for the conception that the capsular zone of the organism is largely composed of this carbohydrate substance, is part of the defense mechanism of the cell, and is the site of its initial contact with antibody, then these soluble bacterial polysaccharides acquire new significance not only in the serological reactions of the cell, but in the actual processes of infection and immunity in the host.

The specifically reacting polysaccharides also offer much of chemical interest. While it had generally been assumed that only proteins and their derivatives offered the innumerable opportunities of isomerism and subtle changes requisite for substances exhibiting the phenomena of specificity, the discovery of carbohydrates with specific properties is not as astonishing as might appear on first thought. When one considers the number of asymmetric carbon atoms in the pentoses and hexoses, the different possible points of attachment of the lactone bridge, the possibility of α- and β- glucosidic unions at various points in the molecule, and the addition of sugar acids, the analogs of amino acids, to the large number of sugars theoretically capable of entering into the composition of such polysaccharides, it becomes clear that perhaps only among the carbohydrates could another sufficiently large and protean group of substances be found to afford the possibility of specific manifestations.

The development of the field thus opened has barely begun, and it would therefore be idle to speculate on the relationship within this group of specificity to chemical constitution. That

some such relation exists, however, and is reflected by marked chemical changes, has been shown by the isolation of three such different sugar derivatives as the three soluble specific substances of Pneumococcus from three such closely related microörganisms as Types I, II, and III pneumococcus. Also significant is the finding that, even in the case of microörganisms of widely differing origin, such as Pneumococcus Type II and the Type B Friedländer bacillus, a certain similarity in the chemical nature of their specific polysaccharides is accompanied by a corresponding similarity in the immunological properties of the organisms themselves.

So far but one specific polysaccharide, that of Type III pneumococcus, has been investigated in detail, and it is too soon to form a judgment as to whether or not the polysaccharides with specific properties constitute a chemically separate and distinct group of carbohydrates. In this one instance the structure certainly appears different from that of any known non-nitrogenous sugar derivative, although an analogy to the nitrogen-containing chondroitin sulfuric acid (30) is discernible, and perhaps even a distant relationship to the pectins (31). In this connection the finding of a specifically reacting polysaccharide as a constituent of gum arabic is of interest, and it is not impossible that sugar derivatives with specific properties may occupy a position of biological significance among higher forms of life as well as among bacteria.

Finally it must again be emphasized that it is for the future to determine the validity, the generality, and the utility of the deductions which have already been drawn and the inferences which seem possible. The work done thus far, however, has not only brought to light new substances of chemical interest, but has pointed the way to an understanding of confusing phenomena in the immunological relationships of at least three microorganisms of pathogenic importance. The hope that its possibilities are not yet exhausted would, therefore, seem not unduly bold.

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