Respiratory Diseases.

I. Chemical and Immunological Relationships of Cell Constituents of Pneumococci.

(Dr. Avery, Dr. Heidelberger, Dr. Tillett, Dr. Goebel and Dr. Julianelle.)

(a) Chemical Studies on the Soluble Specific Substance of Pneumococcus.

With the resumption in September of active work on the soluble specific substance of Pneumococcus, a study of Type I was begun and at the same time the further purification and characterization of the soluble specific substances of Types II and III were attempted.

In the first place it was found possible to remove all of the nitrogen from the Type II and III specific substances. The Type II substance was dissolved in strong hydrochloric acid and precipitated fractionally with alcohol. The latter fractions contained an impurity of lower optical rotation, while the analytical figures obtained on the nitrogen-free main portions are given under Nos. 25A, 26A, $25A_1$ and 40 in Table I. These products were also free from all but unweighable traces of ash.

A portion of preparation 25A was oxidized by means of nitric acid, yielding saccharic acid. Since Glucosazone had previously been isolated from the hydrolysis products, the presence of saccharic acid under these conditions shows glucose to be the sugar unit from which the specific Type II polysaccharide is built up. The molecule, if we may consider it a single substance, appears also to contain an unidentified weak acid with an optical rotation about that of glucose.

Table I

Prepn.No.	$[\alpha]_D$	C°/o	H°/o	N°/o	Acid No.	Reducing Sugars on Hydrol.	Mol.Wt.	Highest diln. giving ppt. with immune serum.
Type II					Type II			
25A	+70.2°	45.8	6.4	0.0	1302	68.4°/o	1950	1:5,000,000
25C	+72.2°	-	-	0.12	1200	67.6°/o	-	"
26A	+74.0°	-	-	0.0	1240	68.0°/o	-	"
$26A_1$	+72.8°	-	-	0.0	1250	68.2°/o	-	"
40	+73.0°	-	-	0.0	1112	73.°/o	-	"
					Type III			
Type III								
30^I	-31°	-	-	0.0	347	73.3°/o	-	1:6,000.000
30A	-35.0°	-	-	0.0	340	71.0°/o	-	1:5,000,000
33^{II}	-34.0°	-	-	0.0	340	75.5°/o	-	"
$33^{II}{}_B$	-31.0°	-	-	0.0	355	73.0°/o	-	"
					Type I			
Type I					Amino N.			
29B	+301°			5.0	2.7	31.8°/o		1:3,000,000
36	+305°	43.3	5.3	4.8	2.4	27.2°/o		1:6,000,000
37	+295°			4.5	2.6	27.2°/o		1:4,000,000
37B	+307°			4.1	2.5	29.4°/o		1:6,000,000
39	+304°			4.4	2.5	27.1°/o		1:6,000,000

A molecular weight determination gave a value of about 2000, but this was subject to a large error owing to the small observed freezing-point depression.

By the action of pyridine and acetic anhydride the Type II substance was converted in 80 per cent yield into an acetate giving almost theoretical carbon and hydrogen values for a polysaccharide triacetate. (Found C, 50. 4 per cent; H, 5.6 per cent. Calculated: C, 50.0 per cent; H, 5.6 per cent). Unfortunately the acetate was so insoluble that any attempt at crystallization was impossible, as was also a decision as to whether or not it reacted specifically. However, on hydrolysis with dilute alkali, it was reconverted into the soluble specific substance.

The Type II specific substance had previously been precipitated by its antiserum and recovered in part from the precipitate. However, dissociation of the specific precipitate was difficult and the recovered product contained 1 per cent of nitrogen and gave the biuret test. In a repetition of this experiment the immune precipitate was first dissolved by digestion with trypsin, but the specific substance remained firmly bound to the protein degradation products, and it was only after a long and involved purification that the specific substance was recovered, this time in a degree of purity in which it did not give the biuret test, and with only 0.12 per cent of nitrogen. (See 25 Q).

The Type III soluble specific substance, also, was obtained free from nitrogen by repeated reprecipitation as the insoluble free acid. Dilutions of the purified substance as high as 1:3,000,000 reacted with homologous immune serum, giving a final concentration of 1:6,000,000. The other analytical constants were unchanged, as will

be seen by comparing the previously submitted figures with those of Preparations 30 I. and 33II. in Table I. Attempts at further elimination of possible impurities were made (1) by adsorption on aluminum hydroxide, and (2) by precipitation as the insoluble barium salt, in the formation of which the Type III substance differs from that of Type II. In both cases, however, the product recovered agreed in all its properties with the starting material (see 30 A and 33 II B, Table I.) Since the two purification processes differ widely from each other and from the usual method of preparation, it would seem that the Type III substance has been obtained as a fairly definite chemical individual.

When the insoluble form of the Type III specific acid is boiled for several hours with a large volume of water it gradually passes into solution, behaving as if an anhydride, or possible lactone, grouping had been opened. That the change is of some such nature, and that it is reversible, is shown by the fact that when the insoluble substance is dissolved in an excess of alkali the initial rotation of -34° gradually drops to -17°, slowly returning again to almost the original value after the solution is again acidified.

Regarding the constitution of the Type III specific substance, it has been found possible to identify glucose as one of the products of hydrolysis. This has been isolated both as the phenylosazone and the p- bromophenylosazone. Another product of hydrolysis, obtained in larger amount than the glucose, is a strong acid showing the properties of glucuronic acid, but it has not yet been positively identified.

Just as the Type II and Type III specific substances differ

markedly from each other in chemical properties, so the Type I substance differs from the other two. It appears to be a highly dextrorotatory, nitrogen-containing, non-reducing, amphoteric polysaccharide, insoluble at its isoelectric point. It forms soluble salts with both acids and bases but the acid salts dissociated very readily, showing the substance to be a weak base. So far it has not been possible to find a record of the natural occurrence of any other substance with these remarkable properties.

In the isolation of the Type I substance it was found necessary to modify the method of purification used in the other two cases. In separating the substance from the accompanying glycogen and other impurities advantage was taken of its insolubility in dilute acetic acid and of the formation by it of an insoluble barium salt. After removal of the barium the hydrochloride of the specific substance was repeatedly precipitated from alcohol and finally converted into the insoluble, isoelectric form by dialysis.

The product obtained in this way (39, Table I) agrees in its properties with preparations in which the purification by barium was omitted (29B, 36, 37) and with a preparation purified by adsorption on aluminum hydroxide (37B), indicating that it has been obtained in fairly pure condition. From Table I it will also be seen that its specific activity is manifested at as high dilutions as that of the other two type substances.

Regarding the constitution of the Type I specific substance, little can as yet be said. Of the 4.4 per cent of nitrogen in the substance in its present state of purity, 2.5 per cent appears as amino nitrogen by the Van Slyke method, but at the same time the molecule

(if the material available be assumed a single substance) is split by the nitrous acid used, reducing sugars are formed, and the specific reaction vanishes. When the Type I substance is oxidized by nitric acid, mucic acid is formed. The substance also gives the color tests of glucuronic acid, which would, however, give saccharic acid on oxidation, so that it is possible that in its galacturonic acid (which would yield mucic acid on oxidation) is combined through the reducing group with the amino group of an amino sugar acid or acid amide.

Attempts at the more rigorous purification of the substance and the elucidation of its constitution are under way.

A summary of the knowledge at present available of the three type specific substances of the Pneumococcus is given in Table II:-

Table II.

Type	Optical Rotation.	C	H	N	Acd. No.	Reducing Sugars on Hydrolysis.		Mol. Wt.	Highest Diln. giving ppp. with spec. immune serum.
I	+300°	43.3*	5.8	4.4	(270)	27%	(Glacturonic Acid) Amino Sugar Derivative.)		1:6,000,000
II	+73°	45.8	6.4	0.0	340	70%	Glucose	Not 1950	1:5,000,000
III	-33°	42.6	5.6	0.0	1250	75%	Glucose (Glucuronic Acid	-	1:6,000,000

*Theory for $(C_6H_{10}O_5)_X$: C = 44.4 per cent; H = 6.2 per cent

Amino N: 2.5 per cent

(b) Immunological Significance of the Various Cell Constituents of Pneumococcus.

The facts acquired by these joint studies have brought to light new, and significant facts concerning the relationship between the chemical constitution and biological specificty of the bacterial cell. A summary of this work as it relates to the problem as a whole is presented at this time instead of the usual individual reports of the several members of the Staff who have participated in some particular phase of the work.

In an investigation of the immunological relationships of the cellular substances of pneumococcus, two constituents of the cell have thus far been studied. One of these, is the substance precipitated from solutions of pneumococcus by dilute acetic acid, which, although comprising a mixture of other proteins and mucoid may, for the purposes of the present discussion, be referred to as bacterial nucleoprotein. The other constituent, non protein in character, is the so-called soluble specific substance, which is now known to be a carbohydrate of the polysaccharide type. While it is realized that these two substances, important as they are, do not comprise the whole antigenic mosaic of the cell, they are emphasized at this time because they happen to be the first chosen for investigation, and the only ones thus far studied.

Studies of the antigenic and serological properties of these two chemically diverse substances as they exist together in the intact cell and free in a dissociated state, have yielded certain facts concerning the differentiation of the specific types of pneumococci, and regarding the relationship between chemical constitution and bio-

logical specificity of the cell as a whole. For not only do these cellular compounds belong to two wholly different classes of chemical substances, namely protein and carbohydrate, but the carbohydrate constituents of the three fixed types of pneumococci have been found in each instance to consist of polysaccharides of correspondingly different structure. These latter substances possess in addition the unique distinction of reacting specifically with antibacterial serum of the homologous type, although in the free state dissociated from the cell they are devoid of the power of inciting the formation of antibodies upon injection into animals. That is, they are specifically reactive substances but are not true antigens. The protein on the other hand, is antigenic; the serum of an animal immunized with this substance reacts with protein derived from any type of pneumococcus. It is at once obious that as these two components exist in the cell they form an antigenic complex which is different from that exhibited by either substance alone. The serological differentiation of the various types of pneumococci is related therefore not only to chemical and antigenic differences in these component substances, but is associated with the structural character and morphological integrity of the bacterial cell.

Structure of the Cell. Before considering in detail the immunological characters of these two cellular constituents, 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. For undoubtedly cell configuration reflects in some measure the ease with which this organism participates in immunity reactions and the avidity with which it interacts with antibody. Many of these reactions are

surface phenomena, and the nature of the reactive material at the periphery of the cell determines 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 layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type specific substance of pneumococcus. 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. In design, therefore, the cell may be conceived of as so constituted that there is disposed at its periphery a substance which is highly reactive and upon which type specificity depends. The structure and, as will be pointed out later, the morphological integrity of the cell are determinative factors in bacterial specificity.

Chemistry of Specific Substance. Study of the chemistry of the soluble specific substance derived from the three fixed types of pneumococcus has shown that in each instance a distinctly different substance, polysaccharide in nature, is involved. While it is still premature to consider the products obtained as pure chemical compounds, the following data briefly summarize the present state of our knowledge concerning them: the soluble specific substance of Type II pneumococcus appears to be a weakly acidic nitrogen-free polysaccharide made up chiefly of glucose units. Its specific optical rotation is about +74°, and its molecular weight is not less than 2000. In the present state of purity it reacts in dilution of 1:5,000,000 with antibacterial

serum of Type II pneumococcus and does not react with Type I or Type III antisera. The soluble specific substance of Type III pneumococcus is also a nitrogen-free polysaccharide, but differs from the Type II compound in that it rotates the plane of polarized light about 33° to the left and appears to be made up of glucose and glucuronic acid units. It is a strong acid, and separates in insoluble form from solutions strongly acidified with hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with antipneumococcus serum of the homologous type. The Type I soluble specific substance differs from the other two in containing nitrogen as an apparently essential component. In its present state of purity the nitrogen content is 4.4 per cent and the specific rotation +300°. Since the carbon and hydrogen content are close to the theoretical values for polysaccharides, and since reducing sugars appear when the substance is treated with nitrous acid in the determination of the amino nitrogen present, it appears likely that a nitrogenous sugar derivative is involved. The substance is both an acid and a base, and is very sparingly soluble in water at the isoelectric point. In the specific precipitin reaction with homologous antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000.

Although the specific polysaccharide by itself evokes no antibodies upon injection into rabbits, it is specifically reactive with antibody induced by immunization with intact cells. The fact that this specifically reactive carbohydrate is non-antigenic when dissociated 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 as free carbohydrate but in combination with some other substance which confers upon it specific antigenic properties. For, 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.

The immunological relationships of the protein and carbohydrate fraction of the cell are shown in Table I and a graphic presentation of the facts is presented in Fig.I. in which "S" represents the specific soluble substance (carbohydrate) and "P" the protein of the pneumococcus. The symbols used are in no sense interpretative of the mechanism involved, but serve simply to visualize the interaction between these cell constituents and their respective antibodies. The reaction illustrated may be briefly summarized as follows:

A. (Fig. I,A). Immunization with intact cells of virulent type-specific pneumococci give rise to the presence in serum of antibodies which are type specific; such sera specifically agglutinate the homologous type of pneumococcus, protect mice against virulent organisms of same type, and precipitate solutions of the corresponding purified carbohydrate substance. These sera are distinctly type specific. They do not precipitate solutions of pneumococcus protein - (Table I).

B. (Fig 1,B). The specific polysaccharide (S) when isolated from the cell is incapable of inciting antibody formation upon injection into animals. The isolated protein (P), on the other hand, is antigenic and gives rise to an immune serum which reacts with pneumococcus protein regardless of the type from which it is derived. Antiprotein sera do not agglutinate type specific strains of pneumococci and do not precipitate solutions of the soluble specific substance (S) (Table I).

C. (Fig. I,C). Solutions and extracts of pneumococci behave antigenically precisely as do solutions of pneumococcus protein; the dissociation of the antigenic complex which occurs whenever the cell is dissolved results in the liberation of free carbohydrate (S) and free protein (P) in solution. In such solutions and extracts the only constituent which functions as antigen is the protein, for free "S" is non-antigenic. The sera of animals immunized with solutions of pneumococci in which complete dissociation of these cell constituents has taken place, contain only P antibodies, and they exhibit the same reactions as do sera prepared by immunization with protein alone (See Table I.)

It is evident from these facts that morphological dissolution of pneumococci is accompanied by antigenic dissociation, for sera prepared from solutions of disintegrated cells fail to exhibit any of the dominant specific properties which characterize sera obtained by immunization with whole bacteria. Morphological integrity of the bacterial cell, therefore, is requisite for production of type specific antisera. (Compare (a) and (c) Fig.I.)

Pneumococcus and cell constituents		Antibodies demonstrable in serum							
Material used for immunization	Effective antigen	Precipitins		Complement fixation		Agglutinins	Protection in mice	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	+	+	+	+	+	+	+	+

†[SP] = Carbohydrate and protein combined antigen of cell

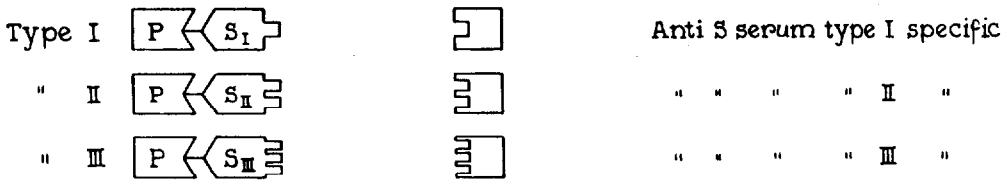
‡S = Free carbohydrate, the soluble, specific substance of cell

‡P = Free protein of cell

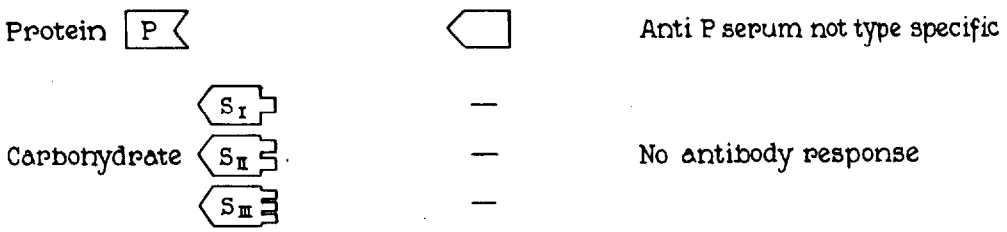
* Free S, as antigen, does not fix complement with immune horse serum; is active with immune rabbit serum

Immunological relationships of protein and soluble specific substance (carbohydrate) of pneumococcus

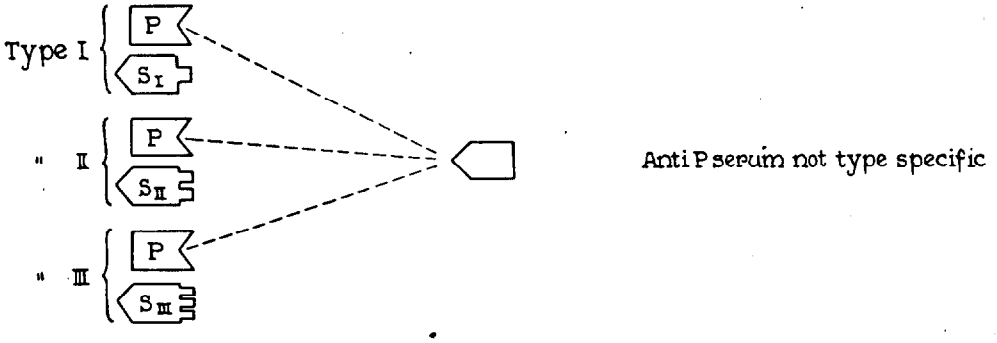
A. Intact cell [PS]



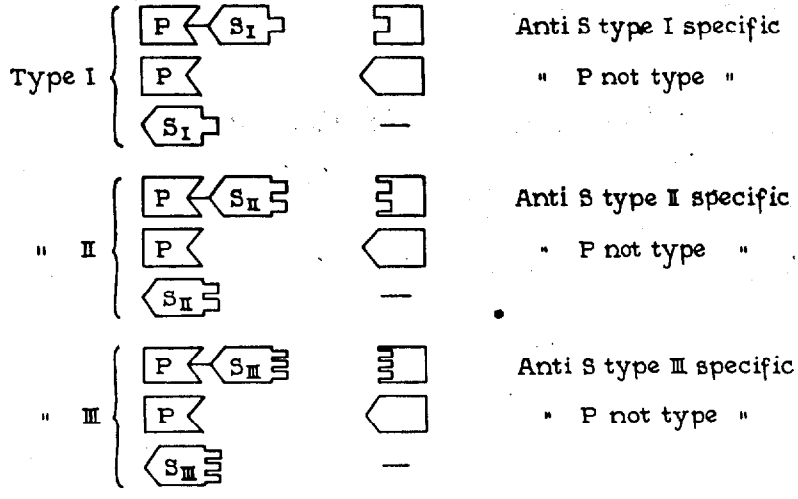
B. Isolated constituents of cell [P], [S]



C. Solutions of pneumococci in which complete dissociation of protein and carbohydrate occurs [P], [S]



D. Suspensions of pneumococci containing intact cells and dissociated cell constituents [PS], [P], [S]



D. (Fig. I,D). It becomes obvious, therefore, that the character of the antibody response is determined by the nature of the 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 suspnnsions contain only intact cells or a mixture of both intact and dissolved cell bodies. Since pneumococci undergo autolysis and dissolution readily, suspensions and, indeed, cultures of these organisms almost invariably contain not only formed elements, but more or less dissociated cell constituents in solution. The predominance of the former in such suspensions stimulates the production of the type specific "S" antibodies, while the occurrence in these same suspensions of dissociated cell protein, provokes the formation of the non-specific protein anti-bodies, (especially if the immunization is carried on for a long period of time as is usually the case in the production of antipneumococcus horse serum.) The 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 a protein substance which is common to all pneumococci. 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. That these two antibodies 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 the titre of specific agglutinins for the homologous culture, or the precipitins for the specific polysaccharide of corresponding type and without loss in the protective value of the serum.

were found in the heart. In one case the rectus muscle presented degeneration of localized groups of fibres. The nuclei were indistinct, the striations lost, and the protoplasm collected into granules which took the eosin deeply.

Sections of the lymph glands, adrenals, and intestines have shown no lesions of note.

V. Purification of Pneumococcus Antibodies.

(Dr. Heidelberger and Dr. Avery).

An attempt has been begun this year to purify the antibodies in anti-pneumococcus serum, bearing in mind both the practical desirability of removing as much unnecessary protein as possible from serum before its administration, and also the solution of the question "To what group of chemical substances do antibodies belong?"

For purposes of orientation a beginning was made by repeating the work of others in this field. Without going into the details of the numerous experiments which yielded negative results, it may be said that in general the ordinary methods used for antitoxin purification did not give a sharp separation of pneumococcus antibodies from the other serum proteins as had previously been determined in 1915, (Avery, J. Exp. Med., xxi, 133,(1915.)) Of the numerous protein precipitants tried, only picric acid gave any evidence of selectivity, and experiments with this substance are being continued.

Most of the work so far, however, has been carried out with modifications of the method employed by Felton (Boston Med.and Surg.J., 1924, 819.) It was necessary to work out the optimum conditions independently, as the directions given by Felton are too vague to be of value. It was found that if Type I anti-pneumococcus serum is precipi-

tated by addition of one volume to 19 volumes of 1/100 potassium dihydrogen phosphate solution, about 90 per cent of the protective antibodies are carried down in the insoluble globulin precipitate. The buffer salt used gives the solution an initial pH of about 4.6, which changes to 5.2 - 5.5 after addition of the serum, thus approximating the isoelectric point or point of lowest solubility of the euglobulin-pseudo-globulin complex with which the antibodies are associated. This fraction can then be redissolved in saline solution and reprecipitated in the same way, resulting in the removal of inactive soluble protein without loss of antibody content. After the precipitate is dissolved again in saline it is found that the dissolved precipitate preserves about 90 per cent of the protective value of the serum, while fully 90 per cent of the total serum protein has been eliminated. For example, New York State Type I antipneumococcus serum Number 267, containing 72 mg. of protein per cc., yielded a concentrate containing 5 mg. of protein per cc. when made up to the original serum volume. In doses of 0.05 cc. this concentrate gave as effective protection as did the original serum, while the remaining serum fraction had only a very weak protective action. It would appear, therefore, that Felton's method in this form is a very rapid and convenient procedure for preparing from anti-pneumococcus serum a crude antibody solution. Further work on this problem is in progress.

VI. Pathogenesis of Pulmonary Infections.

(Dr. E. G. Stillman. Dr. Branch.)

As stated in the previous report, experimental pneumococcus lobar pneumonia has been successfully produced in mice by the inhalation method. In order to produce localization of the infection, partially