

- Van Slyke, D.D., Gasometric determination of urea with urease. Proc. Soc. Exp. Biol. and Med., 1925, xxii, 486-487.
- Hiller, Alma, Linder, G.C. and Van Slyke, D.D., The reducing substances of the blood. J. Biol. Chem., July, 1925, lxiv, 625-638.

In addition the following are in press.

- Hastings, A.B. and Sendroy, Jr., Julius. Studies of Acidosis, XXI. Colorimetric determination of urinary pH.
- Hastings, A.B., Murray, C.D. and Sendroy, Jr., Julius. The first and second dissociation constants of carbonic acid.
- Van Slyke, D.D., Hastings, A.B., Murray, C.D. and Sendroy, Jr. Julius. Studies of gas and electrolyte equilibria in blood. VIII. The distribution of chloride, bicarbonate, and hydrion activity between cells and serum.
- Van Slyke, D.D., Gasometric determination of methemoglobin.
- Van Slyke, D.D., and Vollmond, E., Studies of methemoglobin formation.

Studies concerning acute respiratory disease.

Report of Dr. Avery (assisted by Dr. Heidelberger and Dr. Goebel.)

Chemo-immunological studies of the soluble specific substance of the Pneumococcus and the Friedländer bacillus. It will be recalled that in the last report improved methods of purification of the soluble specific substance of Types II and III pneumococcus were described, as was also the isolation of the analogous substance from the Type I pneumococcus. It was shown that while the products obtained could scarcely be considered pure chemical compounds, widely different methods of purification calculated to remove different types of accompanying impurities failed to change significantly the physical and chemical properties of the substances as isolated. The Type II soluble specific substance thus appears to be a

weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its specific optical rotation is about $+74^\circ$. It reacts at a 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, while also apparently a nitrogen-free polysaccharide, differs from the Type II derivative in many particulars. It rotates the plane of polarized light about 33° to the left. It is a strong acid and is made up of units of glucose and either glucuronic acid or some derivative of this acid. It also 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 Type III antipneumococcus serum.

Table I.

Chemical characteristics of the soluble specific substances of
Types I, II and III Pneumococcus.

Type	Optical rotation	C	H	N	Acid equivalent	Reducing sugars on hydrolysis	Highest dilution giving ppt. with homologous immune serum.
I	$+300^\circ$	43.3*	5.8	5.0†		28 (Galacturonic acid. (Amino sugar derivative.)	1:6,000,000
II	$+74^\circ$	45.8	6.4	0.0	1250	70 Glucose	1:5,000,000
III	-33°	42.6	5.6	0.0	340	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.

The Type I soluble specific substance, on the other hand,

while also polysaccharide in nature, differs from the other two type-specific substances in containing nitrogen as an apparently essential component. 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 when the substance is treated with nitrous acid. Reducing sugars appear at the same time and the specific reaction vanishes, and since the carbon and hydrogen content are close to the theoretical values for polysaccharides it appears likely that a nitrogenous sugar derivative is involved. It is a strong acid and a weak base, and is very sparingly soluble in water at the isoelectric point. Its specific optical rotation is $+300^\circ$, and on oxidation with nitric acid it yields mucic acid. In the specific precipitin reaction with homologous antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000.

In Table I are summarized the available data concerning the chemical differences in the polysaccharide derivatives of pneumococci of Types I, II and III.

The three polysaccharides contain no sulfur or phosphorus and differ from the starch-glycogen group of carbohydrates in giving no color with iodine and in their resistance to the ordinary carbohydrate-splitting enzymes.

It would be of course idle to assume that 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 substances have been isolated from cultures grown in the same medium.

It is believed 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 accepted it may be concluded that the soluble specific substance of each of the three fixed types of pneumococcus is a distinct chemical substance, differing in many striking particulars from the corresponding product elaborated by the other two types, but having in common the properties of polysaccharide structure and of resistance to enzyme action. Each substance breaks down on hydrolysis into reducing sugars, part of which, at least, are peculiar to itself. The Type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acid properties, while of the other two substances, the Type II is a dextro-rotatory weak acid and the Type III a levo-rotatory strong acid. Especially striking is the occurrence of specific substances of such widely differing properties in microorganisms as closely related as the three fixed types of Pneumococcus.

The immunological significance of the specific substances has been discussed by the writers in the last report and has been presented in a recent paper and will therefore not be touched upon at present.

While many of the questions raised in the course of the work are still under investigation it seemed desirable to extend the work to the search for analogous substances in other microorganisms. The Friedländer bacillus was accordingly chosen for study because of the relative abundance of its capsular material and on account of its occasional co-

currence in human pneumonia. At first a strain, referred to hereafter as the E strain, and recovered from a spontaneous epidemic of pneumonia in guinea pigs was used, and this was later found to be serologically related to other strains recovered from human and animal sources.

By procedures essentially the same as those used in the case of the pneumococcus, a nitrogen-free, ash-free polysaccharide with specific properties of a most unusual nature was readily obtained. It differs from the Friedländer carbohydrate reported by Toennissen in giving no color with iodine, and from the specific material obtained by Mueller, Smith, and Litarczek in being nitrogen-free. It is a 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 $+100^\circ$. The polysaccharide itself is non-reducing, but on hydrolysis with mineral acid yields reducing sugars, among which glucose has been shown to be present both by isolation of glucosazone and by oxidation to acid potassium saccharate. The properties of successive preparations are given in Table II.

Table II.

Soluble specific substance of the Friedländer Bacillus (E).

Preparation No.	[α] D	Acid equivalent	Ash	C	H	N	Percentage of reducing sugars on hydrolysis	Highest dilution giving a precipitate with immune serum	
								Anti-Friedländer serum E.	Anti-pneumococcus serum Type II
101	+100.0°	670	0.0	44.61*	6.08	0.0	73.0	1:2,000,000	1:2,000,000
103 I	+102.5°	674	0.0			0.0	72.4	1:2,000,000	1:2,000,000
103 II	+100.0°	704	0.0			0.0	70.0	1:2,000,000	1:2,000,000
104	+100.0°	722	0.0			0.06	73.0	1:2,000,000	1:2,000,000
104 A	+100.0°	685	0.0			0.0	72.0		
105 A	+100.0°	706	0.0			0.66	72.0	1:2,000,000	1:2,000,000
105 B†	+101.5°	674	0.0			0.2	73.0		
105 Ad	+100.0°	716	0.0			0.0	78.0		
25 A	+70.2°	1302	0.35	45.8	6.4	0.0	68.4		1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$ C = 44.4, H = 6.2

† This represented a residue which failed to pass through a Berkefeld filter.

|| A preparation of Type II pneumococcus soluble specific substance.

In the present instance, as in those of the pneumococci, attempts at further purification by precipitation with uranyl nitrate or adsorption on alumina showed carbohydrate and specific function to

be apparently inseparable. The isolation of a fourth specific substance of this nature adds further weight to the growing mass of evidence that the soluble specific substances of microorganisms are often actually polysaccharides.

While the specific substance of the E strain, as purified up to the present, has properties which set it apart from the three analogous substances of the fixed pneumococcus types, there is nevertheless a certain resemblance to that of the Type II pneumococcus, a resemblance extending even to precipitation with Type II antipneumococcus serum.

A comparison of the specific substance obtained from Type II pneumococcus with that isolated from the E strain of Friedländer's bacillus shows that both polysaccharides rotate the plane of polarized light to the right and yield approximately the same percentage of glucose on hydrolysis. In the case of the Friedländer substance the specific rotation is $+100^\circ$, while the Type II pneumococcus substance rotates the plane of polarized light about $+74^\circ$. Both substances have acidic properties, but the Friedländer specific substance has an acid equivalent approximately one-half that of the Type II polysaccharide. Neither of the two products gives glucuronic acid tests as do the specific substances of Types I and III pneumococcus. Both polysaccharides fail to form precipitates when treated with solutions of silver nitrate, copper sulfate, or phosphotungstic acid; both are precipitated by solutions of uranium nitrate and basic lead acetate. Whereas the Type II pneumococcus specific substance gives no precipitate with either barium hydroxide or neutral

lead acetate, the Friedländer polysaccharide is precipitated by both of these reagents.

Because the two specific substances, although of widely different biological origin, resembled each other so closely in some of their chemical properties, the Friedländer polysaccharide was tested with Type II antipneumococcus serum, and a precipitin reaction was found to occur. On the other hand, there was absence of precipitation when this substance was tested with antipneumococcus serum of the other two fixed types. It then became necessary to determine as far as possible the immunological relationships not only of the soluble substances of the E strain of Friedländer's bacillus and of Type II pneumococcus, but of the microorganisms themselves.

The E strain and other serologically related strains of Friedländer bacillus are agglutinated by Type II anti-pneumococcus serum and not by Type I and Type III antisera, while the Type II pneumococcus is agglutinated by E Friedländer antiserum, but not by the antiserum of a strain of Friedländer bacillus serologically distinct from the E strain. Absorption of E Friedländer antiserum and Type II antipneumococcus serum with the homologous organism removes in each instance the agglutinins for both organisms, while heterologous absorption of either serum removes only the agglutinins for the absorbing organism, leaving the homologous agglutinins scarcely diminished in titer. The soluble specific substances of the two organisms under comparison react at practically as high dilutions in the opposite antisera as in the antisera to the organisms from which they were derived. Precipitin absorption parallels the agglutinin absorption.

The facts brought out by the test-tube reactions of agglutination and precipitation find added confirmation in the more final proof of reciprocal protection against infection in the animal body.

The serum of a rabbit immunized with Friedländer's bacillus (Strain E) was first tested for its power to protect mice against infection with virulent cultures of homologous and heterologous types of the Friedländer bacillus.

The protocol given in Table III shows that 0.2 cc. of the anti-Friedländer serum Type E protected animals against 0.1 cc. of a virulent culture of the homologous strain which without immune serum caused death of the mice in doses of one-millionth cc. On the other hand, the same serum afforded no protection in mice against a virulent heterologous Strain Sc, which, by the reaction of agglutination, was shown to be of another type.

Since the E strain of Friedländer's bacillus was found to be agglutinated and its soluble specific substance to be precipitated by antipneumococcus serum Type II, it was of interest to determine whether protection against infection with a biologically different organism was possible by the use of Type II pneumococcus serum. The results of this experiment are given in Table IV.

It is evident from Table IV that mice inoculated with 0.2 cc. of antipneumococcus serum Type II were effectively protected against at least a thousand lethal doses of the virulent E strain of Friedländer's bacillus. As in the agglutinin and precipitin tests the "specific" nature of this protective reaction is shown by the fact that antipneumococcus serum Type I afforded no protection whatever against infection (Table V).

The anti-Friedländer serum prepared by immunization with Strain E protected mice against a hundred thousand lethal doses of another strain (K), which, by agglutination, was classified as belonging to the same type as E. Similarly antipneumococcus serum Type II afforded protection against the K strain just as it did against the E strain; a fact which emphasizes again the immunological similarity of Friedländer's bacillus of this type to Type II pneumococcus. The same protocol shows that antipneumococcus serum Type I is wholly without protective action against this type of Friedländer bacillus. Moreover, Table VI, brings out the fact that protective power against Type II pneumococcus infection is not possessed by anti-Friedländer serum produced by Strain SC., which both by agglutination and protection has been shown to belong to a type different from the effective Type E strains.

The further data presented in Tables VI, VII, and VIII demonstrate the comparable protective power of antipneumococcus Type II and the anti-Friedländer E sera against infection with virulent Type II pneumococci. Indeed the protective potency of the anti-Friedländer serum in pneumococcus infection in two of the three experiments is greater than, and in the third test, equal to that exhibited by the Pneumococcus Type II immune serum itself. This is all the more striking since in the former case immune rabbit serum was used and in the latter the serum of a horse which had received more intensive immunization.

While comparison of the chemical properties of the two soluble specific substances isolated from Pneumococcus Type II and

Friedländer's bacillus (Strain E) reveals many points of resemblance, differences are also found which are too great to be ignored. That the substances in reality are identical and that the observed differences depend only upon impurities present may possibly be the case, but the evidence so far obtained is entirely opposed to this assumption. It has been pointed out that widely differing methods of preparation, calculated to remove different kinds of accompanying inert matter, have yielded strictly comparable products. This fact cannot be taken to indicate that the specific substances as at present isolated are pure chemical compounds, but it at least makes reasonable the assumption that in each instance a large proportion of the adventitious impurities has been eliminated. The view that the specific substance isolated from Pneumococcus Type II and that recovered from Friedländer's bacillus (Strain E) are not identical is further supported by certain of the serological findings, especially those which show that the absorption of agglutinins and precipitins is not reciprocal with the two organisms. 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.

However, 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 steric configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.

In the case of Pneumococcus it has been shown that the polysaccharides by themselves are not antigenic, and it is believed that they become antigenic only when attached to some other substance, probably the protein of the cell. The type-specific character of the antigenic response, however, is dependent almost entirely upon the nature of the polysaccharide and not upon the substance to which it is attached. Therefore, since the specific carbohydrate substance of the Friedländer bacillus (Strain E) and that of Type II pneumococcus possess in common similar chemical properties the antigenic response to each may also be similar even though the proteins or other substances with which they are combined be quite dissimilar. A discussion of the actual number of antigens and antibodies present must be deferred until more facts are available.

A striking and probably analogous example of common antigenic properties in substances of remote biological origin is furnished by the phenomenon of heterogenetic specificity originally described by Forssman. This investigator showed that following the injection of animal tissues of unrelated species common hemolytic antibodies for sheep corpuscles appear. Landsteiner has shown that such heterogenetic antigens consist of two component parts, one a protein, the other probably a lipid substance. Landsteiner and Simms have found that the lipid 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 lipid fraction.

The fact that two biologically unrelated organisms, Pneumococcus Type II and Friedländer's bacillus, (Strain E) possess certain similar serological and antigenic properties suggests that examples of heterogenetic specificity likewise occur among bacteria. In the case of bacteria, however, the specific substance involved, instead of being a lipid, appears to be a polysaccharide. From the results reported 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. This type of immunological correspondence in no way invalidates the systematic classification of bacteria based upon the more usual and general methods of species determination. It is of greater immediate significance in connection with the study of problems dealing with bacteria as disease-producing agents than in the study of bacteria in their genetic relationships.

The work here reported has raised a number of questions, not only regarding the actual structural differences of the soluble specific substances involved, but also regarding the inter-relations and number of antigens and antibodies taking part in the homologous and heterologous "specific" reactions, and the possible application of the cross-protection studies in human therapy. It is hoped eventually to study these points.

In the work on Pneumococcus comment was made on the surprising fact that the analogous soluble specific substances of such closely related organisms as the three fixed types of Pneumococcus should be so strikingly different. After the study of the E strain of Friedländer's

bacillus it appears equally remarkable that the analogous soluble specific substances of such widely different organisms as this strain of the Encapsulatus group and the Type II pneumococcus should be so similar. If - and there is already evidence on this point - it should develop that the type of specificity occasioned by this particular chemical relationship of soluble specific substances is comparatively widespread in nature, it would be a new fact of considerable biological interest.

Table III.

Protective action of anti-Friedländer serum (E) against Friedländer's bacillus of homologous and heterologous types.

Anti-Friedländer Serum E.	Friedländer's Bacillus.			
	Strain E		Strain Sc.	
	Amount of culture.	Result.	Amount of culture.	Result.
<u>cc.</u>	<u>cc.</u>		<u>cc.</u>	
0.2	0.1	S	0.1	D.17
0.2	0.01	"	0.01	" 18
0.2	0.001	"	0.001	" 22
0.2	0.0001	"	0.0001	" 22
0	0.00001	D.40	0.00001	" 30
0	0.000001	" 18	0.000001	" 23

In this and the following tables S indicates survived; D, death, the numerals representing the hours before death of the animal occurred.

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Table IV.

Protective action of antipneumococcus serum Type II against
Friedländer's Bacillus E.

Friedländer's Bacillus E.	Antipneumococcus Serum II.		Virulence controls.
	Amount	Result	
cc.	cc.		
0.001	0.2	S.	
0.0001	0.2	"	
0.00001	0.2	"	
0.000001	0.2	"	
0.0000001	0.2	"	
0.00001	0		D.51
0.000001	0		" 41
0.0000001	0		" 44

Table V.

Protective action of antipneumococcus serum against Fried-
länder's Bacillus (K).

Friedländer's Bacillus K (Type E).	Antipneumococcus Sera.		Anti-Friedländer serum E.	Virulence controls.
	Type I. 0.2 cc.	Type II. 0.2 cc	Rabbit 84 0.2 cc	
0.1		D.42	D.18	
0.01		" 18	S	
0.001	D.18	S	"	
0.0001	" 18	"	"	
0.00001	" 42	"	"	
0.000001	" 18			D.21
0.0000001				" 42
				" 19

Table VI.

Protective action of anti-Friedländer sera against Pneumococcus Type II.

Culture Pneumococcus Type II.	Virulence controls.	Anti-Friedländer serum		Anti-pneumococcus serum Type II (Horse 91 A) 0.2 cc.
		Rabbit 84 D immunized with Strain E. 0.2 cc.	Rabbit 77 D immunized with Strain SC. 0.2 cc.	
cc.				
0.2		D.46	D.20	D.42
0.1		" 46	" 20	" 72
0.01		S	" 20	S
0.001		"	" 20	"
0.0001		"	" 26	"
0.00001	D.36			
0.000001	" 46			
0.0000001	" 46			

Table VII.

Protective action of anti-Friedländer serum E against Pneumococcus Type II.

Pneumococcus Type II culture	Immune sera				Virulence controls.
	Anti-Friedländer E (Rabbit 84).		AntiPneumococcus II (Horse 91 A)		
	Amount	Result	Amount	Result.	
cc.	cc.		cc.		
0.2	0.2	D.19	0.2	D.24	
0.1	0.2	S	0.2	" 48	
0.01	0.2	"	0.2	S	
0.001	0.2	"	0.2	"	
0.00001	0		0		D.20
0.000001	0		0		" 20
0.0000001	0		0		"36

0.0000001 cc. of this culture = 450 colonies.

Table VIII.

Comparative protective value of anti-Friedländer and antipneumococcus serum against Pneumococcus Type II.

Culture Pneumococcus Type II	Immune sera.				Virulence controls
	Anti-Friedländer E (Rabbit 84)		Antipneumococcus Type II. Horse 91 A.		
	Amount	Result	Amount	Result.	
cc.	cc.		cc.		
0.2	0.2	S	0.2	D.44	
0.1	0.2	"	0.2	" 20	
0.01	0.2	"	0.2	" 44	
0.001	0.2	"	0.2	S.	
0.0001	0.2	"	0.2	"	
0.00001	0		0		D,26
0.000001	0		0.		" 26
0.0000001	0		0		" 44

Publications.

Neill, James M., and Avery O.T., Studies on oxidation and reduction by pneumococcus. VI, The oxidation of enzymes in sterile extracts of pneumococcus. Jour. Exp. Med., October 1, 1924, xl, 405-422.

Neill, James M., and Avery, O.T., Studies on oxidation and reduction by pneumococcus. VII. Enzyme activity of sterile filtrates of aerobic and anaerobic cultures of pneumococcus. Jour. Exp. Med., October 1, 1924, xl, 423-427.

Neill, James M., Studies on the oxidation-reduction of hemoglobin and methemoglobin. I. The changes induced by pneumococci and by sterile animal tissue. Jour. Exp. Med., February 1, 1925, xli, 299-313.

Neill, James M., Studies on the oxidation-reduction of hemoglobin and methemoglobin. II. The oxidation of hemoglobin and reduction of methemoglobin by anaerobic bacilli and by sterile plant tissue. Jour. Exp. Med., April 1, 1925, xli, 535-549.

- Neill, James M., Studies on the oxidation-reduction of hemoglobin and methemoglobin. III. The formation of methemoglobin during the oxidation of autoxidizable substances. Jour. Exp. Med., April 1, 1925, xli, 551-560.
- Neill, James M., Studies on the oxidation-reduction of hemoglobin and methemoglobin. IV. The inhibition of "spontaneous" methemoglobin formation. Jour. Exp. Med., April 1, 1925, xli, 561-570.
- Neill, James M., and Avery, O.T. Studies on oxidation and reduction by pneumococcus. VIII. Nature of the oxidation-reduction systems in sterile pneumococcus extracts. Jour. Exp. Med., February 1, 1925, xli, 285-298.
- Avery, O.T. and Morgan, Hugh J., Immunological reactions of the isolated carbohydrate and protein of pneumococcus. Jour. Exp. Med., September 1, 1925, xlii, 347-353.
- Avery, O.T., and Neill, James M., The antigenic properties of solutions of pneumococcus. Jour. Exp. Med., September 1, 1925, xlii, 355-365.
- Avery, O.T. and Heidelberger, Michael. Immunological relationships of cell constituents of pneumococcus. (Second paper.) Jour. Exp. Med., September 1, 1925, xlii, 367-376.
- To be published November 1, 1925.
- Heidelberger, Michael, Goebel, Walther F. and Avery, O.T., The soluble specific substance of a strain of Friedländer's bacillus (First paper)
- Avery, O.T., Heidelberger, M. and Goebel, Walther F., The soluble specific substance of a strain of Friedländer's bacillus. (Second paper). Chemical and immunological relationships of pneumococcus type II.
- Heidelberger, M., Goebel, Walther F. and Avery, O.T., The soluble specific substance of pneumococcus (Third paper.)