A FURTHER STUDY ON THE BIOLOGIC CLASSIFICATION OF PNEUMOCOCCI.

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The biologic classification of pneumococci, according to Dochez and Gillespie,[1] divides these organisms into four main groups. These groups represent well defined types which are distinct and readily differentiated by immunological reactions. In previous communications[2] the varieties of pneumococci, their relation to disease, and their significance in problems of specific therapy have been discussed in detail. The present work is confined to a study of a limited number of strains of pneumococci which, because of certain serological reactions, are closely allied to the second group, and appear to represent distinct subgroups of Pneumococcus Type II.

In classifying pneumococci by serological methods, agglutination and protection experiments have been employed. Agglutination of typical pneumococci of the second group in an immune serum of the homologous type is prompt and characteristic. There is occasionally encountered, however, an organism with which the agglutination reaction in Antipneumococcus Serum II is incomplete and less prompt, often being delayed several hours. The occurrence of strains showing these peculiarities has been frequent enough to direct attention to them, since in the presence of a positive, but atypical agglutination, some confusion may arise in diagnosis. Theoretically, too, it is of interest to determine, if possible, whether or not these variations in agglutinability indicate essential biologic differences.

The facts presented are a result of a study of ten strains of pneumococci, all of which agglutinated in Antipneumococcus Serum II, atypically as described above. Cross-immunity reactions and

[1] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

[2] Cole, Rufus, *Arch. Int. Med.*, 1914, xiv, 56; *New York Med. Jour.*, 1915, ci, 1, 59. Dochez, A. R., and Avery, O. T., *Jour. Exper. Med.*, 1915, xxi, 114.

absorption tests indicate that these organisms possess characteristic group relationships among themselves and a common antigenic relation to the original and typical Type II pneumococcus.

Description of Cultures.—Ten strains of pneumococci were chosen, five of which were isolated from disease and five from the sputum of normal individuals. Table I gives the source and culture designation of the strains studied.

TABLE I.

Pneumococcus.	Source.	Obtained from	Remarks.
J	Acute maxillary antrum	Pus	Recovery.
L	" lobar pneumonia	Lung puncture	"
M	" " "	Blood	Septicemia, fatal.
Jn	" " "	Sputum	Recovery.
W	Primary pneumonia (child)	Lung puncture	Fatal.
As	Normal individual	Sputum	No infection.
Ar	" "	"	" "
F C B	" "	"	" "
S 13	Diabetic patient	"	" "
H	" "	"	" "

These ten strains possess the common characteristic of partial agglutination in antipneumococcus serum of Type II. Culturally and biologically they present all the usual characters of typical pneumococci: such as inulin fermentation, bile solubility, and more or less distinct capsule formation. Their virulence on isolation was distinctly lower than that of the typical Type II organism.

Agglutination as illustrated in Table II establishes a definite relationship between the strains studied and the type organism of Pneumococcus Group II. The reaction of all ten strains in Serum II is distinct, but never as prompt or complete as in the case of the typical Group II organism. With all typical Type II pneumococci the agglutination begins almost immediately and is complete in half an hour, while the reaction of these ten strains is always delayed and often incomplete at the end of the period of observation. It is further evident that these minor agglutinins tend to disappear in the higher dilution and are completely absent in dilution of 1 to 80, at which titer the reaction of the type organism still persists.

This variation in agglutination was at first attributed to the possibility that there might exist, among pneumococci of Group II,

EXPERIMENTAL.

Agglutination Experiments.

TABLE II.

Determination of the Titer of Agglutination of Strains in Antipneumococcus Serum II, Using Antipneumococcus Serum I and Normal Horse Serum (N) as Controls.

Dilution.	1:1			1:10			1:20			1:40			1:80		
Sera.	N	I	II	N	I	II	N	I	II	N	I	II	N	I	II
Culture															
As 11^{5}	−	−	++	−	−	++	−	−	++	−	−	−	−	−	−
L 6^{8}	−	−	++	−	−	++	−	−	++	−	−	+	−	−	−
Jn 8^{9}	−	±	++	−	−	++	−	−	++	−	−	++	−	−	−
M 5^{10}	−	−	++	−	−	++	−	−	++	−	−	+	−	−	−
Ar 9^{6}	−	±	++	−	−	++	−	−	++	−	−	+	−	−	−
J 7^{8}	−	−	++	−	−	++	−	−	++	−	−	+	−	−	−
W 5^{9}	−	−	++	−	−	++	−	−	++	−	−	±	−	−	−
F C B 9^{5}	−	+	++	−	±	+	−	−	−	−	−	−	−	−	−
S 13 12^{8}	−	+±	++	−	−	++	−	−	+	−	−	−	−	−	−
H 7^{3}	−	+	++	±	−	+	−	−	+	−	−	±	−	−	−
II 46^{11}	−	−	++	−	−	++	−	−	++	−	−	++	−	−	++
I 115^{3}	−	+	−	−	+	−	−	+	−	−	+	−	−	±	−

The reactions were read after 2 hrs. at 37° C. and over night on ice.

The numerals following the culture indicate the animal passage; the exponent indicates the number of generations removed from the last passage.

TABLE III.

The Effect of Increased Virulence upon the Agglutinability of the Atypical Group II Pneumococcus in Antipneumococcus Serum II.

Antipneumococcus Serum II.	Dilution.			
	1:10	1:20	1:40	1:80
Pneumococcus				
M 0^{9}	++	++	±	−
M 5^{11}	++	++	±	−
L 0^{9}	++	++	−	−
L 6^{11}	++	++	±	−
Jn 0^{9}	+	±	−	−
Jn 10^{3}	++	+	−	−
F C B 1^{16}	++	−	−	−
F C B 11^{3}	++	−	−	−
II 46^{11}	++	++	++	++

The numerals after the culture indicate number of mouse passages.

The exponent indicates the number of generations removed from the last passage.

strains of poor agglutinability, analogous to similar conditions among other bacterial groups. It was also thought that, since these organisms were of lower virulence than the type organism, this fact might bear some relation to their agglutinability. Agglutination tests were carried out with certain atypical strains, the virulence of which had been enhanced by repeated animal passage. The effect of the animal passage on the agglutination titer was then determined. Four strains were chosen, the virulence of which was raised by mouse passage until it had attained a point comparable to that of the typical Type II pneumococcus, with a minimum lethal dose of 0.000001 cc. of broth culture.

It is evident from Table III that enhancing the virulence of these strains did not affect their agglutinability. The variations in agglutinations between these strains and the type pneumococcus appear to be not merely differences in agglutinability, but suggest rather the possibility that actual differences in agglutinogenic properties may characterize these organisms. To determine this a univalent immune serum was prepared by immunization of rabbits to each of the ten strains.

Agglutination by Immune Rabbit Sera of the Ten Strains of Atypical Group II Pneumococci and the Results Obtained by Cross-Agglutination.

TABLE IV.

Agglutination of the Ten Strains by Homologous Sera and the Effect of Such Sera on Stock Cultures of Pneumococcus of Type II.

	Immune rabbit sera.									
Sera.	As		Ar		S 13		F C B		H	
Culture.	As	II	Ar	II	S 13	II	F C B	II	H	II
1 : 1	++	−	++	−	++	−	++	−	++	−
1 : 10	++	−	++	−	++	−	++	−	++	−
1 : 20	++	−	+	−	++	−	++	−	++	−
1 : 40	++	−	±	−	++	−	±	−	++	−

Sera.	L		J		Jn		W		M	
Culture.	L	II	J	II	Jn	II	W	II	M	II
1 : 1	++	−	++	−	++	−	++	−	++	−
1 : 10	++	−	++	−	++	−	++	−	+±	−
1 : 20	++	−	++	−	++	−	++	−	+±	−
1 : 40	++	−	++	−	++	−	++	−	+	−

Table IV shows that the rabbit immune atypical sera each agglutinated strongly the homologous strain of pneumococcus, but that none of these sera had any effect on the type culture of Group II. This failure of the antisera of the ten strains to agglutinate the Type II pneumococcus shows differences in the antigenic properties of these organisms and is in striking contrast to the positive reactions of agglutination with these same strains by Antipneumococcus Serum II (Table II). This failure of reversibility of the agglutination reaction would seem, therefore, to be due to actual differences in the agglutinogenic groups of these various organisms.

TABLE V.

Cross-Agglutination.

Immune sera.	Pneumococcus cultures.										
	As	S 13	F C B	Ar	L	J	Jn	W	M	H	II
As	++	−	−	−	++	−	++	−	++	−	−
S 13	−	++	−	−	−	−	−	−	−	−	−
F C B	−	−	++	−	−	−	−	−	−	−	−
Ar	−	−	−	++	−	+	−	+	−	−	−
L	++	−	−	−	++	−	++	−	++	−	−
J	−	−	−	++	−	++	−	++	−	−	−
Jn	++	−	−	−	++	−	++	−	++	−	−
W	−	−	−	++	−	+	−	++	−	−	−
M	+	−	−	−	+	−	+	−	+	−	−
H	−	−	−	−	−	−	−	−	−	++	−
Normal rabbit	−	−	−	−	−	−	−	−	−	−	−
II	+	++	+	+	++	+	++	++	++	++	++

Table V reveals the striking fact that in a series of organisms wholly contained within Group II certain separate relationships are demonstrable. An analysis of this table indicates clearly the existence of three distinct groups which appear to represent subdivisions of the main Group II, in the antiserum of which all members of each of these subdivisions are agglutinated. For purposes of convenience these subdivisions will be referred to as Subgroups II A, II B, and II X, and are shown more clearly in Table VI.

Subgroup II A consists of four strains, the immune reactions of which are specific within the group, being identical with those of all other strains of the group, but bearing no relation to those of Subgroups II B, or II X. Similarly, Subgroup II B consists of three other strains characterized by the possession of specific immunity

TABLE VI.

Subgroup II A.

Immune sera.	Pneumococcus cultures.				
	As	L	Jn	M	II
As	++	++	++	++	−
L	++	++	++	++	−
Jn	++	++	++	++	−
M	++	+	+	+	−
II	+	++	++	++	++

Subgroup II B.

Immune sera.	Pneumococcus cultures.			
	Ar	J	W	II
Ar	++	+	+	−
J	++	++	++	−
W	++	+	++	−
II	+	+	++	++

Subgroup II X.

Immune sera.	Pneumococcus cultures.			
	F C B	S 13	H	II
F C B	++	−	−	−
S 13	−	++	−	−
H	−	−	++	−
II	+	++	++	++

reactions, identical for members of this subgroup alone. The remaining three strains have been placed in Subgroup II X which, like the larger Group IV of the original biologic classification, is peculiar in that it seems to consist of a heterogeneous series of independent strains which do not cross in their immunity reactions with members of the other two subgroups or with each other. All, however, possess the common character of atypical agglutination in Antipneumococcus Serum II. This subgroup, like its prototype Group IV, seems to be infinitely variable, and to be characterized by the absence of cross-immunity reactions and by lower virulence.

Protection Experiments.—The protection of animals against infection is generally conceded to be one of the most specific of immunological reactions and hence one of the most satisfactory methods of classification. White mice were given intraperitoneally graduated doses of pneumococci and at the same time a fixed quantity of immune serum. All animals except the virulence controls received 0.2

cc. of immune serum intraperitoneally. This quantity of Immune Serum II as a rule protects mice against 0.01 cc. of broth culture of the homologous organism which, given alone, kills mice regularly in doses of 0.000001 cc. All animals surviving for five days were considered effectively protected.

In experimental pneumococcal infection the specificity of the protective power of an immune serum is evident only when the culture employed is fully virulent. Of the ten strains of pneumococci it was found possible to raise the virulence of seven by animal passage. The virulence of the other three strains could not be increased sufficiently for use in protection experiments, although they were passed successively through 11, 14, and 18 animals, respectively. They were even then of such low virulence that it was impossible to kill mice with the moderate doses necessary for the successful carrying out of the test. Of these three strains, two belonged to Subgroup II X and one to Subgroup II A.

Further Evidence of the Specificity of Group Relationships by Protection Experiments.

TABLE VII.

Protective Action of Antipneumococcus Serum II.
The Relation between the Specificity of the Protective Action of Immune Serum and the Virulence of the Infecting Organism.
Pneumococcus L (Subgroup II A) after One Animal Passage.

Pneumococcus Lr[1].	Virulence controls.	Serum II.	Serum I.	Normal serum.
cc.				
0.01		S.*	D. 96	S.
0.001	D. 72	"	S.	"
0.0001	S.	"	"	D. 96
0.00001	"	"	"	S.
0.000001	D. 72	"	D. 96	D. 96

Pneumococcus L (Subgroup II A) after Seven Animal Passages.

Pneumococcus L 7[1].	Virulence controls.	Serum II.	Serum I.
cc.			
0.01		S.	D. 18
0.001		"	" "
0.0001	D. 36	"	" 24
0.00001	" "	"	" 36
0.000001	" "	"	" "

* In the tables D. stands for died; S. for survived. The figures represent the number of hours before the death of the animal.

Table VII shows that after increasing the virulence of pneumococcus L (Subgroup II A) to a degree sufficient to apply the test, definite protection was afforded by stock Antipneumococcus Serum II against 10,000 times the minimal lethal dose of culture. The odd survivals and non-specific reactions with the same strain before its virulence was raised by animal passage is evidence of the futility of attempting specific reactions of a protective nature with avirulent organisms. That this phenomenon does not represent a reversion to type brought about by animal passage is evidenced by the fact that the antigenic properties of these organisms remained unaffected by such treatment. Of the seven strains, the virulence of which was increased by mouse passage, Antipneumococcus Serum Type II protected against six, three of which belonged to Subgroup II A and three to Subgroup II B. The seventh strain, an organism of Subgroup II X, although made equally virulent by nine mouse passages, was not protected against by immune serum of Type II.

Cross-Protection Tests with Homologous and Heterologous Sera of the Three Subgroups of Pneumococcus II.

TABLE VIII.

Protective Action of Sera of Subgroup II A against a Pneumococcus of the Same Group, and Failure of Sera of Subgroups II B and II X to Protect against the Same Organism.

Pneumococcus Subgroup II A.	Immune sera.					
	Subgroup II A.		Subgroup II B.	Subgroup II X.	Antipneumococcus Type II.	Virulence controls.
Jn 91	Jn	L	W	F C B		
cc.						
0.01	D. 72	S.	D. 18	D. 18	D. 96	
0.001	S.	"	" "	" "	S.	
0.0001	"	"	" "	" "	"	D. 18
0.00001	"	"	" "	" 24	"	" "
0.000001	"	"	" "	" "	"	" 24

Tables VIII and IX demonstrate that an immune serum of Subgroups II A and II B protects against any organism of the homologous subgroup, but fails to protect against any strain of the other two subgroups. Table X emphasizes the individual character of organ-

TABLE IX.

Protective Action of Sera of Subgroup II B against a Pneumococcus of the Same Subgroup and Failure of Sera of Subgroups II A and II X to Protect against the Same Organism.

Pneumococcus Subgroup II B.	Immune sera.					
	Subgroup II B.		Subgroup II A.	Subgroup II X.	Antipneumococcus Type II.	Virulence controls.
W 61	W	J	Jn	F C B		
cc.						
0.01	D. 26	S.	D. 18	D. 18	D. 96	
0.001	S.	"	" "	" "	S.	
0.0001	"	"	" "	" "	"	D. 18
0.00001	"	"	" "	" "	"	" "
0.000001	"	"	" "	" "	"	" "

TABLE X.

Protective Action of Serum of Subgroup II X against the Homologous Organisms Only and Failure of Sera of Subgroups II A and II B and Antipneumococcus Serum II To Protect against the Same.

Pneumococcus Subgroup II X.	Immune sera.					
	Subgroup II X.		Subgroup II A.	Subgroup II B.	Antipneumococcus Type II.	Virulence controls.
F C B II1	F C B	S 13	Jn	W		
cc.						
0.01	D. 18	D. 18	D. 18	D. 18	D. 18	
0.001	S.	" "	" "	" "	" "	
0.0001	"	" "	" "	" "	" "	D. 18
0.00001	"	" "	" 22	" "	" "	" "
0.000001	"	" "	" "	" 36	" "	" "

TABLE XI.

Lack of Protective Power of Immune Sera of Subgroups II A, II B, and II X against Typical Pneumococcus II.

Pneumococcus Type II.	Immune sera.			
	Subgroup II A.	Subgroup II B.	Subgroup II X.	Virulence controls.
II 461	Jn	W	F C B	
cc.				
0.01	D. 18	D. 18	D. 18	
0.001	" "	" "	" "	
0.0001	" "	" "	" "	D. 18
0.00001	" "	" "	" "	" "
0.000001	" "	" "	" 26	" "

isms of Subgroup II X. A serum produced by immunization with any given strain of this type protects against that particular organism and against no other. As previously noted, there is a complete lack of crossing in the immunity reactions of the individual members of Subgroup II X. Table XI shows that in protective action, as in agglutination (Table IV), the immune sera of Subgroups II A, II B, and II X have no effect on the typical pneumococcus of Type II. That these immunologic reactions between the original Type II pneumococcus and organisms of the subgroups are not reversible seems to indicate degrees of difference in antigenic characters. While the serological specificity of the subgroups definitely separates one from the other, nevertheless their immune reactions with the antisera of the typical Type II organism indicate that they are all biologically related. The correlation of these subgroups is further proven by absorption tests.

Absorption Experiments.—The phenomenon of specific absorption of agglutinins from an immune serum by the homologous organism was first described by Castellani.[3] This investigator found that from a polyvalent serum produced by immunization with two microorganisms of different species, the agglutinins for either one could be removed by fractional absorption with the homologous strain, while in a serum thus exhausted, the antibodies for the second organism remained intact. It has been shown also that in a univalent serum against *Bacillus typhosus* not only are agglutinins present for that organism alone, but that in the same serum there also exist partial or minor agglutinins for bacilli which are biologically similar, and which fall within the same general group. Absorption of a typhoid immune serum by *Bacillus typhosus* removes not only the agglutinins for that organism, but completely exhausts the serum of its minor antibodies for the closely allied organisms. This reaction, however, is not reversible, for removal of the partial agglutinins by absorption with a member of the intermediary species leaves the antibodies for *Bacillus typhosus* practically undiminished. The significance of this phenomenon in bacterial classification is obvious, and its applicability to the present study is evident in the following protocols.

[3] Castellani, A., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1902, xl, 1.

Specificity of Absorption Reaction. Absorption of Antipneumococcus Serum I with Pneumococci of Groups I and II.

Technique.—Specific absorption. Antipneumococcus Serum I. 2 cc. of serum were diluted with 3 cc. of salt solution. To the 5 cc. of diluted serum the live, washed bacterial residue of 150 cc. of a twenty-four hour broth culture of Pneumococcus I was added, allowed to stand in contact over night in the ice box, then centrifuged, the clear supernatant serum pipetted off, and passed through a Berkefeld filter.

Non-specific absorption. Antipneumococcus Serum I was absorbed with Pneumococcus II. The technique was the same as above.

Control. Antipneumococcus Serum I diluted, and, without the addition of any bacteria, filtered by the same technique.

TABLE XII.

Specific and Non-Specific Absorption of Agglutinins from Antipneumococcus Serum I by Pneumococci of Groups I and II.

Agglutination.

Culture pneumococcus, Group I.	Antipneumococcus Serum I.		
	Specific absorption with Pneumococcus I.	Non-specific absorption with Pneumococcus II.	Control. Serum unabsorbed.
	−	++	++

TABLE XIII.

Protective Power of Antipneumococcus Serum I after Absorption with Pneumococci of Groups I and II.

Protection.

Culture pneumococcus, Group I.	Virulence controls.	Antipneumococcus Serum I.		
		Specific absorption with Pneumococcus I.	Non-specific absorption with Pneumococcus II.	Control. Serum unabsorbed.
cc.				
0.01		D. 18	S.	S.
0.001		" 20	"	"
0.0001	D. 18	" 24	"	"
0.00001	" "	" "	"	"
0.000001	" 20	" 48	"	"

Tables XII and XIII demonstrate the specificity of the absorption reaction with pneumococci of the fixed Types I and II. Saturating an immune serum of Group I with pneumococcus of the same type completely exhausts that serum of all its agglutinins and protective antibodies, while absorption of the same serum with organisms of Group II does not appreciably diminish these immune substances for pneumococci of Group I.

Absorption of Antipneumococcus Serum II with Pneumococcus II and Organisms of Its Subgroups II A, II B, and II X.

Technique.—To 10 cc. of undiluted Antipneumococcus Serum II was added the washed bacterial residue from 150 cc. of an eighteen hour broth culture of the given strain of pneumococcus. Before being added to the serum the bacteria were killed by heating at 56° C. for forty-five minutes. The serum mixtures were incubated in the water bath for two hours at 37° C. and allowed to remain in contact over night in the ice box. The clumps of agglutinated bacteria were whirled out by centrifugation, and the clear supernatant serum was pipetted off. This serum was absorbed a second time by the same technique, tested for the absence of agglutinins, and called exhausted serum.

TABLE XIV.

Cross-Agglutination Reactions with Antipneumococcus Serum II Exhausted by Absorption with Type Strains of Pneumococcus II and Its Subgroups II A, II B, and II X.

Antipneumococcus Serum II. Absorbed by	Pneumococcus.										
	Subgroup II A.				Subgroup II B.			Subgroup II X.			Group II.
	Jn	As	L	M	W	Ar	J	S 13	F C B	H	Type II.
Subgroup A, Jn	−	−	−	−	++	±+	++	++	+	++	++
" B, W	++	++	++	±+	−	−	−	++	+	++	++
" X, S 13	++	++	++	±+	++	++	++	−	+	++	++
Group II	−	−	−	−	−	−	−	−	−	−	−

Table XIV shows that specific absorption of Antipneumococcus Serum II with the typical Type II pneumococcus removes all the agglutinins, not only for the homologous organism, but also all the partial agglutinins for its subgroups, II A, II B, and II X. In other words, specific absorption of Antipneumococcus Serum II completely exhausts it of both major and minor agglutinins. Conversely, however, absorption of the same immune serum with a representative strain of Subgroup II A removes the minor agglutinins for members of that subgroup only, leaving intact the antibodies for the Type II pneumococcus and its other subgroups, II B and II X. Similarly, absorption of Antipneumococcus Serum II with any member of Subgroup II B takes out the agglutinins for all the Subgroup II B organisms, but leaves unaffected the antibodies for the Type II pneumococcus and its subgroups, II A and II X. The lack of cross-immunological reactions among the heterogeneous organisms within

Subgroup II X already noted in the previous agglutination and protective experiments (Tables VI and X) is again evident in the absorption tests. Saturation of Immune Serum II with a pneumococcus of Subgroup II X robs the serum of its agglutinins for that individual strain only, and for no other.

The results obtained by absorption experiments with Antipneumococcus Serum II, a serum produced by intensive immunization of the horse with a single strain of the typical Type II pneumococcus, corroborate the same specific groupings obtained by the cross-immunological reactions of agglutination and protection with immune rabbit sera of the individual strains.

DISCUSSION.

The biologic classification of the pneumococcus distinguishes four distinct groups. These types are based upon well defined immunologic differences. The accuracy with which these groups may be differentiated and the constancy of their relative frequence in disease and health emphasize the importance of their recognition in clinical and epidemiological studies. The exactness with which the large number of strains studied have conformed to type indicates the extraordinary uniformity and comparative fixity of the specific groups. These distinctive differences in antigenic properties not only offer a reliable method for the more exact determination of the varieties of pneumococcus, but afford the only rational basis for the study of immunotherapy in pneumococcal infection.

The second group of pneumococci of the original classification consists of highly virulent organisms which are responsible for about one-third of all cases of lobar pneumonia of pneumococcus origin. Organisms of this group produce infections which are clinically severe, and the mortality of which is about 35 per cent. The serological reactions by means of which Group II was originally identified are sharply defined. So characteristic and prompt is the agglutination of any strain of this type in Antipneumococcus Serum II that any deviation from the normal reaction is quickly recognized. The isolation of an occasional strain of pneumococcus which agglutinates atypically in Antipneumococcus Serum II led to an attempt to determine the nature of these organisms and their relation to the

type pneumococcus of Group II. Of the ten strains studied five were isolated from disease and five from the sputum of normal individuals. These organisms all exhibited the usual cultural and biochemical characteristics of the pneumococcus; namely, inulin fermentation, bile solubility, and varying degrees of capsular development. The facts developed by this study indicate the existence of pneumococci which are biologically similar and closely allied to the typical organism of Group II. These organisms possess partial antigenic characters common to the Type II pneumococcus, but they vary from the typical representative of this group by a diversity of relationships among themselves, and by a lack of the reversibility of their immune reactions with the type organism. Because of these variations these organisms may be classified as subvarieties of Pneumococcus Group II. All strains of the three subgroups thus far recognized are agglutinated by Antipneumococcus Serum II, but the diminished intensity of the reaction serves to distinguish them from typical II pneumococci. The incomplete reaction of agglutination of these subvarieties in the immune serum of Type II is apparently similar to the diminished reactions which occur in many immune sera with other organisms closely allied to the type used in producing the serum. Such reactions occur only in the higher concentrations of immune sera and may be attributed to the so called minor agglutinins. In such cases, also, a non-reversibility of the immune reaction has been noted. For instance, a potent typhoid immune serum may agglutinate *Bacillus coli* in the higher concentrations, but an anticolon serum may not affect *Bacillus typhosus*. All strains of these subgroups of pneumococci are partially agglutinated in the higher concentrations of Antipneumococcus Serum II, while conversely the immune serum produced by any strain of these subgroups fails to react with the typical II pneumococcus.

In addition to the partial agglutination of these strains in Antipneumococcus Serum II and the absence of reverse immunity reactions, these subvarieties are further characterized by certain interrelationships of a definite antigenic nature, by virtue of which they may be classified into at least three subgroups, which have been called Subgroups II A, II B, and II X. It has been shown in the preceding protocols that a given member of either Subgroup II A or

II B is characterized by the possession of immunity reactions identical with those of all other strains of the homologous subgroup, and that these reactions are specific only within the group. Subgroup II X is peculiar in that it seems to consist of a heterogeneous series of independent strains which do not cross in their immunity reactions with members of the other two subgroups or with each other. This subgroup, like its prototype, Group IV, of the original biologic classification, is of lower virulence, infinitely variable in its composition, and lacking in cross-immunity reactions. As has been noted, serum Type II fails to protect against organisms of this subgroup, and inasmuch as specific protection is regarded as the ultimate criterion for classification, it is doubtful whether organisms of Subgroup II X are of sufficiently close relationship to be included within Group II.

The specificity of these subgroups, II A and II B, as tested by the immunity reactions of agglutination and protection is further confirmed by the phenomenon of absorption. Saturation of Antipneumococcus Serum II with a typical Group II pneumococcus removes all the agglutinins both for the type organism and its three subgroups. Absorption of the same serum, however, with a member of either Subgroup II A or II B removes only the partial agglutinins for the homologous subgroup, but leaves intact the antibodies for the typical II pneumococcus and the other subgroup. Absorption of Antipneumococcus Serum II, on the other hand, by any member of Subgroup II X takes out the antibodies for that particular strain only, and for no other.

In the present discussion no attempt is made to interpret the experimental data in terms of their phylogenetic significance. Whether the subvarieties of the second group of pneumococci represent strains which have acquired independently certain adaptive characters, or whether they are related to each other and to the fixed type by the lineage of common descent is interesting. However, the limited nature of the present study precludes the formulation of any hypothesis as to origin.

SUMMARY.

1. At least three subgroups of Pneumococcus Type II may be recognized by specific immune reactions. They have been called Subgroups II A, II B, and II X.

2. That the organisms of these three subgroups are biologically related to Pneumococcus Type II is shown by the following facts: (a) Agglutination with Antipneumococcus Serum II. (b) Protection with Antipneumococcus Serum II, except Subgroup II X. (c) Absorption of Antipneumococcus Serum II with typical Type II pneumococcus removes the antibodies for all subgroups. (d) Absorption of Antipneumococcus Serum II with a member of Subgroups II A or II B removes only the antibodies for the homologous subgroup. Absorption of Antipneumococcus Serum II with any given member of Subgroup II X removes the antibodies for that particular strain only.

3. That the three subgroups, although biologically related to Pneumococcus Type II, possess, nevertheless, specific differential characters which separate them one from another, is evidenced by the following facts: (a) The organisms of any subgroup are not agglutinated by the antisera of the other two subgroups. (b) They are not protected against by the sera of the other subgroups. (c) They do not absorb from Antipneumococcus Serum II the specific immune bodies of the other subgroups.

4. Subgroups II A and II B are characterized by immunity reactions identical within the respective group.

5. Subgroup II X consists of heterogeneous strains which do not cross in their immunity reaction with each other or with Subgroups II A or II B.

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