TYPE SPECIFIC ANTIGENICITY WITHIN A SINGLE SPECIES OF BACTERIA, USING TYPE II AND TYPE III *DIPLOCOCCUS PNEUMONIAE*

John Bonner

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Dr. James J. Marion

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CHAPTER I

INTRODUCTION

Antigenicity, the property of an organism or molecule to stimulate the production of specific antibodies upon its introduction into an animal body, is one of the most vital phenomena in nature to aid in the survival of a species and its individual members. If it were not for this, the animal body would not be able to cope with the onslaught of foreign material, especially bacterial, and be able to set up defense mechanisms to aid in its survival. This property of many foreign substances of being antigenic, thus causing defense antibody formation, may well have been one of the determining factors of evolutionary success or failure.

Antigens, in the sense they shall be used throughout this paper, are foreign proteins, or potentially foreign protein material, which when introduced into animal bodies stimulate the production of specific antibodies. Antibodies are in turn modified soluble proteins which result from the introduction of these antigens. Not only are antibodies specific for their

antigen, but many times the specificity is that which is called type specificity, i.e. responsive only to certain characteristic categories, types, within the species itself. The type specificity which is discussed in this paper is that of types within the species Diplococcus pneumoniae Weichselbaum on the basis of a polysaccharide carbohydrate capsular material it possesses.

The term species, as used in bacteriology, refers "theoretically to a single kind of bacterium, all individual cells of which are identical or nearly so."(1) This vague definition of a species is used for lack of a better one, since characteristics used to differentiate species in the higher organisms such as ability to breed freely in nature and the production of fertile offspring are not able to be utilized. Even this very general definition used in bacteriology often falls short since the theoretical identity of cells is not often found. But because of its general acceptance, the above will be the sense in which the term species is used.

The organism used in my investigation is one traditionally used in this type of work, Diplococcus pneumoniae.

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(1) FROBISHER, JR., M. Fundamentals of Microbiology. W. B. Saunders Company, Philadelphia and London p. 134. 1953

Its popularity in this field is one due both to convenience and importance. It is a convenient subject since it is easily obtained, routinely cultured, though fastidious in its cultural characteristics, and usually characteristic in its antigenicity. Its importance stems mainly from its pathogenicity, being a short time ago one of the most incidious etiological agents known (2), and still one commonly encountered in epidemiology. Although, due to use of antibiotics and sulfonamides, the disease incidence caused by this organism has fallen, it is still dangerous, and always potentially a great killer. Thus the organism is both a convenient and worth-while subject.

Diplococcus pneumoniae Weichselbaum (also known as Streptococcus pneumoniae by British workers and usually universally referred to as pneumococcus) is a gram positive, encapsulated organism usually encountered in groups of two, but often in longer chains characteristic of Streptococcus. It produces α hemolysis on solid blood media, but also may be propagated on certain other media. The organism is commonly

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(2) SMITH and CONANT. Zinsser's Textbook of Bacteriology. p. 278 ff. Appleton-Century-Crofts, Inc. 1952

referred to as being lancet shaped, although this is not ordinarily evident in common laboratory microscopy. Pneumococcus may possess a capsule which is the basis of its antigenic properties. Upon culturing on artificial media, especially those lacking whole blood, the organism looses its capsule, and its accompaning virulence. The capsule may be regained upon use of suitable techniques, such as animal passage. In such procedures, the species remains stable, the rough non-capsulated form always reverting to the capsulated form of the same species and type (3).

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(3) HARRISON, J. H. Antigenic variation in protozoa bacteria. Annual Review of Microbiology. 1:19. 1947.

CHAPTER II

HISTORY

Subtypes were found to exist within Diplococcus pneumoniae by Neufeld and Handel (4) in the early part of the century. Four types were described, I, II, III, and IV. (5). The group IV, which was of a heterologous nature, was further separated until at the present time there are seventy-seven recognized types. (6).

Recognition of specific types of Diplococcus pneumoniae has resulted also in use of this organism in studies of variability (transformation) of living organisms, which has opened up an entirely new field of research. Utilizing the knowledge of pneumococcal types, basic relationships in genetics have resulted from the successful transformation of bacterial types. This work promises to answer many of the questions pertaining to species development and maintainance, and also may allow evolution to be more fully undersfood.

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(4) NEUFELD and HANDEL. Immunitats. 3, 1959. 1909.

(5) DOCHEZ, A.R. and GILLESPIE, L.J. Journal of American Medical Association. 61:727. 1913.

(6) SMITH and CONANT. Zinsser's Textbook of Bacteriology. p.280 Appleton-Century-Crofts, Inc. 1957.

The steps which led to the principles of immunology of pneumococcus, which form the basis of determining type specific antigenicity, have a long and complicated background. As with most research, long and tedious work, including much work based on misinterpretations of the experimental results, took place from the organism's discovery in 1875 by Klebs until now. Many phases of its biology are still being speculated on, and valuable research progressing.

In 1886, Foa and Bordoni-Uffreduzzi succeeded in the immunization of rabbits by subcutaneous injection, first with attenuated cultures, and then with ever-more virulent cultures at three or four day intervals (7). These results soon attracted the attention of other investigators who continued the work. In the 1890's, Foa and Carbone stimulated an immune response in rabbits using soluble products of pneumococcus, and also sought to chemically isolate the antigen, which at that time was almost universally thought to be a toxin. Unfortunately, the remaining results of these two investigators were vague and tend to be misleading.

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(7) WHITE, B. The Biology of Pneumococcus. Pp. 19 ff. New York-The Commonwealth Fund. London, Humphrey Milford, Oxford University Press. 1938.

The Klemperers, the forefathers of antipneumococcic serum therapy, in 1891 obtained serum from immunized rabbits and produced an extremely high level of immunity (8) by intravenous injection. This was found to be of greater value than the subcutaneous route. But their greatest contribution was the discovery that the young of immunized mother rabbits were most often passively protected, a finding which led to the testing of rabbit serum as a therapeutic agent. After self-testing, the Klemperers administered the serum to patients and obtained successful results. Their explanation of serum action was on an anti-toxic basis, which we now know to not be valid. But they succeeded in isolating the new protein element from the blood which were the antibodies produced in response to the pneumococcus.

Krause and Pansini (9) found that varying colony, capsule, and virulence states, which also alter antigenicity, took place upon culturing on artificial media, but were restored on animal passage. These variations were misinterpreted as mutations from

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(8) No units of measurement had yet been devised for use in this field.

(9) KRAUSE W. and PANSINI, S. Untersuchungen uber den Diplococcus pneumoniae und verwandte Streptokokken. Ztschr. f. Hyg. u. Infektionskr. 11:279. 1891.

pneumococcus to Streptococcus, and that both species arose from a common saprophytic Streptococcal form. This now is what is known to be bacterial dissociation.

Agglutination ability of pneumococcus by immune serum was observed by Metchnikoff (10). This is now easily explained on the basis of current immunology, and serves as one of the tests for bacterial identification, both qualitatively and quantitatively.

In 1892, Mosny (11) found that the organism was able to live and multiply in vitro in the presence of weak immune serum. This showed that the antipneumococcal serum possessed no bactericidal action as had been postulated. He also noted that agglutination occurred in his cultures. But still no true interpretations had been made concerning the phenomena.

This needed interpretation of the defense mechanism against pneumococcus in vivo came when Issaeff (12) recognized that phagocytosis played a major role. This was concluded since phagocytosis was observed,

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(10) METCHNIKOFF, E. Etudes sur l'immunite! 4e memoire. L'immunite des cobayes vaccines contre le vibrio Metchnikowii. Ann. Inst. Pasteur. 5(8): 465. August 1891.

(11) MOSNY, E. Recherches experimentales sur la vaccination contre l' infection pneumonique et sur sa querison. Arch. d. Med. Exper. et D'anat. Path. 4:195. 1892.

(12) ISSAEFF, B. Contribution a l'etude de l'immunite' acquise contre le pneumocoque. Ann. Inst. Pasteur. 7(3):260. March 1893.

and also since neither bactericidal nor antitoxic action could be demonstrated in the immune serum.

Emmerich (13) interpreted the action of the immune serum to be the combination of two proteins, globulin from the blood combining with a protein toxic substance. Although this view was not correct, it was definitely a step forward in understanding this mechanism.

Serological classification was brought closer as a result of the investigations of Bezancon and Griffon (14) of Metchnikoff' s phenomenon, which they named "agglutination." They found that serum of patients after pneumococcal infection acquired agglutination ability. From this they concluded, " that from the standpoint of agglutination there exist several races of pneumococci, which behave as though different microbes." Unfortunately this specificity was not again investigated until Neufeld and Handel in 1910.

Denys in 1897 (15), after increasing pneumococcal virulence by animal passage, found that serum produced

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(13) EMMERICH, R. Ueber die Infection, Immunisirung und Heilung bei crouposer Pneumonie. Ztsch. f. Hyg. u. Infektionskr. 17:167. 1894.

(14) BEZANCON, F., et GRIFFON, V. Pouvoir agglutinatif du serum dans les infections experimentales et humanines a pneumocoques. Part I and II Compt. rend. Soc. de biol. 49:579. June 1897.

(15) DENYS. Recherches sur le serum antipneumococcique. Ann. Soc. med.-chir. d'Anvers. 2(2):51. February 1897.

in rabbits by the organism was preventive, curative, and "neutralized" the "toxin" of pneumococcus. This convinced Denys that the serum was not bactericidal , but that it contained a substance which stimulated the leukocytes to phagocytize the organism.

But no matter what the problem, some standard which serves as a basis of comparison to express quantitative measurements must be established. The standardization, historically, of antiserum was established by Mennes (16) in his research. The "unit" he arbitrarily established was the smallest amount of serum which, when mixed with ten times the least fatal dose of pneumococci and injected into the peritoneum, would bring about the survival of a test rabbit. Although this system is not used often now, it has served as a valuable means to aid in the interpretations of investigative results.

As was explained earlier, Bezancon and Griffon were the first to show that the pneumococcus was not a homogeneous group, but that on the basis of agglutination there were "races of pneumococci which behave as though different microbes." These "races" are what

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(16) MENNES, F. Dan antipneumokokken-serum und der mechanismus der immunitat des kaninchens gegen den pneumococcus. Ztschr. f. Hyg. u. Infektionskr. 25:413. 1897.

are now termed "types." That such types existed was a source of great interest and mystery to the investigators. Kindborg (quoted by Neufeld and Schnitzer) belived that the pneumococcal antibodies were "strain-specific." Eyre and Washbourn (17) surmised that "There exist varieties of pneumococci which at present are only distinguished by the action of antipneumococcus serum."

Dochez and Gillespie (18) divided pneumococcus into four groups by protection and agglutination methods. These groups were Types I, II, III, IV. Types I and II contained over 60 per cent of the strains then being tested, Type II was the so called Pneumococcus mucosus (19), and Type IV was a heterologous grouping containing all the strains not already included in Types I, II, or III. Lister (20), working independently in Africa at the same time,

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(17) EYRE, J.W.H., and WASHBOURN, J.W. Further experiments with Pane's antipneumococcus serum. British Medical Journal. 2:1247. November 4, 1899.

(18) DOCHEZ, A.R. and GILLESPIE, L.J. A biologic classification of pneumococci by means of immunity reactions. Journal of American Medical Association. 61(10):727. September 6, 1913.

(19) This name has now been abandoned.

(20) LISTER, F.S. Specific serological reactions with pneumococci from different sources. Publication of South African Institute of Medical Research. 1(2):1. December 1913.

had worked up a similar scheme of classification of the organism, which, though not expressed in the same schematic representation, was found to be equivalent to that of Dochez and Gillespie.

The American classification proved to contain most of the pneumococci associated with disease and of a strictly parasitic nature in Types I, II, and III. Type IV were those which were of minor importance epidemiologically.

In the early 1920's, some order evolved in the classification of pneumococci as research alleviated the confusion which was first prevalent. Cooper (21) deserves the major credit in clarifying the situation. It was also found that the use of rabbits in the immunology was superior to the use of horses, the animal most used during this period, as the agglutinating serum from rabbits was more selective than that of horses. Also Type XIV horse serum had the drawback of tending to agglutinate human red blood cells when used a therapeutic serum, since an antigenic relationship between this serum and the human erythrocytes

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(21) COOPER, G. M., MISHULOW, L., and BLANE, N. E. Studies on acute respiratory infections. II. A study of the serological relationships of pneumococci from the upper respiratory tract with special references to common colds and influenza conditions. Journal of Immunology. 6(1):25. January 1921.

of all four major blood groups resulted in agglutination of the red blood cells. Type XIV rabbit serum often, but not at all times, showed agglutination ability with group A and AB human erythrocytes. This factor plagued physicians in the treatment of Type XIV pneumonia cases, but rabbit serum was used because it proved to be safer than horse serum (22).

Similar attempts to classify pneumococci in Japan failed (23); the interpretation was that the organisms had undergone variation on artificial medium. This was later shown to be false by Megrail and Ecker (24) who demonstrated that the organism had a type stability under conditions in which other organisms showed variability in agglutination.

Work continued until in 1932 there were thirty-two types demonstrated (25), in 1952 there were

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(22) BOYD, W. C. Fundamentals Of Immunology pp. 122, 123. Interscience Publishers, Incorporated, New York. 1945.

(23) TAKAMI, T. Tohoku Journal of Experimental Medicine. 6(1/2):41. June 1925. 6(3/4): 248. September 1925.

(24) MEGRAIL, E., and ECKER, E. E. Stability of pneumococcus types in sterile abscesses. Journal of Infectious Diseases. 35(1):1. July 1924.

(25) WHITE, B. The Biology Of Pneumococcus. p. 115 New York - The Commonwealth Fund. London-Humphrey Milford-Oxford University Press. 1938.

seventy-two (26), and in 1958, seventy-seven (27). At the present time this work is still continuing, and more progress will most likely be reported soon. The methods of classification now most used in these investigations are agglutination and specific protection of animals.

Besides immunological, other methods of classification have been used. Three large significant groups have been demonstrated by Thompson (28) by determining the electrophoretic rate of migration of pneumococci. This is known as the Electrophoretic Potential Method, but has no apparent advantages over serological classification.

The classification of pneumococci has a long and complex history, but much has been learned. There remain many factors under investigation which should result in information valuable not only in bacteriology, but also in other related fields such as genetics and chemistry.

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(26) SMITH and CONANT. Zinsser's Textbook of Bacteriology. Appleton-Century-Crofts, Incorporated. 1952.

(27) ibid. 1957.

(28) WHITE, Op. Cit., p. 118.

CHAPTER III

IMPORTANCE OF CLASSIFYING TYPES
and
OCCURANCE OF TYPES

The question could next be posed whether or not such classification of bacteria is of itself worthwhile. Is it mainly of a theoretical nature, or is it utilitarian?

Pneumococcus classification into types has proven to be of great importance to science. It has served to help define the relations and interrelations of bacteria, especially in the genetic mechanism of bacterial transformation. This has been of exceptional value in the research of species genetics, many believing that it involves the physical principle of life itself.

Epidemiologically, pneumococcal classification has been instrumental in the saving of many human lives in the spectrum of diseases in which this organism participates. Typing has allowed quick and accurate determination of the organism involved, and the use of the most effective type specific antiserums therapeutically. In more recent years, the sulfonamides and antibiotics have to a great

extent replaced antipneumococcal serums therapeutically, but such sera are still of much value. Currently, typing serums are of inestimable value to the epidemiologist and public health physician in the identification of, and use of effective measures against pneumococcal diseases.

Modern chemotherapy has not resulted in a halt in pneumococcal research. The hospital of the Rockefeller Institute of New York has continued its studies, which are perhaps the most fruitful of all institutions participating in research of this type. One of the greatest incentives for continued research is the possible need for basic pneumococcal biological knowledge in the future, present sulfonamide and antibiotic success not withstanding. Despite chemotherapy and the resultant reduced mortality rate, the incidence of pneumococcal infections has not appreciably changed (29). The possibility of a mutant, highly communicable, antibiotic-resistant Diplococcus pneumoniae evolving is great. This possibility is greatly emphasized by the current upsurge of Staphyloccal epidemics in the hospitals of the United States (30). If such an occurrance occurs in

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(29) SMITH and CONANT, op. cit., pp. 275 ff. 1957

(30) MUDD, S. The Staphyloccus problem. Scientific American 200, No. 1. January 1959.

pneumococcus in the future, basic biological knowledge of its characteristics will have to be drawn upon to meet the challenge.

The phenomenon of the existence of types within a bacterial species is by no means unique of pneumococcus alone. Other species (31) are known to have members which exhibit sharp serological differences among each other, but still are undoubtedly members of the same species. This is to be expected within a species, since variation is a law which is applicable in the phylogeny of all organisms.

The hemolytic streptococci have been classified into types on the basis of group specific polysaccharides by Griffith and Lancefield. Staphylococci have been typed not only on the basis of their polysaccharides, but also on type specific proteins. The acid-fast bacteria (mycobacteria), meningococcus, gonococcus, Hemophilia influenzae, Hemophilus pertussis, Brucella, Friedlander bacillus, dysentery bacilli, Bacillus anthrasis, and Salmonella sp. have all been found to be seperable into antigenic types. Serological methods have also been applied to the virusses, similar results having been obtained, especially on the virus of vaccinia.

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(31) BOYD, op. cit., pp. 118 ff.

CHAPTER IV

BASIS OF TYPE SPECIFICITY

The fact that types have been found to exist within the species Diplococcus pneumoniae has been discussed, but the basis for them is in itself a complicated principle. The pneumococci have been shown to all contain the same proteins, which are related if not identical to proteins of the Streptococci, and most have a somatic group specific carbohydrate (32). Immunization with the organisms containing no capsular material, though non-specific antibodies are developed, have little increased protection against the virulent encapsulated form. The types of pneumococci seem to depend solely on the capsular materials, which are acidic polysaccharides frequently possessing acetyl and amino groups. The specificities not only depend on the varied polysaccharide material, but the virulence as well. Heidelberger's discovery that the non-protein precipitable substances were complex polysaccharides gave an important stimulus to

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(32) TILLET, W. S. and FRANCIS, T. Journal of Experimental Medicine. 52:561. 1930. ibid. 52:895. 1930.

(33) HEIDELBERGER, M. Physiological Review. 7, 107. 1927

the chemical investigation of these substances (33). This view has been enhanced by the fact that type specific immunity may be obtained by immunization using extracts of the capsular material. Since only incomplete information is available on the finer structures of these capsules, investigation is currently proceeding and much is left to be learned.

Type III has been investigated more thoroughly than the other types to determine the basis of the specificity of its capsule. This capsule, referred to as S3, was found to be built of aldobionic acid molecules united by a glucoside link (34). The structure is both phosphorous and nitrogen free, and optically levatory. The aldobionic acid molecules' composition have been determined as one molecule of glucuronic acid and one of glucose, united by a glucose link involving the aldehyde group. This aldehyde group is the reducing group of the glucuronic acid and carbon 4 of the glucose. This combination gives cellobiuronic acid (35).

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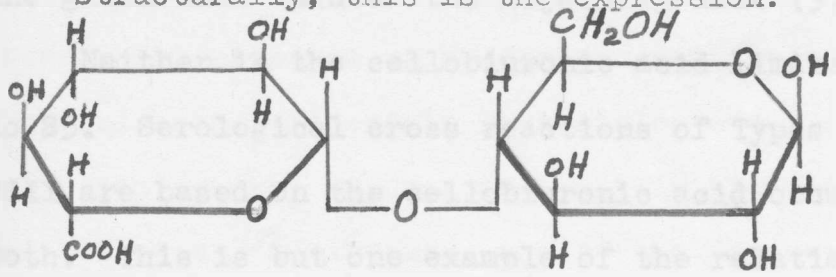
(34) BOYD. op. cit. p. 105

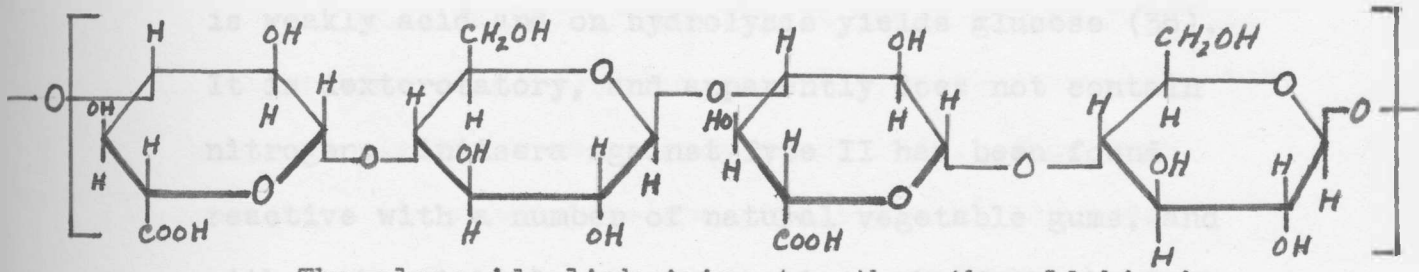
(35) HOTCHKISS, R. D. and GOEBEL, W. F. Journal of Biological Chemistry. 121, 195. 1937.

(36) REEVES, R. E. and GOEBEL, W. F. Journal of Biological Chemistry. 139, 511. 1941

Structurally, this is the expression:



Assuming the β configuration, Reeves and Goebel (36) picture the S3 antigen as this:



The glucoside link joins together the aldobionic acid molecules. This involves the reducing group of the glucose, leaving the glucuronic acid free. This free glucuronic group has important connotations which will be discussed later.

Much is still unknown about the chemical composition of S3, for example it is still uncertain whether the linkage between the cellobiuronic acid units is in the α or β form at position 3 of

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(36) REEVES, R. E. and GOEBEL, W. F. Journal of Biological Chemistry. 139, 511. 1941.

the glucuronic acid of the adjoining unit (37).

Neither is the cellobiuronic acid limited only to S3. Serological cross reactions of Types III and VIII are based on the cellobiuronic acid occuring in both. This is but one example of the relationship existing within the types of the same species.

Chemically, Type II capsular polysaccharide (S2) is weakly acid and on hydrolysis yields glucose (38). It is dextorotatory, and apparently does not contain nitrogen. Antisera against Type II has been found reactive with a number of natural vegetable gums, and with such materials as gum arabic much of the basic knowledge of S2 has been found. There seems to be no reason why some of the basic principles found concerning the specificity of S2 cannot be applied to help explain the mechanism of pneumococcal specificity in general.

S2 is reported to contain five per cent nitrogen as a part of the molecule according to Boyd (39), this being one of the factors which differentiates

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(37) HEIDELBERGER, M., and ADAMS, J. The immunological specificity of Type II pneumococcus and its separation into partial specificities. Journal of Experimental Medicine. 103:2. 189ff. February 1, 1956.

(38) BOYD, op. cit.

(39) BOYD, op. cit., 1945.

S2 from other type capsules, such as S1 and S3. However the presence of a nitrogen component appears doubtful, since Zinsser's Bacteriology (40) specifically states that S2 does not contain nitrogen. Neither does Heidelberger, one of the foremost chemical investigators of pneumococcal specificity, refer to a nitrogen component.

S2 specificity apparently is based on the multiple recurrences of glucuronic acid (41), with rhamnose and glucose playing a relatively minor role. This view has been strengthened since partly hydrolyzed cherry gum, which contains glucuronic acid, and was the most reactive of a number of vegetable gums tested, precipitated up to 44 per cent of the antibody, the antibodies being the globulin protein of the specific antiserum (42). The hydrolysis made the glucuronic units more accessible. Also it was found that the more favorable the distributions of glucose or rhamnose groupings, the more antibody precipitated. This is interpreted not only on the minor part that rhamnose and glucose play in the antigenicity, but also in

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(40) SMITH and CONANT., op. cit., 1957.

(41) HEIDELBERGER and ADAM, op. cit.

(42) MARRACK, J. and CARPENTER, B. R. British Journal of Experimental Pathology. 19, 53. 1938.

their scattered distribution in order not to hinder access to the glucuronic acid. This is believed to emphasize and confirm the importance of multiple reactive units in type specificity.

It was shown by Butler (43) and Stacey that since all of the glucose component in S2 occurs as 1, 4, 6-branch points, the precipitation of the Type II antiserum by the polysaccharide glucoses and by the glycogen components would appear to be due to their possession of such branch points which are suitably located for proper reactivity with the antibody.

Butler and Stacey also isolated the rhamnose derivitive 2, 4-di-O- methyl-L- rhamnose from methylated S2, which indicates that in the capsular structure the rhamnose is linked in positions 1 and 3.

Investigation of the chemical composition of these capsular materials is currently proceeding. This field is now in a complicated and unsettled condition with much valuable work still to be done.

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(43) BUTLER, K. and STACEY, M. Chemical Society Journal. 1537. 1955.

CHAPTER V

IMMUNOCHEMISTRY

The actual principle why Type II and III pneumococcus act as they do is explained on the basis of Immunochemistry, the division of immunology which deals with the chemistry of the antigens and antibodies, and studies the chemical basis on which resistance to disease and immunity operate. It is known that when antigens are introduced into an animal, processes are initiated by the cells producing the blood protein gamma globulin which change the gamma globulin pattern so that the antibody molecule attaches itself to the antigen, the pneumococcal capsular material in this case (44). These changes are specific to the antigen involved, thus an unlimited number of specific antibodies are obtainable, though close chemical affinity often results in cross-reactions.

As described above, the immunochemistry of pneumococcus has been greatly assisted by reacting the antibodies with vegetable gums such as gum arabic. The gums have been used both as complete entities and in

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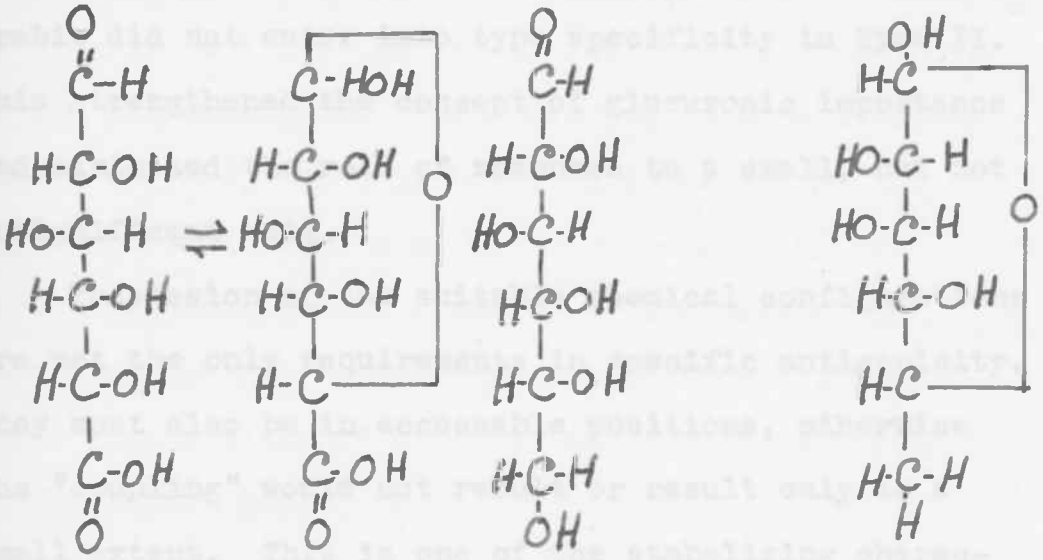
(44) BURNET, M. How antibodies are made. Scientific American. P. 74. 191,5. November 1954.

hydrolyzed conditions. This research has led to the knowledge of the importance of glucuronic acid in many pneumococcal specificities (45).

Glucuronic importance in Type II and III specificities was recognized in 1936 by Goebel (46) (47). The capsular structures were found to characterized by the multiple recurrences of the glucuronic acid components, since substances containing multiple glucuronic acid precipitates much more antibody than the cross reactivity caused by substances containing glucose or rhamnose.



GLUCURONIC ACID GLUCOSE RHAMNOSE

(45) HEIDELBERGER and ADAMS, op. cit.

(46) GOEBEL, W. F. Journal of Experimental Medicine. 64, 29. 1936.

(47) GOEBEL, W. F. and HOTCHKISS, R. D. Journal of Experimental Medicine. 66, 191. 1937.

Using gum arabic (48), it was found that up to 40 per cent of the total antibody was precipitated by high concentrations with gum arabic, this being traced to its glucuronic constituents. This conclusion was reached since the precipitation of other polysaccharides which apparently had the proper glucose and rhamnose linkages as did gum arabic, but containing very little or no glucuronic acid, was an entire magnitude less.

In addition to this, it was found that at least two thirds to three fourths of the rhamnose in the gum arabic did not enter into type specificity in Type II. This strengthened the concept of glucuronic importance and minimized the roll of rhamnose to a small, but not insignificant roll.

Possession of the suitable chemical configurations are not the only requirements in specific antigenicity. They must also be in accessable positions, otherwise the "coupling" would not result or result only to a small extent. This is one of the stabalizing characteristics of antigen-antibody specificity such as in pneumococcus, and also a factor in cross reactivities. Groups have been frequently found to act as hindrances in such reactions. Arabofuranose was found to act as such a hindrance for close approach of Type II anti-

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(48) ibid.

bodies to the glucuronic acid in intact gum arabic, since the intact gum is characterized by a number of labile, terminal arabofuranose residues. Many of these residues are believed attached to the glucuronic acid. If the gum is partially hydrolyzed, the glucuronic acid components become freely exposed, perhaps all of them terminally exposed, and antibody precipitation is much more effficient.

The research of Butler and Stacey (49) indicates that possibly one third of the glucuronic components of Type II (S2) are located on the terminal branches of the polysaccharide. This results in more antibody access to them, which is believed to be one of the most important factors in the high reactivity of most anti-pneumococcal titers. It has been shown that degraded gum with terminal positions accessable react with a titer of at least one hundred times that of the intact gum.

Acacia pycnatha, a gum similar to gum arabic, but containing less rhamnose and about one-third as much glucuronic acid displayed less than one-third of the reactivity of gum arabic. All the evidence seems to indicate the importance of the glucuronic

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(49) BUTLER, K., and STACEY, M. Chemical Society Journal 1537. 1955.

acid in the specificity, acting also in a quantitative manner.

As the still unsettled and incompletely known concept of pneumococcal specificity now stands, it appears that the large proportion of the antibodies in the specific antisera not only require the multiple recurrence of reactable linked carbohydrate components, but these in a suitable and accessable configuration. For Types II and III, this apparently rests on the proper occurrence of glucuronic acid components, and also rhamnose and glucose to minor extents. Certainly in future years, this mechanism will be more completely understood.

CHAPTER VI

EXPERIMENTAL

In order to prove type specific antigenicity of pneumococcus, two white albino rabbits were immunized by an extended inoculation course, one for Type II, the other for Type III. The rabbits weighed 2615 grams and 2725 grams respectively. The demonstration of Type specific antibodies against the organism types used would be the positive results.

Appropriate cultures of the organisms were obtained from the American Type Culture Collection. Also cultures and immune typing serum for identification purposes were obtained through the kindness of Dr. Frank L. Horsfall, Jr. of the Rockefeller Institute, New York, New York.

The pneumococci were cultured on Difco Brain Heart Infusion Broth with defibrinated whole blood added. Increased virulence in preparation for manufacturing the antigen was obtained by repeated animal passage using white mice, the organism being recovered after the animals' deaths by peritoneal washings and heart blood samples.

Antigen was produced using these virulent encapsulated organisms which were transferred from the blood cultures to Brain Heart Infusion Broth without blood and incubated about eighteen hours. The cultures were then centrifuged, the resulting sediment taken up in a solution of 0.5 per cent formalin in normal saline solution. This resulted in the sediment from a 10 c.c. culture being contained in 1 c.c. of suspension.

To insure death of the organisms, the suspensions were allowed to remain at room temperature for five hours. They were then transferred aseptically to sterile vaccine bottles in order to maintain their purity and then refrigerated. All the vaccine for the immunization course was prepared at the same time and refrigerated. This method of vaccine (or more properly bacterin) manufacture was obtained from a paper by Cooper and Watter (50).

Control samples of serum from the rabbits were obtained by cutting a lateral ear vein of each rabbit at its distal position. These control samples tested

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(50) COOPER, G. M. and WATTER, A. W. Application of the Neufeld Reaction to the identification of types of pneumococci with the use of antisera for thirty-two types. American Journal of Public Health. 25:469. 1935.

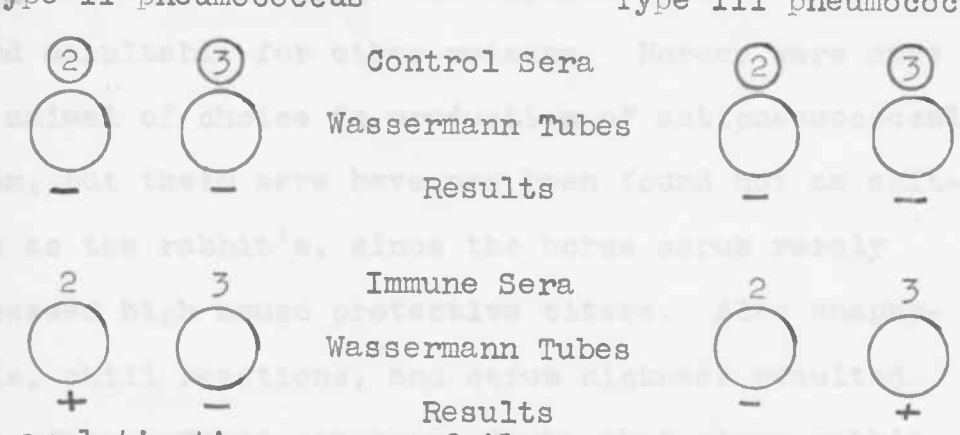
negatively by the Quellung reaction for antibody presence. The immunization course was then carried out on these animals.

Intravenous injections were given on three consecutive days, followed by a rest period of four days. This was continued until the animals had received twelve inoculations, a period of four weeks. The vaccine dosages were: on first day the sediment from 2 1/2 (two and one half) c.c. culture (0.25 c.c. vaccine), on second day sediment from 5 c.c. culture (0.5 c.c. vaccine) and on third day sediment from 10 c.c. culture (1 c.c. vaccine). This schedule was repeated on each of the four weeks. Thus the animals received an ever increasing dosage on each of the three days of inoculation of the week. The lateral ear vein was exclusively used, alternating veins often to avoid their destruction.

At the end of the immunization period, blood serum was obtained from the experimental animals from blood obtained from slashing the central ear artery. The blood was allowed to clot normally, centrifuged, and then serum decanted. Each serum was then used in agglutination reactions with both Type II and Type III Diplococcus pneumoniae.

To eight Wassermann tubes, each containing one c.c. normal saline, one and one-half c.c. of encap-

sulated pneumococci grown on Difco Brain Heart Infusion Broth without blood was added to each tube, resulting with four tubes of Type II and four tubes of Type III. One c.c. of the sera produced in the immunization course was added in turn to two of the individual tubes of each group of four tubes. Control sera obtained from the animals before immunization was added to the remaining tubes to act as controls. The tubes were then incubated for twenty hours, and then stored in the refrigerator. The results were that Type II pneumococcus was agglutinated by the Type II serum, but not by the Type III serum. Type III pneumococcus in turn was agglutinated by the Type III serum, but not by the Type II serum. No agglutination was observed in the control sera tubes. The results are illustrated in the following diagram:



+ = agglutination; − = failure to agglutinate

The above test was confirmed by the Neufeld Quellung Reaction.

These results indicate that the sera produced were type specific for the organism which produced them, and not cross-reactive. This proves the type specificity of Type II and Type III Diplococcus pneumoniae, two antigenic types within the same species of bacteria.

Such serum as produced in this experiment is not only able to be used for identification purposes and other routine procedures, but is also suitable for therapeutic use in treating pneumonia cases. It is by techniques similar to these that anti-pneumococcal serum was produced for hospital use.

Rabbits were chosen as the experimental animal of choice, since mice are so small many animals would be required to obtain any volume of serum, and horses, being not only too large and expensive, have been found unsuitable for other reasons. Horses were once the animal of choice in production of antipneumococcal serum, but their sera have now been found not as suitable as the rabbit's, since the horse serum rarely possessed high mouse protective titers. Also anaphylaxis, chill reactions, and serum sickness resulted more often. There can be no doubt that since rabbit

serum does not display these characteristics as often as horse's does, the rabbit is the most suitable animal (51).

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(51) HORSFALL, JR., F. L., GOODNER, K., MACLEOD, C. M. HARRIS 2D, A. H. Journal of American Medical Association. Pp. 1483 ff. May 1, 1937.

SUMMARY

I. Historical background of the problem was reviewed.

II. Basis of the now seventy-seven recognized types was discussed and shown to be the result of specifically antigenic polysaccharide capsules, the chemical compositions of which are incompletely known.

III. An experimental method of proving type antigenicity was given and results obtained verified the phenomenon.

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