THE EVALUATION AND COMPARISON OF THE IMMUNE RESPONSE IN GUINEA PIGS TO INFECTION WITH PNEUMOCOCCUS TYPE I WHEN TREATED WITH SULPHAMETHAZINE AND WHEN TREATED WITH PENICILLIN

by

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I N T R O D U C T I O N

It has long been known that infection by pneumococci resulted in the development of a type specific immunity to the organism. Until recently, specific serum therapy has been the only means of effectively combatting pneumococcal infections. The advent of antibiotics, however, has now given us another potent therapeutic weapon, and has thus greatly modified the therapy of diseases of pneumococcic etiology. It has thus become of considerable interest and importance to determine whether the presence of various antibiotic drugs in the body modified or influenced the natural immunity response on the part of the host. It was in an attempt to elucidate this question that this study was undertaken.

H I S T O R I C A L R E V I E W

A. NATURE OF THE PNEUMOCOCCUS

The lanceolate Gram positive diplococci, the chief etiological agent in lobar pneumonia, and commonly called Pneumococcus, has the species name Diplococcus pneumoniae, genus Diplococcaceae, tribe Streptococcaceae of the family Lactobacteriaceae (1).

Discovery

It was discovered in 1875 by Klebs (2), but isolated independently in 1881 by Pasteur (3), and Sternberg (4).

Morphology

Morphologically, the organism consists of a pair of oval or lance-shaped cocci, their somewhat flattened proximal ends in apposition and their distal portion pointed. Sometimes single cocci are seen, while at other times single or paired cocci may be arranged in short or even long chains, resembling a string of beads - the "chapelet" originally described by Pasteur. Even when the environment is favourable, there may be many variations from this characteristic form, the individual cocci being round and of varying size (0.5 to 1.25 μ), or elongated to resemble bacilli. In any given preparation, along with typical forms, other members displaying every degree of involution or degeneration may be present, while in aged cultures aberrant forms may be the rule.

Pneumococcus has no spores, no vacuoles, no visible granules, no flagella, and is non-motile. It reproduces by

the primitive method of transverse fission.

Pneumococcus is readily stained with the usual aniline dyes and is Gram positive, although when subjected to the digestive action of leucocytes, or after death and partial disintegration in old cultures, they are easily decolourized and appear Gram negative.

The Capsule

The most distinctive morphological feature of the organism is the capsule, which is most prominent when examined in body exudates, or media enriched with body fluids. The capsule envelops the single, paired, or chained cocci, frequently with a uniform periphery, although sometimes it shows indentations between the twin cells or between the paired individuals in chains.

The capsule was described first by Pasteur (5) as an "aureole", and by Friedlander (6) as a "kaspel".

Modern chemical study of the capsular material has given a rational basis for the serological classification of all pneumococci into definite and specific types. It is this peculiar and complex component of the pneumococcal cell that determines its specific antigenic stimulus and its immunological behaviour in the presence of antibodies.

The first record of a capsule stain is that by Friedlander in 1885 (7). Many satisfactory methods have been devised to demonstrate the capsule. Some widely used are the copper sulphate method of Hiss (8), Buerger (9), and Huntoon (10), while in tissue section, Wadsworth's (11) method may be used.

Cultural Characteristics

Pneumococcus, being a strict parasite, is somewhat fastidious in its nutritional requirements as well as sensitive to the physical and chemical conditions of its surroundings. According to Dernby and Avery (12), culture media should have an initial reaction represented by a pH of 7.8 to 8.0 with an optimal pH of 7.8.

A variety of nutrient materials are necessary for the needs peculiar to this organism. For general purposes, a broth is made of fresh beef heart freed of fat. To this is added 2% peptone preferably with a high proteose nitrogen, a small amount of sodium chloride, usually 0.5 per cent, and glucose up to 1 per cent as a source of carbohydrate (an excess may hasten autolysis).

The growth of pneumococci on all media may be considerably enhanced by the addition of animal or human serum or whole blood, which in addition to contributing some nutritive substances, act as buffers in controlling the reaction, and, being colloids arrest any toxic action of inorganic salts.

Growth takes place most regularly at a temperature of 37.5°C. Development does not usually occur below 25° nor above 41°C. The organism is a facultative anaerobe, and grows both in the presence and absence of oxygen.

Appearance of Growth

In nutrient broth, growth is rapid; in 24 hours the organisms sink to the bottom leaving a slightly cloudy fluid. There is a tendency to form short chains in broth.

Pneumococcus colonies on blood agar plates show a surface which is usually slightly elevated at the center with concentric small raised rings alternating with depressions spreading to the periphery. There is usually a slight halo of haemolysis and methemaglobin formation with a zone of greenish colour about the colony most marked in 48 hours (1).

Bile Reaction

The biochemical reaction taking place in the autolysis of pneumococci is also involved in the solvent action of bile on the pneumococcal cell. It was Neufeld (13), who in 1900, first discovered that bile possessed this unusual property, a property which became of great diagnostic value in differentiating Pneumococcus from Streptococcus and other organisms, and which has since been known as the "Neufeld phenomenon". He found that there was rapid and complete dissolution of the pneumococcal cells on the addition of bile salts in the space of a few minutes.

Biochemical Features

The pneumococcus is a fragile body and contains within itself enzymatic forces that lead to its disruption and disintegration, rob the substrate in which it lives of nutrient substances, and from these substances evolve chemical agents that arrest further growth, cause the death of the organism, and affect the cells of the animal body into which the microbe may find its way.

Intracellular proteases (14), (15) break down the

proteins of the cell and of the substrate upon which the cell feeds into smaller fragments, which in turn are still further reduced in size by the same ferment or possibly by a peptidase. This proteolysis reduces the food supply in the medium, and the products of the digestive action may produce purpura (16), (17) and cause other more or less violent toxic effects in the animal host. These are not true toxins (18), (19), but resemble degraded proteins in their physiological action. Pneumococci are also endowed with saccharatytic enzymes (20), capable of attacking starch, inulin, and glycogen; invertases that convert complex saccharides into simpler sugars, and other ferments split these sugars into acids, while the acid so formed arrests further proliferation of the bacterial cell. Intracellular lipids (21) are converted into fatty acids by pneumococcal lipases and therefore the self destruction of the cell may be complete.

The action of these enzymes may be reversible and by their action or that of some similar agents Pneumococcus is able to build protein, lipids, and somatic and capsular polysaccharides from substances present in the substrate.

Oxygen is essential to the hydrolysis and synthesis of protein, lipid and sugar. Some of the products of oxidation are inimical to the normal functioning of the bacterial cell, and some affect changes in the respiratory mechanism of the blood. Pneumococcus utilizes oxygen to form peroxide (22), (23) that is toxic to the cell. Peroxide destroys labile constituents of the pneumococcal cell, which with the

easily oxidizable intracellular substance continue to form an oxidizing substance contributing to the conversion of oxyhaemoglobin into methemaglobin (24).

Classification of Pneumococci

In 1910 Neufeld and Handel (25) discovered that the pneumococci could be divided into specific groups by immunological methods. Their work was soon confirmed by Cole in 1912 (26), and Dochez and Gillespie in 1913 (27) who differentiated Types I, II, III, and a heterologous Group IV. Subsequently, Cooper and her associates in 1932 (28) separated Group IV into some 30 odd types.

The pneumococci can be typed by the agglutination of the intact organism, by precipitation of the specific capsular polysaccharides, or by swelling of the capsule when the organisms are mixed with the proper type-specific rabbit anti-serum. This latter phenomenon, which was first described by Neufeld in 1902 (29) and redescribed in 1931 (30), is known as the Neufeld or Quellung reaction.

On the basis of cross reactions, there appears to be some relation between organisms of Types II and V, III and VIII, VII and XVIII, and XV and XXX, but the resemblances are not sufficiently close to invalidate the current classification. In nature there appears to be a stability of the types, although transformation of a strain of one type into an organism of another type can be accomplished by appropriate treatment of the culture (31). During the dissociative process, antigenic action may vary from one of strict type specificity to

one merely of the broader species-specificity (32) and decreased virulence. Degraded forms may, if the degenerative process has not been complete, regain all their original morphological, cultural, and immunological characters (33), (34).

Pathogenicity of Pneumococci

(a) Man

Pneumococci frequently lead a vegetative existence in the normal mouth, abiding there without causing any appreciable disturbance in the host. The organisms may be virulent or avirulent, and they may sometimes be found as the predominant species, but, contrary to the older opinion, these so-called normal pneumococci, although possessed of full virulence, rarely cause pneumonia in the individual in whom they temporarily dwell. Buerger (35) by the plate method, detected pneumococci in the mouths of thirty-nine out of seventy-eight normal persons. Brown and Huderson (36) noted a correlation between the incidence of pneumococci in the throats of normal persons and periods of inclement weather.

In lobar pneumonia, the great majority of cases are caused by the Pneumococcus. Pneumococci of Types I and II are accountable for approximately one-half of the cases of lobar pneumonia in all countries from which records are available, except Africa (Heffron 1939) (37), with eight types causing about 75 to 80 per cent of the total number of infections.

Infections in man can be caused by any of the more than 75 serological types of pneumococcus. In lobar pneu-

monia, as far as at present known, the various serological types, are, in order of frequency, I, III, II, VIII, VII, V, IV, XIV, XVIII. In children, pneumococci of the first three types are responsible for the disease in only 36 per cent of the cases, with Types XIV, I, VI, V, VII, IV, IX, XV and XIX occurring most frequently in the order given (38), (39).

The fatality rates of pneumococcal lobar pneumonia have been reported as 40 to 60 per cent for Type III cases, 41 per cent for Type II, 25 per cent for Type I, with types XVIII, VII and VIII next in order of lethal power (37).

In bronchopneumonia, the types responsible for infection in order of incidence are III, VIII, XVIII, X, V, VII, XX, II, XI, and XIV (38). In contrast to extrinsic infection as the causative factor in lobar pneumonia, it would seem that from the data available, bronchopneumonia arises more commonly from intrinsic infection or auto-inoculation (37), (38).

The Carrier State

This may be due (a) to pneumococci implanted by transference from other persons (40) or (b) to organisms arising from pneumococcal disease within the individual (41). The organisms implanted by transference are largely of the heterogeneous types formerly classified as Group IV (42), (43), except in the event that the donor is suffering or recovering from an attack of pneumonia due to the predominant types. The pneumococci that are autogenous in origin are of the type causing the pneumonia.

The carrier state may be brief (transient), prolonged (chronic), or sporadic (chronically intermittent) (43).

(b) Animals

The susceptibility of mice (44) and rabbits (45), (46) to the pneumococcus, has enhanced their usage for experimental purposes in the bacteriological and immunological study of Pneumococcus. Other animals, such as the monkey (47), horse (48), guinea pig (49), rat (48) and dog (50), have all been used experimentally. Susceptibility or resistance depends on peculiarities due to the species, genetic factors, age, weight, environmental conditions, and the physical state of the test animal

Virulence of a pneumococcus for a given animal species may be raised by serial passage through animals of the same species (51), and the enhanced pathogenicity can be maintained by continued animal passage or by the application of suitable "in vitro" methods of preservation (52). On the other hand, the pathogenicity of a pneumococcal strain may be decreased by subjecting the organisms to unfavourable cultural conditions, or by propagating the organisms in media containing increasing amounts of homologous immune serum (53). Pathogenicity or virulence, therefore, is only a relative term, and must be interpreted in the light of the biological characters of the pneumococcal strain and of the functional variables in the animal host.

B. IMMUNITY TO THE PNEUMOCOCCUS

Chemical Constituents of the Pneumococcus

Prior to 1917, none of the constituents of the Pneumococcus had been isolated or subjected to chemical study.

The basis for the immunological differentiation and virulence of pneumococci into types was demonstrated by Dochez and Avery in 1917 (54), to reside in the elaboration during growth of so-called Specific Soluble Substances (SSS) which constitute the capsules of the microorganisms. It was, however, Heidelberger and Avery in 1923 (55), who described the isolation of the Specific Soluble Substance of Type II pneumococcus, which they concluded consisted mainly of a carbohydrate, which appeared to be a polysaccharide built up of glucose molecules and which, in a dilution as high as 1 to 3,000,000 gave a specific precipitin reaction with homologous immune serum. In the immediate following years a great deal of work was published by several authors (56), (57), (58), which contained descriptions of refinements in the methods in preparing these polysaccharides, and of their physical, chemical and immunological properties and differences. Dubos (59) has described the capsular materials as being essentially or exclusively polysaccharides of high molecular weights which often are acidic in nature and frequently possess acetyl and amino groups. From Heidelberger's (60) reviews of the subject, it is apparent that the chief polysaccharide of each type of pneumococcus is chemically distinct. The polysaccharide of Type I pneumococcus contains nitrogen in an amino sugar combination, on hydrolysis yields galacturonic acid and is

dextrorotatory. Type II polysaccharide is a dextrorotatory complex of weakly acidic glucose units and does not contain nitrogen. Type III polysaccharide, also nitrogen free, is laevorotatory and is composed of glucose and aldobionic acid. These complex molecules of glucose and uronic acids do not contain either phosphorus or nitrogen and do not give a colour reaction with iodine.

The capsular polysaccharides are non-toxic, but are of great importance in determining the pathogenicity of the pneumococcus. The capsule of living pneumococci protects the organism from phagocytosis, and the capsular material from dead pneumococci, known as the Specific Soluble Substance or SSS, is so soluble that it diffuses through the tissues where it meets and combines with the antibodies as they are formed by the patient. This antibody neutralizing effect of the polysaccharide explains the nature of the "virulin" which Rosenow (61) extracted from cultures of pneumococci and from the lungs of patients who died of pneumococcal pneumonia.

The capsular polysaccharides act as haptenes or partial antigens when tested in rabbits. In mice and men, capsular polysaccharides are antigenic (62), and have been used successfully to produce active immunity in man against pneumococcal pneumonia, (63), (64), (65).

Tillett and Francis (66) injected specific purified polysaccharides intradermally into patients with pneumonia, and observed the reactions which varied with the immunological status of the patient. The dose was 0.1 ml. of a solution of physiological saline which contained 0.01 mg. of the specific

polysaccharide (67). In the early days of the disease, when there was an excess of specific polysaccharide in the blood and tissues, the test was negative. However, after a spontaneous crisis or after adequate serum therapy, when there was an excess of specific antibodies in the blood and tissues, the intradermal injection of the polysaccharide was followed in 20 to 30 minutes by the appearance of a wheal with pseudopodia surrounded by an area of erythema.

Although the capsular material is essential for virulence and type specificity, it is not necessary for the life and growth of the pneumococcus. The capsules may be removed by cultivation in homologous antiserum (68) with the development of avirulent non-specific pneumococci with colonies having a finely roughened surface.

The capsules may also be destroyed by enzymatic hydrolysis without affecting the viability of the culture (69). From the soil of a cranberry bog in New Jersey, an aerobic, sporulating bacillus was isolated from which an intracellular bacterial enzyme could be extracted, having the capacity to hydrolyze the capsular polysaccharide of Type III pneumococcus only. Injections of the enzyme into mice infected with virulent Type III pneumococcus, exerted a curative action. However, this enzyme is not a therapeutic substance per se, but in its ability to digest the capsule, the non-encapsulated bacteria become highly susceptible to phagocytosis. However, the pneumococci may revert to the capsulated pathogenic state when the enzyme is withdrawn.

Other bacterial enzymes active against pneumococcus

polysaccharides have been found by Dubos (59) in immune sera, in leucocytes, and in animal tissues. These enzymes are so specific that they can differentiate between polysaccharides which give cross reaction in specific antisera, i.e. they are even more specific than are the antibodies obtained by immunization of experimental animals. These enzymes are neither bacteriostatic nor bactericidal; they destroy the capsules without affecting either the viability of pneumococci or their capacity to produce capsular polysaccharide. From the point of view of cellular structure, therefore, it appears that the capsule is not a component essential to the integrity of the cell. It behaves as an excretion product, which because of its viscosity, accumulates around the cell wall, and exerts an anti-phagocytic effect which is one of the conditions of virulence.

The protective capacity of a serum depends on the amount of antibody present which is capable of reacting with capsular polysaccharides. Absorption of a highly protective serum with purified capsular polysaccharides by precipitation, results in loss of its protective power.

The somatic portion of the pneumococci are composed of antigenic proteins and carbohydrates, which are antigenically similar for all types despite the immunological specificity of the capsular polysaccharides. Antibodies to the whole somatic portion of pneumococcal cells, or any fractions of it so far studied show only modest protective power for experimental animals in contrast with the great protection given by antibodies to the capsular polysaccharides (70).

Like β-haemolytic streptococci, the somatic portion of pneumococcus contains a "C" or cellular carbohydrate described by Tillett, Goebel and Avery (71) and isolated in 1931 by Heidelberger and Kendall (72) which appears to be immunologically as characteristic of pneumococcus as a species as are the "C" carbohydrates for the Lancefield Groups of streptococci. MacLeod and Avery (73) found this "C" material was highly antigenic but unrelated to the specific capsular polysaccharides. The studies of Goebel and Adams (74) have shown that the "C" carbohydrate forms a portion of the Forsmann (heterophile) antigen of the pneumococcus which was first isolated in 1931 by Bailey and Shorb (75). McCarty in 1947 (76) isolated the somatic "C" polysaccharide in crystalline form, from pleural and abdominal fluids.

The "C" somatic polysaccharide is species specific, but not type specific, non-toxic in mice - up to 1 mgm., contains no amino nitrogen and is protein free. It also contains 4 per cent phosphorus.

While, for the most part, the pneumococcal capsule is important in the pathogenicity of the organism, the virulence of two well known strains of pneumococcus Type III known as A. 66 (non-virulent for rabbits) and S.$\overline{X}$ - III (virulent for rabbits) both with apparently similar capsular polysaccharide, yet by means of transformation of the capsule, there is no alteration of virulence showing that in this case the pathogenicity depends on the somatic portion and not on the capsule (77).

In 1930 Enders (78) isolated from autolytic products of Type I pneumococcus a new element among the specific polysaccharides which was designated "A" substance. This "A" substance was unstable to heat at pH 9, but stable at pH 4, and was antigenic. Sabin (79), and Wadsworth and Brown (80) also demonstrated the "A" substance. It produced type specific immunity in mice, fixed complement in the presence of Type I antipneumococcic rabbit serum, and while it caused no reaction when injected intravenously, in a dose of one milligram into a normal guinea pig, it evoked fatal anaphylactic shock in guinea pigs passively sensitized not only with Type I antipneumococcic rabbit serum, but with the same serum after removal of precipitin by absorption with the soluble specific substance.

Sevag (81) believing that the procedure hitherto employed in the preparation of the pneumococcal polysaccharides might have disrupted the molecular configuration of the carbohydrate molecule with a consequent loss of essential radicals, used modified methods in their preparation in which alkali was purposely avoided. The result was a highly purified polysaccharide differing chemically from the originally isolated carbohydrate in the respect to the presence of acetyl groups. This acetyl polysaccharide and the "A" carbohydrate on further study seemed to represent the same chemical substance immunologically (82).

Nucleoproteins from the pneumococci are non-toxic and non-specific, but antigenic, and give rise to local and general reactions when injected into a hypersensitive animal

Humphrey (84) found that some types of pneumococci produce measurable amounts of hyaluronidase although there was no correlation between the presence or absence of this enzyme and the virulence of the strain.

Toxins of the Pneumococcus

The mechanical effects produced by the pneumococci cannot account for the severe toxaemia in patients with pneumococcal infections. No exotoxins have been found but endotoxins or autolysates of pneumococci are definitely toxic, and will kill guinea pigs and rabbits in a few minutes after injection (85), (26).

Small amounts of pneumolysin, an oxygen-labile or "O" haemolysin, is liberated from pneumococcus especially on autolysis (24), (86).

Parker (87) has described a necrotizing toxin, and Julianelle and Reimann (17) a proteose-like material which causes purpura in mice (88).

Oram (89) identified a relatively thermolabile leucocidin which was active against the rabbit leucocytes.

Coca's (90) fever-producing toxin is still under investigation.

However, haemolysins, leucocidins, and other specific types of toxins seem to be of minor importance in pneumococcal infections. The symptoms can be explained adequately by the very rapid growth and equally rapid autolysis of pneumococci in the tissues with the consequent liberation of endotoxins in quantity.

Antigenicity of the Pneumococcus

The immunizing properties and type specific antigenic action of the Pneumococcus and its derivatives are directly proportional to the virulence of the culture employed (91), (92) and appear to be the same whether the organism is in a living condition or appropriately killed by heat or by formalin, the integrity of the immunizing principle being better preserved in suspensions of heat-killed than of formalinized pneumococci (93).

Filtrates from fluid cultures and watery or saline extracts of pneumococci, representing only a part of the antigenic components of the pneumococcal cell, are more limited in immunizing properties than the entire cell (94).

Specially prepared broth cultures may exhibit a toxic action in animals, but there is no evidence of specific protective antibodies or the production of pneumococcal antitoxin (95).

The route by which antigens are introduced into the animal body affects both the kind and quantity of specific antibodies produced. The intravenous method is the most effective in producing a high degree of immunity, and strict type specificity of the antibodies. The intraperitoneal, intramuscular, or subcutaneous, have slower absorption rates of antigen respectively, but all can be used successfully to attain an immune response (96). The intradermal route usually stimulates the formation of species specific rather than type specific antibodies (97). Other routes, such as inhalation, insufflation, or injection into the bronchi are much less

The age and physical state of the animal usually determines the quantitative response to antigenic stimuli, and various species of strains may react differently.

Excessive doses of specific capsular polysaccharide may exert an antagonistic effect, since the amount of the soluble specific substance in a vaccine influences the development of an immune state (99).

Antibodies to the Pneumococcus

The introduction into animals of suitable species of pneumococci, and some of their natural components, results in the production by the body cells of specific immune substances demonstrable in the serum, that serve to protect the animal against the invading cocci. The presence of specific humoral antibodies, does not always denote recovery, but their absence usually presages death. These substances can be demonstrated by appropriate serological and immunological reactions. These immune substances comprise agglutinins, precipitins, opsonins or tropins, and complement fixing and protective antibodies.

These various immune reactions are often merely different manifestations of activity by the same substance - antibody. The type of the reaction depends on the method used.

Antibodies are now generally accepted as proteins, and belong to the class of serum globulins.

Measurement of Antibodies

Numerous methods have been devised for estimating

the amounts of antibody in a given antiserum. These may be classified into two general types - "in vivo" and "in vitro" methods.

"In Vivo" methods, measure the relative capacity of an antiserum to protect experimental animals against lethal doses of toxins or virulent bacteria, neutralize cutaneous reactions of toxins, or produce local or generalized passive sensitization. One of the most widely used is the mouse protection test.

"In Vitro" tests fall into several types:

1. Dilution Methods

Immune sera may be compared by using as the end point on titre, the highest dilution at which a detectable reaction, such as precipitation, agglutination, lysis, or complement fixation occurs, when added to a constant amount of antigen.

(a) Agglutination

The specific agglutination reaction takes place between the intact pneumococcal cells, whether living or dead, and the homologous antibody in the serum.

The appearance of agglutinins in the blood during the course of lobar pneumonia was studied by Chickering (100). In the most severe and often fatal cases, agglutinins could not be demonstrated - when they were demonstrable they usually appeared at the time of crisis, varying from one day up to several weeks.

Bull (101) ascribed to agglutinins a decisive part in the resistance of animals to pneumococcal infection.

On the basis of agglutinability, Cooper et al (102) extended the serological classification of pneumococcal types.

The agglutination reaction varies from strict type specificity to a broader species specificity, depending upon the nature of the antigen employed in the production of the immune serum, and that of the haptene participating in the reaction.

(b) Precipitation

The discovery of the precipitin reaction is attributed to Kraus (103). It involves the formation of a precipitate when antibody and soluble antigen are mixed together, followed by separation from solution of an insoluble antigen - antibody complex (104). Either the amount of antiserum or antigen may be varied but it is usual to keep one constant, usually the antigen. The difference between specific precipitation and agglutination arises chiefly from disparity in size of antigen particles. If the antigen has molecular dimensions (in solution) combination with antibody results in precipitation. However, if the antigen consists of suspended particles (centrifugable at 1000 - 2000 r.p.m.) aggregration by antibody results in agglutination.

The precipitin reaction exhibits a high degree of specificity (105) and sensitivity (106).

Neufeld (107), while investigating the agglutination of pneumococci with immune serum, added the clear solution obtained from dissolved pneumococci in bile, to immune serum,

and noted the formation of macroscopic aggregates. The reaction in different serums varied in degree. A marked reaction was characterized by an immediate opalescence, then flakes which became larger, settled to the bottom in the form of a membrane which did not diffuse on shaking.

In 1917, Dochez and Avery (108) demonstrated a specific capsular polysaccharide in the urine of patients with lobar pneumonia. Blake (109) showed a definite relationship between the excretion of precipitin in the urine and its development in the blood. If there were an excess in the urine over the amount in the blood, recovery took place shortly after or coincidentally with the appearance of the antibody, and had great prognostic value in the individual case of lobar pneumonia.

The somatic protein produces species specific precipitins not type specific (110).

The phenomenon of precipitation of the somatic or "C" polysaccharide of Pneumococcus is not limited to the serum of individuals ill with pneumococcal infection, since it may be demonstrated in the serum of patients suffering from rheumatic fever, bacterial endocarditis and lung abscess (111).

In 1931, Avery and Goebel (112) proved that the type specificity of the interaction of Pneumococcus and homologous immune serum was due to the capsular polysaccharide and not to the somatic nucleoprotein.

Morgan (113) demonstrated that a proper balance between antigen and antibody was required to bring about the

phenomenon of precipitation, and similarly in agglutination (114). In 1929, Heidelberger and Kendall (115) published the first of a series of communications on a quantitative study of the precipitin reaction as related to soluble specific substance and homologous immune serum. In 1932 (116), they devised a method for the micro-determination of specific polysaccharide of Type III pneumococcus, and in 1933, Soo Hoo, Heidelberger and Kendall (117) described the micro-estimation of precipitin in antiserum.

By means of the precipitin reaction, it is now possible to determine quantitatively the amount of immune nitrogen (specific antibody). It is usually analyzed by some modification of the micro-Kjeldahl technique developed by Parnas and Wagner (118). Heidelberger and MacPherson (119) have described a method for estimating very small amounts (as little as 10γ) directly as protein.

(c) Complement-Fixing Antibodies

The discovery of complement or alexin emerged from observations in the 1880's by Nuttall (120) and other workers, that blood serum exerts a destructive influence upon bacteria. This bacterial power decreased as the serum aged and was rapidly lost on heating at 56°C (121).

A fundamental capacity of complement is its capacity to participate in antigen-antibody combinations. The uptake of complement by specific precipitates has recently been demonstrated by an increase in weight (122), (123). Since complement is not a single entity but a complex of several serum constituents, and since the extent to which each of these components of complement is fixed by specific precipi-

tates, is not as yet clearly established, the weight increase measured by Heidelberger is tentatively ascribed to the "combining components" of complement.

The method of complement fixation is inferior to other serological methods for the demonstration or measurement of pneumococci antigen and antibody. It has, however, revealed the difference in the nature of specific antibodies in immune rabbit and horse serum (124).

2. Optimal Proportions Methods

These procedures determine the ratio of antigen and serum at which floccules appear most rapidly. They are based upon the assumption that the rate of flocculation is a measure of the combination of antigen and antibody, and will occur most rapidly when the relative proportions are most favourable.

Ramon (125) developed the constant antigen optimal ratio for the assay of diphtheria antitoxin, by adding increasing dilutions of antitoxin to a constant amount of toxin.

Dean and Webb (126) developed the constant antibody optimal ratio for the assay of precipitating sera, by mixing varying dilutions of antigen with a constant volume of serum (antibody) in a series of tubes, the total volume in each tube being constant. The tube in which flocculation first occurs is noted, and the ratio of antigen dilution to antibody dilution in this tube is calculated.

The optimal proportions method is more precise than qualitative dilution methods, but only comparisons of relative antibody content may be made.

These two ratios are not the same (127), and neither optimum appears to correspond necessarily to the point of complete removal of antibody (128). However, the strength of different sera may be compared by determining their optimal proportions points against the same antigen.

3. Quantitative Chemical Methods.

These methods permit measurement of amounts of antibody on a weight basis with a precision conforming to the requirements of analytical chemistry. These methods take advantage of the specificity of immunological reactions, and of the fact that antibodies are proteins. Since on addition of antigen, only antibody is removed from the serum, analysis for nitrogen can provide a measure of the amount of antibody.

To measure the antibody content of a serum (117), (119), an amount of a soluble or particulate antigen is added, sufficient to remove all the antibody as an insoluble precipitate or agglutinate. This usually occurs when a slight excess of antigen is present. The precipitate or the agglutinated bacteria are centrifuged off, washed free from serum protein, and analyzed for nitrogen by the micro-Kjeldahl (118) or other suitable method. Antibody nitrogen is calculated by subtracting the nitrogen of the added antigen from the total nitrogen found.

Host Response to Antigenic Action of Pneumococcus

Nature has provided animals with many natural means for defense against infection. However, once these barriers

are passed and entrance is effected, the offenders are subjected to still further antagonistic forces.

The pathogenic organism is one which arrives with the capacity to survive in a host, and whose multiplication results in injury which, in turn, usually elicits an active response from that host. With the beginning of growth, the products of the pathogen cause injury to the local tissues, arousing an inflammatory response which seeks to localize the injury. This effort at fixation is accompanied by the accumulation of polymorphonuclear leucocytes from the blood stream and phagocytosis begins. This attraction of phagocytes to the bacterial cell is termed positive chemolaxis (129).

The polymorphonuclear leucocytes after engulfing and transporting the bacteria to clearing depots, is unable to digest them. The mononuclear macrophages soon appear and rapidly digest the organisms.

However, virulent organisms as the pneumococcus, may by virtue of its capsule resist phagocytosis, and soon gain entry to the blood stream. Here antagonistic forces encountered may be body temperature inimical to the proliferation of the cocci, normal opsonins which prepare the bacteria for ingestion by the phagocytes, and perhaps natural protective substances (130).

However, with a progressing superiority of the invading organism over the natural localizing and disposing mechanisms, the host would be overwhelmed unless other defenses could be marshalled. To meet this emergency, the body

cells are now actively aroused to activity by the stimulating components of the bacteria and produce humoral antibody globulin specifically designed to neutralize them, and so permit the host to assume the offensive.

In the case of the virulent pneumococcus, the specific globulin fraction combines with the specific capsular polysaccharide resulting in agglutination of the bacteria, which now sensitized can be phagocytosed and digested. If the capsular polysaccharide is in solution, there is precipitation of antibody-polysaccharide complex, or complement is used up during the process of the antibody combining with antigen.

However, the appearance of antibody does not correspond exactly with termination of clinical disease (131). Injured must be removed and healing occur.

After this has been concluded, the cells of the animal seem to be significantly altered, in that they are sensitized and exhibit an accelerated response to a second stimulus from the same antigen (132). This may be permanent or temporary.

In pneumonia, cutaneous reactions to the nucleoprotein are absent until convalescence. Precipitins are demonstrable in the serum, however, throughout the entire course of the infection (133).

The somatic "C" polysaccharide, can be demonstrated subcutaneously within twenty-four hours after onset of the illness. The reaction, while exhibiting an immediate wheal and erythema but only reaches its maximum in six to twelve

hours. This reactivity persists until convalescence, when it disappears (134).

The capsular polysaccharide exhibits a cutaneous response with the beginning of convalescence and the appear-. ance of type specific antibodies (135). This reaction is an indication of specific immunity, but cellular participation seems necessary, for fatal cases may be negative even though specific antibodies are present.

Despite the fact that specific antibodies develop, recovery from pneumococcus pneumonia does not ensure a permanent resistance. This was demonstrated in 1934 by Finland and Winkler (136) in the frequency of Type I pneumonia.

Site of Antibody Formation

A large body of evidence indicated that the reticulo-endothelial system is involved in antibody production. The evidence in support of the role of the lymphocyte in antibody formation has been based on the earlier appearance of antibodies in local lymph than in serum; the higher titre of specific antibody in lymph and lymphocytes from the node on the side inoculated with antigen; the high titre of antibody in lymphocytes as compared to that in lymph or serum, and the high titres of antibody in lymph node extracts (137 - 140).

It has also been shown that adrenal cortical hormones may release γ-globulin as well as antibodies into the circulation through their effect on lymphocytes (141 - 144).

Other investigators have provided evidence that the plasma cells are at least in part responsible for antibody

manufacture (145).

Habel et al (146) have shown that the relatively high antibody content of lymph may be accounted for, at least in part, by physical transfer from blood, and that the ratio of lymph antibody to serum antibody increases when the node has been stimulated by an irritant such as a heterologous antigen. They state that there is little significant evidence in their experiments to indicate that the lymphocytes or lymph node cells per se carry large amounts of antibody.

The amounts of antibody which can be produced in response to minute amounts of antigen are quite large (147), therefore the view that antigen may be permanently incorporated into the antibody manufactured is difficult to conceive.

The role which antigen plays in the manufacture of antibody is not yet known, nor is it certain that the antigen must always be present at the time antibody is formed (148), (149). Whether or not the presence of antigen is necessary for continued synthesis of antibody, it appears certain from isotope tracer experiments, that antibody protein is continually being destroyed at the rate of about one-half every two weeks (150), (151).

Antibody continues to be formed even though the total level is declining, which implies in this circumstance, the rate of destruction exceeds the rate of formation, but the presence of antibodies years after apparent recovery from infection may denote the persistence of antigen, perhaps in a living and potentially infectious state.

C. I CHEMOTHERAPY - SULPHONAMIDES

An ideal chemotherapeutic agent may be defined as one which by inhibiting certain vital functions of the invading microorganism, or neutralizing its products, terminate the disease without causing any toxic effect on the host. The definition presupposes that the point of attack must be on a specific function or structure unique to the microorganism, so that the tissues or organs of the host escape the toxic action.

Until Domagk in 1935 (152) first reported the usefulness of prontosil in haemolytic streptococcal infections, and Trefouel et al in 1935 (153) established sulphonamide as the active part of the molecule, specific antipneumococcal serum therapy had been the only means of combatting pneumococcal infection. Apart from the difficulties met in the variety of specific serological types of sera, preparation, concentration, sensitization etc., sera was powerless to stop the multiplication, invasion, and subsequent toxic activities of the organisms within the infected host, and the mortality rate in pneumococcal infections was still considerably high.

Pneumococci are highly susceptible to most of the sulphonamide derivatives, but sulphonamide resistant mutants may appear, especially if the drugs are administered in sub-optimal dosage for a prolonged period (154), (155).

The similar pharmacological action of the sulphonamides can be ascribed primarily to their common structure, which is a free aromatic amino group para to the sulphonic group. Acetylation of the amino group removes all anti-

bacterial activity.

Absorption, Excretion and Distribution (156).

Most sulphonamides are nearly completely absorbed in three to four hours after oral administration, and more rapidly on parenteral administration. After absorption, they are acetylated in the liver, and excreted as both free and acetylated compounds in the urine. It diffuses readily into all tissues and fluids.

On the basis of the rate of absorption elimination, the lag period before bacteriostasis and the tendency to develop resistant mutant strains, early effective concentration must be maintained, and furthermore, decreases the possibility of toxicity symptoms from prolonged usage.

Mode and Mechanism of Action

The primary action of sulphonamides on bacteria is generally believed to be bacteriostatic, but maybe bactericidal if the concentration is sufficiently high (157), or if accompanied by other unfavourable environmental conditions (158), such as poor cultural conditions, adverse temperatures (159), antibodies, toxic proteolytic products etc. It may also inhibit the growth or functions of cells other than bacteria, and may also vary in its action on bacterial species, strains and even individual organisms (160).

Its action is usually biphasic (161), and inversely related to the size of the inoculum (162) influenced by the structure of the sulphonamide (160), and changes in pH (163). Sulphonamide inhibition is synergized by antibodies (164) and

bacteriophage (165).

In 1940, Woods and Fildes (166), made the discovery that *p*-amino-benzoic acid is an extremely potent sulphonamide antagonist, and from this arose the theory that sulphonamide interferes with the utilization of the substrate aminobenzoic acid in an anabolic reaction by competing with the latter for its enzyme. On further investigation, however, many other substances were found with no *p*-aminobenzoic acid content, e.g. methionine, urethane, glucose, which were shown to be sulphonamide antagonists. The recent theory now held is that *p*-aminobenzoic acid is only a cog in the metabolic apparatus, and sulphonamides prevent *p*-aminobenzoic acid from being synthesized to replace losses or to satisfy growth requirements (167). This explains the lag period in sulphonamide activity, since the normal store of *p*-aminobenzoic acid in the organism is sufficient at first for this to occur.

Sulphonamides inhibit the aerobic and anaerobic respiration of bacteria and other cells whether in a resting state or actively dividing, and so inhibit growth. The identities of the inhibited respiratory enzyme or enzymes, responsible for the growth inhibition are not definitely known, but sulphonamides inhibit certain dehydrogenases (168) and carboxylase (169) which secondarily results in growth inhibition.

Lockwood et al (170) in 1938, demonstrated inhibition of growth, due to destruction of proteases by sulphonamides.

It is now conceded that sulphonamides need undergo no transformation to be bacteriostatic being in the active form per se. It had been previously assumed that sulphonamides had to first undergo some oxidative, transformation before becoming active (171), and while it may be conceded that certain toxic and side effects of the sulphonamides may be caused by small amounts of oxidation products formed"in vivo"(172) and catalase inhibition may play a part in the bacteriostasis of some organisms, the principal mode of sulphonamide action"in vivo"and"vitro"seems to be by some mechanism other than the inhibition of catalase.

"In vivo"and"in vitro", there is a demonstrable lag period (173) and a tendency to primary stimulation of growth, and"in vitro"tests show no loss in concentration of the drug, hence disproving any possibility of fixation or absorption to the surface (174) of bacteria.

II ANTIBIOTICS - PENICILLIN

The term antibiotic designates a product of the metabolism of one microorganism that is antagonistic to the continuation of the normal life activities of another microorganism, when present even in very low concentrations (175).

The antibiotic Penicillin, produced by the mold Penicillium notatum, was first discovered in 1929 by Fleming (176), and purified and tested by Chain and Florey in 1940 (177). This substance has been found to be an unstable acid with the probable formula of $C_{14}H_{19}NO_6$ or $C_{14}H_{17}NO_3.H_2O$. (178).

The dosage until previously had been expressed in terms of Oxford or Florey Units, one Oxford Unit designating

the amount of penicillin, which, when added to 50 ml. of meat extract broth under standard conditions completely inhibits the growth of Staphylococcus aureus (179). Recently, a crystalline salt of penicillin G. has been accepted as the international Standard, of which by definition, one international unit is 0.6 micrograms (180).

The penicillins are virtually non-toxic to animal tissues, and exert a strong selective action against certain types of bacteria (181). There seems to be little evidence in differences in the mode of action of the various penicillins F. G. K. X. etc. (182).

Penicillin is selective in its action, and "in vitro" studies have shown that it causes an actual decrease in the number of organisms, the bacterial action taking place only if multiplication occurs (178). According to Waksman (183), this decrease in number of organisms and their final destruction by an antibiotic probably consists in the interference with certain essential metabolic processes of bacteria. Gale and Taylor (184) have shown that one of the earliest manifestations of bacteriostatic concentrations of penicillin on Staphylococcus aureus is a blocking of the absorptions of the essential metabolite, glutamic acid, while lysine assimilation endogeneous respiration and glucose oxidation and fermentation are essentially unchanged. Evidence from cytochemical studies indicate that penicillin exerts its bacteriostatic action by promoting dehydrogenation of - SH groups to S - S more rapidly than the organisms can restore the active sulphhydryl group (185).

The penicillins are characterized by the fact that they inhibit susceptible bacteria more effectively both "in vivo" and "in vitro" when the environment is most favourable for growth and that their activity does not seem to be affected by the number of organisms present (186).

The structure of penicillin differs from that of any known compound and no specific antagonists are known. However, an enzymatic inactivator penicillinase was discovered by Abraham and Chain in 1941 (187) among the colon-dysentery group. However, sensitivity of an organism to penicillin is not determined primarily by the presence or absence of this enzyme. Tissue extracts, pus, urine and peptones do not interfere with its action (188).

Bacteria are most susceptible to the action of penicillin, when they are in the logarithmic phase of growth, and when they are dividing most rapidly, and are exhibiting the greatest need for oxygen (175). The primary action of penicillin is not upon the respiratory system, as shown by Chain, Duthrie, and Callow (189), in that resting cells showed no decrease in oxygen consumption in the presence of penicillin, although inhibition occurred when growth began.

Hobby, Meyer and Chaffee (190) found that the effect of penicillin may be bacteriostatic or bactericidal depending on experimental conditions, and also that the number of bacteria decreased at a constant rate until 99 per cent were destroyed. No detectable amount of penicillin was absorbed from the solution or destroyed by the organism. However, Johnson (1947) (191) showed that in low concentrations,

penicillin is extracted from the medium by sensitive staphylococci, while resistant cells extracted much less.

Bacteria under the influence of bactericidal concentrations of penicillin undergo morphological changes, with the tendency to produce giant forms, distortion, swelling, lysis, elongation and chain formation (192), (193). This is not specific to penicillin, however, and may be induced by sulphonamides (194) and other drugs.

Resistance to penicillin is usually of a temporary nature, for after several generations, organisms return to their normal rates of growth (195). Strains of organisms that become resistant to one type of penicillin are correspondingly resistant to others (196) since penicillin fastness depends upon the slowing down of metabolic rates.

There is an insignificant lag phase in bactericidal action"in vivo"once effective concentration in the tissues has been attained, in contradistinction to sulphonamides (197).

Like other Gram-positive organisms pneumococci are highly susceptible to the action of penicillin, even though infection has been well established (198). Sub-bacteriostatic concentrations, however, have been shown to enhance metabolic activity and growth of Staphylococcus aureus (199).

Penicillin is considerably less toxic than the sulphonamides in man. Among animals, however, the guinea pig shows a species susceptibility to penicillin (200), (201).

Absorption and Excretion

Rammelkamp and Keefer (202) studied the absorption, excretion, and distribution of penicillin administered by

various routes; i.e. intravenous, subcutaneous, intramuscular, intra-articular (knee joint), intrapleural, oral, duodenal and rectal.

Intravenous injection resulted in a high initial concentration in the blood plasma, followed by an abrupt fall. After intramuscular injection, penicillin was absorbed rapidly and excreted in the urine, but after subcutaneous administration, both processes were retarded. Absorption from intrapleural and intra-articular cavities also was delayed, with corresponding slow excretion: Appreciable amounts of penicillin were found in the pleural and joint cavities twenty-two hours after injection of these sites. Absorption from the duodenum was rapid, but oral and rectal doses were poorly absorbed; little penicillin was excreted in the urine after any of these three methods of administration. In patients with renal failure, the excretion of penicillin was delayed and high concentrations in the blood stream were therefore maintained after intravenous injections. A study of the distribution of the drug in the body showed that very little penetrated the erythrocytes. None was found in the spinal fluid, saliva, or tears of subjects receiving it intravenously.

Eagle, Fleischman, and Musselman (203) showed that for equal doses of penicillin G. intramuscularly the serum levels at a given time were greatest in man, rabbit and mouse respectively, indicative of a significant species difference in the initial distribution of the drug in the body fluids. Excretion however was 91 to 99 per cent in an hour

in the mouse, 65 per cent in the rabbit, and 65 - 80 per cent in man.

One of the greatest disadvantages of penicillin clinically is its rapid elimination from the body. Efforts to retard its absorption and elimination and thus to prolong its action, have been the concurrent use of diodrast, para-aminohippuric acid, beeswax in peanut oil, aluminium monostearate in peanut oil, dextrose, adrenalin, in combination with human serum albumin, etcetera. Much larger doses are given and usually once daily, and effective therapeutic serum levels can be demonstrated (204), (205).

D. IMMUNITY TO THE PNEUMOCOCCUS FOLLOWING THERAPY

The details of the mechanism by which type specific antipneumococcic serum overcomes pneumococcal infections are only partly known. Serum with a high antibody content acts immediately by agglutinating the pneumococci which are then removed by phagocytosis (206). Antibody, by combining with somatic or capsular substance of homologous pneumococci (230), inhibits some of the vital activities of the cells and renders them more susceptible to phagocytosis. The completeness of the phagocytosis, and the ability of the body to dispose of any of the remaining organisms determine whether recovery or death takes place (207). There are conceivably as yet unknown qualities of human blood or tissue cells as well as of pneumococci which may either augment or decrease the curative action of specific immune serum. Also immune serum is powerless to prevent multiplication, invasion, and subsequent malignant activities of the organisms within the infected host, and even though it be rich in passive protective power cannot compensate for active antigenic stimulation in the tissues of the host.

Chemotherapy, however, is ultimately conceived with the triple relationship of drug, parasite and host complex. When the drug is introduced into the host the entire picture may be radically modified through factors of absorption, distribution, excretion and degradation which immediately come into operation and which cannot all be reproduced"in vitro."

The sulphonamide drugs, which are essentially bacteriostatic, act by restraining the growth of the organisms, with reduction in rate of production of endotoxins and antibody neutralizing polysaccharides until sufficient antibody has been formed by the body to assure sensitization and phagocytosis of the pneumococci (208). The participation of specific immunity appears to be necessary for a successful outcome in sulphonamide therapy, (208), (209). As might be expected, the sulphonamide drugs and specific antipneumococcal serum exert a synergistic effect (208).

In the case of penicillin, which is essentially a bactericidal compound when used in full therapeutic dosage, dependence upon specific antibody is less than with sulphonamides, although there is some evidence that penicillin is less active against those pneumococcal types (e.g. II and III) which are weakest antigenically (210).

"In vitro" experiments indicate that factors which determine the effect of sulphonamides on penicillin action include the organisms involved, their number, environmental conditions, susceptibility to both penicillin and sulphonamides, and the concentration of each bacteriostatic agent (211).

An excess of penicillin acts rapidly during the first few hours of multiplication of organisms, and complete sterilization may result before the end of the sulphonamide lag period. If, however, sterilization is incomplete at the end of this lag period, the sulphonamides will decrease the rate of growth provided the organism is sensitive to the

sulphonamide and present in small numbers. Also because penicillin acts predominantly at the time of cell division, a decreased rate of multiplication due to the sulphonamide will also tend to decrease the rate at which penicillin acts (211).

Active immunity indicated by the appearance of an excess of type specific antibodies in the serum, however, is not an essential part of the mechanism of recovery from pneumonia (212), although early appearance is usually favourable and lowers homologous carrier rate (213) as it is assumed to be evidence of an excess over the amount necessary to neutralize antigens present in the tissues (214).

Experimentally, Kneeland and Mulliken (212) (1940) have also shown variation in the amount and time of appearance of antibodies with respect to different sulphonamides, i.e. Sulphathiozole and Sulphapyridine, the former demonstrating an early excess, the latter very little and late. They concluded that sulphapyridine was probably a more powerful antibacterial agent than sulphathiozole. However, there did not seem to be any demonstrable effect on final immunity as seen in tests for type specific agglutinins and skin tests with the homologous type specific polysaccharides, (215) which were of the magnitude of those occurring naturally in the course of untreated pneumonia, and were not affected by drug therapy.

Wood et al (207) (216) (1946) have presented evidence to show that phagocytosis, a major role in the final destruction of pneumococci in the lung, is due neither to the

opsonizing action of the sulphonamides nor to type specific antibody contrary to the opinion held previously by others (217), (218). Also sulphonamide chemotherapy frequently brings about a crisis in pneumonia long before type specific antibodies appear in the patient's serum (219), (220), (221). However, although sulphapyridine may exert a marked improvement in twenty-four hours, if therapy is stopped before antibodies appear (often up till the 8th or 9th day) a relapse occurs (209), (212).

Jersild (222) observed that the rapid bactericidal action of penicillin on streptococci resulted in a concurrent rapid fall in the Erythrocyte Sedimentation Rate (E.S.R.) and the formation of a small amount of antibody, and noted the possibility of relapse and reinfection with the same type of streptococcus in a patient within one month. Welch (223) et al also noted inhibition of phagocytosis by penicillin.

Heidelberger and Anderson (224) in quantitative pneumococcus anticarbohydrate determinations have not been able to show any difference or prevention of the immune response characteristic of spontaneous recovery in lobar pneumonia, bronchial pneumonia or pneumococcic meningitis, during sulphonamide or penicillin therapy (225).

Collen et al (226) clinically were able to show that although patients were afebrile in forty-eight hours, to avoid a relapse, it was necessary to continue penicillin therapy somewhat longer than with sulphadiazine. This has been observed also by Tillet et al (227).

The time of appearance of specific immunity during sulphonamide therapy, as demonstrated by agglutinins and intracutaneous reaction to the homologous polysaccharide varies from two to seven days (228), (229), (214), (210), (209), (231), (174), (221), (232). However, the indisputable evidence of immunity is resistance to reinfection with the homologous infecting organism (232), (174), (234), (235), (214).

Junge and Rosenthal (236) have also shown experimentally that body temperature and oxygen consumption during pneumococcal infection under sulphonamide therapy are influenced by environmental temperature, with a resulting effect on survival and immunity.

MATERIALS AND METHODS

I TEST ORGANISM

A virulent strain of Diplococcus pneumoniae Type I was used throughout the experiments here reported. This lyophilized strain No. 710 was obtained by courtesy of Dr. G. W. Rake, The Squibb Institute for Medical Research, New Brunswick, N. J. and hereafter will be designated S-710-T.I in this report.

This strain was transferred to "L.S." broth, and from this, Brewer's meat and blood-agar plates were inoculated. After 18 hours incubation at 37°C., the organisms were tested for purity and morphology of culture by Gram's Stain. Bile solubility was tested by making a suspension of the pneumococci grown on the blood-agar plates with 0.85 per cent saline in a test tube, then adding an equal amount of 0.5 per cent sodium desoxycholate pH 7.5 and incubating in a water bath at 37°C. for 20 minutes with resulting clearing of the previously turbid solution. Specific typing of the organism was tested by the Neufeld-Quellung reaction using Type I antipneumococcic rabbit serum (E. R. Squibb & Sons, N. Y.) with resulting agglutination of the organisms and swelling of the pneumococci capsules.

Finally "pneumo" broth was inoculated with S-710-T.I, and approximately 0.1 ml. of the concentrated organisms were then aseptically transferred to sterile Kahn tubes and lyophilized for future use in experiments.

The strain was typically Gram-positive, appearing

mostly in pairs of large lancet-shaped cocci and surrounded by definite capsules, others were in short and long chains, others singly. No swollen or involution forms were seen. On the surface of an 18 hour old blood-agar plate, the colonies were smooth, small, flat, somewhat flattened colonies surrounded by a small zone of greenish discoloration in the depths of the media. Capsules were demonstrated by Baker's Method of capsule staining, using Ziehl-Neelsen's Carbol Fuchsin and Nigrosin.

II VIRULENCE FOR MICE

The contents of 1 lyophilized S-710-T.I tube was transferred into 10 ml. "L.S." broth and incubated for 18 hours at 37°C. The tube was then twirled to resuspend the sedimented organisms, and 1 ml. of this was placed into 9 ml. of peptone broth using a 1 ml. K-Exax pipette (T.D.), care being taken that as little as possible of the 18 hour culture remained on the outside of the pipette. This tube was twirled between the palms of the hands to ensure good mixing, and another 1 ml. K-Exax pipette used to transfer this dilution (10^{-1}) into 9 ml. of peptone broth (10^{-2}). This was carried out until 10^{-9} dilution was reached. 1 ml. of each dilution, from an 18 hour culture to 10^{-9} dilution was injected intraperitoneally into 4 white mice, 2 mice placed in each glass jar.

All 4 mice in each dilution up to 10^{-7} died within 48 hours, some in the lower dilutions dying within 24 hours. In 48 hours 2 out of 4 mice died of the 10^{-8} dilution, none

in the 10^{-9} dilution. Dilutions 10^{-7} and 10^{-8} were again checked, with again 100 per cent mortality in the former and 50 per cent mortality in the latter.

The number of organisms in the dilutions were counted as described by Reed and Reed (237). This was done by using the ordinary Pasteur pipettes made from 7 mm. glass tubing. As the size of the drop delivered is governed, other factors being constant, by the external diameter, the tapered portion of the pipette is pushed through the gauge hole 0.047 inch in diameter in a brass square 2 inches square and 1/8 inch thick, until it fits snugly by its own weight. At this site on the upper surface, a small circular scratch is made with a glass file, the pipette removed and with both thumb nails at this mark, broken by pulling apart, so that a clean circular break is made with no ragged edges. These pipettes were then sterilized and plugged at the top with orange cotton to separate them from other Pasteur pipettes. These pipettes delivered 40 drops of water per ml., at a rate of 30 drops per minute. This was calibrated by dropping a counted number of drops into a weighing bottle. Blood-agar plates were placed upside down with the lids half off in the incubator at 37°C. until ripples appeared on the surface. This took approximately one hour, although this depends on the humidity. The pipette was then held vertically and 5 drops delivered on the surface of the plate about 3/4 inch from the periphery. The plates were then gently shaken by a horizontal rotatory movement to spread the drops evenly, and incubated for 18 hours.

The total number of bacteria per ml. of the original material = $\frac{A}{B} \times C \times D$

Where A = number of colonies per plate.

B = number of drops per plate.

C = number of drops per ml.

D = dilution factor.

As the number of colonies developing per drop of fluid added to the plates that can be most satisfactorily counted is between 20 to 40, those dilutions up to 10^{-2} were discarded as they exceeded this greatly. The dilution of 10^{-3} however gave 36, 28, 30, 32, and 33 colonies per drop, making a total of 159 colonies per plate and so a total number of 1,272,000 bacteria per ml. Repetition gave 29, 34, 29, 30 and 33 colonies per drop,making a total of 155 colonies per plate and a total number of 1,240,000 bacteria per ml. Therefore 10^{-7} dilution was assumed to be approximately between 124 and 127 organisms per ml., and 10^{-8} dilution about 12 organisms per ml.

III VIRULENCE FOR GUINEA PIGS

Guinea pigs were chosen for the main experiment as they often suffer from spontaneous pneumococcal infections, as humans are prone to do. The virulence of S-710-T.I in producing clinical fatal pneumococcic peritonitis in guinea pigs was therefore next determined.

Guinea pigs are usually less susceptible to pneumococci than are mice; however, it was necessary to determine an adequate infective dose that would produce a 50 per cent

mortality rate yet sufficient to establish clinical infection.

Two guinea pigs each were injected intraperitoneally with 1 ml. of an 18 hour culture, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Rectal temperatures were taken twice daily and weight daily for a week without the appearance of peritoneal infection, although bacteremia was evident 6 hours after injection of the cultures. These organisms were subsequently grown in "L.S." broth for 18 hours, and 1 ml. injected intraperitoneally into 2 more guinea pigs, again without any mortality. This was repeated twice more, and 5 per cent fresh rabbit serum added to the "L.S." broth in which the pneumococci were grown.

Peritoneal smears taken 6 hours after injection of the pneumococci showed marked phagocytosis by the leucocytes.

Besredka (238) has shown that in the presence of carmine, when given either some hours before or at the same time as the injection of living meningococci intraperitoneally, the apparent virulence of the culture is markedly raised; and also that animals which had previously received an intraperitoneal dose of finely powdered carmine succumbed to a dose of $As_2 S_3$, which normally they would have tolerated. This was similarly shown by Murray (239) who injected carmine into mice at the same time and 96 hours before the intraperitoneal injection of varying doses of meningococci.

An intraperitoneal dose of 0.01 gm. of carmine powder in no way affects the apparent health of mice. Durham (240) and others have shown that carmine is taken up almost completely by the omentum within 15 minutes of intraperitoneal

injection. It is first "agglutinated" into masses by mucin as described by Gengou (241), and then gathered up by the peritoneal folds; the omentum is the principal of these, but the pelvic folds and those in relation to the genital organs are almost equally active.

A stock solution of carmine (G. T. Gurr, London, England), 10 gms. in 100 ccs. sterile saline, was autoclaved for 20 minutes at 120°C. 1 ml. of this solution containing 100 mgm. was injected intraperitoneally into 2 guinea pigs - wt. 538 gms. and 519 gms. (5 - 10 mgm. calculated for average 20 gm. mouse). Peritoneal smears in 24 hours showed macrophages engorged with carmine particles. These smears were stained with 1 per cent gentian violet diluted 1 in 20 with distilled water, and 0.5 per cent acetic acid. The carmine particles appear bright red in a pale blue cytoplasm, the nucleus staining darker blue. No ill effects were noted in the animals at any time.

Using this dosage of carmine, 2 guinea pigs (wt. 646 gms. and 583 gms.) each were injected intraperitoneally with 100 mgm. carmine, then 1 ml. of an 18 hour culture of S-710-T.I pneumococci; both succumbed within 48 hours and peritoneal smears showed leucocytes engorged with carmine particles and very large numbers of extracellular pneumococci. 1 ml. of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of an 18 hour culture were then injected intraperitoneally immediately after 100 mgm. carmine solution, into each of 2 guinea pigs. Both guinea pigs of the 10^{-1} dilution succumbed, one in the 10^{-2} dilution; both with the 10^{-3} and 10^{-4} dilutions survived.

The 10^{-2} dilution was checked in 2 more guinea pigs with a similar result.

This was the dilution used in the following experiments.

IV TYPE SPECIFIC ANTIPNEUMOCOCCIC RABBIT SERUM

In order to have sufficient test sera to devise a suitable technique for measuring antibody production, 3 white rabbits (No. 1. weighing 3440 gms., No. 2. 4760 gms., No. 3. 3470 gms.) were immunized after being kept for one week under observation, according to the schedule acclaimed by Goodner, Horsfall and Dubos (242).

The vaccines were prepared by inoculating 10 ml. "L.S." broth with a lyophilized S-710-T.I pneumococci culture, incubated 18 hours at 37°C., killed at 60°C. for 1 hour in a water bath, centrifuged and resuspended in saline to compare with No. 8 McFarland Nephelometer, then cultured for sterility, bottled and kept in the refrigerator.

As type specific antigenicity of encapsulated pneumococci is closely associated with the Gram-positive structure of the cell, this was checked by Gram's stain and not used after one week.

TABLE I

SCHEDULE OF IMMUNIZATION

Week	Amount of Suspension of Pneumococci injected intravenously				
	Monday	Tuesday	Wednesday	Thursday	Friday
	ml.	ml.	ml.	ml.	ml.
1	0.05	0.1	0.2	0.5	
2	0.1	0.2	0.5	1.0	
3	0.2	0.5	1.0	2.0	
4	Rest				
5	1st bleeding.A.	0.2	0.5	1.0	2.0
6	Rest				
7	2nd bleeding.B.	0.2	0.5	1.0	2.0
8	Rest				
9	3rd bleeding.C.				

Immunization was carried out by giving each rabbit the scheduled dose into the left marginal ear vein using a No. 24 needle and a 1 or 2 ml. syringe as was necessary.

The first bleeding (A) was taken after three weeks of immunization and a week's rest, the second bleeding (B) after a week of maintenance immunization and a week's rest, after which the third bleeding (C) was taken.

The rabbits were bled from the heart. They were held firmly on their back by an assistant in an extended position. The precordial area was shaved and swabbed with Tincture of Iodine (5 per cent). A dry sterile 50 ml. syringe fitted with an 18 gauge needle was used to withdraw about

55 - 60 ml. blood slowly, avoiding negative pressure. It was placed in a dry sterile 250 ml. centrifuge tube, allowed to stand at room temperature for about an hour, then placed in the refrigerator overnight to clot. It was then centrifuged, the serum drawn off, Merthiolate 1:1,000 (Eli Lilly and Co.) was added to ensure sterility.

Test Bleedings and System of Potency Grading

At the time of the scheduled bleedings 1.0 ml. of blood was obtained from each rabbit and placed in a small centrifuge tube.

To 0.2 ml. of serum obtained from this blood is added 0.3 ml. of saline and 0.5 cc. of a 1:20,000 dilution of specific capsular pneumococcus polysaccharide Type I (Preparation # 207, E. R. Squibb & Sons, N. Y.). The tubes were incubated for 2 hours at 37°C. and overnight in the refrigerator. Readings were made then as to the character and quantity of the immune precipitate,as a guide, as to whether the individual rabbit was producing adequate immunized serum.

Goodner et al (242) have prepared a scale for the approximate estimation of Type I antipneumococcic rabbit sera from test bleedings. In the first bleeding (A) rabbit No. 1 sera gave an opaque disc corresponding to Grade 5 in their scheme and of approximately 900 - 1100 mouse protective units per ml. Rabbit No. 2 sera was the same, and rabbit No. 3 gave a heavy opaque loose precipitate corresponding to Grade 7 and of approximately over 2000 mouse protective units per ml.

These 9 sera (3 bleedings from 3 rabbits) were used in "in vitro" methods of measuring antibody potency by:-

(a) Qualitative agglutination tests

(b) Qualitative precipitation tests

(c) Optimal proportions tests

(d) Quantitative precipitation tests

(e) Standardizing each pneumococci Type I antigen used in agglutination tests in the experiment.

(a) Qualitative Agglutination Tests

Halving serial dilutions with 0.85 per cent saline, from a 1 in 2 dilution to a 1 in 2048 dilution of each serum were made in clean dry Kahn tubes, each tube containing 0.2 ml., and the control tube containing 0.2 ml. saline only. 0.2 ml. of antigen No. 1. was then added to all the tubes, which were placed in a water bath at 37°C. for 2 hours, then in the refrigerator overnight at 4°C. The tubes were read for agglutination against a viewing box having a black background with oblique light.

See Figure 1. for agglutination titres of sera.

i. Preparation of Antigen

The antigen used in agglutination tests was prepared from inoculating about 100 ml. "L.S." broth (prepared by the method used in the Department of Bacteriology, McGill University) with about 6 lyophilized S-710-T.I pneumococci cultures. The inoculated broth was incubated at 37°C. for 18 hours, then the organisms killed by heating for 1 hour at

60°C. in a water bath. After centrifuging, the organisms were washed 4 times with 0.85 per cent sterile saline, and finally diluted to a concentration of No. 2 McFarland Nephelometer with 0.85 per cent sterile saline and kept constantly in the refrigerator at 4°C.

The antigen was tested for purity and Gram-positiveness of the organisms by a Gram's stain, capsule swelling with homologous Type I antipneumococcic rabbit serum (E. R. Squibb & Sons, N. Y.) and sterility by culturing in Brewer's medium and an aerobic blood-agar plate. No antigen more than a week old was used throughout the experiments.

ii. Variation in Consecutive Antigen Preparations.

Each new lot of antigen, as it was prepared, was tested for agglutination against the same immunized rabbit Type I antipneumococcic serum. (Rabbit No. 2., 2nd (B) bleeding was arbitrarily chosen). See Figure 2.

Agglutination of Antigen 1. was checked on consecutive days with constant results and negative controls. See Figure 2. Antigen 1.

Antigen 2. was prepared 15 days later in every way identical to Antigen 1., but the agglutination titre given by Antigen 1. could not be duplicated. The pH of Antigen 2. was next varied through pH 5.8, pH 6.0, pH 6.2, pH 6.4, pH 6.6, pH 6.8, and pH 7.0 and all tests placed for 2 hours at 37°C., then ice box overnight. (See Figure 2. Antigen 2.).

The first three agglutination titres of antigen, 2 at pH 6.3 were the original Antigen 2. placed 2 hours at 37°C.

then ice box overnight, ice box only, and room temperature only in that sequence.

All pH's were recorded by the Beckman pH meter which was standardized each time by use of 0.1 M potassium hydrogen phthalate at pH 4.0.

Antigen 3. prepared 9 days after Antigen 2. was pH 6.35 on 3 days of checking against the same serum. However on the first 2 days the titre, while higher, was less in amount than Antigen 1. The following day the test was left at room temperature only for 24 hours with increase in amount of agglutination and more so when left for 48 hours. Vigorous shaking after addition of the antigen however produced the original titre of Antigen 1.

Antigen 4. pH 6.5, prepared 4 days after Antigen 3., was completely non-agglutinable regardless of shaking, temperature or pH variations.

Antigen 5. pH 6.6 gave a titre similar to Antigen 1. Its preparation was the same as originally described for Antigen 1.

Antigen 6. again was completely non-agglutinable. However, this was found to be contaminated with yeast organisms.

Antigen 7. gave good agglutination and was prepared identically to Antigen 1.

Antigens 8. and 9. were non-agglutinable in every dilution.

Antigen 10. showed good agglutination.

Antigen 11. was prepared by washing off the organisms

grown for 18 hours on blood-agar plates with 0.85 per cent saline, then killing at 60°C. for 1 hour in a water bath. This antigen produced complete agglutination from a 1 in 2 dilution to 1 in 16 dilution but nonein 1 in 32 dilution or thereafter.

Antigen 12. was prepared from pneumococcus Type I injected intraperitoneally into mice then recovered shortly before death and grown in "L.S." broth. Agglutination from this antigen was extremely poor.

Antigen 13. was prepared as Antigen 11. and gave an identical agglutination titre.

Antigen 14. was prepared by growing Type I pneumococcus from a lyophilized culture in "L.S." broth 18 hours, then killing with 0.2 per cent formalin at room temperature, then heated for 1 hour at 60°C. in a water bath, the remaining as in preparation of Antigen 1. Agglutination was fairly good with this antigen.

Antigens 15. and 16. were prepared as for Antigens 11. and 13.

Antigens 17 to 20 inclusive were prepared as for Antigen 1, with varying poor to moderate agglutination titres.

The "L.S." broth in which the pneumococci had been growing and each saline washing was tested for Specific Soluble Substance (S.S.S.) by adding it drop by drop to 0.5 cc. of the immunized rabbit antiserum (Rabbit No. 2., 2nd bleeding). Only a very faint trace was detected by a faint cloudiness.

Antigen 21. was prepared as Antigen 1. However, Difco Proteose Peptone No. 3. was used in its preparation

instead of Difco Proteose Peptone No. B. 120 which had been previously used. This antigen gave good agglutination titres up to a dilution of 1 in 64.

Antigen 22. with the original peptone and prepared as Antigen 1. gave no agglutination whatsoever.

Antigen 23. "Pneumo" broth (prepared as in the Department of Bacteriology, McGill University) was used instead of "L.S." broth and prepared as in Antigen 1. Agglutination was good.

Antigen 24. using equal amounts of "L.S." and "Pneumo" broth, and prepared as in Antigen 1. gave good agglutination titres.

Discussion

The variability in agglutination response in different preparations of antigen, using the same organisms, culture media, procedures and serum, remains an enigma for which little explanation can be offered. The serum was checked by using other rabbit sera which had all given checked titres with the original Antigen 1. Variation in the use, size of tubes, temperature, pH, medium, time, shaking on addition of antigen and virulence of the organisms were checked repeatedly, without any one factor appearing to affect the outcome. Capsule swelling could still be demonstrated prior to use of the antigen in all, even those which showed complete absence of agglutination. However, only those antigens demonstrating a similar sensitivity as Antigen 1. were used on the experimental sera.

iii. Culture Media

"L.S." Broth

Finely minced fresh beef heart was freed of fat, one pound to a litre of distilled water, and heated at 75° - 80°C. for 2 hours. It was filtered through paper pulp in a Buchner funnel under slight suction. Phosphates were removed with N/10 NaOH with phenol red indicator (B.D.H.) at pH 9.5.

1.25 per cent Difco Proteose Peptone No. B. 120 was added but no salts. The pH was adjusted to 7.8 using a Lovibond Colorimeter (B.D.H.), then autoclaved at 115°C. for 15 - 20 minutes. It was filtered through paper pulp and pH readjusted up to 7.8. It was then bottled and sterilized at 120°C. for 20 minutes. Finally 0.1 per cent sterile dextrose (Seitz filtered) solution was added (Analar - B.D.H.) just before use.

"Pneumo" Broth

Finely minced fresh beef heart was freed of fat, 500 gms. to 1 litre of distilled water; heated at 75° - 80°C. for 2 hours and filtered through paper pulp in a Buchner funnel, under slight suction. 2 per cent Proteose Peptone (Difco No. B. 120) was added and the reaction adjusted to pH 7.2 with phenol red indicator (B.D.H.) using N/10 NaOH. (This removed certain protein fractions and gave a clear end product rather than a faintly turbid one). It was heated at 120°C. for 10 minutes, then cooled and filtered through paper pulp. The pH was adjusted to 8.0 with phenol red using 10/N

NaOH. It was then heated 10 - 15 minutes at 120°C. and cooled before filtering through paper pulp. The pH was adjusted to 7.8. 0.1 per cent dextrose (B.D.H.) was added, then bottled and sterilized at 120°C. for 20 minutes.

Before using, 0.2 per cent K_2HPO_4 (2 per cent of a 10 per cent sterile autoclaved solution) was added and 5 per cent fresh sterile rabbit serum (not more than 72 hours old).

Brewer's Medium (J. Bact. 1940, 39, 1, 10.)

Any good meat infusion broth.....	1000 ccs.
Dextrose (B.D.H.).....	1 %
Agar (B.D.H.).....	0.05 %
Sodium thioglycollate (B.B.L.).....	0.1 %
Methylene Blue (B.D.H.) (medicinal).....	0.0002 %

To prepare: - Peptone broth was filtered through paper pulp and adjusted to pH 7.6 with phenol red indicator (B.D.H.) and 10/N NaOH. The Bacto. Agar (Difco - granular) was added and autoclaved to melt at 115°C. for 20 minutes. The pH was adjusted back to 7.6. The Sodium thioglycollate, dextrose and Methylene Blue were then added, then poured into screw capped tubes about 1½ to 2 inches above ¾ inch meat.

The meat was prepared by removing any fat from lean meat, minced and an equal weight of meat placed into an equal volume of boiling N/20 NaOH for 20 minutes in order to saponify and hydrolize any remaining fat. Approximately double the volume of distilled water was added and left for about 1½ hours. Any fat was skimmed off and the remaining water drained off

and the meat dried in gauze. It was placed in an incubator at 37°C. to dry for 5 - 10 minutes.

The meat and broth in screw capped tubes were finally autoclaved at 100°C. for 30 minutes, then increased to 120°C. for 20 minutes.

Blood Agar Plates

Beef heart infusion broth was made from minced fresh beef heart, fat free, 454 gms. to 1 litre of distilled water. The infusion was extracted at 75° - 80°C. for 2 hours. To this was added 1.5 per cent Difco Agar (granular), 1 per cent Proteose Peptone (Difco), 0.25 per cent NaCl, 0.02 per cent KCl, and 0.01 per cent $CaCl_2$. It was left overnight to gel.

In the morning, it was melted and adjusted pH to 8.4 with phenol red indicator using 10/N NaOH, and heated at 120°C. for 20 minutes to precipitate phosphates. It was then filtered through paper pulp, pH readjusted to 7.2 using phenol red indicator and N. HCl. It was then heated to 120°C. for 20 minutes and filtered again through paper pulp, the pH checked to 7.2, and bottled in 550 ccs. volumes and sterilized by autoclaving at 120°C. for 20 minutes.

Each stock bottle was remelted at 120°C. for 20 minutes, cooled to 50°C. and 4 per cent sterile citrated human blood added. It was mixed by rotation and poured into sterile Petri plates - about 28 plates from each bottle, then incubated inverted at 37°C. for 24 - 48 hours for sterility.

(b) Qualitative Precipitation Tests

0.5 ml. halving serial dilutions of the immunized antipneumococcic Type I rabbit sera were made in a series of small tubes. To this was added 0.5 ml. of a 1:10,000 dilution of homologous capsular polysaccharide. Type I (Squibb & Sons, N. Y. Preparation No. 207). The tubes were incubated for 2 hours in a 37°C. water bath and final readings made after being placed in the refrigerator overnight.

TABLE II

Rabbit No.	Bleeding	Final Serum dilution showing Precipitation with S.S.S.Type I (1:10,000).
1	1st.	1:32
	2nd.	1:32
	3rd.	1:64
2	1st.	1:32
	2nd.	1:32
	3rd.	1:16
3	1st.	1:16
	2nd.	1:16
	3rd.	1:16

Results Titres were much lower than in agglutination tests on these well immunized rabbit sera. Using halving dilutions, an antigen excess is soon passed, and while the antigen could be used in a greater dilution, this was inadvisable as the density of the precipitate varies accordingly, and in toto the

test is not as sensitive when sera with low antibody content is to be titrated.

(c) Optimal Proportions Tests

The method used was that of Dean and Webb (126). A rough test was first made, making serial halving dilutions with 0.85 per cent saline of the antigen in a series of Kahn tubes. The first tube contained 0.5 ml. of a 1:25,000 dilution of specific capsular polysaccharide Type I (Squibb & Sons, N. Y. Preparation No. 207), the 6th tube 0.5 ml. of a 1:800,000 dilution, and 0.5 ml. of saline in the control.

A 1:10 dilution with 0.85 per cent saline was made of each rabbit serum, and 0.5 ml. added to each tube starting from the highest dilution to the lowest as quickly as possible. The tubes were then thoroughly shaken to ensure adequate mixing, and closely observed in order to note the one in which turbidity followed by flocculation appeared first. The time of appearance of flocculation will depend on the serum dilution. A 1:10 dilution was found to be satisfactory in the rough test on these sera.

TABLE III

GROSS OPTIMAL PROPORTIONS TEST

Rabbit No.	Bleeding	Antiserum Dilution	Dilution of S.S.S.giving First Flocculation	Time Mins.
1	1	1:5	1:100,000	35
	2	"	1:100,000 & 1:200,000	25
	3	"	1:200,000	25
2	1	"	1:100,000	30
	2	"	1:100,000 & 1:200,000	60
	3	"	1:200,000	50
3	1	"	1:100,000	25
	2	"	1:100,000 & 1:200,000	60
	3	"	1:200,000	50

As the optimal proportions point may be expected to be somewhere between the values indicated by the two most rapid tubes, from the antigen and antibody dilutions present in these tubes, it may be calculated what would be a suitable dilution of antigen and antibody for the "fine" test.

The antigen dilutions in the "fine" test were calculated using a harmonic dilution so that the dilution obtained in the rough test would be approximately in the middle tube of a series of 10 dilutions. E.g. Rabbits No. 1, 2, 3, 1st bleedings showed flocculation first with a 1:100,000 dilution of antigen. Therefore in the "fine" test the 1st tube should contain a 1:50,000 dilution of antigen, 2nd tube a 1:55,500

dilution of antigen etc., each succeeding concentration diminishing by 0.002.

In the "fine" test for rabbits No. 1, 2 and 3, 2nd. and 3rd bleedings, the first tube contained a 1:80,000 dilution of antigen, the 3rd tube 1:100,000 dilution and the 7th tube a 1:200,000 dilution of antigen, each succeeding concentration diminishing by 0.00125.

An equal volume of a 1:10 dilution of serum was added to each dilution as in the rough test and conducted in the same way, so that the tube in which flocculation appeared first was the exact dilution of antigen needed for the dilution of sera used.

TABLE IV

"FINE" OPTIMAL PROPORTIONS TEST

Rabbit No.	Bleeding	Antiserum Dilution	Dilution of S.S.S.giving First Flocculation	Time Secs.
1	1	1:10	166,500	30
	2	"	114,200	45
	3	"	100,000	45
2	1	"	71,250	20
	2	"	133,200	45
	3	"	114,200	40
3	1	"	62,500	20
	2	"	114,200	40
	3	"	133,200	40

Since the dilution of antigen which was originally 10 mgm. of the capsular polysaccharide Type I in 10 ccs. 0.85 per cent saline, i.e. a 1:1000 dilution, the carbohydrate reacting with each ml. of sera may be calculated.

TABLE V

Rabbit No.	Bleeding	Mgm. Carbohydrate per ml. sera
1	1	0.06
	2	0.08
	3	0.1
2	1	0.14
	2	0.07
	3	0.08
3	1	0.16
	2	0.08
	3	0.07

See Figure 3.

(d) Quantitative Precipitation Tests

It has been found that one of the immunologically active components of pneumococci of all types, even of unencapsulated forms which are devoid of type specificity is the somatic polysaccharide or "C" substance (72), and that measurable amounts of antibody to pneumococcus "C" substance was contained in nearly all antipneumococcic sera. Therefore in any

quantitative assay, it was necessary to remove anti-C, by a preliminary absorption, since almost all samples of the type specific carbohydrates contain "C" substance as an impurity.

The somatic "C" polysaccharide was prepared by extraction from an "R" strain of pneumococcus Type II. It was essential to use "C" substance derived from another type of pneumococcus other than that being used to inject the experimental animals, for it is as difficult to remove the last traces of type specific antigen from a sample of "C" substance, as to eliminate the last residues of "C" substance from samples of type specific polysaccharide.

The method of extraction used was that of Tillett and Avery (243).

18 Litres of an "R" strain of Type II pneumococcus culture were centrifuged and the bacteria resuspended in physiological saline, frozen and thawed several times in dry ice, acidified with 0.1 N acetic acid, heated to 100°C., and cooled. It was then neutralized with N/10 NaOH, filtered through a Seitz filter and concentrated in vacuo by a water pump to approximately one sixth of the original volume. To this concentrate was added 0.5 ml. of 0.1 N acetic acid, then heated for 15 minutes at 100°C., the protein precipitate removed by centrifuging. To the clear supernatant, 5 volumes of 95 per cent alcohol was added, and allowed to stand overnight in the refrigerator to settle out the precipitate of "C" substance and nitrogenous impurities.

This precipitate was redissolved in saline made slightly alkaline with NaOH, then reprecipitated again with

5 volumes of 95 per cent alcohol, redissolved in about 40 ml. water, made slightly acid and precipitated by 5 volumes of 95 per cent alcohol. This purification was repeated 5 times. The final precipitate was dissolved in 15 ml. distilled water and acidified with 2 ccs. HCl (S.G. 1.18) at 0°C to precipitate protein impurities and the clear supernatant was precipitated with absolute alcohol at 0°C. This procedure was repeated and the precipitated "C" substance washed free of chlorides with 85 per cent alcohol, then finally washed with absolute alcohol and ether. Approximately 25 mgms. of a fine white amorphous powder was obtained which gave the test for "C" substance.

Since complement may add nitrogen to the antigen-antibody precipitates, it was removed by the addition of an antigen and corresponding antibody which is unrelated to the immune system to be analyzed. Heat inactivation may damage antibodies in weak sera. The egg albumen (Ea) and rabbit anti-Ea system was not used as the preparation of the crystalline egg albumen entailed much time and certain difficulties, and other systems seemed simpler.

The typhoid-anti-typhoid system was first tried but was not satisfactory. It was then decided to try horse - anti-horse fresh guinea-pig sera (pooled from 8 to 10 guinea pigs on each test). An anti-horse precipitin serum (rabbit) of an optimal proportions ratio 1:50 was used to determine the greatest dilution of antigen and antibody which would completely remove the complement from an equal volume of undiluted guinea pig serum. As maximum fixation takes place in

antibody excess, ratios varying between 1:4 to 1:16 were tested. Using a ratio of 1:4, fixation of complement was complete with a 1:8 dilution of antigen and not in 1:16; using a ratio of 1:8 and 1:16, fixation was complete in both with a dilution of 1:32 of antigen and **not** in 1:64. Therefore it was concluded that a ratio of 1:8 gave an adequate precipitate for removal and complete fixation of complement without adding too much extraneous globulin.

Method of Quantitative Estimation of Antibody Nitrogen in 9 Rabbit Sera.

The immunized rabbit sera were titrated for presence of complement after storing for one month in the refrigerator. 0.5 ml. of each sera as complement, 0.5 ml. of 1:32 dilution of horse sera, and 0.5 ml. of 1:4 dilution of anti-horse sera were incubated for 2 hours at 37°C. in a water bath, then the haemolytic system added (0.5 ml. 3 per cent sheep's cells and 0.5 ml. of 1:4000 amboceptor) with no haemolysis in any of the nine sera, showing that all complement had degenerated by itself in the ice box.

0.01 ml. of a 1:1000 dilution of Type II pneumococcus "C" substance was added to 2 ml. of each of the immunized rabbit sera in Pyrex centrifuge tubes. The contents of the tubes were thoroughly mixed by twirling, then capped with sterile rubber caps and placed in the ice box overnight. All showed a faint turbidity next morning so they were centrifuged in the cold at about 3000 r.p.m's, then 0.01 ml. of "C" substance added again. After leaving for a week in the ice box, the tubes were again centrifuged and the supernatant poured

off into centrifuge tubes. 0.1 ml. of a 1:10,000 dilution of the homologous type-specific carbohydrate was then added with the almost immediate formation of a precipitate. The tubes were centrifuged in the cold, and 0.1 ml. of the carbohydrate added again. Some formed precipitates again but the tubes were capped and placed in the refrigerator overnight. Next morning they were centrifuged, and 0.1 ml. of carbohydrate added to all. This was continued until no further precipitate was formed (224).

The supernatant was now poured carefully off. A blank was run at the same time by centrifuging 2 ml. sera. All the precipitates were now broken up and washed with chilled saline, and transferred to Kjeldahl flasks, to which were added 2 ml. of sulphuric acid solution containing copper sulphate, and a boling stone (Heugar Co., Phil. Pa.). They are digested for about one half hour after they have cleared. They are then cooled, diluted with distilled water and transferred quantitatively to a Kjeldahl Still and distilled into a wide mouth tube containing 1 ml. of a solution freshly prepared by adding 0.1 ml. of Solution C. (5 ml. of 1 per cent methyelene blue plus 41 cc. of saturated solution of methyl red in 95 per cent ethyl alcohol) ,to 20 ml. saturated boric acid. They were then titrated by a microburette with N/70 hydrochloric acid.

A blank using the reagents was run first and this subtracted from the results obtained from the sera.

TABLE VI

Rabbit No.	Bleeding	Mgm. N_2/2 ml. Sera	Mgm. N_2/ ml. Sera
1	1	.33	.16
	2	.52	.26
	3	.35	.17
2	1	.50	.25
	2	.24	.12
	3	.22	.11
3	1	1.19	.59
	2	.44	.22
	3	.56	.28

See Figure 4.

Results

No attempt has been made to compare antibody estimation of the sera by the different methods employed, since they are an expression of different antibody values. However, the results for individual sera in each method are comparable.

V TYPE SPECIFIC ANTIPNEUMOCOCCIC GUINEA PIG SERUM

As guinea pigs were the animals selected for the experiment, and as the production of antipneumococcic rabbit sera has been shown to be relatively simple to obtain, the same immunizing schedule as used in the production of specific antipneumococcic serum was next started in nine guinea pigs, dosage being given in proportion to weight of the guinea pigs.

Agglutination tests were carried out on the sera of these (see Figure 5.) using the same antigen and dilutions of sera as used with the rabbit sera.

Results

While agglutination was obtained in comparatively high dilutions as for the rabbits, the amount was lower in most, and the maintenance immunizing doses did not increase the titre appreciably.

These "in vitro" results were substantiated by "in vivo" attempts to reinfect the guinea pigs with 10 times the lethal dose along with 1 cc. of carmine without the appearance of clinical infection.

VI IMMUNE RESPONSE TO A SINGLE DOSE OF HEAT-KILLED TYPE I PNEUMOCOCCI IN GUINEA PIGS.

Guinea pigs No. 1 and 2 (Group A.) were bled by intracardiac puncture, then each injected intraperitoneally with 1 ml. of an 18 hour heat-killed S-710-T.I pneumococci culture and immediately after by 1 ml. of a 10 per cent carmine solution. Guinea pigs No. 3 and 4 (Group B.) were treated similarly but given 1 ml. of a 10^{-1} dilution of the 18 hour pneumococci culture, and guinea pigs No. 5 and 6 (Group C.) 1 ml. of a 10^{-2} dilution of the 18 hour pneumococci culture.

They were all bled in 5 days and then in 10 days. Agglutination tests with Antigen No. 1. were done on the sera. (See Figure 6.).

Results

None of the animals show any agglutinating antibodies

in the sera prior to injection of the antigen. In 5 days, the titres while fairly high were very low in amount. In 10 days, the titre in guinea pig No. 1. increased in amount in a 1:32 dilution but fell off somewhat in height. Guinea pig No. 2. showed some increase in amount but not as much as No. 1. Guinea pigs No. 3. and 4. decreased in height but increased in amount, and guinea pigs No. 5. and 6. increased somewhat in amount while maintaining the height of titre.

From these results, a possible idea was obtained that by agglutination tests a detectable titre may be expected in response to a single injection of pneumococci, and particularly to 1 ml. of a 10^{-2} dilution as was to be used in the experiment.

VII PENICILLIN SENSITIVITY TESTS

A rough test was made by inoculating a blood-agar plate with S-710-T.I pneumococcus culture. Dried filter paper discs made to contain dilutions of 0.5, 1.0, 5.0 units of penicillin were placed on the plate, the whole incubated at 37°C. overnight. The sensitivity of the culture was deducted from the area of inhibition surrounding the disc.

S-710-T.I pneumococcus was found to be markedly sensitive to 0.5 units per ml. penicillin.

Quantitative Sensitivity to Penicillin of S-710-T.I

11 tubes and a 12th control tube were placed in a rack. 2 ml. of "L.S." broth was placed in tubes 2 - 12 inclusive, and 3.6 ml. in tube 1. 0.4 ml. of a 10 units/ml. dilution of penicillin was mixed in the first tube and 2 ml.

transferred to the 2nd tube up to the 11th tube, none in the control.

The dilution of penicillin containing 10 units per ml. was made from a 100 units per ml. solution made from weighing a known amount of penicillin accurately. This standard penicillin was crystalline sodium penicillin G. which was obtained from the Laboratory of Hygiene, Ottawa and had a standard potency of 1650 International Units per mgm. This was prepared just before use.

Into each tube 0.1 ml. of an 18 hour S-710-T.I pneumococci culture was added, the tubes shaken and incubated overnight.

The end point was taken as the last tube showing no growth which was the 8th tube which contained 0.0075 units of penicillin per ml.

VIII (a) PENICILLIN LEVELS IN SERUM

The sterile guinea pig serum was tested within 3 hours after collection.

A penicillin standard was first made up. 8 tubes and a control tube were placed in a rack. Into all tubes except the 1st was pipetted 0.5 ml. of a test medium made from sterile skim milk to which had been added methylene blue thiocyanate solution (Standard Methods of Milk Examination, A.P.H.A.) (244).

0.5 ml. of penicillin containing 1 unit per ml. (prepared from penicillin with standard potency - see above) was placed in the 1st and 2nd tubes, and after mixing 0.5 ml.

was transferred from the 2nd to the 3rd tube up to the 8th tube. To all the tubes 1.5 ml. of the test media, seeded with a culture of Bacillus subtilis, were added. The Bacillus subtilis culture was obtained from a strain kept in the refrigerator and subcultured into peptone broth every 10 days. 5 ml. of this culture was used for every 100 ccs. of test media.

The unknown sera was now set up in the same manner as the penicillin standard, 0.5 ml. being placed in the 1st and 2nd tubes and halving dilutions made up to the 8th tube - none in the control. 1.5 ml. of the test media was then added to all the tubes. Both standard and unknown racks were incubated overnight in the incubator.

In 24 hours, both racks were read. The end point of the standard was taken as the last tube in which there was no colour change from green to pink. By comparing this end point with the end point of the unknown, the amount of penicillin in the unknown may be calculated.

VIII (b) PENICILLIN LEVELS IN URINE

In order to compare the excretion of penicillin with the blood levels obtained, each guinea pig was placed in a metabolism box in use in the Department of Bacteriology, McGill University. The urine excreted 24 hours after the 4th consecutive daily intramuscular dose of procaine penicillin G. (crystalline) in oil was collected. (See Experiment IV). Serum penicillin level at this time was less than 0.03 units per ml.

The test was conducted as for serum penicillin levels,

an appropriate dilution being made on an estimated amount of penicillin expected to be present.

The amounts were calculated to be in guinea pig No. 3. 480 units per ml. in a volume of 6 ccs. making a total of 2880 units, and guinea pig No. 4. 200 units per ml. in a volume of 10 ccs. making a total of 2000 units. However, it is probable that some of the urine was lost in collection and therefore the total amount of penicillin excreted was greater.

E X P E R I M E N T A L

The following series of experiments were undertaken in order to determine whether guinea pigs injected with virulent Type I pneumococci intraperitoneally, would demonstrate immunity as demonstrated by circulating antibodies, when the infection should be terminated by sulphonamide and penicillin therapy. In addition, how soon after administration of the infection, and the commencement of either form of therapy, could one demonstrate specific circulating antibodies. Finally, how permanent was the immunity developed, if any, as ascertained by reinfection with the original infecting dose.

Animals

Groups of guinea pigs were chosen that had been under observation for at least a week, and tested in order to determine whether any specific circulating antibodies for the infecting organism, existed prior to infection.

Culture

1 ml. of a 10^{-2} dilution of S-710-T.I pneumococci culture was injected intraperitoneally immediately after the peritoneal injection of 1 ml. of a 10 per cent carmine solution, shown in previous preliminary experiments to produce clinical peritonitis in guinea pigs with a 50 per cent mortality rate.

Determination of Circulating Antibodies

"In vitro" tests for type specific agglutinins were carried out using halving serial dilutions in a series of clean dry Kahn tubes.

0.2 ml. of 0.85 per cent saline was placed in all the tubes including a control. 0.2 ml. of sera was placed in the first tube giving a 1:2 dilution, mixed several times with the pipette, then 0.2 ml. was transferred to the next tube until a dilution of 1:2048 in the eleventh tube, from which 0.2 ml. was discarded. No serum was placed in the control. 0.2 ml. of the heat killed S-710-T.I pneumococci antigen at a dilution corresponding to No. 2 McFarland nephelometer and previously standardized against an immunized Type I antipneumococci rabbit serum, was then added to all the tubes including the control tube. The tubes were then vigorously shaken, incubated at 37°C. in a water bath, and placed in a refrigerator overnight and read the following morning in a viewing box against a black background by oblique light.

"In vivo", 0.1 ml. containing 0.0004 mgm. of the homologous capsular polysaccharide was injected intracutaneously on the abdominal wall after the 20th day post infection bleeding. Control of saline only was given at the same time.

Finally, reinfection with the original infecting dose intraperitoneally was made immediately after the 20th day post-infecting bleeding.

Therapy

I Sulphonamides

(a) Orally sodium sulphamethazine (Ayerst, McKenna & Harrison Ltd., Montreal, Canada) was given

(i) by stomach tube using a 8F rubber catheter

(ii) syringe

(b) Intramuscularly into the thigh muscles veterinary sodium sulphamethazine (Trade Name Sulmet - Lederle Laboratories Division, American Cyanamid Co., N.Y.)

II Penicillin intramuscularly

(a) Procaine penicillin G. (crystalline) diluted in aluminium monostearate 2 per cent in peanut oil. (Special formula of Ayerst, McKenna & Harrison, Ltd., Montreal, Canada).

(b) Aqueous sodium penicillin G. (crystalline - buffered (Lederle Laboratories, N. Y.).

Time of Bleeding

1. Bleeding A. was taken prior to any infection or therapy (control).
2. Bleeding B. was taken after infection and prior to commencement of any form of therapy.
3. Bleeding C. was taken 5 days after bleeding B.
4. Bleeding D. " " 10 " " " "
5. Bleeding E. " " 20 " " " "

Time of Initial Therapeutic Dose

Corresponding groups of sulphonamide and penicillin treated guinea pigs were given their initial dose of therapy 5 hours after infection and 16 hours after infection.

However, only one group of 5 guinea pigs were given their initial dose of sulphamethazine immediately following the infecting organism and carmine given intraperitoneally, and therefore the time of bleeding is slightly altered.

1. Bleeding A. was taken prior to any infection or therapy. (control)
2. Bleeding B. was taken 5 hours after administration of simultaneous infection and therapy.
3. Bleeding C. was taken 16 hours after administration of simultaneous infection and therapy.
4. Bleeding D. was taken 5 days after simultaneous infection and therapy.
5. Bleeding E. was taken 10 days after simultaneous infection and therapy.
6. Bleeding F. was taken 20 days after simultaneous infection and therapy.

Reinfection

First The original infecting dose was given intraperitoneally immediately after carmine intraperitoneally on the same day as the 20 day post-infection bleeding.

Second Reinfection

The same dose was given intraperitoneally at varying periods from 10 - 40 days after the first reinfecting dose.

EXPERIMENT I
The Effect of Sulphonamide Therapy Administered 5 hours after the Inoculation of Type I Pneumococci Intraperitoneally on the Immune Response in Guinea Pigs.

Guinea pigs No. 1 - 8, all weighing between 500 - 600 gms. were infected intraperitoneally with Type I pneumococci and carmine after an initial control bleeding. 1 ml. containing 75 mgm. of a 7.5 per cent sodium sulphamethazine solution was started orally by an 8F rubber catheter 5 hours

after infection and administered four times a day for three days, giving a total of 900 mgms. or 13½ grains.

The catheter was abandoned after the second dose as the animals strenuously objected and in their struggle Nos. 1, 2 and 3 were lost by suffocation and asphyxiation; also when an assistant was not available, as occurred at night, it was almost impossible to introduce the catheter alone.

A 2 ml. syringe without the needle, was next tried. This was placed at the back of the tongue but the dose given was often inaccurate as the animal would occasionally regurgitate part of it, and also show disdain for the taste which was masked somewhat by giving the juice from fresh grated carrots before and after the dose.

Guinea pigs No. 4 - 8 inclusive however survived this form of therapeutic administration.

They were bled according to the schedule previously given. Guinea pig No. 6. however died on the 18th day after infection and peritoneal smear and culture showed no pneumococci and at autopsy there was no evidence of peritonitis. The thorax also was clear. No obvious cause of death could be ascertained from the gross, and treatment had been successful.

Guinea pigs Nos. 9 - 13 inclusive were given 0.4 ml. of sodium sulphamethiazine (Trade Name Sulmet) intramuscularly containing 1.52 grains, 5 hours after infection, then once daily for 5 days. All survived.

Guinea pigs Nos. 14 - 18 inclusive were given 0.5 ml. of sodium sulphamethazine intramuscularly containing 1.9 grains,

5 hours after infection, then 0.4 ml. once daily for two days. All survived.

Results

Agglutination Titres

These are shown in Figure 7.

Bleeding A. showed no agglutination in guinea pigs 1 - 18 inclusive.

Bleeding B. In 5 hours after infection Nos. 1, 2, 4, 5, 7 and 9 - 14 inclusive showed no agglutinating antibodies in any dilution. However, Nos. 3, 6, 8, 15, 16, 17, 18 all showed titres varying from a 1:32 dilution to 1:256 dilution. These were not very high in amount for any animal (≠ agglutination the highest recorded) and all except No. 3 showed marked prezoning.

Bleeding C. In 5 days, all the animals showed some agglutinating titres except No. 4. Guinea pigs No. 5 and 7 for the first time showed an agglutinating titre to a 1:8 dilution. No. 6 had fallen from 1:128 to 1:8. Guinea pig No. 8 had increased from 1:128 to 1:1024. Guinea pigs No. 9 - 14 which had previously shown no titre in Bleeding B. now showed fairly good titres, and Nos. 15 - 18 had either increased or remained the same.

Bleeding D. In 10 days, all the animals showed an agglutinating titre even No. 4.

Nos. 6, 8 - 13 showed little change, while Nos. 5, 7, 14 - 18 showed a marked increase in amount and height in agglutinating titres.

Bleeding E. In 20 days, there was a marked variation in the

agglutinating titres, some showing an increase in amount if not height, some a marked decrease in titre.

TABLE VII

CUTANEOUS TESTS TO TYPE I CAPSULAR POLYSACCHARIDE ON 20TH DAY

Guinea Pig No.	4	5	7	8	9	10	11	12	13	14	15	16	17	18
Positive	o	+	+	±	+	+	o	+	+	+	+	+	+	+
Time(Mins)	-	5	10	10	10	10	-	5	10	10	15	10	5	10
Control	o	o	o	o	o	o	o	o	o	o	o	o	o	o

A positive reaction was taken as an area of erythema surrounding the site of intracutaneous injection, usually appearing within a half hour, with absence of any reaction in the control.

TABLE VIII

FIRST HOMOLOGOUS INFECTION 20 DAYS AFTER PRIMARY INFECTION

Guinea Pig No.	4	5	7	8	9	10	11	12	13	14	15	16	17	18
Survival (1 wk.+)	o	+	o	o	o	o	o	+	+	+	+	+	+	+
Time of Death (Days)	1	-	1	1	1	1	2	-	-	-	-	-	-	-

TABLE IX

SECOND HOMOLOGOUS REINFECTION

Guinea Pig No.	5	12	13	14	15	16	17	18
Time after 1st Reinfection(Days)	20	20	20	29	29	29	29	29
Survival(1 wk.+)	+	o	+	+	+	+	+	+
Time of Death(Days)	-	2	-	-	-	-	-	-

Discussion of Experiment I

Agglutinating antibodies in the sera of 7 out of 15 guinea pigs were demonstrated within 5 hours of infection with pneumococci Type I. However, 5 days after infection and treatment with sulphamethazine all except one animal showed a clearly demonstrable agglutinating titre, and in 10 days all showed a titre which was considerably increased in some animals. However, this was not maintained entirely in all animals by the 20th day.

Two animals, out of 14 surviving infection, did not react to an intracutaneous test with the homologous capsular polysaccharide and these two animals were not able to survive reinfection in 20 days to the original infecting dose.

Four other animals, out of 14 surviving infection, while exhibiting a positive intracutaneous test, did not survive reinfection. All others who did have a positive intracutaneous test did survive the first reinfection.

Only one out of 8 animals which had survived the first reinfection succumbed in 20 days after the second reinfection.

EXPERIMENT II
The Effect of Sulphonamide Therapy Administered 16 hours after the Inoculation of Type I Pneumococci Intraperitoneally on the Immune Response in Guinea Pigs.

Guinea pigs Nos. 1 - 5 were similarly infected with Type I pneumococcus after an initial control bleeding, and treated with the same dose in the same manner as guinea pigs Nos. 1 - 8 in the previous experiment, except that the initial therapeutic dose of sulphamethazine was started 16 hours after infection instead of 5 hours.

Again the mechanical difficulties involved with the rubber catheter, resulted in the loss of No. 1, 2 and 3. Guinea pigs Nos. 4 and 5 after surviving the first dose by catheter were fed orally by syringe and survived.

Guinea pigs Nos. 6 - 10, inclusive, were given 0.4 ml. of sodium sulphamethazine (Trade Name Sulmet) intramuscularly containing 1.52 grains 16 hours after infection, then once daily for 5 days. Guinea pig No. 6 died about 1½ hours after bleeding B. was taken, and on autopsy there was an extensive fibrinopurulent peritonitis due to Type I pneumococcus only and a pregnant uterus. Guinea pig No. 8. died on the 18th day after infection and 13 days after therapy had ceased. On autopsy, there was a haemorrhagic purulent peritonitis which on culture and by the Neufeld Quellung method proved to be Type XIX pneumococcus.

(Unfortunately this was only the beginning of a widespread laboratory Type XIX pneumococcus epidemic prevalent in the spring with a high death toll in guinea pigs). However, had it not been for this unforseen disaster, one could presume that the animal had been clinically well and would have survived 20 days and perhaps even reinfection. (See Figure 8., bleeding C. No. 8).

Guinea pigs Nos. 11 - 15 inclusive were given 0.5 ml. of sodium sulphamethazine intramuscularly containing 1.9 grains 16 hours after infection, then 0.4 ml. once daily for 2 days. Of these 5, Nos. 11, 12 and 15 died, Nos. 11 and 15 died in the afternoon of the initial dose given in the morning and No. 12 was found dead the next morning prior to the second

daily dose. All 3 on autopsy showed extensive peritonitis; peritoneal smear showed very large numbers of pneumococci extracellularly; there was no phagocytosis of the organisms, and the phagocytes were packed with carmine particles only.

The cause of death in these three guinea pigs must be partly contributed to delay in starting therapy as this dosage was given to guinea pigs 5 hours after infection with no mortality, and partly to susceptibility of individual animals. This latter is substantiated in the fact that guinea pigs Nos. 16 - 20 inclusive were given the same amount of therapy 16 hours after infection and survived in toto.

Results

Agglutination Titres

These are shown in Figure 8.

Bleeding A. showed no agglutination in guinea pigs Nos. 1 - 20 inclusive.

Bleeding B. In 16 hours after infection,guinea pigs Nos. 1, 3 - 9, 11, 12, 15 - 20 showed no agglutinating antibodies in any dilution while Nos. 2, 10, 13, 14 show agglutinating antibodies in variable low titres, the highest demonstrated by the ill fated No. 2 to a 1:64 dilution.

Bleeding C. In 5 days, guinea pigs Nos. 4, 5, 7 - 9 still showed no agglutinating antibodies, while Nos. 10, 13 and 14 showed high titres, Nos. 16 - 20 showing moderately high titres, but low in amount.

Bleeding D. In 10 days, guinea pigs Nos. 4 and 7 still show no antibodies, while for the first time No. 5 showed a low titre moderately high in a low dilution of 1:4, and Nos. 8

and 9 show rather high titres. Guinea pigs Nos. 10, 13, 14, 16 - 20 all show titres higher than those given in 5 days, most marked in No. 14.

Bleeding E. In 20 days, guinea pig No. 4 for the first time showed some agglutinating antibodies even if very low. No. 5 had shown an appreciable increase, No. 7 showing no antibodies even now. Guinea pigs Nos. 9, 10, 13, 14, 16 - 20 showed no marked difference to the results obtained at the 10 day bleeding.

TABLE X

CUTANEOUS TESTS TO TYPE I CAPSULAR PNEUMOCOCCUS ON THE 20TH DAY

Guinea Pig No.	4	5	7	9	10	13	14	16	17	18	19	20
Positive	o	+	o	+	+	+	+	+	+	+	+	+
Time(Mins)	-	10	-	10	15	10	5	10	5	5	10	15
Control	o	o	o	o	o	o	o	o	o	o	o	o

TABLE XI

FIRST HOMOLOGOUS REINFECTION 20 DAYS AFTER PRIMARY INFECTION

Guinea Pig No.	4	5	7	9	10	13	14	16	17	18	19	20
Survival (1 wk.+)	o	+	o	+	+	+	+	+	+	+	+	+
Time of Death(Days)	1	-	1	-	-	-	-	-	-	-	-	-

TABLE XII

SECOND HOMOLOGOUS REINFECTION

Guinea Pig No.	5	9	10	13	14	16	17	18	19	20
Time after 1st Reinfection(Days)	10	40	40	32	32	10	10	10	10	10
Survival (1 wk+)	+	+	+	*	+	+	+	+	+	+
Time of Death(Days)	-	-	-	5	-	-	-	-	-	-

* Died 5 days after 2nd reinfection - pure growth of Type XIX pneumococci recovered on culture - No Type I.

Discussion of Experiment II

Only 4 guinea pigs out of 20 demonstrated agglutination within 16 hours of infection and treatment with sulphamethazine, but in 5 days 8 showed some, and in 10 days 11 exhibited titres, and the same number in 20 days.

Two animals who survived until the 20th day after infection failed to demonstrate a positive cutaneous test to the capsular polysaccharide and similarly did not survive reinfection with the original infecting dose. All the others did. On the 2nd reinfection from 10 to 40 days after the 1st reinfection, all survived except one, and the loss of this animal was probably entirely due to an additional unforseen infection, as it lived 5 days after being reinfected for the 2nd time.

EXPERIMENT III
The Effect of Sulphonamide Therapy Administered immediately after the Inoculation of Type I Pneumococci Intraperitoneally on the Immune Response in Guinea Pigs.

Guinea pigs Nos. 1 - 5 were similarly infected with Type I pneumococcus after an initial control bleeding, and

treated immediately after infection with 0.5 ml. of sodium sulphamethazine intramuscularly containing 1.9 grains, then 0.4 ml. once daily for 2 days.

All survived.

Results

Agglutination Titres

See Figure 9.

Bleedings A., B., C., D. of guinea pigs Nos. 1 - 5 inclusive Bleeding E. In 10 days, all the guinea pigs demonstrated a fairly high agglutinating titre.

Bleeding F. In 20 days, guinea pigs Nos. 1 and 2 showed some decrease in titre, and Nos. 4 and 5 some increase. Guinea pig No. 3 on the 19th day after infection succumbed to Type XIX pneumococci peritonitis.

TABLE XIII

CUTANEOUS TESTS TO TYPE I CAPSULAR PNEUMOCOCCUS ON THE 20TH DAY

Guinea Pig No.	1	2	4	5
Positive	+	+	+	+
Time (Mins.)	15	10	5	5
Control	o	o	o	o

TABLE XIV

FIRST HOMOLOGOUS REINFECTION 20 DAYS AFTER PRIMARY INFECTION

Guinea Pig No.	1	2	4	5
Survival (1 wk+)	+	+	+	+
Time of Death (Days)	-	-	-	-

TABLE XV

SECOND HOMOLOGOUS REINFECTION

Guinea Pig No.	1	2	4	5
Time after 1st Reinfection (Days)	30	30	30	30
Survival (1 wk+)	+	+	+	+
Time of Death (Days)	-	-	-	-

Discussion

In as much, as agglutinating antibodies were late in appearing in these 5 animals (not until the 10th day), they all gave a positive cutaneous test with the polysaccharide (one animal died from heterologous Type XIX before the 20th day after infection.). They also showed marked resistance to reinfection with the homologous infecting dose in 20 and 30 days after the primary infection and treatment with sulphamethazine.

EXPERIMENT IV
The Effect of Penicillin Therapy Administered 5 hours after the Inoculation of Type I Pneumococci Intraperitoneally on the Immune Response in Guinea Pigs.

Guinea pigs Nos. 1, 5 - 6 inclusive were infected with Type I pneumococcus after an initial control bleeding. Procaine penicillin G. (crystalline) was diluted in aluminium monostearate 2 per cent in peanut oil so that 1 cc. = 4800 units.

5 hours after infection, 0.5 ml. containing 2400 units were given intramuscularly and repeated daily for 4 days making a total of 9600 units given to each animal.

During the period in which the first two doses were given, the animals seemed to show some clinical improvement but after that began to lose weight rapidly, would not eat and showed signs of lethargy and ruffled vertical fur. A few hours after the 4th dose (4th day) Nos. 1, 2, 5 died. They had lost more than a 1/5th of their original body weight, and on autopsy no peritonitis could be detected, cultures were negative and peritoneal smears showed no pneumococci. Serum penicillin levels done at the time of Bleeding C. (5 days after infection), i.e. 24 hours after the 4th consecutive daily dose on guinea pigs Nos. 3 and 4 showed less than 0.03 units per ml. However, while this may seem somewhat low, the growth of this S-710-T.I pneumococci strain to penicillin was inhibited by 0.0075 units per ml. penicillin. In addition, from the peritoneal smears and cultures the absence of viable pneumococci showed that this dose was sufficiently bactericidal to combat the infecting dose. In spite of this, guinea pigs Nos. 3 and 4 both died on the 6th day after infection.

Guinea pigs Nos. 7 - 11, were given the same dose of penicillin with a total of 9600 units with a similar 100 per cent mortality. Nos. 7 - 10 survived until the 5th day but died within a day or two after. All peritoneal smears showed absence of any exudate or penumococci.

Guinea pigs Nos. 12 - 16, were given a single dose of penicillin G. (crystalline) in 0.85 per cent saline. 0.5 ml. containing 5000 units were given intramuscularly into each thigh. Guinea pigs Nos. 12,14 and 15 died before the 5th post infective day, Nos. 13 and 16 died on the 8th post

infective day. All showed marked body weight loss and a complete absence of pneumococci in the peritoneal smears.

As it now became apparent that the therapeutic agent was probably more toxic than the infecting organism, it was decided to use a very small dose of penicillin G. (crystalline) once only, and taking into account the marked sensitivity of the organism, and the fact that a higher level could be obtained during the early growth phase of the organisms, this form of penicillin would be more efficient in such a critical balance.

So guinea pigs Nos. 17 - 21 were given a single dose of 1000 units of penicillin G. intramuscularly, 5 hours after infection. Guinea pigs Nos. 17, 19, 20 died before the 5th post infective day, but showed only a slight weight loss but moderate numbers of pneumococci were seen on peritoneal smears - all Type I. In this dosage, penicillin did not seem to exert a toxic effect but therapeutically was insufficient to combat infection. Nos. 18 and 21 however managed to survive.

Guinea pigs Nos. 22 - 26 were next given 1000 units of penicillin 5 hours after infection then 500 units the following day. No. 24 died on the 3rd day and No. 23 a few hours after the 10 day bleeding - both however of Type XIX laboratory pneumococci infection and not the infecting Type I. Nos. 22, 25 and 26 survived.

Results

Agglutination Titres

See Figure 10.

In the initial control Bleeding A., none of the guinea pigs showed agglutination in any dilution.

In Bleeding B., 5 hours after infection, only 5 out of 25 guinea pigs showed some agglutinating antibodies, i.e. Nos. 8, 9, 11, 18 and 20, all others showed no titres.

In Bleeding C., 5 days after infection, 7 out of 13 guinea pigs surviving until this time, i.e. guinea pigs Nos. 7, 8, 9, 18, 20, 23 and 25 showed agglutinating antibodies. No. 7 showed antibodies for the first time, but the serum was so fatty that in the lower dilutions, any agglutination if present could not be easily viewed. This was seen in 4 other guinea pigs at this time of bleeding (see later). Nos. 18 and 20 showed extremely good titres.

In Bleeding D., 10 days after infection, only 6 animals were living, all showing very good agglutinating titres.

By Bleeding E., 20 days after infection only 5 animals were alive, all maintaining good titres.

TABLE XVI

CUTANEOUS TESTS TO TYPE I CAPSULAR POLYSACCHARIDE ON THE 20TH DAY

Guinea Pig No.	18	20	22	25	26
Positive	+	+	+	+	+
Time (Mins)	5	5	15	15	10
Control	o	o	o	o	o

TABLE XVII

FIRST HOMOLOGOUS REINFECTION 20 DAYS AFTER PRIMARY INFECTION

Guinea Pig No.	18	20	22	25	26
Survival (1 wk.✓)	✓	✓	✓	✓	✓
Time of Death (Days)	-	-	-	-	-

Guinea pig No. 26 died of Type XIX pneumococcic peritonitis 14 days after the first reinfection.

TABLE XVIII

SECOND HOMOLOGOUS REINFECTION

Guinea Pig No.	18	20	22	25
Time after 1st Reinfection (Days)	29	29	30	30
Survival (1 wk✓)	✓	✓	✓	✓
Time of Death (Days)	-	-	-	-

Discussion of Experiment IV

After infection with pneumococcus Type I 5 out of 25 guinea pigs showed agglutinating antibodies within 5 hours. 5 days after being infected and treated with penicillin, 7 out of the 13 guinea pigs which survived showed agglutinating antibodies, showing a marked increase as the original total of 25 had now dropped to 13. In 10 days, the total had again decreased to 6 all of which exhibited antibodies, and in 20 days, the total now 5, all of which showed an agglutinating antibody titre.

Cutaneous tests were all positive in the surviving 5, all of which survived reinfection in 20 days, and again in 29

to 30 days, except for one more fatality from an epidemic of Type XIX pneumococcic peritonitis.

EXPERIMENT V
The Effect of Penicillin Therapy Administered 16 hours after the Inoculation of Type I Pneumococci Intraperitoneally on the Immune Response in Guinea Pigs.

Guinea pigs Nos. 2 - 5 were infected with Type I pneumococcus after an initial control bleeding. 16 hours after infection, 0.5 ml. (2400 units) of procaine penicillin G. (crystalline) in aluminium monostearate 2 per cent in peanut oil, was given intramuscularly, and repeated daily for 4 days making a total of 9600 units given to each animal. As in the guinea pigs treated with this dose 5 hours after infection, all died before the 5th day after infection and exhibited the same toxic symptoms of anorexia and marked weight loss. In addition the infective dose was an added burden resulting in death of 2 animals Nos. 2 and 3 who were moribund in 30 hours, somewhat before the toxic manifestations of penicillin are first demonstrable. Peritoneal smears showed large numbers of extracellular pneumococci, and phagocytosed carmine particles.

Guinea pigs Nos. 6 - 15 inclusive, 16 hours after infection, were given 1000 Units of crystalline penicillin G. intramuscularly, 16 hours after infection. Nos. 6 and 8 died on the 5th day before bleeding from Type XIX pneumococcic peritonitis not Type I. Nos. 7, 9, 10 survived. Guinea pigs Nos. 11 and 14 died on the 5th day and No. 13 on the 18th day from Type XIX pneumococcic peritonitis. Nos. 12 and 14 only surviving.

It was most unfortunate to have encountered this Type XIX epidemic which wiped out entire rooms of animals kept for tuberculosis work. The room in which the above experimental animals were kept separate, suffered a lower mortality rate in comparison to the tuberculosis animals, but sufficient to impair results in the number of animals used.

Results

Agglutinating Titres

See Figure 11.

As in the preceding experiments guinea pigs Nos. 2 - 15 inclusive demonstrated no agglutinating antibodies in Bleeding A.

In Bleeding B., 16 hours after infection, only 6 out of 14 animals exhibited an agglutinating antibody titre. Nos. 7, 9, 10 were quite high, Nos. 12, 13, 14 appreciably lower.

In Bleeding C., 5 days after infection, titres for all surviving had increased, but decreased by the 10 day bleeding and more so by the 20 day bleeding.

TABLE XIX

CUTANEOUS TESTS TO TYPE I CAPSULAR POLYSACCHARIDE ON THE 20TH DAY

Guinea Pig No.	7	9	10	12	14
Positive	+	+	+	±	+
Time (Mins.)	10	10	10	15	10
Control	o	o	o	o	o

TABLE XX

FIRST HOMOLOGOUS REINFECTION 20 DAYS AFTER PRIMARY INFECTION

Guinea Pig No.	7	9	10	12	14
Survival (1 wk/)	/	/	/	o	/
Time of Death (Days)	-	-	-	1	-

TABLE XXI

SECOND HOMOLOGOUS REINFECTION

Guinea Pig No.	7	9	10	14
Time after 1st Reinfection (Days)	32	32	32	10
Survival (1 wk/)	/	/	o	/
Time of Death (Days)	-	-	1	-

Discussion of Experiment V

16 hours after infection with pneumococcus Type I, 6 out of 14 guinea pigs exhibited agglutinating antibodies. In 5 days after infection and treatment with penicillin, 6 animals which had survived had increased titres, decreasing in 5 out of 6 by the 10th day with 5 surviving to the 20th day, and showing agglutinating antibodies.

Of these 5 all exhibited a positive reaction to the cutaneous test, 4 survived the first reinfection, and 3 the second reinfection in 10 to 32 days.

EXPERIMENT VI
Toxicity of Penicillin in Guinea Pigs in Relation to the Increase of Fatty Acid in the Sera.

As briefly mentioned in Experiment IV, at the time of Bleeding C., 5 days after infection, guinea pigs Nos. 3, 4,

7, 10 and 16 produced serum that was extremely fatty in appearance, varying from the serum of guinea pig No. 7. that formed a solid white clot to guinea pig No. 4 which was slightly milky in appearance. All these animals died soon after.

Fatty acid determinations were made on these 5 sera, according to the method of Stoddard and Drury as modified by Man and Gildea (245).

TABLE XXII

SERUM FATTY ACID DETERMINATIONS IN GUINEA PIGS TREATED WITH PENICILLIN AFTER INFECTION

Guinea Pig No.	3	4	7	10	16
Pneumococcic Peritonitis (Type)	I	I	I	I	I
Penicillin (intramuscularly)	Procaine penicillin in oil 9600 Units	Procaine penicillin in oil 9600 Units	Procaine penicillin in oil 9600 Units	Procaine penicillin in oil 9600 Units	Crystalline Sodium penicillin 10,000 Units
Time Bleeding after infection (Days)	5	5	5	5	5
Serum Fatty Acid (Milk equivalents)	105	30	285	45	127.5
Time of Death after Infection	6	6	6	6	8

Discussion

5 out of 25 guinea pigs infected with pneumococci Type I intraperitoneally and treated with penicillin in 5 hours, exhibited milky sera when bled after 5 days, which when determined

for fatty acid gave results ranging from 30 to 285 milk equivalents. They all died 6 to 8 days after being infected and treated with penicillin.

It was then decided to inject a small number of guinea pigs with large doses of penicillin daily, bleed on the 5th day and determine the fatty acid content of their sera.

In asmuch as guinea pigs receiving penicillin seemed to show a marked weight loss, this factor was eliminated by starving the animals for 4 - 5 days until they had lost about 1/5th of their body weight, then determining the fatty acid content of their sera. They would then be fed until they had regained their original weight (about 1 week) then daily injections of penicillin begun. The normal fatty acid of their sera was first determined before starvation in order to give a control base line.

After the 5 day bleeding, the same dose of penicillin was given intramuscularly daily for two weeks without any mortality or great loss of weight as seen in the experimentally infected animals who received a fraction of the total dose these did.

Results

The fatty acid in the serum at the time of the 5 day bleeding was increased in the animals receiving penicillin; however it was not lethal even in these doses.

TABLE XXIII

SERUM FATTY ACID DETERMINATIONS IN GUINEA PIGS RECEIVING PENICILLIN ONLY

Guinea Pig No.	1	2	3	4
Original Weight (Gms.)	565	615	425	650
Serum Fatty Acid (Milk equivalents)	10	8.75	2.5	5.0
Weight after starvation 4 days	555	520	365	535
Serum Fatty Acid (Milk equivalents)	6.25	7.5	12.5	7.5
Weight after penicillin 5000 x 4 days	510	*	420	600
5 day bleeding Serum Fatty Acid (Milk equivalents)	25	*	20	15

* Guinea pig No. 2 was found dead on the morning it should have been bled. Wt. 515 Gms.

Discussion

The animals which received penicillin after infection showed markedly elevated serum fatty acid and 100 per cent mortality in contradistinction to the animals which received penicillin only which showed only moderately elevated serum fatty acid and a possible 25 per cent mortality.

The infected animals receiving penicillin had in addition to a clinical infection, the dye carmine. The latter has been proven harmless to animals, and the peritonitis produced had been treated with sulphamethazine without the development of a high serum fatty acid. However, whether the combination of trio, i.e. dye, infection and penicillin resulted

in the production of a high serum fatty acid, must be determined by eliminating each factor as a control.

As others have previously reported (246), (247), (248), no gross pathology could be detected on autopsy of these animals dying after receiving penicillin, even those with high serum fatty acid.

Coincidentally, none of the guinea pigs receiving penicillin 16 hours after infection showed fatty serum on the 5 day bleeding.

G E N E R A L D I S C U S S I O N

Throughout the report here presented, there were brief discussions given of the conclusions that could be drawn from the respective findings.

The experimental results have demonstrated that immunity in pneumococcic peritonitis Type I in guinea pigs does not seem to be influenced in the time of its appearance or amount, when the infection is terminated by the use of sulphonamides or by the use of penicillin. Immunity could be demonstrated by "in vitro" methods, and it was further substantiated by "in vivo" methods in both the sulphonamide and penicillin treated guinea pigs.

Individual animals in the groups treated with sulphonamides and penicillin, five and sixteen hours after infection, demonstrated agglutinating antibodies at various consecutive intervals of bleeding. However, the persistence of active immunity, as demonstrated by resistance to reinfection, varied in different animals in each group.

The majority of animals, which showed agglutinating antibodies and a positive intracutaneous test with the amount of antigen used, were able to survive the original infecting dose within a period of twenty days. However, survival of one reinfecting dose did not necessarily ensure immunity to a second reinfecting dose.

In general, there appeared to be a correlation with the agglutinating titre and survival at the time of reinfection. An agglutination titre in the range of 1 in 32 to a 1 in 64

dilution of serum with the constant amount of antigen used, resulted in survival to reinfection in a certain number of animals. A titre higher than a 1 in 64 dilution, ensured survival of all animals to reinfection.

Frequent bleedings were taken, from the time therapy was instituted until a period of twenty days had elapsed after initiation of infection. In this manner, the level of the agglutinating titre was charted as it increased or decreased. In addition,methods, routes of administration, and varying dosages of each form of therapy, were demonstrated in relation to the immune response.

For an arbitrary period of twenty days, animals which received sulphonamide therapy five hours after infection, exhibited the lowest mortality rate. This was higher when therapy was instituted sixteen hours after infection.

Penicillin is toxic for guinea pigs as a species, but in addition, there was very little difference in the mortality rate when penicillin therapy was instituted five or sixteen hours after infection. In this instance, the number of animals that survived and demonstrated agglutinating antibody titres, was not necessarily the actual number that would have demonstrated titres had this added factor of drug toxicity not interfered.

An extremely interesting observation was the fact that in both sulphonamide and penicillin treated animals, agglutinating antibodies could be detected within five hours and sixteen hours. However, this was not evident when sulphonamides were administered immediately after infection. In view

of a lag period of about four hours in the mode of action of this drug, therapy was probably not immediately fully effective, and so this permitted the survival and growth of a slowly immunizing dose of antigen. In this case, circulating antibodies were rather late in appearance, and as a result, this delay in production of antibodies seemed to result in a longer persistence. This was demonstrated by the survival of these animals to two reinfecting doses of the original infecting dose. In view of the small number of animals used in this experiment, it is difficult to evaluate whether this was entirely true. On the other hand, it seems to be evident that an early production of an immune response does not necessarily ensure survival to reinfection.

The detection of antibodies in five hours, appears to be the earliest demonstration on record, (249), (250).

The strain of pneumococcus Type I that was employed was an excellent antigen producer, and the injection of living suspensions resulted in the greatest production of agglutinating antibodies. In guinea pigs, a large dose had to be given in order to establish a 50 per cent mortality, and at the same time was adequate in stimulating the production and development of type specific agglutinating antibodies. However, even with the same immunization schedule, guinea pigs did not develop as good agglutinating titres as rabbits, although the average titres were fairly high.

As regards the method of detection of antibodies, agglutination as a qualitative test, appeared to be extremely sensitive, and an extremely small amount of serum could be

used. The optimal proportions test, for purposes of comparison of individual sera, was also quite satisfactory. However, since a greater volume of sera was required, and in consideration of the frequency of bleeding of each animal, the employment of this method was impractical. Quantitative precipitin tests, besides involving a rather cumbersome technique, required strong sera in addition to a large volume.

The variation in agglutinability of antigen preparations, demonstrated both by the same, or different procedures, was an enigma that has been observed in other organisms. E.g. typhoid. Instead of compensating for this, by assigning to each preparation an agglutination factor, which might be difficult with weak sera, a Standard immunized serum was used, to select antigens of suitably sensitive agglutinability.

Manifestations of penicillin toxicity in guinea pigs, although lacking in gross internal pathology, resulted in loss of weight, ruffled fur, anorexia, and finally death. In addition, a high serum fatty acid, three to four times the normal values, was obtained in some animals. Penicillin therapy, in other animals infected with pneumococci Type I intraperitoneally, induced by carmine, however, demonstrated even higher sera fatty acids. These animals had received initial therapy five hours after infection. In those receiving penicillin sixteen hours after infection, this increase was not so evident. In none of the animals treated with sulphonamides, did the serum exhibit a fatty appearance.

SUMMARY AND CONCLUSIONS

The following conclusions were arrived at from the experimental data here reported, as obtained from the strain of pneumococcus Type I used:

1. The development of an immune response to pneumococci Type I infection in guinea pigs, is not influenced by either sulphonamide or penicillin therapy.
2. Homologous agglutinating antibodies could be demonstrated as early as five hours after intraperitoneal infection with pneumococcus Type I in guinea pigs.
3. Homologous agglutinating antibodies could be demonstrated in both sulphonamide and penicillin treated guinea pigs.
4. The demonstration of agglutinating antibodies was not absolutely parallel with a state of permanent active immunity, as demonstrated by reinfection with the original infecting dose; however, an agglutinating titre higher than a 1 in 64 dilution of serum for a constant dilution of antigen, afforded definite protection.
5. Sulphonamide therapy administered immediately after pneumococcic infection resulted in a delay in the appearance of antibodies, but active immunity was more prolonged.
6. There is extreme variability in the time of development and persistence of effective active immunity.
7. Penicillin shows a species toxicity in guinea pigs with the appearance of increased serum fatty acids.

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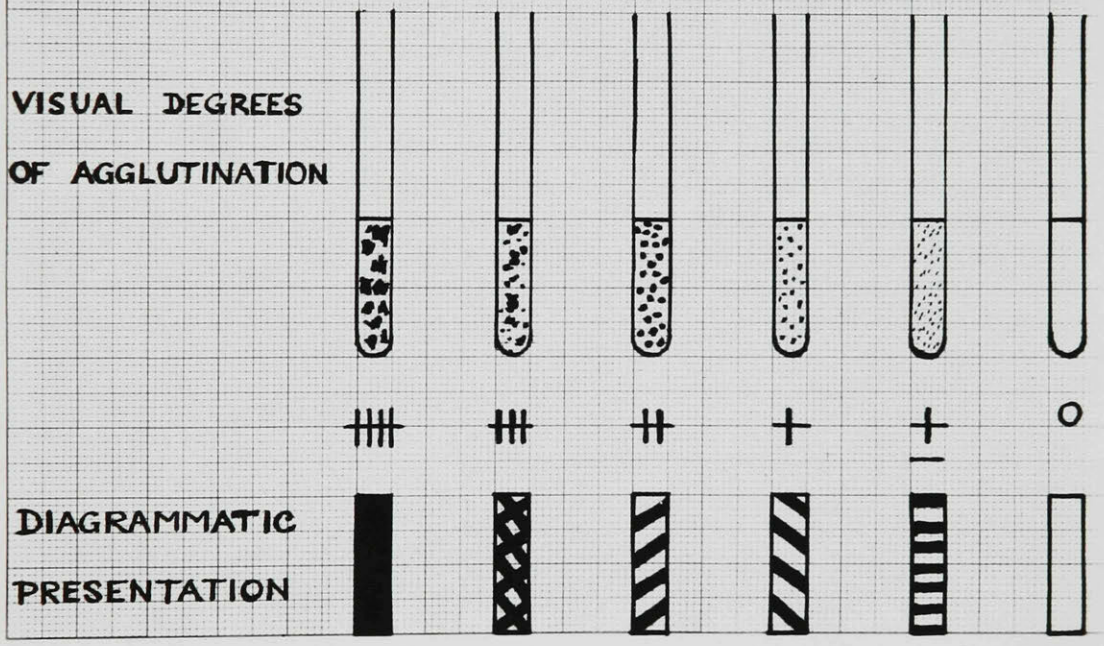

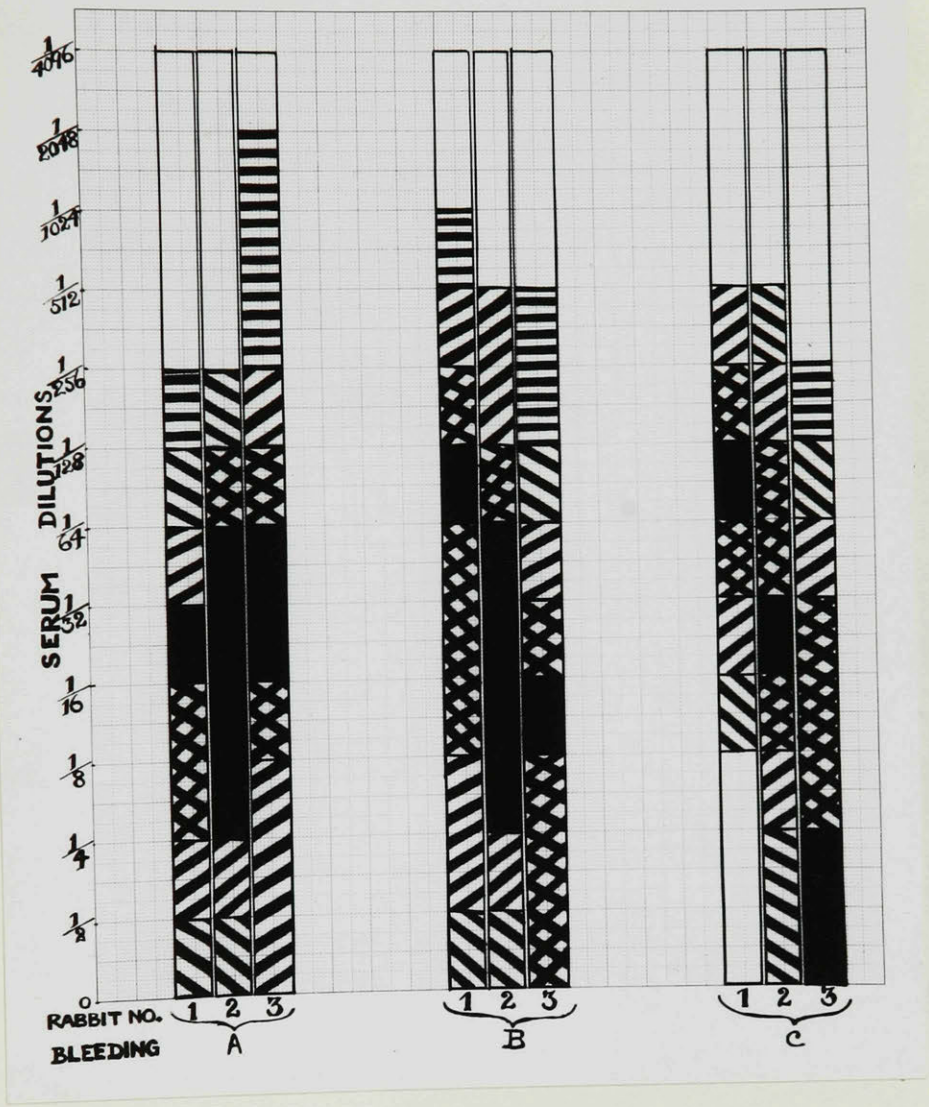

Figure 1. Agglutination titres in immunized Type I pneumococcic rabbit sera. (see text)

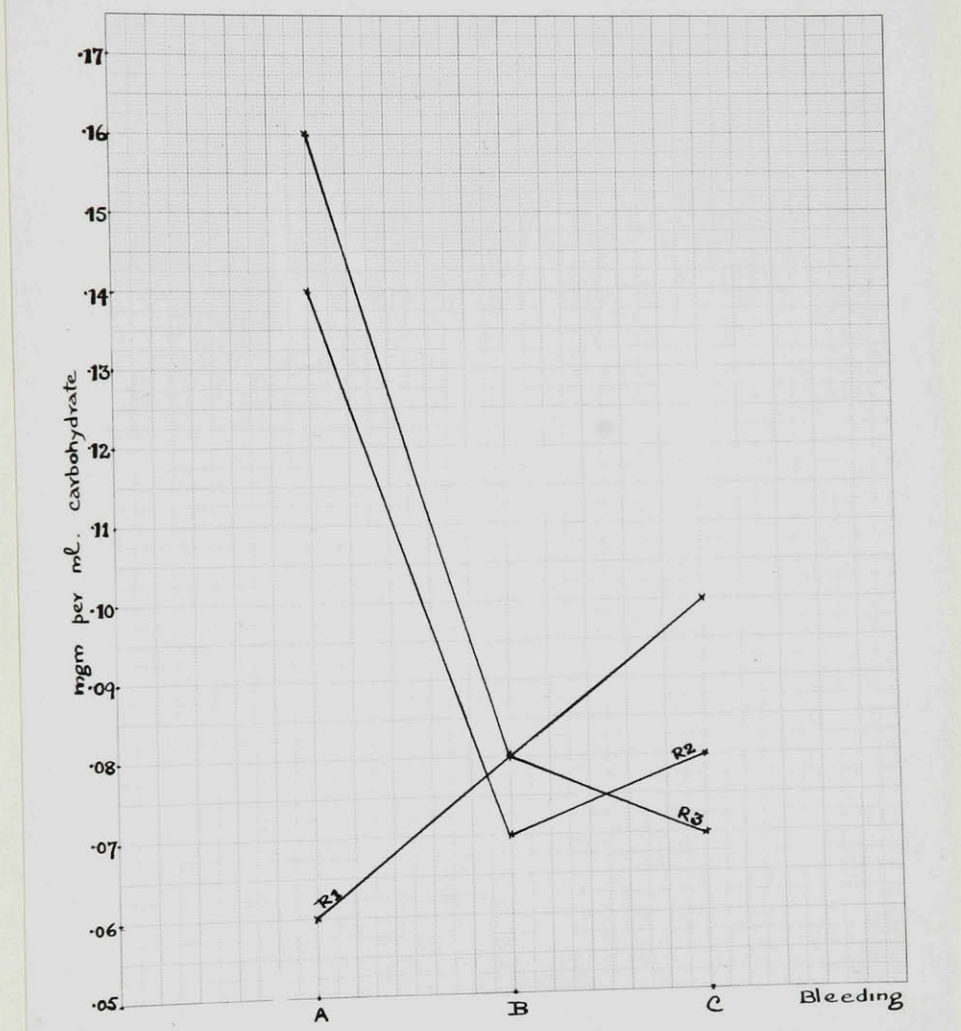

Figure 3. Antibody content (mgm. carbohydrate per ml.) in immunized Type I pneumococcic rabbit sera. (see text)

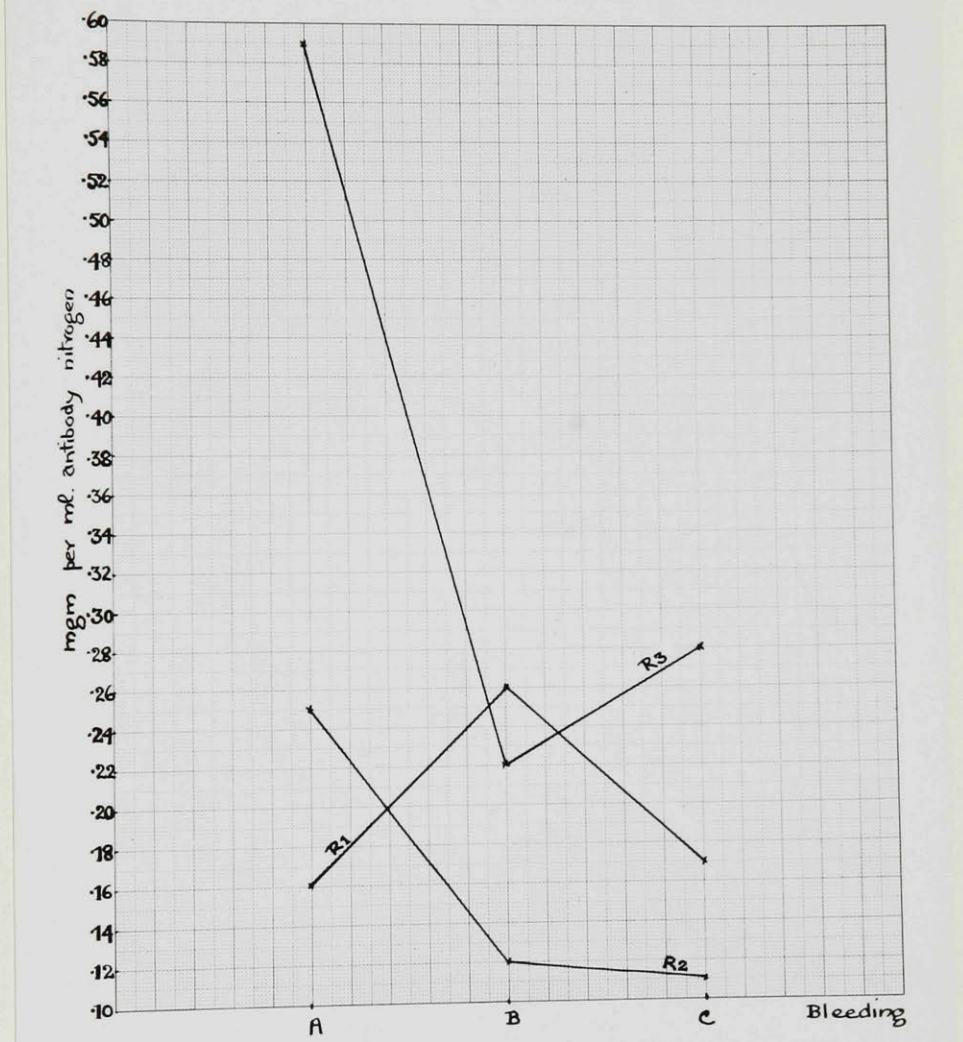

Figure 4. Antibody content (mgm. antibody nitrogen per ml.) in immunized Type I pneumococcic rabbit sera. (see text)

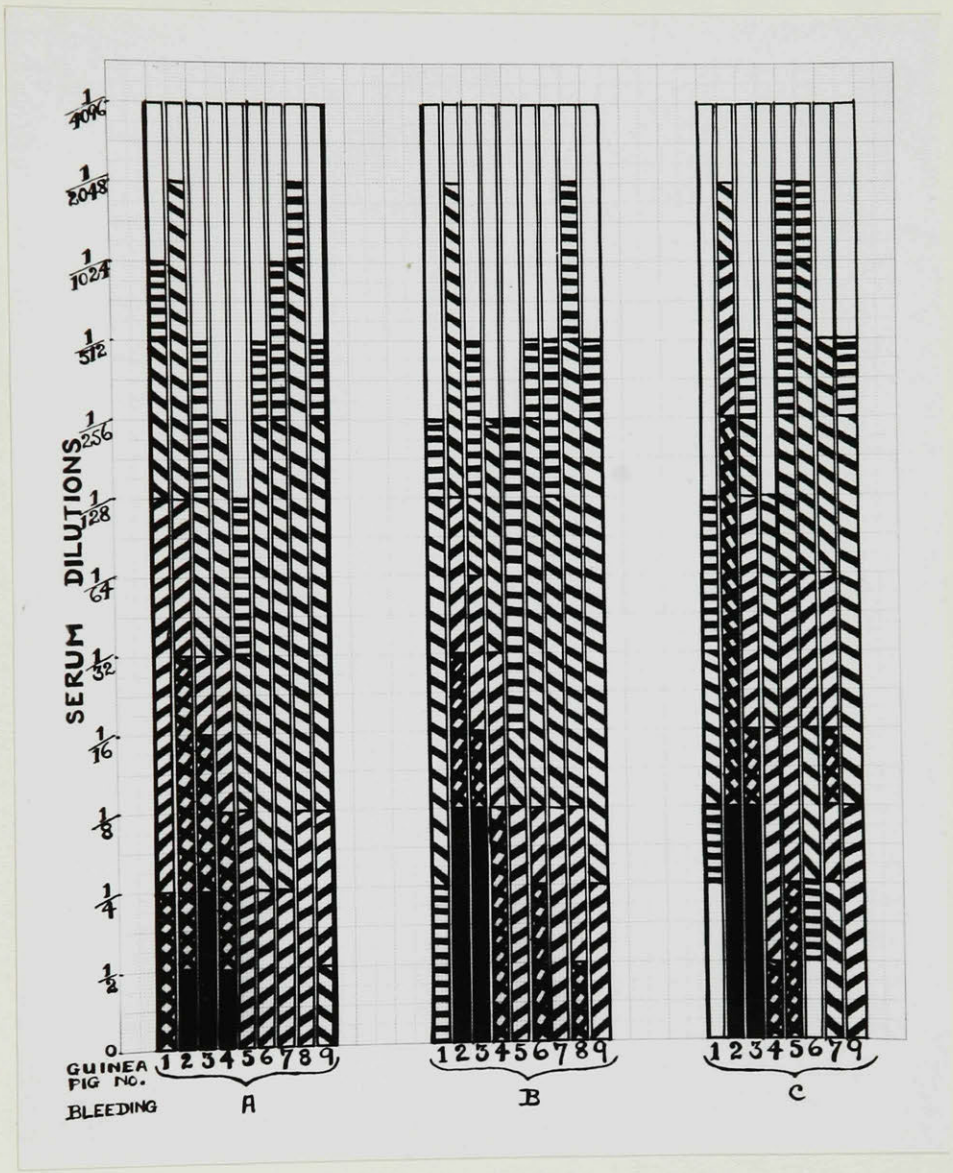

Figure 5. Agglutination titres in immunized Type $\overline{\text{I}}$ pneumococcic guinea pig sera. (see text)

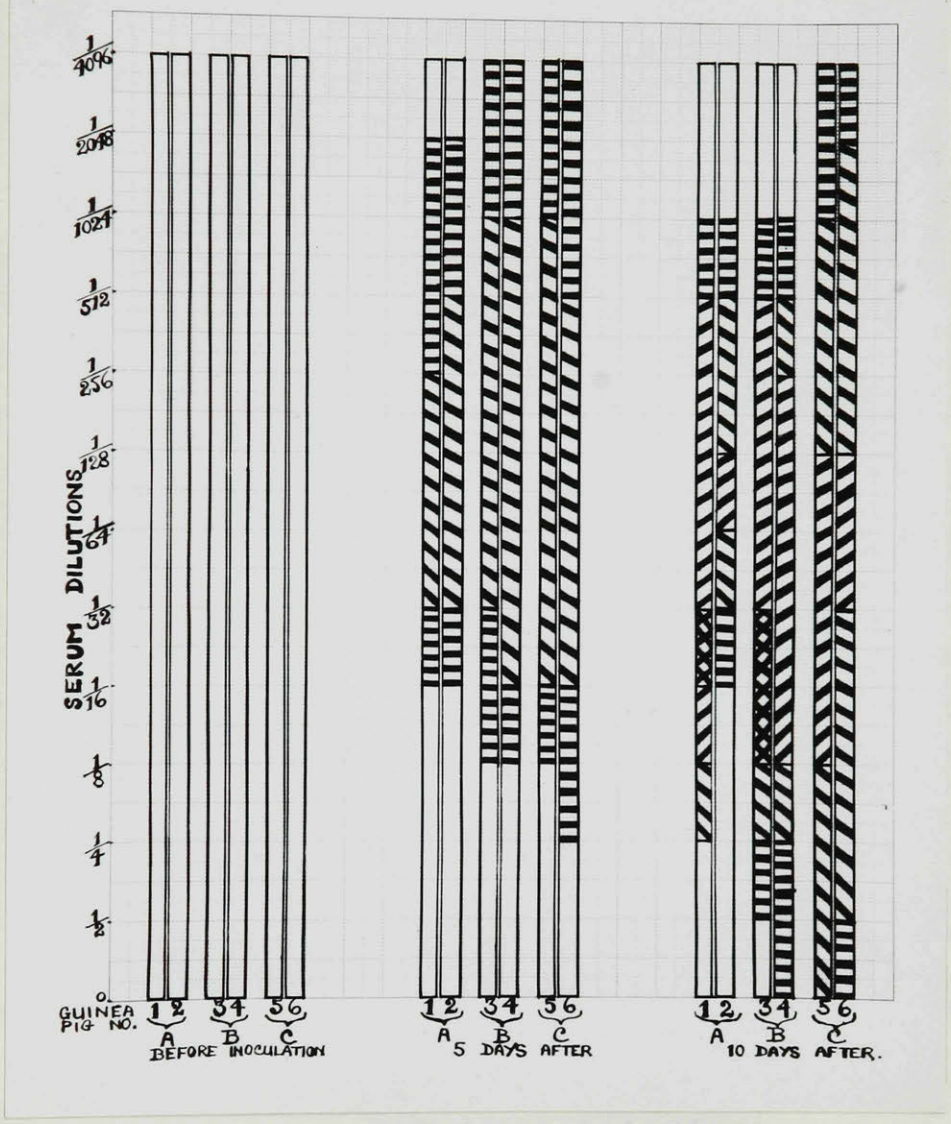

Figure 6. Agglutination titres in guinea pigs immunized by a single varying dose of heat killed pneumococcus Type I.

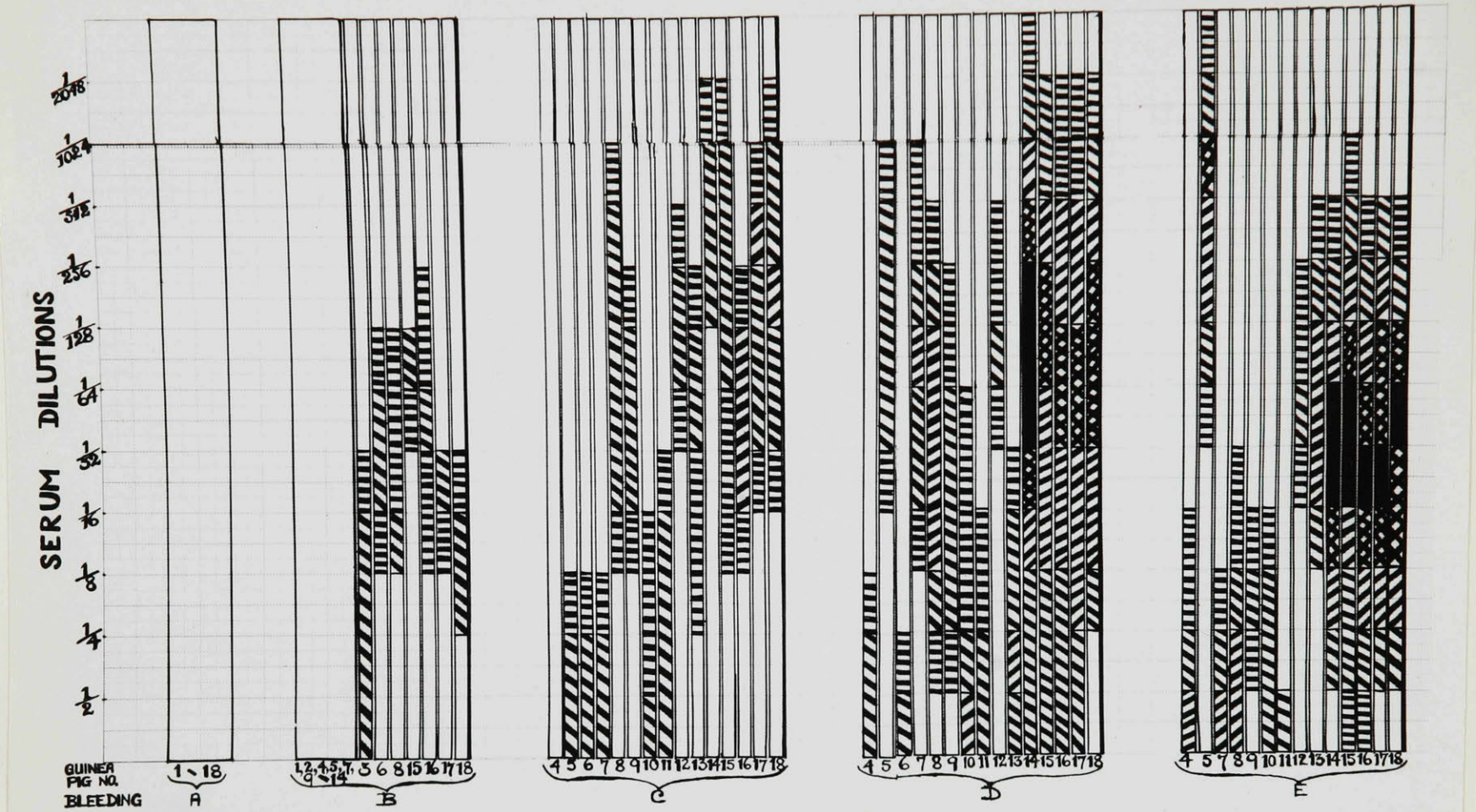

Figure 7. The Effect of Sulphonamide therapy administered 5 hours after the inoculation of Type I pneumococci intraperitoneally on the agglutination titres in guinea pig sera.

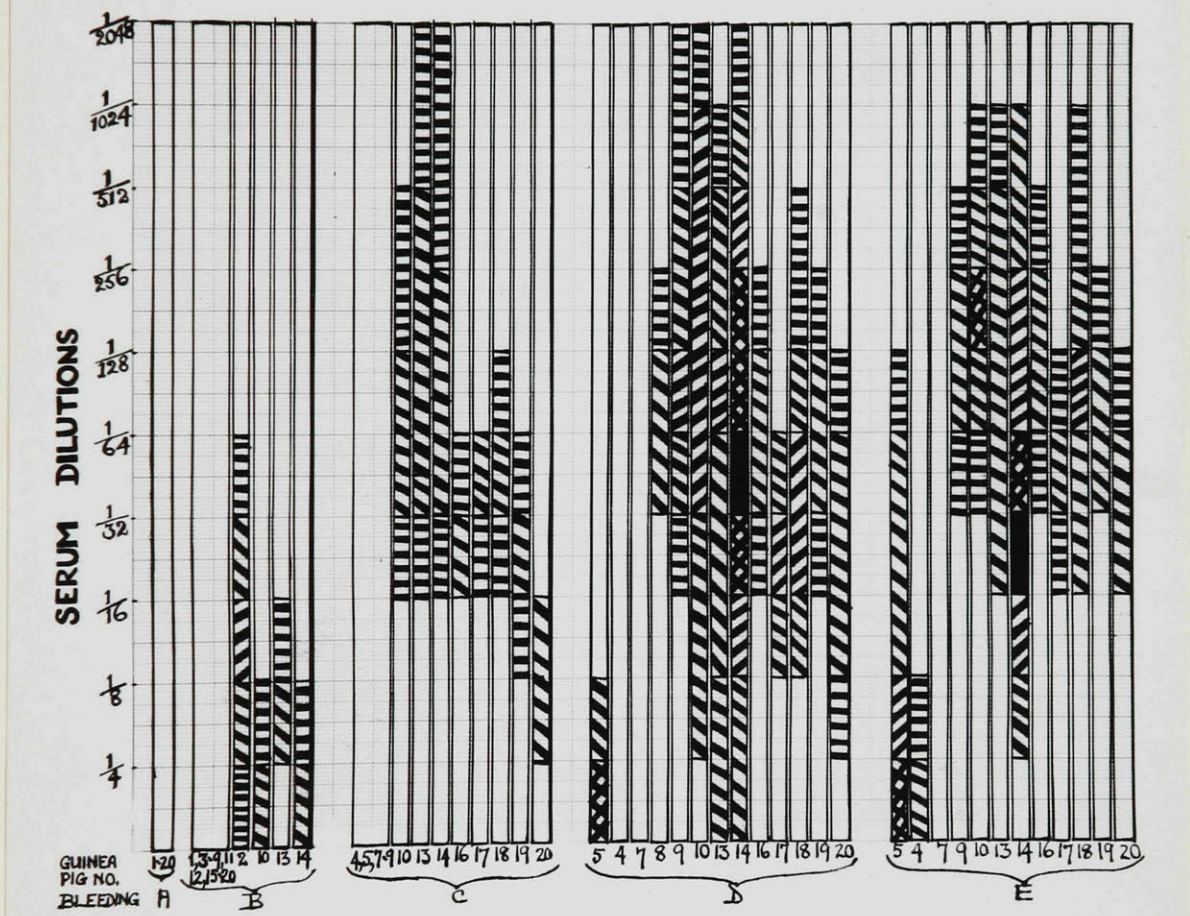

Figure 8. The Effect of Sulphonamide therapy administered 16 hours after the inoculation of Type I pneumococci intraperitoneally on the agglutination titres in guinea pig sera.

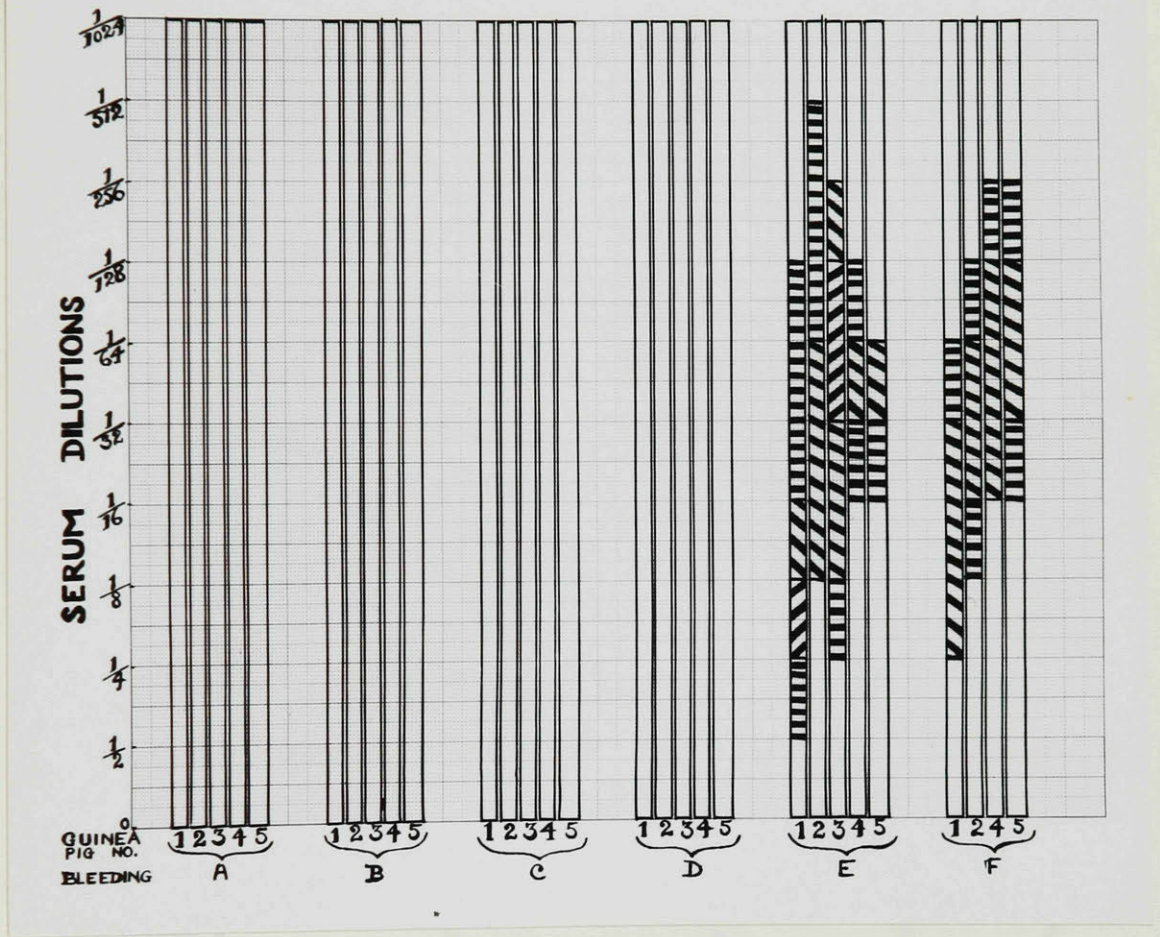

Figure 9. The Effect of Sulphonamide therapy administered immediately after the inoculation of Type I pneumococci on the agglutination titres in guinea pig sera.

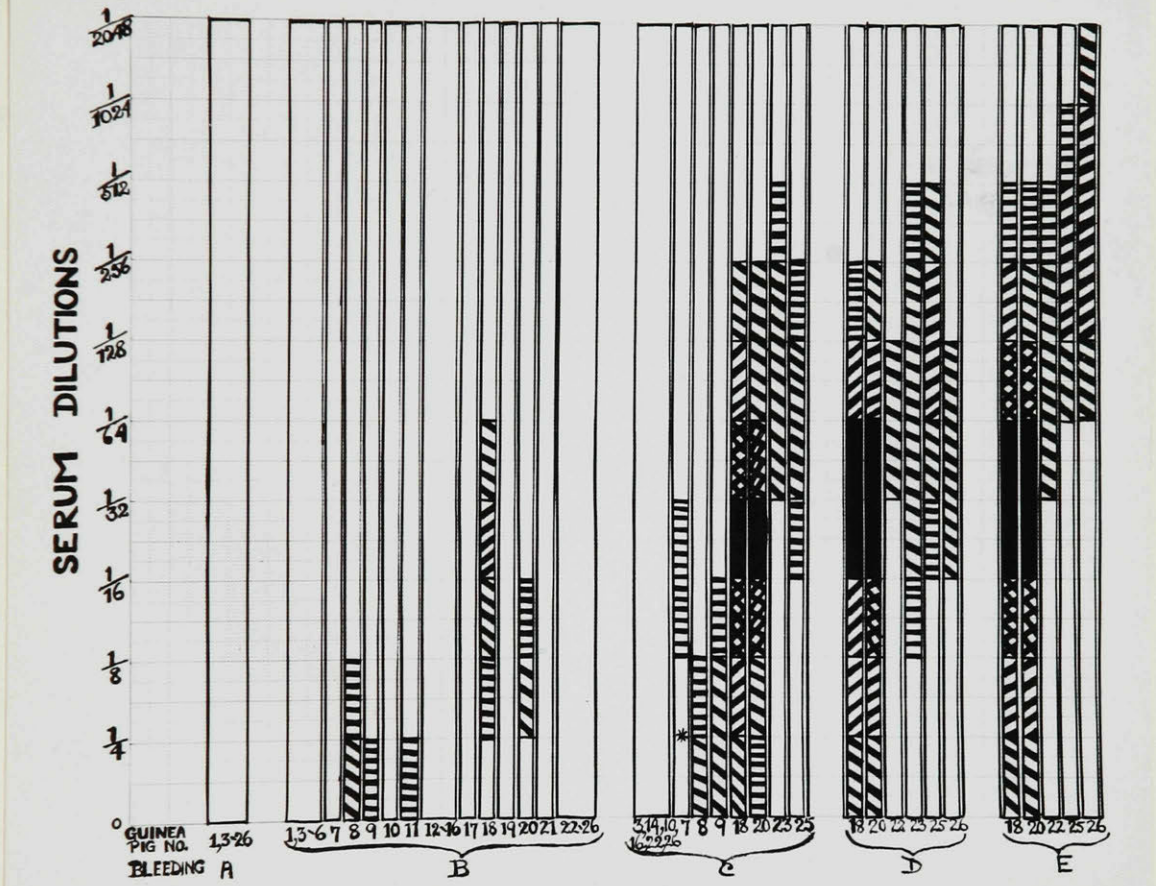

Figure 10. The Effect of Penicillin therapy administered 5 hours after the inoculation of Type I pneumococci, on the agglutination titres in guinea pig sera.

* In these dilutions, agglutination if present could not be detected due to extreme milky appearance of the serum.

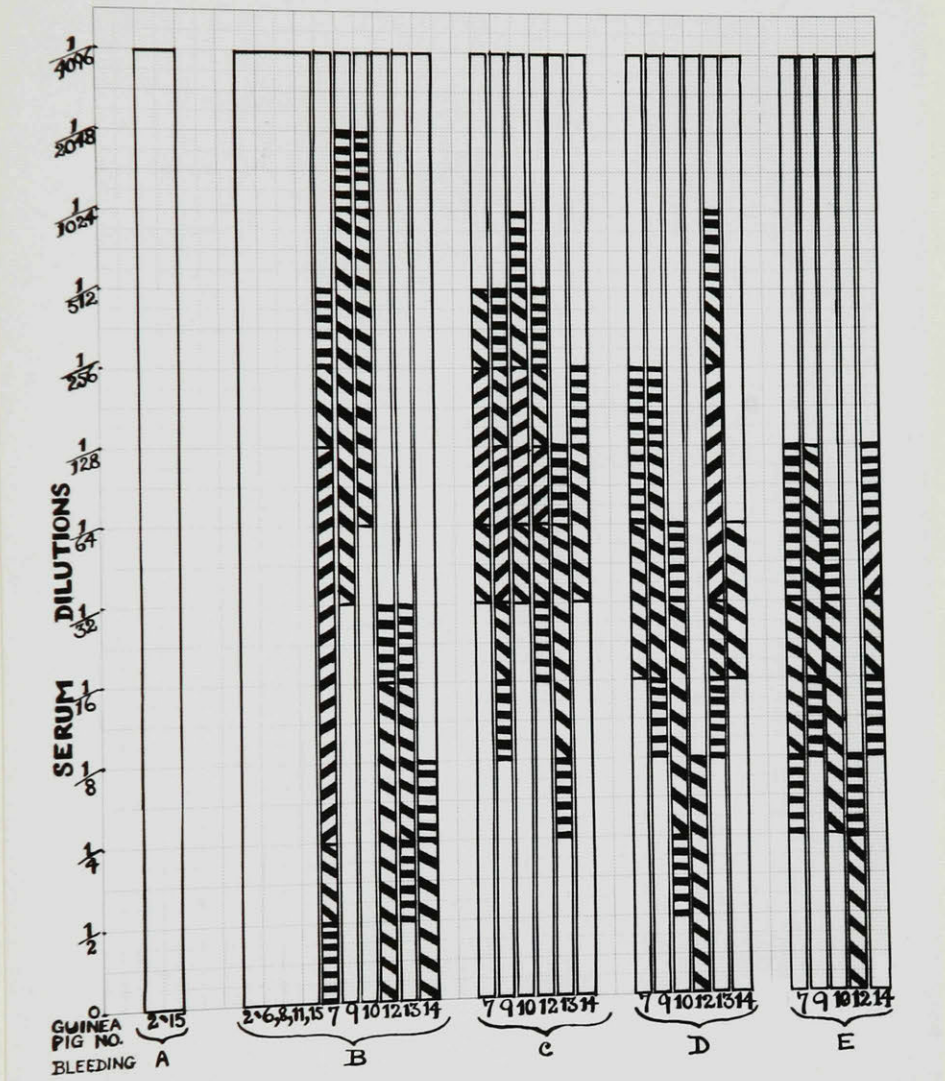

Figure 11. The Effect of Penicillin therapy administered 16 hours after the inoculation of Type I pneumococci, on the agglutination titres in guinea pig sera.