Report of Dr. Avery (with Drs. Stillman, Tillott, Julianelle, Goebel, Dubos, Dawson and Francis).

Studies on Pneumococcus Infection and Immunity.

I. Chemo-immunological Studies:

1. Nature of the type-specific antigen.

2. Synthesis of carbohydrate-protein antigens.

II. Reversion of "R" $\rightarrow$ "S" forms of Pneumococcus and the Interconvertibility of the specific types.

III. Cutaneous reactions in pneumonia patients to the protein and specific carbohydrate of Pneumococcus.

IV. Cutaneous infection of normal and immune rabbits with "R" and "S" pneumococci.

V. Cutaneous vaccination of rabbits with Pneumococcus: the character of the antibody response, the resistance to infection and the hypersensitivity induced in animals.

VI. Reversion of "R" $\rightarrow$ "S" forms of Friedländer bacilli and the interconvertibility of the specific types.

VII. Nature and duration of the immunity produced in rabbits by the inhalation of pneumococci.

VIII. Significance of oxidation-reduction processes in bacterial growth.

IX. Publications.

In the work on Pneumococcus infection and immunity carried on in the bacteriological laboratories of the hospital during the past year in conjunction with the clinical study of pneumonia in the wards, the studies of special interest and those sufficiently supported by experimental data to justify their inclusion in the annual report to
the Board of Scientific Directors are, the chemo-immunological inves-
tigation concerning the specific antigens of Pneumococcus, the results
of which are already yielding more precise knowledge of the chemical
and biological properties, and of the causes and the prevention of
the dissociation and consequent inactivation of these complex antigens,
before and after injection into the animal body; the successful and
original synthesis of sugar-proteins by the coupling of a carbohydrate
radicle in glucosidic union with serum globulin, undertaken with the
hope of thus orientating the antigenic specificity of the protein
molecule by the introduction of an antigenically inert glucoside; the
important confirmation and elaboration of the original observations of
Griffith on the interconvertibility of specific types of Pneumococcus,
establishing the occurrence of fundamental variations in virulent
pathogens, defining conditions under which avirulent organisms may
become virulent and indicating the possible significance of these
adaptive changes in the course of infection and in the epidemiology
of the disease; the discovery of the important fact that in pneumonia
patients, two chemically different constituents of Pneumococcus may
give rise to two distinct kinds of skin reaction, namely the immediate
wheal and erythema type of anaphylactic response elicited by the
intracutaneous injection of a fraction of a milligram of the specific
polysaccharide, and the delayed tuberculin-like type of inflammatory
reaction produced by minute amounts of the bacterial protein, each of
these reactions exhibiting their characteristic specificity and both
suggesting the relationship of hypersensitiveness to the processes and
course of the disease; the observations made on pneumococcus infection
of the skin of normal rabbits and of rabbits immunized with "R" and
"S" strains and the correlation between the kinds of lesions produced
by these virulent and avirulent organisms, thus providing an experimental method of determining more accurately the mechanism underlying the differences between natural resistance and acquired immunity in terms of specific antibodies and cellular reactions; the observation of significant differences in the character of the antibody response of rabbits to intravenous and intracutaneous injection of pneumococci, demonstrating the fact that after introduction into the animal body type-specific strains ("S" forms) under certain circumstances may lose more or less completely the property of stimulating the type-specific antibodies although still retaining unimpaired the property of eliciting the antiprotein antibodies commonly provoked by all "R" cells, - a fact of significance in understanding the phenomenon of antigenic dissociation and in interpreting the character of the immune response to the complex antigen of Pneumococcus; the finding that following intracutaneous vaccination with heat-killed "R" and "S" organisms, rabbits acquire a solid immunity against infection with virulent "S" strains of homologous and heterologous types, and the observation that rabbits treated with "R" cells become resistant to infection in the total absence of demonstrable type-specific antibodies; the observation that intracutaneous vaccination in rabbits may lead to the development of hypersensitivity to the bacterial protein as shown by positive skin and eye reactions; the demonstration of the reversion of avirulent organisms to the virulent forms and of the interconvertibility of the specific types as indicated by the fact that Gram negative bacilli of the Friedländer group may be caused to undergo these transformations by the methods successfully employed in the case of Pneumococci; the production of an immunity of long duration in rabbits by the inhalation method demonstrating the possible importance of the respiratory
route in the acquisition of specific resistance and illustrating the significance which the bacterial flora of the upper air passages may have in determining the reactions of immunity and hypersensitivity to infection; and finally the application of the principles of oxidation-reduction processes to the study of pneumococcus, particularly with reference to the cellular functions of growth, thus bringing to light new facts which are of general biological significance as well as of practical use in the improvement of culture media.

I. Chemo-immunological Studies on Pneumococcus. Pneumococcus Antigen and Synthetic Carbohydrate Antigens. (Dr. Goebel).

The problems of understanding the relationship between chemical constitution, physiological effect and biological specificity, which probably found their origin in the study of the active principles of certain natural drugs, have become so absorbing and so encompassing that they have attracted and held the interest of the chemist, the pharmacologist and immunologist alike. The question of protein specificity in particular, and the possibility of changing specificity by altering the protein molecule through chemical means, have engaged the minds of many investigators. On the other hand, the rôle which carbohydrates play in the phenomena of immunity has only recently been disclosed, despite the fact that the presence of these substances in bacteria and yeast has long been known.

Some years ago, Dochez and Avery observed that filtrates of pneumococcus cultures contained a stable substance which reacted specifically with antipneumococcus serum of the homologous type. This observation led to the isolation and identification of specifically reacting substances from the three specific types of Pneumococcus and from the three types of Friedländer's bacillus. Since then other
investigators have isolated similar substances from various other species of bacteria.

These type-specific substances fall into the class of carbohydrates; they are unusual carbohydrates in that each contains a sugar acid as an integral part of its complex molecule. Immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has named haptens.

One of the striking characteristics of these bacterial carbohydrates is their failure to produce antibodies when injected into the animal organism, though in the state in which they occur in the bacterial cell they are not only type-specific but also effectively antigenic as well. When the pneumococcus cell is permitted to autolysate under sterile conditions it has been found that the autolytic process is accompanied by an increase in amino and non-coagulable nitrogen and in ether-soluble fatty acids. This change is due to the action of liberated intracellular enzymes, protease and lipase, upon the native substances of the cell. When extracts of pneumococci containing the active intracellular enzymes are added to heat-killed pneumococci, lysis of the cells occurs and there is an increase in the non-coagulable and amino nitrogen comparable to the changes accompanying spontaneous autolysis. Furthermore, when extracts containing the active intracellular enzymes are added to emulsions of the alcohol-soluble lipoids extracted from pneumococci, an increase in the ether-soluble fatty acids occurs. When living pneumococci are dissolved by means of sodium desoxycholate and such solutions are incubated at 37°C, an excess of the bile salt has been found to inhibit the action of the pneumococcus protease, but not the action of the lipase. Such solutions, however, no longer yield type-specific antibodies when
injected into animals. It seems, therefore, that the specific immunizing antigen of pneumococcus is impaired by the action of its own lipase. In order that the bacterial polysaccharide may be effective as an antigen it is believed that it must be combined with another cellular constituent, possibly a protein, in ester linkage to form an easily dissociable, complex antigen.

The products of hydrolysis of the specific carbohydrates of Pneumococcus and Friedlander's bacillus have been studied in detail. All except the Type I Pneumococcus carbohydrate yield glucose on hydrolysis. The complex sugars of Pneumococcus Type III and Friedlander bacillus Type A yield on hydrolysis isomeric aldobionic acids (glucose-glucuronic acid) in addition to glucose. Evidence has also been secured which indicates that the specific polysaccharides of Type II Pneumococcus and of Type B and C Friedlander bacillus also contain other aldobionic acids within their molecules.

The selective specificity of encapsulated organisms, such as Pneumococcus and Friedlander's bacillus, seems to depend primarily on the hapten part of the hypothetical complex antigen. In all of the haptens studied thus far, the invariable presence of isomeric aldobionic acids seems to indicate that particularly the acid group (carboxyl group) and its stereo-chemical relationship to other groups of the intact polysaccharide molecule, which in each instance must necessarily be different, has a profound influence in orienting specific antibody response.

In order to understand more fully the rôle of carbohydrates in immune phenomena we turned to the group of mucoproteins, the naturally occurring conjoined carbohydrate-proteins, but without avail, for we did not find the carbohydrates either of ovomucoid or of tendomucoid
to behave as haptens. Nor did mixtures of purified specific soluble substance and protein yield, on immunization, antibodies reactive with the specific carbohydrates. Therefore, for the purpose of studying the role which simple sugars, and in particular sugar acids, of different spatial configuration, such as those which are found in specific polysaccharides, might play in altering the specificity of proteins, it was thought that it might be possible to combine different sugars and their acid derivatives with a given protein and to observe any specific differences in antigenic properties that might result.

The problem thus becomes two-fold, first to synthesize the desired sugar derivative, and second to devise some means of combining the sugar with the protein. Some years ago, Pauli described a test for tyrosin and histidin in proteins by adding diazobenzene sulphonic acid to an alkaline solution of protein. The diazobenzene sulphonic acid combines with the protein, and on acidification the complex is precipitated. This fundamental principle was made use of in combining glucose and galactose to serum globulin. The second problem, that of preparing a sugar derivative adaptable to this principle, was solved by the following synthesis:

\[
\begin{align*}
\text{CH}_2\text{OH} \text{CH} - (\text{CHOH})_3 - \text{C}^\text{H}_\text{OH} & \quad \text{CH}_3\text{COOCH}_3 - \text{CH} - (\text{CHOOCOCH}_3)_3 - \text{C}^\text{H}_\text{OCH}_\text{3} \\
\text{Glucose.} & \quad \text{Pentaacetyl glucose.} \\
\text{H Br} & \quad \text{CH}_2\text{OCOOCH}_3 - \text{CH} - (\text{CHOOCOCH}_3)_3 - \text{C}^\text{H} \\
\text{Tetra-acetyl-bromo-glucose.} & \quad \text{Silver para-nitrophenolate.} \\
\text{CH}_2\text{OCOOCH}_3 - \text{CH} - (\text{CHOOCOCH}_3)_3 - \text{C}^\text{H}_{\text{OC}_6\text{H}_4\text{NO}_2} & \quad \text{Ba} (\text{OH})_2 \\
\text{Tetra-acetyl-para-nitrophenol } \beta\text{-glucoside.}
\end{align*}
\]
The end product of this series of reactions may then be converted into the corresponding diazonium derivative and coupled with protein in the presence of dilute alkali:

\[
\text{CH}_2\text{OH} - \text{CH} - (\text{CHOH})_3 - \text{C}^\text{\,\,\,H} \quad \xrightarrow{\text{HNO}_2} \quad \text{CH}_2\text{OH} - \text{CH} - (\text{CHOH})_3 - \text{C}^\text{\,\,\,H} \quad \xrightarrow{\text{HNO}_2} \quad \text{H}_2\text{O}
\]

Paranitrophenol - $\beta$-glucoside. Paraaminophenol - $\beta$-glucoside.

Thus one may couple practically any sugar (aldose) or its derivative with any protein.

We have thus far synthesized, in addition to intermediate glucosides, paraaminophenol $\beta$-glucoside, and paraaminophenol $\beta$-galactoside. These hexosides have been coupled to pure serum globulin under conditions which do not alter, by denaturation, the protein molecule. Two different synthetic sugar proteins have thus been prepared. They exhibit different chemical properties, and although the protein substrate is in each instance the same, they appear to be different compounds. Animals are being immunized with these synthetic sugar protein antigens. Attempts are also being made to prepare the glucosides of aldobionic acids, the sugar acids found in the specific soluble substances of Pneumococcus, and to combine them with the bacterial protein and to study the antigenic properties of these complex substances.
II. Studies on the Interconvertibility of "R" and "S" Forms of Pneumococcus and the Interconvertibility of Pneumococcal Types. (Dr. Dawson).

In the January (1928) number of the Journal of Hygiene there appeared an article by F. Griffith in which he claimed to have demonstrated the following points: (1) That "R" pneumococci could be reverted into the homologous "S" types by the subcutaneous injection in mice of large amounts of live organisms; (2) that "R" pneumococci could be reverted into the homologous "S" type by the subcutaneous injection in mice of small amounts of live "R" cultures together with the heat-killed deposits of large amounts of the corresponding "S" cultures; and (3) that "R" forms could be transformed into heterologous "S" types by the subcutaneous injection in mice of small amounts of live "R" organisms together with the heat-killed deposits of large amounts of heterologous "S" cultures.

In view of the significance of these findings, experiments were at once undertaken to verify Griffith's results.

(1) The reversion of "R" forms into the homologous "S" type by the subcutaneous injection in mice of large amounts of living "R" cultures. It was found that this was a most effective method of causing "R" forms to revert to the "S" type. Different "R" strains varied in the amount of culture required and one strain (1/192/R) uniformly failed to revert. The results obtained were entirely in accord with those previously obtained by other methods. In all instances in which reversion occurred the "R" forms invariably reverted to the same specific type from which they were originally derived.

(2) The reversion of "R" forms into the homologous "S" types by the subcutaneous injection in mice of small amounts of live "R" cultures together with the heat-killed deposits of large amounts of the corresponding "S" culture. This method was found to be more effective than any
other previously employed in producing the $R \rightarrow S$ change. Success was attained with one culture (1/192/R) which had resisted all previous efforts to effect the transformation. This particular culture had previously been passed intraperitoneally through 105 consecutive mice and still maintained all of its "R" characteristics. (The question of the viability of any organisms in the heat-killed deposits is carefully considered in the next part of the report.)

(3) The transformation of "R" forms into heterologous "S" types by the subcutaneous injection in mice of small amounts (0.25 cc.) of living "R" organisms together with the heat-killed deposits of large amounts (50-100 cc.) of "S" vaccines of heterologous types.

Since the question of the viability of the organism in the vaccines is an all important consideration, the procedures employed in the preparation of the vaccines and the controls used in testing for sterility are given in detail.

The vaccines were heat-killed in sealed ampules totally immersed in water. Fifteen minutes heating at 60° has invariably sufficed to kill all pneumococci but longer periods and higher temperatures have also been used.

**In Vitro Controls:**  
(1) All vaccines have been cultured on blood agar plates and in blood broth.  
(2) Cultures have been made aerobically and anaerobically in blood broth and blood extract dextrose broth.  
(3) Vaccines have been cultured serially in 10 per cent serum broth for 20 transfers.

**In Vivo Controls:**  
(1) An equal number of control mice have been inoculated with the vaccine alone and later sacrificed and cultured at varying intervals.  
(2) Control animals have been inoculated with the vaccine together with other organisms, such as $B$ influenzae and
streptococcus. (3) Mice intoxicated with alcohol have been injected with large amounts of vaccine. (4) In the majority of experiments there survived many test animals in which the transformation did not occur.

In the course of the work additional, and even more convincing, controls were afforded by certain observations which will be later reported.

In a large series of experiments the following points have been established: -

a) The subcutaneous injection into mice of a large amount (50 - 100 cc.) of a vaccine of an "S" culture, (heat-killed at temperatures varying from 60°C up to and including 80°C for periods of from 30 minutes to 2 hours), together with an "R" culture derived from a heterologous type, results in the formation of virulent "S" pneumococci of the same type as that of the vaccine employed. (b) If the vaccine is heated to a higher temperature than 80°C., the "R" culture frequently reverts to the same type as that from which it was originally derived, not to the type of the vaccine. (c) Any "R" organism regardless of derivation potentially has the capacity of elaborating the specific soluble substance of any specific type of pneumococcus, and by the procedure described can be transformed into "S" organisms of a heterologous type. (d) All attempts to effect heterologous transformation "in vitro" have failed. (e) The following conditions are necessary:

a) Large amounts of "S" vaccine are necessary (50-100 cc.).

b) The vaccine must not be allowed to autolyse before being heat-killed.

c) The "R" culture must be well "degraded" else it reverts to its own type.

III. Cutaneous Reactions in Pneumonia Patients to Cell Constituents of the Pneumococcus. (Dr. Tillett and Dr. Francis). Patients acutely ill with and convalescent from pneumonia are being tested by intra-
cutaneous inoculation to determine their reactivity to the protein and carbohydrate constituents of Pneumococcus. The cell fractions used for injection are (1) soluble specific substances (polysaccharides) derived from Types I, II and III, in purified state, and (2) the so-called nucleo-protein fraction, which exhibits the protein-specificity of the species. The polysaccharides and the protein are injected in amounts of 0.01 mgm.

On admission to the hospital each pneumonic patient receives 5 intradermal tests: 1) Type I "S" substance; 2) Type II "S" substance; 3) Type III "S" substance; 4) Nucleo-protein fraction; 5) Salt solution control.

At the same time blood is withdrawn in order to determine the presence or absence in the sera of antibodies reactive with the substances injected. These tests are repeated every few days during the patient's stay in the hospital. Following injection, the patients are observed both for an immediate reaction and for a delayed response which appears after 24 to 48 hours.

Two independent and distinct forms of reactions are noted. The first of these is produced by the protein fraction. Shortly after the injection of the material a slight reddening occurs, and occasionally a slight edema. This subsides rapidly. Beginning at about the eighth hour, erythema, edema and tenderness, in varying degrees, appear at the site of inoculation and then increase up to 20-24 hours when the maximum is reached. In general, the appearance of this form of skin reaction to protein substance is similar to the tuberculin reaction, and its occurrence and course are briefly as follows: During the acute phase of the illness there is no response to the protein material, but following the return of the temperature to normal the reaction becomes positive. As convalescence proceeds the intensity of reaction becomes more marked, and the appearance more striking. Sufficient time has not elapsed since
the beginning of these experiments to determine how long patients continue to exhibit this altered reactivity to Pneumococcus protein. The reaction of normal individuals is also being studied, but as yet sufficient data have not been accumulated to warrant any conclusions.

The second form of reaction has been obtained with the type-specific carbohydrates. During the phase of acute illness there is no reaction. However, coincident with recovery a positive reaction may develop. The character of this type-specific response offers a striking contrast to that following protein injection. Fifteen to 30 minutes following the intradermal inoculation, there appears at the site of injection a wheal-like swelling with intense white edema. Surrounding the wheal a zone of erythema appears which becomes increasingly larger and more intense. The height of reaction occurs between 30 and 60 minutes, after which a gradual regression takes place leaving a firm pale edematous area which may require 24 hours or longer to subside. At the site of injection of the carbohydrates of the heterologous types and of the salt solution, which serve as controls, no reaction appears. This form of typical "wheal and erythema" reaction is of the immediate anaphylactic type and is in sharp contrast to the delayed protein response, which requires 24 hours for maximum reaction and is allergic in character. Positive results have been uniformly type-specific, that is, the reaction has occurred only with the specific carbohydrate homologous to the type of pneumococcus causing the disease. To date it has been strikingly noted in cases of Type I pneumonia treated with Type I antipneumococcus horse serum. In these treated cases, large amounts of Type I antibodies are introduced intravenously. In serum-treated patients the occurrence of a positive reaction to the Type I "S" substance appears to be related to recovery. It seems possible that a positive test might indicate an excess
of antibodies in the blood and thereby serve as an index of the amount of serum therapy required. These reactions are of further interest as an example of the reactivity of the soluble specific substances in human.

It has also been found that, in patients not treated with immune serum coincident with spontaneous recovery, a positive reaction may follow the injection of the homologous "S" substance.

In addition to observations on the skin reactions, sera obtained at the same time have been tested for the presence or absence of antibodies reactive with the test material - i.e. pneumococcus nucleo-protein and specific polysaccharides. From the data so far obtained, no significant difference has been noted in the sera with reference to content of protein precipitins before or after crisis. In other words, there seems to be no relation between the amount of antiprotein-precipitin in the sera and reactivity of skin to this substance. However, there does appear to be a definite correlation between the reaction to "S" substances and the presence of specific antibodies. Although some cases possessing circulating anti-S antibodies do not react, no case has as yet been found which gives a positive reaction in the absence of anti-S antibodies. It is too early in the work to interpret the significance of these results. However, it is a striking fact that two distinct types of reaction to two chemically different constituents of Pneumococcus, may be elicited by skin tests in pneumonia patients; the one, produced by the carbohydrate is type-specific, immediate and anaphylactic in character; the other, produced by the protein, is independent of type-specificity and is analogous to the delayed reaction characteristic of allergic phenomena.

IV. Natural and Acquired Resistances to "R" and "S" Forms of Pneumococcus, (Dr. Tillett). The experimental studies, reported in previous publications, on natural and acquired resistance of rabbits to
pneumococcus infections have indicated that immunity may exist in the absence of demonstrable type-specific antibodies. The fact was established that normal rabbits are capable of withstanding large doses of Type III pneumococci although antibodies reactive with this organism could not be demonstrated in the serum of the normally resistant animal. It has also been shown that rabbits immunized with degraded non-virulent, non-type-specific pneumococci - so-called "R" strains - acquire a considerable degree of resistance against subsequent infection with virulent pneumococci of any of the three specific types. Active resistance was demonstrable under these conditions in spite of the fact that the sera of the immunized rabbits contained no detectable type-specific antibodies capable of agglutinating type-specific organisms, or of precipitating the soluble specific substances, or of conferring passive protection on mice against pneumococcus infection.

Furthermore, it has been shown that, although passive protection of mice is ineffectual, the whole blood or serum of rabbits immunized with "R" organisms is capable of conferring immunity on normal rabbits against any of the three specific types of Pneumococcus. The results of the experiments on active and passive immunity in rabbits immunized with "R" cells reveal characteristics which differ from type-specific immunity; it seems possible that the underlying mechanism is dependent upon factors other than those participating in type-specific pneumococcal reactions. Moreover, the so-called "R" immunity - stimulated in rabbits by "R" cells and effective against virulent "S" pneumococci - has been shown to possess certain characteristics similar to the natural resistance which normal rabbits possess against most strains of Type III. This form of "R" immunity appears to represent an enhancement of these factors which are concerned in the natural resistance of normal rabbits.
During the past year experiments have been carried on in an attempt to
determine more accurately the factors involved in type-specific im-

munity, in "R" immunity, and in natural resistance. For this purpose,
rabbits immunized with type-specific organisms, others immunized with
"R" organisms, together with normal control animals have been infected
by intradermal inoculation. The character of the local lesions and the
course of the resultant blood invasion have been simultaneously followed.
Experiments of this nature have afforded a means of comparing certain
similarities and differences which have been found to exist in these
forms of natural and acquired immunity.

The animals used in these experiments may be divided into three
groups:- A. Normal rabbits. B. Rabbits immunized with "R"

pneumococci. C. Rabbits immunized with type-specific pneumococci.

The pneumococci employed for infection have been as follows:--
1) "R" strains. Degraded, avirulent, and non-type-specific
forms of pneumococci.

2) "S" strains. Two strains of Type III pneumococcus have been
used: one, avirulent for rabbits (designated SA); the other, virulent for
rabbits (designated SV).

3) "S" Pneumococci. Type I and II, virulent for rabbits.

The results of these experiments may be summarized as follows:--

A. Intradermal infection of normal rabbits:-

1. "R" Pneumococci. The skin lesion which develops is small,
discrete, firm, slightly red, and nodular; it appears within the first
24 hours and disappears in three to four days. Repeated blood-cultures
remain sterile. Phagocytosis of "R" organisms by polymorphonuclear
leucocytes is readily demonstrable. The infected animals invariably
recover.
2. "S" Pneumococci, Type III.  
A Strain avirulent for rabbits.

The skin lesion developed is large, edematous, reddish-purple, and within 48 hours may extend to the midline ventrally; marked ecchymosis commonly occurs. After a few days central necrosis and ulceration take place; several weeks is required for complete healing. Blood cultures taken at frequent intervals reveal the presence of organisms varying in number from time to time, and persisting for several days but ultimately disappearing. Phagocytosis of these "S" organisms by polymorphonuclear leucocytes is not demonstrable. Recovery of the animal ensues.

b. Strain virulent for rabbits. The skin lesion produced by these organisms is identical with that caused by the avirulent strain.

It is large, edematous, ecchymotic and extends ventrally to the midline. Blood cultures taken at frequent intervals, show that organisms are present in the circulation a few hours after inoculation; the septicæmia rapidly increases in severity until death ensues from an overwhelming blood infection. No phagocytosis is demonstrable. The infection is invariably fatal.

3. "R" Pneumococci. Type I and II. The skin lesion and blood infection resulting from the injection of the virulent strains is identical with that described for the virulent Type III. The animals die in 36-48 hours.

Intradermal inoculation of rabbits immunized with "R" Pneumococci.

1. "S" Pneumococci, Type III; Virulent for normal rabbits; The skin lesion produced is a large, edematous, reddish-purple inflammation which spreads ventrally to the midline; as in normal rabbits inoculated with "S" organisms ulceration and necrosis set in and several weeks are required for complete healing. Frequent blood cultures demonstrate the presence of organisms in varying numbers for several days. They finally disappear, however, and recovery of the animal ensues. The character of
this lesion, as well as the bacteremia, are similar to that occurring in normal rabbits injected with Type III "S" strain, avirulent for the species.

2. "S" Pneumococci. Type I and II; Virulent for normal rabbits:
The skin lesion and course of blood infection are similar to that described for virulent Type III.

C. Intradermal Infection of Rabbits Immunized with Type-specific Pneumococci. I. "S" Pneumococci; In this group of animals the organisms inoculated were always of a type homologous to the "S" strain used for immunization. The skin lesion produced is small, discrete, firm, slightly red, and nodular. It appears within the first 24 hours, and disappears in three to four days. This lesion is similar to that produced in normal rabbits by the injection of "R" pneumococci. Blood cultures always remain sterile. Phagocytosis of the sensitized type-specific organisms by polymorphonuclear leucocytes is readily demonstrable.

In an analysis of these experiments the reactions to skin infection as observed in normal and immunized rabbits offer certain similarities and contrasts. There appears to be, in one group of animals, a skin infection followed by recovery which may be cited as follows:

1. Reaction and recovery of normal rabbits from infection with "R" pneumococci.

2. Reaction and recovery of type-specifically immunized rabbits from infection with homologous type-specific organisms.

The character of the skin lesion in these two instances is the same. A small, insignificant nodule develops which disappears in a few days. Organisms are never present in the blood stream, and prompt
phagocytosis is demonstrable in each instance. Recovery depends primarily upon phagocytic activity. The type-specific organisms being sensitized by type-specific antibodies are quickly disposed of. The "R" pneumococci possessing no capsule can be ingested as such. Natural and acquired resistance to pneumococcus infection, as exemplified in these two instances, indicates a close similarity in the mechanism of recovery.

In another group of animals, the reaction and recovery differ from the first group and may be cited as follows:

1. Reaction and recovery of normal rabbits from infection with "S" pneumococci, Type III, avirulent for the species.
2. Reaction and recovery of "R" immunized rabbits from infection with virulent type-specific organisms I, II or III.

The character of the skin lesion in these two instances is similar. A large, edematous, ecchymotic, purplish, spreading inflammatory reaction follows the intradermal inoculation of the organisms. Ulceration and slough ensue and several weeks are required for complete disappearance of the reaction. Organisms may be present in the bloodstream in varying numbers for several days before their final disappearance. In neither instance can phagocytosis by leucocytes be demonstrated.

Natural and acquired resistance to pneumococcus infection, as exemplified in these two instances, also indicates a close similarity in the mechanism of recovery. The degree of the inflammation, however, and the duration of the infection differ considerably from the mild lesion and transient infection described in the first group. Consequently, it seems not unlikely that the underlying mechanism is different in each instance.

In the first group, prompt phagocytic activity appears to be the
initial reaction involved while in the second group the factors are as yet not understood. In so far as the work has progressed it seems unlikely that the primary reactions are due solely either to antibodies, as ordinarily understood, or to phagocytosis. However, the recent work of Griffith has shown that "R" pneumococci possess the capacity to change into any of the specific types. Consequently, it may not be impossible that "R" cells possess some increment capable of inciting a response specific for each type of pneumococcus and that this is responsible for the broad immunity resulting from immunization with "R" cells.

The work has not as yet progressed sufficiently to justify any conclusions as to the nature of the so-called non-type-specific resistance.

V. Intracutaneous Vaccination of Rabbits with Pneumococcus. I. Antibody Response. (Dr. Julianelle). Suspensions of heat-killed pneumococci were injected into the skins of rabbits at intervals of seven days during a period of 10 to 14 weeks, in doses such that the total amount of bacterial substance injected was at least equivalent to that employed in the routine intravenous immunization of rabbits. The antigens studied were Types I and III and a capsule-free "R" strain derived from a Type II organism. The sera of the rabbits immunized in this way were tested for the presence of agglutinins, precipitins and protective antibodies.

The sera of rabbits immunized intradermally with Type I failed in more than 85 per cent of the animals to show any type-specific antibodies. They failed to agglutinate the corresponding type of pneumococcus, and they failed to precipitate the homologous specific polysaccharide. In rare instances, passive protection was conferred upon white mice against infection by Type I, but in such cases the protection afforded was not great. In the remaining rabbits, although type-specific antibodies were present, the titre as measured by agglutination
was low ranging from 1:1 to 1:20.

On the other hand, none of the rabbits immunized with Type III by way of the skin, showed any demonstrable type-specific antibodies. Their sera showed a complete lack of type-specific agglutinins, precipitins, and protective antibodies.

Although in the majority of instances, the type-specific pneumococcus failed to stimulate type-specific antibodies when introduced into the skin of rabbits, the serum of all the animals studied contained a high titre of the antiprotein, species-specific antibody, agglutinated "R" cells, and precipitated solutions of protein derived from any type of Pneumococcus. The intracutaneous inoculation of rabbits with "R" cells stimulated the production of only the species-specific antibodies. In the majority of instances therefore, the results of immunization with type-specific Pneumococci cannot be distinguished from those obtained by immunization with "R" variants.

Later rabbits were immunized with nucleoprotein solution and a purpura-producing extract in the manner described above. In no instance were type-specific agglutinins or precipitins demonstrated and no protective antibodies were found. The sera of these rabbits, however, possessed species-specific antibodies.

II. Resistance to infection. It may be stated briefly that following repeated skin inoculation of heat-killed suspensions of Pneumococcus, rabbits acquire a considerable degree of active immunity against infection by virulent strains of both homologous and heterologous types. For example, rabbits immunized intracutaneously with Type I may survive the intravenous injection of two million lethal doses. Similarly, rabbits immunized with Type III may survive a thousand or more minimal lethal doses. Moreover, animals inoculated
intracutaneously with Type III become as resistant to infection with Type I as animals similarly immunized with homologous organisms; and animals immunized with Type I possess a comparable degree of immunity to Type III infection. Even when an "R" strain derived from Type II is used for injection, rabbits acquire a high degree of active immunity to infection with virulent "S" strains of heterologous type.

Although rabbits treated in this manner develop a solid immunity against infection with both homologous and heterologous types, nevertheless the serum of these animals usually confers upon white mice little or no passive protection against infection by even the homologous type.

III. Hypersensitiveness. The intracutaneous injection in normal rabbits of 0.2 cc. of a heated vaccine, representing the bacteria from 2 cc. of broth culture is followed by a circumscribed, slightly raised and indurated nodule, reddish in color and measuring about 1 cm. in diameter. Upon repeated injection of the same amount of bacterial suspension at weekly intervals, the reaction changes in character; the size increases often reaching a maximum of 4 to 6 cm. in diameter, accompanied by a spreading edema and purplish discoloration. The reactions persist longer, often breaking down with the discharge of sterile necrotic material, and a maximum reactivity is reached after about 6 injections. Thereafter, the reactions are less intense and are more quickly resorbed. Two to three weeks after the final injection (8 to 12), the animals may be sensitive to the nucleoprotein and other protein derivatives of Pneumococcus when tested by the skin and ophthalmic reaction.

In the doses employed, the nucleoprotein solution gave no local reaction when injected into the skin of normal rabbits, while extracts
containing purpur-a-producing material caused a faint erythema in about
half of the normal controls. In skin-vaccinated rabbits, on the other
hand, the protein substances induce an inflammatory reaction at the site
of injection which may persist for three to five days.

In testing eye sensitivity in the skin-vaccinated rabbits, the
cornea was anaesthetized and lightly scarified, and one drop of nucleo-
protein or purpura-producing extract was then instilled into the con-
junctival sac. In normal rabbits this procedure causes no visible
reaction. In rabbits sensitized by the intracutaneous method, a defin-
ite reaction occurs within 24 hours which increases in intensity during
the first 24 to 48 hours and disappears after 4 to 8 days. The reaction
consists first of conjunctivitis with dilatation of capillaries at the
sclerocorneal junction, with clouding of the cornea and last of all
development of pannus which occurs only occasionally. The ophthalmic
reaction shows great variation in severity in different rabbits and may
not proceed beyond the stage of corneal turbidity. Of over 50 animals
immunized by the routine intravenous method, none have given a positive
eye reaction, while 90 per cent or more of the rabbits immunized intra-
cutaneously reacted positively when later tested to protein derivatives
of Pneumococcus.

The hypersensitiveness is not elicited in sensitive animals by
instillation in the eye of purified type-specific carbohydrates. The
reaction therefore, is not type- but species-specific.

The hypersensitive state may persist for at least four months.
An interesting point in this connection is the fact that after the
ophthalmic reaction has definitely disappeared, the intravenous in-
jection of nucleoprotein solution often causes a reappearance of the
eye reaction.
VI. The Reversion of "R" Friedländer bacilli to the "S" Forms, and the Interconverstibility of Specific Types. Numerous attempts have been previously made to cause reversion of "R" Friedländer bacilli to the "S" variety by the various methods which have been effective in the case of Pneumococcus. The results of these attempts have been uniformly negative, and the reversion of an "R" form of Friedländer's bacillus to its original specific type had never been attained by "in vitro" methods.

The recent studies of Griffith have demonstrated that, under proper conditions, "R" pneumococci may be induced to revert not only to the "S" antecedent, but even to the "S" form of a heterologous type. The method he employed consists of the simultaneous injection subcutaneously in white mice of the deposit of large quantities of heat-killed "S" organisms and small amounts of living "R" cells. The conversion to a different type, when it does occur, is to the type of the culture employed as vaccine. Subsequent confirmation of Griffith's results in this laboratory, suggested the possibility of the reversion of "R" forms of Friedländer bacillus by the newer technique.

Up to the present time two sets of experiments have been completed. On one occasion, of six mice injected with the deposit equivalent to 40 cc. of Type A culture heated for 30 minutes at 56-60°C together with 0.25 cc. of "R" organisms derived from Type B or Type C, 3 mice yielded cultures containing both "R" and "S" colonies. The S colonies proved to be of the Type A variety. Three mice which received similar quantities of heat-killed bacteria alone were sacrificed after 10 days, and in none of them were any bacteria cultivated from either the site of injection or the heart blood.

On a second attempt, 4 mice each were injected with the deposit
of bacilli from 30 cc. of Type A culture previously heated (30 minutes at 56-60°C), together with 0.25 cc. of "R" culture derived from Type B, Type C and Group X. Two mice of each group yielded cultures containing both "R" and "S" colonies; all the "S" colonies isolated, proved serologically to be of Type A. Four mice injected with the same quantity of Type A vaccine and 0.25 cc. of "R" cells derived from Type A, yielded cultures composed purely of "R" colonies. As controls, 4 mice received the heat-killed Type A deposit alone. They were killed after 10 days and cultures taken from the site of injection and from the heart blood showed no growth.

Since previous results from this laboratory showed that Type II Pneumococcus and Type B, Friedländer's bacillus possess similar antigenic properties, observations were made on the reversibility of "R" Friedländer bacilli when injected subcutaneously into white mice together with heat-killed suspensions of pneumococcus Type II. Three different sets of experiments were carried out, but in no instance was it found that Type II Pneumococcus exerted any influence on the reversion of Friedländer's bacilli to either homologous or heterologous types.

Two sets of experiments were performed to determine the effect of heat-killed cells of Type B, Friedländer's bacillus on the reversibility of "R" Pneumococci. In the first experiment, 3 mice each were injected with the deposit equivalent to 40 cc. of Type B bacilli, heated for 30 minutes at 56-60°C together with 0.25 cc. of "R" forms derived in one case from Type II and in the other from Type III Pneumococcus. Three of the six mice yielded cultures of mixed "R" and "S" pneumococcus colonies and in each instance the "S" colony corresponded to the type from which the "R" strain had been derived.

On a second occasion, 4 mice each were injected with 0.25 cc. of
a culture of "R" cells derived respectively from each of the three fixed
types of Pneumococcus together with the deposit of heated bacteria (56 -
60°C for 30 minutes) of 70 cc. culture of Type B, Friedländer's bacillus.
In no case, however, was reversion of the Pneumococcus variants observed.

Although the data from these observations are insufficient to
warrant any conclusion, they indicate that (1) "R" strains of
Friedländer's bacillus may be converted to the "S" form of a different
type; (2) "R" pneumococci may be reverted to their homologous "S"
type when injected subcutaneously in mice with heat-killed suspensions
of Type B, Friedländer bacillus; and (3) under the circumstances stated,
"R" strains of the Friedländer bacillus have never been observed to
revert to the "S" form when injected together with heated cells of
Type II Pneumococcus.

VII. Immunity Induced by the Inhalation of Virulent Pneumococci.
(Dr. Stillman). Studies of the immune response of rabbits to
repeated inhalations of living pneumococci have been continued. The
character and duration of immunity induced by inhalation varies with the
type of pneumococci used.

It has been demonstrated that rabbits which have been repeatedly
sprayed with Type I pneumococci develop in their serum type-specific
agglutinins and protective antibodies. After the inhalations of
pneumococci are discontinued, the agglutinins rapidly disappear. The
protective antibodies persist for long periods. The sera of two rabbits
which have survived treatment and are now living, almost three years
(990 and 1030 days) after their last exposure to sprayed pneumococci,
still protect mice against infection with large doses (0.01 cc.) of a
virulent culture of the same type as that with which they were originally
sprayed.
The sera of rabbits sprayed 10 times with a strain of "R" pneumococcus, derived from Type II organisms contained no type-specific antibodies. The sera of rabbits repeatedly exposed to sprayed mouse-virulent but not rabbit-virulent Type II pneumococci contained protective antibodies but no demonstrable agglutinins. The same strain of Type II was rendered rabbit-virulent by passage through a series of rabbits. The rabbits sprayed with this strain showed the presence of both protective antibodies and agglutinins in their serum.

Two groups of rabbits have been repeatedly sprayed with a rabbit-avirulent and a virulent Type III pneumococcus. The sera of the rabbits exposed to an atmosphere of avirulent Type III organism contained no type-specific antibodies. Although a large number of the animals died of pneumococcus septicemia following the first exposure to the rabbit-virulent strain, those which have survived repeated exposures have, so far, failed to develop any detectible type-specific antibodies. A series of rabbits were intravenously injected with a heat-killed suspension of the same rabbit-virulent strain. The sera of a number of these animals contained both agglutinins and protective antibodies.

The duration of the immunity in rabbits actively immunized by the inhalation method and by inoculation is being followed.

VIII. Significance of oxidation-reduction processes in bacterial growth. (Dr. Dubos). It has been shown by previous work from this department that Pneumococcus cultures respond very readily to changes in the conditions of oxidation-reduction of the medium and environment in which they are placed. An attempt is being made to analyze the influence of such factors on the growth of Pneumococcus. The following points are being studied:

Initiation of growth, as measured by the minimum number of cells (used
as inoculum) required to initiate growth; the growth curves and lag period; the density of growth, and the number of cells developing per unit volume of medium; and the viability of the cultures.

It has been found that an understanding of the problem could not be obtained without some knowledge of the oxidation-reduction properties of sterile media. When sterile plain broth is kept in the absence of oxygen, this medium exhibits a progressive drift toward highly reducing potentials. This drift has been followed electrometrically and by the reduction of indicators of oxidation-reduction potentials. These indicators are progressively reduced by sterile broth in the order of the electromotive series; the indophenols being reduced most rapidly (in a few hours) and indigo disulfonate more slowly (in several days or weeks). Indicators with an $E'_0$ negative to that of indigo disulfonate were not reduced under any circumstances. These studies also indicate that plain broth contains substances which combine with oxygen to form toxic products and that these oxidized substances may be decomposed by heat.

1) Initiation of growth. The number of cells of Pneumococcus necessary to initiate growth may vary a great deal with the age and condition of the broth. It appears that, in the presence of oxygen, there is formed in plain broth a toxic substance which has a bacteriostatic — and even bactericidal — action on Pneumococcus and related organisms. This action may be checked by addition of certain reducing substances, by incubation of the culture under anaerobic conditions, by addition of blood and by heating the broth previous to inoculation. The action of these procedures may be explained by a reduction or breaking down of the toxic substance. The use of a large inoculum serves the same purpose. It was possible to trace the origin of this toxic substance
to the peptone used in the preparation of the medium. Different peptones behave differently in this respect. Witte's and Difco-protose peptones exhibiting to the smallest degree the ability to form toxic substances in the presence of air. Then precautions are taken to prevent the formation of these toxic substances, or when they are removed by proper means, growth of Pneumococcus and related organisms can be obtained even when one or very few cells are used as inoculum.

2) **Bacteriostatic action of oxidized dyes.** Another evidence of the bacteriostatic action of oxidized substances has been obtained from the study of the action of certain dyes on bacterial growth. Whereas, the growth of many organisms is checked by the presence in the medium of the oxidized forms of certain dyes (indophenols and methylene blue for instance), the reduced forms of the same dyes - even in large concentrations - are without any inhibitory action on growth. There are also indications that the place of the reversible dyes in the oxidation-reduction scale bears a definite relation to their bacteriostatic action for different organisms.

3) **Growth curves and lag period.** Some of Chancey's observations on the growth curve of Pneumococcus can be partly accounted for by the conditions of oxidation-reduction prevailing in the medium. The course of development of the culture may be very much accelerated by the presence of reducing substances in the medium or the maintenance of reducing conditions. The lag period probably corresponds to the time during which the cells are reducing the toxic substances of the medium, in which process some of the organisms are injured and succumb.

4) **Viability.** It has been shown in a previous report that the processes of cellular oxidation markedly influence the death rate of pneumococci. Under conditions of active aeration, pneumococcus
cultures form large amounts of peroxide. It was found that the viability
of the cells is lessened when the peroxide production is increased, while
the cells remain alive longer when the conditions are such that the
formation or accumulation of peroxide is prevented.

The oxidation-reduction system of pneumococcus cells consists of
two essential components. One of them can be removed by washing the
cells repeatedly in saline solution; this process results in a complete
inactivation of the oxidation-reduction system. It has been observed
that pneumococci which have been deprived of their oxidation-reduction
activities by washing in saline solution remain viable longer than the
original unashed cells.

In the course of this work, it has become more and more evident
that an analysis of the oxidation-reduction properties of Pneumococcus
cultures is rendered especially difficult by the fact that plain broth
itself is an active and dynamic system from this point of view. Work
is in progress to develop a synthetic medium the behavior of which can
be interpreted more accurately.

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