Report of Dr. Avery with Drs. Stillman, Goebel, Dubos,

Francis, Kelly, Babers, Goodner, and Alloway.

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Type III Pneumococcus by a Bacterial Enzyme.

1. Methods for obtaining potent, non-toxin preparations

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2. The protective action of the specific enzyme against

Type III Pneumococcus infection in mice.

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I. The decomposition of the capsular polysacchar-
ide of Type III Pneumococcus by a bacterial enzyme. (Drs.
Avery and Dubos).   A microorganism has been isolated, and
from it an enzyme extracted, which decomposes the purified
capsular polysaccharide obtained from Type III Pneumococcus.
This microorganism, a pleomorphic aerobic bacillus, was iso-
lated from a soil rich in hemicelluloses under-going decom-
position, by the use of a synthetic mineral medium contain-
ing the capsular polysaccharide as sole source of carbon,
As already described in a previous report, the endocellular
enzyme extracted from this microorganism exhibits a remark-
able specificity in its action against the capsular polysac-
charide of Type III Pneumococcus.

The work of this year may be conveniently describ-
ed under two main headings -

1. Attempts at obtaining potent and non-toxic enzyme pre-
parations.

2. Protective action of a specific enzyme against Type III
Pneumococcus infection in mice.

Before going on with a description of these stud-
ies, it may be worth while to state that the work has been
controlled by a rapid and fairly accurate method of titra-
tion of the potency of the enzyme preparations in vitro;
this method, based on the existence of a quantitative rela-
tionship between amount of substrate decomposed and amount
of enzyme used, consists in determining the amount of cap-

sular polysaccharide decomposed by a definite amount of enzyme preparation.

1. Attempts at obtaining potent and non-toxic enzyme preparations. The amouhts of specific enzyme obtained from a culture of the bacillus decomposing the capsular polysaccharide of Type III Pneumococcus is not a direct function of the number of cells from which the enzyme is extracted. It is conditioned by a series of factors among which the following have been recognized:-

A. Past history of the culture.

B. Composition of the culture medium, with special reference to - a) The presence or absence of the capsular polysaccharide in the medium; b) The presence or absence of substances acting as a more available source of energy for the bacillus: c) The form in which nitrogen is supplied in the medium; d) the presence of catalysts affecting the rate of decomposition of the capsular polysaccharide; and e) The presence of substances retarding autolysis of the cells.

C. Environmental factors, especially - temperature and conditions of aeration.

In brief, the following points must be observed in order to obtain a maximum yield of enzyme. The culture must be maintained at a high degree of specific activity by continued passages in the specific synthetic medium containing the capsular polysaccharide as sole source of energy. The enzyme is usually not formed, or only in small amounts, un-

less the capsular polysaccharide (or the aldobionic acid derived from it), is present in the medium. However, the concentration of capsular polysaccharide must not be too high; it must be such that the decomposition is completed in 2-3 days at the most. This condition may be accounted for as follows:- The enzyme can be demonstrated free in the medium, as soon as the cells begin to age. In case there is still some polysaccharide undecomposed at this time, the free enzyme decomposes the residual polysaccharide and is therefore neutralized without contributing in any way to the anabolic processes of the cell. This results of course in a loss of both enzyme and capsular polysaccharide. This loss may be reduced in two ways: - a) by increasing the rate of bacterial growth (use of a large inoculum, presence of enough nitrogen, proper conditions of aeration, and the addition of growth catalysts, such as soil extract and yeast extract), and by retarding autolysis and thus preventing the liberation of the enzyme (addition of yeast extract to the medium). Under these conditions, the yield of enzyme has been increased almost tenfold over that previously obtained.

Unfortunately some of the new preparations have exhibited a definite primary toxicity. The production of non-toxic preparations is being attempted along three different lines:- a) modification of the medium used for the growth of the bacillus; and b) modification of the process of extraction of the enzyme; and c) by purification of the

enzyme preparations.

   2. Protective Action of the Specific Enzyme on Type III Pneumococcus Infection in Mice. The earlier studies comprised observations on the action of the enzyme on the capsular sugar of Pneumococcus removed and separated from the bacterial cells. In the natural state in which this specific polysaccharide exists, it forms the capsular structure which determines the antigenic and serological specificity of the cell as a whole, and conditions its power to invade and multiply in animal body. It was of special interest, therefore, to determine what effect the specific enzyme would have upon the encapsulated forms of Type III pneumococcus growing in vitro and in vivo:- whether in a medium containing the active enzyme, the pneumococci would fail to grow at all or would grow merely deprived of their capsules; whether in the body of a susceptible animal, the administration of the enzyme would in any way modify the course of experimental infection with virulent Type III pneumococci. The results of these studies form the subject matter of a paper which is now in manuscript form.

   Briefly, the work shows that, when a sterile extract of the enzyme is added to a culture medium seeded with Type III pneumococci, growth occurs but the bacteria are deprived of their capsules; they are no longer specifically agglutinable, and the soluble specific substance is no longer demonstrable in the culture fluid. Experiments of this

kind have shown quite clearly that the enzyme by itself is neither bactericidal nor bacteriolytic; that by decomposing the specific carbohydrate, it merely strips the pneumococci of their capsules without impairing the viability of the bacteria. Moreover, the action of the enzyme on the living cell does not result in a loss of the function of elaborating the capsular substance, since pneumococci so treated, regain their capsules and continue to elaborate the soluble specific substance when transferred to fresh medium free of the enzyme.

In more recent studies it has been found that the enzyme has a distinct protective action in the animal body against infection with virulent Type III pneumococci. Mice receiving a single intraperitoneal injection of an active preparation of the enzyme may survive infection with a million times the fatal number of virulent organisms. The protection afforded by the enzyme is type-specific; just as in the test tubes the enzyme acts only on the Type III polysaccharide, so in the animal body it is effective only against infection with Type III pneumococcus. A protocol of an experiment illustrating the degree and the specificity of the protective action of the enzyme in mice is given in Table I.

T a b l e I.

Specificity of the Protective Action of Enzyme.

Infecting dose of Pneumococcus	Enzyme (lot 4a) 0.5cc.			No Enzyme		
	Type I	Type II	Type III	Virulence controls		
cc.				Type I	II	III
.1	-	-	S	-	-	-
.01	-	-	S	-	-	-
.001	-	-	S	-	-	-
.0001	D20	D34	S	-	-	-
.00001	D24	D34	S	D22	D36	D34
.000001	D34	D34	S	D34	D36	D34
.0000001	-	-	-	D34	S20	D72

S = Survived. D = Death of animal; the numeral indicates the number of hours before death, or the time at which the animals were found dead.   - = Not done.

The active principle responsible for the protection of mice is destroyed by exposure to 70° C. for ten minutes. The inactivation by heat of the protective power for mice of this enzyme, parallels the loss of its activity when tested in vitro after exposure to temperatures of 60° C. or higher. The fact that heat destroys the power of the enzyme to act either in vitro or in vivo, justifies the assumption that the same principle is involved in the mechanism of both reactions.

Repeated tests have shown that within the limits of the reaction-capacity of the mouse, the protective action of the enzyme is a function of the concentration of the active principle in any given preparation; and further, that the protection afforded by different preparations bears a

definite relation to their activity in decomposing a known quantity of the capsular polysaccharide in vitro. The evidence is that, to be effective in animal protection, the concentration of enzyme in the body must be maintained in excess so that the rate of decomposition of the capsular substance is always greater than the rate of its production by the living bacteria.

Experimental evidence indicates that in mice the enzyme has a curative action when administered in the course of an infection already established at the time of treatment. A single injection of enzyme as late as 18 hours after the onset of infection has brought about the recovery of mice infected with a dose of virulent pneumococci 100 times greater than that fatal for the untreated controls. It must be borne in mind, however, that the curative action of the enzyme suffers the limitations imposed by the variations which occur in the cellular response of the host, since phagocytosis is apparently the important process involved in the protection afforded by the enzyme.

A better understanding of the mechanism under-lying the protective action of the enzyme in infected mice has been gained by following the course of the bacteremia by means of blood cultures and by a study of the cellular reactions in the peritoneal exudates of treated and untreated animals. The results of a typical experiment are graphically illustrated in the accompanying photomicrograph (fig.1).
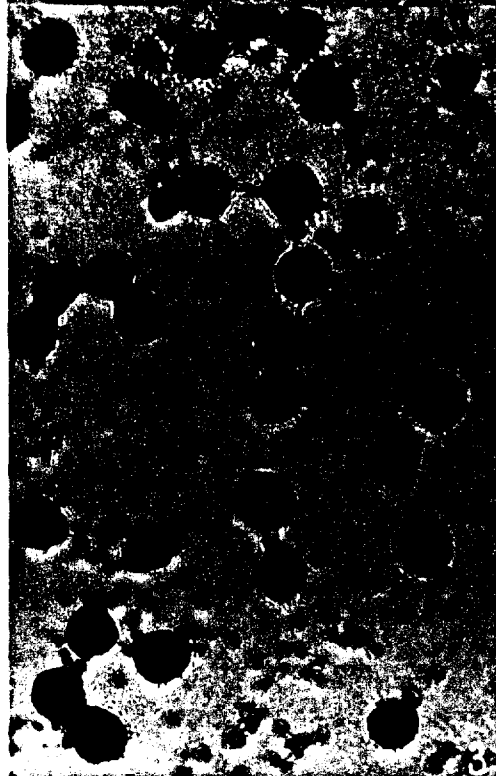
In this experimenta, 12 mice were infected with one million fatal doses of Type III pneumococci. Six of these mice received at the time of infection 0.5 cc. of an active preparation of the enzyme. The other six mice served as untreated controls. At intervals following infection, one mouse of each group was sacrificed and autopsied. Cultures of the heart's blood were made, and stained films of the peritoneal exudate were examined to compare differences in the morphology of the organisms and in the occurrence of phagocytosis in the treated and untreated animals.

Two hours after infection the blood cultures of both the treated and untreated mice were positive. Microscopic examination of the exudate of the control animal at the end of 2 hours shows the presence of many well encapsulated cocci in every field without any evidence of phagocytosis. (fig. 1a). On the other hand, the pneumococci present at this same period in the exudate of the enzyme-treated mouse, (fig. 1b), are smaller in size and devoid of capsules; only the naked cells are visible and many of these have already been taken up by the leucocytes. At the end of four hours, the bacteria are more numerous in the peritoneal exudate of the infected control (fig. 1c); they are surrounded by well defined capsules and none are found within the leucocytes; cultures of heart's blood of the untreated animals indicate a progressively increasing bacteremia. In the treated animals at this period, however, (fig. 1d)

only an occasional naked cell is seen outside the leucocytes and frequently at this time and invariably by the fifth hour the organisms are no longer demonstrable in the blood.

The evidence obtained from repeated experiments of this nature supports the view that the protective action of the enzyme lies in its capacity to decompose the capsular polysaccharide of Pneumococcus Type III. The process of de-capsulation, brought about by the direct action of the enzyme, strips the bacteria of an important defense mechanism and thereby exposes their naked, unprotected bodies to direct attack by the phagocytes of the host. In this sense the action of the enzymes may be said to initiate phagocytosis; not, as in the case of antibodies, by specifically sensitizing the bacteria, but by the process of decapsulation. In the former instance, the reaction is an immunological one, whereby the specific substance of the capsule is altered by union with the type-specific antibodies; in the latter case, the reaction is a chemical one in which the capsular polysaccharide itself is decomposed by the enzyme. Although the mode of action is different in each instance, the end result, so far as the fate of the pneumococcus is concerned, is the same in both instances.

The enzyme, like the type-specific antibody, serves to initiate a protective reaction, the completion of which, however, is ultimately dependent for its successful issue upon the effective cellular response of the host. This study

suggests that the capsule - long recognized as a defense me-
chanism of virulent bacteria - is a decisive factor in deter-
mining the fate of pneumococci in the animal body, and that
this structure is vulnerable to attack by specific agents
other than antibodies.

II. Isolation of other microorganisms decomposing
the capsular polysaccharides of different types of Pneumo-
coccus. (Dr. Dubos). The search for other microorganisms
decomposing the specific polysaccharides of Pneumococcus
types is being continued.

A new spore forming, Gram positive, thermophilic
bacillus, decomposing the capsular polysaccharide of Type
III Pneumococcus, has just been obtained from horse manure.
Nothing is known as yet of the biochemical properties of
this organism except that it decomposes the Type III poly-
saccharide when incubated at 50° C.

From a corn cob compost, there has been isolated
an unidentified microorganism, probably belonging to the
Mucorales, which decomposes the Type II capsular polysac-
charide. This organism does not attack the capsular poly-
saccharides of Type I and Type III Pneumococcus.

It may be recalled that the capsular polysacchar-
ide of Type II Pneumococcus exhibits the same serological
reactions as that of Type B Friedländer bacillus. It is a
remarkable fact that the same microorganism which specifi-
cally decomposes the Type II polysaccharide of Pneumococcus,

also decomposes the immunologically related capsular substance
of Type B Friedländer bacillus. These observations again con-
firm the fact that the capsular polysaccharides determine
the specificity of these serological types.

III. Chemo-immunological Studies on Carbohydrates. (Dr.
Goebel and Mr. Babers). 1. Determination of the molecular
size of the capsular polysaccharide of Type III Pneumococcus.
A preliminary account of the determination of the molecular
size of the specific polysaccharide of Type III Pneumocococcus
was given in the last report. This work has since been com-
pleted. The diffusion coefficient of the sodium salt of the
specific carbohydrate has been found to be 0.0415 Cm.^{2} per
day. From this value the radius of the molecule has been
calculated as 2.975 x 10^{-7}cm. and subsequently a molecular
weight of 118,000 has been ascertained. The molecular weight
of hemoglobin and of the carbohydrate from ovomcoid were de-
termined in control experiments. Values of 67,200 and of
2,200 respectively, were found. These numerical values are
in agreement with the accepted values of other investigators,
reported in the literature.

2. The specific carbohydrate of two strains of
Pfeiffer's bacillus. The specific carbohydrate from a strain
of Type A Pfeiffer's bacillus has been isolated from cultures,
grown on dextrose-agar. The medthod of isolation was similar
to that which has been used in the preparation of other bac-
terial polysaccharides. Three preparations have been secur-

ed which react with homologous immune rabbit serum in dilutions of 1:2,000,000. The carbohydrate has a levo-rotation of -32°, it is acidic, and in the present state of purification contains only slight traces of nitrogenous constituents.

The carbohydrate from a strain of Type B Pfeiffer's bacillus is also being studied. Thus far a material has been secured which possesses only a fourth of the serological activity of that of the Type A strain. This carbohydrate appears to be much less stable than that of Type A. Both the A and B types of Pfeiffer's bacillus elaborate only small quantities of specific polysaccharides, about one-tenth the quantities obtained from Pneumococcus. A more detailed account awaits the preparation of sufficient quantities of the materials for chemical investigation.

3. The somatic carbohydrate of Pneumococcus. The "C" substance, or species-specific somatic carbohydrate of Pneumococcus has been identified as a nitrogenous polysaccharide which yields 30 per cent of reducing sugars, calculated as glucose, on hydrolysis. At the suggestion of Dr. Francis, a phosphorous analysis was made on this substance by Dr. Heidelberger at the Presbyterian Hospital. The carbohydrate contains organically bound phosphorous to the extent of approximately 3.3 per cent. We have also found this material to contain about 5 per cent of nitrogen. The carbohydrate is hydrolyzed by nitrous acid in the cold, with an accompanying loss in specificity. We have attempted to split

off the organic phosphorous of this carbohydrate by means of

a powerful phosphatase, but the enzyme causes no liberation

of inorganic phosphorous.   At present sufficient quantities

of this material are being prepared for Dr. Francis in order

that he may carry out certain clinical studies.

    4. Chemo-immunological studies on synthetic carbo-

hydrate derivatives.   In previous reports we have described

the method for the preparation of synthetic carbohydrate

(hexose)-protein antigens, as well as the preparation of a

polysaccharide-protein antigen which elicit specific immune

responses when injected into animals.   From these studies we

have learned first, that carbohydrates, both simple and com-

plex, can be rendered antigenic when they are coupled to a

protein, and second, that the specific immune response (in

the case of hexoses) is determined by the configuration of

the hexose molecule.   In the case of large molecules, such

as the specifically reacting bacterial polysaccharides, the

ultimate immune response is such that the induced antibody

will not only agglutinate microorganisms from which the car-

bohydrate was derived, but will actually confer passive pro-

tection on mice infected with an homologous type of organism.

We are inclined to believe that in order to secure immunity

against a disease in which the chief immune response is de-

pendent upon the elaboration of anticarbohydrate antibodies,

it is necessary only to have as the antigenic agent, a com-

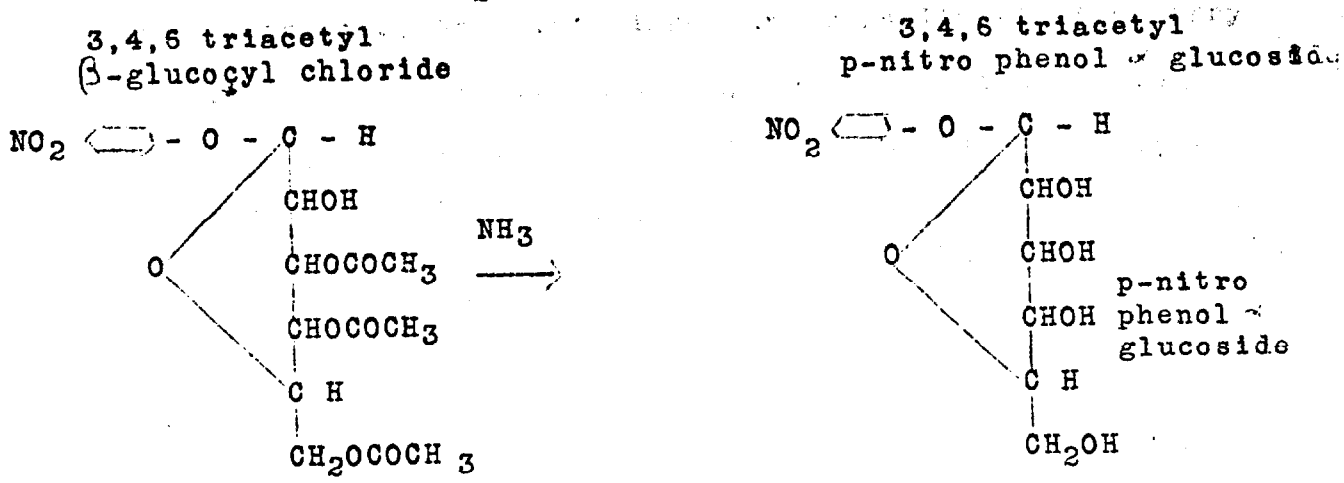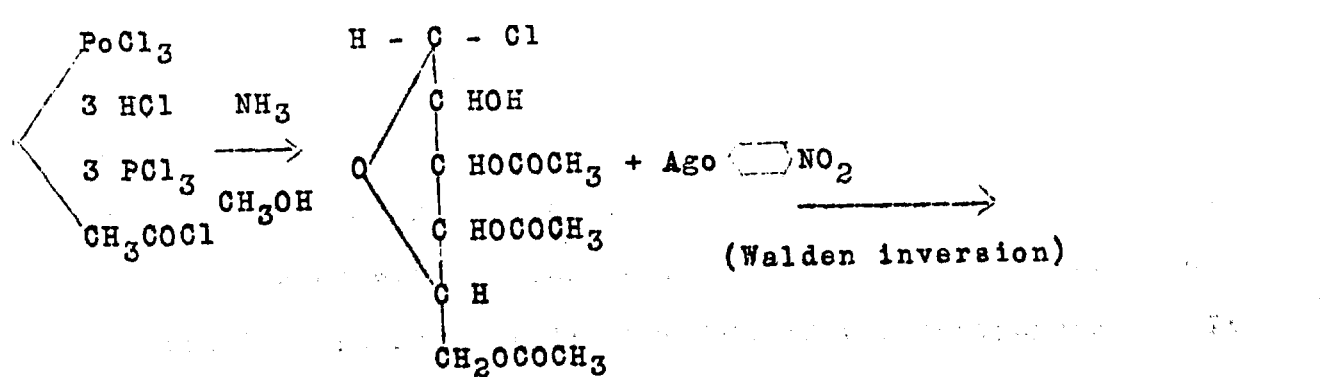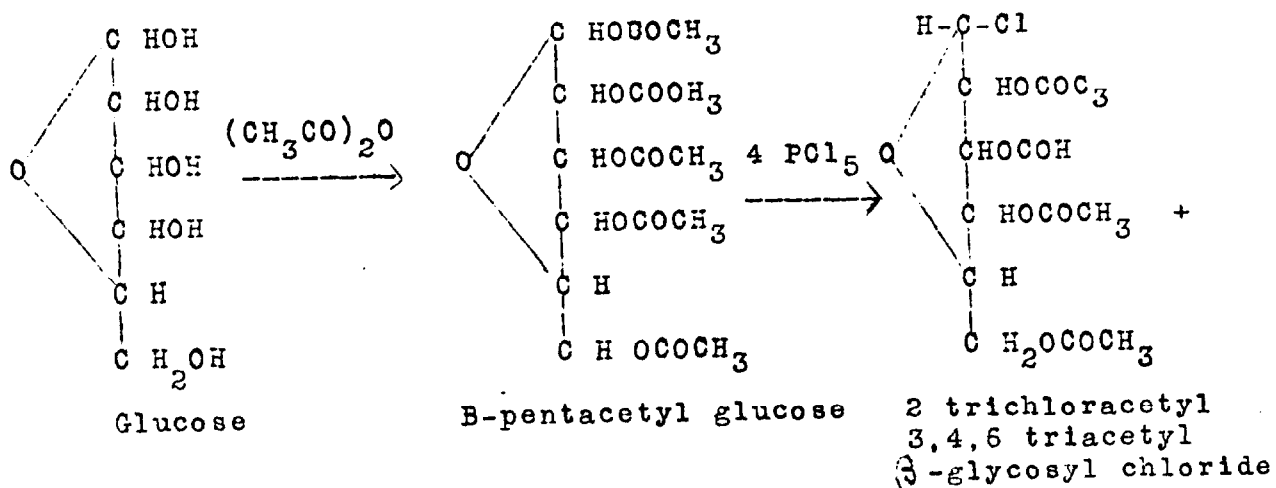bined carbohydrate, of an exact and correct chemical confi-

guration, capable of stimulating the formation of these an-
tibodies.

In a molecule as complex as that of Type III Pneu-
mococcus polysaccharide there are naturally many factors
which enter into the orientation of its ultimate specificity.
We know, for example, that the molecule is studded with
highly polar carboxyl groups. We know that there are three
free hydroxyl groups per unit of hexose, and we know that
the molecule is a polymer of an aldobionic acid (glucurono-
glucose). We do not know, however, the mode of linkage of
one aldobionic acid unit to the next; we do not know whether
the glucurono-glucose is an λ or β glucuronoside, nor do
we even know if an λ sugar can give rise to a different
antibody than its β homologoue.

However, these fundamental problems in the under-
standing of the specificity of immunological reactions in
the case of carbohydrates are not entirely unapproachable,
for we have at our disposal methods based on synthetic chem-
istry, whereby we can analyze the influence, not only of in-
termolecular stereochemical relationships, but the influence
of polar groups in the carbohydrate molecule as well.

a) The synthesis of λ para-amino phenol gluco-
side. We have demonstrated in previous work that the alter-
ation of the stereo-chemical configuration of one carbon
atom in different hexoses, suffices to elicit totally differ-
ent and specific antibody response. Our next problem is to

ascertain whether the α and β glucosides of the same hex-
ose would give rise to separate and distinct antibodies, or
merely to antibodies showing but slight differences. We
have already synthesized the β-p-nitro phenol glucoside of
glucose. The homologous α glucoside has been synthesized
in the following manner.

Glucose → (CH$_3$CO)$_2$O → B-pentacetyl glucose → 4 PCl$_5$ → 2 trichloracetyl 3,4,6 triacetyl β-glycosyl chloride

POCl$_3$ + 3 HCl + 3 PCl$_3$ + CH$_3$COCl → (NH$_3$, CH$_3$OH) → 3,4,6 triacetyl β-glucocyl chloride + Ago ⬡ NO$_2$ → (Walden inversion) → 3,4,6 triacetyl p-nitro phenol α glucoside

NO$_2$ ⬡ - O - C - H ... CH$_2$OCOCH$_3$ → NH$_3$ → NO$_2$ ⬡ - O - C - H ... p-nitro phenol α glucoside ... CH$_2$OH

The nitro phenol glucoside has not as yet been reduced to
the amino homologue. The α nitro phenol glucoside analyses
correctly for carbon and hydrogen. It melts at 220° and has
an optical rotation of +233.9° whereas its isomer, the β -
glucoside, melts at 164° and rotates the plane of polarized
light - 79.6°. We intend to reduce the α compound catlyt-
ically, to couple it to protein and to immunize animals and
finally to compare the antibody thus produced with those ob-
tained by immunization with the β -glucoside-protein complex.

b) Studies on glucuronic acid. We have spoken
above of the occurrence of glucuronic acid in the Type III
Pneumococcus specific polysaccharide, and we have indicated
that the carboxyl groups remain free in the intact molecule.
Not only have we found glucuronic acid in this specific car-
bohydrate, but we have found it in every bacterial polysac-
charide investigated in our laboratory. Furthermore, we
have found it in gum acacia, and we have shown that gum aca-
cia will react with antipneumococcus Types II and III sera
in dilutions as high as 1:6,000,000 of the carbohydrate. It
seems, therefore, that this important constituent of so many
specific polysaccharides must play an important immunologi-
cal role. It is now our belief that the highly polar car-
boxyl group of glucuronic acid, and its relationship to other
polar groups in the carbohydrate molecule, exert a strong
influence in the immune response. In order to test this
conception, we have decided to determine whether there are

differences in the antibodies produced by immunization with

protein complexes of β-p-amino phenol glucoside of glucose

and those of β-p-amino phenol glucoside of glucuronic acid.

$$NH_2 \langle ? \rangle - O - C - H$$
$$\quad HC\ OH$$
$$O \quad HO\ C\ H$$
$$\quad H\ C\ OH$$
$$\quad C\ H$$
$$\quad C\ H_2OH$$

$$NH_2 \langle ? \rangle - O - C - H$$
$$\quad H\ C\ OH$$
$$O \quad HO\ C\ H$$
$$\quad H\ C\ OH$$
$$\quad CH$$
$$\quad C\ OOH$$
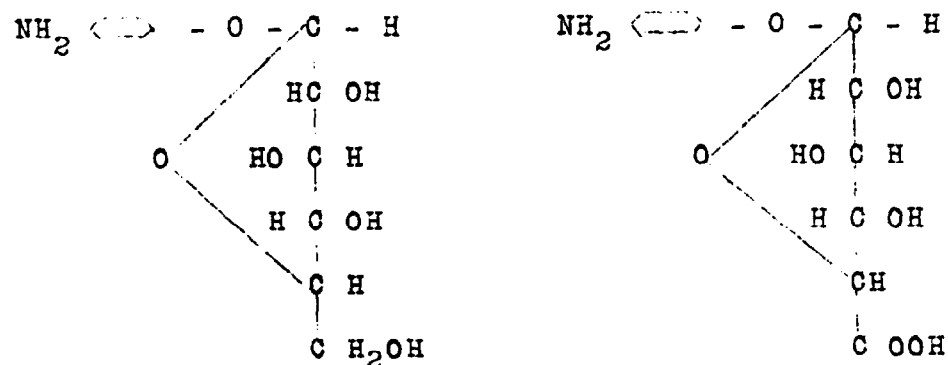
β-para amino phenol glucoside   β-para amino phenol
   glucoside of glucuronic acid

From these two formulae one may see that the ster-

eochemical relationship of these two molecules are exactly

alike. They differ only in that the symmetrical sixth car-

bon atom of the glucose glucoside, a primary alcohol group,

is in the form of a polar carboxyl group in the glucurono-

side. Since the other atomic groupings of these two com-

pounds are exactly alike, save in this one respect, it is

possible to determine directly the influence on the immune

response of the substitution of an acid grouping in a hexose

molecule.

The problem of synthesizing the para-amino phenol

glucoside of glucuronic acid is by no means simple. It is

difficult to secure large quantities of the sugar acid, and

the chemistry of the acetyl and halogen acetyl derivatives

from which these glucosides can be prepared, is unknown.

The method of procedure is as follows:- Glucuronic acid was prepared by feeding 5 grams of borneol daily to each of 6 dogs. The animal organism conjugates the borneol and excretes it in the form of a glucuronic acid glucoside which can be quantitatively recovered from the urine as a crystalline zinc salt. Between 20 and 30 grams of zinc borneol glucuronic acid can be recovered daily from 6 dogs. The zinc borneol glucoside is treated with sulfuric acid to split off the zinc, yielding the borneol glucoside of glucuronic acid. The latter is then hydrolyzed with 0.2 normal sulfuric acid, and the insoluble borneol is separated from the solution of glucuronic acid. After removal of the sulfuric acid by $BaCO_3$ the glucuronic acid can be crystallized as a snow white product. The chemical manipulation can be easily carried out, and the ultimate yield of this rare sugar represents nearly 90 per cent of the source material.

The sugar can be converted to its lactone by crystallization from acetic acid.
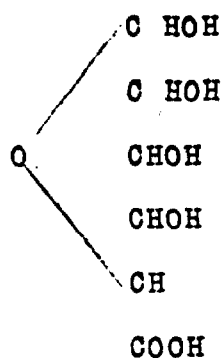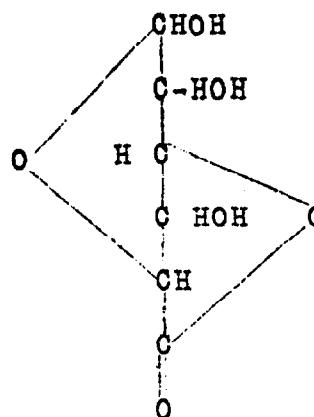
glucuronic acid   glucuron

This derivative has been acetylated and α and β - triacetyl glucuron have been separated by fractional crystal- lization from alcohol. Neither the α nor the β -triacetyl glucuron will yield a stable bromo derivative, but we have secured from both derivatives a stable diacetyl chloroglucuron.

glucuron   triacetyl glucuron

1-chlor diacetyl glucuron
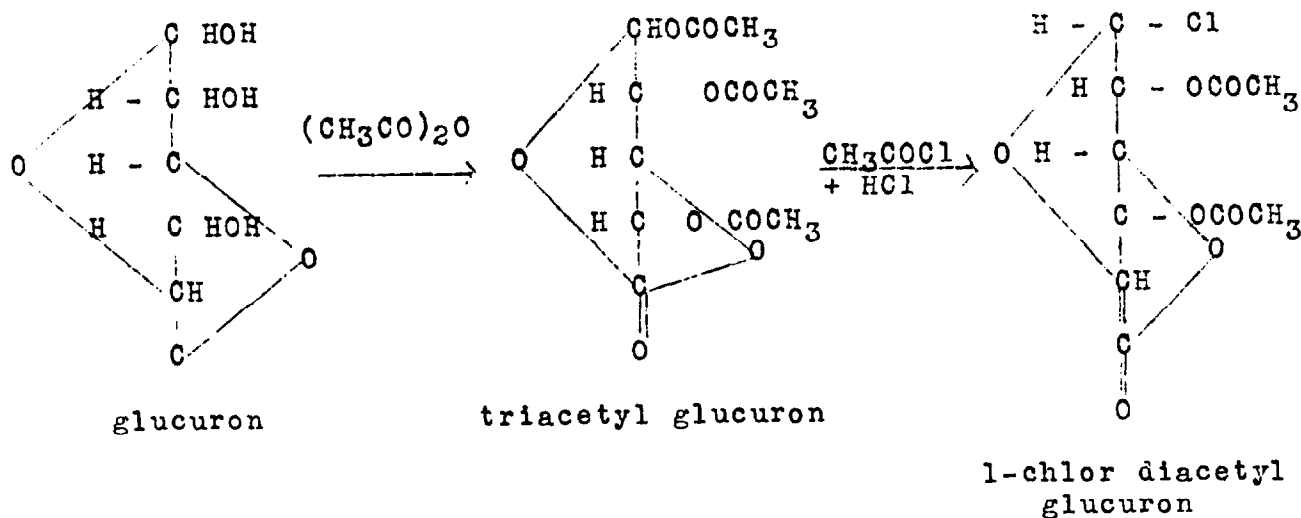
Though yet obtained in a crystalline state, these halogen acetyl derivatives give correct analyses. The syn- thesis has not as yet progressed beyond this point; but the preparation of these two chloro diacetyl glucuron deriva- tives now make possible the completion of the synthesis of the para amino phenol glucoside of glucuronic acid, which we hope to accomplish in the near future.

IV. The Chemical Nature of the Type-specific Capsular Antigen of Pneumococcus. (Drs. Avery and Goebel).

The importance of determining the chemical nature of the type-specific antigen of Pneumococcus becomes evident when one considers that the protective substances in anti- pneumococcus serum are precisely those antibodies which are produced in response to this particular antigen. By speci-

fic union with these antibodies, the capsular substance of
the virulent Pneumococcus becomes so altered that the bac-
terial cell as a whole is rendered susceptible to phagocy-
tosis.   And, so far as our knowledge at present goes, the
therapeutic effect of antipneumococcus serum is largely, if
not exclusively, dependent upon the presence of these type-
specific antibodies.   Indeed, the application of serum thera-
py in Pneumococcus today rests upon our knowledge of these
type-specific relationships.   It would appear, therefore,
that an understanding of the chemical nature of this impor-
tant antigenic constituent of the Pneumococcus might contri-
bute much to the improvement and perfecting of methods useful
in the production of potent antisera.

Interestingly enough, although the exact nature of
this antigen itself eludes us, we have considerable knowledge
of one of its component parts, namely, the type-specific
capsular polysaccharide.   For instance, we know that this
specific carbohydrate is given off from the bacteria growing
in the focus of disease and circulates in soluble form in
the body fluids; that in this form it still retains its capa-
city to unite with and thus to neutralize the immune substan-
ces of the serum; that even in minute amounts it inhibits
phagocytosis, and that because of these reactions its pre-
sence tends to interfere with the normal processes of recov-
ery from the disease.   We know also something of the chemi-
stry and immunological specificity of this capsular substance.

We have succeeded in chemically identifying a polysaccharide
in the capsular substance of each of the different types of
Pneumococcus and have found in each instance that the carbo-
hydrate is as chemically distinct as it is serologically
specific for each type. We have demonstrated the remark-
able specificity of these carbohydrates in the phenomenon of
anaphylaxis and in the reactions they incite in the skin of
convalescents from pneumonia. We have evidence that these
specific carbohydrates, when separated from the bacteria,
still retain unimpaired the property of combining with anti-
bodies, but lose more or less completely their power to in-
cite the formation of these same antibodies when injected
alone into the animal body. This very fact indicates that
in their native state, these specific polysaccharides exist
in the cell not as haptens but as part of a more complex
antigen from which they may be more or less readily disso-
ciated either before or after the injection of the whole
antigen into the animal body. The rate and extent to which
this dissociation occurs apparently varies with the differ-
ent types. The theory of antigenic dissociation and its re-
lation to the production of specific sera have been discuss-
ed in a preceding report. It is referred to here merely to
emphasize the importance of knowing the chemical nature of
the type-specific antigen, and to point out again the prac-
tical bearing which such knowledge might have upon the prob-
lems of active and passive immunity to Pneumococcus infec-
tion. Progress in the solution of this problem has been

impeded by two major technical difficulties:- one, the diffi-
culty of extracting the intact antigen in active form because
of the ease with which dissociation of the complex occurs;
and the other, the difficulty of identifying in such extracts
the particular substance or chemo-specific groups which con-
fer antigenicity upon the capsular polysaccharide.

The first of these difficulties has been largely
overcome, and the second has been attacked through an indi-
rect but promising approach. Following the leads suggested
by the work of Perlzweig and of Day, filtered solutions con-
taining the type-specific antigen in active form have been
obtained by extracting the encapsulated cells at an acid re-
action with heat. The antigenic activity of the extracts
has been tested by injecting them into mice and determining
whether the treated animals have acquired specific immunity
to infection with pneumococci of the homologous type. In an
acid buffer solution (pH 6), the antigen resists heating in
a boiling water bath for ten minutes; two injections total-
ing 0.4 cc. of a solution containing as little as 0.003 mgs.
N per cc. have proved effective in immunizing mice against
an homologous strain of Type I pneumococcus. The extraction
of the antigen, however, is only the first step in the anal-
ysis of its chemical nature. Is the type-specific polysac-
charide bound to a protein, or to some other substance? Is
it even necessary to assume that the effective complex con-
sists of a protein coupled to the carbohydrate? This seems

the most reasonable assumption, especially since we have
found that an artificial antigen prepared by the coupling of
a diazonium derivative of the Type III polysaccharide with
animal protein stimulates in rabbits the formation of type-
specific antibodies which agglutinate living pneumococci of
the homologous type and protect mice against infection with
virulent Type III pneumococci. Despite this analogy, may it
not be that the polysaccharide carrying the specific binding
groups is but part of a still larger and more complex carbo-
hydrate molecule from which the antigenic groups may be eas-
ily split off, leaving the antigenically inert but specifi-
cally reactive polysaccharide intact. In the case of the
pectins for example, partial hydrolysis suffices to split
off pectinic acid from the complex and a polysaccharide then
remains as a product of hydrolysis.

As Wells points out, there is theoretically no
reason why a carbohydrate should not function as an antigen
provided it is colloidal in nature and of sufficient molecu-
lar size. However, to assume that the lack of antigenicity
of the capsular polysaccharides is attributable to denatura-
tion in the process of isolation is to overlook the fact that
the more these substances are chemically purified the more
specifically reactive they become and the more closely they
conform on chemical analysis to the theoretical values for
pure carbohydrates.

The mere prediction of theoretical possibilities

however, is not paramount to the actual identification of
the true antigen. The amount of substance required for chem-
ical analysis is large and this is difficult to obtain. De-
termining whether a given extract contains the effective an-
tigen involves the process of immunization and this requires
time. It seemed possible, however, that an indirect approach
and one which might furnish a clue as to the nature of the
substance to be looked for, might be made by determining if
possible, what enzyme or class of enzymes would destroy the
antigenicity of a bacterial extract known to contain the
whole antigen. Whether for example, the antigenicity of an
extract is destroyed by trypsin, or pepsin, etc., or by li-
pases or nucleases, etc., or whether enzymes, such as pecti-
nase, emulsion, and others which are known to hydrolize par-
tially the more complex carbohydrates would destroy the ef-
fective antigen without impairing the specific binding pro-
perties of the polysaccharide.

In collaboration with Dr. Goebel this work is now
in progress, and the results thus far obtained are promising,
but not as yet sufficiently established to warrant presenta-
tion at this time.

V. The Significance of the Skin Test with Specific
Polysaccharide as a Guide to Serum Therapy in Pneumonia.(Dr.
Francis). During the study of the cutaneous reaction of pneu-
monia patients to the type specific polysaccharides, it was
soon observed that all of the cases of Type I pneumonia which

recovered gave a positive reaction to the Type I polysaccha-
ride. The majority of Type I patients received specific se-
rum therapy, while those suffering from infections due to
Type II and III Pneumococcus do not. Approximately only 50
per cent of the latter group reacts to the specific soluble
substance of the respective types. In none of the patients
was this reaction elicited before the onset of recovery,
even when type specific therapeutic serum had been adminis-
tered. Furthermore, in certain instances, when the patient
still appeared sick and the problem of additional serum
treatment was immediate, a positive skin test was found to
indicate recovery and that treatment could be safely discon-
tinued. In the case of individuals, in whom no positive re-
action was obtained at any time during the course of the
illness, a fatal termination occurred, even though a subsi-
dence of fever, sterilization of the blood of the invading
organisms and a comparatively high concentration of serum
antibodies were observed. Consequently a negative cutaneous
reaction to the polysaccharide was thought to be an indica-
tion for continuation of serum therapy.

The study has been extended with the idea of es-
tablishing the true value of this reaction as a guide to
proper serum therapy. At present, data have been obtained
in 35 cases of Type I pneumonia. Four of these were not
treated with serum but recovered and gave positive skin reac-
tions; they serve as controls for the cases which received

specific treatment.   The results are tabulated below.

| | Recovered | | | Died | |
	Total Number	Positive Skin Reaction	Negative Reaction	Positive Skin Reaction	Negative Reaction
Serum Treated	31	25	1*	0	5
Untreated Controls	4	4	0	0	0

*Empyema

From the table it can be seen that of 26 recovered cases which received serum, only one failed to give a positive response to an injection of the specific polysaccharide and in the exception a purulent complication had developed. In the remaining 25, all of whom gave a positive skin test, recovery took place despite such complications as sterile pleural effusions, delayed resolution, furunculosis, and recurrent acute nephritis.   In 5 fatal cases no skin reaction was obtained at any time.

The extended observations have, therefore, confirmed the impression that the skin test with type specific polysaccharide is a valuable guide in the serum treatment of pneumonia.   Its value lies in the fact that, when positive, it indicates that recovery has begun and serum therapy may be safely discontinued, but a negative response is an indication that the administration of serum should be continued. If, in the face of continued serum therapy with definite clinical improvement a negative reaction persists, a suppurative complication should be suspected.   If none is found serum therapy should be maintained, although the outlook is unfavorable.

VI. Studies on Natural Resistance and on the Im-
munity induced in Rabbits by Injections of R Pneumococci.
(Dr. Francis). A study is being made of the natural resis-
tances of the rabbit to Type III Pneumococcus and of the
form of immunity produced in rabbits by injections of R
pneumococci. The method of Robertson and Sia, using serum-
leucocyte mixtures, has been shown to be quite sensitive in
detecting slight degrees of immunity. For this reason the
same method, as well as a modification employing whole hepa-
rinized blood, was adopted in the present study.

Although the work is in its early stages, several
interesting facts have been revealed. While the normal rab-
bit is resistant to the rabbit-avirulent strain of Type III
Pneumococcus, no pneumococcidal powers against this organism
is demonstrable in the animal's blood. Against the R form
of Pneumococcus, however, the normal rabbit exhibits the
power to inhibit the growth of a large number of organisms.
The blood of a rabbit containing anti-R antibodies, produced
by immunization with R pneumococci, has an even greater bac-
tericidal power for the R organisms than has the normal ani-
mal. In addition, it possesses a moderate but definite ca-
pacity to prevent the growth of the avirulent encapsulated
Type III Pneumococcus. So far no experiments have been done
with the blood of immune rabbits containing type specific
antibodies. The state of immunity has been simulated, how-
ever, by the addition of type specific horse anti-serum to
the blood of the normal rabbit. Under these conditions a

marked pneumococcidal effect on the rabbit avirulent Type III pneumococci is also observed.

Certain conclusions appear justifiable. The normal rabbit, resistant to the avirulent encapsulated Type III Pneumococcus, shows no evidence in the blood of a phagocytic mechanism for its disposal. In the R immune rabbit this capacity has been developed to a moderate degree, while the blood of the animal containing type specific immune bodies has a still greater pneumococcidal power.

VII. The Principles Governing the Agglutinin and Precipitin Reactions. (Dr. Francis). It was observed in the course of precipitin reactions that when a prozone was produced with an excess of soluble specific substance of the pneumococcus, the serum had also lost its capacity to agglutinate the homologous type-specific organism. This observation brought further evidence to indicate the unity of agglutinogen and precipitinogen. Consequently the following experiments were devised in an attempt to correlate the mechanisms of the reactions of agglutination and precipitation.

To an immune serum a suspension of the homologous type specific pneumococcus was added. After agglutination had occurred the compact mass was placed in a dilute concentration of the specific polysaccharide of the same type. Under these circumstances precipitation occurred, with removal of the polysaccharide from solution, indicating that an excess of antibody was attached to the bacterial cells

and was able to unite with additional amounts of antigen, in this case the specific soluble substance. Now, if still more of the free soluble substance was added, the agglutinated organisms became dispersed and remained diffusely suspended in solution. When the bacteria so dispersed were removed from the mixture by centrifugation they were found to be in a normal serological state as shown by the fact that they could be agglutinated again, but only by immune serum for the homologous type of Pneumococcus. Had antibody remained bound to them, the bacteria might conceivably agglutinate spontaneously in heterologous serum, or in salt solution. This, however, was not the case. Similarly, organisms which were added to a serum in which a prozone had been created with specific soluble substance were serologically unaltered.

The results show that the type-specific precipitin and agglutinin reactions are dependent upon the reaction of antibodies with the same substance; in one case, present in the bacterial cell; in the other, chemically purified and separate from the bacteria themselves. The two forms of the type specific substance may be substituted for one another without altering the phase of reaction.

Heidelberger and Kendall recently studied the precipitin reaction quantitatively and concluded that the various phases of reaction could be expressed in terms of the laws of mass action. That is, antibody and specific substance combine in multiple proportions, depending upon the

concentration of the two reagents. The present studies conform qualitatively with theirs, and it seems that the same laws govern both the agglutinin and precipitin reactions.

VIII. Studies on Epidemiology. (Dr. Stillman).

1. Antibody response to immunization by different routes. The work on the epidemiology of pneumonia is being pursued along two lines. Data is being accumulated as to the development of immunity following immunization of rabbits with different types of pneumococci by various routes. The length of time that agglutinins and protective antibodies persist in the serum of rabbits immunized with heat-killed Type I pneumococci has been determined. Agglutinins soon disappeared from the sera of these rabbits. Protective antibodies, on the other hand, could be demonstrated in high concentrations in their sera for prolonged periods. It appears that there is a definite relationship between the total amount of antigen administered and the length of time antibodies may be demonstrated in the blood or rabbits. Furthermore, there is a close relationship between the route of administration of antigen and the character and persistence of the antibody response in rabbits.

2. Production of experimental pneumonia in animals. Work is also in progress on the production of experimental lobar pneumonia in laboratory animals. This has a two-fold object. Once the complicated mechanism of the factors necessary to cause an animal to localize pneumococcus

infection is understood, we will be able to not only treat
the disease of lobar pneumonia in man more efficiently but
also know better what precautions to observe in order to
escape the disease. Although experimental lobar pneumonia
has been successfully produced in mice, the various factors
entering into the production of pneumonia could not be stud-
ied in such a small animal. Ever since Wadsworth claimed to
have produced pneumonia in partially immunized rabbits in
1904, it has been assumed that pneumonia could be induced in
these animals. It has already been shown that pneumonic
consolidation may be produced in partially immunized alco-
holized mice by the inhalation method. But we have been un-
able to cause partially immunized rabbits to localize the
infection which develops following inhalation of virulent
pneumococci. The inability of rabbits to localize the in-
fection may possibly be due to the anatomy of the rabbits'
lung. Pneumococci in rabbits rapidly pass through the pleura
and cause empyema and pericarditis. There seems to be no at-
tempt to confine the infection within the lung itself.

IX. The Antipneumococcus Immune Reactions of
Normal Hog Serum. (Dr. Kelly). It was reported by Bull and
McKee in 1921 that the blood serum of normal chickens when
injected into susceptible animals confers a notable degree
of passive antipneumococcus immunity. They also showed that
the factor responsible for this immunity is associated with
serum globulin, and that it is type specific in action.

In 1924 Robertson and Sia by means of serum-leuco-
cyte mixtures were able to demonstrate the presence of nat-
urally occurring antipneumococcus opsonins in the blood of
resistant animals. By this technic the serum of normal pigs
showed a high content of these opsonins.

Sia in 1929 found that pig serum injected intra-
peritoneally into mice affords these animals a marked degree
of protection against pneumococcus infection. This antipneu-
mococcus protective action was shown by absorption experi-
ments to be type specific in nature.

At variance with the widely accepted theory that
natural immunity is dependent solely on the cellular defense
of the host, are the two examples, here cited, of a natural
immunity to pneumococcus infection that is passively trans-
ferable through the blood serum, and that appears to be
type-specific in nature.

The present study is an attempt to repeat the ex-
periments of Sia, and to further analyze the antipneumococ-
cus immune reactions of the serum of normal hogs and of
other resistant animals. In confirmation of Sia's results
it has been found that the serum of normal hogs when in-
jected intraperitoneally confers upon white mice a marked
degree of immunity to Pneumococcus, Types I and II. 1 cc.,
the optimal protective dose of the serum, usually affords
protection against 10,000 to 100,000 lethal doses of pneu-
mococcus culture. The serum yields its maximal protective

action when injected four to eight hours before inoculation of the mice with culture. The degree of protection against Type II is consistently higher than that against Type I. The serum gives no protection against pneumococcus infection when injected subcutaneously. Under the same conditions the serum from other animals - rabbit, guinea pig, ox, and sheep, - when injected intraperitoneally, have conferred no protection on mice.

The protective principle of hog serum is associated with the serum globulin obtained by precipitation with ammonium sulphate. Hog serum gradually loses its antipneumococcus protective action on standing, so that after two to four months in the ice-box the protective action is entirely, or almost entirely, lost. At room temperature this loss occurs more rapidly. In a paraffin-lined tube, under a vaseline seal the serum loses its protective power in about the same time as it does when unsealed. The protective action of the serum against Type I is lost usually in shorter time than that against Type II. The protective power is considerably reduced by heating the serum to 56° C. for 30 minutes, and is entirely destroyed by exposure to a temperature of 64° C. for the same time. The protective action when lost, either through heat, or standing, is not restored to the serum by the addition of a small amount of fresh normal serum.

The antipneumococcus protective principle for either Type I or Type II can be specifically absorbed. The

quantity of pneumococci per volume of serum necessary for specific absorption, is small. The absorption requires only a short time for completion. Absorption of the serum with avirulent R pneumococci, and with bacteria other than pneumococci, does not consistently alter the protective power of the serum. Absorption with charcoal and Kieselgur does not appreciably lower the protective power.

Sia in 1922 noted that S pneumococci, when sensitized by large amounts of hog serum, show agglutination after separation from the sensitizing serum by centrifugation. It has been found in the present study that hog serum agglutinates S pneumococci. The agglutinated pneumococci from a firm, thin disc similar to that produced by type specific antipneumococcus serum. In comparison with specific antipneumococcus serum large amounts of hog serum are necessary for demonstration of the agglutination. It is necessary also, except occasionally in case of Type II, to use Gate's technic for this demonstration. The agglutinins for Type II are present in hog serum in greater concentration, than are those for Type I. Similar to the antipneumococcus protective action in mice, the agglutinins for S pneumococci are specifically absorbable and are destroyed by heating the serum at 64° C. for 30 minutes.

It has also been observed that avirulent R pneumococci are agglutinated by hog serum. This agglutination is of typical R character. The agglutinated pneumococci

form a granular precipitate leaving the supernatant clear.
The maximum R agglutinin titre is usually 1:250 to 1:500.
So far no falling off of the R agglutinin titre has been
seen even after the antipneumococcus protective action of
the serum is lost.

Although sensitization of living pneumococci with
hog serum does not alter their virulence, heat-killed pneu-
mococci when sensitized by hog serum provoke a very meagre
specific antibody response in rabbits.

With the concentrations used, no change in colony
form has been brought about by growing either S or R, pneu-
mococci in dilutions of hog serum. The R pneumococci cul-
tured in presence of the serum grow in clumps.

X. Pathogenesis of Experimental Intradermal Pneu-
mococcus Infection. (Dr. Goodner). The intradermal pneumo-
coccus infection in rabbits offers an experimental disease
which bears many analogies to lobar pneumonia. The general
nature of this symptom-complex has been studied for a num-
ber of years and is now sufficiently well understood to
serve as a basic method for more specific projects.

With Dr. Rhoads the histological pathogenesis of
this lesion has been studied and found to be comparable to
that of lobar pneumonia in man. The findings are described
in detail in another section. The outstanding feature of
the early lesion is the edema fluid; its accumulation occurs
before any change in the cellular picture, and in the pro-

gressing or moving lesion its passage through the tissue precedes any other sign of reaction between tissue and micro-organism. It seems probable that the advancing fluid carries with it the first infecting organisms and consequently inoculates all tissues which it reaches. The resultant infection appears to be due, not to an active invasion, but rather to a progressive passive inoculation.

Edema fluid has been removed from the developing lesion and its properties studied in vitro. If obtained free from blood and tissue elements such fluid does not clot even after long standing. That this property is not due to a deficiency of fibrinogen is shown by the fact that a firm clot promptly appears on the addition of thromboplastin. Evidence points to a high antithrombic content since the edema fluid has the property of retarding the clotting of normal rabbit blood. The clotting time of the blood in these rabbits infected with pneumococci is prolonged, particularly in severe cases, but the effect is never as pronounced as in the exudative fluid where the concentration of the antithrombic factor appears to be much greater. The nature and source of this antithrombic substance are now being investigated.

A study has been made of the factors which have to do with the development, movement, final localization, and extent of the dermal lesion in the rabbit. The direction of movement is determined by gravity and is independent of the

distribution and course of blood vessels and lymphatics.
The lesion seems always to seek to reach the more directly
dependent area.   The direction of movement may be modified
by changing the position of the animal or by arranging ar-
tificial guiding structures such as scars.   If the skin of
the abdomen is the final site of localization there is a
massive collection of edema fluid.   Such edematous lesions
are not obtained in areas where the skin is tense, such as
over an extremity.   Drainage distance largely determines the
volume of edema fluid accumulating in a given area.

Numerous factors modify the development and rate
of movement.   Perhaps the most striking of those yet observ-
ed is the accelerating or synergistic co-infection with B.
influenzae.   This seems of particular significance since
some form of the Pfeiffer bacillus is found in a large per-
centage of sputa from cases of lobar pneumonia.   If B. in-
fluenzae and pneumococci (Type I) are injected together in-
tradermally the effect is a marked acceleration in the de-
velopment and movement of the lesion.   The lag period of the
pneumococcic infection is almost entirely eliminated.   Other
studies on the associative relationships of these organisms
are now in progress.

I.Intradermal Infection with a Rabbit Virulent Type
III Pneumococcus. (Dr. Goodner).   The character of the in-
tradermal infection with a rabbit virulent strain of Type
III pneumococcus has been studied and found to follow essen-

tially the same course as with Type I, although the disease
is much more severe and the fatality rate higher. Cases
terminated more often by lysis than by crisis. This is pos-
sibly explained by the low grade active immunity which fol-
lows artificial immunization or recovery from the disease.
Recovered animals show but slight resistance to reinfection
even at short intervals. The immunity acquired from a course
of the disease is of sufficient quantitative character to
prevent death after reinfection but not sufficient to pre-
vent a severe infection.

The use of Type III antiserum (by ourself and by
others)in treating experimental infections has yielded poor
results. A selected serum was concentrated by our method
and although high agglutinin and precipitin titers were ob-
tained, this concentrate fails completely to protect mice.
It has, however, a favorable influence in the rabbit infec-
tion, although even in large amounts it seems incapable of
promptly terminating the disease.

The enzyme which specifically decomposes the cap-
sular polysaccharide of Type III pneumococcus is being used
in treating this experimental disease. Preliminary experi-
ments have given encouraging results but many factors are as
yet not sufficiently understood to warrant final conclusions.
The enzyme preparations as obtained at this time possess a
primary toxicity for normal rabbits and are particularly
toxic for infected animals. With certain preparations which

showed the lower range of toxicity the therapeutic effect
was marked.   The blood stream became free of organisms and
after an initial rise the temperature dropped to normal lev-
els.   Repeated injections of the enzyme are necessary to
prevent relapses.

XI. Study of the Transformation of the Specific
Types of Pneumococcus. (Dr. Alloway).   The experiments car-
ried out by Griffith in 1928 for the first time brought con-
vincing evidence that specific types of Pneumococcus are not
absolutely fixed but may, under special conditions of growth,
be transformed one into another.   It had been known previous-
ly that type-specific pneumococci may be degraded into the
so-called common R forms, but it was generally believed that,
if, these forms ever again reverted to type, they always re-
verted to that type from which they were originally derived.

Griffith brought about the transformation of types
by inoculating mice subcutaneously with small quantities of
living R organisms together with large quantities of heat-
killed S cells of a type other than that from the R strain
originally came.   Under these conditions, the R forms resum-
ed the specific properties of the particular S type used in
the heated inoculum.   In other words, the process of rever-
sion may now be specifically oriented at will, depending
upon the type of Pneumococcus used in the reaction-mixtures.

Dawson in 1930, working in this laboratory, con-
firmed Griffith's results and later defined the cultural

conditions under which transformations similar to those orig-

inally induced in the animal body may now be brought about

in the culture tube. Pneumococci which have assumed the

specific characters of the new type _in vivo_, show no tenden-

cy to revert spontaneously to the original type. With the

acquisition of a new specificity, the R forms also regain

the property of virulence, a quality usually not restored by

mere cultivation on artificial media. At present further

studies are in progress with the hope of finding out the

basic factors responsible for these remarkable changes in

biological specificity. Attempts are being made to extract

from type specific pneumococci, the substance, or substances,

responsible for the activation of the R forms. Since the

type-specificity of pneumococci is dependent upon the pre-

sence of the capsular polysaccharide, this substance in pur-

ified form was used instead of the whole cells. The failure

to induce the change under these conditions indicates that

the specific polysaccharide, in a chemically purified state,

does not, by itself, function as the activating substance.

Reference has already been made in this report, to a method

of extracting the capsular polysaccharide in a form in which

it still retains its antigenic property. Since acid extrac-

tion with heat has been found to remove from the cells this

otherwise easily dissociated antigen, the same method was

used in attempts to extract in active form the bacterial

substance which is concerned in the process of transformation.

Extracts prepared in this way, from which formed cells were removed by prolonged centrifugation, have proved active in causing R pneumococci derived from Type II to assume the specific characters of the Type III S cells from which the extract was made. However, when the same extract was passed through a Berkefield filter it lost its activity and was no longer effective in bringing about the change. Although too little is known of the biologically active substances which appear to be specific activators of the R forms of pneumococci, the possibility exists that they may function as a co-zymase or activator of a zymase which exists in an inactive state in all R cells. This hypothesis furnishes at least a working basis and seems the more likely since it is now apparent that every R cell has potentially the capacity to synthesize any one of the type-specific capsular polysaccharides, which one it happens to elaborate being dependent upon a specific stimulus, or activator.

Various methods of extraction are being tried with the hope of obtaining the active substance from the type-specific cells. From such extracts it may then be possible to isolate the active principle and thus to obtain a more definite knowledge of its chemical and biological properties and its relation to the mechanism of this important phenomenon.

XII. Significance of Oxidation-Reduction Processes in Bacterial Growth. (Dr. Dubos). When Pneumococcus is seeded into plain broth, it is necessary to use a fairly large

inoculation in order to insure the initiation of growth.
The work of previous years had established that this "bac-
teriostatic" action of the broth, as expressed by the lack
of growth of very small inocula, can be corrected by a var-
iety of treatments which have in common the development of
reducing conditions in the medium. It has now been shown
that the bacteriostatic substance is present in the peptone
used in the preparation of the broth in which this substance
usually exists in an oxidized form. It appears furthermore
that this bacteriostatic substance can be reversibly oxidiz-
ed and reduced and that the bacteriostatic properties are
exhibited only by the oxidized form, and not at all by the
reduced form. In this respect, it behaves like methylene
blue, gallocyanine and the various indophenols, dyes which
have been shown previously to be bacteriostatic only in the
oxidized form.

Different brands of commercial peptones contain
varying amounts of bacteriostatic substance. This substance,
although its nature is not known, can be titrated by means
of reducing substances, in particular the reduced thio-acids.
The principle of the titration is to determine what amount
of the reduced substance is necessary to correct the toxic-
ity of a certain amount of the bacteriostatic material.
Such titrations have revealed that the bacteriostatic action
can be recognized with concentrations corresponding to as
little as 10^{-5} molar concentration of thioglycollic acid.

These concentrations are of the order met with in catalytic
reactions and one may wonder whether the bacteriostatic sub-
stance of the peptone does not act as anticatalyst in some
phase of the metabolism of the cell.

It was of obvious interest to attempt to obtain
peptone preparations free of this bacteriostatic action.
This has been achieved by two completely different methods.

A. Purification of a brand of commercial peptone.
It has been found possible to remove 90-95 per cent of the
bacteriostatic fraction of Fairchild peptone by precipita-
tion with concentrated hydrochloric acid. The bacteriosta-
tic substance thus precipitated appears as a pigment which
can be reversibly oxidized and reduced. In the course of
this process, the color of the material changes from green
to brown; the bacteriostatic action is associated with the
green (oxidized) form.

As mentioned previously, the acid precipitation
removes only 90-95 per cent of the bacteriostatic material.
Another bacteriostatic fraction is associated with the ace-
tone soluble component of the peptone. A purified peptone
was prepared by removal of these two fractions. With this
purified preparation, a medium could be prepared in which a
positive growth of Pneumococcus was obtained, in the absence
of blood or any reducing substance, with an inoculum as
small as .000,000,1 cc. of culture.

B. Preparation of peptones from pure proteins.

There are indications that the bacteriostatic substance is derived from some constituents of the animal tissues used in the preparation of the peptones, and it was thought that peptones prepared from purified proteins would be devoid of bacteriostatic substance.

In fact, peptones prepared by peptic or tryptic digestion of casein have justified this assumption. These casein digests, when used for the preparation of "plain broth" give media in which the growth of Pneumococcus can be initiated with very small inocula; it has also been observed that the quantity of growth, as measured by the number of cells or amount of bacterial protein synthetized, is much higher with these casein digests than with any of the ordinary peptones which have been tested. Work is in progress establishing the value of these peptones for the growth of other Gram positive cocci, of B. granulosis, of the anaerobic filter passing group, and of strict anaerobes. It may be mentioned that Dr. A. R. Dochez has used these preparations in experiments on the cultivation of virus of the common cold in vitro.

Equally satisfactory results, although not so complete, have been obtained with peptones prepared from other purified proteins (fibrin, globin).

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