Report of Dr. Avery (assisted by Drs. Adams, Beeson, Cattaneo, Curnen, Daddi, Dublin, Dubos, Goebel, Goodner, Heggie, Hotchkiss, C. M. MacLeod, Mirick, and Stillman).

Studies on chemotherapy: Treatment of pneumonia with sulfapyridine alone and in combination with antipneumococcal serum (MacLeod, Mirick and Curnen). The earlier observations on the use of sulfapyridine in the treatment of lobar pneumonia have been confirmed and extended. In pneumonias due to pneumococci other than Type III the drug has been used as the sole specific therapeutic agent, except in a few instances as in patients of advanced age or where the infection has been of great severity. In these cases type-specific antipneumococcal serum has been used in addition. Because of the toxic effects incident to the use of sulfapyridine an attempt has been made to treat each patient with as small a daily dosage of the drug as is consistent with the maintainence of an effective concentration in the blood, and to discontinue its administration promptly following a favorable therapeutic response. The total amount of drug administered per patient has been decreased to an average of approximately 15 grams and cessation of treatment has been possible almost invariably after three days. With this scheme of therapy, relapse of the pneumonia has not occurred except in one patient with Type III infection in whom the disease was complicated by asthma and bronchiectasis. In the clinical treatment of the disease the use of sulfapyridine in lower dosage has decreased the incidence of severe toxic effects without sacrificing in any way the therapeutic value of the drug.

From the comparative results obtained in mice infected with Pneumococcus Type III and treated with sulfapyridine or a combination of the drug and antipneumococcus serum, it was felt that the combined form of

therapy might be more efficacious than either agent alone in the treatment of patients with Type III pneumonia. This opinion was based on the observation that sulfapyridine or immune serum when administered singly is relatively ineffective whereas a combination of serum and drug is synergistic and highly effective in the treatment of experimental Type III infection in mice.

Of 20 patients with Type III pneumonia all but one were treated with both serum and sulfapyridine. Following admission to the hospital sulfapyridine has been administered for a period of twelve to eighteen hours before beginning serum therapy. Despite the presence of an adequate blood concentration of the drug during this initial period, definite evidence of progression of the disease has been observed in five patients. However, in all but one of these a favorable outcome has resulted following treatment with serum in dosage sufficient to produce an excess of circulating Type III antibody. The mortality of patients with Type III pneumonia treated with a combination of serum and sulfapyridine has been much lower than that observed in patients receiving serum alone. During the years 1936-1938, 26 patients with Type III pneumonia were treated with concentrated antipneumococcal rabbit serum alone, and 7 of these died, a mortality of 27 per cent. In the past two years only one patient has died in a group of 20 treated with a combination of serum and sulfapyridine, a mortality of 5 per cent.

Metabolism of a "sulfapyridine-fast" strain of Pneumococcus Type I (MacLeod). The experimental production of "sulfapyridine-fastness" in a strain of Pneumococcus Type I was described in the preceding report. "Fastness" has been shown to be associated with a relatively stable alteration in metabolism without changes in morphology, type-specificity, or virulence of the pneumococcus. Studies of certain metabolic activities of

the parent and drug-fast strains have revealed differences which may be significant in relation to the mechanism whereby sulfapyridine is able to exert a bacteriostatic action on the growth of these microorganisms.

Hydrogen peroxide is formed in cultures of pneumococcus as a product of aerobic metabolism. When grown in a shallow layer of broth exposed to air, the parent strain produces an abundance of hydrogen peroxide while the drug-fast strain under the same conditions forms only a small amount. The relation of peroxide production to carbohydrate metabolism is unknown; however, the decreased formation of peroxide in the drug-fast strain is associated with changes in its dehydrogenase activity.

The dehydrogenase activity of the two strains was studied by determining the ability of cell suspensions to reduce methylene blue in the presence of various substrates. In the preparation of the bacterial suspensions care must be taken to minimize exposure to air in order to preserve the dehydrogenase activity of the cells.

Of the substrates tested, glucose is an active hydrogen donator in the presence of both strains of pneumococcus and no difference is observed in the time required for the reduction of methylene blue. On the other hand, the drug-fast strain shows little dehydrogenase activity for glycerol, lactate or pyruvate, whereas the parent strain dehydrogenates these substances actively.

In the above experiments the dehydrogenase activity of the cell suspensions was tested in the absence of sulfapyridine. When sulfapyridine is added to the reacting system, there is no inhibition of the glucose dehydrogenase activity of either strain. However, the drug greatly inhibits the dehydrogenation of glycerol, lactate, and pyruvate by the parent cells. It would appear, therefore, that the bacteriostatic action of

sulfapyridine may depend in part upon the inhibition of the enzyme systems
associated with the dehydrogenation of glycerol, lactate, and pyruvate.
Furthermore, when a strain of pneumococcus becomes "fast" to sulfapyridine
the dehydrogenation of the same 3-carbon compounds is greatly decreased.
These systems no longer function as actively in the metabolic processes of
the cells of the "fast" strain as they do in those of the parent strain.

The occurrence and nature of a substance which annuls the bacter-
iostatic action of sulfonamide compounds (MacLeod and Mirick). The presence
of a substance in peptone which greatly diminishes the bacteriostatic action
of sulfonamide compounds in vitro has been observed by many investigators.
The occurrence of this material in the peptones used in the preparation of
the usual bacteriological media has made comparative bacteriostatic tests
difficult to interpret, since different lots of media prepared under apparent-
ly identical conditions may contain different amounts of the "sulfonamide
inhibitor". However, the presence of this substance is not restricted to
peptones, since it has now been found to exist in certain body tissues
particularly in those in which autolytic changes have taken place. Purulent
exudates are rich in the substance as are also tissues containing necrotic
lesions. This inhibitor is of importance, therefore, not only with respect
to the in vitro bacteriostatic tests but also in relation to the lack of
bacteriostatic action of these drugs frequently observed clinically in the
presence of purulent lesions.

B. coli has been used as a test organism for the detection of
the inhibiting substance, since it will grow in a simple synthetic medium
of which all the constituents are known. In this synthetic medium certain
of the sulfonamide derivatives exert a pronounced bacteriostatic effect on
B. coli; however, if materials containing the inhibitor are added the

bacteriostatic effect is either partially or completely annulled. On the other hand, complex culture media containing muscle infusion and peptone are customarily used in the cultivation of pneumococcus and streptococcus hemolyticus. Since both of these ingredients contain varying amounts of the sulfonamide inhibitor an accurate determination of the bacteriostatic effect of sulfonamide compounds on organisms in nutrient broth is difficult and often misleading.

It has been found, that fresh liver is free of the inhibiting substance and that an infusion of fresh liver which has been prepared without heating on the alkaline side will support the growth of pneumococcus in the absence of peptone. Moreover, the inoculum necessary for the initiation of growth in this medium is much less than that required in the usual media. In liver infusion, sulfanilamide in a dilution as great as 1:1,000,000 exerts a pronounced bacteriostatic effect upon Streptococcus hemolyticus, and sulfapyridine in the same dilution is bacteriostatic for pneumococcus. On the other hand, in certain lots of peptone-containing media only slight bacteriostatic effect may be observed when these drugs are used in concentrations as high as 1:10,000.

Up to the present time the results of bacteriostatic tests in which a liver infusion medium was used have been highly consistent, and this technique is being used to determine the possible acquisition of drug-fastness in strains of pneumococcus isolated from patients before and after treatment with sulfapyridine.

The presence of the inhibiting factor is associated with the occurrence of autolysis in tissues or exudates. Thus, fresh muscle contains only a small amount of the substance, but if autolysis takes place a great increase in inhibitor occurs. Pancreas and spleen are rich in the material,

but none is demonstrable in fresh liver. As previously mentioned, purulent

exudates obtained from patients with staphylococcal, pneumococcal, and

streptococcal infections, as well as fresh guinea pig liver containing

caseous tuberculous lesions have been found to yield large amounts of the

drug-inhibiting substance.

Because of the importance of these facts in relation to the

bacteriostatic effect of sulfonamide compounds both in vitro and in vivo

an investigation of the biochemical properties of the inhibiting substance

has been undertaken. The active principle is readily dialysable through

cellophane; it is heat-stable at both alkaline and acid reactions but is

destroyed on prolonged heating with strong acid or alkali. It is soluble

in ethyl alcohol, butyl alcohol, and acetone but insoluble in ether. On

the acid side the material can be readily absorbed from aqueous solution

with charcoal. The active substance can be eluted from the charcoal with

hot alcohol and pyridine. Further purification of the eluate can be

effected by precipitation of inert materials with isobutyl alcohol and

acetone.

Studies on a bactericidal agent extracted from cultures of a

sporulating bacillus (Dubos, Cattaneo and Hotchkiss). It was reported last

year that a bactericidal principle had been extracted from cultures of a

sporulating bacillus isolated from soil. The present report describes the

methods of purification of this bactericidal agent, studies on its chemical

nature and properties, and some observations on the mechanism of its action

on susceptible bacteria.

Preparation of crystalline substances which exhibit bactericidal

activity: The active principle is released in solution in autolysates of

peptone cultures of the soil bacillus; it is separated from the culture

media by precipitation at pH 4.7. Extraction of the acid precipitate with alcohol, acetone, or dioxane yields a fraction soluble in these organic solvents which carries all the bactericidal activity of the original material. The bactericidal principle present in the alcohol or acetone solution is practically insoluble in water in the presence of electrolytes; it is precipitated by diluting the alcohol or acetone solution in 10 volumes of aqueous saline. The precipitate thus obtained is free of protein; it carries the bactericidal activity of the original culture and can be desiccated without loss of activity.

Further purification is obtained essentially by adding ether to an alcoholic solution of the protein-free product to precipitate selected fractions. It is necessary to repeat the precipitations a few times before the fractions represent entirely distinct groups of substances. This is true because some of the substances present can modify considerably the solubilities of others.

Inert material is found in fractions soluble in ether (fatty acids and waxes) and in fractions insoluble in absolute alcohol. The bactericidal material is collected in two fractions; 1) material insoluble in a mixture of one volume of alcohol and 15 volumes of ether, and 2) material soluble in the same mixture but insoluble in pure ether. From fraction (1) there were isolated by crystallization from alcohol two crystalline acidic substances, which have been designated graminic acid and gramidinic acid. From acetone solutions of fraction (2) there was isolated a crystalline neutral substance which has been named gramicidin.

From every one hundred liters of culture there were obtained about 10 grams of protein-free product which yielded approximately 6 grams of mixed graminic and gramidinic acids and 1.0 to 1.5 grams of gramicidin,

Bactericidal activity of different crystalline fractions. As
stated above three fractions endowed with bactericidal activity have been
obtained in crystalline form. 0.005 to 0.01 mg. of these substances is
sufficient to kill in vitro 10^9 pneumococci of Group A streptococci in 2
hours at 37^0C; gramicidin is probably twice as active (per weight) as either
graminic or gramidinic acid. Still smaller amounts of either fraction in-
hibit the growth of Gram positive bacteria in nutrient broth. This is
particularly striking in the case of pneumococci which failed to grow in
nutrient broth containing a dilution of 1:1,000,000,000 of the active sub-
stance.

The standard test used for estimating the activity in vivo of
the preparations of bactericidal substance has consisted in determining the
minimal amounts of substance which, when injected intraperitoneally within
30 minutes after infection, will protect mice against 10,000 fatal doses of
Type I pneumococci.

In spite of the great bactericidal activity which graminic and
gramidinic acid exhibit in vitro, these substances appear to be ineffective
in vivo. On the contrary, one single dose of 0.001 to 0.002 mg. of gramici-
din, injected into the peritoneal cavity, is capable of protecting mice
against 10,000 fatal doses of pneumococci or hemolytic streptococci. The
material has been found equally effective against infection with five differ-
ent types of pneumococcus, eleven types of Group A streptococcus, and five
strains of Group C streptococcus. By using larger doses of the bactericidal
agent and repeating the treatment on three consecutive days, we have been
able to protect mice against 1,000,000 fatal doses of pneumococcus and to
cure mice of a well-established infection, even when treatment was admin-
istered 6, 12, and 17 hours after injection of the infective inoculum.

Marked protection has also been obtained against infection with a mouse virulent strain of staphylococcus. Finally, preliminary results obtained in collaboration with Dr. R. B. Little indicate that gramicidin also exhibits some effectiveness when injected into the udder of cows suffering from Group C streptococcus mastitis.

Extraction from cultures of the soil bacillus of a water-soluble form of the bactericidal substance. The results which have just been reported demonstrate that gramicidin when injected into the peritoneal cavity of mice is very effective against infection with pneumococci and streptococci. However, the same substance, when injected intravenously, subcutaneously, or intramuscularly, fails to protect mice against infection with the same organisms. Several possibilities may be invoked to account for this failure: a) the active substance, when introduced into the general circulation, may be eliminated so fast that it never reaches the effective concentration; b) it may be inactivated in some tissues, for instance, by hydrolysis, oxidation, or conjugation; c) the active substance is known to be very insoluble in aqueous media and may therefore fail to diffuse and reach the different foci of infection.

In any case, it is obvious that the insolubility of the material in aqueous media is a great handicap both for experimental studies and for possible use in therapy. It has been recently found that a number of dispersing agents such as sulphonated and sulphated oils, permit the substance to remain in solution in water even in the presence of electrolytes; ox bile also acts as a particularly effective dispersing agent, 2 cc. being sufficient to maintain 10 mg. of gramicidin in solution.

Furthermore it has been possible to extract from cultures of the soil organism which produces gramicidin a form of this substance which is completely soluble in water without the help of dispersing agents. The

soluble preparation is obtained by the following technique. The culture is precipitated in the cold at pH 4.7; the precipitate is resuspended in phosphate buffer at pH 7.5 and allowed to stand in this medium for 48 hours; the cell bodies and other insoluble material are then removed by prolonged centrifugation at 4,000 r.p.m.; the supernatant fluid is precipitated in the cold at pH 4.7; the precipitate is taken up in distilled water, neutralized with dilute NaOH, and filtered through a Berkfeld candle; the filtrate is again precipitated at pH 4.7, washed with saline and kept in the precipitated form at ice-box temperature.

The fraction thus obtained is immediately and completely soluble in neutral aqueous media; it is very effective both in vitro and in vivo. Moreover, in a number of instances, it has been possible to cure mice of pneumococcus peritonitis or septicemia by the subcutaneous injection of this new water soluble fraction.

The method of preparation of the soluble fraction and the technique of its administration to experimental animals have not yet been perfected to the extent that the results are regular; they are, however, unequivocal, and suggest that systematic experimentation would yield a form of the bactericidal substance more efficacious in vivo than are the fractions which have been crystallized.

Mechanism of the bactericidal action. Although the bactericidal substance does not inactivate any of the bacterial hydrolytic enzymes so far tested, it markedly alters the carbohydrate metabolism of the susceptible microbial species. More specifically it has been found that graminic acid inhibits quantitatively the acid production and the reducing power of suspensions of pneumococci, streptococci, and staphylococci in sugar solutions. On the contrary, gramicidin markedly stimulates, at least for three hours, both the acid production and the reducing action. This

contrast in the effect of gramicidin and graminic acid on the metabolic functions of the susceptible species is still the more remarkable when it is remembered that both these substances exhibit _in vitre_ the same bactericidal effect. It is likely that an understanding of the mechanism of these phenomena will throw much light on the nature of the bactericidal action. It may be stated here that other workers have also observed (personal communication) that the two fractions behave differently in other biochemical tests; especially striking is the fact that gramicidin forms very stable monomolecular films with dehydrocholesterol whereas the sterol-graminic acid mixed films can readily be destroyed under the influence of low pressures. In any attempt to explain the bactericidal effect of the substances under consideration, it will be necessary to keep in mind that the bactericidal effect is limited to certain groups of microorganisms. Pneumococci, streptococci, staphylococci, diphtheria, and diphtheroid bacilli, and the aerobic sporulating bacilli, all of which being Gram positive organisms, are extremely susceptible to the bactericidal agent. On the contrary, all the Gram negative bacilli so far tested have been found to be resistant to it. Recent experiments have established that gonococci and meningococci are more resistant than the Gram positive organisms and much more susceptible than the Gram negative bacilli; it is of interest to point out in this respect that although meningococci and gonococci react negatively to the Gram stain, they are very different in all other respects from the Gram negative bacilli, and that many bacteriologists have considered them as intermediary between the Gram positive organisms and the Gram negative bacilli.

How then is the behavior of the different microbial species toward the Gram stain correlated with their susceptibility to the bactericidal

agent? At least three possibilities can be considered at the present time:

a) the resistant species are capable of decomposing the bactericidal sub-

stance;   b) the susceptible species contain a cellular substrate for which

the bactericidal agent has a great affinity;   c) the cell membranes of the

two groups of organisms are different, for instance, the membrane of the

resistant species is impermeable to the bactericidal agent.

Many observations, and a few experimental facts could be presented

to illustrate possible avenues of analysis of this problem. In particular,

it is a striking fact that many substances of varied chemical structure

exhibit differential selectivity with reference to their bactericidal

activity toward Gram positive and Gram negative microorganisms. It appears

that the structure of the bacterial cell, as reflected in its staining

reactions, conditions the bactericidal efficacy of different chemical agents.

A comprehensive survey of the comparative effect of properly selective

chemical groups upon representative bacterial forms might give important

clues concerning the structure of the microbial cell and suggest a rational

basis for the development of antisepsis and chemotherapy.

Studies on the chemical nature of crystalline bactericidal

substances prepared from cultures of a sporulating bacillus (Hotchkiss and

Dubos). The results which have just been summarized show that three bacteri-

cidal substances have been obtained, one of which, gramicidin, has an effect

in vitro and in vivo demonstrably different from that of the other two. It

has already been indicated that gramicidin is a neutral substance whereas

the others are acids. It is to be supposed that further chemical knowledge

about the three substances might make it possible to know what chemical or

physical principles are responsible for the toxic action of these substances

toward Gram positive bacteria and toward higher animals, and why only one of

the substances is effective against intraperitoneal infections in the mouse.
Accordingly a chemical study of these substances has been undertaken.

Gramicidin, which has been the most investigated, appears to
have a molecular weight of about 1400. For substances of this complexity
an empirical formula cannot be determined accurately by ordinary elementary
analyses. The analytical data can be represented by the formula
$C_{74}H_{106}N_{14}O_{14}$ or formulae differing from it by one or two atoms of carbon
or hydrogen or by the difference C_5H_7NO. Upon acid hydrolysis, a quantity
represented by the above formula liberates a) a total of 11 equivalents of
non-volatile acid, b) a total of 11 equivalents of basic groups, c) 10 moles
of $\propto$ amino acid, d) 2.4 moles of l-tryptophane, e) a small quantity of an
aliphatic fatty acid, f) the amino acid leucine (amount not determined).
Although tryptophane is fairly stable in acid when pure or in the absence
of carbohydrates or aldehydes, some destruction does occur and an error may
result especially in items b) and d). The substance contains neither
methoxyl nor acetyl groups; and tyrosine, ammonia and basic amino acids
are not found in the hydrolysate.

The data at this point are sufficient to indicate that gramicidin
is essentially a polypeptide containing 10 molecules of $\propto$ amino acids of
which two or three are tryptophane residues. The remainder of the molecule,
comprising probably 10 to 15 per cent of the weight, is very likely involved
in substituting the terminal carboxyl and amino groups of the peptide chain
to form neutral amides or esters. The nature of the individual amino acids
and, in particular, that of the remaining constituents, not amino acid in
character, is being investigated at present. Derivatives of some of them
have been isolated and their identification is in progress.

Graminic acid and gramidinic acid are similarly built up from
amino acid but contain one free carboxyl group per molecule. They appear

to contain a lower proportion of tryptophane and, furthermore, each of them contains the amino acid tyrosine which is not present in gramicidin. Graminic acid has a molecular weight of about 900 and can be represented by the formula $C_{44}H_{63}N_9O_{11}$ with the same alternative as for the preceding formula. Gramidinic acid has a molecular weight of approximately 1000.

Through the cooperation of Dr. Fritz Lipmann of the Cornell University Medical College, indications have been obtained by the use of Krebs' d-amino acid oxidase that acid hydrolysates of gramicidin and of gramidinic acid have nearly one-half their α amino acids in the form of dextro – the so-called unnatural – amino acids. The fact that the tryptophane isolated from gramicidin had exclusively the natural or levo form indicates that racemization during hydrolysis probably did not occur. It is, therefore, possible to conclude that these bactericidal polypeptides contain a large proportion of dextro-amino acids. As the isolation and identification of the products of acid hydrolysis proceeds it should be possible to determine with certainty which of the amino acids are present in the unnatural form. The presence of dextro-amino acids may prove to be of interest in connection with the mechanism of action of the bactericidal agents, since it is known that peptides of dextro-amino acids are not hydrolyzed by most of the known peptidases and proteinases.

Toxicity for dogs of a preparation of the bactericidal substance (MacLeod, Curnen, and Mirick). The toxicity of a purified protein-free preparation of the bactericidal substance derived from a soil bacillus by Dubos was tested in young, healthy, short-haried dogs weighing from 8 to 12 kgs. An alcoholic solution of the substance was diluted in 20 cc. of a 5 per cent solution of glucose and injected intravenously in daily doses varying from 0.5 to 2.0 mg./kg. of body weight. The injections were continued

for 10 days in the dogs which survived. Clinical records were kept and pathological studies of the organs were made at autopsy.

Seven of the 8 animals, which received from 0.4 to 2.0 mg./kg. died as a result of the injections and in 6 of these death occurred before the course of 10 daily injections was completed. The remaining dogs were sacrificed from 23 to 54 days after the course of injections was begun.

The more prominent signs of toxicity during life were loss of weight, anorexia, fever, anemia, hematuria and the excretion of bile in the urine. In the animals which died acutely marked congestion of the viscera was present and petechial hemorrhages were observed in the heart, lungs, and kidneys. The livers showed acute central necrosis associated with hemorrhage and dilatation of the sinusoids. The spleens were hemorrhagic and pronounced phagocytosis of erythrocytes by the macrophages was present. In the animals which received daily 0.3 mg./kg. or more and which did not die acutely, the changes in the organs were of a more chronic nature. The liver cells showed fatty degeneration which was most marked at the center of the lobules. In these areas there was an increase in reticular tissue but cirrhotic changes were not pronounced. Ascites was present in two of these animals. The only change noted in the organs of the animals receiving 0.2 mg./kg. or less was a slight degree of fatty degeneration of the liver.

Effect of the bactericidal substance on experimental pneumococcus pneumonia in dogs (MacLeod, Ournen, and Mirick). The bactericidal substance was tested in dogs for its efficacy in the treatment of experimental pneumonia. The disease was produced in morphinized animals by the intrabronchial instillation of Type I pneumococci and mucin according to the method of Robertson. Clinical studies comprising bacteriological, hematological and X-ray examinations and the pathological study of the organs removed at autopsy have been carried out in all cases. Over a period of several weeks

groups of dogs were infected, half of the animals in each group receiving treatment and half serving as controls. Therapy was carried out by the intravenous injection of the bactericidal substance in a 5 per cent glucose solution. The amount of bactericidal substance given per day varied from 0.09 to 0.3 mg./kg. of body weight, administered in divided doses. Treatment was begun from 4 to 24 hours after infection except in one group of animals where 0.2 mg./kg. was administered twice during the 24 hours prior to infection and continued daily thereafter.

Twenty-three dogs with experimental pneumonia were treated and an equal number served as controls. The incidence of bacteremia in the treated dogs was 78 per cent and 18 died, a mortality of 78 per cent. Six of the treated animals developed bacteremia after treatment was begun. The incidence of bacteremia in the untreated dogs was 83 per cent and 16 died, a mortality of 70 per cent. The average period of survival in the treated dogs was 77 hours and in the controls 71 hours.

Under the conditions described, treatment with a purified preparation of the bactericidal substance showed no evidence of therapeutic effect upon experimental Type I pneumonia in dogs.

Induced natural resistance (Goodner). Certain epidemiological aspects of pneumonia suggest the possibility of a relation between dietary habits and the incidence or severity of the disease. This suggestion, although vague, and until now rather remote, has prompted exploration into that field of immunology which is not concerned with antibodies or with the specific character of resistance to infectious disease. This field occupies a heterodoxical position except in so far as all workers recognize the existence of a natural resistance not explicable in terms of the antibody theory. In a rather general sense the protective mechanism is

conceived as having a physiological character but the biological mechanisms concerned have not been identified.

As a preliminary test of this proposition, mice were given unaccustomed diets, that is, certain vegetables or fruits were offered as the sole source of nourishment. After a period of a few days the normal diet was restored. The animals were then tested for resistance to Type I pneumococcus infection, 300 minimal infective doses being given by the intraabdominal route. Some of these animals survived although the control animals invariably died. Moreover, it was noted that survival was not associated with all fruits and vegetables but was sharply limited to certain particular items.

These results suggested the possibility that the animals had acquired substances from the foods which had enhanced resistance. Since the results did not correspond with the incidence of well recognized vitamins, attempts were then made to extract the substances responsible. Infusions of the various materials were clarified and injected subcutaneously. In many instances subsequent infection did not lead to death. These extracts could not be shown to be either bacteriostatic or bactericidal. Indeed, it could be shown that, even in animals which survived, the pneumococci persisted for several days, often with increased numbers. The physiological mechanism of the mouse simply seemed to ignore the presence of the pneumococci; no cellular reaction was produced, nor were the animals ill. This corresponds well with our knowledge of natural resistance. For example, the rabbit cannot be "infected" with human tubercle bacilli. It is known, however, that these organisms survive in the rabbit --- the simple fact is that this animal does not react, does not form tubercles, and therefore does not develop tuberculosis.

A survey as to the presence of these protective principles in nature has been extended to over 200 sources. By way of example a few instances may be cited:

	Present	Absent or relatively so
Vegetables	Beet tops Lettuce Parsley Spinach	Beet roots Celery Green beans Tomatoes
Fruits	Orange (California)* Grapefruit Greengage plums	Orange (Florida) Apples Grapes
Grains	Corn* Oats* Wheat*	Alfalfa Millet Rape
Herbs	Boneset Chamomile Delphinium Horehound	Elder flowers Mustard, ground Plantain Sassafras

* Not present in most of the edible derivatives

Early attempts at isolation of the active material eliminated the possibility of proteins and fats. Active fractions always gave a positive Molisch reaction and some reducing sugar after acid hydrolysis. This was suggestive of the glucosides. Many of the source materials were known to yield glucosides of definite composition. A comparison of our results with the available information brought out the suggestive fact that the active extracts were from materials known to yield flavones, flavanols, and flavanones (collectively known as anthoxanthins) with hydroxy groups in the 5,7 positions on the benzopyrone ring structure. Isolations based on this deduction have substantiated the general premise although much remains to be done along this line to show that other substances may not possess this biological property. With feeding, and with the injection of crude extracts, it has never been possible to protect all mice in any group. The reasons for

this are not known but it has been noted that, as purification proceeds, the results have become more consistent.

Pneumococcus heterophile antigen (Goebel). In 1911 Forssman observed that emulsions of guinea pig tissue stimulated the production of lysins for sheep red corpuscles when injected into rabbits just as did sheep erythrocytes themselves. Since this important discovery there have been many investigations concerning the Forssman phenomenon. The Forssman antigen has been found widely distributed in nature, not only in the tissues of animals, but in many microorganisms and in plants as well. The chemical nature of the true Forssman hapten, however, has never been fully elucidated.

In a previous report evidence was presented which established a direct chemical relationship between the blood group specific substances and the capsular polysaccharide of Type XIV Pneumococcus. Through this study it was possible to explain the reason underlying the untoward and frequently fatal reactions accompanying therapy with Type XIV antipneumococcus horse serum. Certain diseases in man notably infectious mononucleosis and serum sickness following therapy in acute lobar pneumonia are accompanied by a marked increase in the hemolytic titre of the serum. Some of the obscure phenomena of intoxication during the acute phase of the infection may possibly be related to the presence of Forssman antigen in the infectious agent. It seems not unlikely therefore that a thorough investigation of Forssman substances as they occur in tissue and in microorganisms may lead to an explanation of their role in infection and resistance. At the same time the results of such a study may reveal the chemical basis underlying the phenomena of tissue and blood group specificity.

We have chosen therefore to investigate the chemical nature of the Forssman hapten in the avirulent or R strain derived from Type I Pneumococcus. The results of the investigations are briefly as follows: The

Forssman antigen as it occurs in pneumococci is thermostable and resistant
to autolysis. The hapten portion of the antigen is partially liberated
during the autolytic process, but filtrates of autolysed cells contain
little or no intact antigen. The latter appears to be bound to the cell
detritus and in this form it is still fully antigenic. The Forssman hapten
is not identical with the pneumococcus "C" or species specific polysaccharide,
for the residue or cell bodies after autolysis give rise in rabbits to
potent hemolytic antisera which are devoid of precipitins for the "C" sub-
stance.

The cellular detritus of autolysed pneumococci is a complex mix-
ture of protein, carbohydrate, and lipids. The protein may be digested away
with trypsin leaving an insoluble material which is rich in Forssman sub-
stance. There is no loss in Forssman hapten following tryptic digestion.
The residue obtained from the enzymatic hydrolysis is composed of an un-
characterized material and some fifty per cent of soluble lipids, which can
be extracted with boiling alcohol and ether. In so doing there occurs a
diminution in the active Forssman hapten, but this loss cannot be accounted
for by the presence of the substance in the lipid extract. The residue left
after extraction with the organic solvents is, however, still rich in Forss-
man hapten. The latter can be separated by extraction with aqueous pyridine.
In this fashion a solution of the Forssman hapten has been obtained. This
material is highly active and appears to be essentially free from protein
and rich in carbohydrate. Further work directed toward the characterization
of this substance is in progress.

Synthetic antigens: Investigations carried out in this labora-
tory on the chemical and immunological properties of bacterial polysaccharides
and artificial antigens containing simple saccharides of known constitution

have revealed many of the factors which govern the immunological specificify

of encapsulated microorganisms. As our knowledge in this field has advanced

we have come to believe that it should be possible to incite antibacterial.

immunity in experimental animals with artificially compounded antigens con-

taining immuno specific groups of synthetic origin rather than with anti-

genically complex bacterial cells as they naturally occur.

     In the report of last year it was shown that sera of rabbits

immunized with an artificial antigen containing the naturally occurring aldo-

bionic acid 4-β-glucuronosido glucose (cellobiuronic acid) agglutinate

Type III pneumococci and confer passive immunity on mice against infection

with multiple lethal doses of virulent Types II, III, and VIII organisms.

A similar antigen containing the synthetic isomeric aldobionic acid 6-β-

glucuronosido glucose (gentiobiuronic acid) failed however to evoke in

rabbits agglutinins for Type III pneumococci or protective antibodies for

Types III and VIII organisms. Thus it was proved that the chemical con-

stitution of the aldobionic acid determines the capacity of the corresponding

artificial antigen to incite antibacterial immune bodies specifically

directed toward pneumococci of Types III and VIII.

     Although gentiobiuronic acid antiserum failed to protect mice

against infection with either Types III or VIII organisms it has now been

found to afford protection against infection with as much as 100,000 minimal

lethal doses of Type II pneumococci. Since this property is shared by

cellobiuronic acid antiserum it is evident that the differences in the inter-

molecular structure of the two aldobionic acids is unimportant in determin-

ing the specificity of antibodies evoked by the corresponding antigens to

Type II pneumococcal infection in mice.

     It has been possible to show that the protective action of

gentiobiuronic and cellobiuronic acid antisera to Type II pneumococcal in-
fection is due to an antibody directed toward the glucuronic acid con-
stituent common to the aldobionic acid antigens. Two artificial antigens
have been prepared; one containing the azo benzyl glycoside of glucuronic
acid and the other that of the isomer, galacturonic acid. These two hexose
uronic acids differ only in the configuration of the fourth carbon atom where
the position of the H and OH group is interchanged.

p-aminobenzyl galacturonide   p-aminobenzyl glucuronide
$$R = -CH_2C_6H_4NH_2$$

This alteration in configuration of but one carbon atom suffices however to
determine the capacity of the hexose uronic acid antigens to evoke anti-
bacterial immunity. The sera of rabbits injected with the glucuronic acid
antigen protect mice against infection with 100,000 minimal lethal doses of
Type II pneumococci, whereas the antiserum to the galacturonic acid antigen
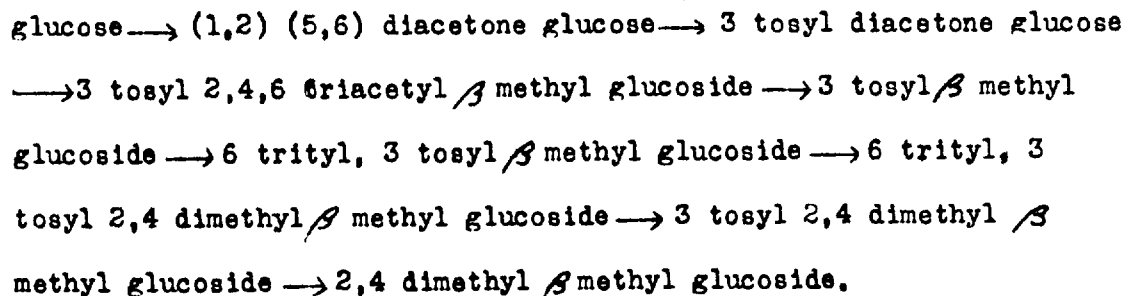is devoid of any protective action.

From the foregoing it is evident that antibacterial immunity can
be incited in rabbits with artificial antigens containing simple saccharides
prepared entirely by chemical synthesis. These observations are of course
directly related to the general problem of the factors underlying the
specificity of bacterial polysaccharides. It is obvious that the specificity
of the latter must in part be determined by the position of the intermolecu-
lar linkages of the simple saccharide constituents and in part by the
stereochemical configuration of the hexose uronic acid constituents as
well.

The structure of the capsular polysaccharide of Type III Pneumo-
coccus (Adams and Goebel). The type specificity of the pneumococcus is
determined by the chemical structure of its capsular polysaccharide. The
relationship between chemical structure and immunological specificity among
the pneumococcus types has been demonstrated by reactions involving synthetic
antigens containing haptens which are sugars of known chemical structure.
Relatively minor changes in the stereochemical configuration or in the
position of linkage bonds in these sugar haptens are reflected in alterations
of the immunological specificity of the serum antibodies invoked by these
antigens and especially in the reactions of these antibodies with the various
pneumococcus polysaccharides. Because of this dependence of immunological
specificity upon the chemical structure of the haptens of artificial antigens,
it was thought that the structure of the capsular polysaccharides, the nat-
ural haptens of pneumococci, was deserving of investigation. Accordingly
the structure of the specific capsular polysaccharide of Type III Pneumo-
coccus has been the subject of research for some time

This polysaccharide is composed entirely of units of cellobiu-
ronic acid (4-glucuronosido-glucose) linked in glycosidic union to form an
extended chain of high molecular weight. At present our problem consists in
the determination of the position of the glycosidic linkage joining the
aldobionic acid units in this capsular substance.

When the capsular polysaccharide is methylated in the conven-
tional manner, the free hydroxyl groups form methyl ethers and the carboxyl
groups form methyl esters. If the methylated polysaccharide is reduced by
catalytic hydrogenation, the ester groups alone are changed by this process
to primary alcohol groups. The reduced polysaccharide yields on acid
hydrolysis the known 2 : 3 : 6 trimethyl glucose (from the glucose moiety

of the aldobionic acid units) and an unknown dimethyl glucose (resulting
from the reduced glucuronic acid portion of the polysaccharide). The most
reasonable configuration for this unknown derivative is 2:4-dimethyl glu-
cose, and on this assumption the synthesis of the latter compound was under-
taken according to the following scheme:

glucose —→ (1,2) (5,6) diacetone glucose —→ 3 tosyl diacetone glucose

—→3 tosyl 2,4,6 triacetyl /3 methyl glucoside —→3 tosyl/3 methyl

glucoside —→6 trityl, 3 tosyl /3 methyl glucoside —→6 trityl, 3

tosyl 2,4 dimethyl /3 methyl glucoside —→ 3 tosyl 2,4 dimethyl /3

methyl glucoside —→ 2,4 dimethyl /3 methyl glucoside.

The synthesis through the stage of 6 trityl, 3 tosyl /3 methyl
glucoside is rigorously exact since this compound yields on acetylation a
crystalline 6 trityl, 3 tosyl, 2,4 diacetyl /3 methyl glucoside having
characteristic physical properties and a correct analysis. The subsequent
steps in the synthesis have produced only uncrystallizable syrups, poorly
characterized and analytically impure. With the end in view of obtaining
some of the intermediates of the synthesis in crystalline form we have
constructed a high vacuum molecular still with which we have distilled un-
stable sugar derivatives of high molecular weight usually considered to be
outside the range of distillability.

A small amount of crystalline 2,4 dimethyl /3 methyl glucoside,
identical with that prepared from the hydrolysis products of the reduced
methylated Type III polysaccharide, has been synthesized according to this
scheme. The isolation of this same derivative both by direct synthesis and
by the hydrolysis of the Type III polysaccharide justifies the formula which
has been ascribed to the latter substance. We feel, however, that until
experimental difficulties encountered in the later stages of the synthesis

are overcome we cannot consider the structure of the Type III polysaccharide to be rigorously proven, although the evidence favors the structure given below.
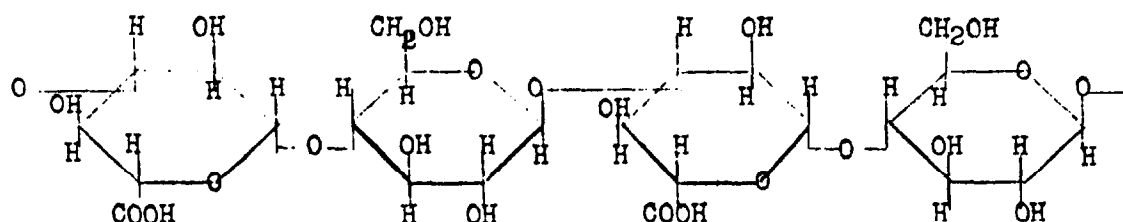
Studies in epidemiology (Stillman). An understanding of the epidemiology of pneumonia necessitates a knowledge of the capacity of organisms to invade, survive and multiply in the tissues once they have been implanted on their surface. The reaction of various laboratory animals to organisms which have been implanted on the mucous membranes of the upper respiratory tract has been studied. Mice have usually been employed, since they readily succumb to infection when the organisms are inoculated directly into the tissues. A comparison of the reaction of freshly isolated strains in the same host does offer an opportunity to study the variations that occur in various strains of the same and different types of organisms. The result of exposing mice to inhalations of freshly isolated strains of pneumococci has been reported. Since Webster has shown that mice may be infected by intranasal instillation of pneumococci, it was considered advisable to try this method as a further means of testing the virulence of freshly isolated pneumococci which had clinically demonstrated their ability to invade and multiply in the human host. From these studies it is apparent that freshly isolated strains of Type I pneumococci have a low virulence for mice when tested by intraperitoneal injection, inhalation or intranasal instillation. Strains of Type II and Type III pneumococci have a higher virulence as measured by each of these three methods. In considering the

virulence of pneumococci, it is important to consider the capacity of this

organism to gain entrance and to become disseminated throughout the body

following implantation on the mucous membranes of the respiratory tract.

Publications

Beeson, P. B., and Goebel, W. F., The immunological relationship of the capsular polysaccharide of Type XIV Pneumococcus to the blood group A specific substance, J. Exp. Med., 1939, 70, 239.

Dubos, R. J., Enzymatic analysis of the antigenic structure of pneumococci, Ergebn. Enzymforsch., 1939, VIII, 135.

Dubos, R. J., Studies on a bactericidal agent extracted from a soil bacillus
I. Preparation of the agent. Its activity in vitro.
II. Protective effect of the bactericidal agent against experimental pneumococcus infections in mice, J. Exp. Med., 1939 70, 1 and 11.

Dubos, R. J., and Cattaneo, C., Studies on a bactericidal agent extracted from a soil bacillus. III. Preparation and activity of a protein-free fraction, J. Exp. Med., 1939, 70, 249.

Goebel, W. F., Studies on antibacterial immunity induced by artificial antigens. I. Immunity to experimental pneumococcal infection with an antigen containing cellobiuronic acid, J. Exp. Med., 1939, 69, 353.

Goebel, W. F., Immunity to experimental pneumococcal infection with an artificial antigen containing a saccharide of synthetic origin, Science, 1940, 91, 20.

Goebel, W.F., Beeson, P. B., and Hoagland, C. L., Chemo-immunological studies on the soluble specific substance of pneumococcus. IV. The capsular polysaccharide of Type XIV Pneumococcus and its relationship to the blood group A specific substance, J. Biol. Chem., 1939, 129, 455.

Goodner, K., Horsfall, F. L. Jr., and Bauer, J. H., The neutralization of pneumococcal capsular polysaccharide by the antibodies of type-specific antisera, J. Immunol, 1938, 35, 451.

Hotchkiss, R. D., and Dubos, R. J., Fractionation of the bactericidal agent from cultures of a soil bacillus, J. Biol. Chem., 1940, 132, 791.

Hotchkiss, R. D., and Dubos, R. J., Chemical properties of bactericidal substances isolated from cultures of a soil bacillus, J. Biol. Chem., 1940, 132, 793.

MacLeod, C.M., Chemotherapy of pneumococcic pneumonia, J. Am. Med. Assn., 1939, 113, 1405.

MacLeod, C.M., Metabolism of "sulfapyridine-fast" and parent strains of Pneumococcus Type I, Proc. Soc. Exp. Biol. and Med., 1939 41, 215.

MacLeod, C.M., and Daddi, G., A "sulfapyridine-fast" strain of Pneumococcus Type I, Proc. Soc. Exp. Biol. and Med., 1939, 41, 69.

Reeves, R. E., Saccharolactone methyl ester, J. Am. Chem. Soc., 1939, 61, 664.

Smadel, J. E., Lavin, G. I., and Dubos, R. J., Some constituents of elementary bodies of vaccinia, J. Exp. Med., 1940, 71, 373.

Stillman, E. G., and Schulz, R. Z., Difference in virulence of various types of pneumococci for mice, J. Infect. Dis., 1939, 65, 246.

In Press

Beeson, P. B., and Goebel, W. F., Immunological cross-reactions of Type B Friedländer Bacillus in Type II pneumococcal horse and rabbit serum, J. Immunol, 1940.

Dubos, R. J., The adaptive production of enzymes by bacteria, Bact. Rev., 1940, 4.

Erf, L. A., and MacLeod, C. M., Increased urobilinigen excretion and acute hemolytic anemia in patients treated with sulfapyridine, J. Clin. Invest. 1940,

MacLeod, C. M., Mirick, G. S., and Curnen, E. C., Toxicity for dogs of a preparation of a bactericidal substance derived from a soil bacillus, Proc. Soc. Exp. Biol. and Med., 1940.

Stillman, E. G., The viability of pneumococci in dried rabbit's blood, Susceptibility of mice to intranasal instillation of various types of pneumococci, J. Infect. Dis., 1940.