

Report of Dr. Avery (assisted by Drs. Adams, Coburn, Curnen,  
Dubos, Goebel, Goodner, Hotchkiss, MacLeod, Mirick and Stillman)

Relation of the presence of the C-reactive protein to other evidences of disease activity (Curnen, Mirick and MacLeod). It has been shown previously that the presence of the C-reactive protein in the blood of patients during a variety of infections closely parallels the clinical course of the disease. During the acute phase of illness the precipitation test with C polysaccharide is most marked, and diminishes in intensity as convalescence is established. A specific antiserum prepared in rabbits by immunization with the C-reactive protein of human blood has provided another means of testing for the presence of this abnormal protein. As previously pointed out the immunological reaction with immune rabbit serum is far more sensitive than the precipitation test with the C polysaccharide in detecting small amounts of the reactive protein. For example, if a patient's serum known to contain the reactive protein is diluted 1:40 the precipitation test with the C polysaccharide may not be demonstrable. However, if the same acute phase serum is diluted 1:600 the presence of the reactive protein can still be detected by means of the specific antiserum (anti-CP serum). Use has therefore been made of the immunological reaction in determining how long the reactive protein persists in the blood of patients during the course of convalescence.

An attempt has been made to correlate the presence of the reactive protein during the acute disease and in convalescence not only with the state of the lesion itself but also with various clinical tests used as a measure of disease activity, particularly the erythrocyte sedimentation rate and leucocyte count. In a series of patients admitted to this hospital suffering from various acute infections of the respiratory tract, frequent determinations of the erythrocyte sedimentation rate have been made, both during the acute phase of

disease and in convalescence. Estimations of the amount of C-reactive protein have also been made on the same specimens of blood by the precipitation test with the C polysaccharide and the immunological reaction with anti-CP serum. The C-reactive protein is present in the blood of pneumonia patients soon after the onset of the disease. In some cases its presence in considerable amount precedes the increase in sedimentation rate. Likewise, in certain severe infections where the sedimentation rate remains normal, the precipitation reaction with the C polysaccharide may be strongly positive. In a number of instances late in convalescence when the patient's clinical condition has apparently returned to normal, the sedimentation rate has remained rapid, while only small traces of reactive protein were present in the blood as measured by the reaction with anti-CP serum. The reaction with the test carbohydrate becomes negative in patients with pneumonia when the temperature returns to normal. However, if tested with anti-CP serum the presence of the reactive protein may be demonstrated in the blood for a variable period after the reaction with the C polysaccharide has become negative. In certain other patients whose sera have not reacted at any time with the C polysaccharide, the presence of the reactive protein may be readily detected with specific antiserum.

The lack of parallelism between the titer of the C-reactive protein and the increased sedimentation rate applies also to the leukocyte count. The reactive protein has invariably been demonstrated in the blood during infections associated with leukocytosis, and in a general way the presence and amount of this protein parallels the degree of leukocytosis. Moreover, in lobar pneumonia, as the leukocyte count falls during convalescence, so also the amount of reactive protein in the blood diminishes, although it may be detected for a considerable period after the leukocyte count has returned to normal. In this connection it is worthy of note that in acute infections associated with leukopenia the reactive protein may be demonstrable in the blood.

From the data thus far obtained it appears that the titer of C-reactive protein in the blood during acute infections parallels both the clinical course and the activity of the lesion more closely than does either the erythrocyte sedimentation rate or the leukocyte count. The findings also suggest that the detection of the reactive protein by means of the C polysaccharide and anti-CP serum may be useful clinically as an index of disease activity, particularly during infections of a polycyclic nature such as rheumatic fever, tuberculosis, etc.

Development of drug-fastness in pneumococci isolated from patients treated with sulfonamide drugs (Mirick and MacLeod). Strains of pneumococci encountered clinically show considerable variation in sensitivity to the bacteriostatic action of the sulfonamide drugs. In a few instances it has been reported that drug-fastness may develop during the course of treatment but an adequate study of this important phenomenon has not been made by the more precise techniques recently developed in this laboratory.

The presence in ordinary culture medium of inhibitors to the sulfonamide drugs may so affect the outcome of bacteriostatic tests that the actual sensitivity of various strains may be impossible to determine by the usual cultural methods. To overcome this difficulty an infusion of fresh liver has been used in the present experiments, since this medium is not only free of sulfonamide inhibitor but is capable of supporting the luxuriant growth of small inocula of pneumococci. The bacteriostatic tests have been performed by seeding a standard inoculum of young, actively growing, pneumococci into tubes of the liver infusion containing varying dilutions of sulfapyridine.

Pneumococci have been isolated from twenty-two patients with pneumonia before or early in treatment as well as after receiving a full course of a sulfonamide drug, and the respective strains have been compared for their sensitivity to sulfapyridine. In ten instances there was an increase in drug

resistance of the organism isolated after treatment, averaging a fourteen-fold increase in the concentration of drug required for complete bacteriostasis. In eight cases there was no change. In four cases the culture obtained after treatment was more susceptible to sulfapyridine than the original, bacteriostasis being effected by one-third as much drug as was necessary for the strain when first isolated. An exception was a case of pneumococcal endocarditis where the final culture was fifty times more susceptible than the original. The greatest increase in drug resistance was seen in those cases receiving prolonged and continuous treatment.

A comparison of the in vitro test and the clinical response of the patient showed that eleven strains of pneumococci from thirteen patients who responded poorly to the drug grew well in a concentration of 1/10,000 sulfapyridine and five of these in 1/5,000 indicating definite drug-fastness of these strains. The strains isolated after treatment from twenty out of twenty-two patients who showed a good clinical response to the drugs were inhibited by a dilution of sulfapyridine of 1/20,000 or higher.

The difference in drug sensitivity shown in vitro between three susceptible and three resistant strains of *Pneumococcus* Types I, III, and IV was experimentally confirmed in vivo by comparing the therapeutic response of infected mice to sulfapyridine treatment. Further studies are planned to determine the conditions under which pneumococci develop resistance to the sulfonamide drugs, and the nature of the alterations brought about in the microorganisms when this phenomenon occurs.

Studies on sulfonamide inhibitors by the use of a soil bacillus capable of destroying para-aminobenzoic acid (Mirick). The action of the sulfonamide drugs both in vitro and in the animal body appears to be closely related to the occurrence of substances which inhibit their bacteriostatic effect. It has been

shown in this laboratory that inhibitory substances are present not only in the tissues and in certain fluids of the animal body, but also in bacteria. In some tissues, notably liver and kidney, inhibitor is not demonstrable until autolysis has taken place or the tissue has been subjected to mild acid hydrolysis. The inhibitor present in urine is likewise not demonstrable unless acid hydrolysis has been used.

Yeast and all the bacterial species studied have been shown either to contain inhibitor in the cells or else to produce such a substance in the culture medium in which they have been grown. The amount of inhibitor produced by bacteria varies considerably from one microbial species to another and between different strains of the same species. A strain of Pneumococcus Type I which had been rendered sulfapyridine-fast in vitro has been shown to produce approximately ten times as much inhibitor as the susceptible parent strain from which it was derived. However, the increased production of sulfonamide inhibitor by pneumococci is not the only factor involved in drug-fastness, since some strains which have become relatively fast in vivo do not produce any more inhibitor than do susceptible strains.

Woods has shown that p-aminobenzoic acid acts as a powerful sulfonamide inhibitor, and Rubbro and Gillespie have recently isolated this compound from yeast. It has been suggested by Fildes and Woods that p-aminobenzoic acid is an essential metabolite for various bacteria and that the sulfonamide drugs act by competitive inhibition of the enzyme systems concerned with its metabolism. More recently it has also been shown that p-aminobenzoic acid is an accessory growth factor for Clostridium acetobutylicum, as well as for chicks, and its role as an anti-grey hair factor for rodents has been demonstrated by Ansbacher.

Although extracts of various organs and of bacterial cells exert a pronounced inhibitory effect on the sulfonamide drugs, it by no means follows

that the active substances exhibiting this property are in all instances identical with p-aminobenzoic acid. Certain of the chemical properties of the naturally occurring inhibitors lend support to this view. Consequently a method was sought which would differentiate between the inhibitors from various sources without recourse to the laborious procedures of chemical isolation and identification. Although by the very sensitive diazo reaction, p-aminobenzoic acid may be detected in concentrations as small as one part in a million, this compound is active as a sulfonamide inhibitor in a dilution 100 times greater than that which can be detected by diazotization. Moreover, the diazo reaction is not specific for p-aminobenzoic acid.

The possibility suggested itself that there might occur in nature microorganisms possessing enzymes capable of so altering p-aminobenzoic acid that it no longer is active as a sulfonamide inhibitor. We have isolated from alkaline soil a bacterium capable of modifying p-aminobenzoic acid so that this compound no longer gives the diazo reaction and is completely inactive as a sulfonamide inhibitor. Following the technique so successfully employed by Dubos in the adaptive production of other bacterial enzymes, the isolation was accomplished by inoculating alkaline soil into a simple basal medium to which p-aminobenzoic acid was added as the sole source of carbon and nitrogen.

The soil bacterium capable of annulling the action of p-aminobenzoic acid is a small, motile aerobic, Gram negative bacillus. It is not pathogenic for mice, ferments no sugars, and produces an alkaline reaction in litmus milk. It grows abundantly and exerts its action on p-aminobenzoic acid in a synthetic mineral medium containing an acid hydrolysate of casein, as well as in ordinary laboratory media. Its action is not entirely specific, however, since it readily attacks acetylated p-aminobenzoic acid, novocaine, and also acts, although at a slower rate, on o-aminobenzoic and p-aminophenylacetic acids. It is inactive on the meta form as well as the methyl and ethyl esters of p-aminobenzoic acid,

arsanilic acid, sulfanilic acid, and the sulfonamide drugs.

Sulfapyridine 1:500,000 is bacteriostatic for the soil bacillus, and although it attacks p-aminobenzoic acid it is worthy of note that the latter compound is itself bacteriostatic for this bacillus. This statement at first glance appears paradoxical. However, a similar phenomenon has been observed in this laboratory in the case of the inhibition of growth of staphylococcus by hydrogen peroxide despite the fact that these bacterial cells contain an active catalase capable of decomposing this toxic compound.

The action of the bacillus on p-aminobenzoic acid is apparently accomplished by means of adaptive enzymes. Washed bacterial cells which have been grown in inhibitor-free synthetic medium are inactive. However, if cultivated in the presence of p-aminobenzoic acid the cells now contain potent enzymes capable of attacking this compound.

The action of the soil bacillus on some of the naturally occurring sulfonamide inhibitors has been studied. The inhibitory action of yeast extract is destroyed, confirming the evidence of others that yeast cells contain p-aminobenzoic acid. However, the inhibitor found in the cells of Streptococcus hemolyticus (Group C) is only slightly affected, and that occurring in peptone broth is not altered. In liver infusion devoid of free sulfonamide inhibitor, the growth of the soil bacillus gives rise to a potent inhibitory substance. In the latter instance, the inhibition is apparently not due to the presence of coenzymes, which have been shown to be inhibitory under certain circumstances, since the inhibitor formed in liver infusion is not inactivated by treatment known to destroy coenzymes.

The nature of the mechanism is unknown whereby the soil bacillus inactivates p-aminobenzoic acid and certain related compounds. However, the change produced is not due to acetylation or to decamination alone.

The observations thus far made indicate that the use of this soil bacillus in which the enzyme systems have been specifically oriented, affords a very useful method for the further study of the nature and action of the sulfonamide inhibitors and for the detection of minute amounts of p-aminobenzoic acid.

The effect of sulfapyridine upon the development in rabbits of specific immunity to pneumococcus (Curnen and MacLeod). It has been maintained that the effectiveness of the sulfonamide drugs in the therapy of pneumococcal infections in man is unrelated to the development of specific immunity to the invading microorganism. The evidence for this view is based upon observations that at the time of defervescence circulating antibodies are rarely demonstrable in the blood of patients treated with the drug.

Previous experiments in this laboratory have indicated that effective sulfapyridine therapy of experimental pneumococcal infection in mice is dependent upon development of a specific immune response. If drug therapy is discontinued before sufficient time has elapsed for active immunity to develop the animals do not survive.

The apparent discrepancy between the clinical and experimental observations was thought to be attributable to differences in the methods used for detecting the immune response. In patients recovering from pneumonia the development of immunity is recognized by employing serological techniques to demonstrate antibodies in the circulating blood. In animals, however, the presence of active immunity may be determined experimentally by infection at an appropriate interval following previous infection or prophylactic vaccination. Numerous studies have shown that under such circumstances active immunity to pneumococcal infection appears in both mice and rabbits before type specific antibody is demonstrable in the blood.

The present study was undertaken in order to establish whether or not



the administration of sulfapyridine alters the development of immunity to pneumococcus in rabbits and to compare the relative sensitivity of various methods for the detection of the early immune response. The experiments were carried out in rabbits because of their suitability for the study of both active resistance and the presence of humoral antibodies. The animals were first vaccinated and after intervals of 48, 72, and 96 hours thereafter comparable groups were infected to determine the degree of active resistance that had developed. Prior to vaccination and at intervals of 24 hours until just before infection, blood was drawn for serological studies which included tests for agglutinins, precipitins, and mouse protective antibodies.

Each rabbit was vaccinated intravenously by a single injection of heat-killed Type I pneumococci equivalent to 10 cc. of broth culture. Half of the vaccinated rabbits in each of the groups previously designated received sulfapyridine during the period when immunity might be expected to develop, that is, coinciding with and following vaccination. The drug was given by stomach tube in divided doses totalling 4.5 grams for each animal. Administration of the drug was scheduled to terminate so that at the time of infection no remaining "free" sulfapyridine could be detected in the circulating blood.

All vaccinated animals including those which had and those which had not been given sulfapyridine as well as an appropriate number of unvaccinated and untreated controls were infected by the intradermal injection of a rabbit virulent strain of Type I Pneumococcus. The infecting dose was invariably fatal for the control animals. The course of infection including the occurrence and degree of bacteremia was observed and recorded for each rabbit.

Regardless of the interval between vaccination and infection, there was no significant difference in the immune response of the rabbits which

had received sulfapyridine as compared with those which had not. Rabbits infected as early as 48 hours after vaccination irrespective of whether or not they had received sulfapyridine showed that active immunity of a type specific nature was already present at this time. Although the course of experimental infection was usually severe and accompanied by bacteremia 80 per cent of these animals survived. Further development of active immunity was manifested in the rabbits infected 72 hours after vaccination by a milder clinical course and a lower incidence of bacteremia, although the survival rate was the same as in the preceding group. Of the rabbits infected 96 hours after vaccination few showed bacteremia and all survived. Although demonstrable antibodies had not appeared in the sera of rabbits earlier, practically all of the sera obtained 96 hours after vaccination conferred protection on mice, and about half of these sera contained agglutinins and a few contained precipitin in low titer for the type specific polysaccharide.

From these observations it is evident that in rabbits the immune response to pneumococci is not influenced by the administration of sulfapyridine. Moreover, the development of active immunity considerably precedes the earliest appearance of antibody in the circulating blood. The experimental findings lend further support to the view that in man effective sulfapyridine therapy is intimately associated with the development of active immunity.

Epidemiological studies (Stillman). In continuing an epidemiological study of the dissemination of disease producing types of pneumococci, experiments have been carried on to determine how long these organisms will survive after being rapidly dried in the frozen state. It was found that the desiccated cells may remain viable for at least three years provided moisture is prevented from coming in contact with the dried material. More recently,

living organisms have been recovered from a specimen of dried cells preserved in hermetically sealed vials for a period of five years. In all instances the recovered strains have exhibited all the distinguishing characteristics of the original culture including capsule formation, type specificity, and virulence. In this connection it is interesting to recall that although pneumococci are not generally regarded as capable of surviving long under the naturally more rigorous conditions of the outside world, they have been isolated directly from dried pneumonic sputum which has been exposed to air and diffuse light at room temperature for from 4 to 8 weeks. Pneumococci preserved in dried rabbit blood and similarly exposed have been recovered a month later without suffering any loss in virulence or type specificity. Duplicate specimens similarly dried but stored in the cold have yielded living pneumococci when cultivated a year later. The capacity of individual strains of the same type to survive under these conditions varies, and there are distinct differences in this regard between different types of pneumococcus.

Studies on capsular synthesis by pneumococci (MacLeod and Avery).

The capacity of pneumococci to grow and produce disease in the animal body is conditioned by, if not wholly dependent upon, the activity of the particular group of enzymes concerned in the synthesis of the cell capsule. Capsular synthesis is most highly developed and the product of its activity most pronounced in cells best adapted to growth in the animal body. The presence in the capsule of a chemically unique and serologically reactive polysaccharide confers upon the cell a highly selective specificity which makes possible the differentiation of sharply defined and specific types within the species. The enzymes responsible for capsular synthesis can be reversibly inactivated by known changes in environmental conditions without impair-

ing the viability of the microorganisms. The selective inactivation of this particular function results in the loss of capsules, together with the consequent loss of type specificity and invasive properties. Under these conditions highly pathogenic microorganisms are reduced to a state in which they are no longer capable of inducing disease in animals highly susceptible to fatal infection with the originally encapsulated parent strain.

Important and essential as capsular synthesis is to the pathogenicity of pneumococci in the living host, this function is not vital to the life and growth of the microorganism outside the animal body, since cells in which the formation of capsules has been inhibited are still capable of carrying on the vegetative processes of metabolism and multiplication in artificial media. Capsule formation may be regarded therefore as an adaptive mechanism whereby the bacterial cell seeks to protect itself against the defense reactions of the host.

It has long been recognized that pneumococci can exist as in encapsulated or non-encapsulated forms which in terms of bacterial dissociation are referred to respectively as S and R variants. Under suitable conditions this form of dissociation ( $S \rightleftharpoons R$ ) is reversible and may occur spontaneously or be experimentally induced in response to environmental influences which favor either activation or inhibition of the capsule-producing enzymes. By appropriate methods these changes can be brought about in vitro. For example, it is possible to inactivate capsular synthesis in pneumococci of any specific type and to derive thereby the non-encapsulated R variant; conversely, it is also possible to reactivate these same R cells under conditions such that they invariably revert to the original type and regain all the specific characteristics of the parent strain from which they were derived.

In the study of the mechanism of capsular synthesis the phenomenon

of the transformation of specific types of pneumococcus lends itself more readily than does the direct form of reversible dissociation. Griffith first showed in mice that encapsulated cells of one specific type may be transformed into other specific types through the intermediate stage of the R forms. Dawson found subsequently that transformation may be effected in vitro without the use of animal inoculation, and Alloway succeeded in causing transformation by growing R cells in a medium containing small amounts of a filtered extract prepared by dissolving S pneumococci by means of desoxycholate. It is possible therefore to study the properties of the specific activator of capsular synthesis in a soluble form entirely free of bacterial cells, and to define the conditions under which it exerts its greatest activity.

In the preparation of potent extracts it is essential to work under conditions which limit as far as possible the action of the autolytic enzymes of the pneumococcal cells. Autolysis, whether occurring spontaneously or brought about by the action of the extracted enzymes, is known to destroy the transforming principle. Since the autolytic system is a mixture of various enzymes an attempt was made to determine if possible what particular enzyme is involved in breaking down the activity of extracts. For this purpose selective substances known to inhibit certain enzymes were tested.

MacLeod first showed that the transforming substance was not impaired when cell autolysis takes place in the presence of fluoride. Although under these conditions cell disintegration occurs, such autolysates are still potent in inducing transformation. Apparently fluoride selectively inhibits the enzymes responsible for the destruction of the transforming principle without impairing the activity of the enzymes which bring about cell lysis.

Cell-free extracts of encapsulated pneumococci prepared by dissolving the bacteria in sodium desoxycholate at low temperature are immediate-

ly heated at 65°C. for 30 minutes in order to destroy the bacteriolytic enzymes. Sodium desoxycholate is removed by precipitating the extract in alcohol, in which the desoxycholate is soluble. The alcohol precipitate is then dissolved in saline at a slightly alkaline reaction and the bacterial protein removed by shaking repeatedly with chloroform according to the method of Sevag. Extracts prepared in this manner contain considerable amounts of nucleic acid. The latter substance may be almost completely removed by digestion with crystalline ribonuclease without affecting the transforming potency.

Provided the various purification procedures are carried out within a fairly narrow pH range, little of the specific transforming activity is lost, and protein-free and lipid-free extracts have now been obtained which will effect the complete transformation of R cells to the S form when used in amounts corresponding to 1.0 cc. or less of the original culture from which the extract was prepared.

The transforming activity of purified extracts has been found to be resistant to the action of the crystalline proteolytic enzymes, trypsin and chymotrypsin. A purified phosphatase preparation from swine kidney did not affect the transforming principle. Phosphatase preparations from rabbit bone and dog intestine, on the other hand, quickly destroyed the transforming activity. In addition to their phosphatase activity both of the latter enzyme solutions showed considerable esterase activity, whereas the kidney phosphatase preparation was free of this enzyme. In this regard it is of interest that sodium fluoride, which is known to inhibit the action of esterases, prevents the destruction of the transforming principle by the bacteriolytic enzymes of pneumococcal cells. As noted above the transforming activity is not affected by digestion with crystalline ribonuclease.

Normal human serum contains an enzyme which is capable of quickly inactivating the transforming principle. On the other hand, serum obtained from patients during the acute phase of various infections does not destroy the activity of potent bacterial extracts. The lack of activity of acute phase serum is probably attributable to the presence of an antiferment which inhibits the activity of the serum enzyme.

From the evidence obtained by the use of various enzyme preparations it is not held that the specific transforming principle is necessarily of the nature of an ester, inasmuch as other enzymes may have been present. However, the evidence obtained by the use of sodium fluoride as an enzyme inhibitor when taken in conjunction with the other studies, suggests that the transforming principle may be an esterified compound.

Whatever may ultimately prove to be the nature of the transforming principle, one of its most striking characteristics is the type-specificity of its action on R cells. The mechanisms involved in the phenomenon of transformation and the nature of the activating principle are still undetermined. However, the results indicate that all R cells in which retrogression has not advanced too far, possess a potential but inactive system of enzymes capable of synthesizing any of the type specific polysaccharides. Once the capsular function has been specifically activated, the newly transformed cells continue to synthesize the same capsular material and retain their type specificity through innumerable transfers on artificial media.

The study is being continued with the hope that knowledge of this important cellular mechanism may lead to a better understanding of the principles involved in certain induced variations of living cells, not only of *Pneumococcus*, but also those of other biological systems. Furthermore, it is possible that knowledge pertaining to the nature of the substances which serve

as activators and inhibitors of the capsule-producing enzymes might afford a specific approach to the suppression of the capsular function, upon the activity of which the pathogenicity of pneumococcus depends.

The reduction of Fe<sup>+++</sup> iron as a measure of bacterial metabolism (Dubos). Bacterial metabolism has often been studied by following the rate of reduction of methylene blue or other reducible dye; this method does not lend itself to quantitative analysis and also suffers from the fact that dyes are toxic for several types of enzymes. It is known that the Fe<sup>+++</sup>  $\rightleftharpoons$  Fe<sup>++</sup> system possesses a very high redox potential and can therefore respond to most of the oxidation-reduction reactions associated with metabolism.

Inorganic Fe<sup>+++</sup> forms with  $\alpha\alpha$ 'dipyridyl, a red complex which permits very sensitive and accurate determination of the former substance. We have found that, in the concentrations required to titrate the iron reduced by bacteria,  $\alpha\alpha$ 'dipyridyl does not exhibit any toxicity for these cells. It is possible, therefore, by adding Fe<sup>+++</sup> and  $\alpha\alpha$ 'dipyridyl to a cell suspension, to obtain a continuous record of the reduction reactions going on in the system. In fact, it has been found that the production of the red Fe<sup>+++</sup> -  $\alpha\alpha$ 'dipyridyl complex in a metabolizing cell suspension is proportional to time and to the number of cells and can serve as a quantitative measure of metabolism.

Bactericidal substances produced by aerobic sporulating bacilli (Dubos, Hotchkiss, and Little). The culture of aerobic sporulating bacillus which produces the bactericidal substances described in previous reports has now been identified as a strain of B. brevis. In fact, several different strains of this species have been isolated from soil, sewage, cheese, etc., or obtained from culture collections and all have been found to exhibit antagonistic activities toward other microorganisms. Cultures of B. brevis



have yielded two crystalline products, gramicidin and tyrocidine, both of which are endowed with bacteriostatic and bactericidal properties.

Tyrocidine is a polypeptide with a free amino group; it is bactericidal for all microorganisms so far tested, with the possible exception of the tubercle bacillus. In fact, it behaves like a general protoplasmic poison, analogous to the cationic detergents which it resembles by its basic character and ability to depress surface tension. Although very active in vitro against Gram positive and Gram negative organisms, tyrocidine is completely inactive in vivo against Gram negative infections, and affords only very limited protection against infection of mice with pneumococci.

As stated in earlier reports, gramicidin exhibits a remarkable selective activity against Gram positive bacteria and, when used under the proper conditions, retains much of its activity in vivo. Gramicidin injected intraperitoneally into mice exerts a protective and curative action against streptococcus and pneumococcus peritonitis; its efficacy has also been demonstrated in the case of a naturally occurring localized infection, namely bovine mastitis. This disease, caused by group B streptococci, remains localized in the udder of the infected animal. It has been found that the injection of gramicidin directly into the infected quarter through the teat canal often results in the rapid, complete and permanent disappearance of the streptococci. Particularly successful results were obtained when gramicidin was injected in emulsion with mineral oil, thus minimizing the irritation resulting from the treatment.

Although gramicidin is therefore effective in vivo for the local treatment of localized Gram positive infections, it is practically ineffective when administered by the intravenous, subcutaneous, or intramuscular routes; as will be shown later, the lack of effectiveness by systemic administration

may be due to the presence in the blood of a substance - cephalin - which is a specific inhibitor of gramicidin.

Studies on the chemical nature of bactericidal substances prepared from cultures of *B. brevis* (Hotchkiss and Dubos). The bactericidal material produced by *B. brevis* contains the neutral substance gramicidin and a weakly basic substance designated as tyrocidine. Both gramicidin and the hydrochloride of tyrocidine have been obtained crystalline and in highly purified form. Substances previously described as graminic and gramidinic acids have been recognized as tyrocidine, crystallizing without the full complement of hydrochloric acid.

Tyrocidine is apparently a polypeptide containing a free amino group and having a carboxyl group combined as an amide ( $-\text{CO}-\text{NH}_2$ ). The chloride content of the hydrochloride indicates a molecular weight of about 1300. Of approximately eleven amino acid residues, one is tryptophane and one tyrosine.

Work has continued on the chemical nature of gramicidin. A molecular weight of approximately 1400 was found by use of a physico-chemical method which may have entailed an error of considerable proportion. Consequently, in the chemical degradation of gramicidin by hydrolysis, an attempt has been made to obtain more accurate indications of the molecular weight. The tryptophane analysis together with other results allow a choice of two values of the order of 1000 and 1500. A number of statements can be made which will be true of either unit: Cyclic tryptophane nitrogen atoms and aliphatic amino nitrogen bound in peptide linkages account for the total nitrogen present in the molecule. One amino group per molecule is derived from a substance which is not a typical  $\alpha$ -amino acid; the remainder belongs to  $\alpha$ -amino acids (present in part as l- and in part as d- or "unnatural" amino acids as previously reported). One half of the  $\alpha$ -amino acid residues

- and one half the weight of gramicidin - is accounted for by the known units, tryptophane, leucine and serine. Analysis shows that the remainder must be aliphatic mono-amino mono-carboxylic acids. The nature of these, and especially of the amino compound which is not an  $\alpha$ -amino acid is being investigated at present.

The mode of action of tyrocidine and gramicidin (Dubos, Coburn, and Hotchkiss). In spite of their common origin and similarity in chemical structure, tyrocidine and gramicidin differ profoundly in biological activity and in the mechanism of their action upon the susceptible bacterial cells. The addition of sufficient amounts of tyrocidine to cell suspensions immediately results in an irreversible inactivation of the oxidizing enzymes. Tyrocidine also causes the lysis of many bacterial species (also of frog eggs and protozoa) apparently as a result of the destruction of cellular respiration.

The action of gramicidin is of a much more subtle nature. Gram positive bacteria treated with amounts of this substance sufficient to render them unable to grow on ordinary media, still continue to respire and do not undergo lysis. The effect of gramicidin on cellular respiration has been followed by determining the rate of reduction of methylene blue or indo-phenol, the production of inorganic ferrous ion in media to which ferric salts had been added, the oxygen uptake in the Warburg technique, the production of acid. Only one type of result will be mentioned at this time. It has been found that gramicidin causes an immediate stimulation of the reduction of  $Fe^{+++}$  by staphylococci resuspended in appropriate substrates; this stimulation persists for several hours even in the presence of a large excess of gramicidin. It is of interest to remark that  $0.02 \mu g$  of gramicidin added to 24 cc. of cell suspension, causes a marked stimulation of production of

Fe<sup>++</sup> ions; in other words gramicidin still exerts its physiological action in a dilution of 10<sup>9</sup> and can therefore be counted as one of the most active biological substances.

It has been found recently that cells which had lost the ability to grow in meat infusion peptone broth or agar as a result of treatment with gramicidin recover their ability to grow when placed in solutions of "amino acid cephalin". Serum exerts the same effect by virtue of its cephalin content, and a preparation of soya bean cephalin has also proven active. On the contrary, serum proteins, peptones, lecithin, cholesterol, fatty acids fail to restore to cells treated with gramicidin the ability to grow on ordinary media. The gramicidin- cephalin relationship may therefore be of a rather specific nature.

A digression is necessary at this time to put on record another type of biological material which can also neutralize the effect of gramicidin. It will be recalled that all Gram negative bacterial species so far tested appear resistant to this substance. In order to account for this resistance, we have wondered whether the Gram negative species do not contain a cellular component capable of neutralizing gramicidin. It is known that Gram negative microorganisms contain endotoxins which, in some cases, have been found to consist of a complex of polypeptide - polysaccharide - phospholipid. This complex (endotoxin) prepared from the cells of Shigella dysenteriae and Escherichia coli, both Gram negative species, has proven very effective in inhibiting the effect of gramicidin. Whether this inhibitory action is due to the presence of the phospholipid cephalin in the endotoxin molecule cannot be considered established, but it is interesting to remark that the cellular structure which remains after removal of the endotoxin is without effect upon gramicidin action.

As stated above cephalin, and the endotoxin of Gram negative species, restore to cells treated with gramicidin the ability to grow in ordinary media; they also neutralize the effect of gramicidin on the metabolism of susceptible cells, for instance bringing back to normal the rate of reduction of  $Fe^{+++}$  to  $Fe^{++}$  by staphylococcus which had been stimulated by gramicidin.

The hemolytic action of tyrocidine and gramicidin (Dubos and Hotchkiss). Mammalian erythrocytes washed free of serum immediately undergo hemolysis when tyrocidine is added to the cell suspension, in physiological saline or isotonic glucose solution. Gramicidin also causes hemolysis of washed erythrocytes resuspended in physiological saline, but the hemolytic action is very slow, requiring several hours. Furthermore, the hemolytic effect of gramicidin is completely inhibited when small amounts of glucose, or of cephalin, are added to the physiological saline. It appears possible, therefore, that the hemolytic action of gramicidin is of a peculiar order and is associated with some effect of gramicidin on the metabolism of the red cell. Studies of the effect of this substance on the respiration of erythrocytes may serve to establish this hypothesis.

All these facts, and others not described here, indicate that tyrocidine behaves like a general protoplasmic poison. On the contrary, it appears that gramicidin prevents the multiplication of the susceptible cells, not by a gross toxic effect, but by altering the normal course of breakdown of carbon compounds. This interference is reversible and both normal metabolism, and normal growth, are restored by amino acid cephalin. An attempt is being made to define with greater precision the specific metabolic reaction which is disturbed in the presence of gramicidin.

Pneumococcus heterophile antigen: A. Chemical and immunological nature (Goebel and Adams). In the report of last year reasons were outlined

for undertaking an investigation of the chemical and immunological nature of the pneumococcus heterophile hapten and the results of preliminary experimental work on the isolation and properties of this substance. During this past year considerable progress has been made in furthering this problem. Methods for the isolation of the pneumococcal heterophile or F hapten have been perfected; it is now possible to procure in pure chemical form some ninety per cent of the substance originally present in the microorganism. The substance proves to be an unusual type of polysaccharide closely related both in its chemical and immunological properties to the species specific carbohydrate of pneumococcus.

Although the exact chemical structure of the F hapten has not been established many salient facts have been gathered in regard to its nature which enable us to characterize the substance. The material has been isolated as a dextro rotatory amorphous substance soluble in water but insoluble in the usual organic solvents. The hapten contains 43 per cent carbon, 11.0 per cent acetyl, 4.5 per cent of organically bound phosphorus, and 6.0 per cent of nitrogen. The nitrogenous constituent is an acetylated hexosamine, the identity of which has as yet not been fully established. On acid hydrolysis the F hapten yields some 50 per cent of reducing sugars. In addition to an amino sugar the F substance apparently contains a second saccharide possibly d-ribose.

From a comparison of their gross chemical analyses it is difficult to distinguish between the F hapten and the so-called C polysaccharide of pneumococcus. Yet there are points of distinction which can be demonstrated by means other than direct chemical analysis. For example, the percentage of reducing sugars liberated on acid hydrolysis are in each instance different as are the mobilities of the two substances in an electrophoretic field.

In the light of our present knowledge the two substances may be regarded as intimately related chemically, yet differing in that the F hapten contains a chemical grouping not possessed by the C polysaccharide.

A more sharply defined differentiation between the F and C substances may be made through a comparison of their specific immunological properties. Such a study has entailed not only a comparison of the immunological properties of the purified polysaccharides themselves, but an investigation of their antigenic function as they occur in their natural state in the intact bacterial cell. Thus it has been found that the two substances can be readily differentiated by means of immune sera obtained from rabbits immunized with heat-killed "R" pneumococci. It is possible to evoke in rabbits antisera which contain precipitins for the C polysaccharide, but are devoid of heterophile antibodies. Similarly antisera can be procured which have a high hemolytic titer but contain no C precipitins.

The most striking point of differentiation, however, resides in the specific immunological properties of the two purified polysaccharides themselves. The pneumococcus "F" hapten in quantities as small as 0.1 microgram will completely inhibit the lytic action of two units of heterophile antibody whereas two thousand times this amount of the C polysaccharide are required to obtain a similar effect. This startling difference in immunological specificity is believed to reside in subtle differences in the chemical constitutions of the two polysaccharides in question. The nature of these differences is the problem under investigation at the present time and its elucidation will go far toward clarifying our understanding of bacterial heterophile antigens.

B. Physico-chemical nature (Shedlovsky and Goebel). A preliminary electrophoretic study of the pneumococcus C polysaccharide in buffer solution

at pH 7.8 and ionic strength of 0.05 has been made. The material consists of two components one of which predominates. The major component has associated with it the immunological activity whereas the second component is serologically inert. The active component, which appears as a single electrophoretic boundary is somewhat inhomogenous, however, for it shows boundary asymmetry and a considerable degree of reversible boundary spreading. The average mobility of this component in the buffer mentioned was  $2.1 \times 10^{-5}$  cm<sup>2</sup> per volt sec., migrating as an anion. The serologically inactive component was electrophoretically immobile.

A similar study under the same conditions of pH and ionic strength showed the heterophile hapten to be a single and homogenous entity with a mobility of  $1.7 \times 10^{-5}$  cm<sup>2</sup> per volt sec., migrating as an anion. Experiments were made in which the pH was varied between 4.5 and 8.5. In all instances a single sharp boundary was observed thus confirming our impressions from the preliminary study that the F hapten is electrophoretically pure. From these measurements the isoelectric point of the hapten appears to be slightly under pH 3.0.

On the basis of these physico-chemical studies it is possible to differentiate sharply between the cellular (C) polysaccharide and the Forssman hapten of pneumococcus.

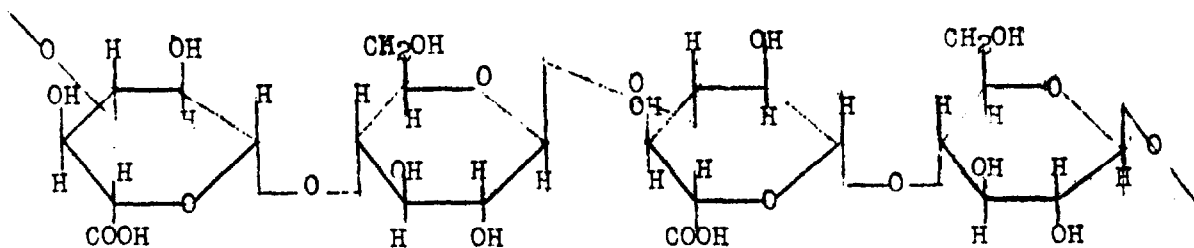
The synthesis of 2:4-dimethyl  $\beta$  methylglucoside and the structure of the capsular polysaccharide of Type III Pneumococcus (Adams and Goebel).  
The dependence of immunological specificity on the chemical structure of the hapten of an artificial antigen has been abundantly demonstrated in this and other laboratories. Since the capsular polysaccharides are the natural haptens determining the antigenic specificity of the various types of pneumococcus it was thought that investigation of their chemical structures would



contribute to the understanding of their immunological specificities. Accordingly the structure of the capsular polysaccharide of Pneumococcus Type III has now been completely established. The validity of the proposed structure depends on the rigorous demonstration of the configuration of 2:4-dimethyl glucose. The  $\beta$  methylglucoside of 2:4-dimethyl glucose has been prepared in pure crystalline form by an alternative synthesis to that previously described (1940 report).

Glucose  $\rightarrow$  diacetone glucose  $\rightarrow$  3 benzyl diacetone glucose  $\rightarrow$  3 benzyl glucose  $\rightarrow$  6 trityl, 3 benzyl glucose  $\rightarrow$  6 trityl, 3 benzyl, 2:4-dimethyl  $\beta$  methylglucoside  $\rightarrow$  2:4-dimethyl  $\beta$  methylglucoside.

The synthesis of this same crystalline derivative of 2:4-dimethyl glucose by two different procedures establishes beyond doubt the structure of this hydrolytic product of the reduced methylated Type III polysaccharide. In consequence the structural formula of the Pneumococcus Type III polysaccharide as proposed in the report of 1940 has been confirmed. This is the first type specific hapten of a pathogenic organism, the structure of which has been fully established. The structural formula of the Type III capsular polysaccharide is as follows.



The independence of the antigenic and enzymatic properties of tyrosinase (Adams). The production of antibodies to enzymes is of interest to the immunologist because the antigen is a protein exhibiting a high degree

of chemical specificity toward another substance, its substrate. When serving as an antigen, the enzyme must also exhibit specificity toward its antibody. It was of interest therefore to determine whether these two types of specificity have any relationship, that is whether the antibody to the enzyme neutralizes the latter's catalytic activity toward its substrate.

The availability of mushroom tyrosinase of relatively high purity in connection with another problem (Schroeder and Adams, report 1941) made it possible to investigate the antigenic properties of this enzyme. Rabbits were actively immunized by repeated intravenous injections of mushroom tyrosinase. The immune sera thus prepared were titered and gave a positive precipitin reaction with tyrosinase at an antigen dilution of 1 part in 400,000 indicating that the enzyme tyrosinase functions as an excellent antigen in rabbits.

Quantitative titration curves of the reaction between antiserum and tyrosinase were found to conform in all respects to those obtained in typical antigen-antibody reactions. In mixtures of tyrosinase and immune serum containing an excess of antibody, the tyrosinase was completely precipitated from solution indicating that the antibody reacted with the enzyme itself and not with a possible accompanying contaminant.

The specific precipitate between antibody and tyrosinase on resuspension in saline was completely active catalytically toward the substrates of tyrosinase, indicating that although the antibody combined with the enzyme to form a precipitate, it did not neutralize the catalytically active group of the enzyme. As in the case of urease and catalase, the addition of various quantities of antibody to the enzyme before addition of substrate did not in any case inhibit the enzyme activity. It is then evident that the prosthetic group of tyrosinase responsible for its activity as an enzyme is not involved

in the reaction between tyrosinase and its specific antibody. Tyrosinase is a "divalent" protein capable of reacting simultaneously with antibody and with substrate.

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