

Report of Dr. Avery (assisted by Drs. Adams, Curnen, Goebel,  
Horsfall, McCarty, MacLeod, Mirick and Stillman)

A study of bacterial virulence as manifested in infections produced by pneumococci (Avery, MacLeod, Horsfall and McCarty). The introduction of a given microorganism into the tissues of a particular animal species initiates a series of interreactions which may progress in different ways. The parasite may fail to survive or multiply in the host; it may survive and multiply for a varying period of time; or it may survive and persist throughout the life of the host. The result is dependent upon the presence or absence of two interrelated characteristics; one possessed by the parasite and termed virulence, the other possessed by the host and designated as resistance. The degree of virulence of the parasite is necessarily defined in terms of a particular host and similarly resistance or its absence on the part of the host is referable to a particular parasite.

Individual animals of a single species differ somewhat among themselves as to susceptibility to infection by a bacterial pathogen although as greater genetic homogeneity is achieved individual differences become less obvious. That there may be marked genetic differences in susceptibility has been demonstrated by the selective breeding of offspring from common parents and the eventual establishment of races which show wide variations in their responses to standard test infections.

More marked, however, are the differences in virulence between strains of a single bacterial species when tested in a host species, the individual members of which are of closely similar susceptibility. Moreover, the morphological variants which arise from an individual strain of bacteria whether they develop as a result of unknown conditions or in response to experimental alterations in environment usually possess varying

degrees of virulence commonly lower than that of the parent strain. Furthermore, in the case of many species of pathogenic bacteria, the property of virulence seems to be dependent upon a delicate balance between certain functions of the cell since in some instances it may be lost or become inactive without demonstrable morphological change or observable alteration in biochemical activities.

Certain biological characteristics of the pneumococcus offer advantages in the use of this microorganism as a tool in the study of bacterial virulence. In the case of pneumococci, virulence becomes manifest only when the cells are encapsulated, that is in the so-called smooth (S) phase. Pneumococci which are devoid of capsules, that is in the so-called rough (R) phase are avirulent even in the most susceptible animal species. It seems obvious that the presence of the capsule is intimately associated with the manifestation of the property of virulence. However, there is reason to think that the capsule is not alone the basic factor responsible for this property.

The pneumococcus capsule is composed largely of polysaccharide, which in each type has a distinct chemical structure. Were the type specific capsular polysaccharide wholly responsible for the property of virulence, all strains of the same type should possess identical virulence if the quantities of polysaccharide elaborated were equal. It has been repeatedly demonstrated that this is not the case. Numerous gradations in virulence are found among different strains of a single type although, so far as is known, their capsular constituents are identical. Even more striking are the differences in virulence between strains of a single type when tested in more than one host species. For example, two strains of Type III pneumococcus may possess approximately equal virulence when tested in mice but

when tested in rabbits one may be highly virulent and the other wholly avirulent. Similarly, Type XIV pneumococcus although it is often associated with severe pneumonia in human beings, and may therefore be thought of as virulent for man, is almost wholly without virulence when tested in mice and rabbits.

In a number of instances it is possible by repeated rapid passages through a susceptible host to enhance markedly the virulence of encapsulated pneumococci for this particular species. There appear to be three possible explanations for this phenomenon. (1) The increase in virulence may be the result of the progressive adaptation of the bacteria to the environment provided by the host. (2) The increase in virulence may be the result of the selection of individual bacterial cells which possess initially the greatest virulence. (3) The increase in virulence may be the result of mutation of the bacteria followed by the selection of cells possessing the greater virulence. Whichever of these explanations may prove correct, in the case of pneumococci it should be pointed out that virulence can be enhanced without any demonstrable alteration in the capsule or in the chemical constitution of the capsular polysaccharide.

The considerations stated above suggest that the property of virulence possessed by pneumococci, although only manifest in the presence of the intact capsule, is also dependent upon some other cellular function. As a hypothesis, subject to experimental trial, it was considered possible that this second basic factor for virulence might be present, although inapparent, in the non-encapsulated or so-called R cell.

It is possible to derive non-encapsulated (R) pneumococci from encapsulated (S) cells by means of a variety of experimental procedures. One of the most regularly effective techniques depends upon the fact that

encapsulated (S) pneumococci grown in the presence of homologous type specific antiserum eventually become non-encapsulated (R) and subsequently remain in this phase even though grown in the absence of antiserum. As has been stated, the induced R variants are wholly devoid of manifest virulence irrespective of the virulence of the S cells from which they were derived.

It is also possible to derive encapsulated (S) pneumococci from non-encapsulated (R) variants by means of one or another of several experimental techniques. One of the least complicated procedures is dependent upon the observation that when grown in the presence of anti R serum induced R variants revert to the encapsulated (S) forms of the same type as that of the parent cells. Encapsulated cells derived from R variants in this manner subsequently remain type specific even though grown in the absence of antiserum. More important, however, is the finding that on reversion from R to S the latter cells manifest virulence in a degree comparable to that of the parent S cells. Thus, when encapsulated Type III pneumococci virulent both for mice and for rabbits are converted to the avirulent non-encapsulated (R) forms the latter cells still possess the virulence factor in a latent form since upon reversion to encapsulated (S) cells unaltered virulence for both these animal species reappears.

Another and more critical approach to the problem of virulence is provided by means of the phenomenon of controlled transformation of pneumococcus types. It will be recalled that non-encapsulated (R) variants derived from one type of encapsulated (S) pneumococci can be caused to revert to S cells of the original type. Of much greater significance is the fact that under certain special conditions non-encapsulated (R) variants can be selectively transformed into encapsulated (S) cells of any desired type. This can be accomplished in vitro during the 24 hour growth of a

single culture of R cells by means of the directional influence of a purified cell-free extract obtained from encapsulated (S) cells of any selected type. Thus, in the presence of appropriate extracts, non-encapsulated (R) cells derived from Type I pneumococci can be caused to produce encapsulated (S) pneumococci of Types II, III, etc.

If the factor for virulence were actually present though latent in the non-encapsulated (R) variants, virulence should again become manifest when these cells are transformed to encapsulated (S) pneumococci of a heterologous type. This has been found to be the case. Thus, two strains of Type III pneumococcus were chosen one of which was virulent and the other avirulent for rabbits. Non-encapsulated (R) variants were obtained from each of these two strains. Then both R variants were transformed by means of a sterile and specific extract prepared from encapsulated Type II pneumococci. The two strains of Type II pneumococci thus derived were then tested for virulence in rabbits. The strain of Type II transformed from the rabbit avirulent Type III strain was found to be avirulent for rabbits. Conversely, the strain of Type II similarly obtained by transformation from the rabbit virulent Type III strain was equally as virulent for rabbits as was the parent culture from which it was derived. These results lend support to the view that the basic attribute responsible for virulence in a particular host species is present though latent in the non-encapsulated (R) cell and that if this factor is present in the parent strain it can be carried through the intermediate R form and will reappear when these cells are transformed to encapsulated forms of a wholly different type. The results of these experiments are graphically presented in Text figures 1 and 2.

Although the evidence suggests that the factor for virulence

## Cellular Factor in Virulence

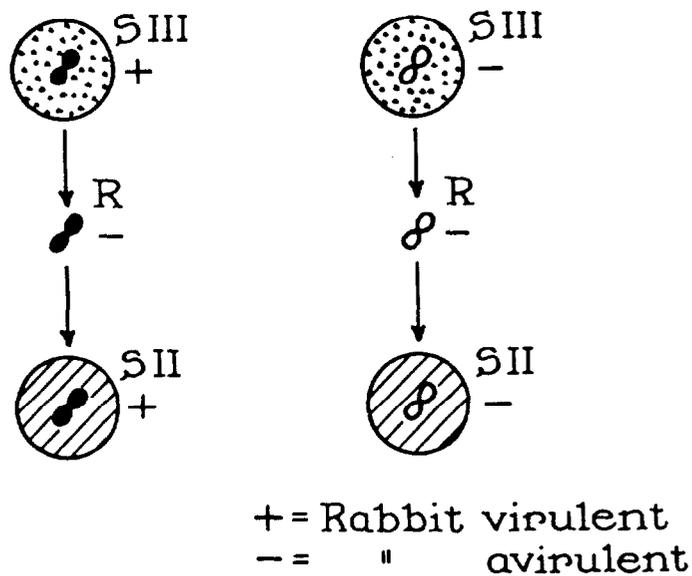


Fig. 1

## Capsular Factor in Virulence

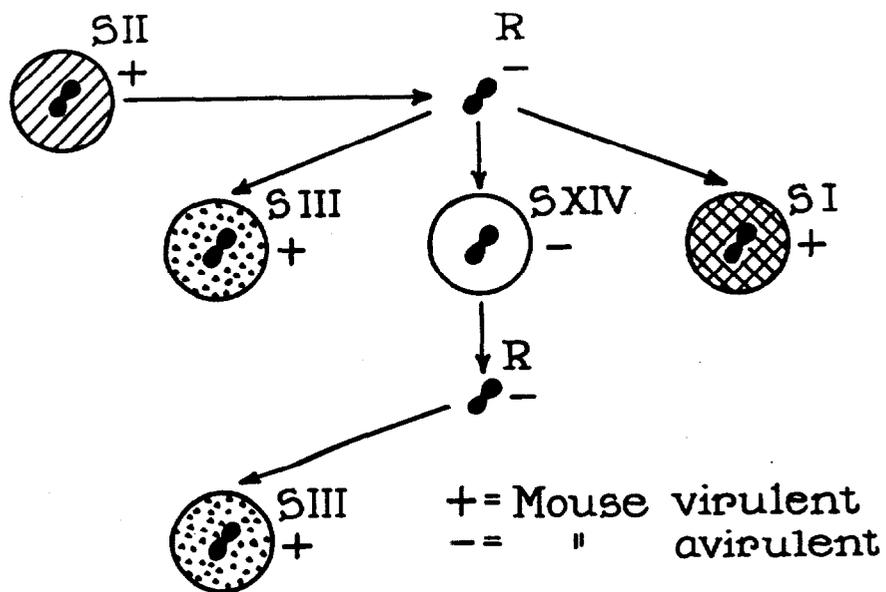


Fig. 2

resides in the R cell there is additional evidence which indicates that the chemical nature of the capsular polysaccharide of S cells may be of considerable importance in determining the extent to which potential virulence may be manifest. Type XIV pneumococci are notably avirulent for both rabbits and mice.

As already mentioned, Type XIV pneumococci, although frequently encountered in human infection and therefore evidently virulent for man, are without virulence for mice. An attempt was made by the use of transforming methods to determine whether the lack of virulence for mice is due to a peculiarity of the capsular polysaccharide rather than to the absence of the cellular factor as in the example illustrated in Text figure 1. To determine this point, a strain of Type II pneumococcus highly virulent for mice was chosen for study. (See Text figure 2). R cells derived from this strain were transformed into Type III, Type XIV and Type I. That the factor for mouse virulence was present in the R cell is evident from the fact that the encapsulated strains of Type I and Type III derived therefrom were equally as virulent for mice as the parent strain of Type II from which they were derived. However, when these same basic R cells were caused to produce the Type XIV capsular material, the S cells thus derived were no longer manifestly virulent. This fact shows that even though the factor for virulence is present in the cell body, the nature of the capsule itself is the limiting factor in this particular instance. To prove that under these conditions the cellular factor is retained within the cell body, an R strain was derived from these same Type XIV cells and transformed into Type III encapsulated cells with the reappearance of virulence.

Finally, it has been possible in a single step to derive from Type I pneumococci of maximum virulence cells which although still encap-

sulated nevertheless have lost completely their virulence for both animal species. This was accomplished by growing Type I cells in a solid medium in the presence of small quantities of sulfathiazole. Cells derived in this manner are fully encapsulated, morphologically and immunologically indistinguishable from the parent cells, and depending upon the experimental conditions used may or may not possess the property of "fastness" to this drug. Despite the possession of capsules these Type I strains may be entirely avirulent. Since the capsular synthesizing function and therefore the production of type specific capsular polysaccharide appears to be unaltered in strains derived in this manner it seems probable that the loss of virulence is the result of alterations in metabolic systems within the cell body not related to the capsular synthesizing system. Serial sub-culture of these strains has not resulted in any augmentation in virulence, nor so far as the experiments have progressed, has rapid serial passage in normally susceptible hosts increased their virulence. It should be mentioned that the repeated cultivation of encapsulated (S) pneumococci in liquid media containing gradually increasing concentrations of sulfonamide has resulted in the development of so-called sulfonamide "fastness". Cells adapted in this manner to growth in the presence of sulfonamides have retained both their type specificity and their virulence in unaltered degree.

The results of these experiments afford additional evidence in favor of the hypothesis that some of the basic factors responsible for virulence reside in the cell body and are unrelated to the capsular system or the chemical structure of the polysaccharide produced.

The available evidence appears to indicate that the manifest virulence of encapsulated pneumococci for a particular host species is dependent upon at least two distinct and separate properties of the

bacterial cell. One factor seems to reside in the cell body and its influence is not demonstrable in the absence of a capsule. The other factor appears to be intimately associated with the capsule, if indeed it is not actually the capsule itself, and its influence is without effect in the absence of the cellular factor. With two interdependent factors responsible for either of the two extremes of virulence, that is full virulence and total absence of virulence, it should be possible theoretically to obtain four distinctly different variants from any type of pneumococcus of known virulence for a given species. There is evidence indicating that this can be accomplished. If the capsular factor is designated as A, the cellular factor as B and the presence or absence of either indicated by + and - signs respectively, a table illustrating the theoretical possibilities can be constructed. In each instance the existence of the predicted variants shown in Table I has been confirmed experimentally. Of the four variants only #1, the cell in which both factors were demonstrable was found to be virulent. Variants #2 and #3, though each was shown to possess only one or the other of the two factors, were avirulent.

Presence of Capsular and Cellular Virulence Factors in  
Variants Derived from Type I Pneumococcus

Variant	Virulence Factors		Manifest virulence	Experimental Confirmation
	A	B		
1	+	+	+	Type I, S cells
2	+	-	-	Type I, S cells derived from #1 in sulfathiazole
3	-	+	-	R cells derived from #1 in anti S serum
4	-	-	-	R cells derived from #2 by spontaneous dissociation

A = capsular factor for virulence; B = cellular factor for virulence

It is hoped that additional investigation may be helpful in the problem of elucidating the nature of the factors concerned with bacterial virulence.

The heterophile antigen of pneumococcus (Goebel and Adams).

Heterophile antigens derived from Gram negative microorganisms have been described by several investigators, but the isolation and identification of such substances from Gram positive bacteria has hitherto not been achieved. During the past two and a half years we have conducted an extensive investigation on methods of isolation, the chemical nature, and immunological properties of the heterophile antigen of pneumococcus. We are now able to report that the nature of this substance has been fully revealed.

The heterophile antigen of pneumococcus has been isolated and characterized as a lipo-carbohydrate intimately related to the intracellular "C" polysaccharide. It differs from the latter in that it contains a lipid bound in firm chemical union to the carbohydrate moiety. The complex has been isolated in its entirety from autolysed pneumococci by an intricate series of chemical fractionations. The substance comprises about one per cent by weight of the bacterial cells and from 0.5 to 0.7 gm. are obtained from the microorganisms from 500 liters of bacterial culture.

The heterophile antigen is a water-soluble non-diffusible macromolecule constituted from an acetylated amino hexose, a second hexose, phosphoric acid and a lipid. Solutions of the heterophile antigen foam on shaking. The antigen is not precipitated from solution by the salts of heavy metals, nor do such solutions give any of the usual protein tests. Spectroscopic examination reveals no gross contamination of the heterophile antigen with nucleic acid or protein, indeed, when examined by electrophoresis the lipo-carbohydrate appears to be quite homogeneous. The hetero-

phile antigen is not destroyed by digestion with ribonuclease, papain, pepsin, trypsin, chymotrypsin, or by phosphatases derived from various sources. Nor is the antigen destroyed by lipases or by the autolytic ferment of the pneumococcus. The substance shows a remarkable stability to the action of H and OH ions at 100° between pH 2.8 and 9.5. Outside these limits the biological activity of the antigen is slowly destroyed and this destruction is accompanied by an hydrolysis of the carbohydrate component and the liberation of the lipid constituent. Thus, it is not possible to sever the union between lipid and carbohydrate by any simple chemical procedure. The union remains firm and the biological activity intact when the antigen is subjected to anything but drastic chemical treatment.

The intimate chemical relationship between the pneumococcus heterophile antigen (F) and the cellular carbohydrate ("C") was described in the report of last year. Further chemical studies have furnished additional evidence of this close relationship. Studies on the kinetics of hydrolysis of the two substances as measured by the increase of a) reducing sugars, b) amino (glucosamine) nitrogen, and c) phosphoric acid give proof of the identity of the common carbohydrate moiety. Hydrolysis of the heterophile carbohydrate with mineral acid results in a destruction of the carbohydrate constituent accompanied by a liberation of the lipid component. The latter has been isolated and found to contain neither nitrogen nor phosphorus and is probably a fatty acid of high molecular weight.

The immunological properties of the heterophile antigen (F) of pneumococcus have been extensively studied and compared with those of the cellular (C) carbohydrate. The heterophile substance has been found to be fully antigenic when injected into rabbits. The purified F lipo-carbohydrate gives rise to antibodies which not only precipitate the homologous antigen

but hemolyse red blood cells in the presence of complement. The C carbohydrate, on the other hand, fails to function antigenically in rabbits. Thus it has been shown that the incorporation of a simple lipid within the carbohydrate molecule endows the complex thus formed with new immunological properties. Antibodies evoked by the heterophile (F) antigen likewise precipitate the C carbohydrate, but to a lesser degree.

The intimate chemical and immunological relationship between the C and F carbohydrates has been further demonstrated by a study of the quantitative precipitin reactions in antipneumococcal rabbit sera containing both C and F antibodies. It has been found that the C carbohydrate, when used as the precipitinogen removes from these sera both the homologous precipitins and hemolytic antibodies simultaneously. On the other hand, when the heterophile (F) antigen is used as the precipitating agent, the hemolytic antibodies are neutralized by small amounts of the F antigen but far larger quantities are required to remove completely the C precipitating antibodies.

Various strains of pneumococci have been investigated for their content of the C and F polysaccharides. One R strain derived from Type III pneumococcus contains a C polysaccharide essentially identical with that obtained from the R variant of Type I. However, the heterophile antigens of the two R strains have been found to be quite different; that of the R strain from pneumococcus Type I evokes hemolysins which are not neutralized by the F antigen of the III R variant. The latter antigen, on the other hand, gives rise to hemolysins which are neutralized by the F antigen of either strain. Thus it has been demonstrated that different strains of R variants irrespective of type derivation may contain antigens, which are chemically related yet immunologically distinct.

In conclusion, it may be pointed out that the lipo-carbohydrate

(F) from pneumococcus shows no toxic effects when injected into mice or rabbits. In this respect the substance is quite unlike the analogous substances obtained from the Gram negative group of microorganisms which show a very high order of toxicity in both these species of animals.

The capsular synthesizing enzymes of pneumococcus (Goebel and Adams). During the past three years several outstanding contributions to our knowledge of the biochemistry of carbohydrates have been made. The in vitro synthesis of glycogen and starch by enzymes derived from mammalian and plant tissue has been described. In addition, there has recently appeared an account of the in vitro synthesis of serologically active dextrans by enzymes derived from microorganisms.

In the report of last year evidence was presented for the chemical structure of the capsular polysaccharide of Type III pneumococcus. In the light of this knowledge we are now in a position to investigate the enzymatic mechanism which brings about its chemical synthesis. During the course of this year preliminary studies have been made with this end in view. The problem has been approached from two angles. The chemical synthesis of certain sugar derivatives which are considered as possible substrates in the enzymatic system has been undertaken and the behavior of these derivatives in the presence of the bacterial enzymes studied. Differences in the metabolism of various strains of pneumococci of the same and different types as well as the metabolism of different R variants derived therefrom are also being investigated. This study has already yielded data on carbohydrate metabolism of pneumococci which it is hoped may prove valuable in gaining an insight into the mechanism of the enzymatic synthesis of the capsular polysaccharides.

Immune response of human beings treated parenterally with the enzyme tyrosinase (Adams). An account of the production in rabbits of precipitating antibodies to the enzyme tyrosinase was included in the report of last year. The sera of rabbits actively immunized with tyrosinase were found to precipitate tyrosinase quantitatively from solution. The immune precipitates were found, however, to possess all the catalytic activity of the tyrosinase contained therein, thus demonstrating that the antibody to the enzyme could precipitate the latter without neutralizing its physiological activity.

The parenteral administration of tyrosinase to patients (Schroeder, report 1942) with essential hypertension has made it possible to study the immune response of human beings to the injection of tyrosinase. Sera obtained from treated patients varied widely in their antibody content some having no precipitins for tyrosinase. The precipitin titers of the human immune sera did not correlate with the dosage of tyrosinase, or the duration of treatment. However, the precipitating antibodies when present reacted with the enzyme tyrosinase in a manner analogous to the reaction of antityrosinase rabbit sera. The enzyme activity was completely precipitated from solution by active immune human sera, but these sera had no neutralizing effect upon the catalytic activity of the enzyme. This observation was of importance in assaying the results of tyrosinase treatment in hypertension. Although patients did develop antibodies to tyrosinase during treatment these antibodies did not alter the catalytic activity of the enzyme in vitro and hence should not affect the physiological activity of the same enzyme in vivo.

The sulfonamide resistance of strains of pneumococci isolated from patients (Mirick). It has been repeatedly demonstrated that pneumococci, following prolonged exposure to the sulfonamide drugs, may acquire

increased resistance to the bacteriostatic action of these agents. A technique has been previously described for the study in vitro of the susceptibility of pneumococci to the sulfonamides. This technique employs fresh liver infusion medium which, unlike ordinary peptone broth, contains no substance inhibitory to the bacteriostatic action of the sulfonamide derivatives. This technique has been applied to a study of strains of pneumococci freshly isolated from patients.

It was considered quite possible that any variations in the sulfonamide resistance of different strains of pneumococci might take place through processes of natural selection rather than as the result of adaptation. For this reason it seemed important to perform the bacteriostatic tests on the largest possible sample of the bacterial population in a culture freshly isolated from the patient. Attention was paid to this principle both in the isolation of strains from patients and in carrying out the in vitro bacteriostatic tests, where relatively large inocula of 5,000 to 20,000 cells were employed.

Eighty-seven strains of pneumococci have been obtained from 57 patients with pneumococcal infections, and tested in the liver infusion medium for sensitivity to the bacteriostatic effect of sulfapyridine. Thirty-eight of the strains were isolated from patients before the administration of any sulfonamide drug. Forty-nine of the strains were obtained after the patient had received sulfonamide therapy. The bacteriostatic tests showed that there is considerable variation in the resistance to sulfapyridine of different freshly isolated strains of pneumococci. On the whole, however, it was clear that the strains isolated after adequate sulfonamide therapy were more resistant to sulfapyridine than were those isolated before the patient had received any drug treatment. Only one of the

38 strains collected before treatment grew readily in the special liver medium containing concentrations as great as 10 to 20 mgs. per cent of sulfapyridine whereas 13 out of 49, or 26 per cent of the cultures isolated after treatment, grew readily in these concentrations of the drug.

In 29 instances, two strains isolated from the same patient have been compared. In 22 cases the first culture was obtained before any sulfonamide drug had been given and the other seven cases had received only a few doses. The second strain from these 29 patients was isolated after the course of sulfonamide therapy had been completed. In fifteen of the 29 cases the second strain showed greater resistance to sulfapyridine than did the original culture. In twelve instances, there was no demonstrable difference in the two strains. In the remaining two instances, the second strain was actually more susceptible to sulfapyridine than was the original strain. Both of these were bacteremic patients, one eventually dying of bacterial endocarditis.

It has often been suggested that the occasional unsatisfactory response of a patient with a pneumococcal infection to proper sulfonamide therapy is due to resistance of the infecting strain to the sulfonamide drugs.

An attempt has been made in 20 appropriate cases to correlate the patient's clinical response to chemotherapy with the sulfonamide resistance of the infecting pneumococcus. The initial strains of pneumococci isolated before treatment from six patients who later showed a poor chemotherapeutic response, were no more resistant to sulfapyridine than were the initial strains from 14 patients who subsequently responded well to drug therapy. On the other hand, the strains of pneumococci isolated after sulfonamide therapy from patients who showed a poor response to the treat-

ment were, on the whole, definitely more resistant to sulfapyridine than were strains obtained after therapy from patients who showed a good therapeutic response. It should be noted, however, that the patient who showed a poor response received much more of the sulfonamide preparation and for a longer period of time. As a result, these strains of pneumococci had a greater opportunity to develop sulfonamide resistance than those strains isolated from patients who received the shorter course of treatment.

It is well known that mice infected with "drug fast" strains of pneumococci respond poorly to sulfonamide treatment. Up to this time no "drug fast" strain of pneumococcus has been isolated from a case of human infection prior to sulfonamide treatment. The occurrence of initially resistant strains of pneumococci or of strains capable of rapidly developing "fastness" have not so far been isolated from cases of pneumonia. Consequently, it has not been possible to relate the occasional poor response to therapy either to initial resistance or acquired fastness of the invading microorganism. However, the capacity of the infecting strain of pneumococcus to develop sulfonamide resistance during the course of treatment may have some influence upon the ultimate effectiveness of drug therapy.

Further studies on a soil bacillus capable of destroying para-aminobenzoic acid (mirick). A soil bacillus has been previously described which can produce adaptive enzymes capable of so modifying para-aminobenzoic acid (hereinafter referred to as PAB) that it no longer gives a diazo reaction and is no longer active as an inhibitor of the sulfonamide drugs. Manometric determinations have shown that when one molecule of PAB is destroyed by the active bacterial cells, about 13.4 atoms of oxygen are absorbed. This is a quantity sufficient to account for nearly complete oxidation of the compound to carbon dioxide, water and ammonia.

A technique has been developed to measure the specific enzymatic activity of a suspension of resting bacterial cells. The number of cells as determined by nephelometric methods, is plotted against the amount of PAB oxidized in 30 minutes at 37°C. Within the limits studied, there is a linear relationship between the number of bacterial cells, whatever their activity, and the quantity of substrate oxidized. This technique has been used to investigate the phenomenon of specific activation. The bacterial cells, even when grown on a synthetic medium containing no PAB, possess a certain small basal capacity to oxidize PAB. The enzyme activity may be greatly increased if PAB is present in the medium during growth. This specific activity of the cells is a function of the concentration of substrate present during their growth. A concentration of 10 mg. per cent causes the bacterial cells to be about fifty times more active than the basal cells. However, an appreciable degree of specific activation of the oxidizing enzymes can be demonstrated in bacilli tested after they had grown for 10 hours in a medium containing as little as 0.1 mg. per cent of PAB.

When active cells, which have been grown in the presence of PAB continue to multiply after the complete oxidation of this substrate, the strain will be found within four to five hours to have returned to its basal activity. Cell division does not seem to be essential for the formation of active enzymes, since it has been found that shaking a washed suspension of the bacilli for only 30 minutes with a solution of PAB results in definite activation of the cells.

The specificity of the enzymatic activity on a number of substances chemically related to PAB has been tested both with respect to the ability of the bacterial cells to attack these compounds during growth and

as to the simultaneous and specific activation of these same cells to oxidize PAB.

The amino group in the para position on the benzene ring seems to be the particular structure which stimulates specific activation of the enzyme system. Both the basal and adapted cells are equally active in attacking substances such as benzoic acid, para-hydroxybenzoic and para-toluic acids, which do not possess an amino group. Moreover, none of these three substances activate the specific enzymes capable of oxidizing PAB. Aniline, which possesses an amino group, but not in the para position, is not attacked and does not activate the specific system. Both the basal and activated cells equally and rapidly split acetylated PAB and glyceryl PAB to the free form of PAB.

Some other related substances are slowly destroyed by the bacillus when incorporated in the medium, and they all probably pass, during the process, through the form of PAB itself. They are all found to activate specifically the PAB system. These compounds are: para-nitrobenzoic acid, which other bacteria have been shown to reduce to PAB, the methyl ester of PAB and novocaine, which probably undergo initial hydrolysis, and para-aminophenyl acetic acid.

The ortho-isomer of PAB is readily destroyed by the growing bacilli but, unlike the other amino compounds studied, does not activate the para enzyme systems. On the other hand, bacilli so grown were found to possess enzymes directed specifically against the ortho compound while maintaining only their basal activity against PAB itself. These two systems are quite independent and may be either separately or simultaneously activated.

Some substances, although closely related to PAB are not attacked

at all by the bacillus. These are: The meta isomer and the ethyl ester of PAB, benzoylated PAB, a compound in which the amino group has been covered; para-aminohippuric acid, a compound in which the carboxyl group has been covered; and two compounds in which the carboxyl group has been altered, namely para-aminobenzyl alcohol and para-aminophenyl alanine.

Sulfanilic acid and the sulfonamide drugs are bacteriostatic but the soil bacilli will grow in high dilutions of these compounds if the culture is seeded with a sufficiently large inoculum. Although growth can be obtained under these conditions, there is no destruction of these agents themselves. The bacterial cells that have been grown in their presence show, not activation, but marked suppression, below the usual basal level of both the para and ortho oxidative enzymes. On the theoretical basis of enzyme competition, it was anticipated that only the para enzymes would be inhibited under these conditions. This suppression can be easily reversed by shaking these cells with either para or ortho-aminobenzoic acid. Under these conditions the corresponding enzyme is readily activated and the isomeric enzyme returns from a suppressed to a normal basal level.

Other workers have shown that tryptophane may be metabolized by some bacteria to ortho-aminobenzoic acid. For this reason tryptophane has been included in the present studies. As was anticipated, it was found that this substance is actively destroyed by the soil bacillus and selectively activates the ortho enzymes. Indole is slowly destroyed by the bacillus but has no effect on either enzyme system. Skatol, on the other hand, which is somewhat bacteriostatic, was found to activate the para enzymes. The significance of this latter observation is not yet understood but none of these substances are inhibitors of the sulfonamide drugs.

The natural occurrence and biological significance of PAB has

never been adequately explored. Use can be made of the soil bacillus and of the techniques described to identify the very small quantities of diazotizable compounds encountered in nature.

By application of these techniques it has been possible to acquire some understanding of the nature of induced resistance to the sulfonamide drugs, acquired by a strain of pneumococcus. The soil bacilli, grown in the liver infusion which is free of sulfonamide inhibitor show no activation of the PAB oxidative enzyme system. If a strain of Type I pneumococcus is grown in this medium, a sulfonamide inhibitor appears which has the solubilities of PAB and also gives the diazo reaction. Moreover, the sulfapyridine-fast derivative of this strain of pneumococcus produces about ten times as much sulfonamide inhibitor and diazotizable material as the parent strain or about one gamma per 25 grams of liver. Finally, this substance is thought actually to be PAB because it is rapidly destroyed by the soil bacilli which have been specifically adapted to oxidize PAB and is only very slowly attacked by cells which have not been so activated. Furthermore, the sulfonamide inhibiting substance produced in liver infusion by pneumococci will itself specifically activate the para enzyme system of the soil bacillus. An extract of pneumococcal cells yields none of this substance. Therefore, the PAB must be either synthesized by the pneumococci or enzymatically released from a bound form initially present in the liver medium.

The discovery of PAB in animal tissues is suggestive evidence that this compound may have a wide biological significance. Nutritional studies are contemplated in rats, freeing the diet from all PAB by use of the soil bacillus in much the same manner that avidin has made possible the study of the syndrome in rats due to a deficiency of biotin in their diet.

The presence of a polysaccharide in the blood during acute infections (Curnen and Mirick). Early in this century several observers reported that growth of pneumococci in the sera of patients with pneumonia results in the formation of a heavy white precipitate, whereas growth in normal sera produces only a slight cloudiness. In 1904 Rosenow demonstrated that the heavy precipitate is due, not to an excessive deposit of organisms as the earlier observers believed, but to the formation of acid during growth of pneumococci in sera obtained from patients with pneumonia. The formation of excess acid and consequent sedimentation of acid precipitable protein does not occur when pneumococci are grown in normal sera nor when streptococci or staphylococci are grown in sera obtained from pneumonia patients. In 1905 Dr. Longcope confirmed and extended these observations. He observed acid formation and sedimentation following growth of pneumococci in the sera of patients with a variety of infectious diseases as well as in sera from cases of acute rheumatic fever, nephritis and uremia. He concluded that "there is.....some substance which makes its appearance in the blood serum under certain conditions and from which the pneumococcus is capable of forming large quantities of acid." He indicated that the substance is not glucose, but implied that it might be carbohydrate in nature. In 1939, Friedemann and Sutliff demonstrated quantitatively that the increased acid formation is more than can be accounted for on the basis of the free glucose present and concluded that "the phenomenon is due to the presence of abnormal quantities of a polysaccharide which supports rapid growth and which is readily fermented by the pneumococcus."

These observations suggested that pneumococci possess an enzyme capable of attacking the particular polysaccharide present in blood during infection. Meyer, Dubos, and Smyth have isolated from pneumococci an enzyme

which specifically decomposes hyaluronide, a polysaccharide found in the tissues of man, animals and in certain bacterial cells. Consequently, a purified preparation of hyaluronidase was isolated from pneumococci by the method of Meyer and its action tested in normal and 'pneumonic' serum. The enzyme preserved in dry state as the protein flavianate was found to be highly active in depolymerizing authentic samples of hyaluronide of both human and streptococcal origin. The specificity of the enzyme preparation used in the present study is shown by the fact that it does not attack any other of the polysaccharides thus far tested including those of pneumococcal origin.

The enzyme was used experimentally in the following manner. The reducing values of deproteinized serum filtrates were determined by the method of Hagadorn and Jensen. The results were expressed as mg. per cent of reducing substance calculated as glucose. Any increase in the reducing value as compared with that of the control was considered as due to activity of the hyaluronidase upon the serum polysaccharide. A similar method was employed for the detection of the polysaccharide in chest fluid and in urine.

The results obtained thus far indicate that a polysaccharide susceptible to hydrolysis by pneumococcal hyaluronidase is present in the sera of patients with pneumonia. The reducing value after enzymatic hydrolysis of sera obtained from pneumonia cases tended to reach a maximum during the acute stage of illness and to decline to lower levels thereafter. The serum of individual patients, however, showed considerable differences in polysaccharide content. Too few normal sera have been tested thus far to establish the normal range and limiting values in healthy individuals.

The polysaccharide has been detected by a similar method in the

urine of about one-third of the pneumonia patients tested. Correlation of the polysaccharide values in serum and urine obtained from patients on the same day indicate that when the serum values are high, the urine values are low, whereas when the urine values are high little or no polysaccharide can be detected in the serum.

A few preliminary investigations in animals have revealed high levels of polysaccharide in the sera of normal rabbits. In this connection it is of interest to recall the original observation of Dr. Longcope that the substance from which pneumococci produce acid during growth was not detectable in horse and beef serum but was present in calf and rabbit serum.

The chemical nature of the serum polysaccharide is indicated by the following evidence. In the present study, it has been found that when a highly specific preparation of pneumococcal hyaluronidase is allowed to act on serum containing the polysaccharide reducing substances are liberated. Meyer and his associates have shown that this enzyme hydrolyzes hyaluronic acid into its components, glucuronic acid and acetyl glucosamine, both of which are reducing substances. In the present study the color reaction for acetyl glucosamine is invariably positive in sera treated with active enzyme and negative in the corresponding controls. Finally, it has been shown that yeast which specifically destroys any free glucose present in the serum has no effect upon pure acetyl glucosamine or glucuronic acid nor upon the split products of the serum polysaccharide after hydrolysis by pneumococcal hyaluronidase. These findings strongly support the view that polysaccharide present in abnormal quantities during acute disease is either hyaluronic acid or a closely related chemical substance. The acidity noted by earlier investigators in serum during growth of pneumococci probably arises from the primary hydrolysis of the polysaccharide by the bacterial hyaluronidase

and the subsequent formation of lactic acid by the bacteria from the glucosamine thus liberated.

Factors effecting reversion of R to S pneumococci in mice (Stillman). It has been suggested that pneumococcus infection may be initiated by the degraded R forms of pneumococci changing in the body into virulent smooth organisms which are associated with pneumonia. As alcohol is known to render mice more susceptible to invasion by pneumococci, the effect of injecting the avirulent R pneumococci into intoxicated mice was tried. In the case of the R variants derived from Types I and II pneumococci no evidence of reversion to the corresponding encapsulated cells was observed to occur under these experimental conditions. But in the case of an R strain derived from Type III pneumococci, virulent cultures of the homologous type were recovered following the injection of degraded avirulent R forms into intoxicated mice. The smooth forms were recovered from mice 2 - 5 days after inoculation.

In view of the incidence of respiratory infections associated with the presence of H. influenzae as a primary or secondary invader, an intensive study of this organism has been undertaken. Serum is being prepared for the 6 specific types so far differentiated so as to be able to classify the various strains for epidemiological studies immediately after recovery.

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