Studies on the transformation of specific types of pneumococcus. (Avery and C. M. MacLeod.) Rarely in the case of a pathogenic microorganism is it possible to correlate many of its biologically specific and pathogenic characters with the activity of a single enzymatic function of the cell itself. This correlation, however, is particularly striking in the case of Pneumococcus. In this instance the bacterial cell possesses a potential system of synthesizing enzymes, the functional activity of which results in the elaboration of a specific substance which is structurally disposed about the periphery of the cell in the form of a capsule. The presence of the capsule confers a new and highly differential type-specificity upon the microorganism, and conditions its capacity to grow and multiply in the tissues of the infected host. This synthesizing function is most highly developed, and the product of its activity most pronounced in cells best adapted to growth in the animal body. On the other hand, cells in which this system of synthesizing enzymes has been spontaneously or experimentally inactivated by exposure to an unfavorable environment are not only deprived of their capsules and type-specificity, but also lose their invasive properties and become relatively non-virulent for animals highly susceptible to infection with the original encapsulated strain.

Important and essential as is the activity of capsular function to the parasitic career of the microorganism
in the living host, it is, however, not vital to the life of the cell growing outside the animal body. For capsule-free cells are still capable in artificial media of carrying on the vegetative processes of metabolism and multiplication after the capsule synthesizing function has been inhibited or suppressed. This retrogressive change from more complex to simpler cell structure, with the resultant loss of type-specificity and virulence, is intimately associated with, if not wholly dependent upon, the inactivation of the system of enzymes concerned in the synthesis of the cell capsule. The function of capsular synthesis may therefore be regarded as an adaptive mechanism or defense reaction whereby the cell seeks to protect itself against the resisting forces of the invaded host.

Thus, pneumococci may exist as encapsulated or non-encapsulated cells which, in terms of bacterial dissociation, are referred to respectively as the S and R forms. The cells of these two forms differ from each other in morphological structure, colony topography and biological behavior, and these differences are in each instance referable to a gain or loss in activity of the capsule-producing enzymes of the cell.

Under suitable cultural conditions, the change from one form of cell to the other is reversible (S⇌R). A type-specific strain of pneumococcus, after serial transfers in a medium containing immune serum of the homologous type, is converted into the R form in which capsular development is suppressed. Similarly, the non-encapsulated, degraded R cells may, by cultivation in anti-R serum, be caused to regain all the distinguishing and type-specific properties which
characterized their parasitic antecedents. Under these conditions, the reversion is invariably back to the homologous type, and is associated with the acquisition of virulence and the re-synthesis of a capsular polysaccharide identical in chemical constitution and type-specificity with that elaborated by the parent strain. Once this change in functional activity has occurred, irrespective of whether the variation induced is progressive or retrogressive in nature, the cells thus altered breed true on subsequent cultivation until they are again subjected to environmental conditions which cause reversal of function and form. From this point of view, the processes of dissociation may be regarded as a response of the cells to environmental influences which favor either the activation or inhibition of the capsule-producing enzymes.

More significant, perhaps, than this direct form of reversible dissociation (S→R) is the phenomenon of transformation of specific types of Pneumococcus. Griffith first showed, by a special technique 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 subsequently found that the transformation of specific types can be brought about in vitro without the use of animal inoculation. Later, Allouay succeeded in causing transformation by growing R cells in a medium containing small amounts of a filtered extract of S cells of a heterologous type. Under these conditions the R cells, irrespective of type derivation, acquired all the type-specific characteristics of S cells from which the extract was prepared. Thus, it has been experimentally
demonstrated that it is possible not only to reactivate the capsule-synthesizing enzymes of an R cell, but actually to so orient and direct the synthesis that R cells, under the influence of a specific stimulus, are caused to elaborate a capsular polysaccharide having the same chemical constitution and serological specificity as that of the type of S cells from which the activating stimulus was derived.

The work begun by Dr. Alloway and continued last year by Dr. Rogers is being actively carried on at present in collaboration with Dr. MacLeod in an attempt to ascertain the nature and properties of the transforming principle present in active extracts of S cells. Extracts thus derived can be freed of intact cells and cellular detritus by filtration through a Berkofeld candle, without loss of activity. The active principle passes the filter, however, only when the extract is adjusted to an alkaline reaction (pH 8.2) before filtration. From crude extracts a large amount of irrelevant protein and other inert material can be removed by adsorption with charcoal without appreciable diminution in the potency of the absorbed extract. The active principle in these partially purified and water-clear extracts does not pass through parchment or cellophane membranes on prolonged dialysis against running water.

The transforming principle in active extracts withstands heating at 60°-80° C. for 2 hours, but is completely destroyed on shorter exposure to higher temperatures. The potency of an extract is lost when the reaction of the solution is adjusted to an acidity equivalent to or greater than pH 4. On the other hand, the transforming principle is stable in
extracts at pH 9, and at this alkaline reaction resists heating to 60° C. for 45 minutes.

Observations of considerable significance have recently been made concerning the effect of certain enzymes on the transforming activity of potent extracts. Then S cells are allowed to undergo spontaneous autolysis, the resulting autolysate is no longer capable of causing transformation, indicating that the active transforming principle is destroyed by the autolytic ferments of the cell. All active extracts contain varying amounts of the specific capsular polysaccharide in solution. It was early discovered, however, that the chemically purified specific polysaccharides are by themselves devoid of transforming activity. Moreover, Rogers showed, and MacLeod has since confirmed the fact, that active extracts of Type III pneumococci, which have been treated with the specific enzyme capable of decomposing the Type III polysaccharide, still possess transforming activity although the presence of the specific carbohydrate is no longer detectable by the precipitin test.

Similarly, all active extracts contain varying amounts of bacterial protein. However, extracts which have been digested by crystalline trypsin and subsequently dialysed to remove the hydrolysed products of digestion are as fully, if not more, active than the original extracts before digestion and dialysis. Unless it is subsequently shown that the split products of enzyme action account for the activity of digested extracts, it may be tentatively concluded that the transforming potency of extracts is due to the presence of some constituent not
destroyed by trypsin or by the specific carbohydrate.

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 Τ cells. The mechanism involved in the phenomenon of transformation and the nature of the activating principle are still undetermined. However, the results indicate that all Τ 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, the particular one produced being determined by the specificity of the activating stimulus. Once the capsular function has been specifically activated, the newly transformed cells continue to synthesize the same capsular material and retain the same type-specificity through innumerable transfers on artificial media without the further addition of the activating substance initially employed to induce the transformation. From these same cells, after prolonged cultivation, a transforming principle of the same specific nature and activity can again be extracted in an amount greatly in excess of that originally added to initiate the change.

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 with specific transformation and induced variations of living cells, not only of Pneumococcus, but also those of other biological systems. Furthermore, the thought suggests itself that were we in possession of knowledge pertaining to the nature of the substances which serve as activators and inhibitors of the
capsule-producing enzymes, the knowledge gained might afford a new approach to a specific attack directed toward the suppression of the capsular function upon the activity of which the pathogenicity of Pneumococcus depends.

The protective action of antipneumococcus serum in mice (Gooch and Horsfall). Typo-specific antipneumococcus horse and rabbit sera have the capacity to protect mice against fatal infection with pneumococci of the homologous type, even when enormous numbers of the latter are administered with the immune serum. There are, however, definite limitations in both the amount of serum and the amount of culture which can be used in order to demonstrate protection. An example of these limitations is shown in Text Fig. 1, which includes results with both rabbit and horse immune sera of approximately the same agglutinative titers. In both instances there is a limiting or titer zone in which the amount of immune serum appears to be insufficient. In both instances, also, one observes the so-called Schwellenwert or upper limit of culture beyond which it appears to be impossible to protect mice irrespective of the amount of serum. The most striking difference between the results with the two sera is that whereas the immune rabbit serum protected mice even when given in large amounts, the administration of immune horse serum in similar quantities resulted in a negative phase or prozone in which no protection was obtained.

The Schwellenwert. The clue to the nature of the Schwellenwert was found in a series of experiments which may be summarized as follows:
<table>
<thead>
<tr>
<th>Amount of serum (cc.)</th>
<th>Rabbit</th>
<th>Horse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>0.4</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.2</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.1</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.05</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.025</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.0125</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.00625</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.003125</td>
<td>••••</td>
<td>••••</td>
</tr>
<tr>
<td>0.00156</td>
<td>••••</td>
<td>••••</td>
</tr>
</tbody>
</table>

- •—Death
- ○—Survival

Fig. 1. Protection Tests with Type I Antineumococcus Horse and Rabbit Sera.

Serum and culture administered simultaneously. Type I Pneumococcus culture possessed a virulence such that 0.000,000,01 cc. produced fatal infection in controls.
1. If, instead of administrating serum and culture simultaneously, the former is given 18 hours in advance of the infecting organisms, a very large proportion of the antibody is obviously no longer available in the peritoneum. In spite of this loss, a considerable degree of protection is obtained if large amounts of serum are used, and it is especially noteworthy that under these conditions a number of mice can be protected against relatively large amounts of culture. The results suggest that the cellular make-up of the peritoneal fluid might have been altered by the presence of the foreign protein.

2. If intraperitoneal injections of sodium nucleinate are given 18 hours prior to the simultaneous administration of serum and culture, the protective action of antipneumococcus serum is markedly enhanced. The Schwellenwert undergoes a considerable shift in that the same amount of serum is now effective against larger amounts of culture.

Since it is known that the previous administration of sodium nucleinate brings about a considerable cellular response, it was considered possible that for each individual animal there is a true Schwellenwert related to the number and nature of the cells in the peritoneum at the time of injection of serum and culture.

After a thorough exploration of the possible technical approaches to the solution of this question, the following technique was adopted: Fifteen minutes after the injection of serum and culture, a sample of peritoneal contents was taken with a small capillary pipette and a smear made. From the
Gram stain the number of pneumococci and of cells, in many contiguous fields, were counted and the ratio of pneumococci to white cells determined. This ratio is obviously a function of the total number of white cells in the peritoneum, providing that the same numbers of pneumococci are injected in each instance. By means of this technique, an extensive study has been carried out, the results of which may be summarized as follows:

1. In mice of the same weight receiving the same amounts of immune serum, there appears to be a limiting ratio of pneumococci per white cell, and if this ratio is exceeded death is the inevitable result. In other words, mice with large numbers of white cells in the peritoneum are much more able to utilize the benefits of the immune serum than are those with few cells. These ratios serve as a basis for prediction of survival or death with an accuracy of about 85 per cent.

2. The limiting ratio in mice of the same weight is modified by the amount of immune serum. For example, with one particular lot of antipneumococcus horse serum, the best protection against 0.1 cc. of culture is obtained with 0.05 cc. Here the limiting ratio is approximately 7.0. With 0.0125 cc. of serum the limiting ratio is 5.4. With 0.2 cc. of serum the ratio is 4.0.

3. In a series of mice receiving the same amounts of serum, but differing in weight, the limiting ratio is a function of weight and becomes smaller as the weight increases.

4. With Dr. D. Miller, it has been possible to show that the latter finding is explained by the fact that very
small animals have a very high proportion of monocytes in the peritoneal fluid and vice versa. There are almost no polymorphonuclear leukocytes in the normal mouse peritoneum.

5. As an outgrowth of the foregoing, and in the light of certain other experimental facts, it can be inferred that both weight and peritoneal cell content can be in theory resolved into a single host factor, that is, the absolute number of monocytes in the peritoneum at the time of infective inoculation. These cells appear to constitute the first line of defense.

To summarize, the Schwellenwert, or limitation of the number of pneumococci which the passively immunized animal can dispose of, is a numerical function of the relation of the number of microorganisms to the number of monocytes in the peritoneum at the time of injection.

The prozone. Referring to Text Fig. 1, it is of some interest that whereas 0.05 cc. of antipneumococcus horse serum protects against 0.1 cc. of culture, larger amounts are less effective. With 0.4 cc. no protection is obtained. This phenomenon is referred to as the prozone, and has been the subject of an intensive investigation. The results may be summarized as follows:

1. The extent of the prozone in the protection test is quantitatively correlated with the agglutinin titer, that is, immune sera with high agglutinin titers show a more extensive prozone than do those with low titers.

2. For each lot of antipneumococcus horse serum, there appears to be a definite amount which gives optimal protection in the range of larger amounts of culture. This
optimal amount is related inversely to the agglutinin titer. Thus, one lot of serum with an agglutinin titer of 1-64 gives optimal protection when 0.2 cc. is used; a second lot with agglutinin titer of 1-256 with 0.05 cc.; while a third lot (concentrated) with a titer 1-512 gives best protection in 0.025 cc. amounts.

3. Failure of protection in the prozone range is associated with a marked decrease in the value of the Schwelenwert. An example of this has already been cited.

4. Failure of protection in the prozone range is associated with an apparent inhibition of phagocytosis. The pneumococci, if unchecked, quickly multiply, hence the early reaction on the part of the body is absolutely essential if protection is to be achieved. In the following table are shown results which bear on this subject:

<table>
<thead>
<tr>
<th></th>
<th>White cells showing phagocytosis during first hour</th>
<th>Pneumococci intracellular at end of first hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent</td>
<td>Per cent</td>
</tr>
<tr>
<td>No serum</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Optimal protective amount of serum</td>
<td>20</td>
<td>62</td>
</tr>
<tr>
<td>&quot;Prozone&quot; amount of serum</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

It would appear that the excess of immune horse serum, by inhibiting phagocytosis, permits the almost unchecked growth of pneumococci.

5. Certain heterologous substances have been injected with optimal amounts of immune horse serum. The addition of normal rabbit serum or of egg white fails to alter the protection.
On the other hand, Type II antipneumococcus horse serum, concentrated antineumococcus serum, lipemic rabbit serum and egg lecithin tend to block protection.

Since the only obvious common component of these unrelated inhibitory substances is lipoid, these findings suggested that it might be possible to remove the inhibitory element in immune horse serum by extracting the lipoids from it. This proved not to be the case, but this theory has led to an interesting series of findings which may be summarized as follows:

1. If raw antipneumococcus horse serum is extracted with alcohol-ether by a special technique which does not involve denaturation of the protein, the extracted serum fails to agglutinate pneumococci and does not give a precipitin reaction with the specific capsular polysaccharide. This result is not obtained with antipneumococcus rabbit serum similarly extracted.

2. The extracted horse serum still possesses an unaltered capacity to protect mice against fatal infection, even though the presence of antibodies cannot be demonstrated by agglutination or precipitation methods.

3. If extracted immune horse serum is injected intraperitoneally into mice and the peritoneal fluid withdrawn after 30 minutes, the recovered fluid possesses the capacity to agglutinate pneumococci. This result suggests that the modified antibody combines with a substance in the peritoneal fluid. A similar "reactivation" can be brought about in vitro by the admixture of the extracted serum and lecithin under certain conditions.
4. These results suggest that the antibody of immune horse serum may consist of a loose combination of protein and lipid, the protein carrying the specific combining groups. It is believed that the lipid element may be responsible for the prozone. Extracted sera still give the prozone, but this may be accounted for by an in vivo recombination.

5. Antipneumococcus sera from various other animal species have been examined from this point of view. The results indicate that the rat and the guinea pig may be grouped with the rabbit, while the goat and the mouse are in the same class as the horse.

6. The results presented in this summary are a further demonstration of the wide differences in the properties of antipneumococcus sera derived from rabbits and horses. Previous observations have demonstrated radical differences in the morphological characteristics of the precipitates with the specific capsular polysaccharides (Schiemann and Casper), the ability to give the Quellung reaction with the pneumococcus capsule (Neufeld and Tulcynski), the property of fixing complement with the capsular polysaccharides (Zinsser and Parker), etc. It is believed that the present work may furnish the clue necessary to the explanation of these differences.

To summarize, it has been shown that high titered antipneumococcus horse serum in amounts above the optimum fails to protect mice, whereas this is not true with antipneumococcus rabbit serum of equivalent antibody content. The results indicate that this failure to protect is due to an inhibition of the phagocytic mechanism by some element of the immune serum.
which has not been absolutely identified, but which is believed
to be liquid in nature.

The relationship of uronic acid to the biological
specificity of bacterial polysaccharides (Görbel). In previous
reports we have described the results of studies in which
certain structural and configurational differences of the
carbohydrate radicals of artificially compounded antisera have
been correlated with changes in immunological specificity.
These studies have been directed particularly to explaining the
factors which determine the specificity of carbohydrates. It
has been found that known differences in the chemical constitu-
tion of the carbohydrate radicals of synthetic antisera are
sharply reflected in the serological specificity of the anti-
sera to which they give rise in the animal body. This work has
now been extended to the naturally occurring bacterial poly-
saccharides. The present report deals with an explanation of
the cross serological reactivity of the capsular polysaccharides
of Types III and VIII pneumococcus on the basis of certain
similarities in the constitutions of the two polysaccharide
molecules.

The specific polysaccharide of Type VIII pneumococcus
has been isolated as a nitrogen-free carbohydrate, and shown to
be built up of units of glucose and glucuronic acid in the ratio
of seven molecules of hexose to two of uronic acid. When the
polysaccharide is hydrolysed with dilute mineral acid, the
hydrolysate is found to contain glucose and an aldobionic
acid—glucose glucuronide.
It has previously been shown that the specific polysaccharide of Pneumococcus Type III is likewise built up of molecules of glucose and glucuronic acid in the ratio of 1:1. When the polysaccharide is hydrolyzed with dilute acid, there is found in the hydrolysate an aldobionic acid, which has been likewise shown to be a glucose glucuronic acid.

It has been experimentally demonstrated that if the free hydroxyl groups of a bacterial polysaccharide are substituted with a chemical radical, the resulting derivative retains its capacity to react in homologous antiserum. If, on the other hand, the highly polar carboxyl group is covered with an ester grouping, the corresponding derivative loses entirely its serological specificity. It is believed, therefore, that the uronic acid constituent, and particularly the spatial relationship of its dominantly polar carboxyl group to other groups in the carbohydrate molecule, plays a very important role in determining the type-specificity of a bacterial polysaccharide. If it were possible, therefore, to show that the aldobionic acids of the specific polysaccharides of Types III and VIII pneumococcus were identical, then the cross serological reactivity of these two substances would be established on a definite chemical basis.

The aldobionic acids derived from the specific polysaccharides of Types III and VIII pneumococcus are both amorphous substances. It therefore becomes necessary to prepare a crystalline derivative of these two sugar acids in order to establish either their identity or dissimilarity. Consequently,
the methyl esters of which were prepared, which, on subsequent acetylation yielded the corresponding crystalline heptaacetates. When obtained in a state of purity these two derivatives were found to be identical in crystalline structure, melting point and specific optical rotation. It may be concluded, therefore, that the structural unit of the specific polysaccharide of Pneumococcus Type III is identical with one of the structural units of the polysaccharide of Type VIII. It is believed, therefore, that the cross serological reactivity of these naturally occurring bacterial polysaccharides may be explained on the basis of the identity in structure of the aldobionic acid nucleus common to both carbohydrates.

It has been found that the specific enzyme which hydrolyzes the capsular polysaccharide of Type III Pneumococcus is elaborated not only when the organism from which the enzyme is derived is grown in the presence of the polysaccharide, but is likewise elaborated when the Type III aldobionic acid itself is used as a substrate. Thus it would appear that if the structure of the aldobionic acid were established it should be possible to produce synthetically a specific substrate for the production of the enzyme. The problem of synthesizing the aldobionic acid involves, however, not only a knowledge of its structure, but a knowledge of the chemistry of glucuronic acid as well.

Because the appropriate source material—an acetohalogen derivative of glucuronic acid—has never been satisfactorily prepared, the synthesis of glucuronides has heretofore never been achieved. In collaboration with
Mr. Babers we have attempted to prepare a series of derivatives of glucuronic acid to be utilized for the syntheses of glucuron-ides and aldobionic acids as well. Thus it has been found that when glucuron, the lactone of glucuronic acid, is acetylated, there are obtained two isomeric triacetates from which a crystalline chlorodiacetyl glucuron can be prepared in excellent yields. It has been found that when this halogen derivative is condensed with methyl alcohol in the presence of silver carbonate the corresponding crystalline methyl glucuronide is obtained. From a study of the kinetics of hydrolysis of this derivative in the presence of extremely dilute acid, and from the fact that alkaline hydrolysis removes but one of the acetyl groups, it must be concluded that this derivative does not represent a true glucuronide with the structure:

\[ \text{I} \]

but a derivative of ortho acetic acid having the following structure:

\[ \text{II} \]
In view of the fact that the aceto halogen derivative of glucuron failed to yield a true methyl glucuronide, it was thought that glucuronic acid itself might furnish the appropriate derivative. Consequently, glucuronic acid was converted into the methyl ester, which, in turn, yielded the two isomeric α and β tetracetates upon acetylation. The latter derivatives were readily converted into the corresponding acetochloro derivative, from which the triacetyl methyl ester methyl glucuronide was prepared. Like the corresponding derivative of glucuron, however, this methyl glucuronide was found to be an orthoacetate, and not a true glycoside.

From the results of the above experiments, it would appear that it is impossible to synthesize the true glucuronides either from the halogen derivative of glucuron, or from that of the methyl ester of glucuronic acid. Further experimental work, however, has revealed the fact that if the molecular weight of the aglucon is increased, and if diacetyl chloroglucuron is condensed with the appropriate alcohol, such as nitrobenzyl alcohol, a glucuronide is obtained having the structure of a true glycoside. Furthermore, it has just recently been found that if the β tetracetyl methyl ester of glucuronic acid is warmed with titanium tetrachloride in chloroform solution, a second crystalline triacetyl chloro glucuronic acid methyl ester is secured which is isomeric with the chloro derivative prepared as previously described. On the basis of certain knowledge pertaining to the structure of this second halogen derivative, it may be anticipated that the latter compound will likewise yield the true glycosides of glucuronic acid. Thus,
the synthesis of the aldobionic acid of Pneumococcus Type III specific polysaccharides awaits the determination of the structure of this bios.

The synthesis of the acidobionic acid common to the specific polysaccharides of Types III and VIII Pneumococcus has not only a practical basis—-that of supplying a synthetic substrate for enzyme production, but a theoretical interest as well. By synthesizing the fundamental building stone of the polysaccharides of Pneumococcus Type III, it is our hope eventually to prepare an antigen, truly artificial, and bearing to the Type III Pneumococcus no relationship whatsoever save one of chemical structure, which will give rise in the host to specific immunity against infection with Type III pneumococci.

Publications.


Stillman, E. G. Duration of demonstrable antibodies in the serum of rabbits immunized with heat-killed Type II and Type III Pneumococcus. *J. Inf. Dis.* 1935. (In Press.)
