Report of Dr. Avery (assisted by Drs. McCarty and Taylor)

Studies on the transformation of types of pneumococcus (Avery and McCarty). During the past year, studies have been continued on the predictable and heritable changes induced in R variants of pneumococci by active desoxyribonucleic acid fractions derived from encapsulated pneumococcal cells. Three papers comprising the work presented in the last report have recently been published. The isolation and purification by Dr. McCarty of the enzyme, desoxyribonuclease (1) has provided a biochemical tool, the use of which has furnished confirmatory evidence of the chemical identity of the transforming substance (2). In addition, the fact that the destructive action of this enzyme on desoxyribonucleic acid is completely inhibited by citrate, has led to the development of an improved method for obtaining much larger yields of the transforming substance from lysates of living pneumococci of several different types (3). Moreover, as will be pointed out later in the present report, the enzyme also provides a means of determining the optimal conditions and length of time required for the uptake of the specific nucleic acid by the R cells in the transforming reaction.

Before discussing the newer work now in progress concerning the nature and biochemical properties of certain environmental factors essential in transformation, it may be well to recall briefly the present status of our knowledge of the transforming substance itself. Accumulated evidence based on the results of innumerable tests of the specificity and biological activity of various preparations, together with data obtained by chemical, enzymatic and serological analysis of the active material, has established beyond reasonable doubt that the active substance responsible for transformation is a specific nucleic acid of the desoxyribose type.

Biochemical studies of environmental factors essential in transformation of pneumococcal types (Avery and McCarty). In the historical development of the problem it is of interest to recall that Griffith, who originally described the transformation phenomenon *in vivo*, was unable to obtain positive results *in vitro*. The first successful demonstration of the reaction in the test tube was carried out by Lawson and Sia in nutrient broth containing anti-R rabbit serum. From that time on, serum or serous fluid of one sort or another has always been used, and has been shown to be an essential factor, since in its absence it is impossible to induce transformation. However, the function of serum is not merely one of enrichment, since the nutrient broth itself contains adequate amounts of accessory growth factors required for the initiation and maintenance of growth. The next advance, which, if successfully accomplished, would undoubtedly throw considerable light on the mechanism of transformation, rests upon finding the solution to the following questions: Why is the presence of serum or serous fluid in the medium essential? Why are some sera capable of supporting transformation, while others utterly fail? What components function as essential factors, how do they act, and what is the biochemical nature of their action in respect to the cellular changes evoked by the specific pneumococcal nucleic acid?

Studies currently in progress indicate that serum factor is composed of at least three essential constituents. These are 1) the R-antibody, which causes agglutination of unencapsulated R pneumococci; 2) a dialyzable constituent; and 3) an additional protein factor occurring in the globulin fraction of the serum. The evidence for assuming that the serum factor depends upon the collective action of the three components is summarized below, together with a description of certain experiments designed to

elucidate the function of each, and the mechanism of their combined effect. Finally, a statement is made of the current working hypothesis of the nature of this mechanism.

1) R-antibody. When unencapsulated R-pneumococci are grown in the presence of R-antibody, large aggregates are formed which settle to the bottom of the tube. The supernatant broth is thus left clear, so that if transformation occurs and encapsulated S cells are formed, the change is readily apparent, since the newly formed S cells, not being affected by the R-antibody, grow diffusely throughout the culture. While this phenomenon has been useful in the technique of the transforming test, the R-antibody appears to do more than merely provide visible evidence of transformation.

In the usual fluid media it has not been possible to induce transformation unless R-antibody is present. However, under special conditions, results have been obtained which give some indication of the role of R-antibody. In a semi-solid medium containing a low concentration of agar (0.2 %), pneumococci grow in colonies rather than diffusely, and loose aggregates are formed not unlike those that result from antibody agglutination, although they do not settle to the bottom of the tube. Transformation of type has been obtained in semi-solid medium containing normal rabbit serum, but wholly lacking in R-antibody. These experiments suggest that the type of colonial growth produced by anti-R is an important factor, and that when this type of growth is simulated by other means, the anti-R can be dispensed with.

Although it cannot be stated with certainty why colonial growth is required, it is possible that local reducing conditions arising in the aggregated cells are of primary importance. This thesis is supported by the results of experiments in which the medium is placed in a shallow layer

not exceeding 1-2 mm. in depth. In the shallow layer, oxidizing conditions are promoted, and even in the usual serum medium containing R-antibody, manifest transformation does not occur. Attempts to reverse the effect of the shallow layer by the addition of reducing agents have not yielded consistent results, but on one occasion transformation was obtained in a group of flasks in which glutathione had been added to the usual serum medium. There is, then, certain evidence that reducing conditions are essential in some phase of the transforming reaction.

To summarize, the R-antibody serves the purpose of causing an apparently essential colonial aggregation of R-pneumococci, which in turn results in local conditions, possibly reducing in character, that are required in the transformation reaction.

2) Dialyzable constituent. Early attempts at salt fractionation of serum factor by the classical methods of protein chemistry yielded totally inactive fractions. Some light has been thrown on these results by the discovery that a dialyzable component of serum is essential. When an active serum is dialyzed against physiological saline, there is a progressive decrease in its efficacy in the transforming system, and if dialysis is sufficiently prolonged, the serum becomes completely inactive. Under these conditions, however, the R-antibody is unimpaired, and no denaturation of protein is apparent.

Serum which has been inactivated by dialysis can be reactivated for use in the transforming system by two procedures which differ in certain important respects. In the first place, if inorganic phosphate is added to the dialyzed serum, and the mixture incubated 1-2 hours, the serum regains its ability to support transformation. The period of incubation of the serum with phosphate is essential. The interaction between phosphate and

the serum appears to be prevented by the presence of nutrient broth, for if the latter is added at the same time as the phosphate, or after a short period of incubation, no reactivation is achieved. In contrast to the reactivation by phosphate, which requires time, immediate reactivation can be achieved by adding to the serum such materials as unheated neopeptone, or tryptic digest of casein. Nutrient broth does not interfere with this effect. The nature of the substance responsible for immediate reactivation has not been determined. However, it has been shown to be a dialyzable substance, and to be precipitable by alcohol.

The available data suggest that phosphate brings about reactivation of dialyzed serum by promoting a chemical or enzymatic reaction, which results in synthesis of the dialyzable constituent of serum factor. On the other hand, the neopeptone, or casein digest, provides a preformed source of the dialyzable constituent, or some related substance which is able to replace it. It is of interest that globulin fractions of serum obtained by ammonium sulfate fractionation, that hitherto were found to be ineffective in the transforming system, can be rendered effective by the addition of unheated neopeptone as source of the dialyzable factor. R-antibody plus unheated neopeptone does not support transformation, however, and this fact is one piece of evidence for the existence of an essential protein constituent other than anti-R.

3) Globulin factor. It has long been recognized that the titer of R-antibody does not parallel the efficacy of the serum in the transforming system. Indeed, some of the most potent sera in terms of ability to support transformation, have been shown to have the lowest titers of anti-R. This fact, together with the evidence cited in the preceding paragraph, has led to the assumption that another essential constituent is present in effective sera. The results of fractionation experiments in which are

used salts such as ammonium sulfate and organic protein precipitants such as alcohol, have established that this additional constituent, as well as R-antibody, occurs in the globulin fraction of the serum.

For the purpose of orienting further research, the possibility has been considered that the globulin factor may be an enzyme. If this is indeed the case, it seems highly probable on general grounds that some organ of the animal body contains the enzyme in much higher concentration than does the serum, and would serve as a more favorable source for possible purification and identification of the enzyme. To test this assumption, a preliminary survey was made of several rabbit organs by preparing simple saline extracts and testing them for the presence of the globulin factor. The procedure used consisted of adding the extract to broth containing a small amount of concentrated rabbit R-antibody and unheated neopeptone as a source of the dialyzable constituent. The broth containing these added components was tested for its ability to support transformation. Positive results indicate that the organ extract has supplied the missing constituent (globulin factor), since the R-antibody and dialyzable factor alone are unable to support transformation. Rabbit spleen proved to be a good source of the globulin factor. To provide larger organs as source material, extracts of calf spleen and calf thymus were then tried, and it was found that thymus is superior not only to spleen, but to the best sera available. Fractionation experiments with thymus extracts are now in progress, in an effort to determine whether isolation and purification of the active globulin component can be achieved. The principal interest in purification is the possibility of determining the nature of the substance and its rôle in the transforming reaction. The results of preliminary studies with organ extracts lend some support to the hypothesis on which the experiments were based, i.e., that the globulin factor of the serum may be an enzyme.

Mechanism of action of serum factor. A series of experiments which were designed to provide a more intimate knowledge of the interaction between the specific transforming substance (pneumococcal desoxyribonucleic acid) and the susceptible pneumococcal cells proved to have an important bearing on the problem of the rôle of serum factor. The customary procedure in demonstrating the phenomenon of transformation is to add the specific desoxyribonucleic acid to the serum medium and to inoculate with a susceptible strain of R-pneumococcus. Transformation becomes apparent after 16 to 20 hours' incubation, but little is known of the course of events during this period of incubation. The purified enzyme, desoxyribonuclease, which specifically inactivates the transforming substance, has been used as a tool in an attempt to study certain phases of this problem.

By adding desoxyribonuclease to the transforming system at various intervals after inoculation, in a concentration known to give almost immediate inactivation of the specific transforming substance, it is possible to determine the length of time required for the transforming substance to be taken up or "fixed" by the susceptible cells. The addition of the enzyme at any time up to 3 or 4 hours after inoculation interferes with the reaction, so that transformation does not occur, and it is, therefore, likely that throughout this period the transforming substance is readily accessible to the desoxyribonuclease. After 4 to 5 hours, on the other hand, the addition of desoxyribonuclease has no observable effect on the course of the reaction. Consequently it appears that growth of the R cells in serum medium for 3 to 5 hours is required before the specific desoxyribonucleic acid is taken up by the cells, and thus protected from enzymatic destruction.

A striking confirmation of the foregoing is provided by experiments in which the R cells are grown in serum medium in the absence of the specific

transforming substance. After 4 to 5 hours' growth under these conditions, the cells are so "sensitized" that when transferred to a medium containing the transforming substance, the latter is taken up in as short a time as 15 minutes. If the transfer is made after shorter periods of growth in serum medium, e.g. 2 to 3 hours, the "sensitization" has apparently not taken place and rapid fixation of the transforming substance cannot be demonstrated. Thus, the events that occur in the first four hours of growth of R cells in the transforming system are independent of the presence of the specific transforming substance. It must be concluded that growth under these conditions alters the cell in some way, or provides suitable environmental conditions so that the interaction between the cell and the transforming substance can take place.

The relation of the above experiments to the rôle of serum factor becomes apparent from the fact that growth of the cells in plain broth, in the presence of R-antibody, or in serum inactivated by dialysis, fails in each case to "sensitize" the cells. Thus, the hypothesis is suggested that the major part played by serum in the transforming system is concerned with a modification of the cell so that the specific transforming substance can be taken up.

A tentative hypothesis of the mechanism of serum factor action. The evidence available at present favors the view expressed above that the rôle of serum factor is concerned with some action on the surface of the susceptible R cell which permits interaction between the cell and the specific transforming substance. A plausible and perhaps useful hypothesis with regard to the mechanism of the serum effect can be based on the assumption that the action is enzymatic in nature. In this view, the enzyme is present in the globulin fractions of active sera, as well as in

extracts of certain mammalian organs. The R-antibody, as a result of the colonial growth of the cells, provides the required conditions for the action of the enzyme. It is suggested that the reducing conditions resulting from this type of growth may be of significance.

The enzymatic system as outlined is not more complex than some already known. Recent work on muscle hexokinase in the laboratory of Dr. Carl T. Cori, provides a rather striking analogy. Muscle extracts lose hexokinase activity upon dialysis, and, as in the case of serum factor, activity can be restored by two means: 1) by incubation with phosphate, or 2) immediately by adding the preformed co-factor, which in this case proves to be guanine. In addition to guanine as co-factor, muscle hexokinase also requires the presence of reduced co-enzyme I (dihydrocozymase). This latter fact is suggestive, in view of the possible rôle of reducing conditions in the action of serum factor.

Although the hypothesis outlined above is admittedly only a tentative one, it has made possible an experimental approach to the problem of defining the significance and essential role of the so-called "serum factor" in the transforming system. While the interpretation may be modified as knowledge increases, the facts thus far obtained indicate that transformation consists of two phases:- 1) an initial phase in which, as the result of the combined action of the serum components, the R cells are rendered receptive and become capable of taking up the transforming substance; and 2) a phase in which a chain of biochemical reactions is initiated within the cells that culminates in synthesis of the capsular polysaccharide, the chemical identity and type specificity of which can be predetermined, depending on type of encapsulated cells used as source of the transforming substance.

Cytochemical studies on localization of desoxyribonucleic acid in pneumococcal cells (Taylor). Genetic and cytological studies of animals and plants have for the most part localized the hereditary units of the cell in the nucleus. Cytochemical studies have shown that the most characteristic component of the nucleus is desoxyribonucleic acid. The active substance inducing transformation of pneumococcal types and determining the specificity of the changes has been found to be a nucleic acid of the desoxyribose type. Since transformation may be described as a change in the heredity of the pneumococcal cell, it is of interest to know whether pneumococcal desoxyribonucleic acid occurs in a formed structure comparable to the nucleus of higher organisms.

Under controlled conditions the Feulgen nuclear reaction appears to be specific for desoxyribonucleic acid. When the Feulgen reaction is carried out on pneumococcal cells fixed with osmium tetroxide vapor, a deeply staining red granule can readily be observed within each cell. This suggests that desoxyribonucleic acid is not evenly distributed throughout the cell, but is partially or entirely localized.

In a medium containing anti-R serum, unencapsulated pneumococci grow in long chains of diplococci. Comparison of the Feulgen positive granules in the individual diplococci of these chains indicates that the granules undergo enlargement and duplication in the growing cells. The data thus far obtained do not warrant the conclusion that Pneumococcus is a nucleated cell, particularly in view of the questioned specificity of the Feulgen reaction.

It has been observed in this laboratory that the Feulgen positive material in the nuclei isolated from animal tissues is rapidly removed by the specific action of highly purified preparations of desoxyribonuclease.

Adaptation of this technique to the study of the cytology of pneumococcal cells is being made, since the enzyme affords a more specific method for identifying the nature of the central granules than do any of the known staining methods available at present.

Publications

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