Report of Dr. Avery

(Assisted by Dr. McCarty).

Studies on the pneumococcus transforming substance. In the last report to the Board two phases of the work in progress on the transforming substance were discussed. First, in an effort to provide additional evidence that the transforming substance is itself a nucleic acid of the desoxyribose type, a study of the enzyme which depolymerizes native desoxyribonucleic acid was undertaken. Secondly, the reversible inactivation of the transforming substance by compounds of known chemical composition was studied with a view to obtaining evidence concerning the nature of the chemical groupings essential to its biological activity. Both of these lines of investigation have been pursued.

Desoxyribonuclease (McCarty). The primary objective in the study of this enzyme has been to obtain it in a purified form as free as possible from other enzymes, and to determine the action of the purified enzyme on active preparations of pneumococcal transforming substance. As mentioned in the last report, the major obstacle to attaining this objective was the fact that the enzyme is rapidly inactivated by proteolytic enzymes. Pancreas is the richest source of desoxyribonuclease, but large amounts of proteolytic enzymes are present in extracts of this organ, and are carried along in fractionation procedures in sufficient quantities so that the nuclease preparations are very unstable in solution. By application of the methods devised by Northrop and Kunitz for the isolation of tryptic enzymes from pancreas, this difficulty has been largely overcome.

As in the procedure of Northrop and Kunitz, fresh beef pancreas is ground and extracted in the cold with 0.25 $\underline{N}$ H_2SO_4. Although the tryptic enzymes are present in this acid extract, fractionation is much

sharper than in the case of watery pancreatic extracts, and separation is readily achieved. Desoxyribonuclease is precipitated from the acid extract at 0.4 saturation with ammonium sulfate and at this concentration the tryptic enzymes are left in solution. The material obtained at 0.4 saturation with ammonium sulfate is subjected to further salt fractionation and finally dialyzed and dried from the frozen state. The dried enzyme is stable and highly active. Tests for the presence of other enzymes reveal that lipase, phosphatase and ribonuclease are entirely absent, and that proteolytic enzymes are present only in very small amounts. On the other hand, the activity of the purified enzyme in depolymerizing desoxyribonucleic acid isolated from calf thymus is extremely high. As little as 0.01 microgram of the enzyme brings about a rapid fall in the viscosity of 5.0 cc. of a suitably buffered solution of the nucleate. This effect, as described previously in the case of crude enzyme preparations, is activated by magnesium ion and inhibited by citrate.

The effect of purified enzyme preparations was tested on the biologically active desoxyribonucleic acid fraction obtained from Pneumococcus Type III. When the pneumococcal nucleate is used as substrate, the enzyme in concentrations of less than 0.01 of a microgram per cc. causes not only loss of viscosity, as observed in the case of calf thymus nucleate, but also a rapid and irreversible loss of transforming activity. It now seems certain that the biological specificity of the desoxyribonucleic acid is an inherent property of the intact molecule in its native, highly polmerized form, and that its structural unity cannot be broken down without loss of transforming activity. In view of the high activity, relative purity and selective specificity of desoxyribonuclease, these results afford strong confirmatory evidence

that the transforming substance is a nucleic acid of the desoxyribose type.

A practical application has been made of the inhibition of desoxyribonuclease by citrate. In the method previously described for preparing the transforming substance from type III pneumococci, the bacterial cells were heated at 65° C. for 30 minutes immediately after collection. This procedure was necessary in order to inactivate the desoxyribonuclease contained in the cells so that the transforming agent would not be enzymatically destroyed in the course of preparation. Extraction of transforming substance from the heated cells was known to be incomplete and much active material remained in the residual cells. It has recently been found that by lysing the pneumococcal cells with desoxycholate in the presence of 0.1 M sodium citrate, complete dissolution of the cells may be obtained and loss of transforming substance is prevented by virtue of citrate inhibition of the desoxyribonuclease. Work now in progress indicates that at least a five-fold greater yield of the active desoxyribonucleic acid fraction is obtainable by this procedure.

Reversible inactivation of the transforming substance (McCarty). The inactivation of transforming substance by ascorbic acid and prevention and reversal of this effect by sulfhydryl compounds was considered in the last report. Subsequent work has resulted in a better understanding of the mechanism of inactivation by ascorbic acid. Catechol, hydroquinone, phenanthrene hydroquinone, and p-phenylenediamine have been found to have an analogous action on the transforming substance. As in the case of ascorbic acid, the inactivating effect of these compounds is catalyzed by cupric ion, and is prevented and reversed by sulfhydryl compounds. All of the compounds mentioned undergo a similar

type of autoxidation, and the evidence indicates that inactivation of the transforming substance is linked with the autoxidation of the inactivating agent. Exclusion of oxygen by carrying out the reaction in an evacuated Thunberg tube completely nullifies the inactivating action of these compounds. Thus, molecular oxygen is apparently necessary in the reaction.

In view of the fact that peroxides are known to be formed in the course of aerobic, copper catalyzed autoxidation of ascorbic acid, hydroquinone, etc., the possibility arose that peroxides were responsible for the inactivating effect. That this is apparently the case is shown by the fact that minute amounts of crystalline catalase completely protect the transforming substance from inactivation by ascorbic acid. Furthermore, the addition of hydrogen peroxide to a solution of transforming substance results in inactivation, but the amount required is considerably in excess of that which would be liberated in the course of autoxidation of the minimally effective concentration of ascorbic acid. It is thus suggested that it is not hydrogen peroxide itself, but some other form of peroxide, susceptible to catalase action, which is responsible for the oxidative inactivation of the transforming substance.

No specific information has been obtained as to the nature of the groups in the molecule of transforming substance which are affected in oxidative inactivation. The fact that this form of inactivation is readily reversed by sulfhydryl compounds suggests that SH groups are present in the transforming substance, and that when these are oxidized to the S-S form, loss of activity results. There is, however, no additional evidence to support this suggestion. On the contrary, iodoacetic acid, which has a specific affinity for sulfhydryl groups, has no effect on the activity of the transforming substance. Attempts to

demonstrate changes in the oxidized transforming substance other than
that reflected by loss of biological activity have so far been unsucces-
ful. For example, preliminary studies reveal no alteration in the
characteristic ultra-violet adsorption spectrum of the pneumococcal
desoxyribonucleate as the result of treatment with ascorbic acid.
Furthermore, the oxidized nucleate is depolmerized by the specific
enzyme, desoxyribonuclease, at the same rate as the native substance.

It is of interest that the two types of inactivation de-
scribed – that is, enzymatic depolmerization and reversible oxidation –
are not without practical significance in the techniques employed in
transformation. Pneumococcal cells contain a desoxyribonuclease which
is released from the cell into the surrounding medium. Furthermore,
hydrogen peroxide is formed by the pneumococcus and can be shown to
accumulate in the medium during growth under aerobic conditions. The
transformation test is carried out in the presence of actively growing
pneumococcal cells, and therefore during the course of the reaction
it is possible for both desoxyribonuclease and hydrogen peroxide to
appear in the medium and destroy the transforming substance before it has
had a chance to act. However, experience has shown that certain cul-
tural conditions can be established which tend to suppress the libera-
tion of enzyme and peroxide into the medium during the initial phase
of interaction between the transforming substance and the rapidly
growing cells. For example, small inocula of young, actively growing
R cells are used, with the result that there is much less tendency
to early autolysis and consequent release of desoxyribonuclease. In
addition, as a result of the presence of R antibodies in the medium,
the cells aggregate and are sedimented early in the course of the test,
and subsequent growth proceeds at the bottome of the tube under

relatively reducing conditions. In this way the formation of hydrogen peroxide may be inhibited. In the light of these facts it is interesting to observe that the cultural conditions found requisite and empirically used in the transforming test as originally devised can now be largely explained in biochemical terms of certain known enzymatic and metabolic processes of the pneumococcal cell. In addition, however, there still remains an unidentified factor, present in the serum, which is also essential in the induction of transformation. The nature of the serum factor is now under investigation.

Publications.

McCarty, Maclyn, Reversible inactivation of the substance inducing transformation of pneumococcal types, (Abstract) J. Clin. Invest., 1944, XXIII, 942.