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

Studies on transformation of pneumococci. In order to demonstrate the transformation of pneumococcal types, the following minimal requirements must be supplied: (1) pneumococci of a competent R strain inoculated into (2), a medium adequate for growth, containing (3) anti-R agglutinins, (4) a factor contained in serous fluids, and in addition (5) a specific transforming principle. A proportion of the pneumococci growing in this system are found to be in the smooth phase, which have been "transformed" to the specific serological type that had served as source of transforming principle. It has been the objective of recent work of this laboratory to achieve maximum definition of the chemical and biological nature of these five factors, and the mode of their interaction.

In recent studies progress in this direction has been made by the application of two principles: First, in the detailed investigation of any single factor, this component is made the limiting one in the system by supplying the other four components in the optimal form and quantity insofar as this is known. Secondly, the success of transformation is judged semi-quantitatively by observing the rate of appearance, and quantity, of the transformed cells produced. These principles have made it possible to assess on a quantitative basis the effect of chemical procedures and treatments that are reflected only ambiguously in an all-or-none end point titration. This approach has led to certain findings which will be discussed first in terms of the transforming principle, then of the serum factor, and finally with respect to the interaction of these two factors.

Chemical nature of the transforming principle (Dr. Hotchkiss). Although previous work of this laboratory has strongly indicated that the purified transforming principle of pneumococcus is a highly polymerized desoxyribonucleic acid, chemical characterization of the material was still in many ways incomplete. Chemical analysis has therefore been undertaken of an initial concentrate (containing approximately 300 milligrams of the finally purified material) representing a pool of several lots of active transforming material derived from 225 liters of Type III pneumococcus culture. The material was investigated throughout, and especially at the later stages of a rather extensive purification procedure. The analytical data are fruitful in suggesting further possibilities for purification, but they will be summarized insofar as they have thrown light on the nature of the material.

The purified material finally recovered shows activity at 0.01 μg per cc., and 0.07 μg per cc. is approximately the level which consistently gives transformation in 50 percent of the tests in a system optimum in other respects. It is of especial interest that during the treatments which this active material has received, its amino acid content (determined by a method to be described later in this report) has steadily fallen until less than 0.5 percent of the nitrogen present is amino acid nitrogen. Although a protein material of exceedingly high activity might still be present, it appears quite unlikely that non-specific fractionation procedures which are successfully removing protein material in general should simultaneously be causing enrichment of a hypothetical active protein. In the last stage of the purification, the amino acid content was reduced by one half. Accordingly, while awaiting further development of this work, it is possible to state

definitely that a highly active purified transforming principle contains not more than 0.6 percent of its weight of protein, and gives promise of being rendered still more nearly free of a proteinaceous constituent.

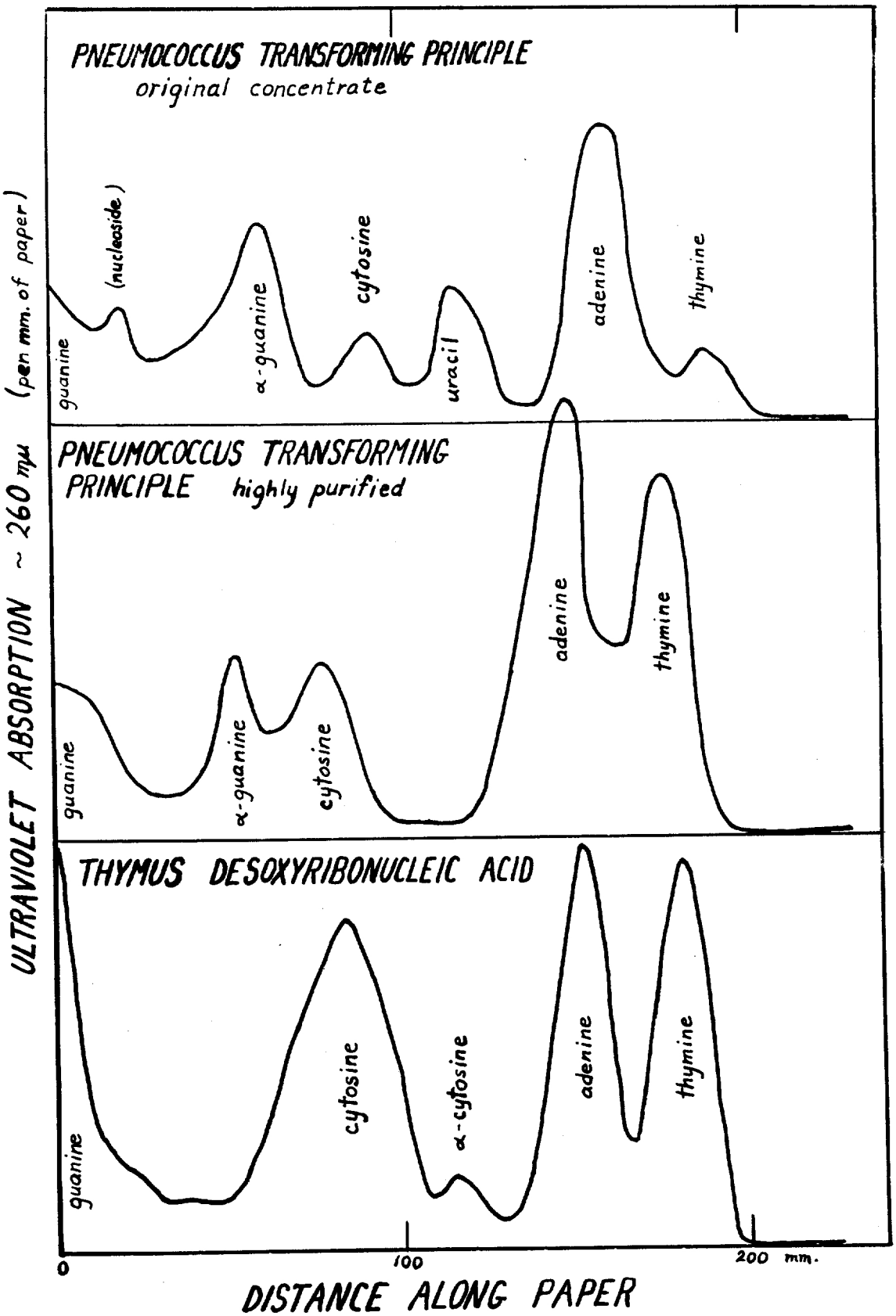
Other analytical data indicate that certain steps in the purification are highly effective in removing inert material. Total nitrogen, organic phosphorus, and ultraviolet-absorbing constituents are removed rapidly during treatment with ribonuclease, and then fall more slowly toward an apparently steady value during many reprecipitations. As should be expected, the nitrogen-to-phosphorus ratio demonstrates neither sensitivity nor reliability as an indication of protein content, but fluctuates considerably during the processing. In the final product, however, this ratio does have the value 1.72, close to the "theoretical" value 1.69 calculated for a desoxyribotetranucleotide.

In contrast to the constituents just mentioned, desoxyribose was but little diminished during the purification process, nearly all of that present in the original crude preparations being found in the highly purified product. Furthermore, the greatly diminished levels of nitrogen, phosphorus and ultraviolet-absorption which remain now bear a relation to the desoxyribose content. However, the ratios are not in all cases precisely the same as those of thymus desoxyribonucleic acid. In particular, the ultraviolet-absorption per unit of phosphorus is about 5 percent lower than that of the thymus preparation.

Present conceptions of the nature of nucleic acids are of two divergent kinds. On the one hand, it has long been stated that all are polymers of one of two simple structures, irrespective of source. Either they are composed of units containing one residue each of adenine, guanine, cytosine, and uracil combined with four ribose and phosphate

residues; or they have the desoxyribonucleic structure made up of one residue each of adenine, guanine, cytosine and thymine, with four desoxyribose and phosphate groups. Contrasting with this view is the opinion that, for example, desoxyribonucleic acids from biologically different sources possess structures characteristic of their sources, differing quantitatively and perhaps even qualitatively from the composition of the classical structure. This latter view derives its support from a few observations of ratios of purine bases deviating materially from the "theoretical" and in one or two reports of the discovery of unusual bases in nucleic acid preparations. Also, the conception that the pneumococcus transforming principle is essentially a desoxyribonucleic acid implies the corollary that even each such preparation from a distinct serological type of pneumococcus must have a unique chemical, or physical, structure. Analyses of nucleic acids other than those from yeast and calf thymus, however, have been too scattered and incomplete to furnish convincing evidence for either of the opposing views.

With the aid of a new analytical tool to be described below, it has been possible to determine quantitatively the individual purine and pyrimidine bases of the various preparations used in this work. "Profiles" of three of them are represented in the accompanying figure. It may be noted that purified transforming principle, like calf thymus desoxyribonucleic acid, contains guanine (and α-guanine: see below), cytosine, adenine, and thymine. Cruder material, on the other hand, contains also uracil, and has a much smaller proportion of thymine. Most significant of these preliminary analyses appears to be the abnormally high content of thymine, one which clearly indicates that at least a portion of the pneumococcus transforming factor must contain two or more


PNEUMOCOCCUS TRANSFORMING PRINCIPLE
original concentrate
guanine
(nucleoside)
α-guanine
cytosine
uracil
adenine
thymine
PNEUMOCOCCUS TRANSFORMING PRINCIPLE highly purified
guanine
α-guanine
cytosine
adenine
thymine
THYMUS DESOXYRIBONUCLEIC ACID
guanine
cytosine
α-cytosine
adenine
thymine
0
100
200 mm.
DISTANCE ALONG PAPER
ULTRAVIOLET ABSORPTION ~ 260 mμ (pen mm. of paper)

thymine residues per tetranucleotide "unit," a definite departure from the classical structure. This pyrimidine may in part take the place of some residues of adenine, a purine base, and of cytosine, both of which appear to be appreciably below the "theoretical" level. The lower content of adenine and higher content of thymine, a base of lower ultraviolet absorption, probably explains satisfactorily the low absorption per unit of phosphorus shown by this nucleic acid as compared with the thymus preparation.

In terms of total base recovered, it may be noted from the figure that uracil, a constituent typical of ribonucleic acids, is completely removed during purification. Calculations show further that adenine and cytosine are reduced during purification, but reveal the noteworthy fact that all of the thymine present in the original material is recovered in the purified preparation.

In summary, these findings force one to the conclusion that the effective steps in the purification of pneumococcus transforming principle are steps which remove proteins and ribonucleic acid, and retain almost without loss the desoxyribose and thymine of the original material. The final product is a desoxyribonucleic acid containing typical constituents, but differs from the classical calf thymus desoxyribonucleic acid in having more thymine, less adenine, less cytosine, and lower ultraviolet absorption per unit of phosphorus.

Chemical methods for the study of nucleic acids (Hotchkiss). Two methods which were developed for the study, the preliminary phases of which have just been summarized, are sufficiently unusual to require some description.

First, the assay of small amounts of amino acids in nucleic

acid preparations is complicated by the fact that adenine partially decomposes to give glycine when heated in acid solution. This side reaction has been controlled by precisely fixing the acid concentration, temperature, and time of hydrolysis in an autoclave. Adenine in all its usual compounds behaves quantitatively identically since it is very rapidly liberated as free base. The other purines and pyrimidines do not give rise to amino acids. Under suitable conditions only 0.5 percent of the nitrogen of an ordinary nucleic acid is converted to glycine. Using the actual adenine content of the nucleic acid preparation as a basis, an accurate correction for the amino acid liberated from adenine can be applied to the total amino acid determined by the Van Slyke ninhydrin procedure.

Secondly, and of far more general interest, a paper chromatographic method has been developed in this laboratory which makes possible the quantitative determination of individual purine and pyrimidine bases in a hydrolysate of one or two milligrams of nucleic acid. Briefly, as in amino acid chromatography, a moist organic solvent is caused to flow evenly along a strip of filter paper bearing at one spot the mixture to be analysed. It is found that a base the solubility of which in the organic solvent is high, relative to that in water, travels as a well-defined spot in the moving solvent, and travels more rapidly the higher its solubility. After a suitable time, the paper contains separated narrow bands of individual, highly purified purine and pyrimidine bases. These substances are detected by virtue of their ultraviolet absorption at 260 millimicrons. Excised segments of the paper strip are soaked in water and the solution is examined in the ultraviolet spectrophotometer. The figure already referred to shows the results of three such analyses,

in which magnitude of absorption is plotted against position along the paper strip.

This procedure is more indirect than the simple color reaction by which amino acids are detected, but it has the enormous advantage that it is quantitative. In addition, experience has shown that individual known bases may be identified positively on the basis of absorption at a few selected wavelengths. Spectrally pure adenine, uracil, thymine, and cytosine have been repeatedly isolated from complex nucleic acids in those regions of the paper strip carrying maximal absorption. Guanine can also be recovered in a virtually pure state in most instances. Furthermore, in case the positions of two substances on the paper do overlap to a certain degree, the individual substances may be quantitatively determined in any binary mixture that may be encountered in practice. This determination can be accomplished by making absorption measurements at three or four wavelengths so chosen that quantitative accuracy is maximal. In addition, special advantage is taken of characteristic shifts in absorption occasioned by the addition of alkali. The total absorption due to a given base is an accurate measure of the total amount of that base present in the original mixture. Recoveries from known mixtures have been within 10 percent of the theoretical.

Applications of this analytical tool, other than that already given, have appeared during the testing of the method. The nucleosides, adenosine, guanosine, cytidine, and the desoxyribosides of thymine and guanine have been isolated as successfully upon paper strips as have the bases themselves. Partial hydrolysates of nucleic acid can be studied, and for example, it has been shown that on acid hydrolysis guanine and adenine are most rapidly split from yeast nucleic acid, followed by

uracil and lastly, cytosine. It is anticipated that the study of enzymatic digests should reveal something of the structure of the nucleic acids, and also the nature of the enzymatic degradations catalyzed by the nucleases.

Finally, any atypical bases present in a specific nucleic acid should be recognizable on a paper strip chromatogram. The figure shows two such bases, "μ-guanine" and "μ-cytosine." The former, judged upon its absorption spectrum is closely related to guanine, and is tentatively regarded as an artifact formed only in the hydrolysis of certain preparations of nucleic acid. Its nature is under further investigation. μ-cytosine is clearly related to, yet distinct from, cytosine, and it has been obtained repeatedly as a minor constituent of hydrolysates of a preparation of calf thymus nucleic acid. It has not been identified with any known compound as yet, but the following suggestive observations might be mentioned: (1) The greater mobility of μ-cytosine than cytosine in butyl alcohol suggests alkyl substitution. (2) The absorption spectrum stands in the same relation to that of cytosine as the spectrum of thymine (5-methyl-uracil) does to that of uracil. (3) Johnson and Coghill in 1925 claimed to have isolated small amounts of 5-methyl cytosine from the nucleic acid of the tubercle bacillus. Identification of these unusual bases which have been revealed on the paper strip chromatogram is being sought, since it should throw further light on the chemical structure of certain nucleic acids.

Biochemical studies of the accessory serum factor (Hotchkiss, Taylor, and Avery). In the preceding report from this laboratory it was indicated that serum albumin of bovine or human origin can replace human serous fluids as an accessory factor (factor 4 in the list enumerated

above) in the transforming system. This fact did not at first appear reconcilable with the observation that serous fluids in general were of extremely variable potency and that normal sera were usually ineffective.

The activity of serum albumin may, however, be obscured by certain interferences. The effect of excessive amounts of the albumin itself will be discussed in a later section of this report. Another kind of interference is found when certain inhibitory substances are added together with albumin. Small amounts of lauric or oleic acid can inhibit transformation in a system containing fifty times their weight of albumin. It is not known whether the fatty acids are themselves toxic or whether they combine with the albumin rendering its chemical groups unable to enter into some other combination essential for transformation. Serum globulin fractions generally have a similar inhibitory effect not clearly due to their content of fatty acids.

As a result of such inhibition, normal sera and many preparations of albumin appear to be lacking in activity. By fractionation, however, active albumin may be recovered from sera which are themselves inactive, and the only active fractions are those containing albumin. Moreover, when serum fractions are tested quantitatively in a transforming system adequate with respect to the other factors, it becomes evident that further treatment of the albumin preparations is often necessary to bring them to full effectiveness. Treatment with trichloroacetic acid, or heating in acid at pH 2.5 have proved to be useful for this purpose. These procedures are such that globulins and probably most low-molecular contaminants are removed, but the albumin itself is not noticeably damaged. Bovine plasma albumin, heated in acid solution, has been adopted as the preferred source of accessory serum factor for

a system in which the activity of transforming nucleic acid can be quantitatively studied.

These facts, and further observations that human serum albumin, purified by several recrystallizations or by a highly selective separation as a mercury derivative (in the laboratory of Professor E. J. Cohn), are highly active as accessory factor, appear to explain negative or conflicting results obtained with cruder preparations. Serum albumins are the only highly effective materials known, but their activity is masked in many sera and serum preparations.

Interaction of serum albumin and transforming principle (Hotchkiss and Avery). Transformation also fails if excessive amounts of an active albumin solution are added to the normal system. This interference can be shown to be due to a slow inactivation of transforming principle on incubation with albumin. Smaller proportions of albumin will have this effect if incubated with transforming principle in the absence of other components of the system. At low temperatures or in the presence of salt, this inactivation is diminished.

Whether this rather striking loss of activity of the nucleic acid is due to a depolymerization, binding of its active groups, or an oxidation, etc., is not known. Its interest here lies in the fact that it is conclusive evidence that two important components of the transforming system under some conditions interact. Further, the interaction is a slow, apparently stoichiometric reaction. When the ratio of albumin to nucleic acid is high enough, the activity of the latter is completely lost even in the presence of the other components of the transforming system. When, on the other hand, salt concentration is raised in the normal system in an attempt to repress all possibility of interaction,

transformation also fails. These observations are compatible with the conception that some reaction of albumin with nucleic acid is important for the success of transformation, but that in its later stages, the reaction leads to inactivation of the transforming principle.

The assimilation of amino acids and of nucleic acid constituents by respiring staphylococci (Hotchkiss). In a previous report it was stated that washed staphylococci, when supplied with glucose and amino acids, do not multiply, but synthesize and accumulate cell protein. The energy furnished by the oxidation of glucose is an essential factor making possible this synthesis. An attempt has been made to learn whether glucose breakdown could also facilitate the coupling of purine and pyrimidine bases to sugar and phosphate with formation of nucleic acids. This it is able to do, but a further interesting requirement must be met before nucleic acid synthesis occurs. A "nucleic acid lack" must be developed, which may be done in at least two ways: (1) the cells may be allowed to respire in the absence of added substrate, in which case they slowly destroy their already existing store of nucleic acid, without losing any protein, or (2) the cells may be given amino acids together with the glucose, in which case they build up protein, to which, apparently, newly formed nucleic acid may be anchored. It is felt that studies of this kind reveal something of the integration of chemical processes in normal growth in general, and of the sequence of events that are very likely occurring during pneumococcus transformation, in particular.

Publications

Hotchkiss, R. D., The assimilation of amino acids by respiring washed staphylococci, Federation Proceedings, 1947, 6, 263.

Hotchkiss, R. D., A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations, Arch. Biochem., 1948, 16, 131.

Hotchkiss, R. D., (Book review) Modern development of chemotherapy, Havinga, E., et.al. Amsterdam, 1946, J. Appl. Physics, 1947, 18, 1135.

McCarty, M., Taylor, H. F., and Avery, O. T., Biochemical studies of environmental factors essential in transformation of pneumococcal types, Cold Spring Harbor Symposia, 1946, 9, 177.