STUDIES ON THE CHEMISTRY OF THE TRANSFORMING ACTIVITY

I. Resistance to Physical and Chemical Agents*

By STEPHEN ZAMENHOF, Ph.D., HATTIE E. ALEXANDER, M.D., and GRACE LEIDY

(From the Department of Biochemistry and the Department of Pediatrics, Columbia University College of Physicians and Surgeons, and the Babies Hospital, New York)

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Avery, MacLeod, and McCarty (5) found that the purified transforming principle of *Streptococcus pneumoniae* has the properties of a highly polymerized desoxypentose nucleic acid (DNA). This conclusion was based mainly on two facts: (*a*) the active preparation consisted of highly polymerized DNA, and substances other than DNA could not readily be detected in it, and (*b*) of many enzymes tested, only desoxyribonuclease (DNase), in minute quantities, could destroy the transforming activity. These results were corroborated by McCarty and Avery (6, 7), and by Hotchkiss (8); in particular, Hotchkiss obtained evidence that the purified and active transforming principle may contain no more than 0.02 per cent protein and that the progressive purification of DNA does not result in any significant decrease of the transforming activity.

Similar evidence was obtained when studying the transforming principles of other bacterial species, *Hemophilus influenzae* (9, 10) and *Neisseria meningitidis* (11, 12).

The above findings have opened the possibilities for more detailed studies of the chemistry of the transforming activity. Avery, MacLeod, and McCarty (5) studied qualitatively the resistance of the transforming activity to various agents; the resistance was much higher in the non-purified than in the purified transforming principle. McCarty (13) studied the inactivation of the transforming principle by ascorbic acid and other self-oxidizing agents, and the reactivation by reducing agents. However, to the authors' knowledge, no other study on the chemistry of the transforming activity has been reported.

The object of the present work is such a study. The purpose of this study is manifold. The first aim is to obtain still more evidence as to the DNA nature of the transforming principle. Another object is to determine safe conditions

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of preparing and handling the DNA having transforming activity. The information so gained can also be applied to DNA preparations of other species which do not lend themselves to the study of the transforming activity. The main object of the study, however, is to obtain information as to the factors of the molecule (bonds, chemically reactive groups, etc.) necessary for its activity as a transforming principle or as a determinant of heredity. The results of one phase of this work are reported here.

Materials and General Methods

Receptor Strain Used for Transformation.—The receptor strain used in this study was the Rd strain of *H. influenzae* described in reference 9.

Donor Strains Used for Preparation of the Transforming Principle.—Two donor strains of *H. influenzae* were used in this study: (1) Strain of type b, as described in reference 9. (2) R strain resistant to 1000 γ/cc. streptomycin and transformed to type b.

The transforming principle from strain 1 was used for all experiments except the determination of minimal amount of DNA necessary for transformation; the transforming principle derived from strain 2 was used only for the above determination and for the studies of the effect of purification (Table I) and of heat (Fig. 1) on transforming activity.

Method for Testing the Activity of the Transforming Principle and the Unit of Activity.—The activity of the transforming principle was tested in various dilutions as described in reference 9 (p. 348) for the transformation to type b and in reference 14 (p. 19) for the transformation to streptomycin resistance. The potency of a preparation of the transforming principle is expressed in this paper in terms of units of transforming activity (UTA). One UTA is the smallest amount of activity necessary to produce uniform transformation (two out of two samples) in the conditions of the test. The number of UTA for a preparation under test is thus obtained by determination of the dilution still giving uniform transformation. The number of UTA per γ DNA is calculated from the amount of DNA in the preparation, determined as described in reference 10.

The typical values so obtained for the intact preparations are given in Table I. Upon inactivation, the lower limit of detectability of the activity in DNA can be estimated from the initial concentration of DNA used for test, it being borne in mind that the conditions of the test (9) introduce 20-fold dilution of the starting material: thus, if the latter contained 1000 γ DNA/cc., the activity of 1 UTA can be still detected in a preparation having 1 UTA/1000/20 γ DNA or 0.02 UTA/γ DNA.

Purification of the DNA Having Transforming Activity.—A method for the purification of the DNA from *H. influenzae* having transforming activity has recently been described (10). The limited volumes of the electrophoresis cells make the method less suitable when larger amounts of the preparation are desired. In this work a new method has been devised as described below; it is based on the observation that when the cells of *H. influenzae* are lysed by desoxycholate and precipitated by ethanol, the resulting fibrous material on extraction with physiological saline yields very little DNA, but abounds in RNA, proteins, and immunologically active substance (polyribophosphate (15)). These impurities can therefore be largely removed, with little loss of DNA, before the main extraction of DNA (with detergent) is undertaken.

In a typical preparation a 6 to 7 hour growth (at 37°C.) on 500 Levinthal agar plates was harvested, washed, and lysed as described in reference 9. Immediately after lysis, the purification was carried out as described below. To the milky suspension of lysed cells 2 volumes of absolute ethanol was added; this resulted in an immediate formation of voluminous fibers and of amorphous precipitate. The fibers floated on top of the mother liquor and could be

easily lifted within 1 hour after addition of ethanol. If left overnight in the original mother liquor, they sink, become friable, and are more difficult to collect.

The collected fibers were well drained, transferred to a sintered glass funnel, washed with three 50 cc. portions of 75 per cent ethanol, and filtered with suction until they exhibited the consistency of moist felt. They were then transferred to a centrifuge cup and stirred for 12 hours in 40 cc. of an aqueous buffer of pH 7.4, 0.14 M with respect to NaCl and 0.015 M with respect to sodium citrate. This buffer will hereafter be referred to as "standard buffer."

The suspension was stored in the refrigerator for 24 hours and then centrifuged at 1800 *g* for 1 hour. The heavily opalescent but transparent supernatant was removed and stored in the frozen state under the designation of extract No. 1. It contained 1.8 mg. of DNA, approximately 107 mg. of RNA, approximately 5 mg. of PRP, and approximately 125 mg. of

TABLE I

Effect of Extraction and Purification on Transforming Activity per Weight Unit DNA

Transformed feature	Extract No.	Purification	Impurities (per cent of DNA)			Activity UTA/γ DNA‡
			RNA	PRP*	Protein	
Production of PRP*	1	None	6000	280	7000	3300
	12	3 × deproteinization and norit	<0.3	<0.3	<0.4	3000
	19	1 × deproteinization	<0.3	<0.3	<0.4	3000
Streptomycin resistance	1	None	6000	280	7000	1300 to 4200
	11	3 × deproteinization	<0.3	<0.3	<0.4	3000

* Polyribophosphate.

‡ Units of transforming activity per γ DNA (see General Methods).

protein. The sediment was reextracted as described above; this procedure was repeated for a total of 8 extractions. The total amount of DNA so extracted was 5 mg.

The sediment remaining after the 8th extraction was deproteinized by the addition of 30 cc. of the standard buffer containing 3.2 cc. of a 4 per cent "duponol C"[1] solution in 45 per cent ethanol (16, 17). The mixture was stirred for 1 hour at room temperature; solid NaCl was added to obtain a final NaCl concentration of 4 per cent. The stirring was continued for 30 minutes and the mixture was stored in the refrigerator for 24 hours.

The mixture was then centrifuged for 45 minutes at 25,000 *g*. The opalescent supernatant was removed and the sediment reextracted as described above; this procedure was repeated for a total of 10 standard buffer-duponol extractions. The final sediment, which contained practically no DNA (as tested by diphenylamine reaction), was discarded.

The supernatants of all the buffer-duponol extractions were now subjected (either separately or combined) to further purifications as described below; the last of these extracts contained the least amounts of impurities (RNA, PRP, and proteins), but had also the lowest

[1] A mixture of sodium lauryl sulfate and other fatty alcohol sulfates manufactured by E. I. du Pont de Nemours & Co., Wilmington.

DNA content. The typical amounts were 11 mg. DNA in the first buffer-duponol extract, and 3 mg. in the last, total 51 mg.

2 volumes of absolute ethanol was added to each extract; the resulting fibers were lifted, washed twice with 75 per cent ethanol, well drained, and redissolved with mechanical stirring in a sufficient amount of the buffer-duponol mixture to obtain a solution containing 300 γ DNA/cc. The deproteinization was then carried out as described before. This procedure was repeated for a total of five to six deproteinizations in the case of the first buffer-duponol extracts, and for a total of three in the case of the last extracts.

The fibers obtained after the last deproteinization were redissolved in standard buffer (final DNA concentration 0.5 to 1 mg./cc.). If at that point the solution was not completely clear, it was filtered through hyflo-supercel (Johns-Manville Corp., New York) as described in reference 16. The solution was purified with charcoal (18), to remove remaining RNA. After centrifugation at 25,000 *g* for 45 minutes the DNA from the supernatant was precipitated by 2 volumes of absolute ethanol, the fibers washed in 75 per cent ethanol, drained, and redissolved in standard buffer in an amount sufficient to obtain the desired concentration of DNA (0.5 to 2 mg./cc.). The final product ("stock solution") was stored in the frozen state at −15°. When so kept, no decrease in activity was observed for at least ½ year. Total yield was 31 mg. DNA. The typical activities are summarized in Table I.

Except as noted, all the operations were performed in the cold, using only glass, lusteroid, or rubber vessels and tools.

Analysis of the DNA Preparations Having Transforming Activity.—The preparations in various stages of purification, and the final products were analyzed for DNA, phosphorus, RNA, protein, and the immunologically active substance, as described in reference 10. In addition, the immunologically active substance, the polyribophosphate, was also estimated chemically, as described in reference 15. A typical analysis of a final product (13th extract) is as follows: DNA, 99.3 per cent; P, 8.8 per cent; RNA, less than 0.3 per cent; Protein, less than 0.4 per cent; immunologically active substance, less than 0.3 per cent (by immunological and by chemical methods as polyribophosphate).

The Determination of Viscosity.—Carried out essentially as described in reference 19; since the flow time depends somewhat on the volume of liquid used, exact volumes of 0.250 cc. were delivered by the use of a microburette with a 1 mm. O.D. plastic delivery tube.

EXPERIMENTAL

Effect of Purification on Transforming Activity of DNA Preparations.—When the transforming principle of *H. influenzae* was purified by electrophoresis, no decrease in the transforming activity per weight unit of DNA was noticed (10).[2] This type determination was repeated for the purification procedure described in this paper. The results are presented in Table I. These results show that the transforming activity is not due to any removable impurities (RNA, protein, PRP); they strongly suggest that the activity is vested in the DNA itself. The results further suggest that at least as far as factors influencing the outcome of all the extraction and purification steps are concerned, the transforming principle behaves like any other DNA molecule in the preparation: were it not so, the activity per weight unit of DNA would change perceptibly in 18 extractions and additional purification steps. It is also to be noted that the principle which causes transformation to the production of

[2] The slight increase in activity noticed in these experiments is not significant in the light of our present experience.

polyribophosphate behaves in this respect similarly to the principle which causes transformation to resistance to streptomycin.

The results in Table I also indicate that none of the steps used in the present procedure (including the deproteinization with duponol, not previously investigated in relation to the transforming principles) causes any inactivation.

Minimal Amount of DNA Preparations Necessary for Transformation and the DNA Content of One Cell of H. influenezae.—For the reasons explained later on it seemed advisable to determine the minimal amount of DNA preparations necessary for transformation. The system of transformation to streptomycin resistance (14) has been used for this purpose because of its convenience in the demonstration of transformed cells. To assure the presence of a maximum number of "sensitive" cells, the total number of cells used was as high as 10^7; a higher number would have introduced a danger of the appearance of a streptomycin-resistant cell by spontaneous mutation (14), and would have increased the viscosity in the small volume of the system to an excessive degree. The total volume employed was 0.02 cc.; the purpose of using such a small volume was to decrease the total amount of DNA without decreasing the concentrations, which would decrease the chances of meeting sensitive cells.

The experiment was performed by introducing into each of several graduated pipettes 0.01 cc. of the bacterial suspension containing 10^9 cells, followed by an equal volume of the solutions containing various concentrations of purified transforming principle derived from streptomycin-resistant strain. After mixing in the pipettes, the culture was incubated for 30 minutes at room temperature and expelled into 2 cc. of Levinthal broth. After 2 hours incubation at 37°C., streptomycin was added to give a concentration of 1000 γ/cc. The cells transformed into streptomycin resistance were detected in the usual way (14). The minimal total amount of DNA necessary to transform at least one cell was found to be 3×10^{-7} γ. In another experiment (20), in which the number of induced streptomycin-resistant cells was counted, the minimal amount of DNA per transformed cell was found to be 10^{-8} γ.

That such low amounts of DNA effect the transformation of *H. influenzae*[3] is undoubtedly due in part to the absence of desoxyribonuclease in the culture. The supernatant from 6 hour old (at 37°C.) cultures of rough strains of *H. influenzae* derived from types a, b, c, d, e, and f and a 48 hour old culture of rough strain derived from type b were tested for the presence of desoxyribonuclease as described in reference 19. No indication of the presence of the enzyme was obtained during 3 to 20 hours' incubation at 30°. This relative absence of desoxyribonuclease even in the 48 hour old (partially autolyzing) cells is one of the advantages of using *H. influenzae* for the preparation of intact transforming principles and for quantitative studies on their activity.

The reported values for the molecular weights of DNA in carefully purified preparations vary between 8.2×10^5 and 7.7×10^6 (21, 22). Recent publica-

[3] The values reported for the transformation of *S. pneumoniae* are 3×10^{-3} γ to 10^{-2} γ DNA (5, 7, 8); these values could be probably lowered by using the methods of analysis described in this paper.

tions (23–27) report 4 to 7 × 10^6 as probable values. With these values, the minimal number of DNA molecules necessary to transform one cell in the system tested would be of the order of 10^3.

It is of interest to compare this minimal amount of DNA with the total amount of DNA in one cell of *H. influenzae*. The reported content of DNA in microbial cells is of the order of 10^{-8} γ for a presumably haploid cell of *Escherichia coli* (28) and 3.3 × 10^{-8} γ for a diploid cell of *Saccharomyces cerevisiae* (29). The DNA content of one cell of *H. influenzae* was estimated, (*a*) on the basis of the total DNA extractable from the growth on 500 plates (56 mg.) as described before, and (*b*) on the basis of total DNA determined by the Schneider procedure (30) modified as follows: The 7 hour growth (at 37°C.) on 40 Levinthal agar plates was harvested and washed as described in reference 9. The sedimented cells were stirred for 10 minutes in 35 cc. of cold 5 per cent aqueous trichloroacetic acid solution (TCA) and centrifuged for 20 minutes at 1800 *g* in the cold; the clear yellowish supernatant, which did not show positive reaction with diphenylamine, was discarded and the sediment was stirred for 10 minutes in 25 cc. of cold 5 per cent TCA and centrifuged. The supernatant was discarded and the sediment was suspended in 10 cc. 5 per cent TCA and heated for 15 minutes at 90°. After centrifugation at 1800 *g* for 20 minutes, the supernatant (10 cc.) and the sediment (0.8 cc.) (washed with 10 volumes of water) were tested for DNA content by the diphenylamine reaction (30). Only the supernatant gave a positive reaction; it corresponded to 440 γ DNA/cc. The total amount was therefore 4.4 mg. DNA per 40 plates or 55 mg. per 500 plates, which is in agreement with the previously mentioned amount (56 mg.). The number of cells per 500 plates was found to be 2.7 × 10^{12}. The DNA content of one cell of *H. influenzae* was therefore of the order of 2 × 10^{-9} γ DNA, which is somewhat lower than the previously mentioned values reported for larger microbial cells.[4] The minimal amount of DNA hitherto found necessary for transformation is therefore only five times higher than the total amount of DNA per cell. (This estimate is, of course, independent of the actual size of the DNA molecule.)

The above finding invites speculation. If one assumes that each molecule of DNA in a haploid cell is different, then only very few molecules of a kind are necessary to effect transformation; that is, practically all molecules of this kind are active. If, in addition, practically every DNA molecule in the cell is a potential transforming principle, then the physical and chemical behaviors of the bulk of the DNA preparation are representative of the physical and chemical behaviors of the active molecules. Obviously, more evidence is needed until such a view can be fully accepted.

[4] If the molecular weight of DNA is taken as 4 to 7 × 10^6 (mean 5.5 × 10^6), the number of molecules of DNA per cell would be of the order of 200. On the basis of radiation experiments Lea (31) has estimated that the number of genes in bacterial cell is 250; this agreement may be purely accidental.

The Effect of Various Agents on Transforming Activity

Heat.—The effects of heat on viscosity and on activity of the preparations of transforming principle were studied.

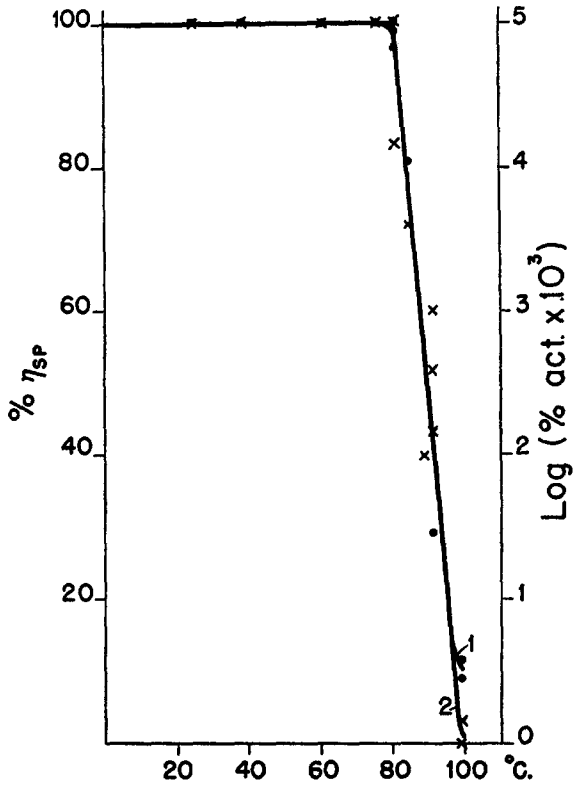

FIG. 1. Stability of the transforming principle preparation to heat. ●, viscosity (curve 1); ×, transforming activity (curve 2). The ordinates indicate the specific viscosities (all measured in standard buffer at 23°C.) as percentages of maximum viscosity, and the transforming activities as the logarithm of (percentage × 10^3) maximum activity; the abscissa indicates the temperature to which the sample was exposed for 1 hour prior to measuring viscosity and activity.

Purified DNA solutions transforming to PRP production or to streptomycin resistance in standard buffer, containing 170–585 γ DNA/cc., were tested for viscosity at 23°C. and for activity. The solutions were divided into 0.6 cc. portions; each portion was heated in a stoppered test tube on a constant temperature bath for a period of 1 hour, cooled to 23°C., and subjected again to viscosity and activity determinations. The results are represented in Fig. 1.

It will be seen that the viscosity and the activity are practically unaffected by 1 hour heating to temperatures as high as 76–81°C. These stabilities are

much greater than those previously reported for the activity of the purified transforming principle of *S. pneumoniae* (5) and for the viscosity of DNA of calf thymus (32, 33). That this discrepancy is not due to the species' difference is shown by the fact that when the human DNA and calf thymus DNA were prepared and tested under conditions similar to those used in this paper, they exhibited a similar stability of viscosity (34). This great stability is undoubtedly partly due to the avoidance of steps to be discussed later, which could lessen the stability (initial enzyme action, drying). However, the possibility of a stabilizing effect of the citrate buffer (compare reference 35) cannot be excluded. When the heating of the transforming principle was repeated in similar buffers in which the citrate was replaced by glycyl-glycine (final pH 7.5) or phosphate (final pH 7.31) of the same concentrations, the activities decreased, as shown in Table II. On the other hand, replacement of citrate by another

TABLE II

Effect of Various Buffers on Stability of Transforming Principle to Heat*

		log (per cent activity × 10^4)			
		Citrate	Glycyl-glycine	Phosphate	EDTA
Control		5	5	5	5
Heated 1 hr. at	64°C.	5		0	5
	76°C.	5	1	0	4
	81°C.	5	0	0	3

* Each buffer was 0.14 M with respect to NaCl and 0.015 M with respect to citrate, glycyl-glycine, phosphate, or ethylenediamine tetraacetate (EDTA). DNA content 420 γ/cc.

chelating agent, ethylenediamine tetraacetate, "sequestrene,"[5] did not result in such a substantial decrease of stability. Whether this stabilizing action results from the reported presence in DNA of magnesium (36), which would form non-ionized complexes with citrate and other chelating agents, remains a matter of conjecture.

The stability to heat depends on pH and on ionic strength. These subjects are discussed later.

It can be seen from Fig. 1 that when the activity starts to decrease due to heat, the temperature corresponds rather closely to the temperature at which the viscosity of the bulk of the DNA preparation starts to decrease. This is further evidence that the behavior of the active molecules is similar to the behavior of the average DNA molecule of the preparation. Although the temperatures at which the viscosity and the activity begin to decrease closely correspond to each other, the amounts of decrease do not: an inspection of Fig. 1 reveals that the decrease of viscosity roughly follows the logarithm

[5] Obtained from Alrose Chemical Co., Providence.

of the decrease of activity. One obvious reason for this discrepancy is that while the heat-altered molecule may become completely inactive, it may still retain considerable asymmetry and thus contribute to the viscosity of the preparation. A mathematical analysis of these relations does not appear to be possible at the present moment.

The change in viscosity[6] of a DNA solution may be due to the change in asymmetry of the molecules or to the change in their association (37–39). It has recently been suggested (22, 26, 27) that the decrease in viscosity of DNA solution upon mild H^+ treatment is due to the change in asymmetry caused by the contracting of the molecule rather than by actual depolymerization. This may also be true for the mild heating. The contraction may be made possible by the breakage of labile bonds (such as hydrogen bonds) under the action of thermal oscillations (40, 41). At higher temperatures, actual depolymerization may occur (32).

As can further be seen from Fig. 1, the temperature coefficients of the (biological) inactivation rates between 85 and 95°C. are very large, leading to large energies of activation (42).

The inactivation by thermal oscillations is not notably influenced by the associations between molecules. When the heating experiments were repeated at concentrations of transforming principle as low as 0.17 γ DNA/cc., the stability of the activity to heat was practically the same as when the concentration was 170 γ DNA/cc.

H^+ and OH^- Ions.—

To study the effect of H^+ and OH^- ions, 0.5 cc. portions of the stock solution of transforming principle were precipitated by 2 volumes of alcohol, the resulting fibers lifted, well drained, and redissolved in buffer solution to obtain concentration of 530 to 560 γ DNA/cc. The DNA contents were measured by the diphenylamine reaction (10), and small corrections were applied to adjust the viscosity data to the same concentration. The buffers used (all 0.1 M) were citrate buffers for pH 6 and below, borate for pH 8, 9, and 10, and phosphate for pH 7, 11, and 12. Immediately after preparation each solution was placed in the viscosimeter at 23°C. and the viscosity measured during 2 hours.

During this period the viscosities remained constant in the region of pH 6 to 10, slowly decreased to 99 to 98 per cent of initial value at pH 5, 4.1, and 3.3, slowly decreased to 46 per cent of initial value at pH 12, and rapidly (within the first 20 minutes) decreased to 58 per cent of initial value at pH 2.35. At the end of the 2 hour period the samples were immediately tested for transforming activity. The results are represented in Fig. 2. The pH reported refer to the final values of the solutions of DNA in the buffer used.

It will first be seen that in respect to both viscosity and activity, DNA of *H. influenzae* is completely stable over a wide range (compare reference 5) symmetrical with respect to pH 7.4; this pH corresponds closely to normal pH of the human blood. It has been shown (37) that in yeast cells, whose

[6] Inasmuch as the viscosities could not be extrapolated to a zero velocity gradient, no attempt was made to calculate the sizes of the molecules.

cytoplasm has much lower pH, the region of the stability of its DNA is shifted towards lower pH. This need to preserve the DNA molecules in their active form is obvious if they are to serve as determinants of heredity.

It can be seen in Fig. 2 that the pH values on both acid and alkaline sides, at which the viscosities begin to decrease, correspond rather closely to the pH values at which the activities begin to decrease. This is further evidence that the behavior of the active molecules is similar to the behavior of the average DNA molecule of the preparation. As in the case of heat, the correspondence

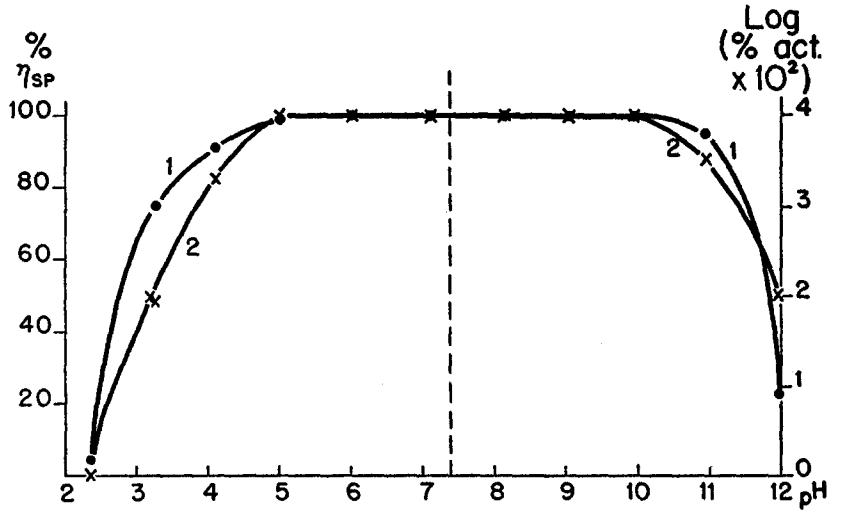

FIG. 2. Stability of the transforming principles preparation to pH changes. Curve 1 (●) viscosity; curve 2 (×) transforming activity. The ordinates indicate the specific viscosities as percentages of maximum viscosity, and the transforming activities as the logarithm of (percentage × 10^2) maximum activity; the abscissa indicates the pH to which the sample was exposed for 2 hours at 23°C.

refers to the pH at which the viscosities and the activities start to decrease, and not to the extent of the decrease.

The pK values reported for primary phosphate in nucleotides and nucleic acids (43, 44) are less than 1. Thus, when the primary phosphate groups are half undissociated, the DNA is completely inactive (Fig. 2). From the above pK value one can calculate that at pH 5 (threshold of stability) less than 10^{-4} of all primary phosphate groups remain undissociated. Assuming again molecular weight of the order of 5.5×10^6, that is approximately 17,600 mononucleotides, one arrives at the conclusion that in the active molecule less than 2 primary phosphate groups remain undissociated at any time. It is therefore to be understood that whenever "desoxyribonucleic acid" is mentioned in this paper, actually desoxyribonucleate is meant.

The above discussion is not meant to imply that the suppression of dissociation is the actual cause of inactivation at low pH. The formation of acid is reversible whereas the inactivation is not. The irreversible changes in the molecule must therefore be held responsible for inactivation. As mentioned in connection with heat, it has been recently suggested (22, 26, 27) that the decrease of viscosity upon mild H^+ treatment may be due to an irreversible change in association (38) or to the change of asymmetry of the molecule when the molecule contracts; this may be due to the breakage of very few labile bonds, such as hydrogen bonds (44–49) or bonds between the phosphate and the first carbon of the sugar (50). The inactivation of the transforming principle may indeed be due to the irreversible breaking of these bonds. However, it is also possible that at low pH the inactivation is due partly to the removal of purine bases (which may be associated with the breaking of hydrogen bonds to these purines). It was therefore of interest to estimate the amount of this "depurination" at the moment at which the DNA largely loses its activity. The decrease of activity in 0.1 M citrate buffer (final pH of the solution = 3.18) in 4 hours at 23°C. has been determined as described before. The decrease was more than 100-fold (Fig. 2).

3 cc. of a similar solution containing 1.9 mg. DNA/cc. was placed in a dialysis bag and dialyzed at 23°C. on a vibrator against 5 cc. of the same buffer. The bag (18/32 inch nojax, The Visking Corp., Chicago) was previously washed for 22 hours on the vibrator at 23°C. in 500 cc. dilute HCl at pH 2.55, then overnight against running water, and again 24 hours on the vibrator in 2 liters distilled water. This procedure removed the substances which are released by the bag and which have an ultraviolet absorption. As controls similar bags were used containing buffer (pH 3.18) alone or DNA in 0.1 M citrate buffer of pH 7.2; the latter was dialyzed against buffer of pH 7.2. At intervals, aliquots of the outside fluids were taken and their U.V. absorption measured. After 49½ hours, the outside fluids were evaporated to dryness, redissolved in 0.04 cc. 1 N HCl, and subjected to paper partition chromatography (51) using water-saturated butanol as solvent.

From the differences in U.V. absorption at 263 and 290 mμ, and at 240 and 290 mμ, the adenine and the guanine contents of the outside fluids were calculated as described in reference 52. The absorptions in the outside fluids of the control bags were insignificant. The values so obtained for total adenine were 1.43 γ at 4 hours, 16 γ at 28½ hours, and 31 γ at 40 hours; the release of adenine (at least in this experiment, in which the speed of dialysis should be also taken into consideration) is thus not strictly proportional to time. The amounts of guanine were negligible. After 49½ hours, the amounts detectable by paper chromatography were 25.8 γ adenine and 13.8 γ guanine. From known composition of the DNA of *H. influenzae* (53) one can thus calculate that in 4 hours at pH 3.18 the amount of adenine released (average of both methods) is 0.16 per cent of total adenine content, and the amount of guanine, if any, is 0.13 per cent of total guanine (the latter is calculated by extrapolation from 49½ hours). Assuming again the mean molecular weight of DNA

to be 5.5×10^6 (approximately 5600 molecules of desoxyadenylic acid and 3200 molecules of desoxyguanylic acid), one arrives at 9 molecules of adenine and 4 (if any) molecules of guanine released at pH 3.18. If the molecular weight of DNA is only 10^6, the amounts are of the order of 1 molecule. Thus, almost complete inactivation of the transforming principle may occur when just 1 or a few molecules of purines are removed from a DNA molecule. Needless to say, this estimate is valid only if the active molecule indeed behaves like the average DNA molecule.

Ionic Strength.—Two methods were used for testing the influence of ionic strength on viscosity and activity.

In one method, 0.8 cc. portions of the stock solution of the transforming principle containing 300 γ DNA/cc. were precipitated by 2 volumes of absolute ethanol, the resulting fibers lifted, washed successively in five 5 cc. portions of 75 per cent ethanol, well drained, and redissolved in aqueous NaCl solutions of desired ionic strength or in distilled water and kept at 6° or 30°C. The transforming activities of all samples were then measured. For the measurement of viscosity under standard conditions, 0.1 cc. of 10 per cent NaCl solution was added to each portion, the DNA precipitated by the addition of 2 volumes of absolute ethanol and redissolved in 0.8 cc. standard buffer. The concentrations of DNA so obtained were checked by the diphenylamine reaction. The viscosities were then measured at 23°C. as described before. The second method differed from the first only in that the sample was dialyzed (on the vibrator) against two 2 liter portions of aqueous NaCl solution of desired ionic strength (or distilled water) at 6°C. instead of being dissolved in that solution.

The results obtained by both methods were essentially the same; they are represented in Table III. It can be seen that the viscosities in standard buffer were not affected by previous exposures to solutions of higher and lower ionic strength. On the other hand, the activities were reduced by the exposure to lower ionic strength (compare also reference 5) but not by higher ionic strength. These results can be interpreted as follows.

It has long been known that the addition of salt to aqueous solutions of DNA decreases their viscosity (54, 55, 45). It has been suggested (56–59) that the increase of viscosity in distilled water is due to actual increase in the length of the molecule, presumably by stretching (uncoiling) caused by repulsion of anions of the DNA molecule in the absence of salt. An inspection of the viscosity data of Table III might suggest that this change is entirely reversible; however, the persistent loss of activity proves that this is not the case. While the changes in the viscosity above the concentration of physiological saline are indeed reversible, the stretching caused by lower concentrations produces irreversible inactivation. This may be due to breakage of few vital bonds, such as hydrogen bonds, in a molecule rendered more vulnerable by distilled water (33); the degree of thermal inactivation increases with temperature (Table III). The damage is not large enough to cause noticeable change of viscosity (under standard conditions), but it can be demonstrated by a loss of stability to heat under standard conditions (34) or in distilled water (33, 34).

Dehydration.—The study of the effect of dehydration on the integrity of the active molecule is of some interest not only from a theoretical point of view, but also because of the widespread practice of "preserving" the DNA in a dried state and using dried DNA for x-ray diffraction and electron microscope studies.

TABLE III

Effect of Exposure of Transforming Principle to NaCl Solutions of Various Ionic Strengths

Ionic Strength	Exposure	Temperature	Viscosity* per cent of maximum	Activity per cent of maximum
	hrs.	°C.		
2.0	168	6	100	100
2×10^{-1}	504	6	100	100
2×10^{-1}	6	30	100	100
2×10^{-2}	24–48	6	96	10–20
2×10^{-3}	24–48	6	97	1–20
$<10^{-6}$	24–48	6	96	0–10
$<10^{-6}$	4	30	98.5	2.4

* In standard buffer at 23°C.

TABLE IV

Effect of Dehydration of Transforming Principle at 23°C. on the Transforming Activity

Drying method	A	B	C	D	E
Storage, *days*	0	9	4	4	4
Activity, UTA/γ DNA	3000	1500	600	30	0.3

Drying methods: A, control, not dried. B, 75 per cent ethanol, air, stored over $CaCl_2$. Moisture content 1.08 per cent. C, 75 per cent ethanol, vacuum over P_2O_5. Moisture content 2.06 per cent. D, 75 per cent ethanol, air stored over saturated NaCl. Moisture content 28.35 per cent. E, 100 per cent ethanol, ether, vacuum over P_2O_5. Moisture content 4.25%.

0.2 cc. portions of the stock solution containing 1800 γ DNA/cc. were precipitated by addition of 2 volumes of absolute ethanol; the resulting fibers were lifted, washed in two 5 cc. portions of 75 per cent ethanol, well drained, and kept at 23°C. in desiccators: (*a*) over $CaCl_2$ at atmospheric pressure; (*b*) over P_2O_5 in a vacuum; (*c*) over saturated NaCl solution (relative humidity 75 per cent) at atmospheric pressure, as suggested by Signer and Schwander (60). After storage, each sample was divided into two parts: one was redissolved in standard buffer to obtain a concentration 400 γ DNA/cc., and subjected to determination of transforming activity, and another was dried at 75°C. over P_2O_5 in a vacuum, to determine the moisture content. The results are represented in Table IV.

As can be seen from Table IV, drying under the conditions of the procedure suggested by Signer and Schwander (60) and preservation in these conditions for 4 days at 23°C. (28.35 per cent residual moisture) results in a 99 per cent inactivation. The most commonly used procedure of drying over P_2O_5 in a vacuum at 23°C. and the preservation under these conditions for 4 days

(2.06 per cent residual moisture) results in an 80 per cent inactivation. Parallel experiments with calf thymus DNA (34) revealed that drying also results in decrease of viscosity and stability to heat. Drying in air and storage in air over $CaCl_2$ may be less injurious. The drying of the purified transforming principle from the frozen state in the lyophile apparatus has also been reported to result in a loss of activity (5). Such procedure usually involves dialysis prior to drying, and the reported inactivation may represent the combined effect of both these processes. It is to be stressed again that the unpurified transforming principle may exhibit higher resistance towards low ionic strength and drying.

One of the commonly used methods of "preservation" of DNA includes drying the fibers with absolute ethanol and with ether. Samples of DNA dried in a similar way lost their activity (Table IV) and stability to heat (34) even more completely than the samples dried in similar conditions but without absolute ethanol and ether. This deterioration may partly be due to the presence of peroxides in ether or to some other unknown factors.

Nothing is known about the nature of changes accompanying the dehydration of DNA. Breaking of few vital labile bonds, such as hydrogen bonds, during the change of the native configuration of the molecule caused by dehydration, seems probable. The over-all effect may be actually due to several causes: small instantaneous injury; slow injury by thermal oscillations in a molecule rendered more vulnerable; slow injury by progressive dehydration.

Desoxyribonuclease.—As mentioned before, desoxyribonuclease was found to destroy the transforming activity. In this work a quantitative study of this phenomenon was made using very small amounts (6, 8) of the enzyme so as to observe the beginning of the process.

To 1 cc. of the stock solution of transforming principle, containing 530 γ DNA per cc. of standard buffer, 0.4 cc. of the enzyme solution was added at time zero. The latter was prepared by dissolving crystalline desoxyribonuclease in aqueous solution containing gelatin (0.035 per cent) and $MnCl_2$ (0.012 M) and diluting in a similar gelatin-$MnCl_2$ solution until the concentration of the enzyme was 3×10^{-4} γ/cc. The final concentration of the enzyme in the DNA solution was therefore 8.5×10^{-5} γ/cc. Mn^{++} was used as activator (6) because Mg^{++} would have formed a non-ionized complex with the citrate in the buffer. The DNA-enzyme solution was immediately placed at 30°C., and a 0.25 cc. portion of it introduced into the viscosimeter which was also kept at 30°C. While the drop of viscosity was being observed during the period of 140 minutes, 0.5 cc. aliquots were periodically removed from the main portion and immediately precipitated by 2 volumes of absolute ethanol. The resulting fibers from each aliquot were lifted, washed 5 times in 5 cc. portions of 75 per cent ethanol, well drained, and redissolved in a 0.5 cc. portion of standard buffer. This procedure destroyed or removed the enzyme completely; solutions so prepared suffered no further loss of viscosity or activity on incubation at 30°C. The solutions were immediately tested for their transforming activity. The results are represented in Fig. 3.

It can be seen in Fig. 3 that amounts of enzyme as low as 8.5×10^{-5} γ/cc. are sufficient to destroy the activity, partially in 28 minutes and completely in

138 minutes, thus confirming previous findings (6, 8). It is highly improbable that such action could be due to an enzyme other than desoxyribonuclease itself. If, for example, the crystalline desoxyribonuclease preparation contained traces of proteolytic enzymes, the amounts involved would be of the order of 10^{-6} γ/cc. and such low amounts of proteolytic enzymes are not known to

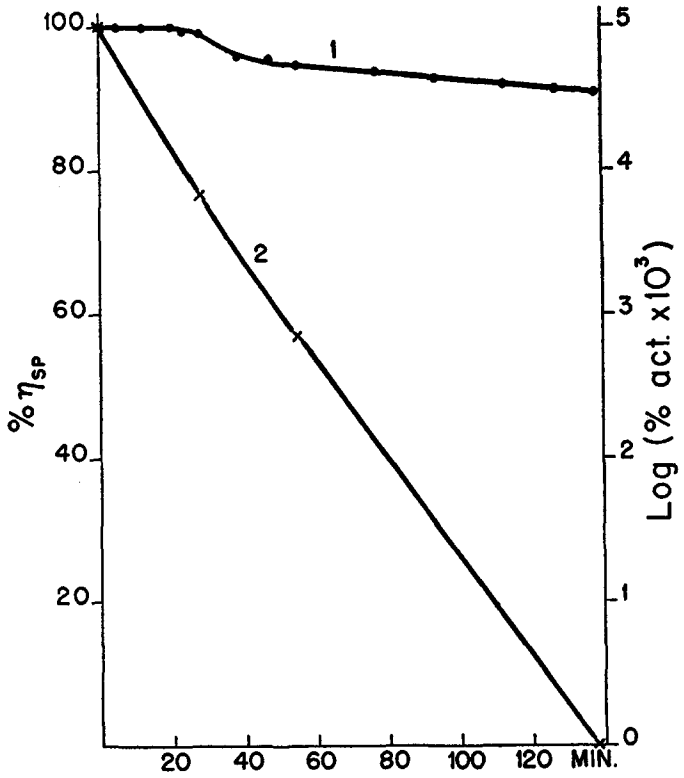

FIG. 3. Stability of the transforming principle preparation to crystalline pancreatic desoxyribonuclease. Curve 1 (●) viscosities; curve 2 (×) transforming activities. The ordinates as on Fig. 1; the abscissa indicates time of incubation with 8.5×10^{-5} γ/cc. desoxyribonuclease at 30.1°C.

destroy the biological activities of protein; crystalline trypsin, chymotrypsin, and ribonuclease in much higher concentrations do not destroy the transforming activity (5, 6, 8). Thus the previous findings (5–11) that the DNA itself is essential for the transforming activity is confirmed.

Further inspection of Fig. 3 reveals that the drop of viscosity at the beginning of inactivation is insignificant. It will be seen that when the concentrations of enzyme are as low as those used in this experiment, an initial lag period in the depolymerizing action of the enzyme can be demonstrated. A

similar result was obtained when calf thymus DNA was used as substrate (34). Despite the lag in depolymerization, the enzyme does exert some action in this period as evidenced by the marked decrease in activity. It is probable that the initial action of the enzyme is to break a few vital weak bonds such as hydrogen bonds, which results in inactivation, but not yet in demonstrable decrease of the asymmetry of the molecule. This statement is again valid only under the assumption that the active molecule (whose activity, but not viscosity, can be measured) behaves in this respect like an average DNA molecule (whose viscosity, but not activity, can be measured). The evidence that the average DNA molecule does indeed undergo some change in this lag period has been obtained by the demonstration of the loss of stability to heat of the DNA preparations exposed to such initial action of the enzyme (34).

Other enzymes (ribonuclease, proteolytic enzymes) have also been shown to exert on their substrates a "denaturing" action prior to actual degradation (61, 62).

Deamination.—In an attempt to obtain some information on the importance of various chemically active groups of the active molecule for the activity, a study was made of the effect of mild deamination on the transforming activity.

To 0.6 cc. portions of the stock solution containing 1 mg. DNA/cc. were added 1.8 cc. portions of the mixture of 3 parts of 0.1 M citrate buffer of pH 5.5 and 1 part of an 8.7 M (saturated at 23°C.), 4.35 M or 1.76 M aqueous solution of $NaNO_2$. The resulting pH was 5.3 (constant throughout the experiment), and the final concentrations of $NaNO_2$ were 2.18 M, 1.09 M, or 0.44 M, respectively. The solutions were incubated at 23°C.; 0.25 cc. portions were introduced into a viscosimeter which was also kept at 23°C. At intervals, 0.5 cc. aliquots were removed from the main portion and immediately precipitated by 2 volumes of absolute ethanol; the resulting fibers from each aliquot were lifted, washed 5 times in 5 cc. portions of 75 per cent ethanol, well drained, redissolved in a 0.5 cc. portion of standard buffer and subjected to determination of transforming activity. The results are represented in Fig. 4.

It can be seen that incubation in 2.18 M or 1.09 M $NaNO_2$ resulted in a rapid inactivation of the transforming principle. However, the viscosity remained constant, showing that the average DNA molecule was but slightly altered.

The deamination of adenine, guanine, and cytosine by nitrous acid, even at low pH, is known to proceed much more slowly than the deamination of amino acids (63). The deamination of these bases in a high polymer is still slower (64, 65). No studies of deamination at pH 5.3 have been reported, presumably because at this pH less than 1 per cent of free HNO_2 exists in equilibrium with NO_2^- (66). This pH has been chosen in the present experiment to slow down the process of deamination and to avoid the inactivation which would be caused by greater acidity (Fig. 2). It was of interest to estimate the extent of deamination of DNA that had occurred at a time when the transforming activity had decreased 1000-fold (2 hours in 2.18 M $NaNO_2$ at pH 5.3). To save material, this rough estimate was made with calf thymus

DNA prepared by a procedure (34) similar to that used for transforming principle.

To 24 cc. portions of a stock solution of calf thymus DNA, containing 1 mg. DNA/cc., were added 72 cc. 0.1 M citrate buffer of pH 5.5, 24 cc. of 8.7 M (saturated) $NaNO_2$ solution

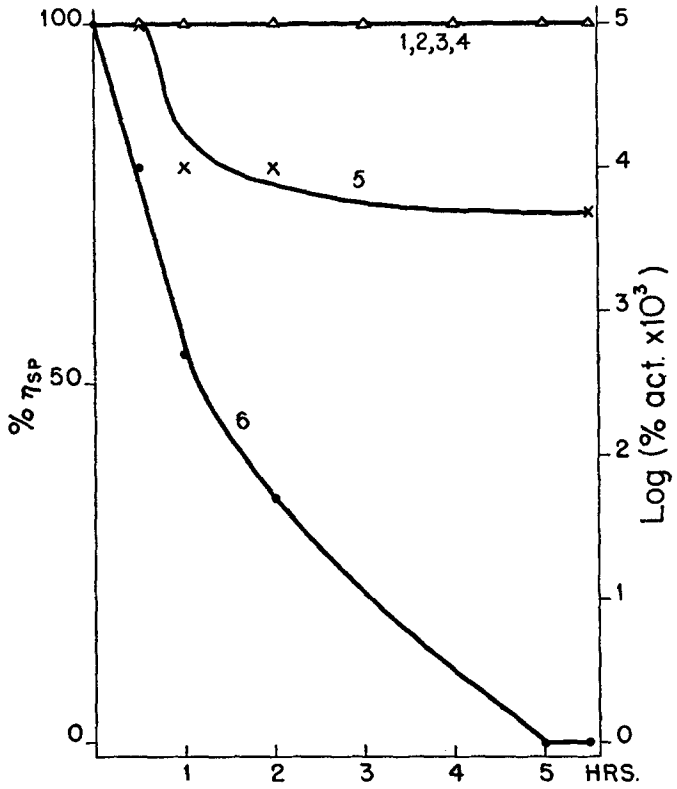

FIG. 4. Stability of the transforming principle preparation to $NaNO_2$ at pH 5.3. Curve 1, viscosities after incubation in 2.18 M; curve 2, viscosities after incubation in 1.09 M; curve 3, viscosities after incubation in 0.44 M; curve 4, activities after incubation in 0.44 M; curve 5, activities after incubation in 1.09 M; curve 6, activities after incubation in 2.18 M $NaNO_2$. The ordinates as on Fig. 1; the abscissa indicates time of incubation at 23°C.

and 0.3 cc. of 0.2 per cent ethyl mercurithiosalicylate. The resulting solution (pH 5.3) was incubated at 23°C. for 0 hours (control), 5 hours, and 50 hours. The solutions were then immediately placed in the dialysis bags and each was dialyzed, on the vibrator, against 2 liters of ice cold tap water for 2 hours and against running tap water for 48 hours. To the resulting 146 cc. from each bag 72 cc. of 1.7 M NaCl solution was added and the DNA precipitated by addition of 2 volumes of absolute ethanol. The resulting fibers were lifted, washed successively in five 20 cc. portions of 75 per cent ethanol, well drained, and subjected to 1 hour hydrolysis at 100°C. in a sealed tube, in 2.4 cc. of 1 N H_2SO_4. The solution was then neutralized to pH 7 by careful addition of 3 N NH_4OH and evaporated to dryness in a vacuum over P_2O_5 at 23°C. The solids were redissolved in 0.18 cc. of 1 N HCl. This solution, in 0.02 cc. portions,

was subjected to "adsorption" paper chromatography, using water as a solvent and Whatman filter paper No. 3 essentially as described in reference 53. The spots containing adenine with guanine, easily located by means of an ultraviolet lamp, were extracted and the adenine and guanine contents determined as described previously.

The hypoxanthine with xanthine spots were not visible, but their location was estimated by means of a guide strip containing sufficient amounts of pure hypoxanthine and xanthine; the former were extracted and the base content estimated as described before.

The amounts of hypoxanthine and xanthine after 0 and 5 hours deamination were below the limit of detection. At 50 hours, the amounts of hypoxanthine and xanthine were 3 per cent of the corresponding contents of adenine and guanine.[7] On the basis of interpolation from 50 hours to 2 hours (which would give a complete inactivation of transforming principle of *H. influenzae*), the amount of deamination of each base at complete inactivation would be of the order of 0.12 per cent. Again assuming the molecular weight of DNA to be 5.5×10^6, *i.e.* 5600 adenine and 3200 guanine molecules (53), one finds that only about 5 molecules of adenine or guanine have been deaminated in a molecule which shows 1000-fold reduction of its transforming activity. If the molecular weight of DNA were 10^6, this inactivation would have occurred when only one molecule of adenine or guanine had been deaminated. This would indicate that practically all the primary amino groups must be intact for activity.

The above estimate is tentative. Several assumptions had to be made, the validity of which is uncertain at the present moment. It is, for instance, not known whether the degree of deamination of the active molecule is the same as that of the average molecule of DNA used as a basis for this determination. The inactivating action of $NaNO_2$ other than through deamination also cannot be entirely excluded, although in general neutral salts were not found to inactivate the transforming principle. Again, if the inactivation occurs because of deamination, it is not known whether this is due to the resulting absence of amino groups or to the resulting breakage of hydrogen bonds to these groups.

Formaldehyde.—Formaldehyde has been found to be a mutagenic agent (67, 68). The inactivation of viruses, enzymes, and antibodies by formaldehyde has often been studied (for a review see reference 69). It is well known that formaldehyde reacts with the free amino groups of amino acids. It was conceivable that the primary amino groups of purines and pyrimidines would also react with formaldehyde; to the authors' knowledge, this reaction has never been studied.

0.15 cc. of 1 M or 12 M aqueous formaldehyde solution (or water in the control) was added to the 0.3 cc. portions of the stock solution, containing 525 γ DNA/cc. The resulting solution (0.33 M or 4 M with respect to formaldehyde; pH 7.2 and constant throughout the experiment) was incubated at 30°C.; 0.25 cc. portions of it were introduced into a viscosimeter which was

[7] The estimation of deamination by volumetric methods (amino nitrogen) proved less dependable because of the errors caused by fast deamination of traces of protein present.

also kept at 30°C. After specified time intervals the solutions were combined and immediately precipitated by 2 volumes of absolute ethanol; the resulting threads were lifted, washed 5 times in 5 cc. portions of 75 per cent ethanol, well drained, redissolved in 0.35 cc. portions of standard buffer and subjected to determination of transforming activity. The results are represented in Fig. 5.

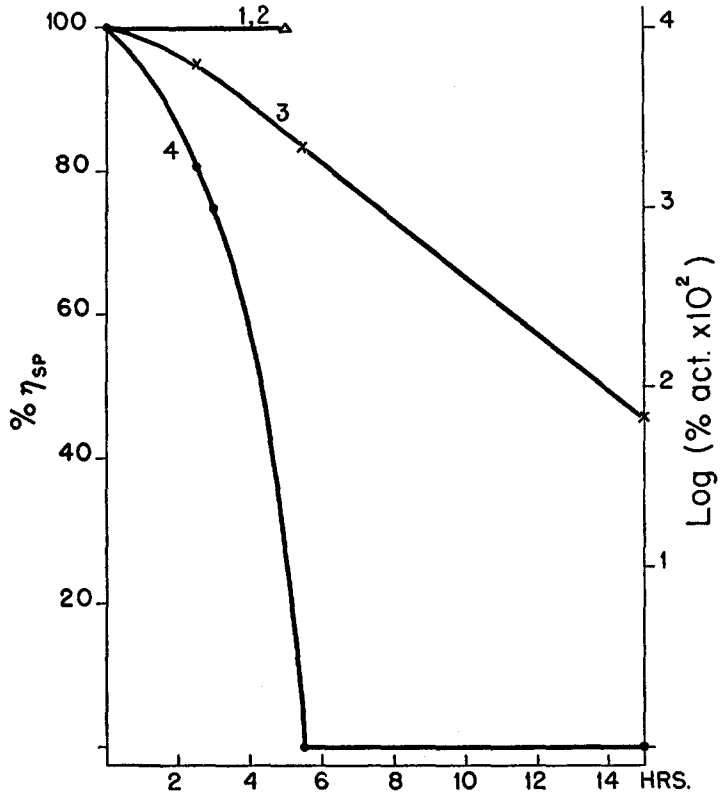

FIG. 5. Effect of formaldehyde on viscosity and transforming activity. Curve 1, viscosities after incubation in 0.33 M formaldehyde; curve 2, transforming activities after incubation in 0.33 M formaldehyde; curve 3, viscosities after incubation in 4 M formaldehyde; curve 4, transforming activities after incubation in 4 M formaldehyde. The ordinates as on Fig. 2; the abscissa indicates time of incubation at 30°C.

It will be seen that incubation in 4 M formaldehyde causes both inactivation and decrease of viscosity. A probable interpretation of these findings is that formaldehyde reacts slowly with the primary amino groups of adenine, guanine, and/or cytosine, and that in this reaction enough labile bonds (hydrogen bonds?) are broken to cause a decrease of asymmetry of the molecule, either by its collapsing, or by actual decrease of molecular weight. The inactivation caused by this reaction is again indicative of the importance of free amino groups for the activity of the transforming principle. However, here too the

breaking of hydrogen bonds as a main cause of inactivation cannot be excluded.

The estimation of the amount of formaldehyde bound at the moment of complete inactivation (5½ hours) involves difficulties. The bound formaldehyde can be removed by prolonged dialysis, or, more efficiently, by treatment with acids, and this theoretically could be used for the estimation of the liberated formaldehyde by means of the Schiff reagent or by weighing the insoluble product of reaction with dimedone. However, a large and variable part of the total detectable formaldehyde remains absorbed, despite repeated washings with 75 per cent ethanol and ether; this circumstance makes the estimation of small amounts of the chemically bound formaldehyde rather unreliable.

Ferrous Ion and Hydrogen Peroxide.—Ferrous ion has recently been found to be a potent mutagenic agent (70). The mechanism presumably involves the formation of free radicals, which are also suspected to be a cause of mutagenic and lethal actions of radiations (71–73). In the presence of H_2O_2, ferrous ion forms Fenton's reagent (74), the strong oxidative action of which seems also due to formation of free radicals (75, 76). In the absence of added H_2O_2, the production of free radicals may proceed by means of the self-oxidation of ferrous ion. In this respect the ferrous ion may be similar to the self-oxidizing substances reported by McCarty to inactivate (reversibly) the transforming principle of *S. pneumoniae* (13). Peroxide solutions have been reported to depolymerize DNA (77–79) or to have no effect (77, 80). It was therefore of interest to study quantitatively the inactivation of transforming principle of *H. influenzae* by ferrous ion and/or hydrogen peroxide.

To 0.24 cc. portions of stock solution containing 392 γ DNA/cc. were added 0.03 cc. of freshly prepared solutions of $FeSO_4$ in standard buffer (or 0.03 cc. of standard buffer alone) and 0.03 cc. of freshly prepared solutions of H_2O_2 in standard buffer (or 0.03 cc. of standard buffer alone). The concentrations of $FeSO_4$ and H_2O_2 were chosen so as to obtain the final concentrations indicated in Table V. The solutions were placed in a viscosimeter at 23°C. and incubated for 3½ hours. No change of viscosity was observed during this period in any of the solutions tested. Immediately after incubation, the samples were serially diluted and tested for transforming activity. The results are summarized in Table V.

It can be seen that the H_2O_2 alone caused only 10-fold inactivation of the transforming principle when present in concentration of 4.4×10^{-3} M, and none in concentration of 3×10^{-4} M. On the other hand, the ferrous ion caused a 10-fold inactivation of the transforming principle even in concentrations as low as 10^{-5} M and complete inactivation in concentrations between 2.8×10^{-5} M and 2×10^{-4} M. The rate of inactivation did not increase in the presence of 6×10^{-4} M H_2O_2. Since this concentration of peroxide is at least 10 times higher than in the standard buffer used for experiment (as estimated by the reactions with titanium sulfate and with potassium chromate), one has to conclude that the action was sufficiently strong when Fe^{++} self-oxidized even

in the absence of typical Fenton's reagent (although of course the latter greatly increases the rate of inactivation). The nature of the reactions involved is not known. The reaction with 10^{-5} to 10^{-4} M Fe^{++} which inactivates without decrease of viscosity, may be an oxidative deamination of a few nitrogenous bases and/or breaking of a few vital labile bonds (hydrogen bonds), the extent of the damage being insufficient for demonstrable decrease of the asymmetry of the molecules. An extensive damage including depolymerization, deamination, dephosphorylation, splitting of bases, and even breakage of sugar and of purine and pyrimidine rings can be produced by Fenton's

TABLE V

Effects of Ferrous Ion and of Hydrogen Peroxide on Transforming Activity. Exposure for 3½ Hours at 23°C. DNA 314 γ/cc.

Experiment No.	Concentration of Fe^{++} M	Concentration of H_2O_2 M	Activity UTA/γ DNA
1	0	0	5000
2	0	4.4×10^{-3}	500
3	0	3×10^{-4}	5000
4	2×10^{-7}	3×10^{-4}	5000
5	2×10^{-6}	3×10^{-4}	5000
6	3×10^{-6}	0	5000
7	1.25×10^{-5}	0	500
8	2×10^{-5}	3×10^{-4}	100
9	2×10^{-5}	0	100
10	2.8×10^{-5}	0	50
11	2×10^{-4}	3×10^{-4}	0
12	2×10^{-4}	0	0
13	2×10^{-4}	4.4×10^{-3}	0
14	1.3×10^{-4}	3×10^{-4}	0

reagents of various strengths. The identification of the products of such strong reactions has recently been reported (81), but it is doubtful whether the same kinds of products are involved in the mild reactions which are reported here or which occur during sublethal mutagenic treatment by free radicals.

Non-Inactivating Agents.—A number of agents tested under conditions described below were found to cause no irreversible change (compare also references 5 and 7) of activity or viscosity of the purified preparations of transforming principle (300 to 700 γ DNA/cc. of standard buffer, unless specified otherwise):

Protein Denaturing and Sterilizing Agents.—Aqueous NaCl solutions (2 M for 1 week at 6°C.) (Table III); 75 per cent ethanol (7 processes of precipitation at 23°C., and storage at 6°C. for 1 week); chloroform–*n*-pentanol mixture 3:1 (1 week, with shaking, at 6°C.); sodium desoxycholate (0.4 M for 4 hours at 23°C.); sodium dodecyl sulfate (0.4 M in standard buffer at 6°C. for 2 weeks); dinitrofluorobenzene (46.5 μM emulsified in 0.4 cc. of 0.2 M bicarbonate buffer, pH 8.5, containing 125 γ DNA; shaken 5 hours at 24°C.); ethyl mercurithiosalicylate

(merthiolate) (0.01 per cent at 23°C. for 2 hours and at 8°C. for 24 hours); formaldehyde (0.33 M at 23°C. for 5 hours and at 6°C. for 42 hours); phenol (0.65 M at 50°C. for 1 hour). The latter agent has been reported to denature DNA (82) as well as proteins.

Agents Reported to Have Mutagenic Action (83–89).—Acriflavine (neutral) (0.2 M; 12 hours at 6°C.); formaldehyde (in conditions mentioned above); sodium desoxycholate (as mentioned above); urethane (0.75 M; 4½ hours at 23°C.); NH_4^+ (1.5 M in 0.2 M borate buffer; final pH 8; 4 hours at 23°C.); phenol (as mentioned above); adenine (4.5×10^{-3} M), guanine and guanazolo (1.7×10^{-4} M), cytosine (3.4×10^{-2} M), thymine (1.8×10^{-2} M) (all 2 hours at 23°C. and 1 hour at 50°C.).

The remarkable resistance of the transforming principle to agents which strongly denature protein is again an indication that the proteins are unlikely to form a functional part of the active molecule. The resistance to strong sterilizing agents gives an indication that a virus or a so called L form of bacteria is unlikely to be responsible for the activity of the transforming principle.

It has recently been suggested (90) that the processes leading to mutation and death are essentially the same, with the exception that the latter is accompanied by more extensive molecular changes. The lack of demonstrable action on DNA may be due to any of the following possibilities:—

1. The agent used is not a mutagen for this DNA (91).
2. The agent is a mutagen but the suggestion (90) that a stronger mutagen is lethal is not true for this particular mutagen.
3. The agents need the presence of the cell to show any action, either mutagenic or lethal.

Obviously much more work will have to be done before any conclusions on these subjects can be reached.

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SUMMARY

The transforming principles of *Hemophilus influenzae* have been purified by a new method including fractional extraction. The active molecule behaves in these extractions like the bulk of the DNA preparation. The minimal amount of DNA necessary for transformation appeared to be of the same order of magnitude as the amount of DNA in a single cell.

Quantitative study has been made of the resistance of transforming activity to various agents. When subjected to heat, the temperature at which the activity starts to decrease corresponds rather closely to the temperature at which the viscosity of the bulk of the DNA preparations starts to decrease. Similar correspondence was found when the transforming principle was subjected to pH changes. This is further evidence that the behavior of the active molecules is similar to the behavior of the average DNA molecule of the preparation. The activity is reduced by exposure to low ionic strength and by dehydration. Desoxyribonuclease in concentrations less than 10^{-4} γ/cc.

is able to destroy the activity; a lag period during which the activity but not the viscosity decreases has been observed. $NaNO_2$ at pH 5.3, HCHO and 10^{-5} M Fe^{++} reduce or destroy the activity; the importance of intact amino groups in the DNA molecule for the activity is discussed. Several protein-denaturing, sterilizing, and mutagenic agents have been found to have no effect on the transforming activity.

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