STUDIES ON THE RESISTANCE OF DESOXYRIBONUCLEIC ACIDS TO PHYSICAL AND CHEMICAL FACTORS*

by

STEPHEN ZAMENHOF, GERTRUDE GRIBOFF AND NICASIO MARULLO

Department of Biochemistry and Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

At present it is not known whether an even most carefully isolated desoxypentose nucleic acid (DNA) can in all respects be identical with the DNA as it existed in the living cell[5]. However, such a carefully isolated DNA preparation may exhibit certain features which it had when it was in the living cell. The most obvious and the most important of these features is the proper biological activity. Such a DNA preparation may be called "functionally intact", and its properties are of more interest than the properties of the products of an undetermined degree of "denaturation"; in particular, the studies of the functionally intact DNA preparation may lead to correlation between the function and the structure.

One proper biological activity which can be demonstrated in certain DNA preparations is their transforming activity[6-9]. In such preparations one can indeed study the physico-chemical properties and the biological activity, and determine the correlations between these two properties. A phase of such a study has been presented in a recent publication[10].

Unfortunately, at present only few bacterial DNA preparations lend themselves to the assay for transforming activity, and the studies of other DNA preparations are open to the criticism that the starting material was a product of degradation. Some of the properties studied, such as chemical composition, are less likely to change upon mild "denaturation" (for a recent review, see[2]); others, such as resistance to various physical and chemical agents, will be greatly altered[10].

While a completely satisfactory study of such DNA preparations cannot be offered at present, advantage can be taken of the fact that the DNA of different species, although chemically different[11,12], do exhibit similarities in their resistance to various agents**. It is shown in this paper that isolation of any DNA under conditions which would not inactivate another DNA having transforming activity may lead to a "functionally intact" product. Methods for such isolation, with the addition of an active transforming principle to serve as a marker, are described. Further, a study of the resistance of DNA to various agents has provided a sensitive physical method for detecting the injury to DNA molecule.

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** An exception has been found in the species (*Saccharomyces cerevisiae*) in which lower pH of the physiological conditions is accompanied by a greater resistance of DNA towards hydrogen ions[5].

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EXPERIMENTAL

Materials

Human DNA and ox DNA* were prepared by a modification of the method used for preparation of the transforming principle of *Hemophilus influenzae*[10]; final degradative steps (drying, dialysis against water), commonly used in the isolation of DNA, are avoided. The procedure is described below.

Crude transforming principle of Hemophilus influenzae, type b, used as a marker, was the fifth extract as described in[10].

Stock solution of purified transforming principle was the finally deproteinized 12th to 15th extract as described in[10].

Crystalline desoxyribonuclease (DNAse) was obtained from Worthington Biochemical Sales Company, Freehold, New Jersey.

Methods

Analytical

The phosphorus, nitrogen, DNA, RNA and protein contents were determined as described in[10] and [14]. The determinations of viscosity and of biological (transforming) activity were carried out as described in[10].

Preparation of Human DNA

Human spleen (normal) from a living adult was frozen within one hour after splenectomy and stored at —30° for two days. 120 g of this tissue, freed from fat, was washed in five 100 ml ice-cold portions of 0.1 *M* sodium citrate (pH 7.4), or 0.1 *M* ethylenediaminetetra-acetate (EDTA)** (pH 7.35). The mixture was then homogenized in a cooled Potter-Elvehjem glass tissue grinder with addition of 600 ml ice-cold sodium citrate or EDTA as above. The homogenate was centrifuged for 30 min at 1800 *g*, and the supernatant discarded. The residue was now stirred in 800 ml citrate or EDTA and centrifuged as before. This operation was repeated two more times. To the final residue were added 250 ml of 2 *M* aqueous NaCl solution, in order to extract DNA, and 5 ml of crude transforming principle, containing 51 γ DNA/ml, to serve as a marker. The mixture was homogenized in a cooled tissue grinder for 3 min and then left in the cold for two days. The material was centrifuged for one hour at 1900 *g*. To the opalescent supernatant 2 volumes of absolute ethanol were added at a rate of 150 ml per hour, with constant slow swirling of the recipient flask; the resulting fibers were lifted, washed in 75% aqueous ethanol, drained and redissolved in a cooled tissue grinder in 200 ml 0.14 *M* aqueous NaCl solution made 0.015 *M* with respect to sodium citrate (pH 7.1), hereafter called "standard buffer". To this solution was added 1/9 vol. of 5% "Duponol"*** in 45% aqueous ethanol[15,16], and the mixture was stirred for one hour at room temperature. Solid NaCl was then added, to obtain a final concentration of 5% NaCl, the mixture stirred for another $^1/_2$ h at room temperature, and then left overnight at 4°. The mixture containing precipitated Duponol was then centrifuged for one hour at 31,000 g; to the clear supernatant 2 vols. alcohol were slowly added, as described before; the resulting fibers were lifted, washed in 75% aqueous ethanol, redissolved in 100 ml standard buffer with 1/9 vol. 5% Duponol, and the mixture stirred for one hour; solid NaCl was then added to obtain 5% NaCl solution, and the mixture stirred, stored and centrifuged, as described before. To the final clear supernatant 2 vols. absolute ethanol were slowly added, as described before, the fibers lifted, washed in 75% ethanol, and redissolved in 40 ml standard buffer to yield a clear, very viscous solution, containing 1490 γ DNA/ml. Total yield 60 mg, of which less than 0.3% was *Hemophilus influenzae* DNA. The final solution was active as transforming principle in *Hemophilus influenzae* (qualitative test) and remained so for at least 3 months, when stored in the frozen state at —15° ("stock solution"). Unless otherwise stated, this method of storage was used throughout this work, and all the steps were performed at 0–4°, using only glass, rubber or plastic tools and vessels.

A sample of human DNA was also prepared using an identical procedure but omitting DNA of *Hemophilus influenzae*. Except for the lack of transforming activity in the latter preparation, the two samples were indistinguishable and were analysed jointly.

For analysis, 5 ml of the stock solution were dialysed against running water for 24 h and against 2 liters distilled water on the vibrator at 0–4° for another 24 h. The solution was then dried from the frozen state in a vacuum. A portion of this product was dried over P_2O_5 in a vacuum at 75° and analysed, as described before. DNA 101%, RNA less than 0.3%, Protein less than 0.2%. P 8.7%, N 14.2%, N/P 1.63.

* Compare[12,13].

** Two commercial preparations of EDTA were used: a. "Sequestrene" (Alrose Chemical Co., Providence, R.I.), and b. "Versene" (Bersworth Chemical Co., Framingham, Mass.).

*** A mixture of sodium lauryl sulfate and other fatty alcohol sulfates manufactured by E. I. Du Pont de Nemours & Co., Wilmington, Del.

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Preparation of Ox DNA

The procedure used was similar to the one for the preparation of human DNA with the following exceptions:

Calf thymus glands served as starting material; not more than 30 min after removing the glands from the animal they were frozen on dry ice and stored for 2 days at —30°. It was noticed that, if the glands were frozen later than 2 h after removal, the deproteinization was accomplished with greater ease, but the final product was less stable to heat; such material might therefore have been already injured by the enzymes.

50 g of frozen, fat-free tissue were reworked, as described before for human spleen (without transforming principle), but using 4 times larger volumes for extraction and deproteinization, because of the higher DNA content in thymus tissue. The finally deproteinized material was redissolved in standard buffer to yield a clear, very viscous solution containing 1 mg to 1.5 mg DNA/ml. Total yield 600 to 950 mg*. The solution was stored in the frozen state at —15° (stock solution).

Another sample of 5 g tissue was reworked, as described above, but using proportionally smaller volumes; 5 ml of crude transforming principle were added during the preparation, as described for human DNA. Yield 100 mg, of which less than 0.2% was *Hemophilus influenzae* DNA. The final solution was active as transforming principle in *Hemophilus influenzae* (qualitative test), and remained so for at least 3 months, when stored in the frozen state at —15°.

For analysis, an aliquot of the stock solution of ox DNA was dialysed and dried, as described before for human DNA. DNA 96%, RNA less than 0.3%. Protein less than 0.2%; P 8.77%, N 14.8%, N/P 1.68.

The fact that the transforming principle was active in the final preparation suggests that also ox DNA was not injured from the moment of extraction throughout all purification steps. To obtain *quantitative* information as to the possibility of injury in the initial stages, the following test was performed, in which the excess of ox DNA over *H. influenzae* DNA had been reduced.

30 mg fresh calf thymus, free from fat, were washed in 50 ml 0.1 *M* EDTA, pH 7.1. To the tissue were added 2 ml of crude transforming principle solution of 60 γ DNA per ml of 0.1 *M* EDTA (pH 7.1), containing 4% NaCl and 0.5% Duponol. The tissue was homogenized in a cooled Potter-Elvehjem glass tissue grinder and the homogenate tested for DNA content and transforming activity (quantitative test). This homogenate, which contained 129 γ ox DNA and 60 γ *H. influenzae* DNA per ml, had the same activity per γ *H. influenzae* DNA as the original solution of transforming principle, which was not in contact with the thymus gland.

These results indicate that in the above conditions the DNA remains uninjured during extraction; indication is also obtained that in the conditions of the test the transforming activity of DNA of *H. influenzae* is not inhibited by twice the amount of ox DNA. To obtain quantitative information on this subject for higher ratios of ox DNA/*H. influenzae* DNA, the following experiment was performed.

Stock solution of ox DNA was mixed with stock solution of purified transforming principle to obtain 230 γ/ml ox DNA and 1 γ/ml *H. influenzae* DNA. No decrease of activity per γ DNA of *H. influenzae* was observed as compared with the control in which ox DNA was omitted. This indicates that in the conditions of this quantitative test the ox DNA, even in 230-fold excess, does not inhibit the transforming activity of the DNA of *H. influenzae*, and that the method of adding the latter DNA to the former indeed may furnish information as to the degree of injury suffered by the DNA molecules.

Resistance to physical and chemical factors

Heat. The resistance of human and ox DNA to heat was studied as follows.

Stock solutions of DNA were diluted with standard buffer to concentrations of 360–1000 γ DNA/ml and the viscosity determined, as described before. Each solution was then divided into 0.6 ml portions; each portion was heated in a stoppered test tube on a constant temperature bath for a period of one hour, cooled to 23°, and subjected again to viscosity determinations. The results are represented in Fig. 1. It will be seen that the stabilities to heat of human, ox and *H. influenzae*[10] DNA are similar under similar conditions. The somewhat lower stability of human DNA might be due to the fact that the desoxyribonuclease activity was higher in spleen (starting material for human DNA) than in thymus[17] or in *H. influenzae*[10], with the resulting slight injury to human DNA during preparation. It is also possible that the biopsy material (human

* We are grateful to Dr. ALBERT SCHRAGE for the preparation of one batch of ox DNA.

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DNA) was not as fresh as the material obtainable from the slaughterhouse (ox DNA). Another alternative, the differences in stability of DNA of different species, is less probable in view of the striking similarities in stability of the DNA preparations from species as distant as ox and *H. influenzae;* however, some slight species differences in stability cannot be entirely excluded at the present moment.

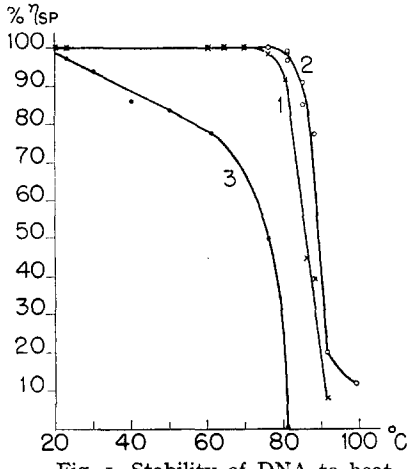

Fig. 1. Stability of DNA to heat. Curve 1: human DNA in standard buffer (see text); Curve 2: ox DNA in standard buffer; Curve 3: ox DNA in distilled water. The ordinate indicates the specific viscosities (all measured at 23°) as percentage of maximum viscosity; the abscissa indicates the temperatures to which each sample was exposed for one hour prior to measuring viscosity.

The stabilities to heat are much higher than previously reported for ox (calf thymus) DNA[18,19]. The examination of Fig. 1 reveals that the viscosities are practically unaffected by heating of one hour to temperatures as high as 76° to 81°.

These temperatures were found convenient for detecting the injuries to the DNA molecule; as will be seen later on, the DNA preparations injured by enzyme, drying or dialysis, lose their stability to heat in this range of temperatures. This circumstance may be a partial explanation of the low resistance to heat reported in the literature. However, the influence of a stabilizing action of the citrate buffer[20] and other chelating agents should not be overlooked. When the heating for one hour was repeated in similar buffers, in which the citrate or other chelating agent was replaced by phosphate (final pH 7.3) of the same concentration, the viscosity (ox DNA) decreased considerably (Table I). Similar results were obtained when testing for activity of DNA of *H. influenzae*[10].

TABLE I

EFFECT OF VARIOUS BUFFERS ON STABILITY OF OX DNA TO HEAT

		Per cent viscosity drop		
		Citrate	*EDTA*	*Phosphate*
Heated 1 h at	69° C	0	0	19.2
	81° C	0–3	0	39.4

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

These results may be interpreted as indicating that the stabilizing action of the citrate and other chelating agents is a result of the removal of the magnesium ions, suggested[21] and reported[22] in the DNA molecule; however, the possibility of a depolymerizing action of phosphate buffer at higher temperatures cannot be excluded. When the heating experiment in standard (citrate) buffer was repeated in the presence of added Mg^{+2} ion in five-fold excess over the stoichiometrical amount bound by citrate, a decrease of heat stability was not observed.

The stability to thermal oscillations depends on ionic strength[19]. This subject will be discussed later.

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The change in viscosity* of a DNA solution may be due to a change in asymmetry of the molecules or to a change in their associations[23-25]. It has recently been suggested[26,27] that the decrease in viscosity of DNA solution upon mild H^+ treatment is due to a change in asymmetry, caused by the contracting (collapsing) of the molecule rather than by actual depolymerization. This may also be true for the mild thermal oscillations. The contraction may be made possible by the breakage of labile bonds (such as hydrogen bonds) under the action of thermal oscillations[28,29]. At higher temperatures, actual depolymerization may take place[18].

Ionic strength. The following method was used to test the influence of ionic strength on viscosity and stability of DNA.

10 ml of the stock solutions containing 1 mg ox DNA/ml were dialysed for 24 h against running water at 12° and 48 h against two 2 l portions of distilled water at 5°, on a vibrator. To the contents of the bags 0.1 vol. of 10% aqueous NaCl was added, the solution precipitated with 2 vols. absolute ethanol and redissolved in standard buffer to obtain a concentration of 430 γ DNA/ml, checked by diphenylamine reaction.

TABLE II

SPECIFIC VISCOSITIES (η_{sp}) AT 23° OF OX DNA DIALYSED OR DEHYDRATED AND REDISSOLVED, IN % OF CONTROL (STOCK SOLUTION)

DNA Content 430 γ per ml Standard Buffer.

Treatment	*Dialysis*	*Drying*			
		a	*b*	*c*	*d*
Moisture content after drying, %	—	2.76	28.35	< 1	5.3
η_{sp} %	100	100	84.6	83.5	64

See text for the description of treatments.

The viscosities at 23° were then measured, as described before. 0.6 ml portions were then heated for one hour to temperatures specified, cooled to 23°, and the viscosities measured again. The results are represented in Table II and Fig. 2 (Curve 2).

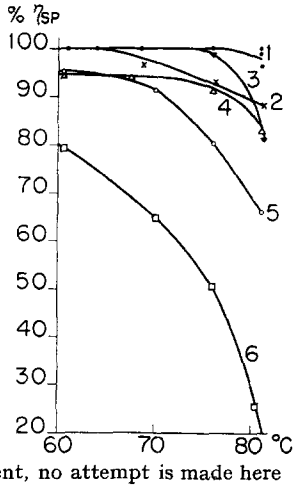

Fig. 2. Stability of ox DNA to change in ionic strength and to dehydration. Curve 1: control (stock solution); Curve 2: dialysed; Curve 3: dried in vacuum over P_2O_5 for 2 days (moisture content 2.8%); Curve 4: dried over saturated aqueous NaCl solution for 2 days (moisture content 28.3%); Curve 5: dried in a vacuum over P_2O_5 for 1 day and then at atmospheric pressure over $CaCl_2$ for 30 days (moisture content less than 1%); Curve 6: dried with absolute ethanol and with ether and stored in a vacuum over P_2O_5 for 2 days (moisture content 5.3%). The ordinate indicates specific viscosities (at 23°) as percentage of viscosities of non-heated samples; the abscissa as in Fig. 1.

* Inasmuch as the viscosities are not measured at o velocity gradient, no attempt is made here to calculate the sizes of the molecules.

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It will be seen that the viscosity in standard buffer is not affected by previous low temperature exposures to lower ionic strength (Table II). Indeed, solutions of DNA in distilled water were used by many workers to investigate the properties of the "intact" DNA. The loss of biological activity upon exposure to lower ionic strength[10] indicates, however, that changes might have occurred. In an attempt to demonstrate these changes also by methods other than biological, these solutions (now in standard buffer) were tested for heat stability. The inspection of Fig. 2 (Curve 2) reveals that, as compared with the non-treated samples, the stabilities to heat (at 76° and 81°) of the treated samples were indeed lost. Thus, the exposure to low ionic strength injures the DNA. These results can be interpreted as follows:

It has long been known that the changes of salt content influence the viscosity of the DNA solutions[30–32]. It has also been suggested[33–36] that the changes are due to actual changes in the length of the molecule, presumably by influencing the stretching (uncoiling) caused by repulsions of anions of the DNA molecule. The results presented here and in[10] suggest that for the ionic strengths lower than the physiological conditions, these changes are irreversible. This may be due to the breakage of a few vital labile bonds, such as hydrogen bonds, during the stretching. That the DNA molecule, while in distilled water, is more vulnerable to thermal oscillations has been shown by MIYAJI AND PRICE[19]. Their experiment was now repeated by exposing the previously described dialysed solutions (prior to salt addition), diluted with distilled water to the concentrations 560 γ DNA/ml, to various temperatures. The resulted are represented in Fig. 1 (Curve 3).

Heating in distilled water for the period of one hour resulted in much less pronounced decreases of viscosity than those reported by Miyaji and Price for the period of 20 min, presumably because the less injured starting material was used in the present experiments; nevertheless, the vulnerability of DNA solutions in distilled water was confirmed. In particular, it is to be noted that some injury occurs even at room temperature.

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

5 ml portions of the stock solution containing 1 mg ox DNA/ml were precipitated by addition of two vols. of absolute ethanol. The resulting fibers were lifted, washed in two 5 ml portions of 75% ethanol, well drained and stored at 23° in desiccators, a. over P_2O_5 in a vacuum (2 days); b. over saturated NaCl solution (humidity 75%) at atmospheric pressure, as suggested by SIGNER AND SCHWANDER[37] (2 days); c. over P_2O_5 in a vacuum (one day), followed by storage at atmospheric pressure over $CaCl_2$ (30 days); d. dried with absolute ethanol and ether prior to storage over P_2O_5 in a vacuum for 2 days. After storage, each sample was divided into two parts: one was redissolved in standard buffer to obtain a concentration of 430 γ DNA/ml, and subjected to determination of viscosity and heat stability, as described above; another part was dried at 75° over P_2O_5 in a vacuum, to determine the moisture content. The results are represented in Table II and in Fig. 2 (Curves 3–6.)

As can be seen from Fig. 2, drying in general reduces the stability of DNA to heat. In addition, the viscosities of samples dried under SIGNER AND SCHWANDER conditions, stored over $CaCl_2$ for one month, or dried with absolute ethanol and ether, were lower

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than those of the non-dried samples (Table II). The most damaging process seems to be the commonly used method of drying the fibers with absolute ethanol and ether. This deterioration may be due to more rapid dehydration, to the presence of peroxides in ether or to other unknown factors.

The various degrees of loss of stability to heat follow the loss of biological activity[10]. It must be concluded that drying does indeed cause irreversible changes in the DNA molecule.

The drying of the *purified* DNA from the frozen state in the lyophilization apparatus has also been reported to result in a loss of biological activity[6]. The impure DNA was more stable. Lyophilization is usually preceded by dialysis, and the reported inactivation may represent the cumulative effect of both of these processes. When this problem was reinvestigated on the similarly dried ox DNA, an unusual behaviour towards thermal oscillations was noticed.

Samples of ox DNA, lyophilized as described above (but not dried over P_2O_5 at 75°), were stored over $CaCl_2$ at atmospheric pressure for a period of 4 months or 2 years. These samples were dissolved in standard buffer to obtain concentrations 0.3 to 1.5 mg DNA/ml, and were subjected to the stability determinations at various temperatures. The results are represented on Fig. 3. It will be seen that the viscosities of samples so dried are considerably lower than those of the non-dried controls.

Upon closer examination it was found that these viscosities (unlike in the intact DNA) are not constant, but increase at a rate of 1 to 2% per hour.

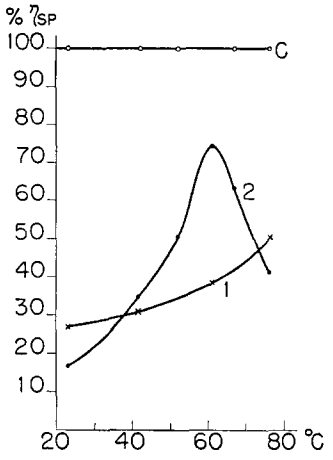

Fig. 3. Viscosities of lyophilized ox DNA. Curve C: control (stock solution, not lyophilized); Curves 1 and 2: lyophilized DNA stored for 4 months (Curve 1) or 2 years (Curve 2) over $CaCl_2$ at atmospheric pressure and redissolved in standard buffer. The ordinate and the abscissa as in Fig. 1.

As the behaviour of the solution is not thixotropic[5], the increase of viscosity could not be due to random repolymerization. When heated (and then returned to 23°), an unusual considerable *increase* of viscosity, rather than decrease was observed (Fig. 3). The increase is more pronounced with the increased temperature of heating. It is conceivable that the drying of dialysed solution from the frozen state, followed by storage, brought about a change in asymmetry or in associations between molecules, and that this change is slowly reversed by temperature oscillations. A complete reversal is never achieved (Fig. 3; compare also loss of biological activity[6]); thus, DNA so dried is also irreversibly degraded.

Desoxyribonuclease. It was recently reported[10] that the desoxyribonuclease, even in concentrations so low as to cause practically no drop of viscosity of DNA, is able to produce a demonstrable destruction of biological activity. It seemed important to investigate whether these changes can also be demonstrated by phenomena other than the loss of biological activity. As shown below, the loss of stability to heat was again found appropriate for the detection of these changes in the DNA molecules.

To 10 ml portions of the stock solutions of human or ox DNA, containing 650 γ DNA per ml standard buffer, 4 ml of the enzyme solution were added at time 0. The latter was prepared by dissolving crystalline desoxyribonuclease (Worthington Biochemical Sales Co., Freehold, N.J.) in aqueous solution, containing 0.035% gelatin and 0.02 molar

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with respect to $MnCl_2$, and diluting in similar gelatin-$MnCl_2$ solution until the concentration of the enzyme was $6 \cdot 10^{-5}$ to $3 \cdot 10^{-4}$ γ/ml (depending on the activity of the particular batch of the enzyme furnished by the manufacturer); final concentrations of the enzyme in the DNA solution were therefore $1.7 \cdot 10^{-5}$ to $8.5 \cdot 10^{-5}$ γ/ml. The Mn^{+2} served as activator[38], since Mg^{+2} could not be used in view of the presence of citrate in the buffer. The DNA-enzyme solution was immediately placed at 30.1°, and 0.25 ml of it introduced into a viscosimeter also kept at 30.1°. While a drop of viscosity was observed during the period of 190 to 270 min. (Fig. 4), 4 ml aliquots were periodically removed from the main portion and immediately precipitated by 2 vols. ethanol; the resulting fibers were lifted, washed successively in 5 ml portions of 75% ethanol, well drained and redissolved in 3 ml portions of standard buffer. This procedure was found completely to inactivate or remove the enzyme, as the solutions so prepared suffered no further loss of viscosity or activity[10] on incubation at 30.1°. The solutions were then tested for their stability to heat at various temperatures, as described before. The results (mean values of 2 to 3 tests for each point) are represented in Figs. 5 and 6.

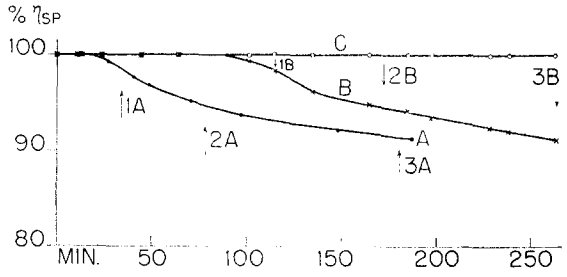

Fig. 4. Stability of human DNA (Curve A) and ox DNA (Curve B) to crystalline pancreatic desoxyribonuclease. Curve C: controls (human and ox DNA) without desoxyribonuclease. Arrows indicate removal of samples (1A–3A: human DNA; 1B–3B: ox DNA) for stability test (Figs. 5 and 6). The ordinate indicates specific viscosities as percentage of maximum viscosity; the abscissa indicates time of incubation with $1.7 \cdot 10^{-5}$ to $8.5 \cdot 10^{-5}$ γ/ml desoxyribonuclease at 30.1°. DNA content 465 γ/ml.

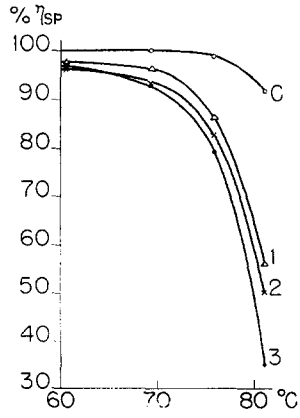

Fig. 5. Stability to heat of human DNA injured by desoxyribonuclease. Curve C: control (not exposed to desoxyribonuclease); Curves 1–3: samples removed at the moments indicated by arrows in Fig. 4. The ordinate and the abscissa as in Fig. 1.

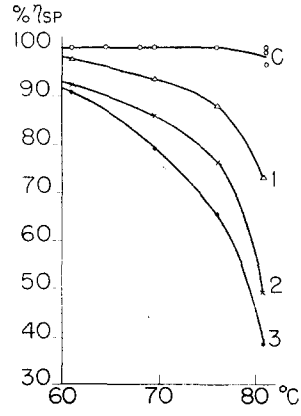

Fig. 6. Stability to heat of ox DNA injured by desoxyribonuclease. Explanation as in Fig. 5.

Figs. 4–6 reveal the following. With low concentrations of enzyme as in this experiment, an initial lag period in the depolymerizing action of the enzyme can be demonstrated. A similar result was obtained for DNA of *H. influenzae*[10]. Despite the lag in depolymerization, the enzyme does exert some action in this period as evidenced

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by the loss of activity[10] and a marked decrease in heat stability (Figs. 5 and 6), which become more accentuated with the length of the period of exposure to enzyme.

As suggested above, human DNA preparations might already have suffered slight injury by DNAse. This injury might be the explanation of a shorter lag period for human DNA (Curve A, Fig. 4) than for ox DNA (Curve B).

The lag in depolymerization may be explained by a hypothesis that the initial action of the enzyme is to break a few vital weak bonds such as hydrogen bonds, with considerable loss of heat stability but no marked loss of the asymmetry of the molecule. Other enzymes (ribonuclease, proteolytic enzymes) have also been shown to exert on their substrates a "denaturing" action prior to actual degradation[39,40].

Formaldehyde. The inactivation of viruses, enzymes and antibodies by formaldehyde has often been studied. One of the reactions involved in proteins is of course with the primary amino groups. It has been reported that the primary amino group of cytosine in "thymic acid" also reacts with formaldehyde[41].

In the present work the influence of formaldehyde on highly polymerized DNA has been studied.

To the 0.4 ml portions of stock solutions, containing 560 γ DNA/ml, 0.2 ml of 12 *M* formaldehyde in standard buffer (or standard buffer alone in the control) was added. The resulting solutions (4 *M* with respect to formaldehyde; pH 7.2 and constant throughout the experiment) were incubated at 30.1 in the viscosimeter. The results of the viscosity measurements are represented on Fig. 7.

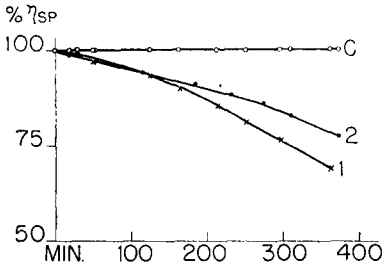

Fig. 7. Stability of human DNA (Curve 1) and ox DNA (Curve 2) to formaldehyde. Curve C: control (human and ox DNA not exposed to formaldehyde). The ordinate as in Fig. 4; the abscissa indicates time of exposure to 4 *M* formaldehyde at 30.1°. DNA content 373 γ/ml.

It will be seen that incubation in 4 *M* HCHO causes gradual decrease of viscosity in both human, and ox DNA. Parallel study on DNA of *H. influenzae*[10] revealed a similar loss of viscosity, and also loss of biological activity. A probable interpretation of these findings is that the 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 collapse or by actual decrease of molecular weight.

Ferrous ion and hydrogen peroxide. Peroxide solutions have been reported to depolymerize DNA[42–44], or to have no effect[45,42]. The discrepancy might be due to the presence or absence of impurities such as ferrous ion. The latter, in concentrations as low as 10^{-5} to 10^{-4} *M*, has recently been found to destroy the transforming activity of DNA of *H. influenzae*[10]. Ferrous ion is also known to be a potent mutagenic agent[46]. The mechanism presumably involves the formation of free radicals. In the presence of H_2O_2, ferrous ion forms FENTON's reagent[47], which produces oxidative free radicals. In the absence of added H_2O_2, the production of free radicals may proceed by autoxidation of ferrous ion[48,49]. It was therefore of interest to investigate the influence of ferrous ion — with or without added peroxide — on DNA, using concentrations of ferrous ion sufficient to cause not only biological inactivation[10], but also a detectable change in physical properties of DNA.

To 0.5 ml portions, containing 570 γ ox DNA per ml standard buffer, 0.3 ml of freshly prepared $8 \cdot 10^{-3}$ *M* $FeSO_4$ solution in standard buffer were added, and the

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mixtures were incubated in the viscosimeter at 23°, as described before. When the experiment was performed in the presence of added peroxide, the latter was introduced when diluting the DNA solutions.

The results are represented in Fig. 8. It can be seen that the ferrous ion in concentration as low as $3 \cdot 10^{-3}$ *M* (final) caused a rapid decrease of viscosities of DNA (Curve 1). The rate of decrease did not change when the experiment was repeated in the presence of $6 \cdot 10^{-4}$ *M* H_2O_2. Since this concentration of peroxide is at least 10 times higher than in the standard buffer used for the 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^{+2} autoxidized even in the absence of typical Fenton's reagent, although the latter, of course, greatly increases the rate of inactivation. When the experiment was repeated in the presence of $2 \cdot 10^{-2}$*M* H_2O_2 (which in itself caused only slow decrease of viscosity, Curve 2), the viscosity decrease (Curve 3) was much faster (especially in the beginning) than the sum of decrease due to Fe^{+2} and H_2O_2 taken separately.

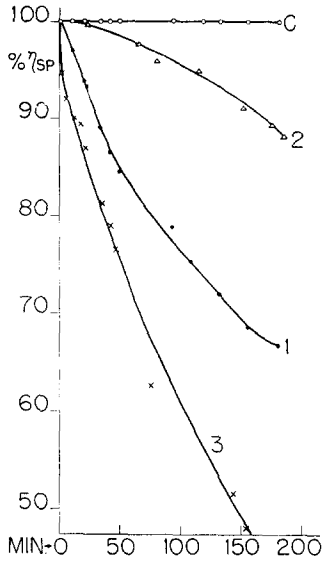

Fig. 8. Stability of DNA to Fe^{+2} and H_2O_2. Curve C: control (ox DNA not exposed to Fe^{+2} or H_2O_2); Curve 1: ox DNA exposed to $3 \cdot 10^{-3}$ *M* Fe^{+2} with and without addition of $6 \cdot 10^{-4}$ *M* H_2O_2; Curve 2: ox DNA exposed to $2 \cdot 10^{-2}$ *M* H_2O_2; Curve 3: ox DNA exposed to $3 \cdot 10^{-3}$ *M* Fe^{+2} and $2 \cdot 10^{-2}$ *M* H_2O_2. The ordinate as in Fig. 1; the abscissa indicates time of exposure at 23°. DNA content 357 γ/ml.

The nature of the reactions involved is unknown. 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 strong Fenton's reagents. The identification of the products of such strong reactions has recently been reported[50], but it is doubtful whether the same kinds of products are involved in the mild reactions investigated here, and in those which occur in the cell.

Other agents. Numerous agents tested in conditions described below have been found to have no irreversible effects on viscosity nor on heat stability of DNA. These agents were also previously found to have no effect on transforming activity[10]. Some of them were used in this work for the preparation of DNA; others have been reported as having denaturing, mutagenic or carcinogenic action.

Aqueous NaCl solutions (2 *M* for one week at 6°); 75 % ethanol (7 processes of precipitation at 23° and storage at 6° for one week); chloroform-*n*-pentanol mixture 3:1 (one week, with shaking, at 6°); sodium desoxycholate (0.4 *M* for 4 h at 23°); sodium dodecyl sulfate (0.4 *M* in standard buffer at 6° for 2 weeks); ethyl mercurithiosalicylate ("Merthiolate") (0.01% at 23° for 2 h and at 8° for 24 h); NH_4^+ ion (as 1.5 *M* NH_4Cl, adjusted to pH 8, at 23° for 4 h); formaldehyde (0.33 *M* at 23° for 5 h and at 6° for 42 h); phenol (0.65 *M* at 50° for one h); urea (0.1 *M* at 76° for one hour) (the last two agents have been reported to denature DNA[51], possibly by action on the less intact starting material); acriflavine (neutral) (0.2 *M*; 12 hours at 6°); urethane (0.75 *M*; 4½ h at 23°); adenine ($4.5 \cdot 10^{-3}$ *M*), guanine and guanozole ($1.7 \cdot 10^{-4}$ *M*), cytosine ($3.4 \cdot 10^{-2}$ *M*), thymine ($1.8 \cdot 10^{-2}$ *M*) (all purines and pyrimidines 2 hours at 23° and

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one hour at 50°). In all experiments the DNA used was ox DNA 400 to 700 γ per ml of standard buffer.

The resistance of DNA to the strong protein denaturing agents (including heat, as described above) suggests that the hydrogen bonds in the DNA molecule may be stronger than those in most proteins, or that more of these bonds have to be broken to achieve "denaturation".

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SUMMARY

The human and ox DNA were prepared by methods one of which included the addition of DNA having transforming activity, to serve as a marker of "non-denaturation". The DNAs so prepared were tested for their resistance to heat, ionic strength, dehydration, DNAse, HCHO, H_2O_2, Fe^{+2}, and various other mutagenic or protein denaturing agents. No significant species differences in resistance were found. The resistance to heat was found to be considerably higher than in the preparations in which the injurious effects of low ionic strength, dehydration or minute traces of DNAse were not excluded. This loss of stability to heat in specified conditions was found to be a sensitive criterion of injuries to the DNA molecule. The parallel findings of the loss of transforming activity of *Hemophilus influenzae* DNA are compared.

RÉSUMÉ

Les acides désoxyribonucléiques humains et bovins ont été préparés par des méthodes dont une comprenait l'addition d'acide désoxyribonucléique ayant une activité transformante pour servir de traceur de "non-dénaturation". Les acides désoxyribonucléiques préparés de cette manière furent examinés au point de vue de leur résistance à la chaleur, à la force ionique, à la déhydration, à désoxyribonucléase, à HCHO, à H_2O_2, à Fe^{+2}, et à divers autres agents mutagéniques ou dénaturant les protéines. Il n'a pas été trouvé de différences d'espèce significatives dans ces résistances. La résistance à la chaleur a été trouvée considérablement plus grande que pour les préparations où les effets nocifs, causés par une force ionique trop faible, par la déhydratation ou par des traces menues de désoxyribonucléase, n'ont pas été exclus. Cette perte de stabilité à la chaleur dans des conditions déterminées peut être un critère sensible de dommage à la molécule de l'acide désoxyribonucléique. Les résultats parallèles de la perte d'activité transformante de l'acide désoxyribonucléique d'*Hemophilus influenzae* ont été comparés.

ZUSAMMENFASSUNG

DNA vom Menschen und vom Rind wurden nach Methoden hergestellt, von denen eine den Zusatz von DNA mit Transformations-Aktivität einschloss, die als Markierer für die Abwesenheit von "Denaturierung" dienen sollte. Die Stabilität der so hergestellten DNA gegen Hitze, Ionen-Stärke, Entwässerung, DNase, HCHO, H_2O_2, Fe^{+2} und verschiedene andere mutagene oder Protein denaturierende Agenten wurde untersucht. Es wurden keine erheblichen Gattungsunterschiede in diesen Stabilitäten gefunden. Die Stabilität gegen Hitze war bedeutend höher als in den Präparaten, in denen die schädlichen Wirkungen von zu niedriger Ionen-Stärke, von Entwässerung oder von geringen Spuren von DNase nicht ausgeschlossen waren. Es zeigte sich, dass dieser Verlust von Stabilität gegenüber Hitze ein empfindliches Merkmal für Beschädigung des DNA-Moleküls ist. Die parallelen Resultate des Verlusts an Transformations-Aktivität beim DNA von *Hemophilus influenzae* wurden verglichen.

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