Micrococcal Nuclease as a Probe of DNA Conformation*

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The action of micrococcal nuclease on native and denatured DNA has been studied with the object of using this enzyme as a "probe" to examine certain aspects of DNA conformation. As previously shown by Dirksen and Dekker [*Biochem. Biophys. Res. Commun. 2*, 147 (1960)] under identical conditions, denatured DNA is hydrolyzed much faster than native DNA by this nuclease. Examination of early hydrolysis products suggests that the site of initial attack of the enzyme depends on the conformation of the substrate; at 60° denatured DNA is attacked essentially at random while the initial attack on native DNA is in regions rich in deoxyadenylic and thymidylic acid residues. It is also shown that at any temperature the rate of hydrolysis of both native and denatured DNA decreases in a first-order fashion with time, as a consequence of the thermal inactivation of the enzyme. Under certain salt and temperature conditions the enzyme is partially protected against this thermal inactivation when *native* DNA serves as substrate. Both denatured DNA and random-coil polyadenylic acid are ineffective in this respect. The "protective effect" of the native DNA structure may be markedly enhanced by the addition of small quantities of bovine serum albumin, ribonuclease, or polylysine.

It has long been known (*e.g.*, see Linderstrøm-Lang, 1952) that native proteins are generally more resistant than their denatured counterparts to attack by proteolytic enzymes. This doubtless arises from the fact that in native proteins residues adjacent to the potentially susceptible peptide bonds are restrained by the secondary-tertiary structure of the protein from assuming an optimal orientation with respect to the enzymatic site. In globular proteins the degree to which these factors affect the rate of cleavage varies greatly from one bond to another, the complexity of the over-all structure making the conformation about almost every bond different from that about any other. However, it has recently been shown with certain fibrous proteins (Harrington *et al.*, 1959; von Hippel and Harrington, 1959; Mihalyi and Harrington, 1959; von Hippel *et al.*, 1960) and polypeptides (Miller, 1961, 1962) in which the conformational environments of all bonds in the "crystalline" portions are basically similar, that one can obtain quantitative information about the secondary structure of these macromolecules by using appropriate proteolytic enzymes as "conformational probes." For example, it was found that the manner in which the enzyme collagenase attacks collagen is strongly dependent upon the conformation of the molecule, the native helical structure being hydrolyzed much more slowly than the denatured random-coil form, and with very different enthalpies and entropies of activation (von Hippel and Harrington, 1959; von Hippel *et al.*, 1960). Since DNA is also a macromolecule characterized by a relatively simple, regularly repeating secondary structure, it seemed that certain features of the DNA structure might also be profitably investigated using an "enzymatic probe" technique.

The following points were considered in selecting the enzyme "micrococcal nuclease," first isolated from *Staphylococcus aureus* (or *Micrococcus pyogenes*) by Cunningham *et al.* (1956), as the principal probe to be used in this investigation: (1) It seems to operate primarily as an endonuclease (Alexander *et al.*, 1961; Sulkowski and Laskowski, 1962). (2) It seems to be relatively nonspecific in terms of the nucleotide sequences it will attack, though showing some preference for phosphodiester bonds on the 5′ side of deoxyadenylic or thymidylic acid residues (Rushizky *et al.*, 1960; Roberts *et al.*, 1962). (3) It is a small and very heat-stable enzyme (Cunningham *et al.*, 1956; Alexander *et al.*, 1961), suggesting that one might conduct enzymatic digestions at or near the temperature of the DNA helix-coil transition. (4) Finally, this enzyme had already been shown (Dirksen and Dekker, 1960) to be sensitive to substrate conformation, in that it had been found to attack heat-denatured DNA much more rapidly than native DNA under certain conditions.

This observation of Dirksen and Dekker (1960), coupled with the other favorable properties of the enzyme, suggested that micrococcal nuclease might well serve as a conformation-sensitive probe of DNA, and therefore we initiated an extensive investigation of the action of this enzyme on native and denatured DNA and on certain simple polynucleotides.

MATERIALS AND METHODS

Enzymes.—The micrococcal nuclease used in these studies was prepared from cultures of *S. aureus* by a modification of the procedure of Cunningham *et al.* (1956). The enzyme was carried through the second ammonium sulfate fractionation step of Alexander *et al.* (1961), redissolved in a small volume of water, and frozen as a concentrated stock solution having a specific activity of 9300 units (see Alexander *et al.*, 1961). Preserved in this form, the enzymatic activity remained completely unchanged over a period of at least 18 months. For use in experiments, 0.1-ml aliquots of this concentrated solution were diluted with 7.9 ml of distilled water. This diluted enzyme solution contained ~0.05 mg of protein per ml, and is the standard stock solution referred to in the text. Rates of enzymatic hydrolysis are expressed in terms of μmoles

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of internucleotide bonds hydrolyzed per minute by 100 μl of this stock solution.

Micrococcal nuclease is completely inactive in the absence of ionic calcium (Cunningham *et al.*, 1956; Alexander *et al.*, 1961). Since our nuclease preparations retained some activity when tested without added calcium, we assumed that the stock solution was slightly contaminated with calcium ions. The addition of 2 μmoles of ethylenediaminetetraacetic acid (EDTA)[1] per ml of enzyme stock solution reduced this activity to zero, suggesting that the stock contained $\sim$2 μM/ml of endogenous Ca^{++}. Since all our experiments were carried out in the presence of excess calcium, this contamination did not present a problem.

One aliquot of the enzyme stock was purified further by carrying it through the DEAE-cellulose chromatography procedure described by Alexander *et al.* (1961). The most active fraction removed from the column showed a specific activity five times greater than that of the starting stock solution.

DNA and Polynucleotides.—Most of the experiments were carried out with highly polymerized calf thymus DNA (lots 596 and 599) purchased from the Worthington Biochemical Corp. This DNA was prepared by a slight modification of the Zamenhof (1957) procedure and air-dried from ethanol; the preparations used had protein contents of 1.5% (lot 596) and 0.4% (lot 599), respectively. Some early experiments were performed with highly polymerized DNA purchased from the Sigma Chemical Co. (preparation described in Felsenfeld and Huang, 1961). Critical experiments were repeated with calf thymus DNA prepared in this laboratory. This DNA was isolated from fresh glands by the method of Kay *et al.* (1952) as modified by Felsenfeld and Huang (1961). It contained 0.3% protein and had a median sedimentation coefficient ($s^\circ_{20,w}$) of 23.4 S in 0.1 M NaCl. No significant differences were noted between experiments carried out with the Worthington and the Sigma DNA preparations, and those performed with the DNA prepared in our laboratory.

Solutions of native DNA were made by dissolving the dried fibrous DNA in neutral 0.05 M NaCl by gentle continuous stirring at 5°. Approximately 4 days of stirring were required to effect complete solution. Native DNA solutions prepared in this way from both the commercial and the noncommercial material were found to be fully native by the criterion of Shack (1958), showing no change in absorbancy at 260 mμ when a DNA solution in 0.01 M NaCl was also made 0.01 M in $MgCl_2$. Native DNA solutions prepared from Worthington DNA and the DNA made in this laboratory were also compared by the optical melting point dispersion method of Felsenfeld and Sandeen (1962) and were found to exhibit identical melting profiles.

The heat-denatured DNA used in these studies was prepared in three different ways: (1) A suspension of DNA in distilled water was made by gently hand-grinding the dried material with water in a Teflon-glass homogenizer. The suspension was then heated at 100° in a sealed tube for 30 minutes to completely dissolve and denature the DNA. (2) Dried DNA was dissolved directly (without prior grinding) by heating at 100° in a sealed tube for 30 minutes. (3) Native DNA solutions in 0.01 M NaCl were heat-denatured just prior to use by heating at 100° in a sealed tube for 30 minutes. Recooled solutions of denatured DNA prepared in all three ways were found to be completely denatured by the Mg^{++} criterion of Shack (1958) (see previous paragraph) and all gave apparently identical results in the enzymatic experiments.

Polyadenylic acid was purchased from Miles Laboratories and put into solution (by gentle hand-grinding in a Teflon-glass homogenizer) in 0.05 M NaCl adjusted to *p*H 8.5. Under these conditions polyadenylic acid exists largely in the single-strand, random-coil form (Steiner and Beers, 1961).

All DNA (and polyadenylic acid) solutions were made up to a final concentration of 1 mg/ml.

Assay of Enzymatic Activity.—The enzymatic hydrolysis of DNA was monitored either by a *p*H-stat method or by the perchloric acid precipitation technique of Alexander *et al.* (1961). In the former case, the equipment consisted of a Radiometer automatic titrator (TTT-1b) and titrigraph (SBR2C) equipped initially with a single combination calomel-glass electrode (GK2025B), and subsequently with separate glass (G202B) and calomel (K401) electrodes. The use of the combination electrode was discontinued when it was discovered that the amount of KCl which leaked into the reaction mixture from this electrode during a run affected the activity of the enzyme. With the separate calomel electrode (K401) this was not a problem. For experiments above 60°, a high temperature glass electrode (G302B) was used. Reactions were run in a thermostated cell ($\pm$ 0.05°) and temperatures were measured directly in the cell by means of a thermistor arrangement. A constant stream of prepurified nitrogen (bubbled through NaOH and H_2SO_4 and then saturated with water at the temperature of the experiment) was passed over the solution to exclude CO_2. Before adding enzyme, the system was always incubated for a short time to test the efficacy of this procedure and to establish a base line of NaOH uptake in the absence of enzyme. In this system, no NaOH was required to maintain the *p*H constant at values below *p*H 8.7, even with 2 mM NaOH as titrant. The NaOH used as titrant was prepared fresh daily from a concentrated standard solution.

Protein Concentration Measurements.—The protein content of the various DNA solutions was measured by a modification (Gellert *et al.*, 1959) of the Folin-biuret method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The protein concentration of the micrococcal nuclease preparations was estimated spectrophotometrically by the OD_{260}/OD_{280} ratio method of Warburg and Christian (1941), as described by Layne (1957).

Characterization of Early Hydrolysis Products.—In experiments designed to determine the composition of the regions of native and denatured DNA attacked initially by micrococcal nuclease, aliquots of reaction mixture were withdrawn from the *p*H-stat cell at various times after adding enzyme and the reaction was stopped by quickly expelling the mixture into a solution containing excess EDTA. Cold perchloric acid, to a final concentration of 1.4%, was then added and the acid-insoluble nucleic acid removed by centrifugation (10 minutes at 10,000 $\times$ *g*, 2°). The ultraviolet absorption spectrum of the supernatant, containing the acid-soluble oligonucleotides released by the enzyme, was measured (at 5°) using a Cary Model 14 recording spectrophotometer equipped with a 0–0.1 OD slide-wire, and the OD_{260}/OD_{280} ratio was determined. Fredericq *et al.* (1961) have shown that the spectrum (220–330 mμ) of calf thymus DNA denatured in 0.1 M acetic acid at *p*H 3 is essentially identical with that calculated for the constituent mononucleotides, and

[1] Abbreviations used in this work: EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; BSA, bovine serum albumin; poly A, polyriboadenylic acid; DDNA, denatured DNA, NDNA, native DNA.

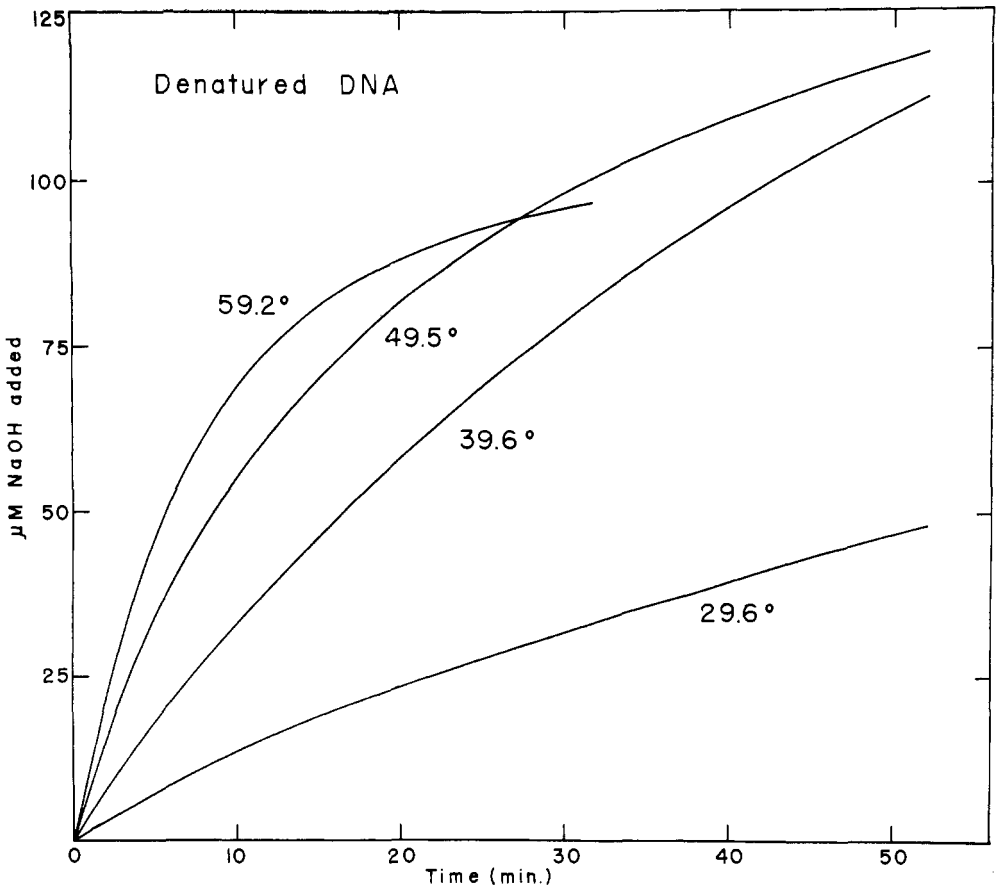

FIG. 1.—*p*H-Stat kinetics of hydrolysis of denatured DNA by micrococcal nuclease at 29.6°, 39.6°, 49.5°, and 59.2°. All runs made with 2 mg of DNA in a total volume of 7.15 ml. Solution was 35 mM in NaCl and 1.4 mM in $CaCl_2$. The *p*H was held constant at 8.5 with 1.7 mM NaOH. Fifty μl of enzyme stock was used for each run.

that the OD_{260}/OD_{280} ratio under these circumstances constitutes a direct measure of the base ratios of the DNA sample if one assumes Watson-Crick pairing (A=T and G=C). We determined that the spectra of a mixture of mononucleotides at *p*H 3 (0.1 M acetic acid) and *p*H 1 (0.14 M perchloric acid) are nearly identical, and thus used the method of Fredericq *et al.* (1961) to estimate the base composition of the perchloric acid–soluble oligonucleotides released during the early phases of enzymatic attack (again assuming A=T and G=C in the acid-soluble fragments). Spectral measurements were made at 0°, since the spectra in perchloric acid changed rather rapidly with time at room temperature and above, presumably as a consequence of progressive depurination.

Chemicals and Reagents.—Ribonuclease A (lot 6018), lysozyme (lot LY577) and deoxyribonuclease II (lot DA609) were obtained from the Worthington Biochemical Corp. Bovine serum albumin (crystalline) was purchased as a concentrated standard solution from the Armour Pharmaceutical Co. Poly-L-lysine-HBr (lot PLH 6101, η_{sp} = 1.26, 1% in dimethylformamide) and poly-L-aspartic acid (lot PAA 6002; $\overline{M}_w$ = 7000–8500) were manufactured by the Yeda Research and Development Co., Ltd., Rehovoth, Israel, and purchased from Schwarz BioResearch, Inc. Polyethyleneglycol ($\overline{M}_w$ = 20,000), manufactured by the Dow Chemical Co., was obtained through the generosity of Prof. Walter Stockmayer. Spermine·4HCl was purchased from Calbiochem. All these materials were readily soluble in water or dilute salt solutions except the polyaspartic acid, which was initially dissolved in a dilute NaOH solution.

All chemicals employed were of reagent grade, and were used without further purification.

RESULTS

Initial Rate of Hydrolysis.—The kinetics of the attack of micrococcal nuclease on denatured and native DNA at four different temperatures are shown in Figures 1 and 2. In all runs the substrate was present in severalfold excess over the amount required to saturate the enzyme, and the initial rate of hydrolysis was proportional, as expected, to the amount of enzyme added. Typical initial rate (k_0) data, obtained at 40° and 60° with 2 mg of native DNA and varying amounts of enzyme, are compiled in Table I. It is clear that the initial rate per unit enzyme is essentially independent of enzyme concentration at both temperatures (and also at 40° at double the NaCl concentration). A similar series of experiments using 2 mg of denatured DNA (DDNA) as substrate under the same conditions (35 mM NaCl) also showed no change in k_0 as the amount of enzyme added was varied between 10 and 100 μl. Average values of k_0 (DDNA) of 4×10^{-2} μM/min/100 μl (40°) and 15×10^{-2} μM/min/100 μl (60°) were obtained. Within these limits of enzyme concentration changes in amount or concentration of substrate, volume, or geometry of the *p*H-stat cell, etc., had no effect on the k_0 obtained with either substrate. At both temperatures the initial rate of hydrolysis of denatured DNA per unit enzyme is substantially greater than the initial rate for native DNA at the same temperature. This result is in qualitative accord with the findings of Dirksen and Dekker (1960) under similar solvent conditions.

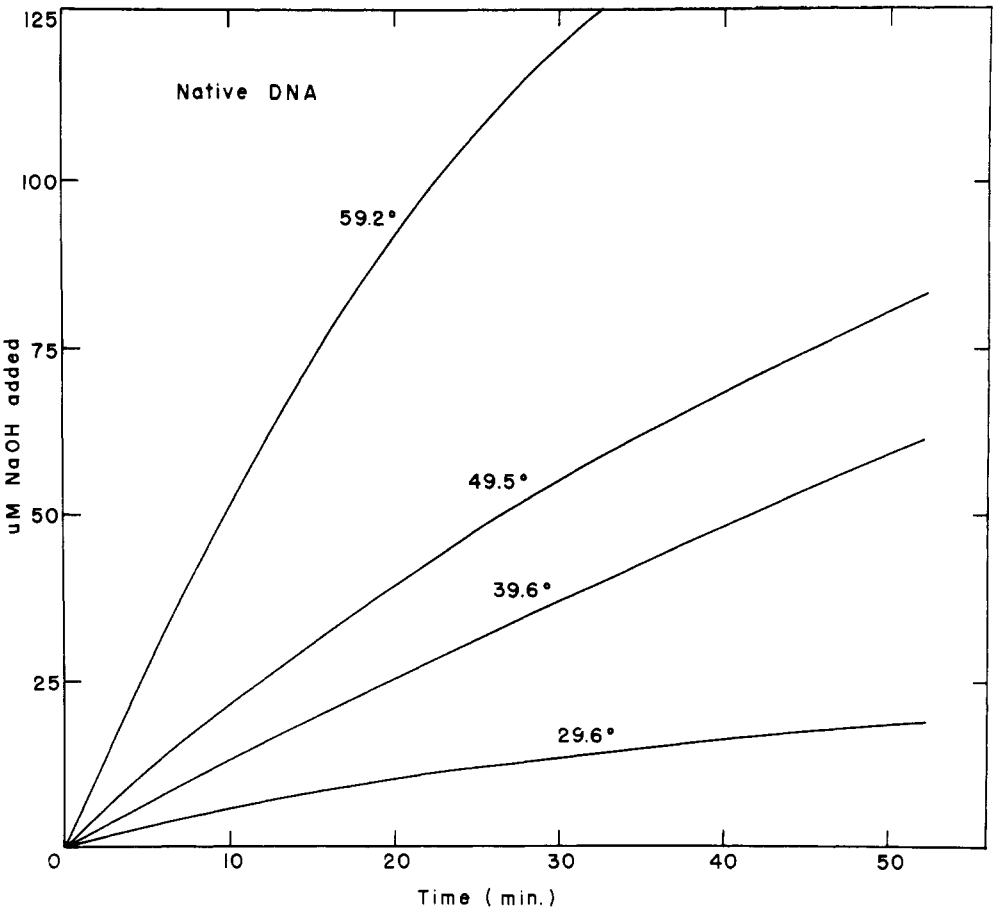

FIG. 2.—*p*H-Stat kinetics of hydrolysis of native DNA by micrococcal nuclease. Same temperatures and conditions as in Fig. 1. One hundred fifty μl of enzyme stock was used for each run.

TABLE I

EFFECT OF VARYING AMOUNTS OF ENZYME ON THE INITIAL RATE OF HYDROLYSIS OF NATIVE DNA, AND ON THE RATE OF INACTIVATION OF THE ENZYME[a]

Amt. Enzyme (μl)	40°		60°	
	k_0 (μM/min/100 μl)	k_0 (μM/min/100 μl)[b]	k_0 (μM/min/100 μl)	k_i (min^{-1})
20	0.52×10^{-2}	0.70×10^{-2}	1.7×10^{-2}	$> -50 \times 10^{-3}$
30	0.50	0.68	1.3	> -50
40	0.52	0.56	2.6	-35
60	0.59	0.50	2.7	-20
90	0.51	0.64	2.3	-19
120	0.49	0.74	2.0	-18
150	0.45	0.70	2.8	-16
200	0.54	0.64	4.2	-15

[a] Experimental conditions: 1.4 mM $CaCl_2$, 35 mM NaCl, *p*H 8.5 (maintained with 2.0 mM NaOH), total volume = 7.15 ml. Total amount DNA in cell = 2 mg = ~7 μM internucleotide bonds. [b] Same conditions except NaCl concentration is 70 mM.

The effect of temperature between (30° and 65°) on the initial rate per unit enzyme (k_0) with both native and denatured DNA as substrate, is shown in the form of an Arrhenius plot in Figure 3. Most of the data fall on two straight and essentially parallel lines, illustrating that over this entire temperature range and under these solvent conditions the energies (and enthalpies) of activation for the hydrolysis of both substrates are constant and essentially the same (see Table II). These data also show that over this range of temperatures denatured DNA is hydrolyzed 6.8 ± 0.6 times faster than native DNA.[2] Values of Q_{10} (35°–45°) have also been calculated and are summarized in Table II.

Heat Inactivation of the Enzyme.—The remarkable resistance of micrococcal nuclease to heating (Cunningham *et al.*, 1956; Alexander *et al.*, 1961) during the early stages of its preparation led us to study the effect of high temperature on the activity of the purified enzyme. If the enzyme is permitted to act on denatured DNA at fairly high temperatures (e.g., 60°) the rate of hydrolysis falls off fairly rapidly with time (see Fig. 1). If the data for the 59.2° run shown in Figure 1 are replotted as the logarithm of the number of bonds hydrolyzed per unit time versus time (Fig. 4),

[2] At 60° (Fig. 3) the points for native DNA start to swing over toward the denatured DNA line (dashed curve). At approximately 70° the points for both substrates become essentially coincident. This suggests that at 70°, under these solvent conditions, the susceptible bonds of native and denatured DNA become equally accessible to the enzyme.

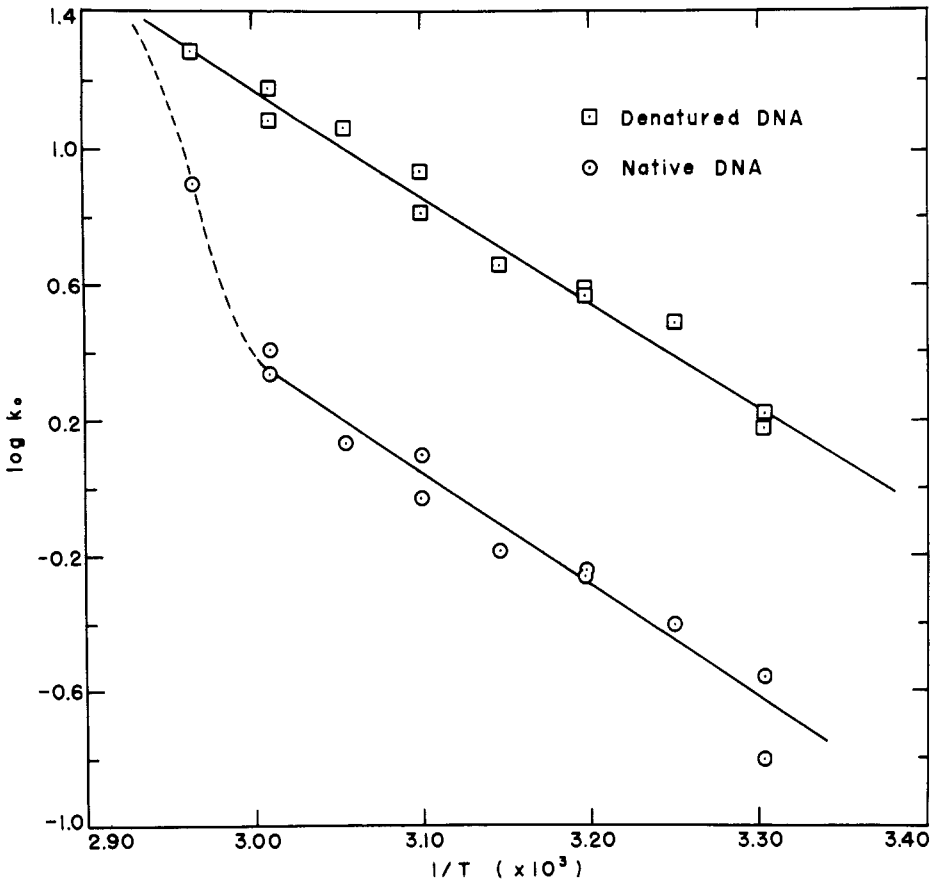

FIG. 3.—Arrhenius plot showing the effect of temperature on the initial rate of hydrolysis of native (O) and denatured (□) DNA by micrococcal nuclease. Initial rate in units of μM NaOH added/min/100 μl of stock enzyme solution. Conditions as in Figs. 1 and 2.

TABLE II

"THERMODYNAMIC" ACTIVATION PARAMETERS FOR THE INITIAL RATE OF HYDROLYSIS OF NATIVE AND DENATURED DNA BY MICROCOCCAL NUCLEASE AND FOR THE RATE OF THERMAL INACTIVATION OF THE ENZYME AT 30–60°

	Initial Rate (k_0)		Rate of Inactivation (k_i)	
	NDNA	DDNA	NDNA	DDNA
ΔE_a^* (kcal/mole)	+15.1 (± 1.5)	+14.1 (± 1.4)	+17.3 (± 2.0)	+17.8 (± 2.0)
$\Delta H_{\ddagger}^{\ddagger}$ (kcal/mole)	+14.5 (± 1.5)	+13.5 (± 1.4)	+16.7 (± 2.0)	+17.2 (± 2.0)
Q_{10}(35–45°)	2.2 (± 0.2)	2.1 (± 0.2)	2.5 (± 0.2)	2.5 (± 0.2)

it may be seen that the decrease in the activity of the enzyme with time follows an exponential decay curve (for at least two to three half-times) as expected for a simple first-order inactivation process. That this decrease in activity with time is actually due to a denaturation of the enzyme rather than to the accumulation of inhibitory reaction products or exhaustion of the substrate is evident, both because the extent of inactivation is clearly not related to the extent of digestion of the substrate (compare the experiments at various temperatures in Fig. 1) and because, after adding a small aliquot of enzyme and following the kinetics until all activity is gone, one can add another aliquot of the same size and obtain the same inactivation kinetics.

If one compares the kinetics of micrococcal nuclease operating on denatured and native DNA (Figs. 1 and 2) it is immediately clear that the rate of inactivation of the enzyme with native DNA as substrate is much smaller than with denatured DNA as substrate at the same temperature. This is quantitatively demonstrated in Figure 4, which shows that inactivation of the enzyme in the presence of native DNA also follows first-order kinetics, but that the process is much slower. Since with both substrates inactivation of the enzyme seems to be a simple first-order reaction, it can be described quantitatively in terms of a first-order rate constant (k_i) obtained from the slope of lines such as those shown in Figure 4.

Values of k_i have been obtained with both native and denatured DNA as substrate under a variety of experimental conditions. As with k_0, the inactivation constant (k_i) for denatured DNA does not vary with the amount of enzyme or substrate added to the *p*H-stat cell (as long as substrate remains in excess). An average k_i (DDNA) of $\sim -50 \times 10^{-3}$ min^{-1} was obtained with this substrate at 60° under the conditions of Table I (35 mM NaCl).

With native DNA (NDNA) as substrate, the situation is somewhat more complex. As the last column of Table I shows, k_i obtained with very small amounts of enzyme in the presence of native DNA is of the same magnitude as k_i (DDNA). However, as the amount of enzyme added is increased, k_i (NDNA) decreases rapidly, and with amounts of enzyme in excess of approximately 60 μl becomes essentially constant at approximately one-third the value found at all levels with denatured DNA. Thus it appears that *under these conditions the native DNA structure*

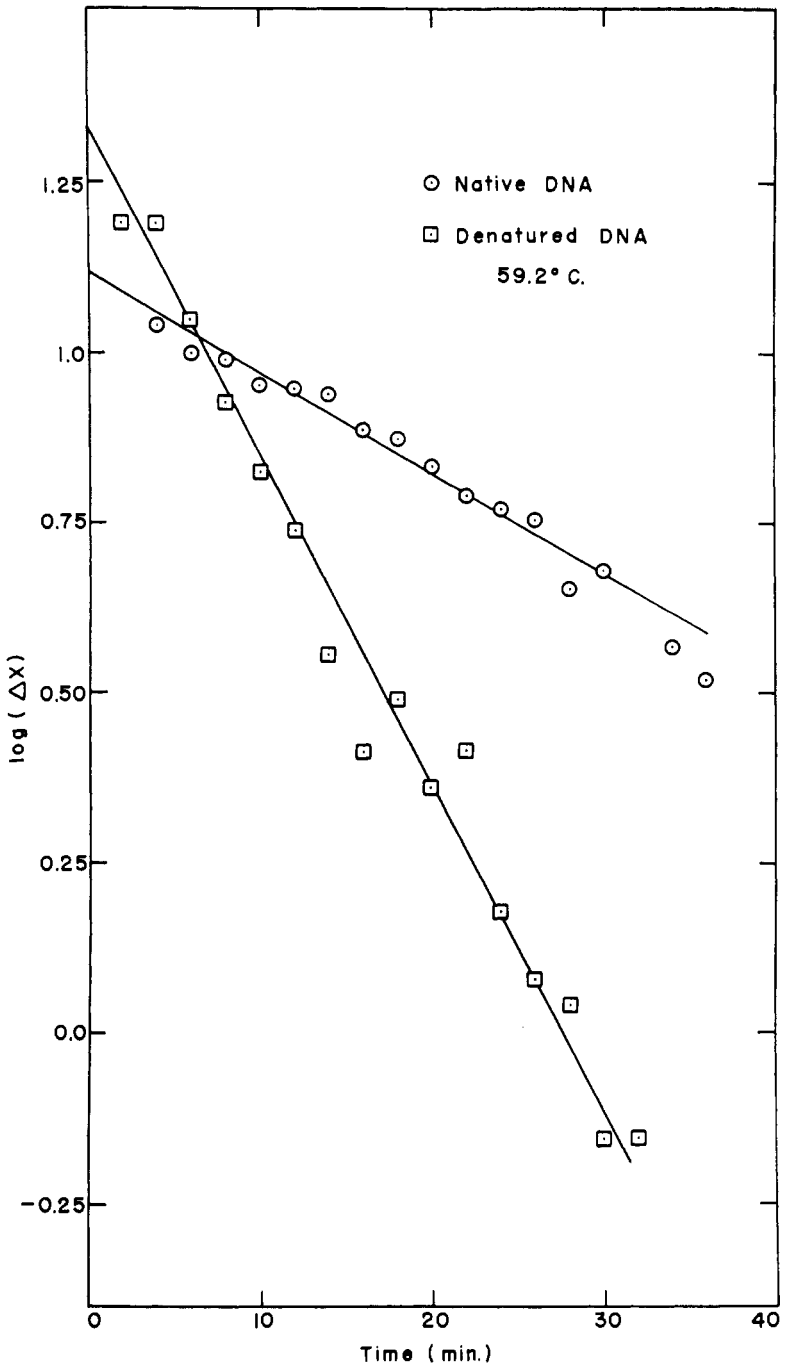

FIG. 4.—First-order plot of the hydrolysis of native (O) and denatured (□) DNA by micrococcal nuclease. The logarithm of the amount of NaOH added in successive 2-minute intervals is plotted against time after the addition of the enzyme. Data taken from Figs. 1 and 2, 59.2° runs.

interacts with the enzyme to protect it against thermal inactivation.

In Figure 5 the effect of temperature on the inactivation constant (k_i) is presented as an Arrhenius plot for both native and denatured DNA, and again the points for the two substrates fall on two straight and essentially parallel lines. Thus for this process also, the enthalpies of activation for both substrates are constant and approximately the same over the 30° to 65° temperature range (see Table II). From Figure 5 we may also calculate that k_i obtained with denatured DNA as substrate is 2.3 ± 0.2 times greater than k_i obtained with native DNA for all temperatures examined.

Thus it appears that we are faced with two phenomena which seem to be critically dependent on the conformation of the DNA molecule: (1) The rate of hydrolysis of denatured DNA is considerably greater than that of native DNA under the same conditions; and (2) the presence of native DNA as substrate partially protects the enzyme against thermal inactivation. In order to demonstrate more fully that the DNA structure alone is responsible for these observations, and in order to differentiate between factors that affect the substrate and those that affect the enzyme, a number of additional experiments were undertaken.

Effect of pH.—The effect of *p*H on the initial rate of hydrolysis of native and denatured DNA by micrococcal nuclease at 40° is shown in Table III. This enzyme is essentially inactive at *p*H values below *p*H 7.5, and the activity increases progressively as the

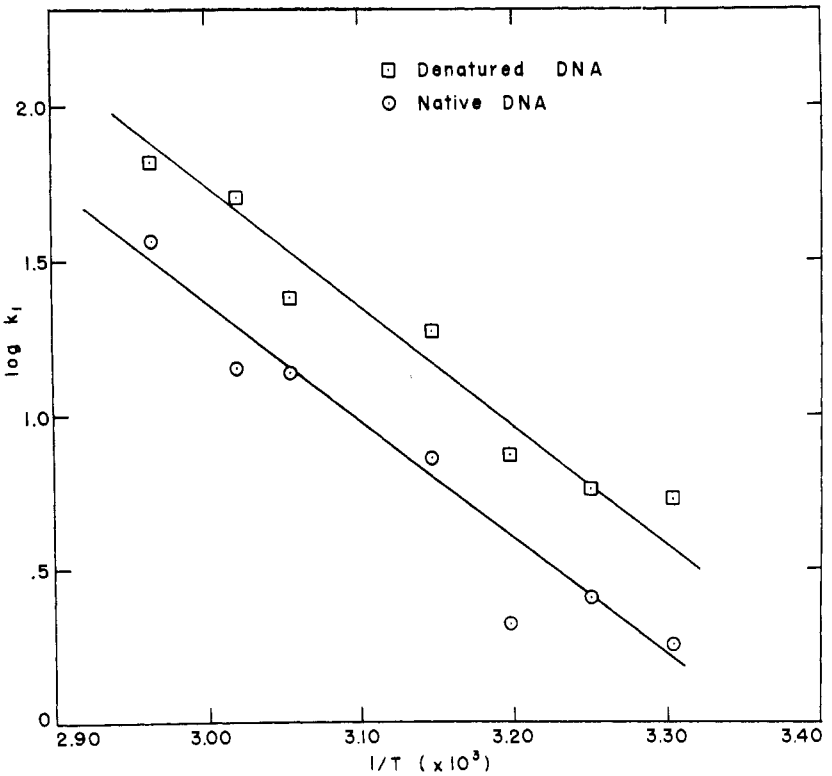

FIG. 5.—Arrhenius plot showing the effect of temperature on the rate of inactivation of micrococcal nuclease during the hydrolysis of native (O) and denatured (□) DNA. Conditions as in Figs. 1 and 2.

solution is made more alkaline, at least to *p*H 9.[3] As Table III also shows, the ratio k_0 (DDNA)/k_0 (NDNA) is essentially constant at all the *p*H values examined, suggesting that *p*H changes in this range do not influence the rate of hydrolysis by affecting the conformation of the substrate, but operate directly on the enzyme.

With both substrates, the rate of inactivation of the enzyme increases markedly with increasing *p*H. Qualitatively similar results were obtained at 60°.

Effect of Ca^{++} *and* Na^{+}.—The experiments described to this point were all performed in 1.4 mM $CaCl_2$ and 35 (or 70) mM NaCl. Tables IV and V show the effect (on k_0 and k_i at 60°) of varying the concentrations of these electrolytes with either native or denatured DNA as substrate. It is clear that the effects differ dramatically: Increasing the calcium concentration increases k_0 (DDNA) only slightly, but almost doubles k_0 (NDNA) (in going from 1.4 mM to 5.6 mM $CaCl_2$). On the other hand, increasing [Ca^{++}] decreases k_i (DDNA) considerably while leaving k_i (NDNA) almost unchanged.

Increasing [Na^{+}] has quite different effects, bringing about only a very slight increase in k_0 (DDNA) but a large decrease in k_0 (NDNA), accompanied by a marked decrease in k_i (DDNA). The k_i (NDNA) first decreases (in going from 14 mM to 35 mM NaCl) and then increases (in going from 35 mM to 140 mM NaCl). It should be noted that at concentrations of sodium ion in excess of 100 mM (at 60°), native DNA is totally ineffective in protecting the enzyme against thermal inactivation. Yet the ratio of the initial rates [k_0(DDNA)/k_0(NDNA)] is greater than at the lower sodium ion concentrations at which the native DNA structure seems to protect the enzyme. As [Ca^{++}] increases, k_i (DDNA) also moves toward k_i (NDNA), though in this case as a consequence of a progressive decrease in k_i (DDNA).

[3] These findings seem to support the *p*H-optimum at 10.3 found for this enzyme by Alexander *et al.* (1961), rather than the optimum at *p*H 8.6 reported earlier by Cunningham *et al.* (1956).

TABLE III

INITIAL RATE OF HYDROLYSIS OF NATIVE AND DENATURED DNA BY MICROCOCCAL NUCLEASE AS A FUNCTION OF *p*H[a]

*p*H	k_0 (μM/min/100 μl) Denatured DNA	k_0 (μM/min/100 μl) Native DNA	k_0 (DDNA)/k_0 (NDNA)
7.5	1.1×10^{-2}	$\sim 0.1 \times 10^{-2}$	—
8.0	2.3	0.51	4.5
8.5	3.6	0.93	3.9
8.8	4.8	1.2	4.0
9.0	5.6	1.3	4.3

[a] Experimental conditions: temp = 40°, 35 mM CaCl, 1.4 mM $CaCl_2$; volume = 7.15 ml; maintained with 2.0 mM NaOH.

These results on the effect on k_0 of altering the NaCl concentration are in accord with the observations of Dirksen and Dekker (1960). These workers also found (at 25° and *p*H 8.6) that increasing the concentration of NaCl from 44 to 200 mM led to a slightly accelerated rate of attack on heated-denatured calf thymus DNA, but markedly decreased the rate of hydrolysis of native DNA. However, in addition, they reported an anomalously large Q_{10} (25–35°) = 4.9 in 0.2 M NaCl. Since this observation is quite at variance with our results at lower ionic strength (Table II and Fig. 3), we have examined the temperature dependence of k_0 (and k_i) for both native and denatured DNA at 140 mM NaCl. The results obtained for k_0 are very similar to those shown in Figure 3. Linear and parallel Arrhenius plots were obtained for both native and denatured DNA over the entire 30–60° temperature range. For native DNA under these conditions, $\Delta E_a^* = +12 \pm 1$ kcal/mole and Q_{10} (35–45°) = 1.9. For denatured DNA, $\Delta E_a^* = +11 \pm 1$ kcal/mole and Q_{10} (35–45°)

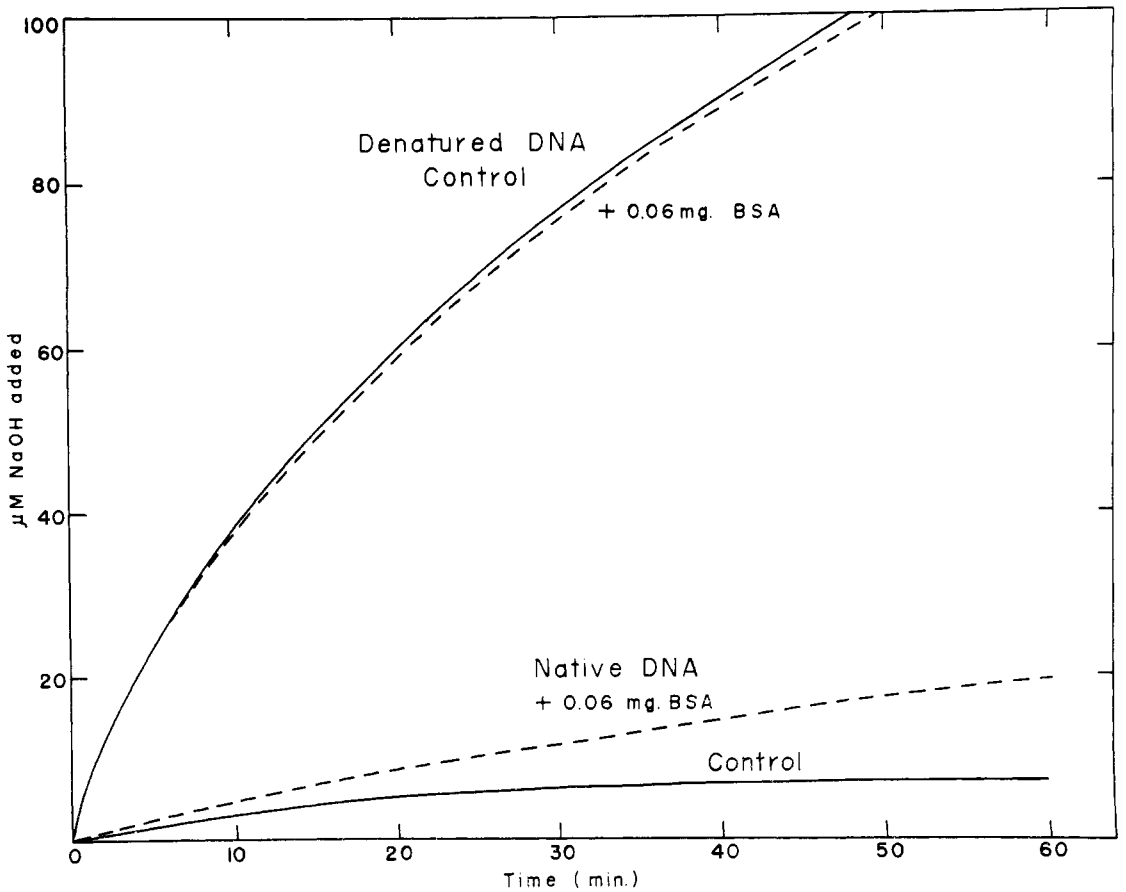

FIG. 6.—*p*H-Stat experiments comparing the protective effect of added bovine serum albumin on micrococcal nuclease operating on native and denatured DNA. Solid curves, DNA only; dashed curves, DNA + 0.06 mg BSA. For all runs: 60°, 2 mg DNA, 30 μl enzyme stock, 35 mM NaCl, 1.4 mM $CaCl_2$, total volume = 7.15 ml, *p*H 8.5.

TABLE IV

EFFECT OF VARYING $CaCl_2$ CONCENTRATION ON INITIAL RATE AND RATE OF INACTIVATION OF MICROCOCCAL NUCLEASE OPERATING ON NATIVE AND DENATURED DNA[a]

Molarity $CaCl_2$	Denatured DNA		Native DNA	
	k_0 (μM/min/100 μl)	k_i (min^{-1})	k_0 (μM/min/100 μl)	k_i (min^{-1})
1.4 mM	14×10^{-2}	-38×10^{-3}	2.2×10^{-2}	-19×10^{-3}
2.8	15	−35	2.9	−22
4.2	17	−31	3.4	−19
5.6	17	−30	3.6	−20

[a] Experimental conditions: temp = 60°; 35 mM NaCl; *p*H 8.5 (maintained with 2.0 mM NaOH); total volume = 7.15 ml; native DNA runs, used 100 μl enzyme stock; denatured DNA runs, used 30 μl enzyme stock. Total amount DNA in cell = 2 mg = ~7 μM internucleotide bonds.

= 1.8. The average ratio of k_0 (DDNA)/k_0 (NDNA) obtained over this temperature range was 13.4 ± 0.4. Thus our results at high ionic strength are very similar to those obtained at lower NaCl concentration, except for the increased difference in the initial rate of attack on the two substrates.

In a further attempt to account for the large Q_{10} (NDNA) obtained by Dirksen and Dekker, we repeated the experiments duplicating their conditions as closely as possible; 0.43 mg DNA, *p*H 8.6, 10 mM $CaCl_2$, and 200 mM NaCl in 9 ml total volume at 25° and 35°. For each of the native DNA runs we used 800 μl of our nuclease stock, and 80 μl for each of the denatured DNA experiments. Under these conditions it is possible that the enzyme was not entirely saturated with substrate, especially in the native DNA experiments, but nevertheless a Q_{10} of 2.3 was obtained for native DNA and 2.8 for denatured DNA. Ratios of k_0 (DDNA)/k_0 (NDNA) of 37 (35°) and 30 (25°) were obtained, in reasonable accord with the findings of Dirksen and Dekker. However, we have been unable to reproduce the high Q_{10} (NDNA) which they reported.

Since the protective effect of native DNA seems to be abolished at 60° in 140 mM NaCl (Table V) we also examined the dependence of k_i (NDNA) and k_i (DDNA) on temperature at this ionic strength. In this case a linear Arrhenius plot was *not* obtained with native DNA; rather the ratio k_i (DDNA)/k_i (NDNA) changed markedly with temperature, increasing from approximately unity at 60° to 1.9 at 55°, and to approximately 3.0 at all temperatures below 50°. Thus at this ionic strength the full protective effect of the native DNA structure seems to manifest itself only at temperatures of 50° or less.

The kinetics of some of these salt effects are also interesting. For example, we have found that if the digestion is performed in 140 mM NaCl, the salt being added at any time *before* the enzyme, the inactivation

TABLE V

EFFECT OF VARYING NaCl CONCENTRATION ON INITIAL RATE AND RATE OF INACTIVATION OF MICROCOCCAL NUCLEASE OPERATING ON NATIVE AND DENATURED DNA[a]

Molarity NaCl	Denatured DNA		Native DNA	
	k_0 (μM/min/100 μl)	k_i (min^{-1})	k_0 (μM/min/100 μl)	k_i (min^{-1})
14 mM	11×10^{-2}	-46×10^{-3}	3.1×10^{-2}	-27×10^{-3}
21	—	—	2.7	−23
35	13	−41	2.0	−19
70	14	−32	2.1	−27
105	16	−28	1.7	−33
140	15	−29	1.7	−35

[a] Experimental conditions: temp = 60°; 1.4 mM $CaCl_2$; *p*H 8.5 (maintained with 2.0 mM NaOH); total volume = 7.15 ml; native DNA runs, used 100 μl enzyme stock; denatured DNA runs, used 30 μl enzyme stock. Total amount DNA in cell = 2 mg = ~7 μM internucleotide bonds.

TABLE VI

EFFECT OF VARIOUS ADDITIVES ON THE INITIAL RATE AND RATE OF INACTIVATION OF MICROCOCCAL NUCLEASE OPERATING ON NATIVE AND DENATURED DNA[a]

Compound Added	Denatured DNA		Native DNA	
	k_0 (μM/min/100 μl)	k_i (min^{-1})	k_0 (μM/min/100 μl)	k_i (min^{-1})
None (control)	10×10^{-2}	-35×10^{-3}	1.5×10^{-2}	-12×10^{-3}
BSA (0.13 mg)	12	−28	2.0	~ 0 (or +)
BSA (1.3 mg)	30	−25	3.0	~ 0 (or +)
Ribonuclease A (0.15 mg)	—	—	1.8	− 6
Ribonuclease A (0.5 mg)	9	−20	2.0	—
Ribonuclease A (1.0 mg)	9	−30	2.0	− 2
Poly-L-lysine (0.06 mg)	11	−27	2.0	− 2
Poly-L-aspartic acid (0.06 mg)	10	−28	1.6	−13
Polyethyleneglycol (0.06 mg)	—	—	1.4	−16
Spermine (0.3 mg)	12	−23	2.0	−12

[a] Experimental conditions: temp = 60°; 1.4 mM $CaCl_2$; 35 mM NaCl; *p*H 8.5 (maintained with 2.0 mM NaOH); total volume = 7.15 ml. Total amount of DNA in cell (native or denatured) = 2 mg = ~7 μM internucleotide bonds. All numbers above represent an average of from 2 to 7 runs (±10–20%). All runs with NDNA contain sufficient enzyme to attain "fully self-protected" state (see text).

rate constant at 60° is immediately that due to the completely "unprotected" enzyme. If, however, most of the salt is added *after* the enzyme, increasing the concentration of NaCl abruptly from 35 to 140 mM, the inactivation rate changes only gradually (over a period of several minutes) from the protected to the unprotected situation.

Effect of Added Proteins and Polypeptides.—The k_i (NDNA) data presented in Table I suggested that a certain minimal amount of enzyme is needed to achieve maximum protection against thermal denaturation. This observation seemed reminiscent of the earlier finding of McCarty (1945), who noted, in the course of an investigation of the enzyme deoxyribonuclease I, that a certain amount of protein (e.g., gelatin) had to be added to the assay mixture containing native DNA to make the specific activity of the enzyme independent of the amount of enzyme added. In the absence of added protein he found that the apparent specific activity with very small amounts of enzyme was much lower than that obtained with larger amounts (see McCarty, 1945, Figs. 1 and 2). These observations led us to test the effect of adding small amounts of protein on the kinetics of the DNA-micrococcal nuclease system. The results were dramatic. The addition of approximately 0.05 mg of ribonuclease, lysozyme, deoxyribonuclease II (acid deoxyribonuclease, inactive above approximately *p*H 5.5) or bovine serum albumin decreased the rate of thermal inactivation of small aliquots of micrococcal nuclease operating on *native* DNA markedly, but had no effect on k_i (DDNA), or on either k_0 (NDNA) or k_0 (DDNA). Figure 6 shows comparative experiments demonstrating this point. Hydrolysis of native DNA continues in the presence of added albumin long after activity has completely disappeared in the control; and, as the upper curves show, this amount of added albumin had little or no effect on the micrococcal nuclease-*denatured* DNA system.

We then proceeded to a quantitative examination of the effect of various concentrations of a number of different additives. Results with bovine serum albumin (BSA) and ribonuclease A, in terms of k_0 and k_i, are summarized in Table VI. It is clear that the addition of 0.13 mg of albumin brings about *total* protection of the enzyme, the inactivation rate constant [k_i (NDNA)] being reduced to zero (or actually becoming positive, in that for the first 10–15 minutes of digestion the rate of hydrolysis actually seems to increase with time). Under these conditions no decrease in the rate of hydrolysis is observed for periods in excess of 60 minutes, during which time up to 1.5 μmoles (~30%) of the internucleotide bonds have been cleaved. In terms of reducing k_i (NDNA), ribonuclease seems to be somewhat less effective than serum albumin.

The effects of poly-L-lysine (a polycation), poly-L-aspartic acid (a polyanion), polyethyleneglycol (a neutral polymer), and spermine (a small polycation) were also examined (see Table VI). Of these, only

TABLE VII

INITIAL RATE OF HYDROLYSIS AND RATE OF INACTIVATION OF MICROCOCCAL NUCLEASE OPERATING ON MIXTURES OF NATIVE DNA, DENATURED DNA, AND POLYRIBOADENYLIC ACID[a]

Substrate	k_0 (μM/min/100 μl)	k_i (min^{-1})
Native DNA	2×10^{-2}	0
Denatured DNA	15	-25×10^{-3}
NDNA-DDNA 1:1	5.6	− 9
Poly A	6	−18
NDNA-poly A 1:1	3.5	− 2.5
DDNA-poly A 1:1	10	−13

[a] Experimental conditions: temp = 60°; 2 mg total substrate used in each run, all solutions contained 0.09 mg/ml BSA, 1.4 mM $CaCl_2$, 35 mM NaCl, total volume = 7.15 ml; *p*H 8.5 (maintained with 2.0 mM NaOH); used 30–100 μl enzyme stock in each run. All numbers represent an average of 2–5 experiments (values are $\sim\pm$10–20%).

poly-L-lysine seemed to have any significant effect on k_t (NDNA). The effects of these additives on k_0 (NDNA), k_0 (DDNA), and k_i (DDNA) were also determined and listed in Table VI. In general the effects on these parameters are minor, though note that k_t (DDNA) is somewhat decreased by all these additives, and that large quantities (1.3 mg) of BSA seem to increase both k_0 (DDNA) and k_0 (NDNA) considerably. But the striking effect of these additives is clearly on k_t (NDNA).

Experiments with Mixtures of Native DNA, Denatured DNA, and Polyadenylic Acid.—In comparing the effects of the enzyme preparation on native and denatured DNA, we have implicitly assumed that the effects of a single enzyme are being observed. It is well known that preparations of nucleases may contain other enzymes capable of acting on DNA, and thus, in the present case, it was important to demonstrate directly that the same enzyme is involved in the attack on both native and denatured DNA. One approach to this question was taken by further purifying an aliquot of the enzyme stock by means of DEAE-cellulose chromatography, as described under Materials and Methods. A 5-fold increase in the specific activity of the enzyme was achieved, and a number of the critical experiments described in previous sections were repeated using this purified enzyme. In all cases the results obtained were not significantly different from those described above, demonstrating that if more than one enzyme were present in the stock no further fractionation was achieved by this purification step.

Further suggestive evidence comes from the *p*H-activity studies described in an earlier section, where it was shown that the ratio of activity toward denatured DNA to the activity toward native DNA was essentially the same at all *p*H values examined.

In addition, we have carried out a series of experiments in which the enzyme was permitted to act on both native and denatured DNA, these substrates being added to the *p*H-stat cell either simultaneously or sequentially. In all such experiments the *p*H-stat cell contained enough DNA to saturate all of the enzyme at all times. Qualitatively we found, as expected for a single enzyme, that when an aliquot of enzyme was permitted to act on native DNA and then denatured DNA was added subsequently, the rate of hydrolysis and the rate of thermal inactivation both increased abruptly. In the converse experiment (adding native DNA after the enzyme had started to operate on a solution of denatured DNA) we found that both the rate of hydrolysis and the rate of thermal inactivation decreased when native DNA was added. These findings suggested that one could quantitate the distribution of enzyme between the two substrates by obtaining values of k_0 and k_t with a system containing a mixture of native and denatured DNA. The results of such experiments, carried out in all cases in the presence of 0.65 mg of BSA (0.09 mg/ml in the *p*H-stat cell) are tabulated in Table VII. Clearly both k_0 and k_t for the mixed system are intermediate between the values of these constants for native and denatured DNA, in both cases falling slightly closer to the native DNA values. These experiments appear to demonstrate quite convincingly that the same enzyme operates on both substrates, and that in a competitive situation the enzyme is distributed between the substrates in a definite proportion.[4]

These results suggested to us that these same procedures might also be extended to polyribonucleotides, with two ends in view. First, it seemed that if the protection against thermal denaturation of the enzyme observed in the presence of native DNA were indeed due only to the native conformation, then k_i obtained with polyriboadenylic acid in the random-coil form should be closely similar to k_i (DDNA). Polyriboadenylic acid exists in the random-coil form at *p*H 8.5 under the ionic conditions used (e.g., see Steiner and Beers, 1961). We found that k_t (poly A) and k_i (DDNA) were indeed essentially identical at 40°, 50°, and 60° (Table VIII; these experiments were run without BSA). The data of Table VII show that k_t (poly A) and k_i (DDNA) measured in the presence of BSA at 60° are also relatively similar. At all temperatures, with or without added BSA, the initial rate (k_0) for the hydrolysis of polyriboadenylic acid seemed to be about one-half that for denatured DNA (see Table VII).

TABLE VIII

RATE OF INACTIVATION OF MICROCOCCAL NUCLEASE OPERATING ON DENATURED DNA AND POLYRIBOADENYLIC ACID[a]

Temperature (°C)	k_t (DDNA) (min^{-1})	k_t (Poly A) (min^{-1})
59.2	-33×10^{-3}	-27×10^{-3}
49.5	−13	−13
39.6	−5	−5

[a] Experimental conditions: 1.4 mM $CaCl_2$, 35 mM NaCl, *p*H 8.5 (maintained with 2.0 mM NaOH); total volume = 7.15 ml, 2 mg DDNA or poly A in cell.

Second, it seemed that enzymatic experiments using mixtures of polydeoxyribonucleotides and polyribonucleotides as substrate might serve to illuminate the question of whether the deoxyribonuclease and ribonuclease activities characteristic of micrococcal nuclease preparations are both due to the same enzyme (e.g., see Laskowski, 1961; Alexander *et al.*, 1961). The results of such experiments are tabulated in Table VII and show quite conclusively that intermediate values of k_0 and k_i are obtained with mixtures of polyadenylic acid and either native or denatured DNA, confirming the view that the activities of this preparation toward DNA and RNA both reside in the same enzyme molecule.

These findings were also confirmed qualitatively by experiments in which an aliquot of polyadenylic acid was added to the cell during the digestion of either NDNA or DDNA, with the expected effects on both

[4] For a similar analysis of the distribution of an enzyme between a native and a denatured substrate, see the discussion of the collagenase-collagen-gelatin system by Seifter *et al.* (1959).

TABLE IX

COMPOSITION OF THE PERCHLORIC ACID-SOLUBLE OLIGONUCLEOTIDES RELEASED DURING THE INITIAL PHASES OF DIGESTION OF NATIVE AND DENATURED DNA BY MICROCOCCAL NUCLEASE[a]

Material	OD_{260}/OD_{280}	$dA + T$ (%)
Total DNA (chemical analysis)[b]	—	58 ± 2
Total DNA[c]	1.405	55.4
Final digest	1.45 ± 0.04	58 ± 1
DDNA (avg: 2–15% digests)	1.48 ± 0.03	60 ± 1
NDNA (avg: 2–15% digests)	1.65 ± 0.06	72 ± 4

[a] Hydrolysis conditions: 60°, 1.4 mM $CaCl_2$, 35 mM NaCl, *p*H 8.5, 3 mg DNA, 200 μl enzyme stock (for NDNA runs) or 50 μl enzyme stock (for DDNA runs). Reaction stopped with EDTA. [b] From Chargaff (1955). [c] From Fredericq *et al.* (1961). Measured on denatured DNA in acetate buffer (see Materials and Methods).

the rate of hydrolysis and the rate of inactivation of the enzyme.[5]

Site of Initial Attack of Micrococcal Nuclease on DNA.—In view of the differences between the kinetics of hydrolysis of native and denatured DNA by micrococcal nuclease, we have attempted to determine whether native and denatured DNA are attacked by the enzyme at different loci with respect to local nucleotide composition. We have used the method of Fredericq *et al.* (1961) to estimate the composition of the perchloric acid–soluble oligonucleotides liberated during the early phases of digestion of both native and denatured DNA at 60° (for description of the method see Materials and Methods), and have found marked differences between products liberated from native and denatured DNA. In each run sequential aliquots were taken from the *p*H-stat cell after approximately 2, 4, 6, 8, 10, and 15% of the potentially susceptible internucleotide bonds had been hydrolyzed. Average results, derived from several runs of this type, are presented in Table IX and show that the oligonucleotides released from native DNA during the initial phases of digestion contain substantially more deoxyadenylic and thymidylic acid residues than those released from denatured DNA during the same portion of the reaction. In fact, the composition of the early digestion products derived from denatured DNA does not seem to differ much from the average composition of the DNA molecule as a whole (see Table IX).[6]

[5] In some of the experiments with denatured DNA or polyadenylic acid as substrate, a small fraction (~5%) of the total activity did not disappear with the same first-order rate constant which characterized the loss of most of the activity (e.g., Fig. 4), but seemed to be considerably more stable with respect to thermal denaturation. However, the presence of this amount of a still more heat-stable form of the enzyme should have essentially no effect on any of the measurements or conclusions presented here.

[6] It should be re-emphasized (see Materials and Methods) that the base ratio calculations made in Table IX are based on the assumption that $dA + dG = T + dC$ in the acid-soluble fragments liberated by the enzyme. If this is not the case, a minimal interpretation of the OD_{260}/OD_{280} ratio data of Table IX would suggest that the initial attack on denatured DNA releases acid-soluble oligonucleotides containing the various bases in a proportion that is very similar to that characteristic of the entire DNA molecule, while the oligonucleotides released initially from native DNA contain a substantial excess of *d*A. [The OD_{260}/OD_{280} ratios which apply to the individual residues at *p*H 3 (and *p*H 1) are: *d*A, 4.67; *d*G, 1.43; *d*C, 0.46; T, 1.35; calculated from Fredericq *et al.*, 1961.]

Preliminary experiments were also carried out with native DNA in the presence of bovine serum albumin, polylysine, and spermine to see whether these additives, which in some cases seem to help protect micrococcal nuclease against thermal denaturation in the presence of native DNA (see Table VII), might bring about an alteration in the composition of the oligonucleotides released during the early phases of enzymatic attack. So far the OD_{260}/OD_{280} ratios obtained in such experiments have not been significantly different from those obtained with native DNA alone.

In these runs the ratio of nucleotides released to internucleotide bonds broken was only about 2, suggesting that the acid-soluble oligonucleotides released by the enzyme in these experiments were largely dinucleotides.

DISCUSSION

The major experimental results of this study may be summarized as follows: (1) Under identical conditions, the micrococcal nuclease–catalyzed hydrolysis of native DNA proceeds much more slowly than that of denatured DNA (as previously shown by Dirksen and Dekker, 1960). (2) The site of initial attack of the enzyme depends on the conformation of the substrate; at 60° denatured DNA appears to be attacked essentially at random, while the initial attack on native DNA occurs in regions rich in deoxyadenylic and thymidylic acid residues. (3) The rate of attack of the enzyme can be markedly altered (in quite different ways for native and denatured DNA) by changes in the concentrations of NaCl and $CaCl_2$. (4) Under certain salt and temperature conditions the enzyme is partially protected against thermal denaturation when *native* DNA serves as substrate. (5) This "protective effect" of the native DNA structure may be markedly enhanced by the addition of small quantities of certain macromolecular additives. A careful consideration of the details of these phenomena seems capable of providing some insight into both the mechanism of action of this nuclease and the effect of various changes in the solvent environment on the detailed conformation of the DNA molecule.

It is noteworthy that, despite the considerable difference between the initial rate of attack of the enzyme on denatured and native DNA at any particular temperature, the energies (and enthalpies) of activation for this process are essentially the same for both substrates over the entire 30–60° temperature range ($\Delta H^{\ddagger} \simeq$ 14 kcal/mole) and very similar to values generally obtained for simple chemical reactions or for the hydrolysis of random-coil proteins and polypeptides by proteolytic enzymes. This finding contrasts markedly with the results of a similar study of the collagenase-collagen-gelatin system (von Hippel and Harrington, 1959; von Hippel *et al.*, 1960) where the large values of $\Delta H^{\ddagger}$ (and $\Delta S^{\ddagger}$) associated with the hydrolysis of the native collagen structure suggested that the rate-limiting step was the distortion (or "opening") of the structure to permit the formation of an active enzyme-substrate complex. Thus the present results suggest that the rate-limiting step for the hydrolysis of both native and denatured DNA by micrococcal nuclease is the actual cleavage of the internucleotide bond, or some adsorption or desorption step which does not involve an enzyme-induced distortion of the native DNA structure.

The results of the experiments on the composition of the regions initially attacked by the enzyme in native and in denatured DNA suggest that at 60° the denatured structure is attacked essentially at random, while

the enzyme attacks the native molecule in regions rich in deoxyadenylic and thymidylic acid residues.[7] This doubtless follows from the fact that such regions are less extensively stabilized than the rest of the molecule, as indicated by their lower melting temperature (Geiduschek, 1962; Felsenfeld and Sandeen, 1962).

It is also interesting to consider certain quantitative aspects of the protection against thermal inactivation afforded the enzyme by the native DNA structure. Complete "self-protection" (see below) is achieved with 60–100 μl of nuclease stock (Table I). In terms of protein content this corresponds to less than one enzyme molecule (estimated molecular weight = 12,000; Alexander *et al.*, 1961) for every 10,000 nucleotide pairs. And 0.13 mg of BSA, the amount required to achieve complete protection of the enzyme at 60° in the presence of native DNA (Table VI) corresponds to about one BSA molecule for every 2000 nucleotide pairs under the conditions of our experiments. Since the radius of a BSA molecule is about 25 A, and the enzyme molecule is even smaller, it is difficult to conceive any explanation of the protective effect which does not involve either a very nonrandom binding of enzyme and BSA to the nucleic acid (with the enzyme and albumin molecules attached to native DNA close to each other in a way that somehow stabilizes the enzyme), or a "long-range" modification of the native DNA structure, which leads to protection of the enzyme as a "long-range" consequence of the interaction of BSA with the DNA. Both these types of mechanisms involve cooperative action, and it is not possible to distinguish between them at present.

The nonrandom binding hypothesis would involve some type of specific protective three-component complex of native DNA, nuclease, and added protein. Possible long-range mechanisms might arise from the dynamic state of the DNA structure. It appears likely that the double helix is normally subject to waves of opening and closing ("breathing") which result in the continuous fluctuation of a given base pair between an "open" and a "closed" hydrogen-bonded configuration. Such "breathing" can account for example for the anomalously rapid exchange of the hydrogens involved in interchain hydrogen-bonding in DNA (e.g., see Bradbury *et al.*, 1961). The binding of an enzyme or protein molecule to the double helix might modify this behavior, and thus make its effect felt at a point many nucleotide pairs removed from the binding site. Localized fluctuations of base-pairs between an open and a closed state might also be crucially involved in controlling the accessibility of the native structure to the nuclease and, in a similar way, might play a role in making native DNA accessible to the various polymerases during DNA replication and messenger-RNA synthesis (e.g., see Bollum, 1963).

The actual nature of the protection provided the enzyme by the native DNA structure is not clear, though it seems reasonable to interpret our results by assuming that the enzyme is at least partially protected against thermal inactivation when it is bound to native DNA in some specific way. The studies with mixed substrates summarized in Tables VII and VIII support this view, and suggest that native DNA is somewhat more effective than denatured DNA in competition for the enzyme. Particularly in terms of k_i, the values obtained for the mixed systems lie considerably closer to the value expected with native DNA alone than to those usually found with either denatured DNA or polyadenylic acid as the sole substrate (see Table VII). This suggests that in these mixed systems a major portion of the available enzyme is involved in a "protected" association with native DNA.

The effect on the micrococcal nuclease–DNA system of adding protein or other macromolecules is of two distinct types which should be clearly differentiated. (1) Very small quantities of these materials (0.02–0.05 mg) have no effect on the system operating with denatured DNA as substrate, but bring very small amounts of enzyme ($\sim$30 μl) working on native DNA into the "fully self-protected" state.[8] Almost all macromolecular additives tested (e.g., BSA, ribonuclease, deoxyribonuclease II, lysozyme, and polylysine, as well as additional micrococcal nuclease itself) were effective in this sense. (2) Larger amounts (> 0.05 mg) of certain of these additives produce an additional effect, increasing the observed "protection" well past the "fully self-protected" level and bringing k_i (NDNA) to values close to zero (see Table VI). This phenomenon turned out to be more demanding in terms of the macromolecule used; among those tested only BSA, ribonuclease, and polylysine were found effective.[9]

The mechanisms whereby these additives bring about their effects are also not established, though as a first approximation one might speculate that (at low ionic strength) areas of the macromolecules which are positively charged at *p*H 8.5 might interact with the negatively charged backbone phosphates of native DNA to form specific DNA-protein (or DNA-polypeptide) complexes. Specific complexes at low ionic strength have been observed between DNA and compounds such as spermine (Tabor, 1962; Mandel, 1962), protamine (Alexander, 1953), α-chymotrypsin (Hofstee, 1962), bovine serum albumin (Zubay and Doty, 1957), and ribonuclease (Hofstee, 1962). We may also note that DNA polymerase is almost totally inhibited at univalent salt concentrations greater than 0.1 M, perhaps because these salt concentrations impair the ability of the enzyme to react with the "primer" DNA (Bollum, 1963). We are currently engaged in optical melting point dispersion studies, using an improved version of the method of Felsenfeld and Sandeen (1962) to study the effect of the addition of various of these macromolecules (and electrolytes, e.g., NaCl and $CaCl_2$) on the melting temperatures of specific sections of the native DNA molecule. So far we have found that binding of native ribonuclease to DNA at low ionic strength markedly *destabilizes* the "*d*A-T"-rich regions of the native DNA molecule (Felsenfeld *et al.*, 1963).

The thermal inactivation of micrococcal nuclease is somewhat unusual, in that linear Arrhenius plots are obtained for the inactivation rate constants with both native and denatured DNA as substrate over the entire temperature range examined (Fig. 5). The magnitude of the activation parameters (Table II) suggests that a relatively simple alteration in the structure of the

[7] It should be pointed out, however, that since a heterogeneous mammalian DNA was used in these experiments, one cannot discriminate on the basis of these experiments between initial selective hydrolysis of particular "*d*A-T" rich zones within each DNA molecule, and initial attack on selected whole molecules which are particularly rich in "*d*A-T" pairs.

[8] We define "full self-protection" as used here as the reduction of k_i to the plateau value reached with relatively large amounts of enzyme ($\sim 15 \times 10^{-3}$ min^{-1}).

[9] It should be noted that the DNA itself contains some protein "contaminants." The concentration of these contaminants varied (from one DNA preparation to another) between 4 and 20% (w/w) of the BSA concentration required to obtain maximum protection. Yet, within the limits of error of the experiments, variation of the contaminant protein concentration over this range had little effect on the protective behavior of the DNA structure.

enzyme molecule must constitute the rate-limiting step in the inactivation. A highly cooperative event, involving the rupture of a number of bonds, is ruled out by the linearity of the Arrhenius plot over at least the 30° temperature interval investigated.

In conclusion, it seems that considerable insight into certain aspects of local nucleic acid conformation can be obtained by using enzymes as structural probes, once the mechanism of action of the particular nuclease involved is fairly well understood. Also, one might utilize specific properties of particular nucleases to bring about controlled alterations in the structure of nucleic acids. For example, by taking advantage of the unusual heat stability of micrococcal nuclease and the considerable difference in the rate with which it attacks native and denatured DNA, one might use it to attack partially melted DNA (at temperatures within the helix $\rightleftarrows$ coil transition region) and thus break the molecule into fairly specific pieces by cleaving through partially melted "*d*A-T"-rich regions.

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