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The Effect of Electrolytes on the Stability of the Deoxyribonucleate Helix[1]

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The stability of DNA secondary structure in aqueous solutions of 1:1 electrolytes has been studied. It has been found that concentrated solutions of several of these salts are powerful denaturing agents, lowering the thermal denaturing temperature at neutral *p*H by as much as 60°. The effect is due mainly to the anions: CCl_3COO^-, CF_3COO^-, CNS^- and ClO_4^- are particularly potent denaturing ions at high salt concentration, and at a concentration of 4 *M*, denaturing power increases in the order CHO_2^-, Cl^-, $Br^- < CH_3COO^- < I^- < ClO_4^- < CF_3COO^- < CNS^- < CCl_3COO^-$. On the other hand, $(CH_3)_4NCl$ does not denature DNA even at a concentration of 15.7 molal. When dependence of thermal denaturation on salt concentration, *p*H, DNA composition and molecular weight is studied in $NaClO_4$ solutions, it is found that (1) DNA is optimally stable at neutral *p*H, (2) the dependence of thermal stability of DNA on base composition is considerably greater in concentrated $NaClO_4$ (7.2 *M*) than in buffered 0.15 *M* NaCl, (3) in concentrated $NaClO_4$ thermal transitions are broadened even in very homogeneous viral DNA preparations, and the rapid reversibility of partial denaturation can be strikingly demonstrated. On the basis of these experiments two proposals are put forward and discussed. They are: (1) that the thermal helix ⇄ coil transition *in each molecular species* of a DNA preparation can be made to occur over a relatively wide range of temperatures, and that this is due, in part, to intramolecular heterogeneity of nucleotide composition; (2) that this class of denaturing agents are "hydrophobic bond" breakers and that they act in this manner, at least in part, by virtue of their effect on the structure of water.

The study of protein and nucleic acid denaturing agents derives its impetus from two directions. On the one hand these are useful reagents that permit the manipulation of chemical and biological reactivity in a general or specific, reversible or irreversible manner depending on the nature of the system. On the other hand, secondary and tertiary structure stabilities have been classified in terms of hydrogen-bond and other origins according to the effectiveness of various denaturing agents such as urea, lithium bromide, guanidinium hydrochloride and formamide, to name but a few. It is our purpose to report on our study of a class of denaturing agents whose action on nucleic acids has received relatively little attention.[2] This communication deals with the effect of concentrated 1:1 electrolyte solutions on DNA secondary structure stability. A number of salts have been surveyed; in one or more of these, the dependence of secondary structure stability on ionic concentration, *p*H, DNA composition and molecular weight also has been determined, together with some details of the transition such as its breadth and reversibility.

(1) Research supported by a grant of the United States Public Health Service (C-5007).

(2) (a) C. F. Emmanuel, *Biochim. et Biophys. Acta*, **42**, 60 (1960); (b) K. Hamaguchi and E. P. Geiduschek, *Abstr. Biophys. Soc.*, Feb. 1961, St. Louis, FB10.

I. Experimental

A. DNA.—Salmon testis and sea urchin (*Strongylocentrotus drøbachiensis*) testis DNA were prepared by the method of Simmons.[3]

DNA from bacteriophage T2 (kindly given to us by L. Kozloff) was purified according to Mandell and Hershey.[4] One sample of T2 DNA, prepared by this procedure, was subsequently precipitated twice from 0.3 *M* sodium acetate, 0.01 *M* Versene, with ethanol.

DNA from *Ps. fluorescens*, *E. coli*, *S. marcessens* and *Ae. aerogenes* was isolated by a method combining aspects of the DNA isolation procedures of N. Simmons[5] and A. S. Jones,[6] which may be summarized as: Bacteria were lysed in sodium xylene sulfonate–deoxycholate–Versene, after which proteins were precipitated in the cold at *p*H 4.2 and removed by centrifugation. After reneutralization, nucleic acids and contaminants were precipitated with isopropyl alcohol. There followed a further deproteinization with sodium xylene sulfonate and two more alcohol precipitations of nucleic acids. This crude material was then redissolved and DNA fractionally precipitated as its cetyltrimethylammonium salt. Finally, several cycles of solution and alcohol precipitation yielded white fibrous preparations of DNA, which were stored at 4–6°. All preparations of DNA from the above four sources followed this outline, with minor variations of procedure. As an example, a detailed description of the preparation of DNA from *Ps. fluorescens* is given:

(3) N. S. Simmons, as quoted by V. L. Stevens and E. L. Duggan, *J. Am. Chem. Soc.*, **79**, 5703 (1957).

(4) J. D. Mandell and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960).

(5) N. Simmons, unpublished work (1956).

(6) A. S. Jones, *Biochim. et Biophys. Acta*, **10**, 607 (1953).

50 g. of packed cells is washed twice with 0.1 *M* NaCl, 0.05 *M* Versene, *p*H 7. They are then lysed in 100 ml. of 27% sodium xylene sulfonate, 4.5% sodium deoxycholate, 0.01 *M* Versene, *p*H 7.5, agitated in a Waring Blendor for 45 sec. and left at room temperature for 30 minutes; 300 ml. of iced 0.05 *M* Versene, 0.05 *M* NaCl is then added, and the gelatinous suspension is kept at 4–6° for 6 hr. Acidification to *p*H 4.2 in ice, with rapid stirring, causes a heavy precipitate to appear. This is removed by centrifuging and discarded. The supernatant is reneutralized to *p*H 7, iced and an equal volume of iced isopropyl alcohol added. The massive, fibrous precipitate is collected on a stirrer, washed with cold ethanol and redissolved in 100 ml. of 0.01 *M* Versene. 150 ml. of 40% (w./v.) sodium xylene sulfonate in 0.01 *M* Versene, *p*H 7.5, is then added at room temperature. The subsequent addition of 750 ml. of cold 0.01 *M* Versene causes the appearance of a marked turbidity. After standing, in the cold, for 8 hr., this suspension is centrifuged at 15000 g. for 10 hr. and the sediment is discarded. An equal volume of isopropyl alcohol (at 0°) is added to the clear supernatant, the resulting precipitate is washed with ethyl alcohol and redissolved in 120 ml. of 0.01 *M* Versene, *p*H 7. Sodium acetate is added to 0.3 *M*, with subsequent precipitation at 0° with an equal volume of ethyl alcohol. The fibrous precipitate is washed with iced ethyl alcohol and acetone and air dried in the cold; yield, 625 mg. of mixed nucleic acids, contaminated with polysaccharides.

From this material, DNA is purified by fractional precipitation at 25° as the cetyltrimethylammonium salt. The entire preparation of crude nucleic acids is dissolved in 150 ml. of 0.01 *M* Versene, NaCl (to 1.0 *M*) and 4.38 g. of cetyltrimethylammonium bromide (CTAB) added with stirring. The solution is maintained at 25°, while water is added slowly, with rapid stirring.[7] As the NaCl concentration approaches 0.60–0.65 *M*, a heavy precipitate containing DNA and CTAB appears. The NaCl concentration is adjusted to 0.6 *M* and the precipitated CTAB–DNA centrifuged off. This material is now redissolved in 125 ml. of 3 *M* sodium acetate, *p*H 7. DNA is precipitated with an equal volume of ethyl alcohol and washed in 90% alcohol. It is then redissolved in 100 ml. of 1 *M* Na acetate, 0.01 *M* Versene, *p*H 7.5 and reprecipitated with ethanol, then with two subsequent ethanol precipitations from 0.3 *M* NaAc. Finally the DNA fibers are washed in ice-cold 95% ethanol and acetone, pressed out between sheets of filter paper and dried on a water aspirator; yield, 250 mg.; analysis, 16.00% N, 9.42% P (basis dry weight), ϵ (P) = 6800 cm.^{2}/mole P at 259 mμ, RNA less than 0.5%. For the determination of ϵ(P), phosphorus was determined colorimetrically by Bartlett's method.[8] RNA content was estimated by the method of Webb.[9]

In addition, we wish to express our thanks for the following gifts of DNA: *M. lysodeikticus* DNA prepared, according to a procedure described by Marmur,[10] by W. F. Dove; Bacteriophage T7 DNA prepared, according to the method of Mandell and Hershey,[4] by D. M. Freifelder; and *D. pneumoniae* DNA prepared by L. Cavalieri according to his method.[11]

Sonically degraded samples of salmon DNA were prepared as follows: 0.1% DNA solutions in 10^{-4} *M* S,2-aminoethyl isothiouronium bromide hydrobromide (AET), 0.01 *M* NaCl, were freed of dissolved oxygen by several cycles of evacuation and flushing with pyrogallol washed N_2. They were irradiated for varying periods of time in a Raytheon 150-watt 9-kc. sonic oscillator. DNA then was precipitated with ethanol, rinsed in ethanol and acetone and dried.

B. Other Materials.—Salts available as analytical grade reagents were used without further purification. Sodium trichloroacetate and trifluoroacetate were made by neutralizing the corresponding acids (analytical grade and Eastman Kodak b.p. 79–81°, respectively). KI solutions were decolorized with charcoal and kept under N_2. Tetramethylammonium chloride (Eastman Kodak) was used without further purification.

Unless otherwise noted, stock solutions of salts were made with 0.1 *M* Versene buffer. Most of the *p*H adjustments were made with standard calomel and glass electrodes.[12] More recently we have used a thermostated calomel electrode with an additional 0.1 *M* NaCl-filled salt bridge for *p*H measurements on solutions of anions whose potassium salts have limited solubility. Concentrated salt solutions were filtered through sintered glass filters, and DNA stock solutions were clarified by high speed centrifugation.

C. Methods.—Viscosities were measured in 4-bulb multigradient suspended level capillary viscosimeters modeled after the design of Schneider.[13] These had 160 cm. capillaries of 0.4–0.5 mm. radius and covered a range of maximum shear gradients from 30 to 200 sec.$^{-1}$ in water at 25°.

Optical rotations were measured in a Rudolph 80S photoelectric polarimeter equipped with a thermostated cell compartment. The 4358 Å. line of the Hg spectrum was selected with interference and auxiliary filters.

Temperature-dependent absorbance measurements were made in Beckman DU spectrophotometers equipped with dual thermospacers. Unless otherwise noted, absorbances (A) are not corrected for thermal expansion of solvent.

For measurements on the irreversible component of denaturation, solutions were prepared by equilibrating 20–60 minutes at a selected temperature and rapidly cooled (quenched) in ice. They were then equilibrated at 25° for the appropriate measurement. For absorbance measurements, this process was repeated several times on a single solution at successively higher temperatures; a considerable economy of materials could be achieved in this way. On a number of occasions we assured ourselves that the denaturation and irreversibility of solutions so handled were not dependent on prior thermal history.

Solutions were prepared in such a way as to eliminate the possibility of denaturation during mixing: (a) DNA was dissolved, at 0–5°, in 0.01 *M* NaCl; (b) when preparing DNA solutions containing high concentrations of electrolytes, the latter were added dropwise with stirring to DNA stock solutions maintained at 0–5°.

D. Presentation of Data.—Three types of measurement—viscosity, optical rotation at 436 mμ ($[\alpha]_{436}$) and absorbance at 259 mμ (A_{259})—have been applied to two different classes of thermal denaturation experiments: (a) measurements of denaturation at the maximum or ambient temperature (which we designate as the "d-assay"), and (b) measurements of the irreversible changes that have resulted when solutions have been equilibrated at a given elevated temperature, quenched and reequilibrated at 25° (which we designate as the "i-assay"). The resulting thermal denaturation profiles are shown, for example, in Figs. 2, 3 and 8.

We define f_t, the *transition fraction* in thermal denaturation, as

$$f_t = \frac{q_T - q_O}{\Delta q_{max}}$$

where q is a property (such as $[\eta]$, $[\alpha]_{436}$ or A_{259}) of DNA, the subscripts T and O referring to an experiment at an elevated temperature and a reference temperature at which q is temperature independent and $f_t = 0$, respectively. The quantity f_t depends at a given temperature on the type of denaturation experiment and is, accordingly, further designated as f_{td} or f_{ti}.[14] The temperature at which $f_t = 1/2$ is designated at $T_{1/2,d}$ or $T_{1/2,i}$ corresponding to the d- or i-assay, respectively. $T_{1/2,d}$ has been called the "denaturation" or "melting" temperature by others and denoted by T_m.[16,17] The temperature range over which f_{td} changes

(7) It would be preferable to substitute 0.01 *M* NaCl for water at this step.

(8) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).

(9) J. M. Webb, *ibid.*, **213**, 107 (1955).

(10) J. Marmur, *J. Molec. Biol.*, **3**, 208 (1961).

(11) L. Cavalieri, *Biophys. J.*, **1**, 301 (1961).

(12) All *p*H values cited in this paper refer to measurements made at 25°. The *p*H at the denaturation temperature will of course be somewhat different depending, in this respect, on the heat of ionization of the buffer acid. Slightly different values of $T_{1/2}$ are therefore to be expected from experiments in which buffers with different ionization heats are used.

(13) N. Schneider, Thesis, Harvard University, 1953.

(14) The question of the relation of f_{td} to the average fraction of disordered nucleotide pairs in a DNA sample is explored in another communication.[15]

(15) E. P. Geiduschek, manuscript in preparation (1961).

(16) J. Marmur and P. Doty, *Nature*, **188**, 1427 (1959).

(17) H. R. Mahler, B. D. Mehrota and C. N. Sharp, *Biochem. Biophys. Res. Comm.*, **4**, 79 (1961).

from 0.25 to 0.75 (*i.e.*, the central half of the transition) is called the transition half-width and denoted by δ.

II. Results

A. A Survey of the Effect of 1:1 Salts on the Stability of the DNA Helix.—The thermal denaturation of sea urchin DNA (strongylocentrotus, 37 mole % GC) in many 1:1 salts at 4 *M* concentration, *p*H 7, has been followed viscometrically. Viscosity measurements (mostly at a single concentration in the range of 5 to 11 × 10^{-2} mg./ml.) were made on solutions heated 60 minutes at the indicated temperature and plunged into ice. The reduced specific viscosity of sea urchin DNA solutions, prepared in this manner showed no time dependence. Figure 1 illustrates the results of a number of these experiments. The midpoints of such irreversible thermal denaturation curves, $T_{1/2,i}$, are listed in Table I for all the salts which thus far have been investigated. At this concentration there are only minor differences in effect of the cations Li^+, Na^+ and K^+ on helix stability. (Note, for instance, the slightly lower $T_{1/2,i}$ of $LiClO_4$ relative to $NaClO_4$.) Among the anions, on the other hand, great variations of denaturing power are observed.

We also found that 15.8 molal $(CH_3)_4NCl$ does not act as a denaturing agent. For salmon DNA, $T_{1/2,d}$, is 85° at *p*H 7, compared with 86° in 0.15 *M* NaCl. In other words, the tetramethylammonium ion is no better a denaturant than the alkali metal ions.

From the data of Table I, we may construct a "chaotropic"[18] series for anions (at a salt concentration of 4 *M*)

$$CCl_3COO^- \gg CNS^- > CF_3COO^- > ClO_4^- > I^- > CH_3COO^- > Br^-, Cl^-, CHO_2^-$$

The properties of those salts which act as strong denaturing agents—$NaCCl_3COO$, $NaCF_3COO$, $NaClO_4$ and KCNS—have been investigated more fully. Before describing these experiments, we digress to establish the *p*H of maximum thermal stability and to consider the relation of the d- and i-assays to each other.

TABLE I

DENATURATION OF DNA (SEA URCHIN, 37% GC) IN 4 *M* AQUEOUS SALT SOLUTION, 0.01 *M* VERSENE, *p*H 7

	$T_{1/2,i}$[a]		
	Li^+	Na^+	K^+
CNS^-	..	68	76
I^-	78.8	76.4	81.8
Br^-	89.5	89.1	91.1
Cl^-	91.6	90	..
ClO_4^-	67.4	74.0	..
Acetate	..	83.5	86.7
Formate	..	92.6	89.6
CCl_3COO^-	..	(<25)[b]	..
CF_3COO^-	..	(63)[b]	..

[a] From viscosity measurements on quenched solutions. [b] $T_{1/2,d}$; from optical rotation and ultraviolet absorbance measurements at the ambient temperature.

B. The *p*H of Maximum Stability.—The thermal stability of DNA has a relatively broad maximum at *p*H 7–8 both in 0.1 *M* NaCl and 7.2 *M* $NaClO_4$. As we show in Table II, the *p*H of maximum stability is somewhat lower in the latter medium. In 0.1 *M* NaCl, viscosity measurements of heated and quenched solutions were used to estimate $T_{1/2,i}$, whereas denaturation in 7.2 *M* $NaClO_4$ was measured at the ambient temperature (A_{259}) yielding $T_{1/2,d}$.

(18) Tending to disorder.

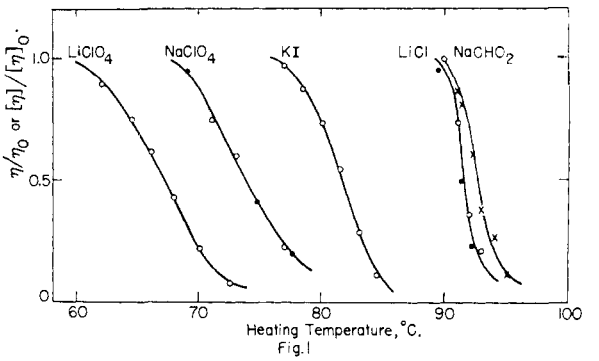

Fig. 1.—Thermal denaturation in 4 *M* $LiClO_4$, $NaClO_4$, KI, LiCl and Na formate, Versene, *p*H 7. Viscosity (at 25°) of solutions heated to stated temperature for 30–60 minutes and quenched, relative to viscosity of unheated control: O, X: measurements at a single DNA concentration (c = 5 to 11 × 10^{-2} mg./ml.); data plotted as ratios of reduced specific viscosities, η/η_0. ●: extrapolated to zero DNA concentration; data plotted as ratios of intrinsic viscosities, $[\eta]/[\eta]_0$. DNA: sea urchin (37 mole % GC).

TABLE II

DEPENDENCE OF $T_{1/2}$ ON *p*H

Sea urchin DNA (37% GC) in 0.1 *M* NaCl, 0.01 *M* Versene			Salmon DNA (43% GC) in 7.2 *M* $NaClO_4$ 0.05 *M* Versene	
*p*H	$[\eta]$ of DNA heated to 89.9° and quenched[a]	$T_{1/2,i}$, °C.	*p*H	$T_{1/2,d}$,[d] °C.
6.25	11.0	87[b]	6.26	43.9
7.1	35	89.9[c]	7.05	44.3
8.1	49	90[b]	7.80	44.0
9.1	16.3	88[b]	8.67	42.3
			9.35	35.7

[a] Solutions heated 60 minutes at 89.9° and quenched (89.9° is the $T_{1/2,i}$ of sea urchin DNA at *p*H 7.1). [b] Estimated from the experiments shown in column 2. [c] Determined by measuring the temperature dependence of intrinsic viscosity, $[\eta]$, at 25°, of samples which have been heated for 60 minutes and quenched. [d] Determined by absorbance measurements at the ambient temperature.

C. Measurement of the "Transition Fractions" f_{td} and f_{ti} by Means of Viscosity, Optical Rotation and Absorbance.—For d-assay experiments (measurement at the ambient temperature) we have made measurements of specific rotation and absorbance. That these yield consistent values of f_{td} is strikingly shown in Fig. 2.

For i-assay experiments, absorbance or specific rotation measurements generally yield somewhat lower values of f_{ti}, than those calculated from viscosity data. Nevertheless, differences between $T_{1/2,i}$ estimated from the various measurements, are not large (Fig. 2), while on the other hand, $T_{1/2,i}$ is considerably higher than $T_{1/2,d}$, as illustrated in Figs. 2 and 8. It is especially interesting to note that no *irreversible* change in A (or $[\alpha]$) can be detected until denaturation at the ambient temperature, for the entire sample, is well advanced. This relationship between the

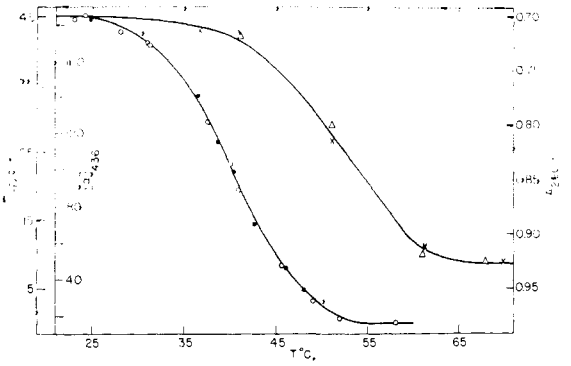

Fig. 2.—Thermal denaturation of sea urchin DNA (37 mole % GC) in 7.2 M $NaClO_4$, 0.1 M Versene, pH 7: ●, absorbance at 260 mμ (A_{260}), measured at T (d-assay); O specific rotation, at 436 mμ, ($[\alpha]_{436}$) measured at T (d-assay); X $[\alpha]_{436}$ measured at 25° on solution equilibrated at T and quenched (i-assay); Δ reduced specific viscosity, ηsp./c., measured on solutions equilibrated at T and quenched (i-assay); DNA concentration 5 to 11 × 10^{-2} mg./ml.

two types of denaturation experiment can be represented conveniently on an "irreversibility plot" (Fig. 3), in which f_{td} is plotted against f_{ti}.

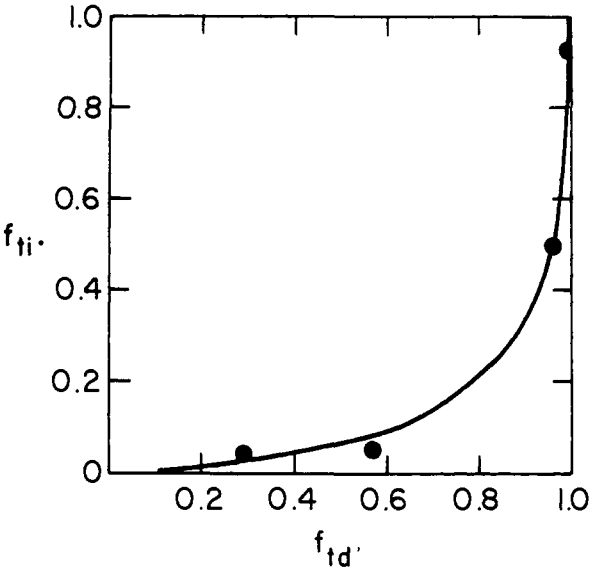

Fig. 3.—"Irreversibility plot" of sea urchin DNA denaturation in 7.2 M $NaClO_4$: f_{td}, transition fraction measured at T (d-assay); f_{ti}, transition fraction measured at 25° after heating to the temperature T, which yields the corresponding value of f_{td}, and quenching (i-assay). See section I D for a definition of f_{ti} and f_{td}. Data taken from Fig. 2.

D. Dependence of Thermal Stability on Electrolyte Concentration.—The variation of $T_{1/2,d}$ with concentration of $NaClO_4$, $NaCF_3COO$, KCNS is shown in Fig. 4 for sea urchin DNA. While the variation of $T_{1/2}$ with salt concentration is approximately linear at higher molarities of salt, a maximum in $T_{1/2,d}$ is observed in the range of 0.1–1.0 M salt. At salt concentrations below 0.1 M the thermal stability decreases once more, an effect attributed to electrostatic repulsions between imperfectly shielded phosphate groups.

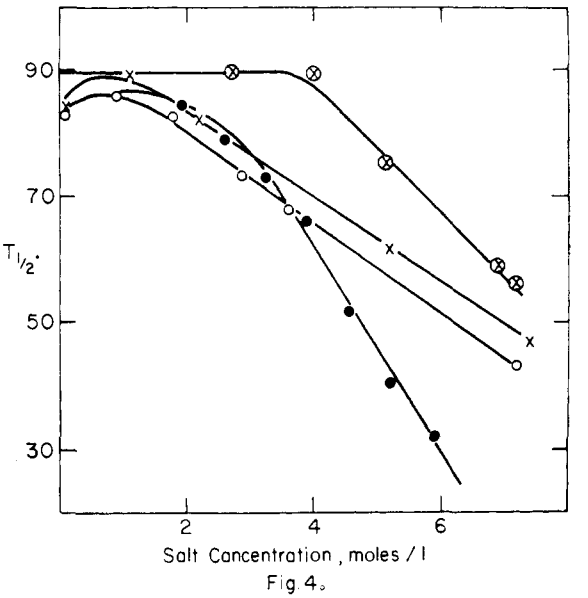

Fig. 4.—Variation of denaturation temperature with salt concentration. Sea urchin DNA (37 mole % GC), Versene buffer, pH 7. ●, $NaCF_3COO$, $T_{1/2,d}$, $[\alpha]_{436}$, d-assay; O, $NaClO_4$, $T_{1/2,d}$, $[\alpha]_{436}$, d-assay; X, KCNS, $T_{1/2,d}$, $[\alpha]_{436}$, d-assay; ⊗, LiBr, $T_{1/2,i}$, $[\eta]$ measured at 25°, after having been heated at T and quenched, i-assay.

Less extensive data on $T_{1/2,i}$ in LiBr are also included, and show that, in this case, no substantial denaturing effect is encountered at salt concentrations below 4 M.[19]

The statistical mechanical problem of the influence of ligand interactions on helix-coil transitions has been solved recently for homopolymers.[20] Predictions, based on a simple model which considers the interaction of single solute molecules with sites participating in helix stabilization (*e.g.* a peptide — C—O, or a purine ring), are that $T_{1/2,d}$ should vary linearly with the concentration of the *chaotropic* agent.[21]

The data of Fig. 4 do show a linear variation of $T_{1/2}$ with salt concentration at higher concentration. Were the strong deviations from this linearity at lower concentration attributable entirely to electrostatic destabilization, then extrapolation of the linear portions to zero concentration should yield a common intercept, which would be interpreted as $T_{1/2,d}$ of DNA at infinite ionic strength. However, no such common intercept exists, and we conclude that the particular statistical model which has been developed thus far is not applicable to our experimental system. In section IIIC, we argue that at least part of the decrease in $T_{1/2,d}$ must be due to changes in the structure of the solvent.

(19) The behavior of DNA in concentrated LiBr has previously been investigated by Emmanuel.[2] Our results for this solvent do not parallel that prior study. We find that $[\eta]$ decreases as the salt concentration is raised but do not detect the "transition" in $[\eta]$ previously described. Thus, our viscosity data, though not in agreement with the previously reported viscosities, are consistent with the previously reported absorbance data. We find that, in 6.8 M LiBr, the intrinsic viscosity of DNA is not affected by 30 minutes' heating to 45°, *i.e.*, DNA in 6.8 M LiBr at 25° is not partly "heat" denatured.

(20) R. F. Steiner and R. F. Beers, "Polynucleotides," Elsevier, Amsterdam, 1961, Ch. 9.

(21) L. Peller, *J. Phys. Chem.*, **63**, 1199 (1959).

Figure 5 shows the variation of specific rotation $[\alpha]_{436}$, and reduced specific viscosity with $NaCCl_3COO$ concentration at 25°, *p*H 7. An abrupt, irreversible decrease of $[\alpha]$ occurs at approximately 3.2 *M*. The optical rotation actually changes sign and denatured DNA has $[\alpha]_{436}$ −45° in 3.9 *M* $NaCCl_3COO$. Thus sodium trichloroacetate is the strongest denaturing agent of its type which has been found thus far. One question arising from this experiment concerns the origin of changes of $[\alpha]$ occurring between 0 and 2 *M* $NaCCl_3COO$. These could be due to (1) partial helix-coil transition, (2) asymmetric binding of $NaCCl_3COO$ (and water) to helical DNA or (3) changes in the geometry of the DNA helix. The same problem arises in interpreting our observations on DNA in $NaClO_4$ and KCNS. We have found that $[\alpha]_{436}$ decreases as the concentration of either salt is raised[22] (Fig. 6). The intrinsic viscosity of sea urchin DNA at 25°, *p*H 7, also decreases in $NaClO_4$: it is 55 dl./g. in 0.5 *M* NaCl, 43 dl./g. in 3.6 *M* $NaClO_4$ and 33 dl./g. in 7.2 *M* $NaClO_4$. However, the extinction coefficient of sea urchin DNA at 25° is only 2% higher in 7.2 *M* $NaClO_4$ than in 0.1 *M* NaCl.

If these changes were due to partial helix-coil transition then this criterion would be satisfied: the helix-coil transition is highly coöperative in all these solvents (Figs. 1, 2); for a set of values of the variables of state (*p*H, temperature, salt concentration) corresponding to partial denaturation, the configuration of DNA will, in general, be sensitive to *all* changes of the environment. On the contrary, we find that $[\alpha]$ and $[\eta]$ in 4.1 *M* KCNS, for example, are almost independent of temperature between 25° and 55° (actually a slight *increase* in $[\alpha]$ is observed). In 7.2 *M* $NaClO_4$ $[\alpha]$ and A_{260} do not change between 20 and 25°. The decrease of $[\alpha]$ and $[\eta]$ with increasing $NaClO_4$ and KCNS concentration (Fig. 6) must, therefore, be due to a process quite different from that which occurs in the thermal helix-coil transition. By analogy the same argument applies to DNA in 0–2 *M* $NaCCl_3COO$.

The fact that both rotatory power and viscosity decrease substantially rules out the possibility that the viscosity changes are entirely due to disaggregation or to the flexing of small, partly disordered sections of DNA molecules. Rather, the viscosity decrease, which reflects increased flexibility of the DNA helix, must arise from alterations of secondary structure, solvent packing or both of these. Such changes would contribute to changes of $[\alpha]$, as would the preferential asymmetric binding of one or the other solvent component. It is noteworthy that these changes are not coöperative.

E. Dependence of Thermal Stability on DNA Composition and Sharpness of Transition.—The variation of $T_{1/2,d}$ with the average nucleotide composition of DNA is shown for 7.2 *M* $NaClO_4$ and 6.5 *M* $NaCF_3COO$ in Fig. 7. Excluding T2 DNA, the average deviation of $T_{1/2,d}$ for 10 DNA's in 7.2 *M* $NaClO_4$ is only ± 0.4°. On the other hand, the stability of both the preparations of T2 DNA examined is abnormally low. This relatively low stability of secondary structure in concentrated $NaClO_4$ is evidently due to the presence of glucosylated hydroxymethylcytosine in place of cytosine

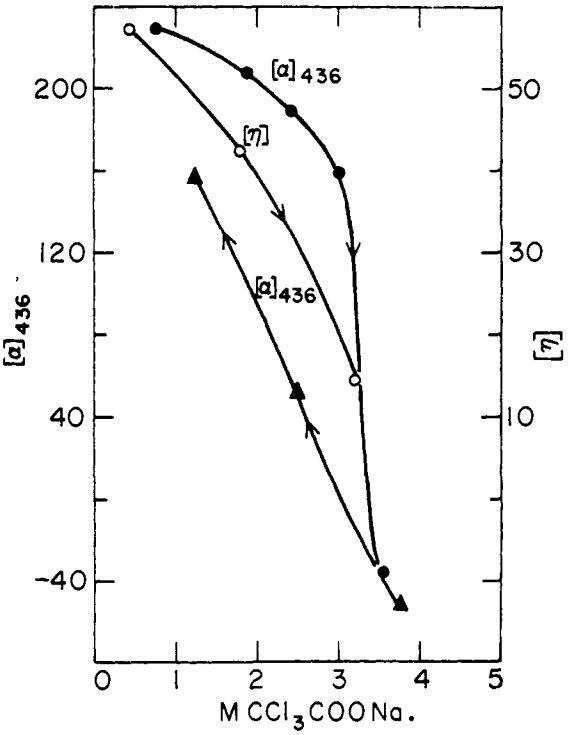

Fig. 5.—Denaturation of sea urchin DNA in sodium trichloracetate at 25° and *p*H 7. Dependence of rotatory power and viscosity on salt concentration: ● specific rotation, $[\alpha]_{436}$; O intrinsic viscosity, $[\eta]$; ▲ $[\alpha]_{436}$ of DNA previously exposed to 3.8 *M* $NaCCl_3COO$, 25°.

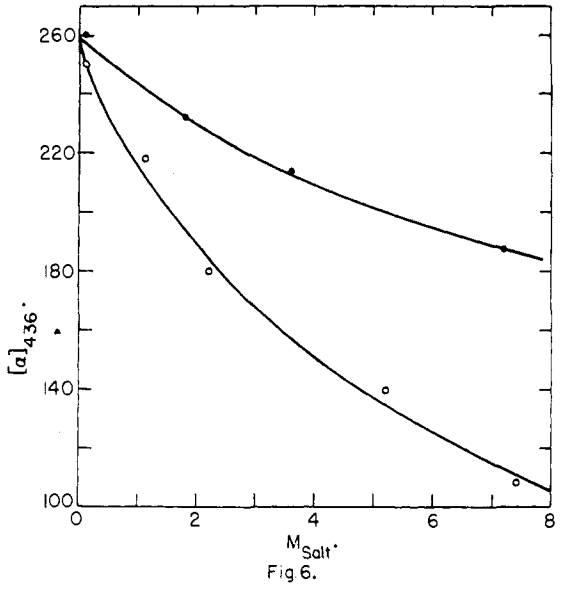

Fig. 6.—Variation of specific rotation of sea urchin DNA with salt concentration at 25°, *p*H 7: ●, $NaClO_4$; O, KCNS.

(22) $[\alpha]$ is not corrected for effects of solvent refractive index. Changes of $\frac{3}{n^2+2}[\alpha]$ with salt concentration are somewhat *more* pronounced than those shown in Fig. 6.

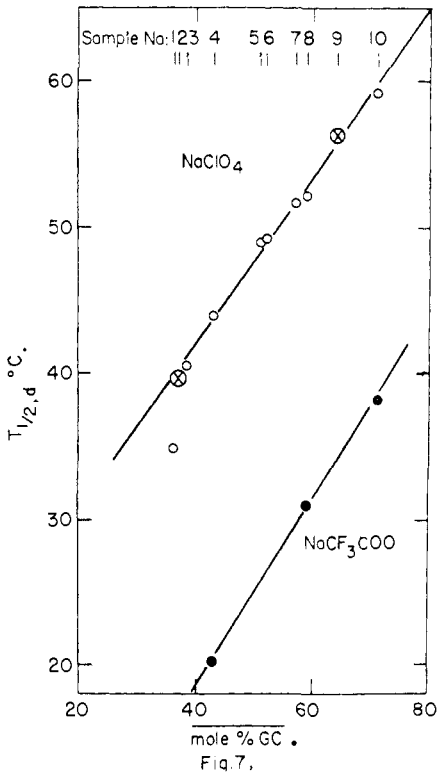

Fig. 7.—Dependence of thermal stability of the DNA helix on average nucleotide composition in 7.2 *M* $NaClO_4$, 0.1 *M* Versene *p*H 7 and 6.5 *M* $NaCF_3COO$, 0.01 *M* Versene *p*H 7. Samples are identified in Table V. O 7.2 *M* $NaClO_4$, A_{259}; × 7.2 *M* $NaClO_4$, $[\alpha]_{436}$; ● 6.5 *M* $NaCF_3COO$, A_{259}.

in phage DNA. That it is *not* due to chemical reaction with perchlorate is shown by

(a) the invariance of thermal stability to the age of T2 DNA–$NaClO_4$ solutions.

(b) the ability of T2 DNA to renature (*i.e.*, to undergo slow, kinetically second order, reformation of ordered secondary structure after heating and quick cooling[23]) in this solvent after repeated cycles of heating and quenching.

In both the solvents examined in Fig. 7, the dependence of $T_{1/2,d}$ on the GC content is much greater than in 0.15 *M* buffered NaCl (Table III). In the absence of any other effects one would therefore expect the thermal transition of *inter*molecularly heterogeneous DNA's to be broadened. While this expectation is realized (Fig. 2, Table IV), broadening is also shown by the intermolecularly very homogeneous bacteriophage T2 DNA (Fig. 8). Such transition broadening can arise from *intra*molecular heterogeneity, or from changes of the thermodynamic parameters describing the thermal transition. The evidence that intramolecular heterogeneity of base composition plays an important role in determining *reversibility* of denaturation is presented elsewhere.[15] If such intramolecular heterogeneity is present, at least part of the transition *broadening* must be due to it.

F. **Dependence of Thermal Stability on Molecular Weight.**—The ability to correlate the stability

(23) P. Doty, J. Marmur, J. Eigner and C. Schildkraut, *Proc. Natl. Acad. Sci. U. S.*, **46**, 461 (1960).

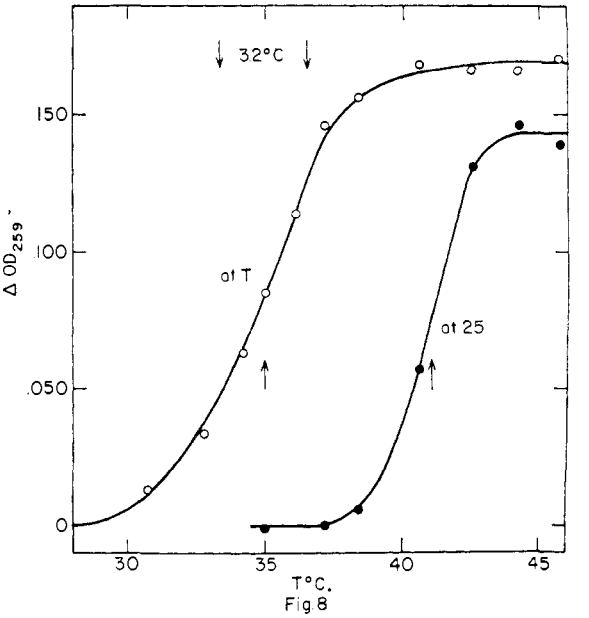

Fig. 8.—Thermal denaturation of bacteriophage T2 DNA in 7.2 *M* $NaClO_4$, *p*H 7: O A_{260} measured at *T*; ● A_{260} measured at 25° on solutions equilibrated at *T* and quenched. *Only values of* A_{260} *extrapolated to zero time at 25° are shown* (*i.e.*, the relatively slow "renaturation"[23] or "Type II reversibility"[35] has been eliminated from this measurement).

TABLE III

DEPENDENCE OF THE DENATURATION TEMPERATURE ON DNA COMPOSITION IN SEVERAL SOLVENTS

Solvent	$dT_{1/2,d}/dGC$[a]	Ref.
6.5 *M* $NaCF_3COO$, Versene *p*H 7	+0.60	This work
7.2 *M* $NaClO_4$, Versene *p*H 7	+ .56	This work
0.15 *M* NaCl, citrate *p*H 7	+ .41	16
51% (v./v.) methanol, 10^{-3} *M* Tris *p*H 7, 10^{-3} *M* NaCl	+ .35	15
81% (v./v.) methanol, 10^{-3} *M* Tris *p*H 7, 10^{-3} *M* NaCl	Approx. .0	15

[a] Change in $T_{1/2,d}$ for a change in guanine–cytosine content of 1 mole %.

TABLE IV

BROADENING OF THE DNA[a] THERMAL DENATURATION TRANSITION IN $NaClO_4$

Medium (Versene, *p*H 7)	$T_{1/2,d}$, °C.	δ, °C.
0.15 *N* NaCl	86	3.1
1.8 *M* $NaClO_4$	83	3.5
3.6 *M* $NaClO_4$	68	5.1
7.2 *M* $NaClO_4$	43	8.4

[a] DNA: sea urchin (37 mole % GC).

of DNA secondary structure with composition is impressive. It is even more remarkable when one considers that there is now general agreement as to the transition temperatures, $T_{1/2,d}$, of DNA's prepared by different methods, in different laboratories, possessing different molecular weights. This variation is also great among the DNA samples used in assembling the data of Fig. 7 as shown by the macromolecular data of Table V.

One reason for this well-behaved nature of $T_{1/2,d}$ is presented here. We have found that the thermal stability of DNA is rather insensitive to molecular

TABLE V

MACROMOLECULAR PROPERTIES OF DNA PREPARATIONS

Sample	Name	Sedimentation constant[a] $S_{20,w}$	Concn., %	[η] dl./gm. in 0.1 *M* NaCl	M_w[e]	Nucleotide composition Mole % GC	Ref.
1	Bacteriophage T2	39.0[b]	0.002	..	.....	35	24
2	*Strongylocentrotus*	23.3[c]	.002	55	.....	37	This work
3	*D. pneumoniae*	13.7[c]	.002	..	1.7×10^{6}[f]	38.5	25
4	*Salmon*	22.6[c]	.003	61	5.9×10^{6}	43	26
5	*E. coli*	16.1[c]	.004	33	.....	51	25
6	Bacteriophage T7	29.0[cd]	...	..	.....	52	27
7	*Ae. aerogenes*	22.1[c]	.002	52	.....	57	25
8	*S. marcescens*	17.4[c]	.002	34	.....	59	25
9	*Ps. fluorescens*	20.1[c]	.003	41	.....	64	25
10	*M. lysodeikticus*	25.1[b]	.002	..	.....	71	24

[a] Determined in 30 mm. Kel F cells in a Spinco model E ultracentrifuge using ultraviolet absorption optics at 23–26°. Corrected to the viscosity and buoyancy of water at 20° assuming the partial specific volume of all DNA's to be 0.55 cm.³/g. Sedimentation carried out in two different media. [b] 0.1 *M* NaCl. [c] 0.9 *M* NaCl; rotor speed 42,040 r.p.m. except bacteriophage T2 which was sedimented at 31,110 r.p.m. [d] Private communication D. M. Freifelder. [e] Independent light scattering measurement. [f] Private communication L. Cavalieri.

weight (Fig. 9). Samples of salmon DNA covering a range of molecular weights have been prepared by sonic irradiation. We assume $[\eta] = K_\eta M^{1.1}$ in calculating the molecular weight scale shown in Fig. 9.[28] For the range of M from 0.6 to 6 million, $T_{1/2,d}$ changes by only 0.5 (±0.5)°, and δ remains constant. For DNA samples ranging in average molecular weight from 1 to 60 million (*i.e.*, approximately 0.17 to 10 times the molecular weight of the unirradiated preparation used for the experiments of Fig. 9), the range of $T_{1/2,d}$ is estimated as 0.7 (± 0.5)°.

It is a direct consequence of the above argument that molecular weight heterogeneity can account for only a small fraction of the transition breadth. For example, consider a DNA sample having a "most probable" molecular weight distribution, for which one half of the sample consists of molecules whose weights lie between 0.48 and 1.35 M_w (M_w is the weight-average molecular weight). The species corresponding to these limits will have their $T_{1/2,d}$ at respectively 0.2° below and 0.1° above the $T_{1/2,d}$ of the entire sample. Therefore, the contribution of molecular weight heterogeneity to δ in this solvent is 0.3°,[29] less than one-tenth of the observed transition half width of the most homogeneous DNA (T2) and about 4% of δ for this salmon DNA.

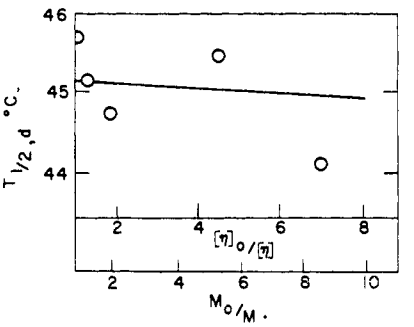

Fig. 9.—Molecular weight dependence of the transition temperature, $T_{1/2,d}$, of salmon DNA (sample C) in 7.2 *M* $NaClO_4$, *p*H 7. Molecular weight calculated on the assumption that the molecular weight and intrinsic viscosity are related by [28]

$$\frac{M}{M_0} = \left(\frac{[\eta]}{[\eta]_0}\right)^{1.1}$$

The weight-average molecular weight (light scattering) M_0 of the unirradiated sample is 5.9×10^6. $[\eta]$ is the intrinsic viscosity at zero shear gradient.

III. Discussion

A. Concentrated Aqueous Salt Solutions as Denaturing Agents.—One practical result of the experiments described in this communication is to demonstrate the use of several DNA denaturing media which have a number of useful properties. $NaClO_4$ and $NaCF_3COO$ recommend themselves particularly as solvents which are transparent, or nearly so, at 260 mμ and in which DNA denaturation occurs at low temperatures. Subsequent use, both in this work and other experiments, has centered mainly on 7.2 *M* $NaClO_4$. This is a solvent medium in which transitions are relatively broad. It should therefore be useful for the more precise and easily controllable fractionation and fractional inactivation of biological markers in transformation experiments. The perchlorate ion does not react with DNA under the conditions of our experiments. Since denaturation occurs at far lower temperatures in this medium, there is less opportunity for the rupture of purine–sugar and phosphoester bonds that occurs at higher temperatures or low *p*H. In addition, perchlorate (as do thiocyanate and trichloroacetate) binds strongly to proteins and may be expected to dissociate these contaminants from their DNA ligands.

As a consequence of the lower transition temperatures, the tendency to form *disordered* secondary structure links at 25° is greatly reduced in 7.2 *M* $NaClO_4$. The absorbance of irreversibly denatured salmon DNA at that temperature is therefore much greater in 7.2 *M* $NaClO_4$ than in 0.1

(24) J. Josse, A. D. Kaiser and A. Kornberg, *J. Biol. Chem.*, **236**, 864 (1961).

(25) A. N. Belozherski and A. S. Spirin, "The Nucleic Acids," E. Chargaff and J. N. Davidson, eds., Vol. III, Academic Press, Inc., New York, N. Y., 1959, Ch. 32.

(26) E. Chargaff, *ibid.*, Vol. I, Ch. 10.

(27) R. L. Sinsheimer, *ibid.*, Vol. III, Ch. 33.

(28) P. Doty, B. B. McGill and S. A. Rice, *Proc. Natl. Acad. Sci. U. S.*, **44**, 424 (1958).

(29) The maximum absorbance increase on denaturation is independent of molecular weight in this range.

M NaCl. The concentrated perchlorate solvent behaves, in this respect, like low ionic strength aqueous media. In other respects, however, denaturation in 7.2 *M* $NaClO_4$ and 0.15 ionic strength saline-citrate are quite comparable. One may, for instance, observe the slow "renaturation" of bacteriophage DNA which has been heated several degrees above its transition temperature ($T_{1/2,i}$), rapidly cooled in ice and reëquilibrated at 25°

The regularity of the variation of secondary structure stability with average composition of DNA has been commented upon in section II E and interpreted (section II F) in terms of the molecular weight - independence of $T_{1/2,d}$. In fact, we have shown elsewhere that enzymatic degradation (with DNAase I), sufficient to lower the intrinsic viscosity by a factor of three and introduce many breaks in each polynucleotide chain, lowers $T_{1/2,d}$ (of salmon DNA in a methanol–water solvent) by only 1°.[30] One cannot, therefore, expect that measurement of $T_{1/2,d}$ should provide a sensitive assay of macromolecular integrity.[31]

However, in low ionic strength media, where interactions of DNA with protein and divalent metal ions are strong, $T_{1/2,d}$ may serve as a partial index of such contamination.

B. Is DNA Denaturation an All-or-None Process?—The problem of helix-coil transitions in homopolymers has been solved by a number of investigators. There is general agreement that the transition from an ordered to a disordered form in very large molecules may range from very broad to all-but-infinitely sharp, depending on the value of the two parameters representing the strength of individual secondary structure links, and the tendency for ordered segments to be clustered together. Numerical values of these parameters can be derived from experiments on the helix-coil transition of homopolymers of known (or sufficiently high) molecular weight. In the case of DNA, the situation is more complex. Most preparations of DNA contain a large number of different molecular species having a range of *average* base composition, representing the various molecular species of the genetic material of the cell and, possibly, their mechanical degradation products. Each molecular species, in turn, has a non-uniform distribution of nucleotide pairs. The problem is to decide the extent to which each of these three categories of variables—thermodynamic, intramolecular heterogeneity and intermolecular heterogeneity—determine the form of the transition.

One further precaution must be observed in comparing transition theory with experiments on DNA denaturation. It is important to confine attention to those experiments involving measurements at the ambient condition (d-assays). Bacterial transformation, equilibrium density gradient centrifugation and other i-assays are not strictly applicable to the available theory. Their analysis is further complicated by the need to establish the mechanisms of irreversibility. In general, it appears that the behaviour of DNA in i-assays is strongly determined by a number of other factors in addition to the average thermodynamic properties and composition of the sample.[34-36]

It is found, in this work and elsewhere,[16] that the breadth of the transition (*cf.* Figs. 2 and 8) increases as the known *inter*molecular heterogeneity increases. This, and other considerations, have led to the proposal that the sharpness of the transition *in each molecular species* is at, or beyond, the limits of the experimental methods currently employed.[23] The experiments reported here provide evidence to the contrary: (1) The transition for even the most homogeneous DNA's (*e.g.*, phage T2, Fig. 8) is relatively broad in 7.2 *M* $NaClO_4$. Moreover, the breadth of the transition does not change over a considerable molecular weight range so that the transition breadth of the parent sample cannot be assigned to mechanical degradation. (2) Irreversibility of denaturation does not set in until the transition, as observed in the d-assay, is well advanced (Figs. 2,3,8). The effect is particularly pronounced in DNA's with a low *average* GC content and occurs in many different solvents and in acid, as well as heat, denaturation.[37,38] We interpret the latter result as demonstrating the existence of states of DNA in which many molecules have substantial disordered regions but none is completely denatured. The question of whether this breadth of the transition in each molecular species is wholly, or in part, due to intramolecular heterogeneity is considered in connection with experiments that will be reported elsewhere.[15]

C. Stabilization of the DNA Helix in Water.—In this final section we discuss the problem of why salts containing certain large anions denature DNA.

It seems certain that these anions are not, in the usual sense, hydrogen bond breaking agents[39]: (1) their denaturing power increases in the order $Cl^- < Br^- < I^-$; (2) trichloroacetate is a much stronger denaturing agent than trifluoracetate, acetate is a very poor denaturing agent; yet the heats of hydrogen bond formation of the corresponding acids (in CCl_4) are all comparable: −5.2, −4.4 and −5.4 kcal./mole H bond respectively[40]; (3) thiocyanate is a much stronger denaturant than any of the carboxylate ions; (4) several of these salts are much more powerful DNA denaturing agents than those prototypical hydrogen

(30) D. M. Freifelder, P. F. Davison and E. P. Geiduschek, *Biophys. J.*, **1**, 389 (1961).

(31) Chemical and photochemical modification of purines and pyrimidines in DNA does, however, change $T_{1/2,d}$.[30,32,33]

(32) E. P. Geiduschek, *Proc. Natl. Acad. Sci. U. S.*, **47**, 950 (1961).

(33) J. Marmur, W. F. Anderson, L. Matthews, K. Berns, E. Gajewska, D. Lane and P. Doty, *J. Comp. Cellular Physiol.* (1961), in press.

(34) W. Ginoza and B. H. Zimm, *Proc. Natl. Acad. Sci. U. S.*, **47**, 639 (1961).

(35) W. Guild, personal communication.

(36) E. P. Geiduschek, *Fed. Proc.*, **20**, 353 (1961).

(37) L. Cavalieri and B. H. Rosenberg, *J. Am. Chem. Soc.*, **79**, 5352 (1957).

(38) E. P. Geiduschek, *J. Polymer Sci.*, **31**, 67 (1958).

(39) *I.e.*, they do not themselves react as H-bond donors and acceptors with purines and pyrimidines, in water solution. Needless to say they may indirectly affect the formation of H-bonds whenever the existence of the latter is also partly determined by other interactions.

(40) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," Appendix B, W. H. Freeman and Co., San Francisco, Calif., 1960.

TABLE VI

SALTING-OUT OF NEUTRAL, BASIC AND ACIDIC NON-ELECTROLYTES BY ELECTROLYTES

Non-electrolyte	Cations	Anions	Method[a]	Ref.
Benzene	$(CH_3)_4 < Li < K < Na$	$I < ClO_4 < Br < Cl$	S	50
Acetone	Na	$ClO_4 < I < Br < Cl < CH_3COO < HCOO$	S, D	51, 52
Butanol	Mg	$CCl_3COO < CF_3COO < ClO_4$	S	53
Aniline	$Li < K < Na$	$I < Br < Cl$	S	51
Benzoic acid	$(C_2H_5)_4N < (CH_3)_4N \ll NH_4 < K < Na < Li$	$C_6H_5SO_4 < CCl_3COO < CNS < ClO_4 < I < Br < Cl$	S	54, 55

[a] S = solubility, D = distribution between two liquid phases.

bond breakers, urea[41] and guanidine hydrochloride; they are matched only by certain organic solvents such as NN′ dimethylformamide (DMF), formamide and dimethyl sulfoxide,[43–45] of which one, DMF, stabilizes the helical form of polypeptides.[46]

It is also interesting to note that the differential stability of guanine–cytosine rich (GC) relative to adeninethymine rich (AT) DNA's is greater in $NaClO_4$ and $NaCF_3COO$ than in dilute NaCl–citrate. In Table III comparison is made with mixtures of methanol and water which are also denaturing solvents, and in which the differential stability is less than it is in dilute NaCl–citrate. That the difference in the contributions of AT and GC nucleotide pairs can be varied so greatly, and in both directions, by substances which are not, in the usual sense,[39] hydrogen bond breaking agents, strongly suggests that the difference in the stabilities of AT and GC-rich DNA helices does not arise exclusively from hydrogen bond contributions.

Since these 1:1 electrolytes are denaturing agents which lower the thermal transition temperature, $T_{1/2,d}$, by as much as 60°, they must be affecting interactions which contribute substantially to the stability of DNA secondary structure. As the basis for a description of these interactions, we have looked for correlations between the properties of these salts in DNA denaturation and their effects on water as well as on the water solubility of non-electrolytes. We conclude that this denaturing action is due to effects on the structure of the solvent. The argument is presented below.

The idea that the action of electrolytes on proteins (and other "colloids") is due to "dehydration" is a venerable one. In the absence of a direct interaction between the added electrolyte and the macromolecular solute, changes in the properties of the latter should be simply correlated with changes of the thermodynamic activity of water. Accordingly, in Fig. 10, we compare $T_{1/2,i}$ with the thermodynamic activity a_w of water in 4 M solutions of various salts.[47] There is no correlation between $T_{1/2}$ and a_w; consequently

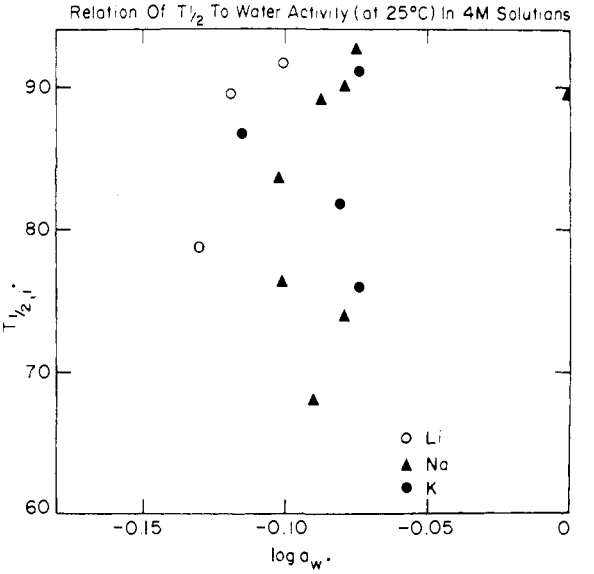

Fig. 10.—Thermal stability of DNA and the thermodynamic activity of water (a_w). $T_{1/2,i}$, taken from Table I, are restricted to 4 M salt solutions. a_w calculated from tabulated values of osmotic coefficients.[48,49]

the helix-coil transition cannot be represented in terms of a reaction of simple stoichiometry between secondary structure ligands and water. Needless to say, this does not exclude a stability-determining role for water. It merely excludes the possibility that the helix-coil transition can be represented by the simple equation

$$\text{purine:::pyrimidine (helix)} \pm H_2O \rightleftharpoons \text{purine, pyrimidine (coil)}$$

On the other hand, a comparison, between the denaturing action of the anions and the salting-out properties of their alkali metal salts yields some very close parallels. Table VI shows data on the salting-out of non-electrolytes by a variety of salts. There is a very strong general correlation with the DNA chaotropic series: those substances

(41) However, Levy and Magoulas[42] have shown that urea does not weaken the intramolecular hydrogen bonding of bifumarate and bimaleate ions in water solution.

(42) M. Levy and J. Magoulas, Abstr. ACS Meeting, Mar. 1961, p. 20-C.

(43) T. T. Herskovits, manuscript in preparation (1961).

(44) G. K. Helkamp and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **83**, 138 (1961).

(45) E. D. Rees and S. J. Singer, *Arch. Biochem. Biophys.*, **63**, 144 (1956).

(46) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).

(47) Osmotic coefficients are tabulated in refs. 48 and 49. In several cases these had to be extrapolated to the higher concentrations of our experiments.

(48) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd Ed., Reinhold Publishing Co., New York, N. Y., 1958, p. 285.

(49) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Academic Press, Inc., New York, N. Y., 1955.

(50) W. F. McDevit and F. A. Long, *J. Am. Chem. Soc.*, **74**, 1777 (1952).

(51) F. A. Long and W. F. McDevit, *Chem. Revs.*, **51**, 119 (1952), have reviewed this subject at length. Most of the references of Table VI are taken from their bibliography.

(52) A. Dobry, *J. Chim. Phys.*, **50**, 49 (1953).

(53) J. Duclaux and C. Cohn, *ibid.*, **50**, 243 (1953).

(54) H. Freundlich and A. N. Seal, *Kolloid Z.*, **11**, 259 (1912).

(55) E. Larsson, *Z. Physik. Chem.*, **153**, 299 (1931).

which salt out non-electrolytes most weakly and particularly those which deviate strongly (toward salting in) from electrostatic theories of salting,[51] also denature DNA most effectively. For these anions, the purely electrostatic salting-out is opposed by a tendency to solubilize non-electrolytes. A similar interaction with purines and pyrimidines would, of course, change the relative stabilities of the helical and disordered configurations of DNA and consequently alter the transition temperature.

However, the result obtained with $(CH_3)_4NCl$ (section II A) is important in showing that this cannot be the sole basis of the denaturing action of salts. The tetramethylammonium ion salts *in* a variety of acidic, neutral and basic non-electrolytes yet it is entirely without effect on the stability of DNA secondary structure.

It is known that tetramethylammonium ion and the strongly denaturing anions differ in their (a) partial molal heat capacities and entropies in dilute solution and (b) effects on the dielectric relaxation spectrum of water. These differences have been interpreted in terms of effects on the structure of water, the anions acting as "structure breakers" while tetramethylammonium ion (and ions with large alkyl side chains) are classed as "structure formers."[56,57] The effect of salts on the n.m.r. spectra of water protons is also of interest in this connection (Table VII). Cl^-, Br^-, I^- and ClO_4^- shift the proton magnetic resonance of water to higher field strengths (H), ClO_4^- being the most effective in this regard. The effect has been interpreted as showing the tendency of these anions, increasing with the ionic radius, to disrupt the hydrogen bonding of water.[58] By contrast, we have found that $(CH_3)_4NCl$ has almost no effect on the nuclear magnetic resonance frequency of water protons. Comparison with NaCl shows that, relative to sodium ion, $(CH_3)_4N^+$ decreases H. Yet, polarization effects alone would produce exactly the opposite result, *i.e.*, $(CH_3)_4N^+$ would increase H relative to Na^+. The data of Table VII are therefore consistent with the view that, relative to sodium ion, $(CH_3)_4N^+$ increases hydrogen bonding of water molecules and acts as a "structure former."

Insofar as the denaturing anions exert their effect on DNA through their modification of the structure of water, they can be classed as *hydrophobic bond breaking agents*. The structural details of these effects are not, at present, established. The interpretation which has been offered above is only qualitative. In fact, until recently there has been little information about the structure of water in concentrated electrolyte solutions, on the basis of which to give these qualitative notions precise structural definition. It is undoubtedly for this reason that the specific details of previous speculations about the function of water in stabilizing the secondary structure of DNA and proteins have proved to be more provocative than accurate.[61-63] However, current structural studies[64,65] may ultimately provide the basis for more fruitful model building.

TABLE VII

CHANGE IN THE NUCLEAR MAGNETIC RESONANCE FREQUENCY OF WATER PROTONS BY SOME SALTS

Salt	δ/M[a]	Ref.
NaCl	+0.58	58
NaBr	+ .78	58
NaI	+ .92	58
$NaClO_4$	+1.32	58
$(CH_3)_4NCl$	0.00	This work[59]

[a] $\delta = \frac{H_{soln} - H_{H_2O}}{H_{H_2O}} \times 10^7$ where H_{soln} and H_{H_2O} are the magnetic field strength at resonance for the solution and pure water respectively; M is the molarity of the salt.[60]

Acknowledgments.—We wish to thank Drs. P. Gerhardt, A. Holtzer, D. Rosenthal and E. L. Swan for most helpful advice, Dr. R. L. Phillips for a gift of *Aerobacter aerogenes*, Prof. L. Kotin for measuring NMR spectra and Messrs. De V. Gallup, R. J. Thornton and A. Daniels for technical assistance.

(56) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).

(57) H. S. Frank and W. Y. Wen, *Discussions Faraday Soc.*, **24**, 133 (1957).

(58) J. N. Shoolery and B. J. Alder, *J. Chem. Phys.*, **23**, 805 (1955).

(59) 2.9 and 15.8 molal solutions of $(CH_3)_4NCl$ in dilute neutral aqueous buffer were measured against a benzene standard, on a Varian Model 4300 B spectrometer equipped with a superstabilizer and operating at a frequency of 40 megacycles per second. Sample and standard were contained in a standard concentric cylindrical tube assembly (Wilmad Glass Company, Landisville, New Jersey). The outer annulus contained pure benzene. Measurements were kindly made by Dr. L. Kotin.

(60) On comparing the listed experimental data of Shoolery and Alder with Table III of ref. 58, we conclude that *molar* rather than *molal* shifts of proton resonance have been calculated by the authors.

(61) B. Jacobsen, W. A. Anderson and J. T. Arnold, *Nature*, **173**, 772 (1954).

(62) W. F. Harrington and J. Schellman, *Compt. rend. Lab. Carlsberg*, **30**, 167 (1957).

(63) W. Kauzmann, *Adv. Protein Chem.*, **14**, 1 (1959).

(64) G. W. Brady, *J. Chem. Phys.*, **33**, 1079 (1960).

(65) H. C. Fernandez-Moran, Abstracts 18th Annual Meeting, Electron Microscope Soc. Am., Aug. 1960.