[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Studies on the Structure of Nucleic Acids. X. On the Mechanism of Denaturation[1]

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Various physical properties of calf thymus DNA have been determined from viscosity, light-scatterng, flow dichroism and spectral measurements. The DNA samples used were prepared by a number of procedures. The principal aim of the investigation was to elucidate the nature of the irreversible changes which occur in DNA solutions of low ionic strength or at low *p*H values. The following conclusions have been drawn: 10^{-3} *M* salt is sufficient to prevent irreversible change at the usual *p*H (~6.5) of a DNA solution; however, no salt is required to prevent irreversible change if the *p*H of the solution is *ca.* 8; the titration of guanine appears to be responsible for the irreversibility. The extinction coefficient of undenatured DNA, E_{260}(P), based on the phosphorus content, is 6300 in 10^{-3} *M* (or higher) salt; the value (in water) approaches 8800 if denaturation has occurred. The changes conferred by raising or lowering the *p*H are similar, if not identical, to those conferred by decreasing the ionic strength at constant *p*H. This is taken to mean that internal fields raise the pK_a's of certain amino groups, which then accept protons (and break hydrogen bonds) at sufficiently low ionic strength. Viscosity and dichroism measurements indicate that the molecule is somewhat contracted at high salt concentrations, and that it collapses as a result of denaturation.

Introduction

It has become abundantly clear in recent years that the physical and biological characteristics of deoxyribonucleic acid (DNA) are greatly dependent on the method of isolation from the tissues and the manner of preparing and handling the DNA solutions directly prior to measurements. The literature is replete with discrepancies, most of which are probably attributable to the two causes just mentioned. Before similarities or differences among DNA's can be considered seriously, or indeed before any absolute basis for DNA structure can be established, it will be necessary to reassess earlier data in the light of such newly discovered variables as the ionic histories of the samples.[2] The present investigation deals with the mechanism of denaturation and its structural implications, based on data obtained with DNA samples prepared in a number of ways. Salt and *p*H effects have been studied by means of spectra, viscosity, light scattering and flow dichroism measurements, and it has been shown that the physical properties are greatly dependent upon the method of preparing the DNA solutions.

Experimental

Materials.—The S and D samples were isolated from calf thymus. Sample TF was isolated from streptomycin-resistant pneumococcus, according to the method of Avery, MacLeod and McCarty[3] and was stored at 0° in physiological saline. Samples S-I, S-II and S-III were isolated according to the method of Schwander and Signer.[4]

Anal. S-I: N, 13.03; P, 8.20; Na, 6.96; H_2O, 16.6. S-II: N, 13.42; P, 7.97; Na, 7.70; H_2O, 21.2. S-III: N, 14.67; P, 8.61; Na, 7.65; H_2O, 10.48.

Sample D-I was prepared according to the procedure of Kay, Simmons and Dounce.[5] D-II, D-III and D-IV were also prepared using this method but are different in that they were never dissolved in water as prescribed[5] but rather in 0.005 *M* salt, and were not washed with acetone at the end of the procedure. D-IV differs from D-II and D-III in that the fibers were never dried but were dissolved in sterile 0.005 *M* salt and stored at 0° in concentrated solution.

Anal. D-I: N, 13.19; P, 8.42; Na, 7.44; H_2O, 17.74. D-II: N, 11.32; P, 6.74; Na, 9.26; H_2O, 16.59. D-III: N, 11.00; P, 6.75; Na, 9.53; H_2O, 16.83.

All water used was distilled and then deionized by running through a "deeminac" column. DNA concentrations were based on dry weight calculated from the analyses.

Methods.—All spectral measurements were made with a model DU Beckman Spectrophotometer at 260 mμ. Quartz plungers were used, when necessary, to decrease the light path from 1 cm. to 0.1 or 0.01 cm.

I. **Flow Dichroism.**—A description of the instrument and the method of measurement is given in the preceding paper.[6] All measurements reported in the present paper were made with light of wave length 260 mμ, polarized in the direction parallel to the flow lines. Dichroism is here defined as $D = (R - A_{\parallel})/R$, where R is the optical density of a randomly oriented solution (at rest) and $A_{\parallel}$ is the optical density of the solution when oriented by flow. Dichroism as thus defined is proportional to the dichroic ratio $A_{\perp}/A_{\parallel}$ (where $A_{\perp}$ is the absorption of light polarized perpendicular to the flow lines), which is also determined by R and $A_{\parallel}$. Comparison of dichroism values at a single shear rate shows differences in chromophoric arrangement, but, for more explicit information, one must compare them at equal degrees of orientation. In this paper, dichroism at 9480 sec.$^{-1}$ is used for the purpose of merely indicating change; otherwise the calculated dichroism at complete orientation is used (D'_{∞}). The methods of calculating D'_{∞} and θ' (the apparent rotary diffusion constant), as well as discussion of the effects of orientation, molecular shape and internal structure of the DNA molecule on dichroism will be found in the preceding paper.

II. **Viscosity. Rotating Cylinder Viscometer.**—The (outer) rotating cylinder viscometer was constructed essentially according to the design of Eisenberg and Frei.[7] It differed mainly in employing a null method for the electrostatic balancing of the viscous torque. A regulated high-voltage d.c. power supply[8] was set at about 3000 v. A series of precision (0.1%) resistors was used as a voltage divider so that any voltage between 0 and 2000 could be tapped in stages of 2000, 200, 20 and 0.2 v. The fine control was accomplished by means of a potentiometer calibrated to 0.02 v., but not used to less than about 0.2 v. The power supply was set and checked regularly with a Beckman model G *p*H meter by tapping off 500 mv. with the potentiometer.

The operation of the viscometer was checked by determining the viscosities of a number of standard glycerol solutions which had been determined in an Ostwald viscometer. The relative viscosities using the two methods agreed to within 1%. The precision of the rotating cylinder viscometer was ±0.004 for relative viscosities. The solvent used was checked at least once a day. The relative viscosities of the DNA solutions were obtained by dividing

(1) This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, Public Health Service, Grant No. C-471, and from the Atomic Energy Commission, Contract No. AT(30-1)-910.

(2) R. Thomas, *Biochem. Biophys. Acta*, **14**, 231 (1954).

(3) O. T. Avery, C. M. MacLeod and M. McCarty, *J. Exptl. Med.*, **79**, 137 (1944).

(4) H. Schwander and R. Signer, *Helv. Chim. Acta*, **33**, 1521 (1950).

(5) E. R. M. Kay, N. S. Simmons and A. L. Dounce, THIS JOURNAL, **74**, 1724 (1952).

(6) L. F. Cavalieri, B. Rosenberg and M. Rosoff, *ibid.*, **78**, 5235 (1956).

(7) H. Eisenberg and E. H. Frei, *J. Polymer Sci.*, **14**, 417 (1954).

(8) Purchased from Atomic Instrument Co., Springfield, Massachusetts.

(voltage)^2 for the DNA by (voltage)^2 for the solvent at each shear rate; this is based on the relation

$$\beta\eta = kV^2$$

where β is the shear rate, η, the viscosity, k, the instrument constant, and V, the voltage.

Determinations were usually made between the shear rates 0.3 and 17 sec.$^{-1}$. Four or five different gear ratios were used for intermediate shears. In some instances the range of shear rates was 0.15–28 sec.$^{-1}$. When shear dependence was still observable at low shear rates, the reciprocal of the relative viscosity *vs.* the shear rate was plotted and extrapolated to zero shear rate.

The shear rate is a function of the annular space between the inner and outer cylinders, and when this space is small the shear rate is constant. In our case the space was 2 mm. and small corrections were applied. The equations relating to these corrections are given in the appendix.

Method of Measurement.—After the power supply had warmed up, the voltage was calibrated with the *p*H meter. Solvent was always determined first to check the behavior of the instrument. Serial dilutions were frequently used to obtain various DNA concentrations down to a relative viscosity of about 1.05. The maximum error in specific viscosity at this point was ±4%. The temperature was maintained at 25.4 ± 0.1°.

The question of adsorption, particularly at low DNA concentrations, was checked many times. After the voltage for a low DNA concentration was determined, the solution was removed from the viscometer and fresh solution from the same stock was added without rinsing the instrument. This was repeated several times and in all cases the voltages agreed within experimental error; the same agreement was found when the viscometer was cleaned between determinations.

III. **Light Scattering.**—The apparatus employed was a Brice–Phoenix light scattering photometer. The instrument was calibrated with a standard sample of Cornell polystyrene (0.5 g./100 cc.) in toluene. A turbidity of 3.40×10^{-3} cm.$^{-1}$ and a dissymmetry $Z = 1.21$ were obtained using unpolarized blue light (4360 Å.). Angular intensity measurements between 35 and 135° were made on solutions contained in a hand-blown erlenmeyer-type cell which in turn was calibrated against a rectangular cell using several concentrations of Ludox. The positioning of the cell was checked by measuring the angular envelope of a fluorescein solution and was constant to within ±1%.

DNA solutions in 0.20 M NaCl were centrifuged for 24 hr. at 20,000 g in specially constructed polythene tubes containing an inclined ledge to minimize convection. The contents were removed with clean pipets. Solutions were examined for cleanliness by means of a strong light beam and a hand lens at low scattering angles. Dust-free solvent was obtained by filtering under slight positive pressure through a millipore filter. Concentrations of DNA solutions were determined by optical density measurements. All concentrations were within the range 0.16–0.04 mg./ml.

A Brice–Phoenix differential refractometer was employed in measuring the refractive index increment, dn/dc, of DNA in 0.20 M NaCl. The value obtained was 0.191.

Light scattering data were handled by the conventional Zimm plot; the equations of the double linear extrapolation

$$HC/\tau = 1/M + 2BC$$

and

$$HC/\tau = 1/M\left(1 + \frac{16}{3}\pi^2\frac{\bar{r}^2}{\lambda_2}\sin^2\theta/2\right)$$

were used to obtain the molecular weight and the root-mean-square end-to-end chain length.

Results and Discussion

We will present here the results of three series of experiments. The first deals with undenatured S-I DNA solutions; the second, with denatured S-I DNA solutions; the third, with solutions of other DNA preparations. The term "denatured" is used here, for lack of a more explicit word, to designate material which has undergone irreversible physical change; undenatured DNA solutions are ones in which no apparent irreversible change has taken place.

1. Undenaturated DNA.—All measurements discussed in this section were performed on solutions made by dissolving S-I DNA in 10^{-3} M NaCl and adjusting the salt concentration, when necessary, by adding solid sodium chloride to the solution.

The intrinsic viscosity of undenatured S-I DNA is higher, beyond experimental error, in 10^{-3} M than in 0.2 M NaCl (Fig. 1, curves E and A),[9] indicating that the molecule is slightly more contracted in the higher salt. The slopes of the reduced specific viscosity curves (at zero shear) show that concentration-dependent interactions are greater in lower salt; they are still present, however, even in 1 M salt. Since the electrical charges are very probably screened at this salt concentration, the residual interactions are probably due to hydrodynamic factors relating to the flexibility of the molecule. Conway and Butler, and Butler, *et al.*,[10] obtained curves with much lower slopes in salt. Their data, however, is not comparable to ours, since their thymus DNA must have been denatured during preparation by extensive dialysis against distilled water, washing with acetone, and drying *in vacuo* over P_2O_5 (see below). They report no difference in intrinsic viscosity in water, 10^{-3} and 0.1 M salt. It is obviously impossible to obtain water curves for undenatured DNA[2,11] unless the measurements are made at about *p*H 8 (see below), which would present experimental difficulties. But our results at high and low salt concentrations need not be considered contradictory to theirs, since their extrapolations rely on points where the relative viscosity is extremely low and their error is of the order of our differences.

Flow dichroism measurements also indicate that the molecule is collapsible. It can be seen in Table I (rows 1 and 2, 3 and 4) that in 10^{-3} M salt the dichroism at infinite shear (D'_∞) is higher than in 0.2 M salt. Since D'_∞ is actually a measure of the angle(s) between the chromophores and the flow lines when the molecules are completely oriented, a higher D'_∞ value is consistent with the view that kinked molecules are straightened out when salt is removed. The possibility that the higher dichroism is due merely to a (more favorable) internal rearrangement of the chromophores is less likely in view of the lower apparent rotary diffusion constant (extrapolated to zero concentration), θ'_0, in 10^{-3} M salt (55 sec.$^{-1}$, compared to 115 sec.$^{-1}$ in 0.2 M salt), which indicates a longer molecule. Table I (compare values of D'_∞ in rows 1 and 3, 2 and 4) also indicates, by a similar argument, that the molecule becomes more distended as the DNA concentration increases.

At finite concentrations, changes in the apparent rotary diffusion constant (θ') reflect changes both of molecular length and of interactions (the latter may be eliminated by extrapolation to zero DNA

(9) See also Fig. 7B, which shows similar, and perhaps more clear cut, curves for a different DNA sample.

(10) B. E. Conway and J. A. V. Butler, *J. Polymer Sci.*, **12**, 199 (1954); J. A. V. Butler, B. E. Conway and D. W. F. James, *Trans. Faraday Soc.*, **50**, 612 (1954).

(11) J. Pouyet, *Compt. rend.*, **234**, 152 (1952), compared only a water solution and a single concentration of salt. His viscosity results are therefore not comparable to ours.

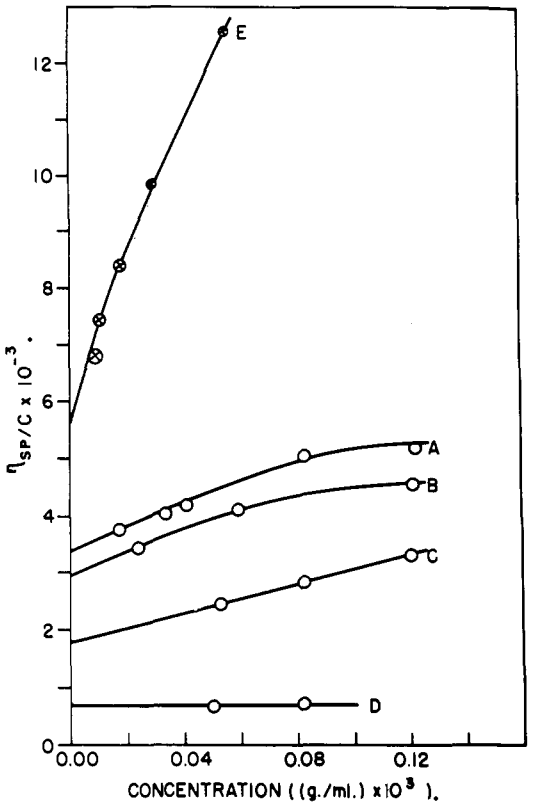


Fig. 1.—Reduced specific viscosity, extrapolated to zero shear, of S-I DNA: O denotes 0.2 M NaCl; ⊗ denotes 10^{-3} M NaCl. Curves A and E, undenatured; curves B, C and D: denatured by exposure of DNA to water in concentrations of 0.82×10^{-3}, 0.42×10^{-3} and 0.08×10^{-3} g./ml., respectively.

concentration). The relative values of θ' in Table I and of D'_∞ (which reflects only length changes) support the conclusions drawn from viscosity data as to the salt dependence of molecular length and intermolecular interactions. A more detailed treatment of dichroism data is presented in the preceding paper.

TABLE I

APPARENT ROTARY DIFFUSION CONSTANTS AND DICHROISM VALUES OF S-1 DNA

	DNA, g./ml. $\times 10^3$	Dissolved in	Measured in	θ' (sec.$^{-1}$)[b]	D'_∞[a]
1	1.16	10^{-3} M NaCl	10^{-3} M NaCl	30	0.322
2	1.16	10^{-3} M NaCl	2×10^{-1} M NaCl	75	.258
3	0.33	10^{-3} M NaCl	10^{-3} M NaCl	45	.241
4	.33	10^{-3} M NaCl	2×10^{-1} M NaCl	100	.198
5	.33	H_2O	10^{-3} M NaCl	100	.215
6	.33	H_2O	2×10^{-1} M NaCl	115	.154

[a] Dichroism extrapolated to infinite shear. [b] Apparent rotary diffusion constants, calculated from the shear-dependence of the dichroism (see preceding paper).

The highest dichroic ratio (calculated for complete orientation) obtained on S-I DNA was 2.5, which is greater than that found by Seeds and Wilkins[12] for semi-crystalline air-dried films of thymus DNA, containing about 30% water.[13] Our value of 2.5 was obtained for a solution of 1.3 mg./ml. of DNA in 10^{-3} M NaCl. Since the molecules in such a solution may not be completely extended rods, as they are in fibers, the intrinsic dichroic ratio[14] is probably even greater than the dichroic ratios calculated from the experimental data, perhaps as great as the maximum value of 4.7 found by Seeds[15] for films at high humidity. At any rate, it is reasonable to assume that the undenatured DNA molecule is intrinsically at least as dichroic in solution as in the usual air-dried solid state. Unfortunately it is impossible to make any calculations of the chromophoric angles without knowing the exact shape of the molecule under stress.

From the foregoing experiments it may be inferred that the DNA molecule in 10^{-3} M salt is extended as a result of both inter- and intramolecular interactions, both of which decrease with the addition of salt. The lesser extension of the molecule in salt has no effect on its extinction coefficient, which, at 260 mμ, is 6300 (based on phosphorus content) in both 10^{-3} and 1 M salt. Figure 2, curve B, shows that no deviation from Beer's law was found over a very wide concentration range. The titration behavior (Fig. 4, curves D and E) is similar to that Thomas[2] reported for undenatured DNA. The optical density of dilute solutions increases by a maximum of about 40% when the pH is lowered.

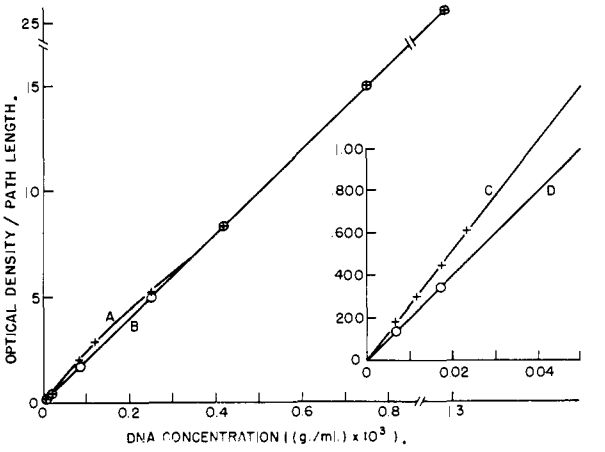


Fig. 2.—Beer's law: O denotes undenatured S-I DNA; X denotes S-I DNA dissolved in water. The curves in the inset (C and D) are magnifications of curves A and B at low concentrations, where Beer's law holds in both cases. The extinction coefficient of undenatured DNA is constant under all conditions that do not produce denaturation, regardless of the salt concentration.

II. Denaturated DNA.—The measurements in this section were made largely on S-I DNA dissolved in deionized water before adding salt.

It has been shown by Thomas[2] that the optical density of DNA in solution increases when the salt concentration falls below a certain value. This value is somewhere between 10^{-3} and 10^{-4} M for

(12) W. E. Seeds and M. H. F. Wilkins, *Disc. Faraday Soc.*, **9**, 417 (1950).

(13) These films, oriented by shearing a viscous gel, had almost the same dichroic ratio (1.7) as fibers which were known from X-ray measurements to consist of completely extended molecules (*cf.* ref. 12).

(14) Defined as the dichroic ratio in the crystalline state.

(15) W. E. Seeds, *Progr. Biophys. Biophys. Chem.*, **3**, 27 (1953).

NaCl (Fig. 3) and is much lower for $MgCl_2$. The change which takes place in low salt is irreversible, since the back salt titration does not follow the same path as the forward. We have found that the extent of the rise in optical density which occurs upon lowering the salt concentration is a function of the DNA concentration (compare curves A, C and D, Fig. 3). This is probably related to the contribution of counterions to the ionic strength, and to the small amount of residual salt present in DNA after precipitation from salt solution. Curve E, Fig. 3, shows the optical density in water for various DNA concentrations, plotted against the corresponding counterion concentration; this curve is very similar to curve A.

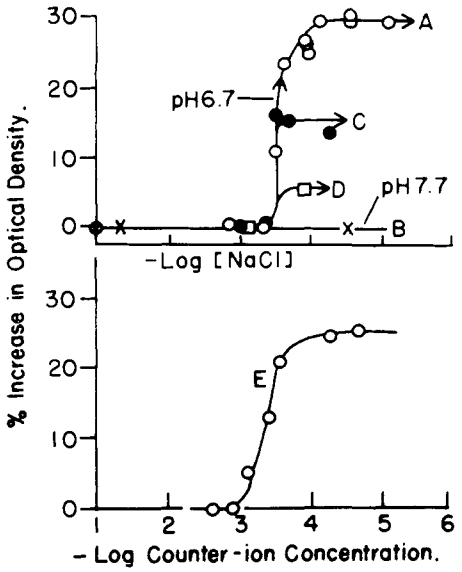


Fig. 3.—Spectral salt titrations at various S-I DNA concentrations. Curves A and B, 0.017 × 10^{-3} g./ml. DNA; curve C, 0.12 × 10^{-3} g./ml. DNA; curve D, 0.25 × 10^{-3} g./ml. DNA. Curves A, C and D show the increase in optical density (based on the optical density of undenatured DNA) when the salt concentration is lowered in the neutral *p*H range (6.5–7). Curve B is a typical salt titration at *p*H 7.7. In curve E, each point represents the optical density increase when a different concentration of DNA (0.75 × 10^{-3} to 0.0069 × 10^{-3} g./ml.) is exposed to water, plotted against its counterion concentration.

The extinction coefficient of DNA in water is thus higher than that of DNA which has never been exposed to water. Figure 2, curves A and C, show that Beer's law is valid in water only for concentrations below about 0.08 mg./ml., where $E_{260}(P)$ is 8200. The back addition of large amounts of salt to denatured DNA solutions lowers the extinction coefficient, but not to coincidence with that of undenatured DNA.[16] Due, perhaps, to the varying ion content of water and of DNA fibers, the reproducibility of measurements on denatured DNA is not exact; however, no significant time dependence has been observed.[17]

An important exception has been found to the foregoing description of denaturation. No denaturation occurs if DNA is dissolved in water of *p*H 8–9, so that the *p*H of the resulting dilute solution is about 7.5–8. This is shown by its extinction coefficient ($E_{260}(P)$ = 6300) and also by viscosity and dichroism measurements (see below). Acid titration of this solution then shows two sharp spectral rises, one at *p*H 7 and the other at *p*H 4.5 (curve A, Fig. 4). One may therefore conclude that water denaturation involves the addition of protons to DNA, as does acid denaturation, and that the spectral change at 260 mμ evidences a macromolecular structural change, since it does not occur upon titration of mononucleotides.

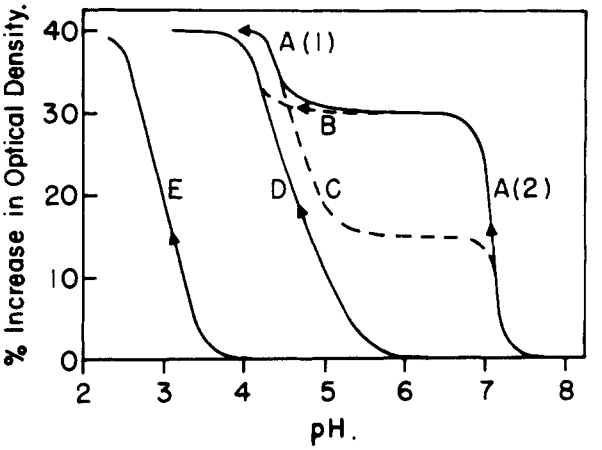


Fig. 4.—Spectral titration of S-I DNA. The optical density increase is based on undenatured DNA, $E_{260}(P)$ = 6300. Curve A, in water; curve B, titrated in water to *p*H 6.7 (dissolving in water results in a *p*H close to 6.7), then NaCl added to 10^{-3} *M*; curve C, in $10^{-3.6}$ *M* NaCl; curves D and E, in 10^{-3} and 0.2 *M* NaCl, respectively (therefore undenatured). Curves A, B and C were obtained for 0.018 × 10^{-3} g./ml. of DNA; curves D and E were obtained for all DNA concentrations measured (0.018 × 10^{-3} to 0.78 × 10^{-3} g./ml.).

Some insight regarding the titratable groups and field effects in DNA may be realized by examining the results obtained by varying the ionic strength and *p*H. If the *p*H of DNA in water is lowered from 7.5 to about 6.5, and then made 10^{-3} *M* in salt before continuing the titration, the second optical density rise (curve A(1), Fig. 4) is shifted downward to a lower *p*H (curve B, Fig. 4). This shift also occurs when salt is added (10^{-3} *M*) immediately after solution at *p*H 7.5, but, in addition, the optical density rise at *p*H 7 disappears (curve D).[18] This optical density increment now appears at the lower *p*H, *i.e.*, curve D is the sum of the two incre-

(16) Beaven, *et al.*, in Chargaff and Davidson, "The Nucleic Acids," New York, N. Y., 1955, p. 527, reviewing the literature, report that Beer's law holds in salt. Two of their references, however (J. Pouyet, G. Scheibling and H. Schwander, *J. chim. phys.*, **47**, 417 (1950); and E. R. Blout and A. Asadourian, *Biochim. Biophys. Acta*, **13**, 161 (1954)) find agreement with Beer's Law only at low DNA concentrations; the third reference (P. D. Lawley, Ph.D. Thesis, Nottingham University, 1953) was unobtainable. All three appeared at a time when DNA was routinely dissolved in water by many investigators.

(17) Thomas (*cf.* ref. 2) has not found the time dependence which is attributed to him by Beaven, *et al.* (*cf.* ref. 16, p. 258).

(18) Unless the initial *p*H is known, titration curves such as those of E. R. Blout and A. Asadourian (*Biochim. Biophys. Acta*, **13**, 161 (1954)) are impossible to interpret. Furthermore, their spectral data cannot be compared to ours, since their DNA solutions were denatured to unknown extents.

ments, (1) and (2), which constitute curve A of Fig. 4. There appear then to be two types of titratable groups. Those represented by curve A(1) are only slightly affected by small amounts of salt, while those represented by curve A(2) are strongly dependent on salt. It would further appear that the latter lose their separate identity in salt, if curve D of this figure be taken as representing a single group. This appears reasonable, since, once curve D appears, further addition of salt merely shifts the entire curve to lower pH values (curve E, Fig. 4).

To determine the difference between the groups titrating at (1) and at (2) in curve A (Fig. 4), let us consider the manner in which A(2) becomes indistinguishable from A(1) to give curve D. Curve A is obtained in $10^{-4.1}$ M NaCl or less, curve D, in $10^{-3.0}$ M. Curve C, obtained in $10^{-3.6}$ M salt, is an intermediate stage between A and D. (It is difficult exactly to reproduce measurements in this range, since small variations in salt content are critical.) The DNA concentration in all three cases is 0.018 mg./ml. It can be seen that the optical density increment at pH 7 decreases. This decrease at pH 7 appears as an increase at pH 4.5, so that the total increment remains constant. Although the relative sizes of the increments change, the pH's at which they occur do not shift. The same behavior occurs when varying concentrations of DNA are dissolved in water to give counterion concentrations between 10^{-4} and 10^{-3} M (calculated from the P analysis of the sample); curve E, Fig. 3, where the pH is just below 7, shows how the size of the A(2) increment varies.[19] Thus, in Fig. 4, curves A and C are concentration-dependent, but curves D and E are not significantly so, since the contribution of counterions to the total ionic strength is much larger in the first case than in the second.

These results suggests that the A(2) groups experience a change which lowers their pK_a' in one step from 7 to that of the A(1) groups, and that the number of A(2) groups thus shifted increases until, when the sodium ion concentration reaches 10^{-3} M, all have shifted and the titration curve (D, Fig. 4) has only one point of inflection. This can be accounted for in a rather straightforward manner. On the reasonable assumption that it is the amino groups of DNA that accept protons, the existence of pK_a shifts indicates the presence of field effects arising from charged phosphate groups acting on nearby amino groups. In the center[20] of a polyelectrolyte molecule, the density of charged phosphate groups is high, and therefore the counterions do not diffuse from this region even in water solution. These phosphate groups, therefore, exert a field diminished in strength, apparently capable of raising the pK_a's of the pertinent (A(1)) amino groups no higher than about 4.5. The counterions of peripheral phosphate groups, however, readily diffuse away from the molecule when the ionic strength of the medium is low, leaving a region of net charge where unshielded phosphate groups may exert a strong field, apparently capable of raising the neighboring (A(2)) amino pK_a's to about 7. The higher the ionic strength and the polyelectrolyte concentration, the smaller the region with net charge, and thus the fewer groups titrating at pH 7. In 10^{-3} M NaCl, which is greater than the counterion concentration in DNA solutions vulnerable to water denaturation (Fig. 3), we must assume that no significant net charge exists (except that on external phosphate groups not involved in field effects) and that all amino groups are subject to fields of equal strength. That is, there are no longer any large local variations in the sodium ion concentration. Further addition of salt increases the screening of charged phosphate groups uniformly and asymptotically, so that progressively larger amounts of salt are required to produce comparable pK_a' shifts.

Regardless of the pH at which it takes place, the titration of certain amino groups appears to permit the occurrence of irreversible macromolecular alterations to a greater or lesser extent, depending on the number of groups titrated. Evidence for such macromolecular changes is presented in some of the experiments which follow. Table II lists the molecular weights and the root-mean-square end-to-end distances, calculated from light scattering in 0.2 M NaCl, of DNA denatured to varying extents by exposure of various concentrations to water. Denaturation does not affect molecular weight, and the root-mean-square end-to-end distance shows no significant change except at high dilution (0.017 mg./ml.), where it has decreased almost to half its original value. This measurement was made on a DNA solution which had been diluted with water to 0.017 mg./ml., concentrated to 0.2 mg./ml. under high vacuum at 0°, and then made 0.2 M in NaCl by adding solid salt. A typical Zimm plot of the scattering data is shown in Fig. 5.

TABLE II

LIGHT SCATTERING RESULTS FOR S-1 DNA DENATURED TO DIFFERENT EXTENTS

The third column is the percentage increase in optical density which occurred upon exposure to water at the concentration in column two. The last column is the root-mean-square end-to-end distance. Light scattering measurements were made after adding salt to 0.2 M.

Stock soln.	DNA concn. in water, g./ml. × 10^3	Increase in optical density, %	Mol. wt. × 10^{-6}	$(\overline{r^2})^{1/2}$, Å.
a	..	0	2.8	4700
b	0.21	10	2.8	4700
c	.083	25	2.6	4000
d	.017	30	2.7	2700

Viscosity is more sensitive than either light scattering or optical density to the changes in molecular shape which accompany denaturation. The intrinsic viscosity in 0.2 M salt drops progressively

(19) It was found convenient to make use of this fact in obtaining DNA solutions at various degrees of denaturation. DNA was diluted with water until the desired optical density increment was obtained, then adjusted in concentration by diluting further with salt, or by concentrating under vacuum.

(20) The existence of field effects necessitates elaboration of the Watson–Crick model (*Nature*, **171**, 737 (1953)) for the DNA molecule. The relatively large distances between phosphates and amino groups in the double helix would not permit field effects. However, if several double helices were intertwined to form a tight aggregate, phosphate groups could approach the amino groups of adjacent strands closely enough to cause the observed pK_a' shifts (L. F. Cavalieri and A. L. Stone, THIS JOURNAL, **77**, 4699 (1955); **78**, 353 (1956)). Such a model would have both internal and peripheral phosphate groups.

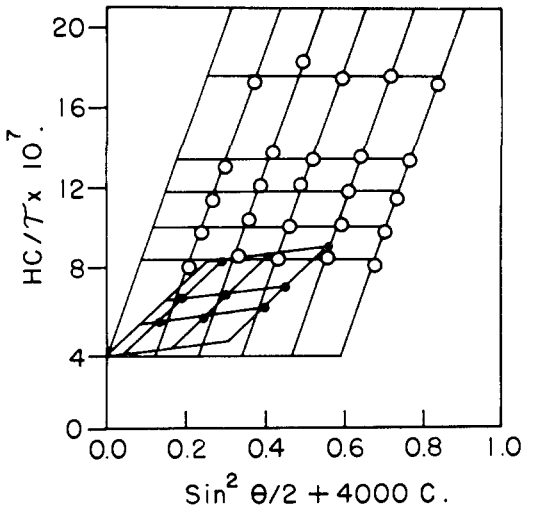


Fig. 5.—Typical Zimm plot of light scattering data, showing angles from 35 to 60°. The large grid is undenatured S-I DNA, 0.03 × 10^{-3} to 0.15 × 10^{-3} g./ml. in 0.2 *M* NaCl. The small grid, superimposed for comparison, shows part of the scattering data between 35 and 60° for water-denatured S-I DNA. The results are given in Table II, rows a and d.

as the degree of denaturation is increased by initial dilution in water from 0.75 to 0.08 mg./ml. (Fig. 1). Evidently the molecule starts becoming more flexible long before the optical density starts to rise. The lowest intrinsic viscosity shown indicates a contraction of the molecule to about 60% of its former length. The intrinsic viscosity in 10^{-3} *M* salt of denatured DNA (0.05 mg./ml. dissolved in water) is also lower than that of undenatured DNA (3200 and 5600, respectively), but the drop is much smaller than that in 0.2 *M* salt (3400 to 700), indicating that intramolecular repulsions are quite effective in preventing the contraction of even highly denatured molecules. It can be seen from the slopes of the curves in Fig. 1 that intermolecular interactions decrease on denaturation. Viscosity measurements also confirm the absence of water-denaturation in the vicinity of *p*H 8. The reduced specific viscosity curve for DNA (0.05 mg./ml.) dissolved in water of *p*H 9.0 (final *p*H of the solution, 8.4) and then made 0.2 *M* in NaCl was identical with that for undenatured DNA (curve A, Fig. 1), from which it follows that *the increased ability of the denatured molecule to contract is a consequence of proton addition and not directly of exposure to low ionic strength.*

The flow dichroism values of solutions prepared similarly to the one just described (*p*H 8.4) are also identical with those of equivalent solutions dissolved initially in 10^{-3} *M* salt. However, when 0.08 mg./ml. of DNA is dissolved in water, or when the *p*H of an undenatured DNA solution in 10^{-3} *M* salt is lowered to 3.8 or raised to 12.3 (in each case the accompanying optical density increase is just maximal), dichroism completely disappears (see Table III). Light scattering measurements on one of these solutions (see Table II, row c) have shown that the molecule has contracted only slightly. Consequently, the loss of dichroism appears to be intrinsic, that is, it must result not merely from coiling of the molecule, but from randomization of the sugar–base bond angles. In the denatured state then, the hydrogen bonds between bases must be broken, so that the bases are no longer held in rigid order[21] but are free to move at random. This would imply the destruction of the rigidity of the molecule, and, presumably, a change in length. However, since the light scattering measurements just noted show no significant change in length, we must assume the existence of residual hydrogen bonds, possibly non-chromophoric. Further denaturation (by further dilution in water) causes these to break as well, and light scattering then shows that the molecule has crumpled (Table II, solution d).

The results just discussed refer to highly denatured DNA solutions. To gain further insight into the mechanism of denaturation, the early stages were examined. The dichroism decreases rapidly as denaturation proceeds (see Table III). Figure 6 shows that the fall of dichroism with *p*H parallels but precedes the rise of optical density in any given salt. Note that dichroism shows the titration of a small number of groups at *p*H 7, even at a high DNA concentration (0.78 mg./ml.) where no optical density rise occurs at this *p*H. Dichroism then, like viscosity, is much more sensitive than optical density to the early stages of denaturation. Since these two sensitive measurements are functions of the molecular extension while the optical density increment measures only chromophoric disturbances, an increase in kinking must result from the titration of only a low percentage of the chromophoric amino groups. This change in length is apparently within the experimental error of light scattering measurements.

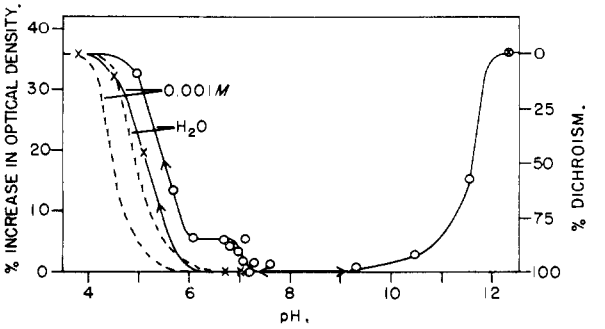


Fig. 6.—Decrease in dichroism and increase in optical density during titration. The scale on the left applies to the dashed curves; that on the right, to the solid curves: O denotes titration of 0.78 × 10^{-3} g./ml. S-I DNA in water; X denotes 0.78 × 10^{-3} g./ml. S-I DNA dissolved and titrated in 10^{-3} *M* salt. Dichroism was measured at 9480 sec.$^{-1}$ after adding salt to make the concentration 0.2 *M*.

The apparent rotary diffusion constant of partially denatured DNA is very small in water, much larger in 10^{-3} *M* salt, and slightly larger still in 0.2 *M* salt. Comparison with the θ''s for the same concentration of undenatured DNA (Table I, rows 3, 4, 5 and 6) shows that θ' for denatured DNA is greater at both salt concentrations. This supports

(21) J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737 (1953); M. H. F. Wilkins, A. R. Stokes and H. R. Wilson, *ibid.*, **171**, 738 (1953); R. E. Franklin and R. G. Gosling, *ibid.*, **171**, 740 (1953).

TABLE III

DICHROISM AND OPTICAL DENSITY AS A FUNCTION OF DENATURATION

Dichroism decrease = percentage decrease of dichroism, based on the dichroism of solution A (undenatured), in the same salt, as 100%. The dichroism values are those measured at a shear rate of 9480 sec.$^{-1}$.

Stock soln.	S-1 DNA, g./ml. × 10^3	Initial solvent	Resulting pH	pH then adjusted to	Optical density increase, %	Salt added before dichroism measurement	Dichroism at 9480 sec.$^{-1}$	Dichroism decrease, %
A	0.78	10^{-3} *M* NaCl	6.7		0	None	0.212	0
A	.78	10^{-3} *M* NaCl	6.7		0	2×10^{-1} *M* NaCl	.140	0
B	.78	H_2O, *p*H 9	7.5		0	2×10^{-1} *M*	.140	0
C	.78	H_2O			0	2×10^{-1} *M*	.117	16
D	.78	H_2O, *p*H 4	5.7		8	2×10^{-1} *M*	.0875	37
E	.78	10^{-3} *M*, *p*H 3.5	5.1		10	None	.0717	66
E	.78	10^{-3} *M*, *p*H 3.5	5.1		10	2×10^{-1} *M*	.0612	57
F	.078[a]	H_2O	6.7		23	None	.011	95
F	.078[a]	H_2O	6.7		23	2×10^{-1} *M*	.012	91
G	.78	10^{-3} *M* NaCl	6.7	3.8	36	2×10^{-1} *M*	.00	100
H	.78	10^{-3} *M* NaCl	6.7	12.3	33	None	.00	100

[a] Concentrated under vacuum to 0.78×10^{-3} g./ml. before measuring dichroism.

TABLE IV

PROPERTIES OF VARIOUS DNA PREPARATIONS

Sample	Mol. wt.[a] × 10^{-6}	$(\overline{r^2})^{1/2}$[a] (Å.)	Intrinsic viscosity (cc./g.), $[\eta]_{\theta=0}$ in 0.2 *M* NaCl	E_{260}(P)	$\frac{D'_\infty}{0.78 \times 10^{-3}}$ g./ml. DNA in 0.2 *M* NaCl	θ'	θ_0'[b]	D_∞[b]
S-I	2.8	4700	3400	6300	0.235	87	115	0.170
S-I denatured[c]	2.7	2700	700	8200	.00			
S-II	5.5	7300	3900	6300	.242	100	133	.170
S-III	4.0	7000	2500		.324	95		
D-I	5.4	5900	5700		.312	75		
D-II	6.4	7100	4900	6400	.335	38		
D-III	6.8	7900	4900	6400	.330	40	54	.175
D-III denatured[d]			4200	8040				
D-III denatured[c]			780		.00			
D-IV	6.2	7700	4900		.340	35		

[a] From light scattering in 0.2 *M* salt. [b] Extrapolated to zero concentration in 0.2 *M* salt. [c] Denatured by dissolving 0.018 mg./ml. in water. [d] Denatured by dissolving 0.08 mg./ml. in water.

the conclusion drawn from viscosity data that intermolecular interactions are reduced by denaturation. (Although θ' is also sensitive to length, it has already been mentioned that light scattering shows the length of partially denatured DNA molecules to be substantially unchanged.)

III. Various Samples of DNA.—The results of a comparison of DNA samples prepared by various methods are presented in Table IV and Fig. 7. Samples S-I, S-II and S-III were prepared identically according to the method of Schwander and Signer,[4] yet few of their properties are the same. Samples D-I–IV were prepared according to the method of Kay, Simmons and Dounce,[5] except that, in the cases of II–IV,0.005 *M* NaCl was used wherever water was prescribed, and acetone was not used to wash the fibers; in addition, D-IV was stored as a concentrated sterile solution in 0.005 *M* salt, rather than as air-dried fibers. The measured properties of D-II–IV are almost identical, indicating that air drying is not deleterious and suggesting that the use of dilute salt solutions in place of water increases the reproducibility of the preparation procedure. Sample D-I, in contrast, appears to be somewhat "denatured" (note the lower slope of its viscosity curve, Fig. 7A, and its lower dichroism and higher θ', Table IV).

The rotary diffusion constants, θ, of S-I, S-II and D-III in 0.2 *M* salt were obtained by extrapolating θ' to zero concentration. The specially prepared D-III sample has a much lower θ than the S samples; D_∞ (at zero concentration), however, is nearly the same in all three. This indicates that although the lengths may vary, the internal structures are essentially the same.

Certain inconsistencies between the properties reported in Table IV cannot be explained, unless it be admitted that each method is selective in its measurement and that the results are therefore weighted differently. For example, intrinsic viscosities calculated from the Flory–Fox equation using light scattering data depend on assumptions regarding the polydispersity and degree of flexibility of the molecules. While some of the calculated intrinsic viscosities agree with the experimental values, most do not. The most striking disagreement was found for the samples with the highest molecular weights, presumably the least degraded. The use of the Flory–Fox equation therefore appears to be unjustified.

All the DNA samples investigated are subject to denaturation (or further denaturation), although the extent of the resulting damage may vary. Data on D-III DNA dissolved in water at concentrations of 0.08 and 0.018 mg./ml. (Table IV and Fig. 7B) show that the intrinsic viscosities and the

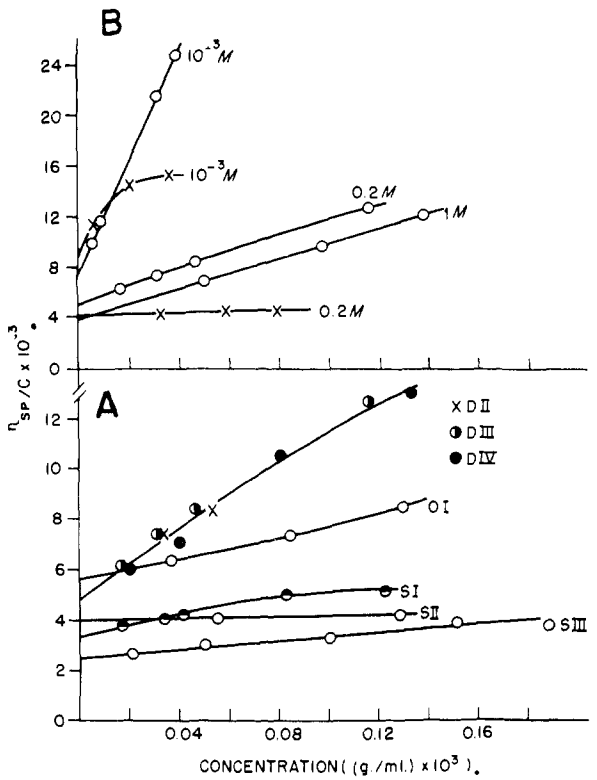


Fig. 7.—Reduced specific viscosities at zero shear: A, various DNA samples in 0.2 M NaCl. A single line was drawn through the points for D-II, D-III and D-IV, since variations among them were small: B, D-III DNA; O denotes undenatured DNA; X denotes DNA denatured by dissolving 0.08 $\times$ 10^{-3} g./ml. in water before adding salt.

slopes of the η_{sp}/C *vs.* C curves decrease in both 10^{-3} and 0.2 M salt, the extinction coefficient rises 26%, and the dichroism disappears.

Sample TF, prepared from streptomycin-resistant pneumococcus according to the method of Avery, MacLeod and McCarty[3] and never exposed to less than physiological salt, lost most of its transforming activity when exposed to water (Table V). In various salt concentrations above 10^{-3} M, however, its activity was constant.[22] These results suggest that the denaturation discussed in this paper is biologically significant, and that experiments performed on denatured material may be meaningless in elucidating the function of DNA.

TABLE V

TRANSFORMING ACTIVITY AND NaCl CONCENTRATION

Activity based on that in 0.145 M NaCl, DNA concentration 0.01 $\times$ 10^{-3} g./ml.

Minimum salt exposure, M	Transforming activity, %
0.145	100
.031	100
.011	100
.0015	100
.0003	10

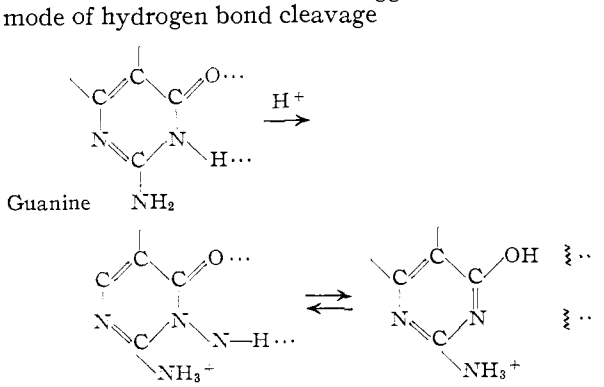
(22) S. Zamenhof, H. E. Alexander and G. Leidy, *J. Exp. Med.*, **98**, 373 (1953), using a different transforming principle, found a gradual decrease in activity with decreasing ionic strength below 0.145 M. Perhaps in dialyzing against 0.02 and 0.002 M salt, the pH of their solutions was lowered sufficiently to cause denaturation.

In order to avoid this possibility, precautions against denaturation should be taken both while isolating DNA and while preparing it for various measurements. Our results suggest that preparation procedures should be modified by substituting 10^{-3} M NaCl for water, and by washing the fibers in ethanol only and drying them in air. Undenatured DNA solutions at neutral pH must be prepared by stirring in no less than 10^{-3} M NaCl. It should be recognized that in experiments in which the ionic strength is varied, the lower limit in the case of sodium salts is in the vicinity of 10^{-3} M; comparison of results obtained in lower salt concentrations with results in 10^{-3} M or higher salt is invalid. DNA may be denatured in many ways: by heat, low X-ray doses, drying, etc., as well as by exposure to unstable pH regions (*e.g.*, by dissolving in water, by raising or lowering the pH, or by dialyzing against media of low ionic strength). It is possible that denaturation, by whatever means, is synonymous with hydrogen bond cleavage, and that the protective effect of salt in many of these cases is due to a pK shift, as described above.

Conclusion

It appears reasonably certain that the titration of certain amino groups, accompanied by hydrogen bond cleavage, constitutes what has been termed denaturation.[2] This may be brought about either by lowering the pH of the solution, or, equivalently, by raising the pK_a of the groups at constant pH (*i.e.*, by removal of salt). The spectral titrations suggest that only one type of group is responsible for the optical density increments. The pK'_a of this group varies from about 7 to 2.5, depending on field effects, which in turn depend on the salt concentration. We suggest that this group is guanine. In another paper[23] we present independent potentiometric data which supports this view. There it was shown that the acid titration of groups above pH 3.3 (in 0.1 M salt) did not result in denaturation, and that below this pH the extent of denaturation closely paralleled the titration of guanine.

For acid denaturation, we suggest the following mode of hydrogen bond cleavage



For alkaline denaturation, the hydrogen bond cleavage is different, presumably involving the removal of protons from the ring nitrogen of thymine, as well as of guanine.

(23) L. F. Cavalieri and B. H. Rosenberg, *Biochim. Biophys. Acta*, in press.

Appendix

The general expression for the flow behavior of solutions is $\beta = f(\tau)$, where β = rate of shear, and $f(\tau)$ is some function of the shear stress τ. The problem of viscometry is the determination of the form of this function from experimental measurements. In the case of Newtonian fluids, the function becomes τ/η, where η is the viscosity.

For a concentric cylinder viscometer

$$\beta = r\frac{d\omega}{dr} = f(\tau)$$

where ω is the angular velocity at a distance r from the axis of rotation. Integration of this equation between the limits of r_1 and r_2, the radii of the inner and outer cylinders, respectively, gives

$$\int_{r_1}^{r_2}\frac{f(\tau)}{r}dr = \omega_0$$

where ω_0 is the angular velocity of the outer cylinder. Since the viscous torque $M = 2\pi L\tau r^2$ = constant, where L is the length of the inner cup, we have $-d\tau/dr = 2\tau/r$ and $\tau_2 = k\tau_1$, where $k = (r_1/r_2)^2$. The integral now becomes

$$\omega_0 = {}^1/_2\int_{k\tau_1}^{\tau}\frac{f(\tau)}{\tau}d\tau$$

Differentiation of this equation with respect to τ_1 gives

$$\frac{d\omega_0}{d\tau_1} = \frac{1}{2\tau_1}[f(\tau_1) - f(k\tau_1)]$$

This expression contains the solution for the desired function.

An additional differentiation and expansion of $f(k\tau_1) = f(\tau_1 + h)$ in a Taylor's series, neglecting derivatives higher than the second, allows an approximate solution of $f(\tau_1)$ from the two differential equations. If the difference in radii is a small fraction of the average radius, one obtains

$$f(\tau_1) = \frac{2}{1+k}\left(\frac{2k\omega_0}{1-k} + \tau_1\frac{d\omega_0}{d\tau_1}\right)$$

The general relation for relative viscosity is

$$\frac{f(\tau_1)_{solvent}}{f(\tau_1)_{soln.}} = \eta/\eta_0$$

If the solvent is Newtonian, then

$$f(\tau_1)_{solvent} = \frac{2\omega_0}{1-k}$$

and

$$\eta/\eta_0 = \frac{\left(\frac{\omega_0}{\tau_1}\right)_{solvent}(1+k)}{2k\left(\frac{\omega_0}{\tau_1}\right)_{soln} + (1-k)\left(\frac{d\omega_0}{d\tau_1}\right)_{soln}}$$

A plot of β *versus* V^2 (voltage squared) for solvent and solution enables the terms to be evaluated. The term $d\omega_0/d\tau_1$ is found at any point by graphical differentiation.

It is clear that the error committed in neglecting the non-equality of the two radii will be greater the more pronounced the curvature of the β *versus* V^2 plot, and in the direction of higher apparent relative viscosities. In the present work, for solutions only slightly shear dependent, and for $k = 0.823$, the correction amounted to about 5%.

Acknowledgment.—The authors wish to thank Dr. Rollin Hotchkiss and Marian Dowling for help with the transforming factor, Mr. Arthur Grayzel for preparing the DNA samples, and Dr. George Bosworth Brown for helpful discussions and suggestions.

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On the Bjerrum Relation and the Formation of Ion Pairs

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The Bjerrum relation for the ratio of the ionization constants of a dibasic acid or general bolaform electrolyte is derived from a consideration of the Grand Partition Function. The derivation emphasizes the physical processes occurring and enables the relationship between the depression of the secondary ionization constant of a dibasic acid and the formation of ion-pairs to be studied. The interrelation between ion association in solutions of polyelectrolytes and the properties of bolaform electrolytes is briefly discussed.

I. Introduction

It is well known that the primary (K_1) and secondary (K_2) dissociation constants of a dibasic acid usually differ by more than a factor of four. If the ionization processes at the two acid groups were independent K_1/K_2 would be exactly four, since both the un-ionized and doubly ionized species have symmetry numbers of two and the singly ionized intermediate has a symmetry number of unity. A theoretical explanation of the deviation of K_1/K_2 from the value 4 was given by Bjerrum[2] in terms of the electrostatic forces operative within the molecule. Subsequent refinement of this model by Kirkwood and Westheimer[3] has shown that the concepts originally introduced are adequate to account quantitatively for the observations.

In recent years, interest in this problem has been reawakened from two diverse points of view. Fuoss and co-workers[4-7] have extensively studied the properties of a class of compounds known as bolaform electrolytes. (It will be recalled that a bolaform electrolyte is one in which the charges are

(1) Junior Fellow, Society of Fellows, Harvard University.

(2) N. Bjerrum, *Z. physik. Chem.*, **106**, 219 (1923).

(3) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938).

(4) R. M. Fuoss and D. Edelson, THIS JOURNAL, **73**, 269 (1951).

(5) R. M. Fuoss and V. H. Chu, *ibid.*, **73**, 949 (1951).

(6) H. Eisenberg and R. M. Fuoss, *ibid.*, **75**, 2914 (1953).

(7) O. V. Brody and R. M. Fuoss, *J. Phys. Chem.*, **60**, 156 (1956).