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

Studies on the Structure of Nucleic Acids. VIII. Apparent Dissociation Constants of Deoxypentose Nucleic Acid[1]

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The ultraviolet spectrum of DNA is discussed and a basis is developed for calculating the apparent dissociation constants (pK_a') of the nuclear hydroxyl and NH_3^+ groups of the deoxycytidylic and deoxyguanylic acid moieties. From the effect of salt on the pK_a's it is concluded that there are charged phosphate groups near the various amino groups, possibly resulting from the intertwining of two or more double helices with one another. The effect of pH changes on the macromolecular structure of DNA is also discussed.

The dissociation constants of calf thymus deoxypentose nucleic acid (DNA) isolated in a mild manner were first determined by Hammarsten[2] and since that time a number of other investigators[3-6] have obtained essentially similar results. Recently, Lee and Peacocke[7] have shown that the values of the dissociation constants are greatly affected by salt, although they made no attempt to calculate these values. The present paper reports the results of a spectrophotometric titration which permit a possible correlation of the apparent dissociation constants with specific groups in the DNA molecule.

Experimental

The sample of calf thymus DNA (sodium salt) was prepared according to the method of Schwander and Signer.[8] Its composition is as follows: adenine 8.8%, guanine 7.6%, cytosine 5.3%, thymine 7.7%, N 13.42%, P 7.97%, water 21.3%, $E_{1\,cm.}^{1\%}$ 201 (H_2O, pH 6.8, 260 mμ), molecular weight 5.8 million (light scattering). Crystalline beef pancreatic deoxyribonuclease was purchased from the Worthington Biochemical Laboratory.

Method of Measurements.—Stock solutions (1 mg./ml.) were made as follows: ten mg. of DNA was allowed to swell overnight in 1 ml. of water. Nine ml. of water was then added gradually, while stirring. Appropriate amounts of the stock solution were delivered by means of a microburet into 3 ml. of solvent contained in 1 cm. silica cuvettes. Measurements were made with a Model DU Beckman Spectrophotometer with an accuracy of ± 0.002 unit on the optical density scale. Cell corrections were determined immediately before adding the DNA to the cells. The addition of salt, sodium hydroxide and hydrochloric acid was also accomplished by means of a microburet, using increments of about 0.001 ml.; solutions were stirred with a stream of nitrogen. The concentrations were: sodium chloride, 5 M; hydrochloric acid, 0.1 and 1.0 M; sodium hydroxide 0.1 M. pH measurements were carried out with a Model G Beckman pH meter.

Results and Discussion

In general, a plot of optical density change *versus* pH yields a typical sigmoid curve whose mid-point is the pK. The procedure is repeated at a number of wave lengths and under optimal conditions the pK will be identical for all wave lengths. For a molecule such as DNA, however, it is necessary to know the concentrations of the component nucleotides and the spectra of the individual nucleotides as a function of pH in order to be able to assign apparent dissociation constants to the component nucleotides. The difficulty apt to be encountered is that macromolecular interactions alter the optical density changes. In the case of DNA it appears that these interactions can be assessed and corrections can therefore be made.

Advantage was taken of the fact that at 260 mμ the optical density of the individual nucleotides does not change with pH in the acid region.[9] For the DNA macromolecule at 260 mμ, however, changes are observed[5] and these have been attributed to alterations in macro-structure. In the present investigation the increments with pH at 260 mμ have been taken as a blank and subtracted from those occurring at 250, 280 and 290 mμ. In Fig. 1, curves A, B and C represent titrations carried out in water; A′, B′ and C′ were carried out in 0.1M salt. In the acid range the increments at 280 mμ (curves B and B′) are due principally to the deoxycytidylic acid moiety with only minor contributions from the other component nucleotides. The mid-points of these curves have therefore been assigned as the pK_a' values of deoxycytidylic acid in the DNA molecule. At 250 and 290 mμ both deoxycytidylic and deoxyguanylic acids contribute to the increments. The contribution of each was calculated from the concentration of DNA, its nucleotide analysis and the known increments of the mononucleotides as a function of pH, using the assigned pK_a' value. pK_a' values in the alkaline range were calculated similarly. Since the spectrum of deoxyadenylic acid remains nearly unchanged with pH, its pK_a' in DNA could not be calculated.

The open circles in Fig. 1 are the corrected experimental points, while the unbroken lines are theoretical curves based on the pK_a''s given in Table I. It will be noted that these pK_a''s are about 2 units higher than those of the individual nucleotides and that the values calculated for these three wave lengths are in good agreement both in the presence and absence of salt. These calculations include the implicit assumption that the entire wave band is uniformly effected by the alterations in macrostructure. This seems plausible since it is highly improbable that pK_a''s calculated for three wave lengths would agree if this were not so. It should be noted that any given pK determined in this manner may not represent all the groups present;

(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) E. Hammarsten, *Biochem. Z.*, **144**, 383 (1924).

(3) J. M. Gulland, D. O. Jordan and H. F. W. Taylor, *J. Chem. Soc.*, 1131 (1947); D. J. Cosgrove and D. O. Jordan, *ibid.*, 1413 (1949).

(4) R. Signer and H. Schwander, *Helv. Chim. Acta*, **32**, 853 (1949).

(5) L. F. Cavalieri, THIS JOURNAL, **74**, 1242 (1952).

(6) J. Shack and J. M. Thompsett, *J. Biol. Chem.*, **197**, 17 (1952).

(7) W. A. Lee and A. R. Peacocke, *Res. Suppl.*, [6] **2**, 155 (1953).

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

(9) See Beavan, Holiday and Johnson, "Nucleic Acids," Chargaff and Davidson, Academic Press, New York, N. Y., 1955, p. 513.

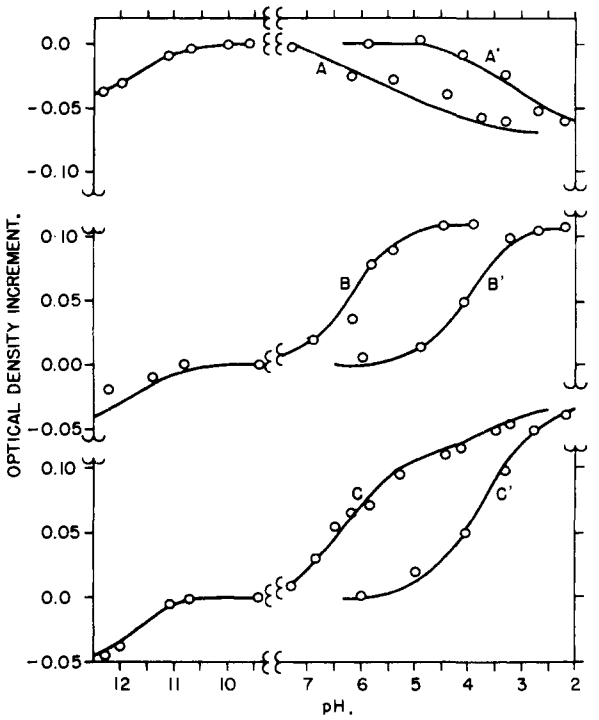

Fig. 1.—Determination of the apparent dissociation constants. The increments shown at 250 (curve A), 280 (curve B) and 290 mμ (curve C) were obtained (for various *p*H values) by subtracting the increments at 260 mμ from those observed at 250, 280 and 290 mμ. Curves A, B and C represent titrations in water; A′, B′ and C′ represent those carried out in 0.1 *M* salt.

hence potentiometric data need not necessarily agree. Since our curves are clear-cut we feel that any differences between our interpretations and those that have been drawn from potentiometric data may be due to differences in the DNA concentrations employed in the determinations.

It can be seen from Table I that 0.1 *M* salt lowers the apparent dissociation constants by about 2 units, a situation which is indicative of a field effect; a similar salt effect has been observed potentiometrically.[7] In DNA the singly charged phosphate groups may serve as field sources. That such a field effect is capable of operating in a polynucleotide system has been demonstrated in the following manner. A solution of DNA was treated with DNAase and magnesium chloride. The resulting mixture of mono-, di- and oligonucleotides was titrated spectrophotometrically; the pK'_a of the deoxycytidylic acid moiety was found to be 4.7, and the pK'_a of the deoxyguanylic acid moiety was 2.8. In 0.1 *M* salt the pK'_as were 4.3 and 2.5, respectively. The oligonucleotides contain significant amounts of tri-, tetra- and pentanucleotides and the phosphate groups in these derivatives are capable of virtually touching the various amino groups. It appears reasonably certain, therefore, that field effects can account for the fact that the pK'_a values are higher than in the respective mononucleotides.

It should be emphasized that in DNA a field effect may occur either within a single chain or between chains, depending on the rigidity of the structure. The field should operate most effectively through the DNA molecule, where the dielectric constant is lower than that of the solvent. However, in the double helical structure[10] the smallest distance between a phosphate group and an amino or hydroxyl group is about 7 Å.[11] and the molecule would have to be greatly kinked in order to permit a closer approach of a phosphate from one part of the chain to an amino or hydroxyl group in another part. Light scattering measurements[12] show that the molecule behaves like a relatively stiff coil. Under these circumstances it is improbable that a phosphate group can exert a field effect on a purine or pyrimidine within the same molecule. A simple modification suffices to make the proposed model[10] consistent with the existing data. We suggest that there exist in solution packets of double helices arranged in such a manner as to permit close approach of the charged phosphate groups to the various amino groups. Further, this structure cannot be one resulting from mere association, or it would dissociate at the high dilutions used for the determinations. The packets must therefore represent a structure with integrity, not readily alterable with environment; for example, one involving double helices entwined about each other, providing thereby an opportunity for intimate interaction. Such a structure would also provide the heterogeneity of phosphate groups which is required by dye binding data[13,14] but unaccounted for by the simple helix model, where the phosphates are symmetrically disposed about the long axis and provide no opportunity for electrostatic heterogeneity. The interrupted double strand model proposed by Dekker and Schachman[15] cannot account for our results, but would be consistent with them if intertwinning were also postulated.

DNA was found to possess an apparent stability at low *p*H values. Titrations were carried out by adding very small increments of acid or alkali. Back titration from about *p*H 2.3 to neutrality gives pK'_a's for deoxycytidylic and deoxyguanylic acids of 5.3 and 3.7, respectively (Table I). The decrease over the values obtained in the forward titration may be accounted for on the basis of the salt effect arising from salt formed on neutralization of the hydrochloric acid. This suggests that the structure has not changed materially from an electrostatic point of view as a result of the gradual decrease in *p*H in the forward titration. However, the macro-structure has been altered since the back-titration curve at 260 mμ (Fig. 2, curve B) is markedly different from the forward.[16] Similarly, the back-titration curve differed from the forward in

(10) J. D. Watson and F. H. Crick, *Nature*, **171**, 739 (1953).

(11) M. H. F. Wilkins, A. R. Stokes and H. P. Wilson, *ibid.*, **171**, 739 (1953).

(12) M. E. Reichmann, S. A. Rice, C. A. Thomas and P. Doty, THIS JOURNAL, **76**, 3047 (1954).

(13) L. F. Cavalieri and A. Angelos, *ibid.*, **72**, 4686 (1950).

(14) L. F. Cavalieri, A. Angelos and M. E. Balis, *ibid.*, **73**, 4902 (1951).

(15) C. A. Dekker and H. K. Schachman, *Proc. Natl. Acad. Sci.*, **40**, 894 (1954).

(16) Cleavage of hydrogen bonds due to high local concentrations of acid is minimal since increments of 0.001 ml. were added to 3 ml. of DNA solution.

TABLE I

APPARENT DISSOCIATION CONSTANTS DETERMINED SPECTROPHOTOMETRICALLY

	λ, mμ	Water	0.01 *M* NaCl	0.1 *M* NaCl	Back-titration[a]
Nucleotide moieties in DNA					
Deoxycytidylic acid (NH_3^+)	250	6.0	5.2	3.8	5.2
	280	6.2	5.1	4.0	5.3
	290	6.3	5.1	4.0	5.3
Deoxyguanylic acid (NH_3^+)	250	4.0	3.7	2.7	3.7
	280				
	290	4.0	3.6	2.7	3.7
Deoxyguanylic acid (OH)	250	11.6[b]			
	280	11.6			
	290	11.6			
Thymidylic acid (OH)	250	11.6			
	280	11.6			
	290	11.6			
Mononucleotides					
Cytidylic acid	(NH_3^+)	4.2			
Guanylic acid	(NH_3^+)[c]	2.4			
Adenylic acid	(NH_3^+)	3.7			
Guanylic acid	(OH)[c]	9.3			
Thymidine	(OH)[c]	9.4			

[a] The salt concentration as the result of forward (water) and back-titration was 0.008 *M*. [b] Shack and Thompsett[6] observed a sharp break at *p*H 11.8 for 230 and 260 mμ. However, those results include both ionization and macro-structure effects. [c] Potentiometric (*cf.* D. O. Jordan "Nucleic Acids," Chargaff and Davidson, Academic Press Inc., New York, N.Y., 1955, p. 459).

the presence of 0.1 *M* salt. It has been shown[17] that the size and shape at *p*H 6.5 remain unchanged as a result of exposure (by dialysis) to acid (*p*H 2.6) in 0.2 *M* salt. Altogether, these results tend to suggest that the changes in macro-structure are minute. In terms of the double helix it appears that small changes in the relative positions of the chromophores within the helix may occur without grossly affecting the outer contours of the molecule.

It is pertinent to consider the origin of the optical density increments at 260 mμ. Curve A of Fig. 2 shows the forward titration at 260 mμ.[18] The shoulder at ~ *p*H 6 appears to be due to changes in macro-structure involving the amino groups of deoxycytidylic acid while the sharp increase at ~ *p*H 4 is probably associated with the amino groups of deoxyguanylic acid, based on the pK'_a values given in Table I. The fact that the increase at ~ *p*H 4 occurs at ~ *p*H 3 in 0.1 *M* salt supports the view that deoxyguanylic acid is involved since the salt effect on the pK'_a of this component is similar (Table I). The steepness at ~ *p*H 4 in curve A, Fig. 2, indicates an abnormal titration process. It would appear that as neutralization begins, with the attendant cleavage of hydrogen bonds, the molecule collapses before titration is actually completed. If this is so, it may be inferred that the amino groups of deoxyguanylic acid play a critical role in the macro-structure of DNA.

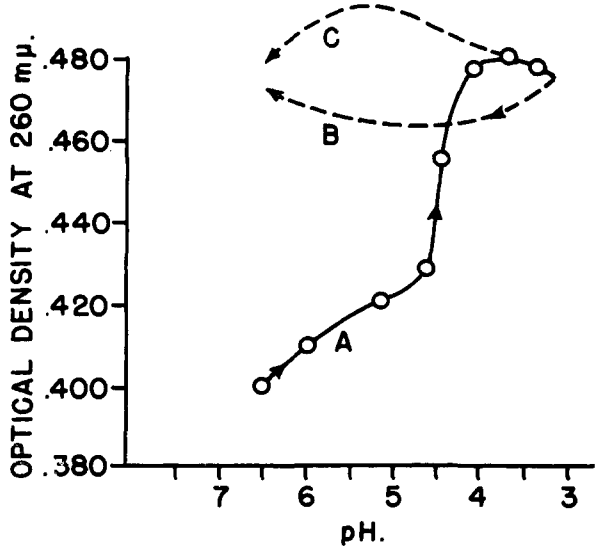

Fig. 2.—Effect of *p*H on optical density at 260 mμ: A, typical changes as function of *p*H; B, typical back-titration curve; C, back-titration curve at low DNA concentrations (optical density ~0.1).

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(17) M. E. Reichmann, B. Bunce and P. Doty, *J. Poly. Sci.*, **10**, 109 (1953).

(18) Similar studies have been carried out by E. R. Blout and A. Asadourian, *Biochem. Biophys. Acta*, **13**, 161 (1954); R. Thomas, *ibid.*, **14**, 231 (1954); and G. Frick, *ibid.*, **8**, 628 (1952). Although there is essential agreement in some areas, there are also discrepancies. The latter must be attributed to the different methods of isolation of the DNA. It cannot be stated with certainty as to which if any, of the materials are degraded.