ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE SECTION ON PHYSICAL CHEMISTRY, NATIONAL INSTITUTE OF MENTAL HEALTH, BETHESDA, MARYLAND]

Aggregation of Acridine Orange Bound to Polyanions: The Stacking Tendency of Deoxyribonucleic Acids

BY A. L. STONE AND D. F. BRADLEY

RECEIVED MAY 2, 1960

The strength of the dye–dye interaction (stacking tendency) between acridine orange (AO) cations bound to neighboring sites on the polyanion deoxyribonucleic acid (DNA) has been measured experimentally in terms of a stacking coefficient and several other parameters derived from the spectrum of the bound dye. The stacking coefficient was found to be uniformly low for native DNAs obtained from a variety of sources ($K = 1.25 \pm 0.07$) and to increase upon denaturation ($K = 2.66 \pm 0.34$ for half-denatured DNAs and $K = 6.30 \pm 0.50$ for 100°-heated DNAs). These results together with previous findings demonstrate that the strength of the dye–dye interaction is a function of the conformation of the polymer to which the dye is bound.

Introduction

Many organic dyes which do not obey Beer's law have long been thought to form dimers and higher aggregates in solution. In certain cases (*e.g.*, thionine,[1] methylene blue[1] and acridine orange[2]) this theory has been rigorously tested by studies of the variation of absorption with concentration and temperature and has been validated.

Color changes similar to those accompanying aggregation in solution also occur when certain of these dyes are bound to a number of different polyelectrolytes. Michaelis[3] and others[4-7] have proposed that the dye molecules are held sufficiently closely to one another on the surface of the polymer to allow them to interact to form aggregates similar to those found in solution. In recent publications,[8,9] this theory has been extended by the discovery that although these dyes aggregate on many polyions, the strength of the dye–dye interaction between neighboring dye molecules depends markedly on the particular polymer to which the dye is bound. The terms stacking and stacking tendency were introduced both to indicate that the interaction is maximal when dyes are ordered in card-pack fashion and that the strength of the interaction varies with the dye and polymer involved. If the dyes are bound to fixed sites on the polyions, the differences in the stacking tendencies of a dye bound to different polymers could be explained readily in terms of differences in the distance and angle between neighboring binding sites. Evidence for this interpretation was obtained[10] by showing that the stacking tendency of AO on DNA increased markedly with heat treatment of native DNA which causes the polymer to undergo a helix → coil conformational transition.

Further evidence for this interpretation could be obtained by demonstrating that all polymers with identical conformations have the same stacking tendency with a given dye. This communication gives the results of studies of the stacking tendencies of AO on 24 DNA preparations from various sources isolated by a variety of methods. Experimental and theoretical methods for evaluating the stacking tendency are described and discussed in detail.

Materials and Methods

Dyestuff.—Acridine orange (AO) obtained from National Aniline Co. was 2× recrystallized as the free base from ethanol–water by dropwise addition of NaOH less than 0.1 *M* in concentration. The precipitate was washed with water and dried under vacuum (m.p. 180–181.5°, Beilstein, 181–182°). The equivalent weight was determined by potentiometric titration with 0.100 *M* HCl in 1:1 methanol:H_2O. The average of 5 determinations was 264 ± 3 (f.w. = 266). Molar extinction coefficients were measured in a Cary Model 14 Spectrophotometer on solutions prepared by weighing aliquots of the dye, adding sufficient 0.100 *M* HCl to neutralize and filling to volume with standard buffer (10^{-3} *M* sodium cacodylate ($(CH_3)_2AsO_2Na$) + 2×10^{-4} *M* HCl *p*H 6.7). Experiments were carried out in the region of 10^{-5} *M* where the extinction coefficient of AO varies with concentration. The relation between the optical density (OD) and molarity of AO was found to be

$$\text{Molarity AO} = \frac{\text{OD (492 m}\mu\text{)}}{56000 - 4300\ \text{OD (492 m}\mu\text{)}} \quad (1)$$

The molar extinction at the absorption maximum (492 mμ) extrapolated to zero concentration is 56000. Although Zanker[2] has reported a higher value, 61000, at zero concentration in *p*H 6.0 acetate buffer at the maximum (20400 $cm.^{-1}$ = 490 mμ), the extinction coefficient from the present work (55700) is in good agreement with Zanker's observed value (57600) at the lowest concentration he employed (1×10^{-6} *M*). Stock solutions of AO were spectrophotometrically stable over periods of many months in the dark at 4°. Solutions were centrifuged at 3000 r.p.m. in the International Clinical Centrifuge (Model CL, #213 head, Will Co.) for several minutes before each experiment.

Deoxyribonucleic Acids. A. Normal Mammalian Deoxyribonucleates.—Calf thymus DNA samples *C.T.M.* (Mann Research Laboratories, M.A., highly polymerized), *C.T.S.* (Sigma Chemical Company, highly polymerized) and *C.T.W.* (Worthington Biochemicals, highly polymerized) were prepared by the method of Mirsky and Pollister[11] using

(1) E. Rabinowitch and L. F. Epstein, *J. Am. Chem. Soc.*, **63**, 69 (1941).

(2) V. Zanker, *Z. physik. Chem.*, **199**, 225 (1952).

(3) L. Michaelis, *Cold Spring Harbor Symposium on Quantitative Biology*, **XII**, 131 (1947); *J. Phys. Colloid Chem.*, **54**, 1 (1950).

(4) P. D. Lawley, *Biochim. Biophys. Acta*, **19**, 328 (1956).

(5) W. Appel and G. Scheibe, *Z. Naturforschung*, **13b**, 359 (1958).

(6) A. R. Peacocke and J. N. H. Sherrett, *Trans. Faraday Soc.*, **52**, 261 (1956).

(7) M. D. Schoenberg, C. N. Loeser and J. L. Orbison, personal communication; for a review of the subject of metachromasia in isolated dye–polymer systems see M. Schubert and D. Hamerman, *J. Histochem. Cytochem.*, **4**, 168 (1956).

(8) D. F. Bradley and M. K. Wolf, *Proc. Natl. Acad. Sci. U. S.*, **45**, 944 (1959).

(9) D. F. Bradley and M. K. Wolf, in "Neurochemistry and Nucleotides and Amino Acids," R. O. Brady and D. B. Tower, John Wiley and Sons, Inc., New York, N. Y., 1960, p. 89–108.

(10) D. F. Bradley and G. Felsenfeld, *Nature*, **185**, 1920 (1959).

(11) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).

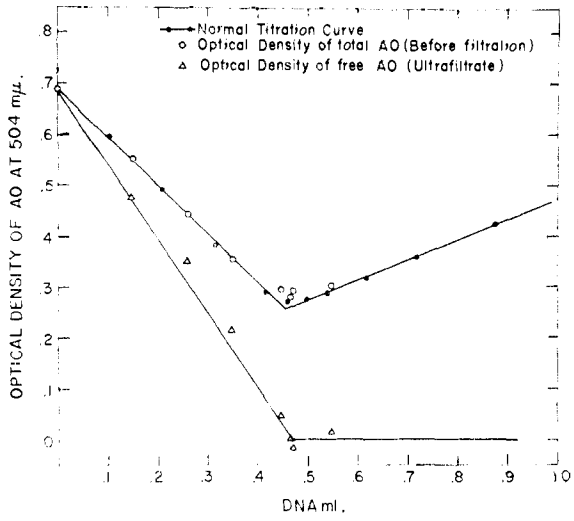


Fig. 1.—Spectrophotometric titration of AO with native DNA: ——·——, normal titration curve obtained under standard conditions; stepwise addition of DNA stock solution, 0.04 mg./ml., to the initial dye solution containing 4.6×10^{-6} mole of AO in 2.4 ml. of buffer (see text for further details); ○, optical density of AO:DNA mixtures at selected points in the titration before ultrafiltration; △, optical density of the free AO in the ultrafiltrate of the corresponding AO:DNA mixtures.

a modified Sevag technique; sample *C.T.D.-1*[12b] was prepared by one of us (ALS) using the method of Kay, *et al.*[13a] Rat intestine DNA, sample *R.I.* (prepared by ALS) and guinea pig testis DNA, sample *G.P.T.* (gift of Dr. Sanford Stone, National Institute of Allergy and Infectious Diseases) were prepared by the method of Kay, *et al.*, modified by the use of 0.001 *M* NaCl instead of distilled water.[13b]

B. Tumor Deoxyribonucleates (previously described[12,14]).—Sample $S180_4$ was obtained from the Crocker Labs., Mouse Sarcoma 180; sample $S180R_2$ was obtained from a 6-mercaptopurine resistant strain and sample $S180Rt_2$ was obtained from the resistant strain after treatment of the mice with 6-mercaptopurine.[13b] An aliquot of $S180R_2$ fibers was taken from the ethanol wash without subsequent acetone washes yielding sample $S180R_{2a}$; sample $S180R_{t2}$ floc was obtained from the first supernatant as a flocculent precipitate.

Sample RC was obtained from RC Adenocarcinoma mouse tumors[13a]; RC_4I and RC_4II were two fractions (fibrous and flocculent, respectively) of DNA isolated[13b] from the same tumor at a later time when growth potential was declining. After tumor bearing mice were treated with 6-mercaptopurine, DNA samples $RCNS_2$ (from growing tumors) and RCS (from regressing, sensitive tumors) were obtained.[13a] Sample *RCS soluble* was a readily soluble fraction of RCS using the standard buffer system. Sample Th (gift of Dr. James Rose, National Cancer Institute) was obtained from a mouse thyroid tumor[12b] and stored in physiological saline solution.

C. Salmon Sperm Deoxyribonucleates.—Samples S.Sp.1 (California Corporation for Biochemical Research) and S.Sp.2 (Mann Research Laboratories, M.A., highly polymerized) were prepared by the method of Emanuel and Chaikoff[15]; sample SS was discussed in previous publications.[8,10]

D. Pneumonococcus Deoxyribonucleate.—Sample Pn, a transforming DNA containing 4.9% RNA and stored in physiological saline, was a gift of Dr. Aaron Bendich, Sloan-Kettering Institute for Cancer Research.

E. Bacteriophage Deoxyribonucleates.—Samples T_4 was prepared from T_4 bacteriophage by one of us (DFB) using a phenol extraction procedure essentially that described by Gierer and Schramm.[16] Sample ϕX from ϕX 174 bacteriophage[17] and stored in physiological saline was a gift of Dr. Robert Sinsheimer, California Institute of Technology.

Preparation of DNA Solutions.—One ml. of standard buffer was added to 5 mg. of DNA and allowed to stand at 4° for 16–20 hr. The resulting transparent gel was further diluted by the stepwise addition of buffer over a period of several hours with gentle manual shaking. Finally, the solution was subjected to mechanical shaking (16 hr. at 3°). In most cases this procedure resulted in apparently homogeneous solutions of 1–2 mg./ml. DNA. With some tumor DNAs (RCS, RC_4I and RC_4II) the gel did not disperse upon addition of buffer. In these cases, in order to bring the samples into solution, the system was subjected to high hydrodynamic shear by passing the gel suspension rapidly through a #30 gauge syringe needle. All stock DNA solutions were centrifuged at 3000 r.p.m. in the International Clinical Centrifuge to remove any insoluble matter and stored in the dark at 4°.

Samples Th, Pn and ϕx which were obtained in salt solutions were dialyzed against standard buffer before use.

In the heating experiments, samples of the stock DNA solutions were heated at 62° and at 100° for 15 minute periods and then cooled rapidly. Previous work[10] had established that Sigma calf thymus DNA is half denatured at 62° (= Tm) under the conditions of these experiments.

Viscosity.—Viscosities were measured with a single-bulb modified Ostwald Viscometer. The sample volume was one ml. with an outflow time for water at 25° of 57 sec. and an average shear of 209 sec.$^{-1}$. Solutions of DNA at concentrations of about 0.04 mg./ml. in *M*/1000 cacodylate buffer were run at 25° to determine the low salt viscosity. Fifty μl. of 5 *M* NaCl in the standard buffer then was added to the sample and the viscosity at high salt (0.25 *M*) determined.

Sedimentation.—Sedimentation studies were made with the Model E Spinco Ultracentrifuge equipped with ultraviolet optics. Optical density tracings of the films were made either with a Spinco Analytrol microdensitometer or an enlarger-Densichron Densitometer arrangement. Dye adsorption on the cell walls was reduced by use of anodized center pieces.

Ultrafiltration.—Dye and dye–DNA solutions were filtered through 50 mμ Millipore filters (VM) using a Swinny hypodermic adapter and Luer-lock syringe. Under these conditions the dye:DNA complexes were retained quantitatively by the filter and the filtrate consisted of a solution of free dye.

Free dye adsorbed onto the syringe surfaces and millipore filter. Therefore, the system was pre-equilibrated with dye so that the amount of adsorbed dye remained relatively constant during the filtration of mixtures containing both free dye and dye:DNA complexes. The concentration of the free dye in the filtrate was corrected for the small changes in the amount of dye bound to the filter and syringe which occurred using control solutions containing similar concentrations of free dye.

Spectrophotometric Titrations.—Spectrophotometric titrations of AO with DNA were carried out (as described previously[10]) in stoppered silica cuvettes. The starting solution contained 4.6×10^{-6} mole of AO in 2.4 ml. of standard buffer and had an optical density at 492 mμ of approximately 1.0. Aliquots of the stock DNA solutions were added stepwise to this solution with calibrated Hamilton microliter syringes. The delivery of the pipets was checked by weighing the cuvette before and after each addition and was generally within ± 0.1 μl. of the rated value. The contents of the cuvette were then stirred by means of a small magnetic stirring bar[18] which remained in the cuvette throughout the titration. The stirring time ranged from 90–180 seconds depending on the volume and viscosity of the solution. The spectrum of the solution was then measured with a Cary 14 Spectrophotometer. Tests were made to establish that the spectral changes which occurred after each increment of DNA were complete and showed no further change with time. At the end of the titration the *p*H and the temperature of the solution were measured (*p*H 6.6–6.7, $T = 27 \pm 1°$).

(12) (a) A. L. Stone, Ph.D. Thesis, Graduate School of Medical Sciences, Cornell University, New York, N. Y., 1959; (b) *ibid.*, p. 43.

(13) (a) E. Kay, N. Simmons and A. Dounce, *J. Am. Chem. Soc.*, **74**, 1724 (1952); (b) *ibid.*, modified by the use of 0.001 *M* NaCl instead of distilled water.

(14) A. L. Stone, *Dissertation Abstracts*, **20**, 1157 (1959).

(15) C. E. Emanuel and I. L. Chaikoff, *J. Biol. Chem.*, **203**, 167 (1953).

(16) A. Gierer and G. Schramm, *Z. Naturforsch.*, **11b**, 138 (1956).

(17) R. L. Sinsheimer, *J. Mol. Biol.*, **1**, 43 (1959).

(18) M. K. Wolf and D. F. Bradley, *Stain Tech.*, **35**, 44 (1960).

As seen in Fig. 1 titrations show an end-point indicated by a minimum value in the volume-corrected optical density of the dye at 504 mμ. With 1.0 mg./ml. DNA solutions this end-point occurs at approximately 14 λ. The titrations are continued beyond this point until 1300 μl. of DNA have been added, that is, to approximately 100 times the amount needed to reach the end-point.

With some samples of DNA a red precipitate appeared before the end-point. To avoid precipitation a dilute solution (0.04 mg./ml.) of DNA was used in the titration. The end-point was reached at about 500 μl., and the titration was carried out to about 3 times the DNA end-point. A separate titration with the concentrated solution (1.0 mg./ml.) was then performed using a sufficiently large initial aliquot (*ca.* 25 μl.) to overstep the region of AO excess and thereby eliminate precipitation (an alternate procedure reduces molecular volume and weight of the 1 mg./ml. DNA sample by hydrodynamic shearing).

Stoichiometry.—The titration end-point was defined as the intersection of the two linear limbs of the experimental curves obtained by plotting the volume corrected optical densities or E_m *vs.* ml. DNA (upper curve, Fig. 1). The titration data were treated as follows: the moles of dye initially present were computed from the optical density and volume of the initial solution using eq. 1; molar extinction coefficients (E_m) of the dye at each point in the titration were computed from the moles of dye and the optical density and volume of each solution. From the volume of DNA added to reach the end-point, and the moles of dye, the molarity of DNA phosphates in the titrating solution was calculated assuming a 1:1 AO:DNA complex (see below). The molar extinction of the DNA in standard buffer (E_p) was calculated from the optical density of the DNA stock solution at 260 mμ and the DNA phosphate molarity computed above. This molarity was also used to compute the ratio of DNA phosphate (P) to AO (D), P/D, for each point on the titration curve.

Spectra of AO:DNA Complexes.—During the course of the titration the visible absorption spectra of the solutions vary with the relative amounts of DNA and dye. The 1:1 AO:DNA complex spectra correspond to the point in the titration at which E_{504} is a minimum. The 1:100 AO:DNA complex spectra are computed from the final experimental point in the titration, corresponding to $P/D \approx 100$.

α/β Ratio.—The free monomeric AO absorption maximum is at 492 mμ. The maximum for the 1:1 AO:DNA complex spectrum occurs at 464 mμ. These absorption bands have been designated as the α and β bands, respectively,[3] and we shall refer to the ratio of their molar extinction coefficients as the α/β ratio. The α/β ratio is a minimum near the titration end-point and increases with P/D. As shown in Fig. 2 a plot of α/β *vs.* P/D is linear and the parameters $(\beta/\alpha)_{P=D}$ $(P/D)_{\alpha=\beta}$ and $d(\alpha/\beta)/d(P/D) = s$ can be computed.

$dE/d(P/D)$.—The extinction coefficient at 504 mμ rises linearly with P/D from the end-point to approximately $P/D = 3$ (Fig. 1). The slope of the best straight line through these points is termed $dE/d(P/D)$.

F Values.—As the P/D ratio increases indefinitely the AO:DNA complex spectrum asymptotically reaches a limiting curve, the 1:∞ AO:DNA complex spectrum. At intermediate P/D the AO:DNA complex spectra lie between the 1:1 and the 1:∞ spectra. The fraction, F, of the total change from 1:1 to 1:∞ at any P/D is given by the expression (*cf.* ref. 8)

$$F = \frac{E - E_{1:1}}{E_\infty - E_{1:1}} \qquad (2)$$

where E, $E_{1:1}$ and E_∞ are the extinction coefficients at a given wave length at any $P/D > 1$, at $P/D = 1$ and as $P/D \rightarrow \infty$, respectively. Unless otherwise indicated the F values are computed at the wave length maximum of the 1:∞ complex (504 mμ) where $E_\infty - E_{1:1}$ and $E_\infty/E_{1:1}$ are maximal. $E_{1:1}$ and E_∞ are obtained from observed E values by extrapolation to $P/D = 1$ and $P/D \rightarrow \infty$ using a plot of E *vs.* $(1 - D/P)^2$.

Stacking Coefficients.—The variation of F with P/D can be expressed in terms of a parameter, K, the stacking coefficient, by an equation previously presented[8]

$$P/D = (1 - F^{1/2})^{-1} + (K - 1)(1 - F^{1/2})^{-1}(1 + F - F^{1/2})F^{1/2} \qquad (3)$$

or

$$K = 1 + \frac{P/D(1 - F^{1/2}) - 1}{F^{1/2}(1 + F - F^{1/2})} \qquad (4)$$

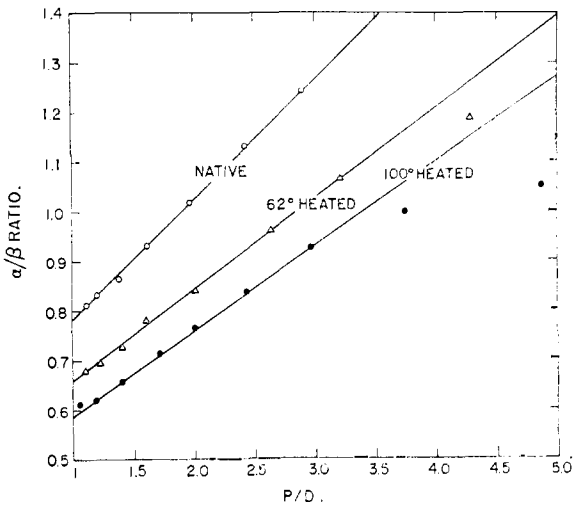


Fig. 2.—Plot of α/β ratio *vs.* P/D: o—o—o, native calf thymus DNA; Δ—Δ—Δ, 62°-heated S180R$_2$ DNA; ·—·—·, 100°-heated calf thymus DNA. The plot yields the following parameters listed on Tables II and III: the slope, $s = d(\alpha/\beta)/d(P/D)$; the reciprocal of the intercept $= \beta/\alpha$ at $P/D = 1$: the value of P/D at $\alpha/\beta = 1$.

K is computed from F and P/D values from a single titration and averaged over the range of F between 0.2 and 0.8. Outside of these limits the computed value of K is sensitive to small errors in the determination of $E_{1:1}$ and E_∞.

Results and Discussion

Formation of an AO:DNA Complex.—A typical spectrophotometric titration of AO with DNA is shown in Fig. 1 (upper curve). The linearity of the titration curve to the end-point indicates that the equilibrium is far on the side of complex formation. This indication was confirmed by ultrafiltration experiments. No free AO was present in ultrafiltrates of titration mixtures at the end-point (Fig. 1, lower curve). Additional evidence was obtained from ultracentrifuge studies which showed that there was no significant amount of non-sedimentable, ultraviolet-absorbing material in end-point titration mixtures (Table I). These data indicate that complex formation is >95% complete at the end-point.

TABLE I

SEDIMENTATION OF AO–DNA COMPLEXES

Sedimentable component in D and E had $\bar{S} = 22.3$ and 24.0, respectively, corresponding to native DNA. In C sedimentation of moving component was complete by the time rotor reached set maximum speed (59780 r.p.m.). M^* is the monomolar concentration, *i.e.*, the concentration of the monomer.

	Solution	Film optical density soln.	Film optical density non-sedimenting component
A	10^{-3} M Cacodylate buffer	0	0
B	2×10^{-5} M AO	0.26	0.26
C	2×10^{-5} M AO + 0.5×10^{-5} M^* DNA	.45	.19
D	2×10^{-5} M AO + 2.0×10^{-5} M^* DNA	.45	.01
E	Same as D + 8×10^{-3} M cacodylate	.53	.21
F	2×10^{-5} M AO + 100×10^{-5} M^* DNA	≫1	.05

TABLE II

STACKING PARAMETERS OF NATIVE DEOXYRIBONUCLEIC ACIDS

Sample	K	β/α[a]	P/D at $\beta = \alpha$	s[b]	$\frac{\Delta\epsilon}{\Delta P/D}$	Extrapolated $\epsilon_{1:1}$	Extrapolated ϵ_∞	ϵ_p	P/D at $F = 0.5$	$\frac{\eta\ 0.001}{\eta\ 0.25}$	ϵ_p from phosphate analysis	Mol. wt. $\times 10^{-6}$	A,[c] %
C.T.S.[d]	1.16 ± 0.14	1.31	1.91	0.259	9200	14000	54700	6000	3.72				
C.T.M.	1.13 ± .11	1.29	1.89	.253	9200	13900	55000	6200	3.66				
C.T.W.	1.11 ± .15	1.30	1.91	.255	9200	13500	55800	6200	3.62				
C.T.D.-I	1.15 ± .12	1.29	1.94	.239	9200	15000	54000	6100	3.70	2.44	6400	5.4	30.1[e]
S.Sp.$_1$	1.18 ± .11	1.39	2.08	.245	8300	13500	55000	7000	3.75	2.97			29.7[f]
S.Sp.$_2$	1.25 ± .18	1.35	2.15	.224	7800	13500	54000	6300	3.89				29.7[f]
R.I.	1.21 ± .10	1.36	2.00	.262	8900	13600	54500	6200	3.89				
G.P.T.	1.37 ± .16	1.32	2.11	.221	7600	14000	54000	6300	4.12	2.24			
Th		1.27	1.85	.253	8600	12800	...	..	..				
S180$_4$	1.26 ± .07	1.33	2.04	.260	8600	13200	54000	6300	3.91	2.65	6300	10.9	31.2[e]
S180R$_2$	1.19 ± .12	1.32	2.02	.237	8800	13500	55000	6200	3.77		6200	10.5	30.6[e]
S180R$_{2a}$	1.23 ± .09	1.32	2.00	.247	8600	13300	54800	6000	3.85				
S180Rt$_2$	1.24 ± .14	1.33	2.15	..	..	14500	54000	6100	3.87	1.48	6600	4.6	30.8[e]
RC$_1$	1.27 ± .21	1.35	2.18	.232	8000	14300	54000	6400	3.93	2.54	6100	4.3	29.8[e]
RCNS$_2$	1.19 ± .17	1.33	2.04	.241	8100	15000	54000	6100	3.77	2.21	5800		30.7[e]
RCS	1.29 ± .14	1.31	2.04	.230	8000	14000	54500	6300	3.93	2.42			31.6[e]
T$_4$	1.33 ± .11	1.43	2.26	.239	8300	13600	55000	6000	4.04	1.98			32.2[f]
Pn	1.54 ± .07	1.37	2.22	.224	7400	14300	55500	6400	4.45				29.8[f]
Mean	1.25	1.33	2.04	.242	8500	13900	54600	6200	3.88		6200		
Mean dev.	±0.07	±0.03	±0.09	±0.11	±500	±500	±500	±100	±0.14		±200		
ϕX	.....	1.60	3.68	0.154	4700			8750					

[a] β/α extrapolated to $P/D = 1$ from α/β *vs.* P/D plot. [b] Slope of α/β *vs.* P/D plot. [c] Adenine/total base × 100. [d] Previously reported.[10] [e] Ref.[12] [f] E. Chargaff, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, pp. 307–371.

TABLE III

STACKING PARAMETERS OF DENATURED DEOXYRIBONUCLEIC ACIDS

Sample	K	β/α[a]	P/D at $\alpha = \beta$	s[b]	$\frac{\Delta\epsilon}{\Delta P/D}$	Extrapolated $\epsilon_{1:1}$	Extrapolated ϵ_∞	ϵ_p	P/D at $F = 0.5$	$\frac{\eta\ 0.001}{\eta\ 0.25}$
Heated 100°										
C.T.S.	6.92 ± 3.50	1.72	3.75	0.179	5200	12100	49300	8400	15.8	
S.Sp.$_1$	5.40 ± 2.43	1.71	4.29	.161	4600	12300	47800	8300	12.2	
S180R$_2$	6.68 ± 3.25	1.71	4.79	.170	4900	12000	47300	7700	19.4	98.0
R.I.	6.20 ± 2.98	1.69	3.74	.155	5200	12700	50300	8300	7.8	
Mean	6.30	1.71	4.14	.166	5000	12300	48700	8100	13.8	
Mean dev.	±0.50	±0.008	±0.40	±0.008	±200	±300	±1100	+300	±3.8	
Heated 62°										
C.T.S.[c]	2.15 ± 0.07	1.48	2.61	0.202	5500	13700	54300	7100	5.61	
S180R$_2$	3.05 ± .42	1.52	2.86	.183	5400	13700	56000	7300	7.33	6.00
S180Rt$_2$	2.77 ± .34	1.55	2.88	.187	5500	13400	55400	7500	6.80	4.63
Mean	2.66	1.52	2.78	.191	5500	13600	55200	7300	6.58	
Mean dev.	±0.34	±0.02	±0.12	±0.008	±0	±100	±600	±100	±0.65	
Heated 100°:										
Native (1:1) R.I.	2.36 ± 0.13	1.51	2.64	0.207	6000	14300	55000	7300	6.02	
Other DNA										
SS DNA[c]	3.49 ± 0.49	1.54	3.68	0.260	4500	13700	53700	7500	8.17	9.85
S180Rt$_2$ floc.	1.99 ± 0.26	1.46	2.75	.180	6100	13000	55600	5700	5.31	
RC$_3$I	.....	1.39	2.39	.200	6100	13500	...	6600		
RC$_3$II	.....	1.39	2.54	.183	5800	13500	...	6700		
RCS soluble	.....	1.45	3.8	.111	4200	13300	...	5900		2.22

[a] β/α extrapolated to $P/D = 1$ from α/β *vs.* P/D plot. [b] Slope of α/β *vs.* P/D plot. [c] Previously reported.[8,10]

Stoichiometry of Complex Formation.—The (E_p)'s of the DNA solutions calculated from the titration data (Table II, col. 9) agree with those calculated from phosphate analyses and are within the expected range for native DNA (Table II, col. 12). This agreement shows that the end-point corresponds to the formation of the 1:1 complex. The titration may be used to measure DNA monomolar concentrations to within a few per cent. with small quantities of DNA (*ca.* 20 × 10^{-6} g.).

The number of titratable sites does not change upon heat denaturation. The E_p's increase upon denaturation (Table III), in agreement with previous work.[10] Single-stranded ϕX174 DNA exhibits many of the properties of heat-denatured DNAs. These data show that E_p values calculated

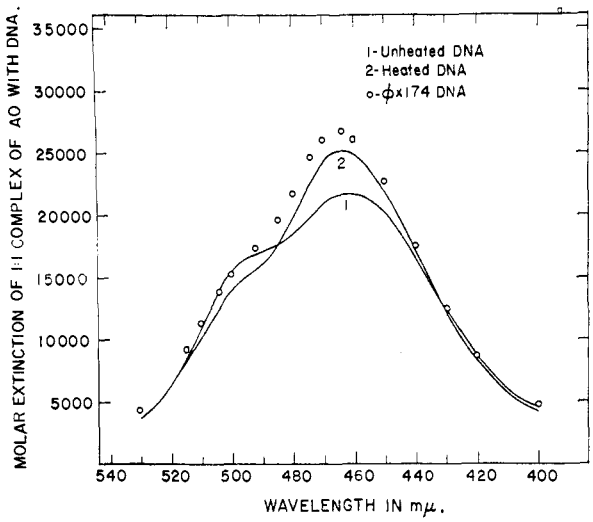


Fig. 3.—Spectra of 1:1 complexes of AO with native and denatured DNAs. Molar extinctions of AO (abscissa) at various wave lengths (ordinate) were calculated for the titration solution with minimum molar extinction at 504 mμ using standard titration procedure of stepwise addition of DNA solution to initial dye solution containing 4.6×10^{-8} mole of AO in 2.4 ml. of buffer (see text for further details). Curve 1, native calf thymus DNA; curve 2, 100°-heated calf thymus DNA; o, ϕX174 DNA.

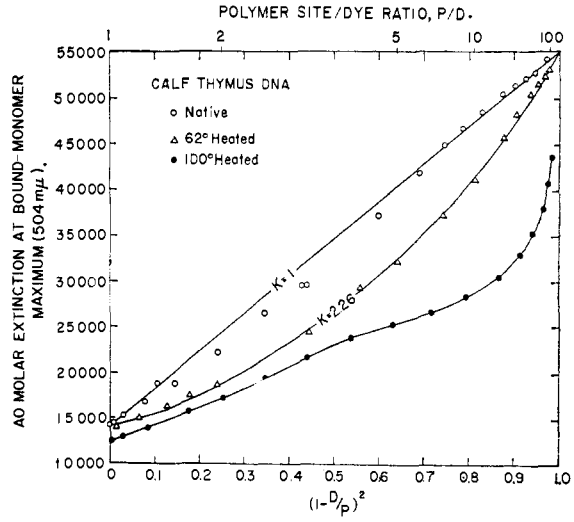


Fig. 4.—The unstacking of DNA-bound AO with increasing polymer site/dye ratio: ———, theoretical curves for $K = 1$ and $K = 2.26$; ·—·—·, experimental curve for 100°-heated calf thymus DNA. Open circles are experimental points for native calf thymus DNA. Open triangles are experimental points for 62°-heated calf thymus DNA.

from the AO titration may be used to determine the extent of denaturation of DNAs (*e.g.*, see Table III, "other DNA").

All of the E_p's reported have been calculated from titration curves at 504 mμ. Titration end-points calculated at other wave lengths, *e.g.*, 464 or 430 mμ, agree with these values within a few per cent.

Spectrum of the 1:1 AO:DNA Complex.—Under the standard conditions of the titrations of the AO spectrum has a maximum at 492 mμ with a molar extinction of 52000 and a shoulder at 464 mμ with an extinction of 39000 (*i.e.*, β/α ratio = 0.75). The 1:1 complex with native DNAs has a shoulder at 492 mμ with extinction of 17000 and a maximum at 464 mμ with extinction of 21600 (β/α ratio = 1.27) (Table IV). Thus complex formation is accompanied by a large hypochromic effect with a relative enhancement of the 464 mμ band.

It can be seen that all native DNAs have the same 1:1 AO:DNA complex spectrum (mean deviation 3%). A convenient parameter for comparing these spectra is $(\beta/\alpha)_{P=D}$ (mean deviation 3%, Table II). Heat denatured and ϕX DNA show different 1:1 spectra and β/α ratios indicating that the spectrum of the 1:1 complex is a function of the degree of intactness of the DNA helical structure (Fig. 3) and that disruption of the structure causes a relative enhancement of the (β) band.

Spectrum of 1:100 AD:DNA Complex.—Titration of AO with DNA in the region of DNA excess causes spectral changes which are complete within approximately 15 seconds. As (P/D) is increased indefinitely (to *ca.* 100:1), the β band present in the 1:1 complex at 464 mμ becomes a shoulder with an extinction of 20000 while a new band appears with a maximum at 504 mμ and extinction of 55000 (β/α = 0.47) (Table IV). Because the spectrum of the 1:100 complex is similar to that of the free-AO monomer except for a 12 mμ red shift, the new band is referred to as the bound-AO monomer band (α'). The difference in wave length between α and β bands in bound-AO (40 mμ) is almost identical with that in free-AO (39 mμ).[2]

The bound-monomer spectra of all native DNAs are the same as that shown in Table IV. The mean deviation of the extinction coefficient (E_∞) at the band maximum is 1% (Table II, col. 8). The E_∞ values reported for 100°-heated DNAs are lower (Table III). This may result from the difficulty in extrapolating to $P/D \rightarrow \infty$ for denatured DNA since the spectrum is changing appreciably at the highest P/D examined (Fig. 4). Experiments with a limited quantity of ϕX DNA ended at $P/D \approx 5$; therefore, a value for E_∞ could not be obtained.

Variation of AO:DNA Complex Spectra with P/D.—A number of parameters have been derived to measure the variation of the complex spectra with P/D: the rate, $dE/d(P/D)$, at which the extinction at a given wave length changes; the rate, $d\alpha/\beta/d(P/D) \equiv s$, at which the ratio of the heights of the α and β bands change; the P/D at which the two bands are equal, $(P/D)_{\alpha=\beta}$; the P/D at which $F = 1/2$; and the stacking coefficient, K.

All of these parameters are the same for native DNAs (mean deviation 4–6%) (Table II). These parameters are also identical for denatured DNAs (Table III), although they differ from those of the native samples. They indicate that the rate at which the spectrum changes with P/D is *less* for denatured than native DNAs.

Values of these parameters for partially denatured DNAs lie between those of the native and denatured forms, and it was found that the values for a 1:1 mixture of native and 100°-heated DNA

TABLE IV

SPECTRA OF ACRIDINE ORANGE AND ACRIDINE ORANGE: DEOXYRIBONUCLEATE COMPLEXES

Extinction coefficient $\times 10^{-2}$

λ (mμ)	360	380	400	420	430	440	450	460	464	470	474	480	485	492	500	504	510	515	530	550
Acridine orange (2×10^{-5} *M*)																				
	16	26	45	77	109	156	229	343	387	429	440	457	486	516	445	362	226	137	25	5
Acridine orange: deoxyribonucleate complex (1:77)																				
	25	27	33	47	58	78	106	165	198	249	273	296	331	426	541	549	448	303	42	9
Acridine orange: native deoxyribonucleate 1:1 complexes																				
Sample																				
C.T.S.		31	42	82	117	162	195	208	208	199	191	176	..	163	151	135	106	82	..	..
C.T.M.	24	30	43	83	121	165	201	214	213	207	197	184	178	173	159	141	109	82	31	12
C.T.W.	22	27	41	82	118	161	196	209	209	203	195	179	172	168	155	139	109	83	33	14
S.Sp.$_1$	26	32	46	87	124	167	202	216	216	208	197	181	170	162	150	136	110	86	40	..
S.Sp.$_2$	25	30	44	84	121	165	199	216	216	210	199	183	175	169	155	139	108	83	33	..
R.I.	25	31	45	85	121	164	197	209	209	202	193	179	171	166	151	136	108	83	36	..
S180$_4$	25	31	44	81	..	158	193	211	211	206	196	183	..	168	153	139	111	..	38	19
S180R$_{2a}$	26	28	43	84	121	164	196	210	210	201	191	176	167	161	148	134	106	80	32	..
RC$_1$	25	..	..	..	124	..	..	..	222	..	..	..	..	178	161	145	111	84	..	13
RCNS$_2$	27	33	50	92	132	181	218	233	233	226	216	199	191	187	170	152	118	88	..	14
RCS	26	32	45	83	118	161	194	209	211	205	196	182	175	169	154	138	109	83	36	18
T$_4$	30	38	53	93	132	178	215	229	229	219	209	192	180	171	157	143	115	88	42	..
Mean	26	32	45	85	123	166	200	215	216	208	198	183	175	170	155	140	110	84	36	15
Mean dev.	±1	±2	±2	±3	±4	±5	±6	±6	±7	±6	±5	±5	±5	±5	±4	±4	±3	±2	±3	±2
φX	27	31	46	87	125	175	227	261	267	260	246	218	196	173	153	139	113	92	43	18
Acridine orange: heat denatured deoxyribonucleate 1:1 complexes																				
62°																				
C.T.S.	21	27	40	81	119	167	209	234	237	229	218	198	184	171	155	138	108	83	34	..
S180$_4$	24	..	..	..	120	..	..	..	251	..	..	..	..	170	149	136	109	85	35	..
S180R$_2$	24	28	43	85	124	175	220	245	248	241	228	205	186	170	153	139	112	89	..	16
180Rt$_2$	24	29	43	85	123	174	217	240	243	236	223	201	184	169	152	137	111	88	..	17
Mean	23	28	42	84	122	172	215	240	245	235	223	201	185	170	152	138	110	86	35	17
Mean dev.	±1	±1	±1	±2	±2	±3	±4	±4	±5	±4	±3	±2	±1	±1	±2	±1	±2	±2	±1	±1
100°																				
C.T.S.	24	31	45	86	124	177	224	252	255	244	229	199	178	156	139	125	102	82	37	15
S.Sp.$_1$	21	26	40	81	118	167	213	242	244	235	221	191	170	150	131	120	97	78	35	..
S180R$_2$	23	29	44	85	123	175	223	254	257	248	232	203	181	161	142	129	105	83	37	13
Mean	23	29	43	84	122	173	220	249	252	242	227	198	176	156	137	125	101	81	36	14
Mean dev.	±1	±2	±2	±2	±2	±4	±5	±5	±5	±5	±4	±4	±4	±4	±4	±3	±3	±2	±1	±1

are identical with those of 50% denatured (62°-heated) DNA (Table III). Values of some parameters for φX174 DNA indicate an even less rapid rate of change of spectrum than for 100°-heated DNA.

Stacking Coefficient.—The stacking coefficients for native DNAs are nearly identical (mean deviation 6%) (Table II) and are close to unity. The small variation in K between the lowest and highest values is reproducible and may signify real differences. The average K values will vary slightly with the range of F values included in the average since there is a slight bias in the deviation of the data from a fit to equation 4, *e.g.*, although the average value of K is 1.25 as $F \rightarrow 1$ the individual values of $K \rightarrow 1.0$.

The numerical value of K differs somewhat with the wave length at which F values are computed. F values are several per cent. lower computed on the long wave length side of the bound monomer band so that K values are correspondingly higher: $K_{504\ m\mu} = 1.25 \pm 0.07$, $K_{510} = 1.62 \pm 0.10$, $K_{515} = 1.74 \pm 0.10$ for native DNA and $K_{504} = 6.22 \pm 0.67$, $K_{515} = 7.38 \pm 0.44$ for 100°-heated DNA. This discrepancy could be due to the small but unequal contributions of dimers and larger aggregates to the spectrum at these wave lengths. K values computed at the band maximum (504 mμ) rather than on the steep shoulder of the band provide a more reproducible standard.

According to the model of Bradley and Wolf[8] dyes bound to specific polyanionic sites in the 1:1 complex interact with dyes on neighboring sites through the same electronic forces which cause the dyes to aggregate in solution. This interaction can alter the energy levels of either the ground or excited states of the dye, or both, causing a spectral shift in the dye (change in energy difference between ground and excited states) and/or a nonrandom distribution of dyes among sites (change in ground state).

As excess sites are added to the system containing the 1:1 complex, the dyes, in dynamic equilibrium with a small pool of free dye in the solution, rapidly distribute among all available sites. A certain fraction, F, of these dyes will by chance

occupy sites with no nearest neighbors. As P/D increases indefinitely the fraction of such isolated dyes increases continuously from 0 to 1 and the spectrum changes continuously from the 1:1 spectrum to the 1:∞ spectrum.

Differences between the 1:1 and 1:∞ spectra result from dye–dye interactions which affect the ground and excited states of the dye to a different extent. The relatively smaller difference between the spectrum of the 1:∞ complex and the monomeric dye in solution (λ_{max} 504 and 492 mμ, respectively) as compared with the 1:1 complex and aggregated dye in solution (λ_{max} 464 and 451 mμ, respectively[2]) can be ascribed to the binding process. Differences between the 1:1 spectra of native and denatured DNAs result from differential effects of dye–dye interaction on ground and excited states of the dye bound to polymers with different conformations of binding sites.

Ground state dye–dye interaction determines the distribution of the dyes (on the sites) among isolated and interacting dyes. If there is no ground state interaction the dyes will distribute randomly, and the fraction of isolates, F, will vary with P/D as shown in eq. 3 with $K = 1$. For a random distribution $F = {}^1/_2$ when $P/D = 3.41$. Equating the fraction of isolates with the fractional change in absorption spectrum defined by eq. 2, we find that (*cf.* Tables II and III) $F = {}^1/_2$ when $P/D = 3.9$ for native DNA, 6.6 for 62°-heated DNA and 13.8 for 100°-heated DNA. Therefore native DNA nearly satisfies this criterion of randomness, while denatured DNAs are non-random. There are fewer isolates in these cases suggesting an attractive ground-state interaction. Differences observed in the rate of variation in spectrum with P/D using other parameters such as $d(\alpha/\beta)/d(P/D)$, $dE/d(P/D)$, $(P/D)_{\alpha=\beta}$ for native and denatured DNAs are explained by a larger ground-state dye–dye attraction in the case of the denatured DNAs.

Lawley treated the problem of a dye (rosaniline) distributing randomly among sites (DNA) when both free and bound dye are present in the system.[4] His data fit the random case with reasonable agreement. The treatment of this problem when all the dye is bound and there is non-randomness in the distribution has been carried out by Bradley and Wolf.[8] The degree of non-randomness is expressed as the parameter K, equal to $e^{-\Delta F/kT}$, where ΔF is the ground state free energy of interaction of a pair of neighboring bound dyes.

A probability interpretation of K may also be given. For example, for $K = 1.25$, a dye will be 1.25 times as likely to bind to any given empty site with one dye-filled neighboring site as any given empty site with no dye-filled neighbors. The average K values reported herein have been calculated from eq. 4 using F values computed from eq. 2. Because both of these equations must be considered as approximations,[19] these K values cannot be considered to correspond exactly with the definition of K in terms of the free energy of dye–dye interaction although they may be used to characterize polymers and to compute approximate values for ΔF.

(19) D. F. Bradley and S. Geisser, in preparation.

The average K value for native DNA (1.25) corresponds to a $\Delta F = -0.065$ kcal./mole of single dye molecules. The spectral shift from 504 mμ for isolated dye to 464 mμ for aggregated dye corresponds to a shift in relative energy levels of ground and excited states of +4.8 kcal. If the entropy change is zero upon aggregation, dye–dye interaction lowers the ground state by 0.065 kcal. and raises the first excited state by 4.7 kcal. These relative changes are approximately what would be predicted on the basis of recent theories of exciton interaction.[20-22]

The stacking coefficients for DNA heated to 62°, the Tm for calf thymus DNA, under these conditions varies from 2.2 to 3.1 (mean 2.66) corresponding to a $\Delta F = -0.24$ to -0.34 kcal./mole AO (mean -0.29). The variation may arise from differences in the Tm values for different samples.[23] DNA denatured in this way behaves like an equimolar mixture of native and denatured DNA, which has a $K = 2.36 \pm 0.13$ giving further support for the conclusions of Hall and Litt.[24]

The stacking coefficient of 100°-heated DNA varies from 5.4 to 6.9 (mean 6.30) corresponding to a $\Delta F = -0.50$ to -0.58 kcal./mole AO (mean -0.55). These values are not as reliable as for native and half-denatured DNA because of the difficulty encountered in extrapolating reliably to E_∞ and the deviation of the experimental curve from the theoretical equation. Using these values and ΔF for native DNA the predicted ΔF for a 50% mixture of native and denatured DNAs is $(-0.07 - 0.55)/2 = -0.31$ kcal./mole AO compared with the observed value of -0.29 for 62°-heated and -0.26 for the synthetic mixture of native and denatured DNA.

Conclusions

Stacking Tendency.—The term stacking tendency refers to all effects of dye–dye interaction and the parameters such as K, $dE/d(P/D)$, $(\alpha/\beta)_{P=D}$, etc., by which they are measured. The stacking tendency will vary with the particular dye and polymer in the complex. Differences in the stacking tendency of a particular dye on a series of polymers is related to differences in the polymers. The terms "stacking tendency of a polymer" or "stacking coefficient of a polymer" is used to refer to the dependence of the dye–dye interaction on the polymer to which the dye is bound.

Acridine orange has been shown to exhibit a wide range of stacking tendencies with different polymers: DNA ($K = 1.16$),[10] ribonucleic acid ($K = 2.9$), acid polyadenylic acid ($K = 12.3$), polyuridylic acid ($K = 109$), basic polyadenylic acid ($K = 161$), heparin ($K = 787$) and polyphosphate ($K = 827$).[8] In addition the changes in several measures of stacking tendency, $(\alpha/\beta)_{P=D}$, $(P/D)_{\alpha=\beta}$ and $dE/d(P/D)$ follow the helix → coil transition in calf thymus DNA sufficiently well to be used as measures of the extent of denaturation in a partially denatured sample.[10]

(20) G. S. Levinson, W. T. Simpson and W. Curtis, *J. Am. Chem. Soc.*, **79**, 4314 (1957).

(21) E. G. McRae and M. Kasha, *J. Chem. Phys.*, **28**, 721 (1958); M. Kasha, *Revs. Mod. Phys.*, **31**, 162 (1959).

(22) I. Tinoco, *J. Am. Chem. Soc.*, **82**, 4785 (1960).

(23) J. Marmur and P. Doty, *Nature*, **183**, 1427 (1959).

(24) C. Hall and M. Litt, *J. Biophys. Biochem. Cytol.*, **4**, 1 (1958).

We have measured the stacking tendencies of twenty-four native DNA samples prepared from various biological sources by different methods and having different nucleotide sequences and compositions, molecular weights and extensions and traces of bound ions, proteins, etc. All of these samples, with the exception of the single-stranded ϕX DNA, show the same stacking tendency regardless of the parameter chosen as a basis of comparison. Using X-ray diffraction techniques, Langridge, *et al.*,[25] have shown that all two-stranded native DNA samples have the same molecular conformation. These data provide further support for the theory that the stacking tendency depends upon the molecular structure of the polyanion to which the dye is bound and in this sense is an intrinsic characteristic of the polyanion.

Recent theories of aggregation[20-22] express the interaction as a function of the distance and angle between the interacting chromophores. Detailed application of these theories to the spectra of the bound dye should permit the calculation of the distance between binding sites in polymer systems where, unlike the case of DNA, this information is not otherwise available. Such calculations will of course determine the distance between sites in the dye–polymer complex which may differ to a limited extent from that of the free polymer in solution.

It should be possible to use the dye method described above to determine the extent of denaturation in any given DNA sample. Under identical conditions the parameters would be expected to be within the range described above. To determine the numerical values of some of the stacking parameters of DNAs under different conditions (small changes in ionic strength, *p*H, temperature, etc., or with a different dye), those parameters for known native and fully denatured samples should be measured and used as standards.

Acknowledgments.—The authors with to express their gratitude to Drs. A. Bendich, J. Rose, R. Sinsheimer and S. H. Stone for their gifts of DNA samples and to Drs. M. K. Wolf and M. Kasha for helpful comments and discussions.

(25) R. Langridge, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton, *J. Biophys. Biochem. Cytol.*, **3**, 767 (1957).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY, PITTSBURGH, PENNSYLVANIA]

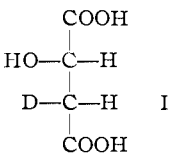
Stereochemistry of Krebs' Cycle Hydrations and Related Reactions

BY OSCAR GAWRON, ANDREW J. GLAID, III, AND THOMAS P. FONDY[1]

RECEIVED DECEMBER 23, 1960

3-Deuterio-L-malic acid obtained from the fumarase catalyzed hydration of fumaric acid in D_2O is shown to have the *erythro* configuration by n.m.r. comparison with stereospecifically synthesized *threo*-3-deuterio-DL-malic acid. The fumarase and aspartase systems thus operate by a *trans* mechanism as do the β-methylaspartase and *cis*-aconitase systems. Brewster's rules are applied to the problem of the stereochemistry of citric acid synthesized in the Krebs cycle and a configuration related to D-malic acid is arrived at. This configuration plus the α_{D_s},β_{L_s} configuration of *d*-isocitric acid permits depiction of the stereochemical pathway of the *cis*-aconitase system and of the reactions of the Krebs cycle. The possibility that *cis*-aconitase exhibits a preferred direction for addition of OH, with concomitant *trans* addition of H, is discussed.

In a previous communication[2] natural[3] 3-deuterio-L-malic acid (I) was shown to have the *erythro* configuration by n.m.r. comparison with



I

stereospecifically synthesized *threo*-3-deuterio-DL-malic acid (II). With this result the stereochemical mechanism of the fumarase and aspartase systems was unequivocally demonstrated to be *trans* and, in conjunction with previously obtained results[4,5] on the *cis*-aconitase system, the stereochemistry of the Krebs cycle from fumaric acid to α-keto-glutaric acid was elucidated.

It is the purpose of this paper to detail proof of the configuration of natural 3-deuterio-L-malic acid and to discuss the stereochemistry of Krebs cycle hydrations and the related aspartase and β-methylaspartase[6] reactions.

The stereospecific synthesis of *threo*-3-deuterio-DL-malic acid (II) was accomplished (Fig. 1) by a *trans* opening[7,8] with lithium aluminum deuteride of the oxide ring of 3,4-epoxy-2,5-dimethoxy-tetrahydrofuran (III) followed by acid hydrolysis to the dialdehyde and oxidation of the dialdehyde to *threo*-3-deuterio-DL-malic acid[9] (II).

Experimental

3-Deuterio-4-hydroxy-2,5-dimethoxy-tetrahydrofuran (IV).—The deuterated tetrahydrofuran derivative was obtained from 3,4-epoxy-2,5-dimethoxy-tetrahydrofuran[7] by the procedure of Sheehan and Bloom[7] utilizing lithium aluminum deuteride[10] in place of lithium aluminum hydride.

(1) National Science Foundation Coöperative Graduate Fellow.

(2) O. Gawron and T. P. Fondy, *J. Am. Chem. Soc.*, **81**, 6333 (1959).

(3) Obtained by the fumarase catalyzed hydration of fumaric acid in D_2O.

(4) O. Gawron and A. J. Glaid, III, *J. Am. Chem. Soc.*, **77**, 6638 (1955).

(5) O. Gawron, A. J. Glaid, III, A. LoMonte and S. Gary, *ibid.*, **80**, 5856 (1958).

(6) H. A. Barker, R. D. Smyth, R. M. Wilson and H. Weissbach, *J. Biol. Chem.*, **234**, 320 (1959).

(7) J. C. Sheehan and B. M. Bloom, *J. Am. Chem. Soc.*, **74**, 3825 (1952).

(8) P. A. Plattner, H. Heuser and M. Feurer, *Helv. Chim. Acta*, **32**, 587 (1949); L. W. Trevoy and W. G. Brown, *J. Am. Chem. Soc.*, **71**, 1675 (1949); W. G. Dauben, R. C. Tweit and R. L. McLean, *ibid.*, **77**, 48 (1955); A. Streitweiser, Jr., R. H. Jagow, R. C. Fahey and S. Suzuki, *ibid.*, **80**, 2326 (1958).

(9) Subsequent to this work, it was found that palladium catalyzed hydrogenation of *cis*-1,2-dicarboxy-ethylene oxide proceeded in a stereospecific fashion yielding *threo*-3-deuterio-DL-malic acid by a *trans* opening. O. Gawron and T. P. Fondy, unpublished work.

(10) Metal Hydrides, Inc.