4. The systems phenylacetylene–methyl acrylate and styrene–diphenylacetylene copolymerize with a rate proportional to the square root of catalyst concentration. In the former case, abnormally high cross-termination is suggested.

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[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

Studies on the Structure of Nucleic Acids. I. Interaction of Rosaniline with Desoxypentose Nucleic Acid[1]

BY LIEBE F. CAVALIERI AND ALICE ANGELOS

Introduction

The question of the structure of nucleic acids as they exist in solution, and in particular as they exist in living cells, has received a considerable amount of attention from various quarters. Organic and biochemical evidence has aided greatly in the elucidation of the nature of the covalent linkages, while physical chemical data, such as electrophoretic patterns, sedimentation rates, titration curves and X-ray studies have been more useful in the understanding of the secondary forces which are of importance in any detailed analysis of structure. Notwithstanding the abundance of data which confronts us, many questions regarding the fine structure remain unanswered. The more important problems deal with the sequence of the nitrogenous bases, the point of attachment in the sugar molecule of the various nucleotides and the extent and nature of the branching. The question as to whether nucleic acids are mixtures of polynucleotides rather than single entities is unanswered and has rendered the problem of structure still more nebulous.

The ability of nucleic acids to bind cationic dyes has been known for some time, but not until recently has any systematic and quantitative attempt been made to study this phenomenon. Thus, Michaelis[2] has observed and correlated spectrophotometric changes of basic dyes in the presence of nucleic acids. More recently the interaction of certain antimalarial drugs with pentose nucleic acid has been discussed.[3] Since the application of binding techniques to the study of protein structure[4,5] has resulted in a measure of success, we felt that a quantitative approach to the problem on hand would yield useful information which could be correlated with existing physico-chemical data.

Experimental

Materials.—The desoxypentose nucleic acid was a sample generously supplied by Dr. Aaron Bendich, prepared from calf thymus by a slight modification of the method of Hammarsten. The material was characterized as to purines, pyrimidines, and phosphorus content by Chargaff and co-workers.[6]

N, %	13.4	Guanine, %	7.4
P, %	8.0	Cytosine, %	4.7
Adenine, %	10.0	Thymine, %	8.4

The molecular weight of thymus DNA has been variously reported, but a value of 35,000 was used for calculations, based on the measurements of Jungner, Jungner and Allgen[7] and Hammarsten.[8] The acid- and alkali-treated samples of DNA were prepared according to Gulland, Jordan and Taylor.[9] Analysis for alkali-treated sample: N, 14.1; P, 9.0; for acid-treated sample N, 13.3; P, 8.0.

The rosaniline was a commercial sample obtained from the Allied Chemical and Dye Corporation. After recrystallization from water, the nitrogen and chlorine analyses indicated 98 to 100% purity. In 0.05 *M* potassium phosphate buffer the extinction coefficient was 79,600 at *p*H 5.6 and 66,300 at *p*H 6.7 (5400 Å.). It was shown to obey Beer's law under the conditions of the study.

Anal. Calcd. $C_{20}H_{20}N_3Cl$: N, 12.48; Cl, 10.51. Found: N, 12.63; Cl, 10.31.

Method.—The binding of dye by DNA was determined by the method of equilibrium dialysis. Experiments were carried out at *p*H 5.6 and 6.7 in 0.05 *M* potassium phosphate buffer. DNA solutions varied from 0.05 to 0.2%. Five milliliters of DNA solution in 0.05 *M* buffer contained in a Visking cellophane bag were immersed in 5 ml. of dye in 0.05 *M* buffer. A group (*ca.* 24) of test-tubes was placed in a shaking device overnight which was sufficient time for equilibrium to be attained. The optical density of the solution of free dye (outside the bag) was determined in a Beckman spectrophotometer, Model DU, at a wave length of 540 mμ and the concentration calculated. Results were reproducible to within about 3%. Experiments were carried out at 3 ± 0.5°, 27 ± 1° and 32 ± 1°. Concentrations of DNA and dye were chosen such that a large proportion of dye was bound with respect to free dye concentration. The amount of dye adsorbed by the cellophane casing at each equilibrium concentration was determined from separate runs and found to be about 20% of the free dye concentration at *p*H 6.7. At *p*H 5.6 the cellophane adsorption ranged from about 30% at low dye concentrations to 15% at high dye concentrations.

Results

The data on the binding of rosaniline hydrochloride by desoxypentose nucleic acid (DNA) are presented in Figs. 1 and 2 and Tables I and II. The figures in the fifth column of Table I represent free dye concentrations to which have been added the values for the casing adsorption. This facilitates the calculations of the figures in

(1) The authors wish to acknowledge the support of the National Cancer Institute of the United States Public Health Service, the James Foundation of New York, Inc., and the joint support of the Office of Naval Research and the Atomic Energy Commission, contract N6-ori-99.

(2) Michaelis, *Cold Spring Harbor Symposia on Quantitative Biology*, **XII**, 131 (1947).

(3) Irvin, Irvin and Parker, *Science*, **110**, 426 (1949).

(4) Karush and Sonenberg, THIS JOURNAL, **71**, 1369 (1949); Karush, *ibid.*, **72**, 2705 (1950).

(5) Klotz and Urquhart, *ibid.*, **71**, 847 (1949).

(6) Chargaff, Vischer, Doniger, Green and Misani, *J. Biol. Chem.*, **177**, 405 (1949).

(7) Jungner, Juugner and Allgen, *Nature*, **163**, 849 (1949).

(8) Hammarsten, *Acta Med. Scand. Supp.*, 196 (1947).

(9) Gulland, Jordan and Taylor, *J. Chem. Soc.*, 1131 (1947).

TABLE I

BINDING DATA FOR DNA

Temp., °C.	DNA concn. × 10^5	One-half of original dye concn. × 10^6	Free dye concn., m./l. (c × 10^6) (from optical density of outer soln.)	Free dye concn. plus casing adsorption (c' × 10^6)	Bound dye, m./l. × 10^6	r	r/c × 10^{-5}
			*p*H 6.7				
3	2.86	4.28	1.04	1.30	5.96	0.208	2.00
		5.98	1.54	1.93	8.10	0.283	1.88
		8.60	2.19	2.74	11.7	.409	1.87
		17.1	4.51	5.64	22.9	.800	1.77
		21.4	5.78	7.23	28.3	.989	1.70
		25.6	7.67	9.60	32.0	1.12	1.47
		34.2	9.84	12.3	43.8	1.54	1.56
	5.72	68.4	11.2	14.0	108.8	1.90	1.69
		76.9	13.6	17.0	119.8	2.09	1.54
		85.5	15.3	19.1	132.8	2.32	1.51
	1.43	8.60	3.05	3.81	9.58	0.670	2.20
		17.1	6.59	8.24	17.7	1.24	1.87
		25.6	10.3	12.9	25.4	1.77	1.71
		42.8	18.2	22.7	40.2	2.80	1.53
		51.3	21.1	26.4	49.8	3.49	1.65
33	2.86	4.28	1.07	1.34	5.88	0.206	1.93
		5.98	1.58	1.97	8.02	.280	1.77
		8.60	2.39	2.99	11.2	.392	1.64
		12.8	3.53	4.42	16.0	.560	1.59
		17.1	5.00	6.25	21.7	.755	1.51
		21.4	6.59	8.24	26.2	.917	1.39
		29.9	9.75	12.2	35.4	1.24	1.27
		42.8	14.0	17.5	50.6	1.77	1.26
			*p*H 5.6				
3	2.86	4.28	0.63	0.94	6.68	0.234	3.72
		5.98	1.03	1.44	9.08	.317	3.08
		8.60	1.39	1.90	13.4	.470	3.37
		17.1	3.28	4.27	25.6	.895	2.74
		21.4	4.52	5.69	31.4	1.10	2.43
		25.6	5.44	6.83	37.6	1.31	2.40
		34.2	7.58	9.44	49.5	1.73	2.28
		42.8	9.56	11.8	62.0	2.17	2.27
		47.0	10.9	13.4	67.2	2.35	2.15
		51.3	12.4	15.2	72.2	2.52	2.03
33	2.86	5.98	1.24	1.67	8.62	0.302	2.43
		8.60	1.79	2.43	12.3	.432	2.41
		12.8	2.85	3.70	18.2	.636	2.23
		17.1	3.90	4.98	24.2	.846	2.17
		21.4	5.12	6.45	29.8	1.04	2.03
		25.6	6.50	8.15	34.9	1.22	1.88
		29.9	7.26	9.02	41.8	1.46	2.01
		34.2	8.86	11.0	46.4	1.62	1.83
		38.9	9.60	11.9	54.0	1.89	1.97
		42.8	11.0	13.5	58.6	2.05	1.87
		51.3	13.2	16.2	70.2	2.46	1.87
			Acid-treated, *p*H 5.6				
3	2.83	15.1	0.80	1.08	28.0	0.99	12.3
		22.6	1.29	1.67	42.0	1.48	11.5
		30.2	1.84	2.29	55.8	1.98	10.7
		37.7	2.55	3.17	69.2	2.44	9.60
		108.5	10.1	12.3	192.4	6.80	6.72
		147.5	16.4	20.0	255.0	9.02	5.48
		211.0	27.8	33.4	355.2	12.6	4.53
28	2.86	17.1	2.10	2.74	28.8	1.00	4.77
		25.6	3.44	4.42	42.4	1.48	4.30
		34.2	4.34	5.50	57.4	2.00	4.62
		42.7	5.56	7.02	71.4	2.50	4.50
		68.4	8.57	10.65	115.6	4.05	4.72
		76.9	9.99	12.3	129.2	4.52	4.52
		85.5	11.0	13.5	144.0	5.03	4.55
		107.0	14.7	17.9	178.2	6.23	4.23
		147.5	20.7	25.9	243.2	8.50	4.11
		211.0	31.9	38.9	344.2	12.0	3.77
			Alkali-treated, *p*H 5.6				
3	2.86	17.1	1.08	1.51	31.2	1.09	10.1
		25.6	1.68	2.26	46.9	1.64	9.75
		34.2	2.46	3.22	61.9	2.16	8.80
		42.7	3.16	4.08	77.3	2.70	8.55
		68.4	5.94	7.42	122.0	4.27	7.18
		107.0	11.1	13.6	186.8	6.52	5.88
		214.0	31.0	38.3	351.4	12.28	3.96
28	2.86	17.1	2.85	3.68	26.8	0.937	3.29
		25.6	4.08	5.22	40.8	1.43	3.51
		34.2	5.60	7.05	54.3	1.90	3.39
		42.7	6.98	8.73	68.0	2.37	3.40
		68.4	11.55	14.2	108.4	3.79	3.28
		76.9	12.50	15.4	122.9	4.29	3.43
		85.5	14.7	17.9	135.2	4.72	3.21
		107.0	19.3	22.6	168.8	5.90	3.06

column six. In Figs. 1 and 2, the amount of dye bound per mole of DNA (r) is plotted as a function of r/c, where c is the equilibrium dye concen-

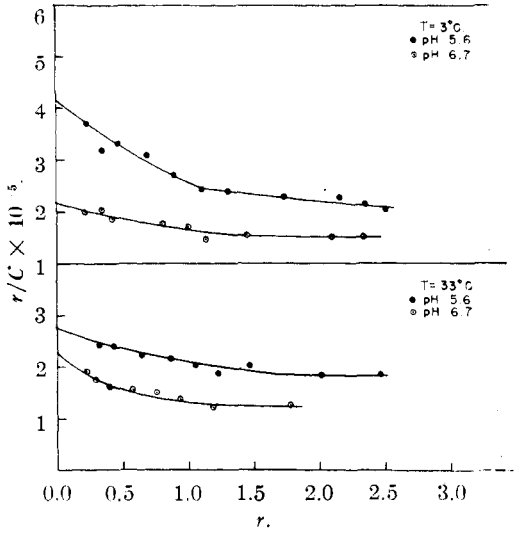


Fig. 1.—Rosaniline–DNA binding, [DNA] = 2.86 × 10^{-5} M.

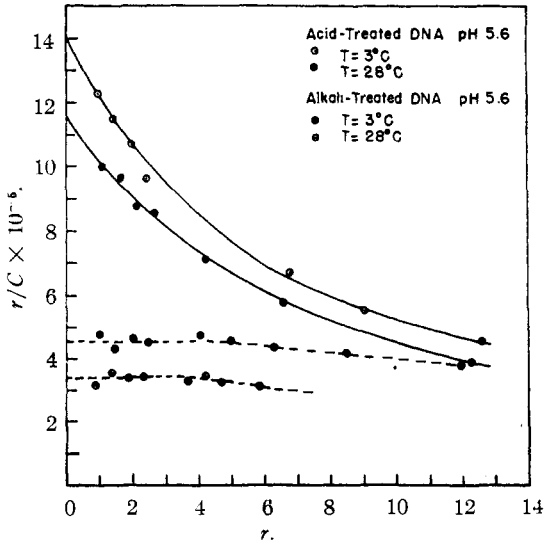


Fig. 2.

TABLE II

THERMODYNAMIC CONSTANTS FOR BINDING OF DNA

pH	6.7		5.6		5.6		5.6	
	Untreated				Alkali-treated		Acid-treated	
Temp., °C.	3°	33°	3°	33°	3°	28°	3°	28°
n_1	0.16	0.28	0.53	0.17	3.0		4.4	
n_2	23.84	23.72	17.47	17.83	27.0	27.0	75.6	75.6
$k_1 \times 10^{-5}$	3.92	3.79	4.27	3.84	2.23		2.36	
$k_2 \times 10^{-5}$	0.065	0.048	0.106	0.115	0.175	0.148	0.047	0.066
ΔF_1^0 (k. cal./mole)	−7.06	−7.80	−7.08	−7.80	−6.75		−6.78	
ΔF_2^0	−4.82	−5.15	−5.10	−5.68	−5.36	−5.83	−4.64	−5.27
ΔH_1^0	−0.24		−0.44					
ΔH_2^0	−1.79		+0.26		−1.57		+2.31	
ΔS_1^0 (E. U.)	+24.7		+24.0					
ΔS_2^0	+11.0		+19.4		+18.8		+25.2	

tration. It should be noted that r and c are independent of volume changes while the bound rosaniline (column six) is not. The binding process was shown to be reversible by immersing the (equilibrated) bags in fresh buffer and calculating r and r/c. The values resulting from the re-equilibration were in good agreement with the expected values. The effect of the concentration of DNA on the binding was examined and r/c was found to be slightly higher at 0.05% as compared to 0.1 and 0.2%. Calculations and thermodynamic constants were obtained according to existing procedures (4,5). Correction for Donnan equilibrium was not applied to the calculations since it was shown to be negligible.

Discussion

The theory and calculations relating to binding phenomena have been adequately described by other workers.[4,5,10] The relationship between the amount of dye bound per mole of DNA (r) and the equilibrium concentration (c) may be expressed by equation (1) and a plot of r/c *vs.* r should yield a straight line. In (1) k' is the intrinsic binding constant and n is the number of sites per molecule. Deviations from a straight line may be due to electrostatic interactions and a correction (equation 2) proposed by Scatchard, based on the Debye–Hückel theory, may be applied.

$$r/c = k'n - k'r \qquad (1)$$

$$(r/c)(e^{2w'r}/e^{w}) = k'n - k'r \qquad (2)$$

where

$$w' = (1 + 1/n)w$$

and

$$w = \frac{\epsilon^2 z^2}{2DkT}\left(\frac{1}{b} - \frac{K}{1 + Ka}\right) \qquad (2')$$

In (2') ϵ is the electronic charge, z the charge on the dye, D, the dielectric constant, k, the Boltzmann constant, T, the absolute temperature, b, the radius of the nucleic acid, a, the distance of closest approach and $K = [4\pi N\epsilon^2/1000DkT]^{1/2}\Gamma^{1/2}$ wherein N is Avogadro's number and Γ is twice the ionic strength of the medium. When electrostatic interactions are negligible, deviations may be due to heterogeneity of binding sites[4] and it is necessary to determine the minimum number of binding constants to express the data in mathematical terms.

In Figs. 1 and 2 it is seen that the binding of rosaniline to DNA does not correspond to the simple equation (1). In the case in which DNA was not exposed to acid or alkali (Fig. 1), the curvature is greatest in the region of r equal to 1 and electrical effects presumably could not account for this since only a small number of dye molecules are bound in this region. The situation is similar in the case of the acid- and alkali-treated samples, although deviation here occurs at higher values of r (Fig. 2). To test the applicability of equation (2) in both cases curves were constructed by plotting $(r/c)e^{2w'r}$ *vs.* r using w values ranging from 0.01 to 0.05. For $w = 0.01$ the curve followed the general contour of the uncorrected curve; that is, there was no decrease in the curvature. For $w = 0.05$ the curve was still non-linear and for large n values the curve drifted upward. Thus it appears that an exponential correction such as that represented by equation 2 is inadequate.

On the assumption that the deviation from linearity is not due to electrostatic effects we attempted to express our data in other terms. Interaction probably occurs at the ionized phosphoric acid residues of which there are essentially two types; namely, those which exhibit primary and those which exhibit secondary dissociations. The view that binding is due to the interaction of the negatively charged phosphoric acid residues and the positively charged dye has been held by Michaelis.[2] We have shown that no binding occurs with the negatively charged methyl orange both by polarographic techniques and equilibrium dialysis. The presence of the two types of phosphoric anions suggested that our data might be interpreted on the basis of at least two binding constants. These constants would actually represent two groups within which other similar constants could exist. We therefore attempted to fit our data into two-constant curves. Values for the n's and k's were obtained by solving equations (3) and (4)

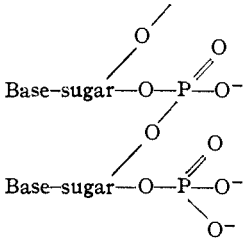
(10) Scatchard, *Annals N. Y. Acad. Sciences*, **51**, 660 (1949).

$$r/c = \frac{n_1k_1}{1 + k_1c} + \frac{n_2k_2}{1 + k_2c} \quad (3)$$

$$n_1 + n_2 = n \quad (4)$$

using the value $(n_1k_1 + n_2k_2)$ as the intercept on the ordinate $(= r/c)$. In general, two points were chosen; one at small and one at large values of r. Minimum values of n which gave the best fit were used. For this reason, we must emphasize that the values of n_1 and n_2 may not represent the actual situation, although, as we will indicate below, they can be correlated with the structure. The ratio n_1/n_2 is of greater importance and probably more significant. In Figs. 1 and 2 the continuous lines represent the theoretical curves. The satisfactory agreement shows that the binding data is expressible by at least two binding constants.

The existence of two types of sites is consistent with the present picture of the structure of DNA. The molecule is believed to be rod-like and (largely) unbranched. The sequence



where Base = adenine, guanine, thymine or cytosine

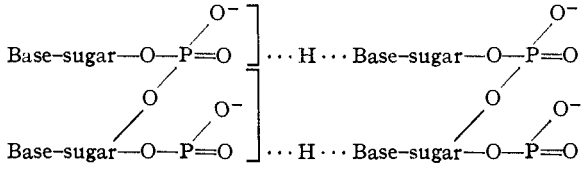
of the nitrogenous bases is unknown and the position of attachment of the nucleotides is not entirely understood. The phosphoric acid is linked to the D-2-desoxyribose by ester linkages and all phosphoric acid residues have been shown to yield primary dissociations together with a small number of secondary dissociations. The latter are believed to be present at the ends of the chains. The molecular weight of DNA (by this method of isolation) has been variously reported to be from about 10,000 to 2,000,000. Recently dipole moment[7] and osmotic pressure[8] studies have indicated the molecular weight to be 35,000. The state of *aggregation* depends on the concentration and on the presence of salts in the solution. Thus an *apparent* molecular weight of 116,900 was observed by Jungner, Jungner and Allgen after dilution of a solution of DNA from 0.1 to 0.05%.

The individual nucleotides differ in the nitrogenous bases and possibly in the position of the phosphoric acid residues. However, it may be assumed as a first approximation that the phosphoric anions are identical at the *p*H values studied. Thus the two binding constants may be correlated with the primary and secondary dissociations. The lower constant (k_2) corresponds to the primary while the higher (k_1) corresponds to secondary dissociations. However, it should not be concluded that small differences in the binding constants within each group do not exist. The region of strong binding occurs presumably at the ends of the rods since only here can the doubly charged anion exist.

The values of n_1 both at *p*H 5.6 and 6.7 are small in comparison to n_2 as would be expected on the basis that the n_1 values correspond to the secondary dissociation. Indeed, Gulland, Jordan and Taylor[9] found that there was at most one secondary dissociation for every 16 atoms of phosphorus, although these workers considered the number to be significantly smaller than one. Based on the assumption that the molecular weight is 35,000[11] there are about 130 atoms of phosphorus present in the molecule. The values of n suggest that about 18% of these are available for binding at *p*H 6.7 and about 15% at *p*H 5.6. The remainder of the sites in these cases may be involved in hydrogen bonding and are thus not available (see below). An alternative explanation for the small values of n is that some of the phosphoric residues lie buried within the molecule, thus creating a steric resistance to binding. Finally, competitive binding with potassium ions could lower the estimated value of n.

Effect of Acid and Alkali on DNA.—Gulland, Jordan and Taylor[9] found that titration of an aqueous solution of DNA with either acid or alkali produced changes in the nucleic acid. Thus, back-titration ether from *p*H 3 or *p*H 12 yielded curves which were different from the original. These workers stated that the back-titration curves indicated the liberation of amino and hydroxyl groups. In the untreated DNA hydrogen bonds were believed to exist between the oxygen and nitrogen atoms of adjacent purines and pyrimidines.

After treatment with acid or alkali we noted a distinct increase in the value of n. At 3° the acid-treated sample exhibited about a four-fold increase, while the alkali-treated DNA showed less of an increase. Since binding probably occurs at the phosphoric acid groups, it would appear that in the untreated nucleic acid the phosphoric anions are united with other groups in such fashion as to prevent the binding of the dye. Thus in addition to the hydrogen bonding suggested by Gulland and co-workers we postulate the type shown. That is, the rod-like molecules are held



together by phosphorus-base hydrogen bonds formed between adjacent molecules. This type of bonding does not invalidate the interpretation of Gulland, Jordan and Taylor, since during titration this bond could be broken by the action of the acid or alkali, thus liberating the phosphoric anions together with the amino and hydroxyl groups.

(11) It must be emphasized that the assumption of any particular molecular weight does not affect the conclusions. The question of the molecular weight could be circumvented by expressing the data in terms of moles of phosphorus per liter.

The values of n_1 in the acid- and alkali-treated samples are 4 and 3, respectively. If there were no branching in the molecule n_1 could not be greater than 2 since the secondary anions could exist only at the ends of the chain. Thus, if we are to attach any significance to the fact that the value of n_1 is greater than 2 it is necessary to postulate one or two branches in the DNA molecule. However, the present data are not definitive in this respect. We might also point out that Gulland, *et al.*,[9] state that the acid- and alkali-treated samples are similar. Our data indicate that the acid treatment results in a more effective liberation of phosphoric anions.[12]

Effect of Temperature.—At higher temperatures the acid- and alkali-treated samples yield similar curves but strikingly different from those observed at the low temperature. The curves at room temperature are approximately linear which indicates that a large proportion of the binding due to secondary dissociations is inoperative. It is of interest to note that these temperature effects are reversible. Thus, dialysis bags were allowed to reach equilibrium in the cold and then re-equilibrated at room temperature. The values resulting from the re-equilibration were identical with those obtained from solutions which had been allowed to reach equilibrium at room temperature directly. The reverse procedure also exhibited reversibility. The character of the high-temperature curves could be explained in either of two ways. The dissociation of the secondary anion might decrease with an increase in temperature, or configurational changes resulting in inaccessibility of the anions might occur.

The k_2 values at 28° for the acid- and alkali-treated samples were obtained by extrapolating the linear portion of the curves and dividing these (n_2k_2) values by the respective n_2's. The close agreement between these k_2 values and those obtained at 3° indicate that all (or nearly all) the binding at the higher temperature is due principally to primary phosphoryl dissociations. It is also noteworthy that there is close correspondence between the k_1 and k_2 values of both the treated and untreated samples.

Thermodynamic Results.—Evidence corroborating the view that the binding of rosaniline occurs at the phosphoric anions is found in the standard entropy changes of the first and second dissociations of phosphoric acid. For example in the binding of dye to the primary phosphoric anion of DNA at *p*H 6.7 and 3°, ΔS_2^0 = 11.0 e. u. and $\Delta H_2^0 = -1.45$ kcal./mole. For the addition of a proton to the H_2PO_4 ion ΔS^0 = 26.1 e. u. and $\Delta H^0 = +5.13$ kcal./mole. The positive entropy in the second instance may be ascribed to the liberation of water molecules during the addition of the proton. Similarly, in the formation of the complex between rosaniline and DNA the positive ΔS^0 is probably due, in part at least, to the liberation of water molecules during the reaction. The negative enthalpy in the case of the complex formation suggests that secondary forces are operative in the binding of dye in addition to the electrostatic effect. The standard entropy and enthalpy changes resulting from the binding to the secondary anions are also similar to those for the addition of a proton to the HPO_4 ion (ΔS^0 = 29 e.u., $\Delta H^0 = -1.15$ kcal./mole).

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Summary

The interaction of rosaniline with desoxypentose nucleic acid has been studied by the method of equilibrium dialysis. The non-linear binding curves have been adequately described in terms of a two-constant equation. The two intrinsic binding constants and the corresponding n values have been correlated with the primary and secondary phosphoryl dissociations. Since the interaction was shown to be reversible, thermodynamic constants were calculated and discussed in terms of structure.

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(12) Slight but definite variations were noted in the binding capacity of the alkali-treated samples. This may have been due to variations in the time of exposure to the alkali.