INTERACTION STUDIES WITH DNA

I. THE BINDING OF ROSANILINE AT LOW RATIO OF CONCENTRATIONS ROSANILINE:DNA, AND COMPETITIVE EFFECT OF SODIUM AND OTHER METAL CATIONS

by

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INTRODUCTION

Several compounds of interest in the field of chemical carcinogenesis yield cations in aqueous solution, which species are expected to interact with the anions of nucleic acids. The effect of metallic cations in displacing bound organic cations has been noted[1,2]. In the present work rosaniline has been chosen as an example of an organic cation able to be bound to the nucleate anion, the degree of binding being estimated by a spectroscopic method. Attention has been paid to the effect of added salts with a view to comparison of the behaviour of rosaniline with that of other organic cations.

For aqueous solutions of rosaniline Beer's Law is obeyed[3], and the same holds true if the solvent be acetate buffer (pH 4.6), aqueous NaCl, or aqueous sodium thymonucleate (DNA) provided this is in large excess (Table I). In phosphate buffers (pH 6.5 or above) fading of the dye occurs, an effect GOLDACRE AND PHILLIPS[4] have ascribed to the slow formation of carbinol base. The colour of dilute (10^{-5} *M*) aqueous solutions also decreases, but more slowly, doubtless due to adsorption of the dye on to the vessel walls. This necessitates the use of freshly prepared solutions.

The change in spectrum of rosaniline due to large excess of DNA (Fig. 1) is to

TABLE I

EXTINCTION COEFFICIENT OF ROSANILINE HYDROCHLORIDE IN WATER (ε°) AND WITH EXCESS DNA (ε')

$R \times 10^5$	4.21	3.475	3.092	2.07		8.42	4.21	3.475	3.5	
$DNA \times 10^3$	—	—	—	—		4.7	2.35	4.84	3.75	
λ $m\mu$					Average $\varepsilon^\circ \times 10^{-3}$					Average $\varepsilon' \times 10^{-3}$
430	8.65	8.67	8.55	8.57	8.61	2.88	2.69	2.82	2.71	2.78
435	10.76	10.79	10.71	10.53	10.70	3.56	3.42	3.49	3.43	3.47
440	12.95	13.11	12.91	12.95	12.96	4.36	4.46	4.32	4.28	4.35
445	15.58	15.78	15.62	15.57	15.64	5.49	5.59	5.40	5.32	5.45
450	18.34	18.52	18.38	18.25	18.37	6.85	7.02	6.80	6.55	6.80
455	21.10	21.15	21.20	20.98	21.11	8.56	8.74	8.54	8.22	8.52
460	23.65	23.86	24.00	23.52	23.76	10.34	10.15	10.65	10.46	10.40
465	26.50	26.80	26.70	—	26.67	12.57	12.16	12.80	12.46	12.50
470	29.60	29.55	29.55	—	29.57	14.90	14.50	15.14	14.86	14.87

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shift the absorption band in the visible to longer wavelengths and decrease the maximum extinction coefficient (for aqueous solutions $\lambda_{max.} = 542\ m\mu$, $\varepsilon = 7.9 \cdot 10^4$, and with excess DNA $\lambda_{max.} = 553\ m\mu$, $\varepsilon = 6.2 \cdot 10^4$). When the concentrations of rosaniline and of DNA (measured in g-equivalents per l, *i.e.* g-atom P per l) are approximately equal, a metachromatic spectrum results. The ratio of concentrations of rosaniline: DNA in the present work was confined to values less than 0.04, and over this range the spectrum of bound rosaniline does not deviate appreciably from that shown in Fig. 1 (curve 2) and Table I.

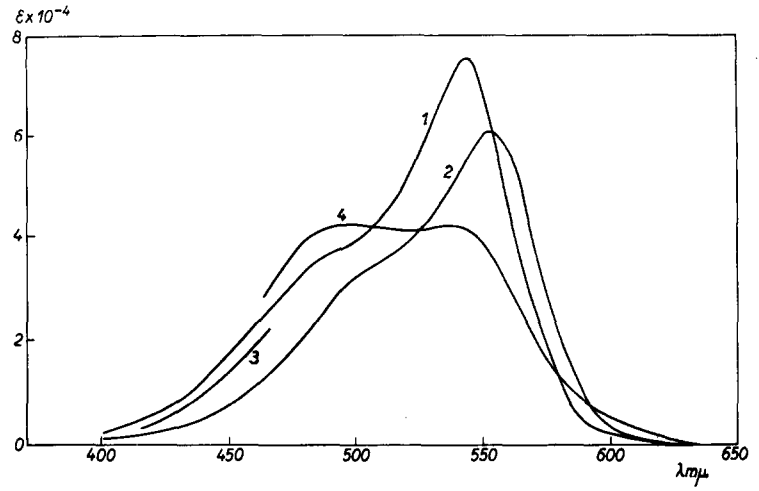

Fig. 1. Absorption spectra of rosaniline hydrochloride. (1) in water; (2) with excess DNA; (3) with excess DNA ($1.12 \cdot 10^{-3}\ N$) and 0.1435 M NaCl; (4) with approx. equivalent concentration of DNA.

Denoting the concentration of rosaniline as R, that of DNA (g-equivalents per l) as DNA, and the proportion of binding sites of DNA (assumed equal to the number of P atoms) occupied by rosaniline as β, we have for the observed optical density due to rosaniline:

$$D = \varepsilon^\circ (R - \beta \cdot DNA) + \varepsilon' \beta \cdot DNA = \varepsilon R \qquad (1)$$

where ε° denotes the molar extinction coefficient of free rosaniline, ε' that of bound rosaniline, and ε that observed.

Hence we obtain for the concentration of free rosaniline

$$R_f = R \left(\frac{\varepsilon - \varepsilon'}{\varepsilon^\circ - \varepsilon'} \right)$$

and for the degree of binding

$$\beta = \frac{R}{DNA} \left(\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'} \right) \qquad (2)$$

EXPERIMENTAL

The DNA (sodium thymonucleate) consisted of two preparations from calf thymus by the method of BUTLER, CONWAY AND JAMES[5]: analyses, T9: P = 7.5 %, H_2O = 16.2 %, N/P = 1.73; T13: P = 7.25 %, H_2O = 19.9 %. Rosaniline hydrochloride

$$[p\text{-}(H_2N \cdot C_6H_4)_2 = C = \text{C}_6\text{H}_4(\text{CH}_3) = \overset{+}{N}H_2]Cl^-$$

(E. Gurr and Co.) was recrystallised twice from water and dried.

Concentrations of DNA were *ca.* 0.1 % and of rosaniline 1 to $8 \cdot 10^{-5}\ M$.

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The spectrophotometer was the Hilger Uvispek with 1, 2 and 4 cm cells, and thermostatted cell-housing.

Generally DNA was dissolved as 0.2 % solution in 10^{-2} *M* NaCl, since lower concentrations of Na^+ enable denaturation to occur at room temperature[6]. However, in absence of added Na^+ excess DNA in water at 21° C showed the effect on the spectrum of rosaniline (Table I) expected from extrapolations to zero concentration of Na^+ from other data (Figs. 2 and 3).

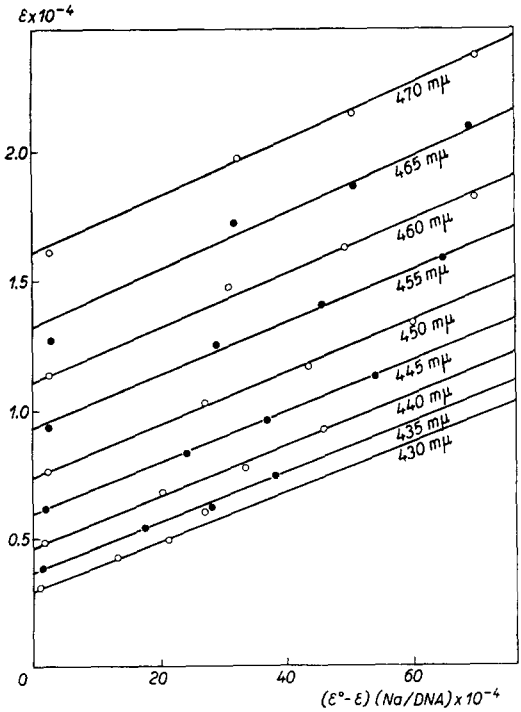

Fig. 2. Displacement of the binding of rosaniline to DNA by added NaCl (*cf.* equation[7] in text).

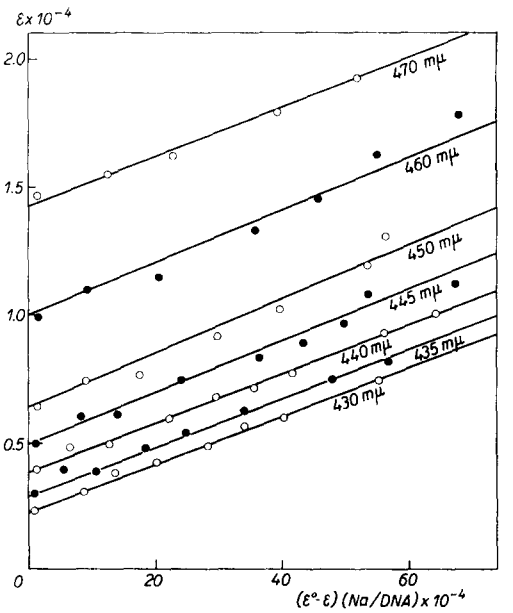

Fig. 3. Displacement of the binding of rosaniline to DNA by added Na acetate, pH 4.6.

TABLE II

DISPLACEMENT OF ROSANILINE BOUND TO DNA, BY Na^+ IONS ADDED AS ACETATE BUFFER pH 4.6

Values of $\left(\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'}\right)$, taken as $= \beta\left(\frac{\text{DNA}}{\text{R}}\right)$ = proportion of rosaniline bound

DNA = $2.42 \cdot 10^{-3}$ (T9), R = $3.52 \cdot 10^{-5}$ *M*, T = 21° C

λ mμ	Na^+ = 0.02	0.04	0.08	0.12	0.2	0.3	0.4	0.6
430	0.92	0.906	0.774	0.684	0.592	0.463	0.413	0.33
435	0.94	0.915	0.780	0.685	0.574	0.45	0.402	0.319
440	0.95	0.91	0.78	0.69	0.581	0.456	0.39	0.31
445	0.945	0.911	0.784	0.699	0.575	0.462	0.388	0.304
450	0.954	0.913	0.784	0.711	0.557	0.44	0.382	0.298
455	0.965	0.91	0.78	0.686	0.558	0.449	0.386	0.31
460	0.965	0.885	0.78	0.68	0.551	0.435	0.395	0.303
470	0.965	0.90	0.784	0.694	0.562	0.443	0.393	0.33
Average	0.95	0.906	0.781	0.691	0.569	0.448	0.391	0.311
log k'_R	3.90	3.62	3.17	2.97	2.74	2.53	2.43	2.27
k_{Na}/k_R	0.0063	0.0060	0.0084	0.0089	0.0091	0.0099	0.0092	0.0088

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The change in spectrum on binding of rosaniline by DNA was partially reversed by addition of metallic salts (for results presented in Fig. 3 and Table II, Na^+ acetate buffer (pH 4.6) was used, for Fig. 2 NaCl, and for Table III various salts as shown). The reversibility of the effect of adding salts was investigated by heating solutions to *ca.* 37° C then cooling to *ca.* 24° C. The change in spectrum due to heating (in the direction of decreased binding of the dye) was found to be reversed (Table III). CAVALIERI AND ANGELOS[7] similarly found the binding of rosaniline to DNA to be reversible, using the method of equilibrium dialysis.

DISCUSSION

The Law of Mass Action may be applied to the binding of small ions or molecules to polymers with a large number of binding sites assumed to possess equal binding affinities[8]. The resultant form is that of LANGMUIR'S adsorption isotherm:

$$\beta = \frac{k'_R (R_f)}{1 + k'_R (R_f)} \tag{3}$$

The binding constant k'_R for rosaniline is strongly dependent on the concentration of added salts. For addition of Na^+ or K^+ ions, k'_R is approximately inversely proportional to the concentration of the added metallic cation, while for the divalent cations Mg^{++} and Ba^{++} approximate proportionality to $(M^{++})^{-1/2}$ is observed. The divalent cations are of the order of thirty times as effective as the monovalent in displacing the binding of rosaniline over the range of concentration employed.

This suggests that the form of LANGMUIR'S isotherm for competitive binding of dye and metal cations would provide a useful correlation of the data, *e.g.* for a monovalent cation M:

$$\beta = \frac{k_R (R_f)}{1 + k_R (R_f) + k_M (M)} \qquad \frac{1}{k'_R} = \frac{(1 - \beta)}{\beta} \cdot (R_f) = \frac{1}{k_R} + \frac{k_M (M)}{k_R} \tag{4}$$

Introducing the relation between extinction coefficient of the dye and its degree of binding (equation (2)) and confining the equation to small values of β:

$$\frac{\varepsilon - \varepsilon'}{\varepsilon^\circ - \varepsilon} \simeq \frac{1}{k_R \cdot DNA} + \frac{k_M (M)}{k_R \cdot DNA} \tag{5}$$

The value of the term $1/k_R \cdot DNA$ is small in the present case, since, with equal concentrations of rosaniline and of DNA of *ca.* 10^{-5} equivalents per l there are marked changes in the absorption spectrum of rosaniline indicating that the greater part of it is bound to DNA[9]. However, with such small concentrations of DNA, in absence of added Na^+ ions, the extinction coefficient of the ultra-violet absorption of DNA is unstable and tends to increase irreversibly, indicative of denaturation of DNA, in the nomenclature of THOMAS[6]. Thus the concept of an equilibrium constant for the binding of a single ionic species to DNA may lack meaning. The reversibility of the displacement of bound rosaniline by metallic cations observed in the present work would appear therefore to be due to the preservation of the secondary-bond structure of DNA, in the form existing in aqueous solution in presence of excess Na^+ ions, the proportion of sites occupied by rosaniline being small through-out. In other words the ion-exchange equilibrium

$$R^+ + NaDNA \rightleftharpoons Na^+ + RDNA$$

appears to be reversible provided almost all the phosphate groups of DNA remain associated with Na^+ ions.

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Application of the Law of Mass Action to this equilibrium yields the result of equation (5) without the first term on the right hand side.

A possible weakness of the concepts employed might appear to be that almost complete association between DNA and Na^+ ions is required. However considerable binding of Na^+ ions has been demonstrated by more direct methods[10,11], and similarly for the case of the synthetic polyelectrolyte polyacrylic acid[12].

In Table III values of the ratio of affinities for binding to DNA of metallic cations and of rosaniline have been obtained from the relations:

$$\frac{\left(1 - \frac{\beta\,\mathrm{DNA}}{\mathrm{R}}\right)}{\frac{\beta\,\mathrm{DNA}}{\mathrm{R}}} \cdot (1 - \beta) = \frac{k_{\mathrm{M}}}{k_{\mathrm{R}}} \cdot \frac{(\mathrm{M}^+)}{\mathrm{DNA}} \qquad (6)$$

for monovalent metal ion M^+, and for divalent M^{++} replacing the concentration (M^+) by $(M^{++})^{\frac{1}{2}}$. In this latter case (M^{++}) represents the concentration of free M^{++} in the aqueous phase. Since it appears that divalent cations are much more strongly bound than monovalent, this is not exactly known. However, with excess of the divalent cation, it is probably a reasonable approximation that this quantity should be taken as the total concentration of M^{++} less that of DNA, which approximation has been employed.

Rearrangement of equation (6), for the case when β is small compared with unity, enables graphical representation of the data (as in Figs. 2 and 3):

$$\varepsilon = \varepsilon' + (\varepsilon^\circ - \varepsilon) \cdot \frac{k_{\mathrm{M}}\,(\mathrm{M})}{k_{\mathrm{R}} \cdot \mathrm{DNA}} \qquad (7)$$

useful when values of ε' cannot be estimated directly. (In the present case values of ε for rosaniline in large excess of DNA in absence of added Na^+ provide a very close approximation to ε', more accurately than the extrapolation.)

To summarize, it may be said that the relations proposed do appear to provide an adequate correlation of the data for the copetitive effect of metallic cations, although it must be remembered that the range of concentrations conveniently employable is somewhat limited.

Some further points which arise from the data presented are as follows:

(a) The binding of rosaniline to DNA at a fixed concentration of added metal cation is weakened by increase in temperature. The effect corresponds to values $\Delta H = 5\ (\pm 1)$ Kals. and $\Delta S = 8\ (\pm 4)$ cals per mole per °C for the displacement of one mole of bound rosaniline by one equivalent of a metallic cation. Such values are in agreement with the concept that in this process a secondary valency, (hydrogen-bond or salt-linkage), between an amino group of rosaniline and a phosphate group of DNA, is broken.

(b) The phosphate groups in question would appear to be almost wholly those of the singly-charged (primary) type, since the binding of rosaniline is essentially similar at pH 4.6 to that in aqueous NaCl (pH *ca.* 6.3). However, if the ratio of affinities for binding of Na^+ and of R^+ were similar for both types of phosphate group, the evidence would remain inconclusive on this point.

(c) At constant concentration of Na^+ ions the binding constant k'_{R} for rosaniline is independent of the concentration of DNA or of rosaniline. This leads to a similar

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TABLE III

DISPLACEMENT OF ROSANILINE BOUND TO DNA, BY NaCl, KCl, $MgSO_4$ AND $BaCl_2$

Values of $\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'}$ = proportion of rosaniline bound

T°C	Metallic Ion	DNA	Concentrations in g-equivalents per litre			$\left(\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'}\right)$ (± 0.02)	log k'_R	k_M/k_R
			$R \times 10^5$	DNA $\times 10^3$	Metallic ion			
38.2	Na^+	T9	3.0	1.0448	0.00364	0.885	3.88	—
38.2	Na^+	T9	3.0	1.0448	0.0252	0.728	3.42	0.0152
38.2	Na^+	T9	3.0	1.0448	0.047	0.533	3.06	0.0192
38.2	Na^+	T9	3.0	1.0448	0.0914	0.407	2.82	0.0166
cooled to 24	Na^+	T9	3.0	1.0448	0.0252	0.777	3.54	0.0114
cooled to 24	Na^+	T9	3.0	1.0448	0.047	0.653	3.28	0.0112
cooled to 24	Na^+	T9	3.0	1.0448	0.0914	0.518	3.02	0.0105
24	Na^+	T9	3.092	0.767	0.00383	0.93	4.11	—
24	Na^+	T9	3.092	0.767	0.0254	0.732	3.56	0.0107
24	Na^+	T9	3.092	0.767	0.0472	0.625	3.35	0.0095
24	Na^+	T9	3.092	0.767	0.0918	0.482	3.09	0.0088
24.7	Na^+	T9	3.02	0.95	0.00457	0.90	3.99	—
24.7	Na^+	T9	3.02	0.95	0.0331	0.694	3.39	0.0124
24.7	Na^+	T9	3.02	0.95	0.0626	0.56	3.14	0.0117
24.7	Na^+	T9	3.02	0.95	0.1252	0.388	2.84	0.0116
38.5	Na^+	T13	3.0	2.345	0.00298	0.925	3.73	—
38.5	Na^+	T13	3.0	2.345	0.0655	0.671	2.94	0.0177
38.5	Na^+	T13	3.0	2.345	0.1283	0.498	2.63	0.0184
38.5	Na^+	T13	3.0	2.345	0.1909	0.373	2.41	0.0206
cooled to 24.5	Na^+	T13	3.0	2.345	0.00298	0.953	3.92	—
cooled to 24.5	Na^+	T13	3.0	2.345	0.0655	0.774	3.17	0.0103
cooled to 24.5	Na^+	T13	3.0	2.345	0.1283	0.582	2.78	0.0131
cooled to 24.5	Na^+	T13	3.0	2.345	0.1909	0.439	2.53	0.0157
24.5	Na^+	T13	3.0	2.345	0.1909	0.446	2.54	0.0152
21	Na	T13	1.035	1.118	0.1435	0.393	2.77	0.0119
21	Na	T13	2.07	1.118	0.1435	0.369	2.72	0.0132
21	Na	T13	4.14	1.118	0.1435	0.406	2.79	0.0113
21	Na	T13	1.035	1.118	0.0045	0.942	4.17	—
21	Na	T13	1.035	1.118	0.027	0.776	3.49	0.0119
21	Na	T13	1.035	1.118	0.0705	0.602	3.13	0.0104
23	K	T13	2.07	1.0395	0.001726	0.979	4.66	—
23	K	T13	2.07	1.0395	0.02888	0.725	3.41	0.0135
23	K	T13	2.07	1.0395	0.0696	0.559	3.09	0.0116
25	Ba	T13	2.07	2.15	0.01074	0.422	2.53	0.0317
25	Ba	T13	2.07	2.15	0.01074	0.443	2.57	0.0292
37.5	Ba	T13	2.07	2.15	0.01074	0.369	2.44	0.0397
cooled to 25	Ba	T13	2.07	2.15	0.01074	0.429	2.55	0.0307
25	Ba	T13	2.07	1.075	0.00537	0.344	2.69	0.031
25	Mg	T13	2.07	1.075	0.005769	0.328	2.66	0.032
21	Mg	T13	2.07	1.03	0.001923	0.449	2.90	0.028
21	Mg	T13	2.07	1.03	0.003846	0.361	2.74	0.034
21	Mg	T13	2.07	1.03	0.00962	0.259	2.62	0.032
21	Mg	T13	4.14	2.06	0.01154	0.375	2.465	0.035
37.5	Mg	T13	4.14	2.06	0.01154	0.286	2.29	0.0525
37.5	Mg	T13	4.14	2.15	0.0079	0.327	2.356	0.058
24	Mg	T13	4.14	2.15	0.0079	0.415	2.52	0.0396

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conclusion to that of (b), *viz.* that the binding sites occupied, up to values of β of *ca.* 0.04, possess the same value of k_{Na}/k_R.

(d) Heat-denaturation of DNA weakens the intrinsic affinity for binding of rosaniline and also the competitive effect of Na^+ ions (Table IV). This suggests that the relevant binding group (primary phosphate) is less available in the heat-denatured form, than in the original form. The model proposed for the structure of DNA in the form of moist fibres by CRICK AND WATSON[13] shows the phosphate groups to be uniformly presented along the exterior of the polymer chain. A similar configuration, held by intramolecular hydrogen-bonds not involving phosphate groups might be thought to persist in aqueous solutions in the presence of excess Na^+ (the undenatured form). It is then conceivable that the effect of heat-denaturation should be to break down this highly ordered structure, and that the phosphate groups should become less available for attachment of bound species in the more random structure resulting, *i.e.* these groups might then be involved to some extent in the formation of secondary bonds intramolecularly.

TABLE IV

EFFECT OF HEAT-DENATURATION OF DNA ON THE BINDING OF ROSANILINE

DNA (T9) denatured by heating a $2.612 \cdot 10^{-3} M$ solution in $9.09 \cdot 10^{-3} M$ NaCl to 100° C for 20 minutes. R = $2.99 \cdot 10^{-5} M$, DNA = $1.045 \cdot 10^{-3} M$.

T°C	Values of $\left(\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'}\right)$			
	$Na^+ = 0.00363$	0.0914	0.358	0.676
24	taken as = 1	0.743	0.588	0.496
38.2	0.720	0.481	0.408	0.357
cooled to 24	0.996	0.773	0.601	0.468

DNA denatured by heating an aqueous solution ($1.51 \cdot 10^{-3} M$ with no added Na^+), R = $2.99 \cdot 10^{-5} M$.

T°C	$\frac{\varepsilon^\circ - \varepsilon}{\varepsilon^\circ - \varepsilon'}$	k'_R
24	0.788	3.398
30	0.744	3.291
39.3	0.699	3.193
55.2	0.585	2.976

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SUMMARY

A spectroscopic method for the study of the binding of rosaniline to DNA (aqueous solutions of sodium thymonucleate) is described. This is employed to investigate the decrease in binding produced by addition of salts (NaCl, KCl, $MgSO_4$, $BaCl_2$, Na acetate buffer pH 4.6), by heating the solutions, and by heat-denaturation of DNA.

RÉSUMÉ

Une méthode spectroscopique d'étude de la fixation de la rosaniline sur le DNA (solutions aqueuses de thymonucléate de sodium) est décrite. Elle a été employée pour l'étude de la diminution de fixation produite par addition de sels (NaCl, KCl, $HgSO_4$, $BaCl_2$, tampon acétate de sodium pH 4.6), par chauffage des solutions et par dénaturation par la chaleur du DNA.

ZUSAMMENFASSUNG

Eine spektroskopische Methode zum Studium der Bindung von Rosanilin an DNA (wässerige Natriumthymonukleat-Lösungen) wird beschrieben. Auf Grund dieser Methode wird die Herabsetzung der Bindung untersucht, welche durch Hinzufügung von Salzen (NaCl, KCl, $MgSO_4$, $BaCl_2$, Natriumazetat-Puffer pH 4.6), durch Erhitzen der Lösungen und durch Hitzedenaturierung von DNA entsteht.

REFERENCES

1 L. F. CAVALIERI, A. ANGELOS AND M. E. BALIS, *J. Am. Chem. Soc.*, 73 (1951) 4902.
2 J. L. IRVIN AND E. M. IRVIN, *J. Biol. Chem.*, 206 (1954) 39.
3 S. E. SHEPPARD AND A. L. GEDDES, *J. Am. Chem. Soc.*, 66 (1944) 1995.
4 R. J. GOLDACRE AND J. N. PHILLIPS, *J. Chem. Soc.*, (1949) 1724.
5 J. A. V. BUTLER, B. E. CONWAY AND D. W. F. JAMES, *Trans. Faraday Soc.*, 50 (1954) 612.
6 R. THOMAS, *Biochim. Biophys. Acta*, 14 (1954) 231.
7 L. F. CAVALIERI AND A. ANGELOS, *J. Am. Chem. Soc.*, 72 (1950) 4686.
8 I. M. KLOTZ, in *The Proteins*, edited by H. NEURATH AND K. BAILEY, Academic Press, 1953, Vol. I, part B, Chap. 8.
9 P. D. LAWLEY, *Biochim. Biophys. Acta*, 19 (1956) 328.
10 J. M. CREETH, J. M. GULLAND AND D. O. JORDAN, *J. Chem. Soc.*, (1947) 1141.
11 J. SHACK, R. J. JENKINS AND J. V. THOMPSETT, *J. Biol. Chem.*, 198 (1952) 85.
12 J. R. HUIZENGA, P. F. GRIEGER AND F. T. WALL, *J. Am. Chem. Soc.*, 72 (1950) 2636.
13 J. D. WATSON AND F. H. C. CRICK, *Nature*, 171 (1953) 737.

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