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Studies on the Structure of Nucleic Acids. II.[1a] Investigation of Pentose Nucleic Acid and Enzyme-resistant Residues

BY LIEBE F. CAVALIERI, STANLEY E. KERR[1b] AND ALICE ANGELOS

The interaction of rosaniline with various fractions of yeast nucleic acid and pentose nucleic acid from beef pancreas has been studied. It is concluded that the binding sites involve the phosphoric acid groups of which about 13% are available for binding. On the basis of the similar intrinsic binding constants and n values, it is suggested that a similar backbone structure exists among the nucleic acid samples studied. The interaction of rosaniline with the ribonuclease-resistant fractions of yeast pentose nucleic acid has been also studied, and the binding capacity of these resistant fractions is of the same order of magnitude as that of the parent nucleic acids. The binding capacity of the n_1-type site (divalent anion) of the resistant fractions appears to be slightly greater than that of the parent PNA. It is suggested that this is due to a decrease in steric inhibition. In the case of the ribonuclease–phosphatase treated nucleic acid, the results of the binding process suggest that only one type of site is involved, and it would appear that this corresponds to the monovalent anion type.

Correlation of the experimental pH titration curves with theoretical curves constructed from the known composition of various samples of nucleic acid, a ribonuclease-resistant fraction and a ribonuclease–phosphatase-resistant fraction indicates that some of the hydroxyl groups of guanine and/or uracil are unavailable for titration and may be covalently bound in a phosphate type bond. In the case of the ribonuclease–phosphatase treated nucleic acid, purine and pyrimidine analyses, periodate oxidation titers and ion-exchange analysis provide additional evidence for such a covalent linkage. Both PNA and the ribonuclease-resistant fraction have been subjected to periodate oxidation, and it appears that a D-riboside phosphate other than the 2′ or 3′ exists in the resistant fraction. Ultraviolet and infrared absorption spectra of both treated and untreated nucleic acids are similar and cannot be used effectively as a means of identification. X-Ray powder patterns suggest that some of the residues remaining after the action of ribonuclease and acid-phosphatase are partially crystalline.

In the present investigation, we have employed the enzymes ribonuclease and acid-phosphatase as tools for the study of the structure of nucleic acids (PNA). Quantitative determinations of the dye-binding process, together with pH and periodate titrations, have been carried out on the parent nucleic acid and on the fractions resulting from the action of ribonuclease and ribonuclease followed by acid-phosphatase.

Numerous investigators have employed these enzymes in a similar capacity.[2-6] One approach has been the electrometric titration of PNA–ribonuclease mixtures for the purpose of ascertaining the extent and mechanism of reaction. There is ample evidence to indicate that cleavage of the D-ribose–phosphate bonds occurs as the result of this enzyme action.[2,5,6] However there is no definite information concerning the specificity of enzyme action on the various types of phosphate linkages in nucleic acids. Thus among what appear to be the purine and pyrimidine nucleoside-2′- and -3′-phosphates[7] the order of cleavage with respect to the 2′- and 3′-position is not known. It is clear, however, that end phosphate groups (singly esterified) are not attacked by ribonuclease. Recently Bacher and Allen[4] have analyzed a number of dialyzable fractions resulting from the action of ribonuclease. These workers found that the products which were liberated at first contained more uracil than cytosine and were of lower molecular weight than those which were liberated toward the end of the digestion. As the reaction proceeded, the purine to pyrimidine ratio increased from a value of less than one to a value greater than one.

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(2) Schmidt, *Cold Spring Harbor Symposia on Quantitative Biology*, **XIV**, 161 (1947).

(3) Carter and Cohn, THIS JOURNAL, **72**, 2604 (1950).

(4) Bacher and Allen, *J. Biol. Chem.*, **183**, 633 (1950).

(5) Loring, Carpenter and Roll, *ibid.*, **150**, 381 (1943).

(6) Allen and Eiler, *ibid.*, **137**, 757 (1941).

(7) Carter, THIS JOURNAL, **72**, 1466 (1950).

The action of acid-phosphatase on PNA or its fragments is of significance. Only a small amount of phosphoric acid is liberated by the action of acid-phosphatase on PNA; however if the nucleic acid is first subjected to the action of ribonuclease, about 25% of the total phosphorus is liberated on subsequent treatment with acid-phosphatase.[2] This has been attributed to the hydrolysis by the ribonuclease of phosphoric acid diester linkages to monoester linkages which are the type attacked by the phosphatase.

Experimental

Materials. (a) **Nucleic Acids.**—Four samples of yeast pentose nucleic acid and one of pancreas nucleic acid were used in this study. A specimen of the former obtained from the Schwarz Laboratories was dissolved in water and brought to a pH of 6.5. The solution was dialyzed (without agitation) for 7 days at 3° against distilled water; the water was changed twice daily. At the end of this period, the contents of the cellophane casing were adjusted to pH 1 with dilute hydrochloric acid in the cold. The precipitate was washed several times with alcohol and then with ether (Sample A). Notwithstanding this dialysis period, dialyzable components were still evident. The amount remaining in the dialysis bags in the binding experiments was determined by nitrogen analysis and spectral measurements; from 10 to 14% of this nucleic acid sample was found to be dialyzable. It contained 0.3% of sodium (flame photometer) and no ammonium salts. A second fraction of the commercial nucleic acid was obtained by adding five volumes of glacial acetic acid to an aqueous solution of the ammonium salt. The precipitate was washed free of acid with alcohol, followed by ether. This material yielded about 10% dialyzable components (Sample B). Sample B contained 0.6% of ammonium salts and no sodium. A third fraction was obtained by adding three volumes of alcohol to the filtrate obtained after removal of the precipitate resulting from the addition of a mixture of glacial acetic acid, acetone and water (2:2:1),[8] to the aqueous ammonium salt. This material contained less than 5% dialyzable components (Sample C). Sample C contained 1.8% of sodium. This was assumed to be present as sodium nucleate in calculating the titration curves. Yeast nucleic acid obtained by means of potassium thiocyanate extraction was obtained through the courtesy of Drs. C. E. Carter and E. Volkin (Sample D) (unpublished).

Pentose nucleic acid from beef pancreas was isolated by a method which has been described.[9] About 10% was lost after dialysis overnight at 3° (Sample E).

(8) Chantrenne, *Bull. soc. chim. Belg.*, **55**, 5 (1946).

(9) Kerr and Scraidarian, *J. Biol. Chem.*, **180**, 1203 (1949).

(b) **Ribonuclease-resistant Fractions.**—A sample of commercial yeast nucleic acid obtained from the Schwarz Laboratories was treated with the crystalline ribonuclease of Kunitz (Worthington) essentially according to the procedure of Loring, Carpenter and Roll.[5] The procedure was modified in that the precipitation of the digestion mixture was accomplished by the addition of glacial acetic acid, acetone and water (2:2:1) rather than with glacial acetic acid alone. This precipitate is designated as Fraction EA. Five volumes of alcohol were added to the filtrate and the precipitate washed and dried as described (Fraction EB). Fraction EA′ represents a second preparation of EA, differing only in that EA′ was exhaustively dialyzed against running tap water. Both samples EA and EB contained components (*ca.* 50%) which were dialyzable through sausage casing in solutions of 0.05 *M* potassium phosphate buffer. For the binding experiments, samples were exhaustively dialyzed against potassium phosphate buffer at the desired *p*H. The material remaining inside the dialysis bag was not precipitated in order to avoid any possibility of cleavage of the nucleic acid into small fragments. The amount remaining in the bag was determined both by nitrogen and spectral analyses. Sample EA′ was found to contain less than 0.3% sodium, as determined by a flame photometer.

(c) **Ribonuclease–Acid-Phosphatase Fractions.**—The product resulting from the action of ribonuclease and phosphatase was obtained according to the method of Schmidt,[2,10] with minor modifications.

Twenty-eight grams of yeast nucleic acid (Schwarz) was suspended in 210 ml. of water and brought to *p*H 5.7 by the addition of 1 *N* sodium hydroxide in the cold. One hundred fifty milligrams of crystalline ribonuclease (Worthington) was added and the mixture was incubated at 37° for 18 hours. It was then diluted to 7 liters with 0.05 *M* sodium acetate buffer, *p*H 5.3. A portion (80 ml.) of acid-phosphatase, extracted from 64 g. of human hypertrophic prostate gland, was introduced. The incubation at 37° was continued for 6 hours at which time about 90% of the total nucleic acid phosphorus was found to be hydrolyzed by 1 *N* acid in 1 hour at 100°, *i.e.*, not more than 10% of the nucleic acid was present as pyrimidine nucleotides. The solution was divided into two portions and the product isolated by two methods. The first was brought to a *p*H of 8 by the addition of barium hydroxide. The precipitate of barium phosphate was removed by filtration and discarded. Traces of barium remaining in the filtrate were removed by the addition of 50 g. of a cation-exchange resin (Amberlite IR-120). After separation of the resin, the solution was concentrated *in vacuo* (25°) to 110 ml. Addition of an equal volume of a mixture of glacial acetic acid, acetone and water (2:2:1) yielded no precipitate, but two additional volumes of ethanol resulted in the precipitation of the product (Sample F). The product was washed with alcohol and ether; 1.24 g. Sample G represents a similar fraction.

The second half of the original solution was dialyzed against running tap water for 3 days to effect the removal of salts and material of low molecular weight. The solution inside the bag was concentrated *in vacuo* (25°) to 100 ml. The addition of an equal volume of glacial acetic acid, acetone and water (2:2:1) yielded 1.64 g. of precipitate (Sample J). Two volumes of alcohol added to the filtrate yielded more material; 2.13 g. (Sample K).

Samples J and K contained less than 0.3% of sodium and negligible amounts of ammonium salts, as determined by flame spectrophotometry and Nessler reagent, respectively.

The rosaniline used in these experiments was identical with that used previously.[11]

Method of Equilibrium Dialysis.—The binding of rosaniline to PNA was determined by the method of equilibrium dialysis.[11,12,13] Five ml. of *ca.* 0.1% PNA solutions contained in cellophane bags were immersed in 5 ml. of dye. The tubes were shaken overnight to attain equilibrium. Experiments were carried out at 3 ± 0.5° and 27 ± 1° at *p*H 5.5 and 6.6 in 0.05 *M* potassium phosphate buffer. Although at *p*H 5.5 phosphate is below its maximum buffer capacity, no changes in *p*H were observed. Phosphate buffer at this *p*H was used in order to obtain a direct comparison with the data at *p*H 6.6. The amount of dye adsorbed by the casing was determined at each concentration. Free dye concentrations (outside the bag) were determined by reading the solutions at 5400 Å. in a Beckman spectrophotometer, model DU. Dye concentrations were chosen such that a large proportion of the dye was bound in order to avoid dealing with small differences between two large numbers. Results were reproducible to within about 3%. A large number of the points shown represent average values from different runs.

TABLE I

ANALYTICAL DATA

MILLIMOLES PER GRAM OF NUCLEIC ACID

Sample	Guanine	Adenine	Cytosine	Uracil	Sum of purines, pyrimidines	N	Total	Phosphorus Hydrolyzable (purine)
				PNA				
A	0.86	0.67	0.53	0.61	2.67	11.2	2.68	
B	.86	.64	.56	.50	2.56	10.3	2.68	
C	.73	.63	.47	.57	2.40	9.88	2.68	
D[a]	.74	.63	.80	.76	2.93			
E	.90	.32	.72	.32	2.26	9.50	2.32	
				Ribonuclease-resistant fraction				
EA	1.14	0.59	0.42	0.31	2.46	13.5	2.60	1.82
EA′	1.27	.66	.27	.26	2.46		2.80	1.84
EB	0.97	.70	.42	.35	2.44	12.1	2.60	1.89
				Ribonuclease–acid-phosphatase fraction				
F	0.97	0.78	0.43	0.25	2.43	12.0	2.04	1.83
K	1.09	.88	.36	.36	2.69	11.6	2.19	1.87
J	1.26	.75	.27	.24	2.52	11.6	2.10	1.93
G	1.38	.57	.35	.20	2.51	11.4	1.74	1.70

[a] This sample was reported to contain about 30% of protein. On the basis that very little interaction occurs between PNA and bovine serum albumin under the conditions of these experiments (unpublished), it appears that no error was introduced.

Method of Titration.—Titrations were carried out on 20- to 30-mg. samples in 10 ml. of solution. The samples were suspended in double-distilled water and dissolved by the addition of potassium hydroxide. The *p*H of the solution was about 7. Titrations were carried out with 0.5 *N* potassium hydroxide and 0.2 *N* hydrochloric acid using a Scholander micro-buret.[14] The system was stirred and kept free of carbon dioxide by a stream of nitrogen. The *p*H of the solution was determined with a glass electrode. In the alkaline range, a special electrode (Beckman Type E) was used to minimize the error. The titration curves were corrected at the extremes of *p*H for the titration of water by the method of Jordan and Taylor.[15] At the extremes of *p*H the titration curves were determined at least three times; the average of these runs was used for plotting purposes. Titration temperature was 23 ± 1°.

Method of Purine and Pyrimidine Analyses.—The determination of purines and pyrimidines was carried out according to the method of Kerr, Seraidarian and Wargon.[16] Most analyses were carried out in duplicate with an estimated error of 5%. The values in Table I are averages.

Infrared and X-Ray Data.—The infrared curves[17] were determined with a Baird Double Beam Recording Infrared Spectrophotometer with an accuracy of about 0.04 micron. All samples were mulled in mineral oil and run against a wire mesh blank. The X-ray powder patterns[17] were determined with a North American Philips X-Ray Diffraction Unit. The powdered samples were loaded in glass capillaries of about 0.5 mm. inner diameter. Filtered copper K alpha radiation was used for an exposure period of 2 hours. Ultraviolet absorption spectra were determined in potassium

(10) The experimental procedure used was kindly furnished by Dr. Gerhard Schmidt (personal communication to Stanley E. Kerr).

(11) Cavalieri and Angelos, THIS JOURNAL, **72**, 4686 (1950).

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

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

(14) Scholander, *Science*, **95**, 177 (1942).

(15) Jordan and Taylor, *J. Chem. Soc.*, 994 (1946).

(16) Kerr, Seraidarian and Wargon, *J. Biol. Chem.*, **181**, 761 (1949).

(17) Kindly furnished by Dr. Carl Clark. We also wish to thank Doctor Clark for discussions concerning the interpretation of the data.

phosphate buffer (*p*H 6.7, 0.05 *M*) with a Carey Recording Spectrophotometer, Model 11.

Periodate Titration.—Periodate titrations were carried out on 50-mg. samples, both at *p*H 7–8 ($NaHCO_3$) and *p*H 4. The alkaline oxidation mixture was allowed to stand for 10 minutes, while that at *p*H 4 remained overnight. The results from the two methods agreed to within 2%.

Ion-exchange Analyses.—The analysis for the alkaline hydrolysis mixture was carried out according to Cohn.[18] Elutions at *p*H 5 were effected with acetate buffer; at *p*H 2–3, with 0.002–0.005 *N* HCl. The Dowex-1 (anion) column was 6 cm. high and 1.4 cm. in diameter; sample, 18 mg.

Results

The data for the interaction of rosaniline with the various nucleic acid samples and the enzyme-treated nucleic acids are contained in Figs. 1 to 3, and Tables II to V. In Fig. 1, the amount of dye bound per mole of PNA (r) is plotted *versus* r/c, where c is the free equilibrium dye concentration. The molecular weight of PNA varies with the source and method of isolation. Since the molecular weight used for the calculations does not essentially alter the conclusions reached, an arbitrary value of 10,000 was selected. In general, this number represents an average value obtained on various samples by several investigators, and the results are based on this molecular weight to afford a better comparison with the previous results obtained with DNA,[11]

TABLE II

BINDING DATA FOR PNA

Temp., °C.	PNA concn., m./l. × 10^6	½ of the original dye concn., m./l. × 10^6	C, free dye concn., m./l. × 10^6 (from optical density of outer solution)	C', free dye, concn. plus casing adsorption, m./l. × 10^6	Bound dye, m./l. × 10^6	r	r/c × 10^{-4}
			*p*H 6.6 (Sample A)				
3	8.7	4.13	1.97	2.46	3.34	0.0384	1.96
		5.74	2.91	3.64	4.20	.0483	1.65
		8.14	4.22	5.27	5.74	.0659	1.56
		12.1	6.55	8.2	7.8	.0897	1.37
	17.4	42.7	18.7	23.4	38.6	.222	1.18
		68.4	31.8	39.8	57.2	.329	1.03
	8.7	76.8	47.3	59.1	35.4	.412	0.868
27	8.7	4.28	2.65	3.32	1.92	.0220	.830
		8.55	5.35	6.69	3.72	.0426	.796
		17.1	11.0	13.8	6.60	.0760	.690
		25.6	17.2	21.5	8.20	.0943	.548
		34.2	22.3	27.8	12.8	.147	.658
		42.7	28.8	36.0	13.4	.154	.535
			*p*H 5.5 (Sample A)				
3	8.7	4.10	1.75	2.26	3.68	0.0425	2.43
		8.15	3.75	4.74	6.82	.0784	2.09
		8.55	4.02	5.09	6.92	.0795	1.98
		16.30	8.55	10.5	11.6	.133	1.56
		17.1	8.73	10.8	12.6	.145	1.66
		25.6	13.6	16.7	17.8	.205	1.51
	17.4	68.4	30.3	36.9	63.0	.363	1.19
		76.9	35.2	43.0	67.8	.389	1.10
		87.5	39.0	47.7	79.6	.456	1.16
27	8.7	8.3	5.0	6.32	3.96	.0455	0.912
		25.1	15.5	18.9	12.4	.142	.915
		42.7	27.4	33.4	18.6	.215	.785
		50.2	32.4	38.2	24.0	.276	.853
			*p*H 6.6 (Sample B)				
3	9.0	3.94	1.69	2.12	3.64	0.0405	2.39
		7.41	3.46	4.33	6.16	.0685	1.98
		10.2	5.23	6.53	7.34	.0816	1.56
		37.5	21.2	26.5	22.0	.245	1.16
			*p*H 6.6 (Sample C)				
3	8.8	3.95	1.49	1.86	4.18	0.0475	3.18
		7.90	3.03	3.78	8.24	.0936	3.09
		12.0	4.88	6.11	11.8	.134	2.74
		32.3	14.2	17.8	29.0	.329	2.31
		36.7	16.0	20.0	33.4	.380	2.37
		36.4	16.6	20.8	31.2	.354	2.14
		43.3	19.6	24.5	37.6	.427	2.18
27	8.8	3.98	2.34	2.92	2.12	0.0241	0.103
		7.92	4.64	5.80	4.24	.0483	.104
		12.0	7.08	8.80	6.40	.0727	.103
		37.6	23.2	29.0	17.2	.195	.842
		66.0	41.2	51.4	29.2	.332	.808
			*p*H 5.5 (Sample C)				
3	8.8	8.41	3.20	4.10	8.61	0.0970	3.03
		16.5	6.55	8.20	16.6	.189	2.89
		25.0	10.4	12.8	24.4	.277	2.67
		33.8	14.4	17.6	32.4	.368	2.56
		50.0	23.6	28.1	43.8	.499	2.11
			*p*H 5.5 (Sample D)				
3	7.0	4.02	1.38	1.91	4.22	0.0603	4.36
		8.05	3.10	4.03	8.04	.115	3.71
		16.1	6.37	8.05	15.9	.227	3.56
		24.1	10.9	13.6	21.0	.300	2.75
		33.1	15.2	18.5	29.2	.416	2.75
			*p*H 5.5 (Sample E)				
3	18.0	3.92	0.816	1.21	5.42	0.0306	3.75
		8.05	2.02	2.62	10.8	.0604	2.99
		16.10	4.85	6.15	19.9	.111	2.29
		24.1	7.65	9.55	29.1	.162	2.11
		40.2	15.2	19.0	42.4	.235	1.54
		48.2	18.2	22.8	50.8	.282	1.55
28	18.0	4.02	1.94	2.57	2.90	0.0160	0.830
		8.05	3.94	4.95	6.20	.0344	.873
		16.1	8.30	10.3	11.6	.0645	.778
		24.1	12.5	15.5	17.2	.0945	.754
		40.2	21.8	25.7	29.0	.161	.740

In the case of the enzyme-treated nucleic acids, no reasonable guess can be made as to size[19] and for these cases the results have been expressed in terms of phosphorus content. However, for comparison, some typical data from the nucleic acid runs have been recalculated on this basis and are included in Table V. In Figs. 2 and 3, the amount of dye bound per mole of phosphorus (r) is plotted *versus* r/c. In all figures the solid lines represent the theoretical curves.

The figures in column 5 in Tables II and IV represent the free dye concentrations to which have been added the values for the adsorption due to the cellophane casing. The binding process was shown to be reversible by immersing the equilibrated bags in fresh buffer and calculating the new r and r/c. The values thus obtained were in good agreement with the expected values. Calculations and thermodynamic constants were obtained

(18) Cohn, THIS JOURNAL, **72**, 1473 (1950).

(19) Bacher and Allen, *J. Biol. Chem.*, **184**, 511 (1950), report values ranging from molecular weight 2000 to 6000.

TABLE III

THERMODYNAMIC RESULTS FOR PNA

Sample	A		A		B	C		D	R	
pH	5.5		6.6		6.6	6.6		5.5	5.5	
Temp., °C.	3	27	3	27	3	3	27	3	3	27
n_1	0.11	.....	0.04	.....	0.05	0.05	.....	0.05	0.07	.....
n_2	3.89	(3.89)	3.26	(3.96)	4.95	1.95	(1.95)	3.95	2.93	(2.93)
$k_1 \times 10^{-5}$	1.87	.....	5.67	.....	5.34	2.24	.....	6.50	4.60	.....
$k_2 \times 10^{-5}$	0.025	(0.026)	0.025	(0.023)	0.018	0.124	(0.062)	0.066	0.0445	(0.033)
ΔF_1^0(kcal./mole)	−6.65	.....	−7.30	.....	−7.28	−6.75	.....	−7.35	−7.15	.....
ΔF_2^0(kcal./mole)	−4.28	−4.68	−4.28	−4.63	−4.11	−5.17	−5.22	−4.82	−4.61	−4.83
ΔH_2^0(kcal./mole)	+0.32	.....	−0.24	.....	.....	−0.57	.....	.....	−2.08	.....
ΔS_2^0 (e.u.)	+16.7	.....	+14.6	.....	.....	+2.08	.....	.....	+9.2	.....
$A \times 10^{-4}$ (ordinate intercept)	2.9	1.0	3.0	0.9	3.6	3.5	0.12	6.0	4.7	0.85

TABLE IV

BINDING DATA FOR RIBONUCLEASE-RESISTANT FRACTIONS

Temp., °C.	Phosphorus concn., m./l. × 10⁴	½ of the original dye concn., × 10⁶	Free dye concn. m./l. (c × 10⁶) (from optical density of outer solution)	Free dye, concn. plus casing adsorption c' × 10⁶	Bound dye, m./l. × 10⁶	r × 10²	r/c × 10⁻⁴
			pH 6.6 (Sample EA)				
3	10.75	8.06	2.88	3.60	8.93	0.832	2.88
		14.8	5.88	7.35	15.1	1.41	2.39
		22.2	10.1	12.6	19.2	1.79	1.77
		32.2	14.4	18.0	28.4	2.64	1.83
		40.7	18.8	23.6	34.3	3.19	1.70
		49.5	23.0	28.7	41.5	3.86	1.68
			pH 5.3 (Sample EA)				
3	10.75	3.97	1.19	1.66	4.62	0.429	3.59
		8.65	2.83	3.65	10.0	0.930	3.29
		12.9	4.47	5.66	14.5	1.35	3.02
		34.3	14.3	17.4	33.7	3.13	2.19
		39.0	16.2	19.6	38.8	3.61	2.23
27	10.75	4.27	2.04	2.72	3.10	0.288	1.41
		8.4	4.05	5.20	6.40	.595	1.47
		12.5	6.26	7.86	9.28	.863	1.38
		32.5	17.3	21.1	22.8	2.12	1.22
			pH 6.6 (Sample EB)				
3	9.82	4.03	1.84	2.30	3.46	0.353	1.92
		7.55	3.59	4.49	6.12	.624	1.73
		11.5	5.95	7.44	8.12	.826	1.39
		32.3	16.9	21.2	22.3	2.27	1.34
		38.3	20.4	25.5	25.6	2.61	1.28
		44.4	24.0	29.9	29.0	2.96	1.23
			pH 5.3 (Sample EB)				
3	9.82	4.28	2.00	2.65	3.24	0.330	1.65
		8.40	4.24	5.36	6.04	.615	1.45
		12.5	6.40	8.04	8.92	.909	1.42
		37.8	21.5	26.1	23.4	2.39	1.11
		42.0	23.7	28.9	26.2	2.67	1.13
	19.64	66.2	29.3	35.5	61.4	3.13	1.07
27	9.82	4.27	2.30	3.02	2.52	0.257	1.11
		8.4	4.96	6.28	4.2	.428	0.87
		12.5	7.60	9.38	6.2	.632	.83
		32.5	20.5	24.4	16.2	1.65	.80
			Ribonuclease-phosphatase treated PNA, pH 6.7 (Sample G), n = 0.087, k = 0.178 × 10⁵				
3	9.24	8.5	4.42	5.52	6.0	0.00648	1.46
		16.3	8.88	11.1	10.4	.01123	1.26
		24.3	13.1	16.4	15.8	.0171	1.30
		34.0	18.9	23.3	20.6	.0223	1.18
		41.5	23.4	29.3	24.4	.0264	1.12
		49.8	28.4	35.5	28.6	.0309	1.09
			pH 5.5 (Sample G) n = 0.156, k = 0.090 × 10⁵				
3	9.25	7.85	4.31	5.46	4.78	0.00517	1.20
		16.2	8.54	10.6	11.30	.0122	1.43
		24.2	13.4	16.4	15.60	.0169	1.26
		32.8	18.1	22.0	21.6	.0234	1.29
		40.8	24.6	29.0	23.7	.0256	1.04
		49.6	29.2	34.4	30.5	.0331	1.13

according to existing procedures.[11,12,13] No correction for the Donnan effect was applied since it was found by calculation to be negligible.

The results of the *p*H titration of samples A, B, C, EA′, F and K are shown in Figs. 4 to 6. In these curves, the continuous line represents the experimental curve while the broken line is the theoretical curve constructed from the analytical data presented in Table VI. The experimental results are expressed in terms of the number of equivalents of acid or base added per 4 gram atomic equivalents of phosphorus. Thus the concentration of nucleic acid was calculated from a unit molecular weight which contained 4 gram atomic equivalents of phosphorus. The zero of equivalents was, for convenience, selected as *p*H 8. The pK_a values used were: for the amino group, guanylic 2.3, adenylic 3.7, cytidylic 4.2; for the hydroxyl groups of guanine and uracil, 10.2.[20] In the case of Sample K, values of 9.36 and 9.46 for the hydroxyl groups of guanine and uracil were used, respectively.

Discussion

It is seen from Figs. 1 and 2 that the interaction of rosaniline with pentose nucleic acid and the ribonuclease-treated acid is not expressed by the simple equation (1).

$$r/c = kn - kr \qquad (1)$$

where k is an intrinsic binding constant, r the number of dye molecules bound per mole of PNA (or phosphorus) and n is the maximum number of dye molecules which can be bound per mole of PNA or phosphorus. The curves remain nonlinear when Scatchard's electrostatic correction[11,21] (equation (2)) is applied.

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

where $w' = (1 + 1/n)w$ and the value of w is based on the Debye–Hückel theory. Thus, since w' was treated as a parameter using a wide range of w' values, it appears that the exponential ex-

(20) Levene and Bass, "Nucleic Acids," Reinhold Publishing Corp., New York, N. Y., 1931, p. 284.

(21) Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

TABLE V

THERMODYNAMIC RESULTS FOR RIBONUCLEASE-RESISTANT FRACTIONS AND PNA

	Resistant fractions						PNA			
Sample	EA			EB			A		B	C
pH	5.3		6.6	5.3		6.6	5.5	6.6	6.6	6.6
Temp., °C.	3	27	3	3	27	3	3	3	3	3
n_1	0.0076		0.0070	0.0018	0.0014	0.006	0.0041	0.0015	0.0018	0.0018
n_2	0.153	0.160	0.153	0.118	0.119	0.174	0.146	0.148	0.184	0.0727
$k_1 \times 10^{-5}$	2.27	.....	3.21	3.12	3.76	1.63	1.87	5.67	5.34	2.24
$k_2 \times 10^{-5}$	0.149	0.0954	0.115	0.112	0.0714	0.068	0.025	0.025	0.018	0.124
ΔF_1^0(kcal./mole)	−6.75	.....	−6.95	−6.94	−7.66	−6.58	−6.65	−7.30	−7.28	−6.75
ΔF_2^0(kcal./mole)	−5.26	−5.45	−5.12	−5.11	−5.29	−4.84	−4.28	−4.28	−4.11	−5.17
ΔH_1^0(kcal./mole)				−15.22						
ΔS_1^0(e.u.)				−0.03						
ΔH_2^0	−2.07			−3.04			+0.32	−0.24		−0.57
ΔS_2^0(e.u.)	+7.92			+7.50			+16.7	+14.6		+2.08
A $\times 10^{-5}$ (ordinate intercept)	4.0	1.5	4.0	1.90	1.37	2.15				

pression[2] does not adequately describe the data. The data were accurately expressed by two-constant equations obtained according to existing procedures.[11,12,22]

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

It should be emphasized that equation (3) states that there are at least two types of sites in these molecules. On the basis of the present data, it cannot be concluded that there are only two types, but rather that there are at least two groups which correspond to the constants n_1k_1 and n_2k_2, respectively. Thus each group could be composed of a series of sites having nearly identical k values indistinguishable by the present technique.

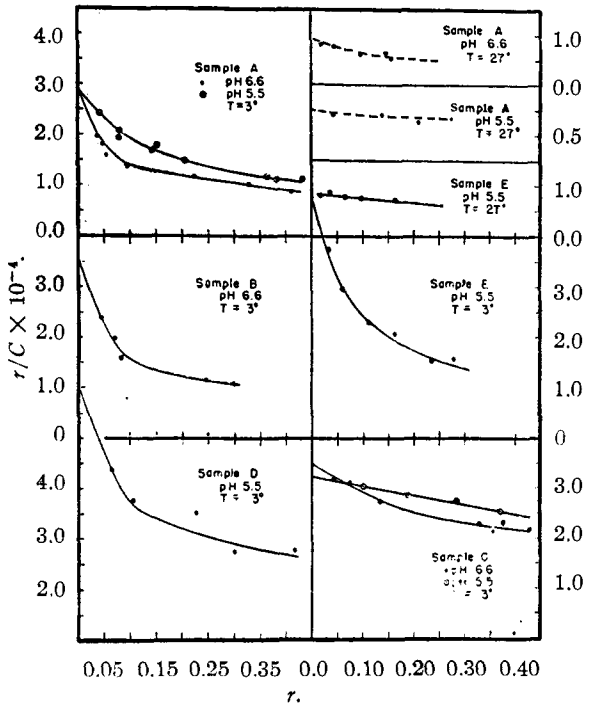


Fig. 1.

In a previous communication,[11] evidence was presented which indicates that the sites of interaction of rosaniline with desoxypentose nucleic acid involve phosphate groups. It is probable that a similar situation obtains in the case of PNA.

(22) Scatchard, Scheinberg and Armstrong, THIS JOURNAL, **72**, 535 (1950).

Since PNA exhibits secondary and primary phosphoryl dissociations, it appears reasonable to postulate, as in the case of DNA, that the two intrinsic binding constants (k_1 and k_2, respectively) represent these two types of anions. We shall make the assumption that at most one molecule of dye attaches to the monovalent anion $((RO)_2\text{—}\overset{\overset{O}{\|}}{P}\text{—}O\ominus)$

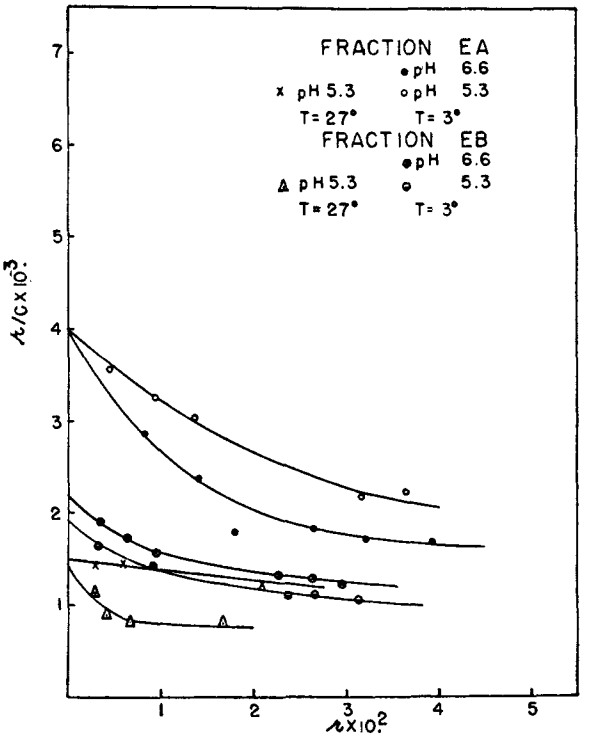


Fig. 2.

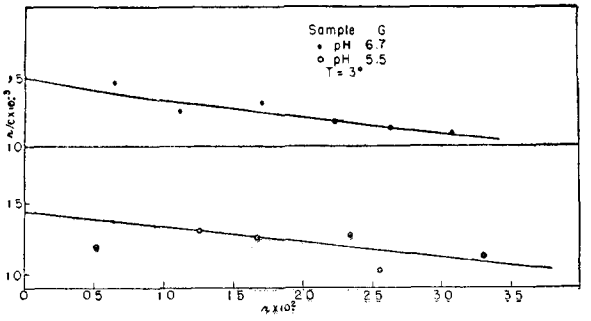


Fig. 3.

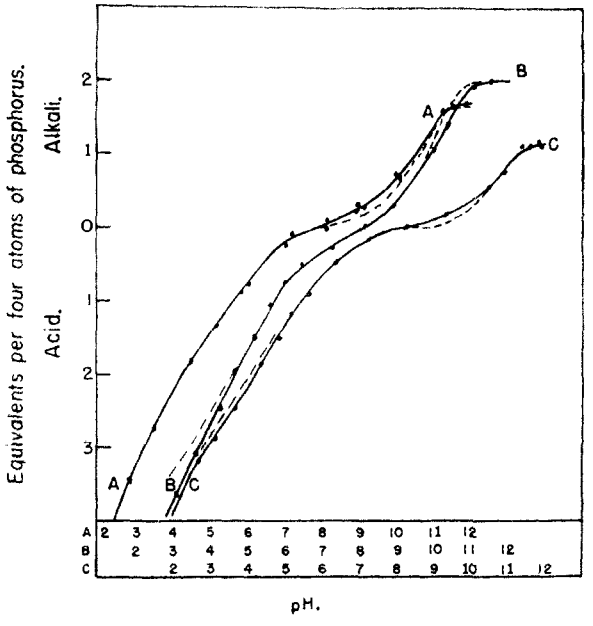


Fig. 4.

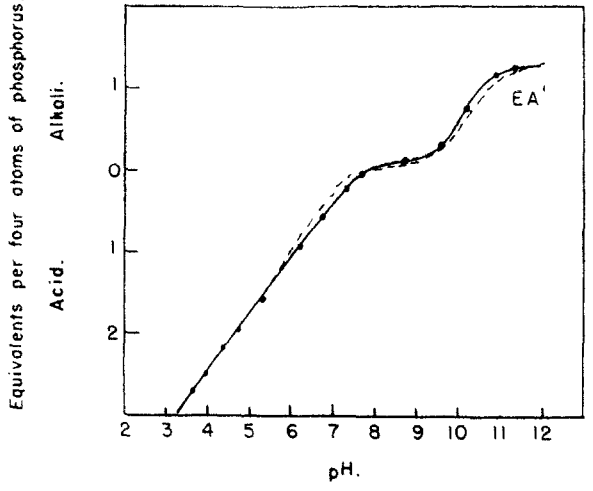


Fig. 5.

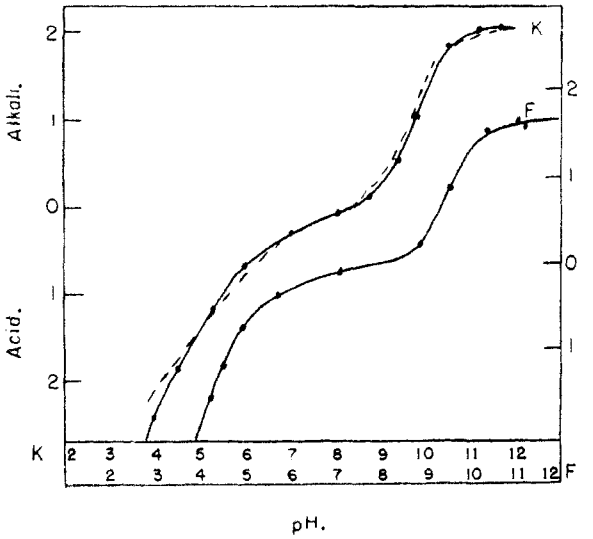


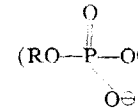
Fig. 6.

TABLE VI

Sample	Analytical data Equivalents/four atoms P		Theoretical curves Equivalents/four atoms P Amino	Nuclear hydroxy[a]	Secondary phosphoryl dissociation
A	Guanine	1.29	1.29	1.29	
	Adenine	1.03	1.03		1.50
	Cytosine	0.79	0.79		
	Uracil	0.92	..	0.31	
B	Guanine	1.29	0.79	1.29	
	Adenine	0.95	.95		
	Cytosine	.83	.83		1.60
	Uracil	.75	..	0.75	
C	Guanine	1.08	0.70	1.08	
	Adenine	0.94	.94		1.20
	Cytosine	.70	.70		
	Uracil	.85	..	0.09	
EA'	Guanine	1.81	0.00	1.40	
	Adenine	0.94	.72		1.80
	Cytosine	.38	.38		
	Uracil	.37	..	0.00	
K	Guanine	2.00	0.00	2.00	
	Adenine	1.61	.66		
	Cytosine	0.66	.66		1.22
	Uracil	0.61	..	0.13	

[a] In all cases (except Sample K) the distribution of the equivalents of the hydroxyl groups between uracil and guanine has been arbitrary since the same pK_a value (10.2) was used for both in the theoretical curves. It is more significant, therefore, to compare total equivalents of guanine and uracil (from analytical data) with the total used for the theoretical calculations.

It would be expected that the divalent anion



binds but one molecule of rosaniline, and more strongly because of the greater negative charge. It is possible, though unlikely, that two rosaniline molecules interact at this site.

Calculation reveals that only about 13% of the phosphoric acid groups of PNA are available for binding. Since a large proportion of the phosphoric acid groups exist as anions at the *p*H values of this study, the low degree of binding at these sites may, in part at least, be due to steric effects. This is a reasonable assumption, based on the knowledge that nucleic acids are polymers composed of nucleotides which are similar in nature, and interaction should be the same for the nucleotides in the absence of other effects. The selective interaction which is observed may therefore result from a union of these similar nucleotides into a branched, network structure such as has been postulated by Fletcher, Gulland and Jordan[23] on the basis of titration curves. The unavailability of sites may also be due to hydrogen bonding of the type discussed previously.[11]

Numerous investigators[24,25,26] have titrated PNA and have determined the number of primary and secondary phosphoryl dissociations. In general, the ratio of secondary to primary has been found to be about $^1/_3$. Our results yield a significantly smaller number for divalent/monovalent, both at

(23) Fletcher, Gulland and Jordan, *J. Chem. Soc.*, 33 (1944).
(24) Levene and Simms, *J. Biol. Chem.*, **70**, 327 (1926).
(25) Jorpes, *Biochem. J.*, **28**, 2102 (1934).
(26) Allen and Eiler, *J. Biol. Chem.*, **137**, 757 (1941).

*p*H 5.5 and *p*H 6.6. The results may be interpreted in either of two ways. The binding at a large number of divalent phosphoric anions may be weak, *i.e.*, the binding constants would approach those of the weaker monovalent anions (k_2), and the k_2 class would thus contain at least two types. The net effect would be to increase n_2 (number of monovalent anion binding sites) and therefore lower the value of n_1/n_2 (divalent/monovalent). Alternatively, the divalent anions may be buried within the molecule and not be accessible to a large molecule such as rosaniline, in contrast to a small entity such as hydrogen ion.

One of the significant features of the present study is the similarity of the binding constants of the various samples of PNA. This similarity is structurally significant and may be interpreted in terms of over-all structure only if we assume that differences in structure would be elicited by this technique. Evidence which lends support to the assumption that the similarity is significant was presented in a previous communication,[11] wherein it was shown that equilibrium dialysis does reveal subtle differences between samples of DNA before and after treatment with acid or alkali. Since the commercial sample of yeast PNA was undoubtedly degraded (by alkali) during isolation, while the pancreas PNA was obtained by mild means, it would appear that drastic treatment does not alter the general pattern or distances between ionized phosphate groups, *i.e.*, one may infer that PNA is merely split into smaller but similar fragments. Further, the fact that several fractions of commercial yeast nucleic acid, yeast nucleic acid extracted with potassium thiocyanate and pancreas nucleic acid all possess similar binding constants and n values is consistent with Gulland's[27] suggestion that a similar backbone structure is involved in all cases studied. The purine and pyrimidine content may vary from sample to sample, but this variation would not be expected to alter the results significantly since the nitrogenous bases are involved only in an indirect manner in the binding process.

It was stated in the experimental section that some samples of PNA yielded dialyzable components. Since these are smaller units of the nucleic acid, it was of interest to examine their binding properties both from the viewpoint of the effects on the present results and the relationship which these smaller entities might bear toward the original nucleic acid. Clearly, equilibrium dialysis is not suited for this purpose. Partition analysis, as developed by Karush,[28] is a simple and rapid technique. Briefly, the method consists in determining the change in the distribution of dye between two immiscible solvents brought about by the (dialyzable) substances which are in the aqueous phase. In our case, the organic phase was composed of hexanol and heptane and the aqueous phase contained phosphate buffer and sodium *p*-toluenesulfonate.[29] It was found that a solution of the dialyzable components exhibited no significant binding. Further, pure samples of the four mononucleotides appeared not to bind rosaniline. From these results it may be concluded that (a) binding distinguishes between the smaller units of nucleic acids and the nucleic acids themselves and (b) the dialyzable components did not introduce significant errors in our data.

It is noteworthy that the intrinsic binding constants (k) of PNA are similar to those of DNA. This suggests that the interaction of dye and acid is essentially the same in both cases and that the ionized phosphate groups are involved. The number of available sites (n) in the case of PNA is about 13% of the total while with DNA there are about twice as many. This effect may be correlated with the greater extent of branching in PNA, a situation which may result in steric interference.

Since the addition of hydrogen ions to phosphate ions is accompanied by positive entropy changes, the present data support the conclusion that the interaction of dye with nucleic acid occurs at the phosphoric acid anions.[11] As in the case of DNA, the positive standard entropy changes indicate that water molecules are liberated during the formation of the complex.

It is to be noted that the binding capacity of the resistant fractions is of the same order of magnitude as that of the original nucleic acid (Table IV). However, comparison of the n_1 value of fraction EA (*p*H 6.6) with that of nucleic acid B (since these were isolated in a similar manner) reveals that there is more binding at the n_1-type sites of EA. EB (*p*H 6.6) and C exhibit the same effect. Before these results are interpreted in terms of structure, it must be recalled that n_1 and n_2 are obtained by treating n as a parameter,[11,12] *i.e.*, minimum values of n which give the best theoretical fit are used. Insofar as this procedure is justifiable, the larger n_1 values are suggestive of a greater extent of binding at the divalent anions, an effect which could result from removal of the interfering portions of the PNA molecule by ribonuclease. With one exception (Sample C) all the nucleic acids studied possess k_2 values of *ca.* 0.04×10^5 while the k_2 values of the ribonuclease treated nucleic acid are, in general, two or three times as large. These results suggest that steric inhibition of binding has been decreased as a result of smaller molecular size, *i. e.*, the "distance of closest approach" to the charged group has been decreased.

The r/c *vs.* r plot for the interaction of rosaniline with the ribonuclease–phosphatase treated nucleic acid (G) is shown in Fig. 3 (Table IV). These curves are linear, though there may be some uncertainty in the region $r < 0.5 \times 10^{-2}$, and it appears that the binding process obeys the simple mass action equation (1). The best straight line was obtained by applying the method of least squares to the results. The value of n was estimated by linear extrapolation to the abscissa and kn, and therefore k, from the ordinate intercept. It should be emphasized that the values of n are significant only insofar as the linear extrapolation is justifiable.[30]

(27) Gulland, *J. Chem. Soc.*, 208 (1944).

(28) Karush, THIS JOURNAL, **73**, 1246 (1951).

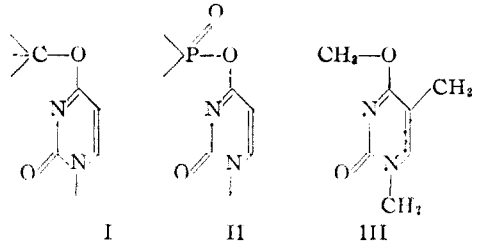
(29) The application of partition analysis to the study of nucleic acid structure is the subject of a forthcoming publication.

(30) The curve at *p*H 6.7 in Fig. 3 has a slight curvature in the region of low r values. However, the n and k values in Table IV were found from the best straight line.

There are two pertinent points to be considered in the evaluation of these results. (a) The maximum binding capacity of G is estimated to be of the same order of magnitude as it is in the parent PNA (A) and the ribonuclease-treated fractions (EA and EB); (b) only one type of site appears to be involved since the simple mass action equation (1) holds. Insofar as binding data reflect the general architecture and charge distribution of the molecule, it is suggestive from (a) that the ribonuclease–phosphatase fraction (G) resembles a nucleic acid. The intrinsic constant, k (Table IV) of G corresponds to k_2 of EA and EB, and it is seen that these values are about two- or threefold larger than those of k_2 of various PNA samples. It is suggested that this increase in binding strength is due to a lower degree of branching in the enzyme-treated materials. Comparisons of the present curves with those obtained with the parent PNA and the enzyme-resistant fractions reveal a general displacement toward lower values of r/c. This may be due to the absence of k_1-type (divalent anion) binding, though the reason for this is not apparent. It may be that the divalent groups are unfavorably situated geometrically. That is, if these groups were remotely situated from the general contour of molecule, their behavior could approach that of the mononucleotides which have been shown not to bind rosaniline.

Titration Data.—Although many investigators have carried out titrations with yeast nucleic acid, correlation of the equivalents of acid or base used with the purine and pyrimidine composition has been impossible because of the unavailability of analytical data. Theoretical curves have been calculated by various workers on the assumption that PNA is a statistical tetranucleotide, *i.e.*, that it is composed of units containing one mole of each of the four nucleotides. This has led to conflicting views concerning the macromolecular structure. Briefly, the present status of the structure of nucleic acids is the following: Nucleic acids are high molecular weight substances composed of nucleotide units. The phosphoric acid residue serves, in part at least, to join the nucleotides through the ribose portion at the 2′- or 3′-position. The view that only 2′-, 3′-linkages are present is open to question and will be discussed later.

It can be seen from Table VI that two nucleic acid samples (A and C) and the enzyme-treated nucleic acids (EA′, F and K) require fewer equivalents of alkali between *p*H 8 and 12 than would be predicted on the basis of the guanine and uracil content. In all cases the discrepancy between the theoretical and the expected equivalents of alkali necessary for the neutralization of the guanine and uracil hydroxyl groups is beyond the experimental error of the analytical procedure used for the determination of these bases. Recently, however, Wiener, Duggan and Allen[31] have carried out *p*H titrations on similar materials. They found the number of hydroxyl groups by titration to be greater than that by analysis. This discrepancy may in part be due to the nature of their curves in the region of *p*H 12 which, as they state, do not level off. Our results suggest that some of the hydroxyl groups are bound in a covalent linkage. Types I and II are the most likely possibilities for a covalent linkage of either uracil or guanine. Since



I II III

PNA is hydrolyzed to mononucleotides on standing in 1 *N* alkali, the linkage chosen must be labile to alkali. A model compound III was found to be stable to 1 *N* alkali, and on this basis type II appears to be more tenable.

The possibility that these nuclear hydroxyl groups are blocked by hydrogen-bonding appeared remote on the basis of back titration curves. It has been shown by Fletcher, Gulland and Jordan,[23] and confirmed by us, that titration with acid from *p*H 12 does not result in the formation of additional free hydroxyl groups. In this back titration about 0.2 equivalent of secondary phosphoryl groups are found in addition to those of the forward titration. This is probably due to alkaline hydrolysis. In the construction of the theoretical curves, the distribution of the equivalents of the hydroxyl groups of guanylic and uridylic acids was arbitrary, since it is not possible to distinguish the two by their pK_a values.

In all cases in the region of low *p*H the theoretical curves could not be made to fit the experimental data when uridylic acid was considered as a titratable group. This suggests that the phosphate group of uridylic acid is triply-esterified, an assumption first set forth by Fletcher, Gulland and Jordan[23] and later questioned.[32] In a number of cases guanylic acid also appears to be unavailable for titration. For example, the theoretical curve for the ribonuclease–phosphatase treated nucleic acid (Sample K) required that all of the uridylic and guanylic acids and part of the adenylic acid[33] be unavailable in order to obtain the best fit. If we are to attach any significance to these results, it would appear that this material, which is a small part of the original nucleic acid, is a highly branched structure.

An interesting feature of the theoretical curve for Sample K is that pK_a values of 9.36 and 9.46 were used for the hydroxyl groups of guanylic and uridylic acids, respectively. These values have been obtained by titration of the respective mononucleotides.[20] However, in other investigations[20,23] a value of 10.2 for both of the acids was used in theoretical calculations since the lower values re-

(31) Wiener, Duggan and Allen, *J. Biol. Chem.*, **185**, 163 (1950).

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

(33) That adenylic acid appears to be unavailable for titration in these samples, whereas in all samples of the original PNA it is available, seems to be contradictory. However, since the present preparations represent about 5 to 10% of the weight of the original PNA and since about one-half the adenylic acid in these is unavailable, it is very probable that the blocked adenylic acid in the PNA (which would be at most about 5% of the total adenylic acid) is not detectable by the titration method used.

sulted in curves which in no way could be made to fit the experimental curve. That the lower values result in a satisfactory fit in the present case suggests that the (structural) features which cause this discrepancy in the parent nucleic acid and the ribonuclease-resistant fraction are no longer operative in these preparations. That is, by virtue of the smaller size, their behavior approaches that of the mononucleotides.

Periodate Titration.—We have found that 4 to 6% of the total D-ribose in various samples of yeast pentose nucleic acid is oxidized by periodate. The mononucleotides isolated from PNA thus far show no periodate uptake. These results are consistent with the view that the phosphoric acid group is linked to the 2′- or 3′-position of the D-ribose[34] in the mononucleotides and that a large part (about 95%) of the D-ribose of nucleic acid contains such groups. On titrating samples EA, EA′ with periodate, we have observed a relatively high uptake (*ca.* 15% of the total sugar content). This indicates that a significant amount of the sugar does not contain phosphate groups at the 2′- or 3′-position. On the other hand, the analytical data (Table I) show that there is sufficient phosphorus present to permit all of the bases to be present as nucleotides. To reconcile these results, it is necessary to assign another phosphate linkage (in addition to the 2′- and 3′-linkages of the nucleotides). If it is assumed that a furanose ring exists, as it does in the mononucleotides isolated thus far, the data are consistent with the presence of a 5′-phosphate linkage. Gulland[35] obtained evidence of a 5′-phosphate by observing the liberation of phosphoric acid on treatment of nucleic acid with a mixture of phosphodiesterase and a highly specific 5′-nucleotidase. However, he was not able to isolate a nucleoside-5′-phosphate and attributed this to a migration from the 5′- to the 2′- or 3′-positions.

The ribonuclease–phosphatase treated nucleic acids, F, G and K, show relatively high uptakes of periodate, namely, 0.88, 0.70 and 0.92 millimole per gram, respectively. In the case of Sample G the analytical data show that there is no pyrimidine nucleotide phosphorus present and that the phosphorus is not present in sufficient amounts to permit all the purines to be present as purine nucleotides, *i.e.*, some purine nucleosidic groups exist. There are 0.55 millimole of pyrimidines per gram of G and 0.25 millimole excess of purines over purine nucleotide phosphorus. If, as is generally believed, the D-ribose exists in furanose form, 0.80 millimole of periodate should be consumed per gram of G since this number would represent the total nucleosidic content. This is in fair agreement with the experimentally observed quantity, 0.70 millimole. Samples F and K contain 0.68 and 0.72 millimole of pyrimidines per gram. In these cases the uptake beyond that of the pyrimidine content cannot be accounted for in terms of purine nucleosidic units and it may be that some of the pyrimidine nucleosidic groups are capable of consuming more than one equivalent of periodate.[36]

(34) Carter, THIS JOURNAL, **72**, 1466 (1950).

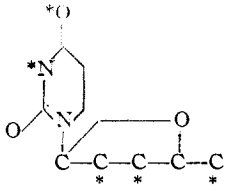
(35) Gulland and Jackson, *J. Chem. Soc.*, 1492 (1938).

(36) This could occur if the D-ribose were in open chain form and the latter is possible if the 1-position is blocked with a phosphate group, in order to prevent cyclization. Periodate oxidation results, similar to ours, have been obtained by Schmidt, Biology Conference on "Current Problems in Nucleic Acids," Oak Ridge, Tennessee, April, 1950, in press.

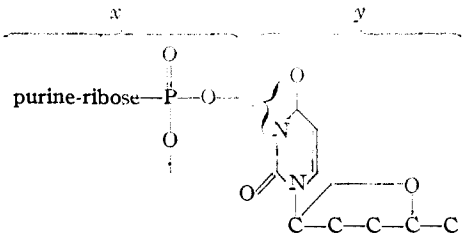
The suggestion that nucleosidic units exist in Samples F, G and K is supported by the results of ion-exchange analyses in conjunction with the analytical data. It is seen from Table I that the hydrolyzable (purine nucleotide) phosphorus comprises nearly the entire phosphorus content. Since these materials contain a considerable amount of pyrimidines, the results suggest that a very large proportion of the uracil and cytosine are present as nucleosidic units. The possibility of the occurrence of pyrimidines as free bases is highly improbable, since these are not liberated by the enzymes. The question as to whether these pyrimidine moieties (nucleosidic groups) are integral components of the enzyme-treated nucleic acid must be considered next. When Sample G was eluted from a Dowex-1 anion column, less than 3% of material was found in the eluate at *ca.* *p*H 5. Since Cohn[18] has shown that nucleosides and free bases are removed from the resin under these conditions, it appears that most of the units in G are joined in such manner as to prevent their ready elution, *i.e.*, in a larger unit. A more extensive ion-exchange analysis was carried out with the exhaustively dialyzed sample K. In this case 2% of nucleosides was found in the eluate at *p*H 5. This material was undoubtedly a mixture, but the ratios of optical densities at various wave lengths suggested that cytidine was a major constituent. Elution with 0.005 *N* HCl liberated 8% of mononucleotides. No attempt was made to determine the separate nucleotides quantitatively. The mixture appeared to contain largely adenylic and cytidylic acids.

Treatment of pentose nucleic acids with 1 *N* alkali at 35° overnight results in a quantitative conversion to mononucleotides. Sample K was subjected to this treatment and 60 to 70% of the original material was recovered. The eluate at *p*H 5 contained about 10% nucleosides, most of which were liberated by the action of the alkali. Elution with hydrochloric acid yielded 2% cytidylic acid, 20% adenylic acid (*a* and *b*), and 29% guanylic acid (*a* and *b*). It is noteworthy that no uridylic acid was obtained. The liberation of nucleosides by alkali is consistent with the conclusion that some of the nucleosidic units are joined by a linkage other than one involving a phosphate to the sugar portion of the pyrimidine nucleosides, since this type would result in the liberation of nucleotides. Whether the nucleosides, which are liberated by alkali, are part of a large nucleic acid molecule cannot be settled on the basis of the present data. However, it appears reasonably certain that they are part of an entity which is larger than a mononucleotide.

There are two general ways in which two nucleoside moieties may be joined. (a) In the absence of a phosphate bond, there could be ether type (C–O–C) linkages either as sugar–sugar bonds or as sugar–pyrimidine bonds



(indicated by the asterisks). Such ether bonds would probably not be hydrolyzed by 1 *N* alkali at 35°. (b) If we consider the union as occurring through phosphoric acid, the most likely structure is



It is highly improbable that the portion *x* is joined to the D-ribose of *y* for the following reason. On mild acid hydrolysis the sugar–phosphate bond of purine nucleotides is cleaved, while that of pyrimidine nucleotides is not. Thus if a sugar–phosphate –sugar bond existed between *x* and *y*, this would most probably yield on hydrolysis a pyrimidine nucleotide. However, the analytical data indicate the presence of only purine nucleotides.

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectra of a number of samples of pentose nucleic acid, ribonuclease-treated and ribonuclease–phosphatase-treated nucleic acid have been determined, and the extinction coefficients calculated on the basis of an arbitrary molecular weight (Table VII). The molecular weight is that which contains four gram atomic equivalents of phosphorus. In this way a theoretical extinction coefficient can be calculated from the known content and extinction coefficients of the individual components and comparison of it with the observed values reveals valid similarities or differences.[37]

There are no striking differences in the spectral curves of the materials investigated. The position of the absorption maximum is nearly identical in all cases (259 mμ), and the curves are superimposable in several instances. It may be concluded, therefore, that the relative changes in purine and pyrimidine composition are too small to be detectable by ultraviolet spectroscopy.

A comparison of the theoretical extinction coefficient with the experimental value (Table VII) reveals that in all cases the latter is lower, an effect which has been noted by other workers.[38] The lower absorbency index of the intact nucleic acid may be attributed to alterations in the chromophoric groups resulting from the union of the components. If all the nucleotides were joined through the sugar phosphates, it would appear unlikely that this, of itself, would affect the intensity of absorption, since these groups are far removed from the absorbing system.[39] However, if the nucleotides in the nucleic acid were in close proximity, secondary interactions of the chromophore with phosphate, amino or hydroxyl groups, might give rise to the observed effect. Another possibility is that some of the chromophoric groups are directly involved in covalent bonds in the nucleic acid in such a way as to decrease their intensities.

TABLE VII

EXTINCTION × 10^{-3} AT 260 mμ

Sample	A	B	C	EA'	EB	F	G	J
Theor.	47.2	44.5	41.9	44.0	45.5	58.2	71.0	60
Exptl.	38.2	36.7	36.3	34.3	40.6	54.2	54.8	48.6

In Fig. 7 the spectrum of nucleic acid (A) is compared to that of a mixture of the four mononucleotides present in amounts identical to those in A. When the extinction coefficients of the nucleotide mixture are reduced to 80.5% of their values, the resulting curve is almost identical with that of A. Since there is no preferential alteration in intensity along the entire wave band, it appears that no one particular nucleotide is responsible for the lower absorption values of A. It may be noted in passing that treatment of nucleic acid A with 1 *N* alkali at room temperature results in about an 18% increase in intensity of absorption which is consistent with the fact that mononucleotides are liberated by this treatment.

Infrared Spectra.—The infrared spectra of the various materials are presented in Fig. 8. As in the case of the ultraviolet spectra, it is obvious that this method does not reveal differences in macromolecular structure. It is noteworthy that the spectrum of a mixture of mononucleotides, particularly in the 8 to 10 micron region, differs from that of the intact nucleic acids. A number of bands have been associated with various groupings, by Blout and Fields.[40]

X-Ray Data.—The X-ray patterns of the parent nucleic acid and the ribonuclease-resistant fraction exhibit broad blurs with a spacing of about 4.0 Å. This may represent the internucleotide spacing.[41] In the case of the phosphatase-treated materials, there are a number of discrete lines (Table VIII). This appears to be the first evidence of crystallinity in the polynucleotides. There is some doubt as to the contamination of Sample G with salts, although comparison with the patterns of a large number of possible contaminating salts revealed no similarities. A sample (J) which was exhaustively dialyzed was found to contain no inorganic materials and about 5% of mononucleotides. The lines of the four authentic mononucleotides do not match those of J. It is possible, therefore, that the lines of Sample J represent crystallinity, though this alone is not a sufficient basis for the crystalline state.

(37) In the cases in which a large proportion of the components are present as nucleosides (G, F, K and J), the theoretical extinction coefficient includes the contributions from the nucleosides. However, since the extinction coefficients of the mononucleosides are nearly identical with those of the corresponding nucleotides, negligible errors would be introduced if the values were interchanged.

(38) Schlenk, "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., 1949, p. 482.

(39) Cavalieri, Bendich, Tinker and Brown, THIS JOURNAL, **70**, 3875 (1948); *ibid.*, **72**, 2587 (1950).

(40) Blout and Fields, *J. Biol. Chem.*, **178**, 335 (1949).

(41) Astbury, *Symp. Soc. Biol.*, **1**, 66 (1947).

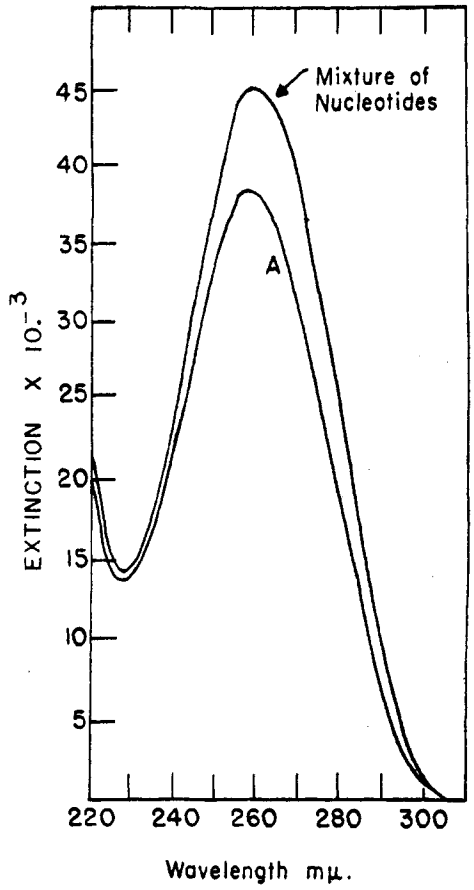


Fig. 7.

TABLE VIII

X-RAY DATA

F	d(A) J	G	F	1/I'[a] J	G
4.44	8.14	4.67	0.2	1.0	0.1
4.00	4.30	4.36		0.2	.1
3.89	4.00	4.00	.2		
3.47	3.87	3.93	.5	.2	.9
3.30	3.56	3.78	.1	.1	.2
3.09	3.45	3.64	.6	.8	.6
2.84	3.28	3.31	.05	.1	.3
2.74	3.01	3.19	.05	.3	.05
2.12	2.58	3.09	1.00	.05	.8
	2.37	2.81		.05	.8
		2.72			.1
		2.64			.1
		2.46			.02
		2.33			.05
		2.22			.05
		2.11			1.0
		2.05			0.02
		1.95			.02
		1.87			.1
		1.75			.02
		1.68			.1
		1.59			.03
		1.53			.03

[a] Intensities by visual examination of the film. I' = intensity of strongest line (= 1).

Suggestions Regarding Structure.—A consideration of the evidence presented leads to a number of definitive suggestions concerning the macromolecular structure of nucleic acids. The ribonuclease–phosphatase treated nucleic acid doubtless represents the simplest of the structures studied and perhaps is the most suitable starting point.

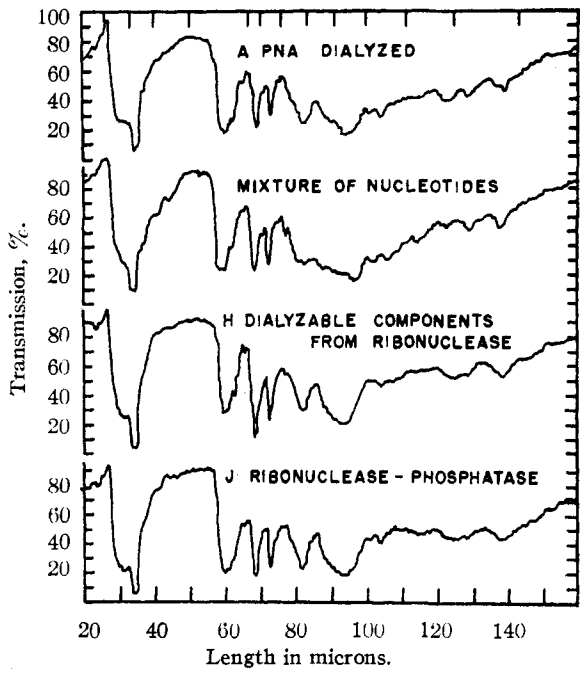


Fig. 8.

The question arises as to whether the product resulting from the treatment of nucleic acid with ribonuclease and phosphatase may still be considered a nucleic acid. Insofar as nucleic acids are defined as high molecular weight polynucleotides, it would appear that the material in question is a nucleic acid on the basis of its non-dialyzability through sausage casing. The binding properties and the deviation between the observed and calculated absorption spectra are consistent with this conclusion. However, these physical properties may reflect only gross structure and any fine structure of the original nucleic acid is probably lost as the result of enzyme action.

One of the most serious difficulties encountered in assigning a structure involves the homogeneity of the specimen. One phase of this problem is the following: The material may be composed of polynucleotides, each polynucleotide containing the four mononucleotides, or each polynucleotide may be composed of but one type of mononucleotide in which case the substance would be a mixture of four polynucleotides. The present data provide evidence for the first possibility, but do not exclude the second. Thus that the pyrimidine nucleosidic groups in Sample J are probably integral parts of the nucleic acid suggests that a bond may exist between the purine nucleotides and pyrimidine nucleotides (or nucleosides), since it is very unlikely that pyrimidine nucleosides are united into polynucleosides. The pyrimidine nucleosidic groups probably exist as side chains on the guanylic and adenylic acid backbone structure. The preponderance of guanine in Samples G, J and K indicates

that at least some of the guanylic acid molecules are adjacent to each other.[31]

The ribonuclease-resistant fraction probably represents that portion of the nucleic acid upon which the phosphatase acts to produce the materials just discussed (G, F, J and K). Comparison of their respective analytical data reveals the major difference to be the phosphorus content. This is consistent with the fact that only singly-esterified phosphates are removed by phosphatase. In the main, therefore, the ribonuclease-resistant fractions and the phosphatase treated material may be considered to be similar.

The relationship of the enzyme-treated materials to the parent nucleic acid is for the most part quite complex. The inability of ribonuclease to completely hydrolyze nucleic acid is one of the more pertinent problems. Linkages other than the usual sugar–phosphate type occur more frequently in the resistant fraction based on the titration results. The phosphate esters of the hydroxyl groups of the pyrimidine ring may be resistant to the enzyme. Although these types might be present in the digestible portion of the nucleic acid, they would not necessarily have to be cleaved by the enzyme, since they could be removed as a part of a larger portion. Implicit in this suggestion is the idea that the resistant fraction is an integral part of a larger molecule, part of which is attacked by enzymes. It is conceivable that the resistant fraction is a separate entity, distinct from the digestible portion.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF KANSAS]

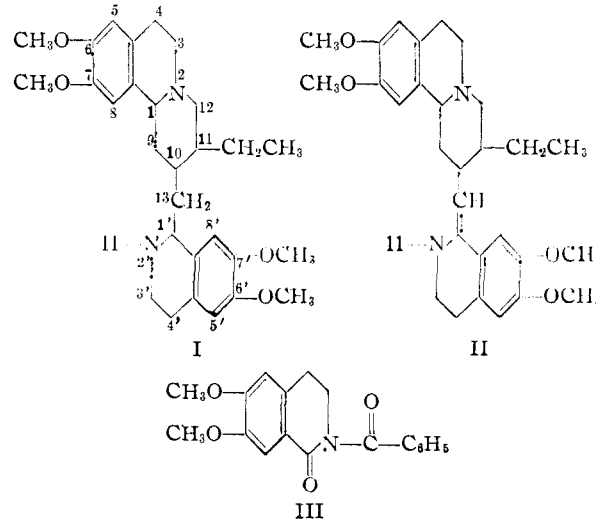
Studies on Emetine[1]

BY ROBERT N. HAZLETT AND WILLIAM E. MCEWEN

Attempts have been made to prepare some new diastereoisomers of emetine and to obtain further evidence concerning the structures of various dehydrogenation products of emetine. Mercuric acetate dehydrogenation of emetine gave a crystalline isotetradehydroemetine together with the known tetradehydroemetine. Catalytic hydrogenation of tetradehydroemetine hydrogen oxalate afforded emetine, isoemetine and two new diastereoisomers. Hydrogenation of either isotetradehydroemetine or isotetradehydroemetine hydrogen oxalate gave emetine as the only isolable product. The production of isoemetine determines the position of one of the ethylenic double bonds in tetradehydroemetine, and the striking similarity of the ultraviolet absorption spectra of tetradehydroemetine hydrogen oxalate and isotetradehydroemetine hydrogen oxalate indicates a close structural relationship between the two isomers. Some new reactions of rubremetinium chloride are described as well as some new Hofmann degradation results on acetylemetine.

Emetine (I)[2,3] having four dissimilar asymmetric carbon atoms is one of sixteen theoretical optical isomers. In addition to emetine only one of these isomers, isoemetine, is known.[4,5] Since isoemetine together with emetine is obtained on reduction of O-methylpsychotrine (II),[4] and since ozonolysis or perphthalic acid oxidation of N-benzoyl-O-methylpsychotrine affords N-benzoylcorydaldine (III),[6] it is known that O-methylpsychotrine has an ethylenic double bond at C_{13}–C_1' and that isoemetine differs from emetine in the configuration of C_1'. The main object of this work was to prepare further optical isomers of emetine starting with known degradation products of emetine. It is to be expected that knowledge of these stereoisomers will facilitate studies on the total synthesis of emetine and on the stereochemistry of the emetine series of compounds.

By dehydrogenation of emetine with four moles of mercuric acetate, Battersby and Openshaw[7] obtained a moderate yield of a substance, formed by the removal of four hydrogen atoms. This compound, tetradehydroemetine, was characterized as the hydrogen oxalate. The substance absorbed two moles of hydrogen on a microhydrogenation,



I II

III

gave rubremetinium chloride on further mercuric acetate dehydrogenation followed by addition of hydrochloric acid, and its ultraviolet absorption spectrum suggested that two ethylenic double bonds

(1) This, together with the work published in THIS JOURNAL, **71**, 1949 (1949), was submitted by Robert N. Hazlett in partial fulfillment of the requirements for the Ph.D. degree at The University of Kansas, 1950.

(2) M. Pailer and K. Porschinski, *Monatsh.*, **80**, 94 (1949).

(3) A. R. Battersby and H. T. Openshaw, *J. Chem. Soc.*, 3207 (1949).

(4) F. L. Pyman, *ibid.*, **111**, 419 (1917).

(5) F. L. Pyman, *ibid.*, **113**, 222 (1918).

(6) P. Karrer, C. H. Eugster and O. Ruttner, *Helv. Chim. Acta*, **31**, 1219 (1948).

(7) A. R. Battersby and H. T. Openshaw, *J. Chem. Soc.*, 867 (1949).