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Studies on the Structure of Nucleic Acids. V. On the Mechanism of Metal and Enzyme Interactions[1]

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Changes in the ultraviolet absorption spectrum of desoxypentose nucleic acid are brought about both by changes in *p*H and by the addition of magnesium ions. It is concluded that the effect caused by magnesium ions is due to alterations in the macromolecular structure of the nucleic acid. Evidence is presented which indicates that Mg^{++} attaches both to phosphate and nuclear amino groups. Inhibition of desoxyribonuclease action by citrate ions, as observed spectrally, does not involve removal of Mg^{++} from the desoxyribonucleic acid. Enzyme–substrate complexes of yeast pentose nucleic acid and ribonuclease have been studied. The formation of complexes probably involves interaction at the ionized phosphate groups, of which there appear to be at least two types. It has been shown that mononucleotides interact with pentose nucleic acid. From the results of the effect on complex formation it is tentatively suggested that inhibition of ribonuclease action by nucleotides occurs during intermediate stages.

The action of ribonuclease and desoxyribonuclease has been widely investigated, particularly from the viewpoint of accumulating information on the covalent linkages cleaved during degradation. In general, the procedures have involved examination of the system after partial or complete reaction. In the present investigation we have focused our attention on the enzyme–nucleic acid complex in the attempt to elicit additional information concerning the mechanism of the process. Since structural characteristics play a predominant role in enzyme action we have examined the system resulting from the interaction of desoxypentose nucleic acid (DNA) and magnesium ion in an attempt to gain an insight into the nature of the accompanying structural changes. In the case of pentose nucleic acid (PNA), the enzyme–substrate system was studied by partition analysis[2,3] under conditions where enzymatic action is at a minimum.

Experimental

Materials.—The cationic dye, trimethyl-*p*-(*p*-hydroxybenzeneazo)-phenylammonium chloride (THPA) and the DNA (sodium salt) used in these experiments were those employed previously.[3] The PNA was obtained from Schwarz Laboratories and it was the material characterized and employed in kinetic experiments.[4] Desoxyribonuclease and ribonuclease were obtained from the Worthington Biochemical Laboratory (isolated from beef pancreas).

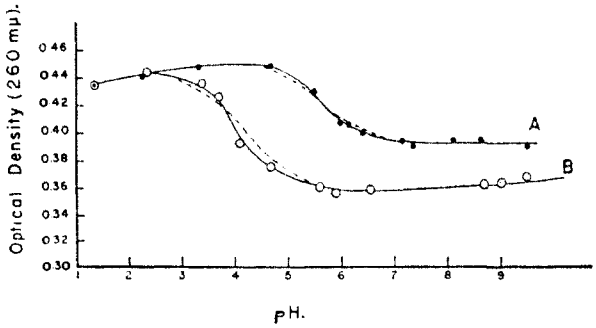


Fig. 1.—Effect of *p*H on spectrum of DNA: curve A, DNA alone; concn. 20 mg./liter H_2O; curve B, DNA (20 mg./liter) in 3×10^{-5} *M* $MgSO_4$.

Methods (a) Partition Analyses.—The dye-binding experiments were carried out by the method of partition analysis[2] used in another investigation.[3] Because of the rapidity, this method has the advantage that enzymatic action is at a minimum. The aqueous phase consisted of 2 ml. of PNA, 1 ml. of enzyme and 1 ml. of dye; the volume of the organic phase was 4 ml. The determinations were carried out at $4 \pm 0.5°$ and the equilibration period was eight minutes. To show that the extent of enzymatic action was at a minimum at this low temperature and for this short interval, the *p*H of a solution of PNA and enzyme was measured and found to decrease only slightly. At room temperature digestion occurred as shown by significant decreases in *p*H. To further demonstrate that little enzymatic action occurred during the partition it was shown that a mixture which had been partially digested at room temperature for 4 minutes possessed a binding capacity much lower than one containing the same original concentration of nucleic acid and which had been in contact with the enzyme at 4° for 4 minutes.

(b) **Spectral Measurements.**—All determinations were carried out with a Beckman spectrophotometer, model DU, with the use of 1-cm. silica cells. In examining the effect of *p*H on the spectrum of DNA the desired *p*H was obtained by adding small quantities of either sodium hydroxide or hydrochloric acid; no other salts were present. The same procedure was used in the determination of the spectrum in the presence of magnesium sulfate.

(c) **Titration Experiments.**—Titrations were usually carried out on 20–30 mg. samples in 10 ml. of distilled water with the use of a Beckman *p*H meter. The normality of the alkali and hydrochloric acid used was about one. Titrations were carried out with a microburet.

Results and Discussion

Part I. DNA

Effect of *p*H on the Spectrum of DNA.—The effect of *p*H on the ultraviolet absorption spectrum of DNA is shown in Fig. 1, curve A. It is seen that there is a significant decrease in the extinction coefficient in the region of *p*H 5–6. At low *p*H values the maximum occurs at 262 mμ; at high *p*H values, 260 mμ. The curve may be considered similar to a titration curve and a pK_a for the reaction

$$DNAH \rightleftarrows DNA + H^+$$

is readily obtained, yielding a value of 5.75. The theoretical curve based on this value is represented by the dotted line in Fig. 1, curve A. In order to ascertain which titratable groups cause the spectral change it is, of course, necessary to know the pK_a values of the nucleotides as they exist in the nucleic acid. For this purpose DNA was titrated electrometrically. The results are contained in Table I. The theoretical number of

(1) This investigation was supported by grants from the National Cancer Institute, of the National Institutes of Health, Public Health Service, and from the Atomic Energy Commission, Contract AT(30-1)-910. The author wishes to thank the Sloan Foundation for a stipend covering the period 1950–1951.

(2) F. Karush, THIS JOURNAL, **73**, 1246 (1951).

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

(4) L. F. Cavalieri, *ibid.*, **73**, 4899 (1951).

equivalents based on the known composition[5] were calculated from the pK_a values indicated in Table I and it is seen that good agreement was obtained. Wide discrepancies between the theoretical and experimental values resulted when the pK_a values of the mononucleotides were used. The ribonucleotides guanylic, adenylic and cytidylic acids have the pK_a values 2.3, 3.7 and 4.2, respectively,[6] and it is highly probable that the corresponding desoxynucleotides have similar values.[7,8] It is likely, therefore, that there are structural features peculiar to DNA which result in the changes in pK_a values. In this connection it should be recalled that in the case of yeast pentose nucleic acid good agreement was obtained using the pK_a values of the mononucleotides.[9]

TABLE I

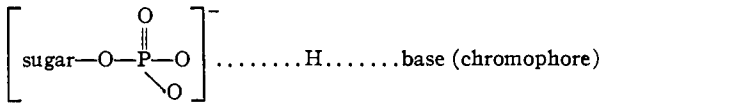
TITRATION OF DNA[a] WITH HYDROCHLORIC ACID

pH	7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5
Equivalents of acid/ mg. DNA × 10^7	0.0	0.35	1.0	1.9	4.2	7.6	11.9	15.4
Calculated equivalents[b] × 10^7		0.41	1.1	1.8	4.4	7.7	11.3	14.3

[a] 2.0 g./liter. [b] pK_a values assigned for the amino groups: guanylic, 3.5; adenylic, 4.3; cytidylic, 4.9; secondary phosphate, 6.0 (5% of the total phosphorus was assumed to be as end groups).

The spectral change (curve A) corresponds to a pK_a of 5.75 and it appears that the addition of a proton to the secondary dissociating group of the phosphate ion is involved since a pK_a value of approximately 6 has been assigned to this group.[6,10] Of the other groups, only the 6-amino group of desoxycytidylic acid has a dissociation constant approaching this value. Since the latter overlaps that of the phosphate group, the observed symmetrical curve would not be expected if both groups were involved.

The spectra of desoxycytidylic and desoxyadenylic acids at pH 4.8 and 6.2 show little or no difference in extinction at 260 mμ.[11] (The same is true for the four ribonucleotides.) Thus, either the chromophoric systems have been modified as a result of having been combined into a large molecule or alterations in macromolecular structure occur with change in pH. The fact that DNA solutions exhibit marked changes in viscosity and double refraction of flow[12,13,14] between pH 3 and 6 indicates the latter. As pointed out above, if secondary phosphoryl dissociations are involved it is possible that hydrogen bonds of the type suggested previously[15] on the basis of dye-binding data may alter the chromophoric groups.



This type of interaction could occur either between adjacent nucleotides in one DNA molecule or between DNA molecules.

Effect of Mg^{++} on the Spectrum of DNA.—The presence of small quantities of Mg^{++} (10^{-6} M) results in a decrease in the extinction coefficient accompanied by a shift of the maximum from 260 to 259 mμ. The results of a "titration" experiment

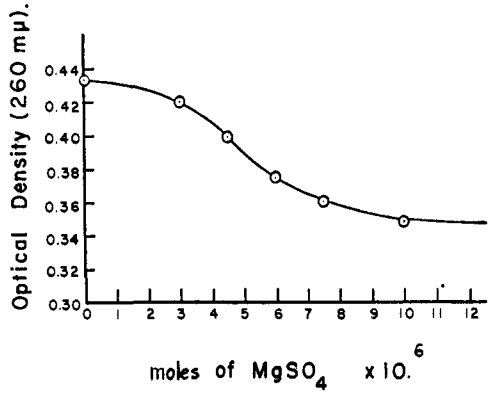


Fig. 2.—Effect of Mg^{++} on spectrum of DNA: the required ml. of $MgSO_4$ (1×10^{-4} M) together with 1 ml. of DNA (2 mg./ml.) were diluted to 100 ml. with water; pH 5.6. At concentrations of $MgSO_4$ higher than those shown in Fig. 2, no spectral changes were noted.

are shown in Fig. 2. Assuming reversibility, it can be seen that K for the reaction

$$DNA - Mg \rightleftarrows DNA + Mg^{++}$$

is 5.0×10^{-6}, indicating that the magnesium ion is strongly bound to the DNA. If more than one type of site were involved this value would represent an average. The question arises as to the nature of the sites to which Mg^{++} attaches. In a previous communication it was shown that Mg^{++} is capable of displacing cationic dyes from DNA.[8] Since these dyes undoubtedly interact with the ionized phosphate,[15,16] it appears certain that magnesium is bound in some way to the groups in question. This was further substantiated when DNA was titrated with magnesium sulfate (Table II), and with hydrochloric acid in the presence of a fixed quantity of magnesium sulfate (Table III). It is seen from Table II that magnesium ion lowers the pH and that throughout the pH range 3–7 smaller quantities of acid are required to reach any given pH (Table III). Similar titration experiments were carried out with the individual ribonucleotides, and in this case the Mg^{++} does not interact perceptibly. Further, neither desoxy-

(5) E. Chargaff, E. Vischer, R. Doniger, C. Green and F. Misani, *J. Biol. Chem.*, **177**, 405 (1949).

(6) P. A. Levene and H. S. Simms, *ibid.*, **65**, 519 (1921).

(7) J. M. Gulland, D. O. Jordan and H. F. W. Taylor, *J. Chem. Soc.*, 1131 (1947), also found it necessary to use pK_a values different from those of the nucleotides.

(8) The fact that the desoxynucleotides are eluted from Dowex 1 in a manner similar to that of the ribonucleotides provides evidence for this belief, E. Volkin, J. X. Khym and W. E. Cohn, THIS JOURNAL, **73**, 1533 (1951). Further, an approximate pK_a for desoxy-5-methylcytidylic acid is 4.3. W. E. Cohn, *ibid.*, **73**, 1539 (1951); the pK_a of cytidylic acid is 4.2.

(9) L. F. Cavalieri, S. E. Kerr and A. Angelos, *ibid.*, **73**, 2567 (1951).

(10) W. D. Kumler and J. J. Eiler, *ibid.*, **65**, 2355 (1943).

(11) These results were obtained by Cohn[9] and confirmed by us with samples kindly furnished by Doctor Cohn. Further evidence along these lines is found in the work of J. A. Little and G. C. Butler, *J. Biol. Chem.*, **188**, 695 (1951).

(12) S. Zamenhof and E. Chargaff, *ibid.*, **186**, 207 (1950).

(13) C. F. Vilbrandt and H. G. Tennent, THIS JOURNAL, **65**, 1806 (1943).

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

(15) L. F. Cavalieri and A. Angelos, THIS JOURNAL, **72**, 4686 (1950).

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

cytidylic nor desoxyadenylic acids show spectral changes on addition of magnesium sulfate. It appears, therefore, that the spectral changes are due to structural alterations of the DNA resulting from the interaction with Mg^{++}. Part of the interaction is suggested to be

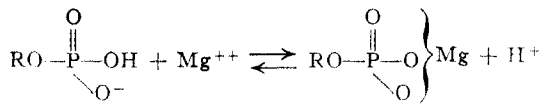


TABLE II

TITRATION OF DNA[a] WITH MAGNESIUM SULFATE

pH	Equivalents of magnesium sulfate × 10^7	pH	Equivalents of magnesium sulfate × 10^7
6.60	0	5.76	72
6.23	3	5.65	102
6.19	6	5.55	132
6.10	12	5.42	162
6.01	24	5.35	192
5.95	36	5.28	222
5.86	54	5.12	342

[a] 25 mg. of DNA dissolved in 25 ml. of water. Magnesium sulfate (0.3 *M*) was added from a microburet capable of delivering 0.0001-ml. The equivalents of magnesium sulfate have been corrected for the titration of water.

TABLE III

TITRATION OF DNA[a] WITH HYDROCHLORIC ACID IN THE PRESENCE OF MAGNESIUM SULFATE (2×10^{-3} *M*)

pH		
In absence of $MgSO_4$	In presence of $MgSO_4$	Equivalents of hydrochloric acid × 10^6
6.70	5.30	0.0
6.00	4.25	1.24
5.50	3.75	2.30
5.00	3.65	4.50
4.50	3.40	7.48

[a] 10 mg. DNA dissolved in 5 ml. of water, one containing the stated amount of magnesium sulfate. Blank titrations were carried out with water and 2×10^{-3} *M* magnesium sulfate and the corrections applied.

The question of interaction of magnesium ions at sites other than the phosphate groups must be considered next. It is conceivable that magnesium may interact with the amino or hydroxyl groups of the purines and pyrimidines to produce structures similar to the chelated complexes. This possibility was subjected to experimental test by observing the spectrum of DNA in the presence of a fixed quantity of magnesium sulfate at *p*H values where the charge of the amino groups is changing; Fig. 1, Curve B. A theoretical curve (dashed curve B) was calculated using a pK_a value of 4.2 and it is seen that there is fair agreement between the experimental and calculated curves. It is apparent that the effect of Mg^{++} increases as the amino groups lose their positive charge. Since the pK_a values of the amino groups obtained theoretically from the titration data are probably no better than ± 0.2 pK unit it is impossible to state with certainty which nucleotides are involved. That some of the amino groups are involved appears certain from the behavior in the presence of formaldehyde. Formaldehyde is known to block the 6-amino group of desoxycytidylic acid[7] and it is seen from Table IV that formaldehyde is capable of obliterating the effect of Mg^{++}. We may conclude that the 6-amino group of desoxycytidylic acid is involved in the nucleate complex and possibly that of desoxyadenylic acid. Assuming that formaldehyde does not alter the phosphoric acid groups, it appears then that two sites (amino and phosphate) are necessary to produce the magnesium complex as observed by its spectrum. It will be noted that curve B (Fig. 1) shows very little change between *p*H 5 and 6 in contrast to the relatively large effect observed in the absence of Mg^{++} (curve A). This suggests that the magnesium ion attaches to the phosphate groups exhibiting secondary dissociations and prevents the *p*H effect represented in curve A, a view which is in harmony with the fact that Mg^{++} results in the liberation of protons. It is also of interest that at the high concentration of formaldehyde (3.7%) the optical density of DNA is lowered (in the absence of Mg^{++}).

TABLE IV

EFFECT OF FORMALDEHYDE ON SPECTRUM OF DNA–Mg SYSTEM

In all cases the concentration of DNA was 20 mg./liter; magnesium sulfate concentration 3×10^{-5} *M*.

	Optical density at 260 mμ				
	In absence of formaldehyde		Formaldehyde		
pH	Water	$MgSO_4$	Water	$MgSO_4$	%
4.0	0.448	0.404	0.451	0.448	1.3
4.8	.446	.372	.408	.408	3.7

On the basis of this evidence it is possible to indicate at least part of the nature of the magnesium nucleate complex. From X-ray data on the ribonucleosides presented by Furberg[17] it is unlikely that magnesium of a magnesium nucleotide chelates with the amino or hydroxyl group of the pyrimidine ring. The fact that no changes in the spectra of desoxycytidylic and desoxyadenylic acids occur in the presence of Mg^{++} is consistent with this suggestion. In DNA it would appear, therefore, that the phosphate of one nucleotide is joined to the amino group of another through a magnesium atom. Whether this is an inter- or intramolecular bond cannot be stated with certainty. It is reasoned from X-ray data[17,18] that the nitrogenous bases lie parallel to each other and perpendicular to the length of the axis (as a stack of coins). Furberg[17] states that the sugar molecules are nearly perpendicular to the planes of the nitrogenous bases and hence parallel to the length of the molecule. If the DNA molecule is relatively rigid, the phosphate groups would be far removed from the nuclear amino or hydroxyl groups and the magnesium bridge would then be intermolecular. On the other hand, Furberg[19] has suggested that one structure of DNA may involve spiraling of the sugar phosphates around the nitrogenous bases. Such a structure might give rise to intramolecular complexes.

Interaction of DNAase.—Desoxyribonuclease (DNAase) does not interact with DNA either in the presence or absence of Mg^{++} as shown by the

(17) S. Furberg, *Acta Chem. Scand.*, **4**, 751 (1950).
(18) W. T. Astbury, *Symp. Soc. Biol.*, **1**, 66 (1947).
(19) Thesis, *cf.* M. J. Fraser and R. D. B. Fraser, *Nature*, **167**, 761 (1951).

dye-binding technique. If the enzyme attaches at all to the nucleic acid, it is probably not at the ionized phosphate groups capable of binding dye. To explore further the DNAase–DNA–Mg system the spectrum was observed under various conditions. When DNAase[20] is added to the magnesium nucleate at room temperature the spectrum reverts to that of DNA in the absence of Mg^{++}; the rate of change depends upon the enzyme concentration. The change could be due to: (1) enzymatic action, (2) the formation of an enzyme–substrate complex, (3) removal of Mg^{++} from the DNA. If DNAase were merely removing Mg^{++} from the DNA, further additions of this ion to the system should result in the spectrum of the original magnesium nucleate. This was not observed experimentally. To distinguish between the first two possibilities it was noted that the enzyme caused no spectral changes when added to the magnesium nucleate in the cold; however, when the solution was allowed to reach room temperature the change was observed. This suggests enzymatic action. Further evidence which points to the first possibility is found in the effect of fluoride and citrate ions. These ions are known to inhibit the action of DNAase.[21] When DNAase was added to magnesium nucleate in 0.003 *M* ammonium citrate at room temperature no spectral change was observed. Fluoride ion was much less effective in inhibiting the reaction, a finding which agrees with that of McCarty.[21] It appears highly probable, therefore, that the change brought about by DNAase is one due to enzyme action. The significance of these spectral effects will be discussed below. It should be emphasized that neither citrate nor fluoride cause any change in the spectrum of magnesium nucleate. Thus the inhibition mechanism, as observed spectrally, cannot involve simply the removal of Mg^{++} from DNA to form, for example, an undissociated magnesium citrate complex. The failure of citrate to alter the spectrum contraindicates the formation of a complex on the DNA molecule.

Mechanism of DNAase Action.—When DNAase is added to magnesium nucleate at room temperature the spectrum reverts to that of DNA in about ten minutes. This change is paralleled by a drop in viscosity which also occurs during about the first ten minutes.[21,22] Kunitz[22] found that the formation of acid-soluble products proceeds at a lower rate and that the rate of liberation of phosphoric acid groups is the lowest. Those results suggest that the first action involves cleavage of the macromolecule into submacromolecules. This is in contrast to a mechanism whereby very small units would be produced from the large molecule as a first step.

The extinction coefficient of DNA is about 25% lower than that calculated from the composition and the individual extinction coefficients of the components.[8] This is probably due, in part at least, to secondary interactions within the large structure. Upon digestion, the optical density of the mixture gradually increases and becomes approximately equal to the calculated value.[22] Thus it would appear that the hydrolyzed units are so small that secondary interactions are at a minimum. The suggestion[11,23] that the final mixture is composed largely of tetra- or pentanucleotides is consistent with the spectral findings though the question of the exact size of the units must be unanswered for the present. It is of interest that our ratio of Mg^{++}/P, which is $^1/_5$, suggests cleavage into pentanucleotides. It is conceivable that this ratio is not fortuitous. The fact that optimal enzyme activity occurs at higher magnesium ion concentrations[21,22,24] may have to deal with such properties as electrolyte concentration and have nothing at all to do with the enzyme action *per se*. An example of this effect is seen in the case of ribonuclease action where salts enhance the activity though the enzyme requires no metal activator.[25,26]

Part II. PNA

Interaction with RNase.—The quantity of RNAase bound to PNA was determined by observing the increase in the dye concentration in the upper phase on addition of enzyme. The fact that RNAase is capable of displacing a cationic dye from PNA represents evidence that the enzyme interacts at the ionized phosphate groups. To determine the extent of RNAase binding, r *vs.* r/c curves were plotted for varying amounts of enzyme (Fig. 3, Table V). Here r is the number of dye molecules bound per mole of PNA and c is the free equilibrium dye concentration.[9,27,28]

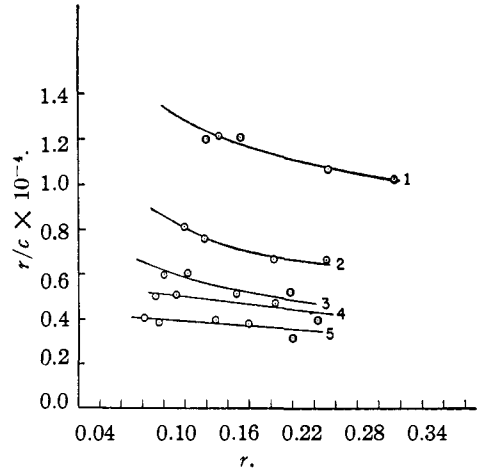


Fig. 3.—Binding of THPA to PNA in the presence of enzyme: curve 1, no RNAase; curve 2, 0.004 mg./ml.; curve 3, 0.008 mg./ml.: curve 4, 0.016 mg./ml.; curve 5. 0.040 mg./ml.; temp. 4 ± 0.5°; *p*H 6.0.

A molecular weight of 10,000 was assumed for PNA. For purposes of comparison r values at the same free dye concentration were chosen to determine the decrease in r with increasing enzyme concentration. Two such series are plotted in Fig. 4. It is

(20) At the concentration used in these experiments (1 γ/ml.) the enzyme did not contribute to the spectrum at 260 mμ.

(21) M. McCarty, *J. Gen. Physiol.*, **29**, 123 (1946).

(22) M. Kunitz, *ibid.*, **33**, 349 (1949).

(23) F. G. Fischer, H. Lehmann-Echternacht and I. Boettger, *J. prakt. Chem.*, **266**, 79 (1941).

(24) N. Weissman and J. Fisher, *J. Biol. Chem.*, **178**, 1007 (1949).

(25) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1940).

(26) J. P. Greenstein, C. E. Carter and H. W. Chalkley, *Cold Spring Harbor Symposia on Quantitative Biology*, **XII**, 82 (1947).

(27) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(28) I. M. Klotz and J. M. Urquhart, *ibid.*, **71**, 847 (1949).

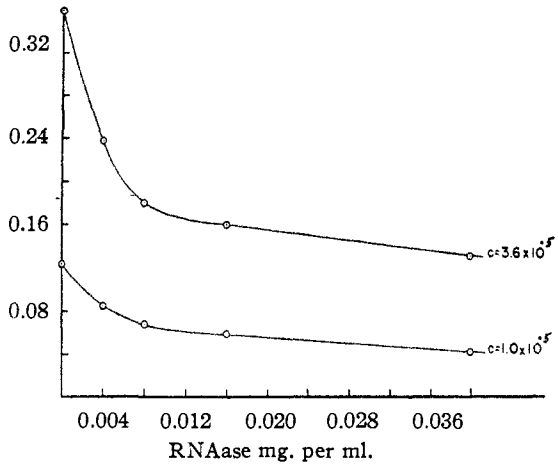


Fig. 4.—Dependence of r on enzyme concn.

seen that the greatest descent occurs in the region up to 8 γ of RNAase per ml. This corresponds to a molar ratio of enzyme/PNA of $^1/_{370}$, when 15,000 is used as the molecular weight of RNAase.[25] It should be emphasized that this ratio is not necessarily a measure of the total amount of bound enzyme. It is apparent from the nature of the curves in Fig. 4 that beyond a low concentration of enzyme, relatively little displacement occurs on further addition of enzyme. The results are consistent with the presence of two types of phosphate groups, such as were suggested on the basis of dye-binding experiments.[9]

Since the value of 8 γ reduces the ordinate value ($= r/c$) by about one-half, an approximate binding constant for RNAase is $1/8 \times 10^{-3}/1.5 \times 10^4 = 18.7 \times 10^5$, where 8×10^{-3} is the concentration of enzyme (g./l.) and 1.5×10^4 is the molecular weight of RNAase.

Interaction of RNAase with the Resistant Fraction.—It is generally known that RNAase hydrolyzes yeast PNA to the extent of about 80%.[29,30] The remaining 20% represents a resistant fraction which is not acted upon on further additions of enzyme. It is of considerable significance that RNAase does not interact with this fraction to form a complex as it does with the parent nucleic acid.

In comparing the resistant fraction to the parent nucleic acid, there are a number of features which should be noted. Evidence has been presented[9] which indicates that the resistant fraction is a more highly branched molecule. This structural effect is associated with a decrease in the number of titratable groups. Further, the periodate titration data indicate[9] that there is a larger proportion of nucleoside-5′-phosphates in this fraction. These differences may be responsible for the different behavior toward RNAase.

Interaction of Nucleotides.—Zittle[31] has shown that the mononucleotides exert an inhibiting influence on the action of RNAase. We thought it of interest to examine the effect of cytidylic and adenylic acids on the RNAase–PNA complex. To accomplish this it was necessary to determine the binding of these nucleotides to PNA as shown by the displacement of the dye (THPA).[32] It is seen from Table VI that adenylic acid complexes

TABLE V

BINDING OF THPA TO PNA IN THE PRESENCE OF RNAase

PNA concn., 2×10^{-4} M; temp. 4 ± 0.5°; pH 6.9

Dye concn. in aqueous phase $\times 10^5$	Concn. in upper phase $\times 10^5$	Concn. in lower phase $\times 10^5$	Bound dye $\times 10^5$	r	$r/c \times 10^{-4}$
In absence of RNAase					
5.00	1.61	1.00	2.39	0.119	1.19
6.25	1.97	1.26	3.02	.151	1.20
10.00	3.08	2.22	4.70	.235	1.06
12.50	3.76	2.85	5.89	.294	1.03
0.004 mg. RNAase/ml.[a]					
5.00	1.87	1.19	1.94	.097	0.815
6.25	2.33	1.56	2.36	.118	.755
10.00	3.64	2.72	3.64	.182	.669
12.50	4.38	3.48	4.64	.232	.666
0.008 mg. RNAase/ml.					
5.00	2.06	1.34	1.60	.080	.596
6.25	2.51	1.70	2.04	.102	.600
10.00	4.15	2.88	2.97	.148	.512
12.50	4.74	3.70	3.96	.198	.522
0.016 mg. RNAase/ml.					
5.00	2.16	1.41	1.42	.072	.502
6.25	2.62	1.80	1.83	.091	.506
12.50	4.87	3.95	3.69	.184	.465
16.70	6.55	5.69	4.46	.223	.393
0.040 mg. RNAase/ml.					
5.00	2.26	1.51	1.23	.061	.404
6.25	2.80	1.96	1.49	.075	.383
10.00	4.17	3.25	2.58	.129	.390
12.50	5.12	4.20	3.18	.159	.379
16.70	6.87	6.00	4.00	.200	.333

[a] It was shown that at these enzyme concentrations no interaction with THPA occurred.

TABLE VI

COMPETITIVE BINDING OF ADENYLIC ACID[a]

		Concn. in upper phase $\times 10^5$	Concn. in lower phase (free) $\times 10^5$	Bound THPA $\times 10^5$		$r/c \times 10^{-4}$
A	THPA alone	2.65	2.37			
B	THPA, adenylic acid	2.57	2.28	0.150	0.0075	0.0329
C	THPA, PNA	1.30	1.07	2.63	.131	1.22
			(1.13)		(.137)	(1.19)
D	THPA, PNA, adenylic acid	1.36	1.13	2.51	.125	1.11
E	THPA, PNA, RNAase	2.16	1.41	1.42	.071	0.502
			(1.13)		(.064)	(.567)
F	THPA, PNA, adenylic acid, RNAase	2.23	1.46	1.31	.065	.447
			(1.13)		(.058)	(.514)

[a] Carried out as described[3]; pH 6.9, temp. 4 ± 0.5°. Adenylic acid concentration 1.15×10^{-3} M, PNA concentration 2×10^{-4} M. RNAase concentration 1.06×10^{-6} M. Original dye concentration in aqueous phase 5×10^{-5} M.

(29) H. S. Loring, F. H. Carpenter and P. Roll, *J. Biol. Chem.*, **169**, 601 (1947).

(30) C. A. Zittle, *ibid.*, **168**, 119 (1946).

(31) C. A. Zittle, *ibid.*, **160**, 527 (1945).

(32) In a previous paper[3] it was stated that the nucleotides did not displace rosaniline from PNA. In those experiments the precision was low because of adsorption of rosaniline on the glassware. In the present investigation the reproducibility was of the order of 1% and the small effect could be detected.

to a small extent with the dye and successfully competes with the dye for PNA sites. Actually the competition is slightly greater than shown in the table since the r/c for the PNA–adenylic acid system (D) is increased by an amount bound by the adenylic acid itself. The competitive effect persists in the presence of enzyme. For comparison, values of r at the same free dye concentration have been calculated (values in parentheses, Table VI). The per cent. drop in r resulting from the addition of adenylic acid is nearly identical for the PNA and PNA–RNAase systems (C–D and E–F). Since RNAase causes the same per cent. drop in r in the presence of adenylic acid (C–E and D–F) it appears that the enzyme–substrate complex does not involve sites which combine with adenylic acid. Since the complex under discussion is probably the first of several formed during the course of the hydrolysis, it may be tentatively suggested that inhibition of the enzymatic action occurs during intermediate stages.

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Diels–Alder Reactions of Maleimide[1]

BY FRANCIS EARL RAY, EUGENE SAWICKI AND OLIN H. BORUM

An attempt was made to condense acrolein, acrylonitrile and crotonic acid and its ester with 2,5-dimethyl-1,5-hexadiene-3-yne or with 1,1'-dicyclohexenylacetylene without success. In investigating the relative reactivity of maleic anhydride and maleimide, it was found that the latter was much more active in condensations with anthracene and 2,5-dimethylfuran This suggested that maleimide might react where the other compounds failed. Success was achieved in condensing 1,1'-dicyclohexenylacetylene with maleimide to yield $\Delta^{6a7a,1a12a}$-tetradecahydrochrysene-5,6,11,12-tetracarboxdiimide.

The determination of the spatial configuration of naturally occurring and synthetic steroids is a problem which requires much further research. Luttringhaus[2] has shown that it is possible to bridge the molecule by linking, for example, the 2,6-positions of naphthalene. The present work was undertaken in the hope of obtaining similar compounds from stereoisomeric forms of polynuclear substances.

In 1940 Butz, Gaddis, Butz and Davis[3] heated 2,5-dimethyl-1,5-hexadiene-3-yne with maleic anhydride and obtained 1,5-dimethyl-2,3,4,6,7,8-hexahydronaphthalene-3,4,7,8-tetracarboxylic acid (anhydride). Similarly, Joshel, Butz and Feldman[4] by treating 1,1'-dicyclohexenylacetylene and maleic anhydride obtained tetradecahydrochrysene-5,6,-11,12-dicarboxylicanhydride. It was not determined whether the anhydride groups were *cis* or *trans*.

Much time was spent in unsuccessful attempts at reaction of such dienophiles as acrolein, acrylonitrile or crotonic acid with dienynes. Under comparable conditions maleic anhydride readily formed an adduct. Maleimide was then investigated as a dienophile in the Diels–Alder reaction. The dipotassium salt of the adduct could then, conceivably, react with a polymethylene dihalide to form a large ring compound.

In the formation of 1,1'-dicyclohexanolacetylene, an ether-*insoluble* high melting by-product was obtained. Analyses and molecular weight determinations indicated the compound to be $C_{16}H_{24}O_2$. As the 1-cyclohexanolacetylene type of compound is a known by-product in the Kazarian synthesis, it is possible that $C_{16}H_{24}O_2$ is a dimer of 1-cyclohexanolacetylene. A study is being made to elucidate the structure of this by-product.

Tuot and Guyard[5] attempted to prepare the dienyne from 2,5-diphenyl-3-hexyne-2,5-diol. They found that the diol was very sensitive to acids, only resins being obtained. We prepared 2,5-diphenyl-1,5-hexadiene-3-yne in rather poor yield by dehydration in an inert atmosphere.

Maleimide was prepared by a modification of the procedure of Plancher and Cattadori.[6] Blomquist and Winslow[7] and Harvey[8] were successful in bringing about its reaction with cyclopentadiene. A comparison of maleic anhydride and maleimide seemed to favor maleimide as the more active dienophile. For example, anthracene refluxed with maleic anhydride for 15 minutes gave a quantitative yield of the adduct as reported by Clar,[9] while the reaction of anthracene with maleimide in boiling xylene gave a violet exothermic reaction after two minutes of refluxing.

When maleic anhydride was treated with 2,5-dimethylfuran in dry ether at 0°, an adduct started to separate after four to six hours standing.[10] For the same reaction with maleimide, crystals started to separate *immediately*. In all cases, however, a practically quantitative yield of adduct was obtained.

This evidence of the reactivity of maleimide suggested that it might be suitable for our purpose. It is of course less desirable than a monobasic com-

(1) Supported by Office of Naval Research, Contract NR-055-225-N9onr-97300.

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