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

Studies on the Structure of Nucleic Acids. III. Kinetics of the Alkaline Hydrolysis of Yeast Pentose Nucleic Acid[1]

BY LIEBE F. CAVALIERI

The kinetics of the alkaline hydrolysis of yeast pentose nucleic acid have been investigated by means of a continuous stirred flow reactor. This technique allows the direct measurement of the rates of formation of the various nucleotides at different degrees of hydrolysis. Complex rate curves were obtained indicating that nucleic acid can yield nucleotides by a mechanism involving simultaneous reactions of both the direct and consecutive types. An adequate theoretical curve was constructed for cytidylic acid by treating the velocity constants as parameters. The reaction mechanism employed for the derivation of the equations was essentially: nucleic acid → nucleotides; and nucleic acid → nucleotides + intermediates → nucleotides. Assignment of the various constants to particular groups was not attempted. On the basis of the velocity constants, there appear to be at least two types of phosphate linkage present in pentose nucleic acid, one much more labile than the other. Further, the results could not be expressed quantitatively by assuming that the nucleic acid sample investigated consisted of one type of molecule.

One of the principal features which distinguishes desoxypentose nucleic acid from pentose nucleic acid is the lability of the latter toward alkali to produce mononucleotides. It is probable that this profound difference in chemical behavior is associated not only with the type of phosphate bond but also with the manner in which the phosphate groups are united.[2,3,4] On *a priori* grounds it might be expected that the action of alkali on pentose nucleic acid would involve consecutive stages. Indeed when the reaction is studied by following either the change in the ultraviolet absorption spectrum or the alkaline titer for the hydrolysis of phosphate esters no simple rate law expresses the results in satisfactory fashion. It was felt, therefore, that the reaction could best be studied in a stirred flow reactor as described by Denbigh[5,6] and Hammett.[7,8,9] Under these circumstances the instantaneous rate of formation of each nucleotide is measured directly, regardless of the order or mechanism of the reaction. The nature of this process will be discussed below.

Experimental

Apparatus.—The apparatus used was essentially like that of Saldick and Hammett.[8] Two one-liter reservoirs were connected to the reactor flask (0.14 liter) by means of glass tubing. Variation in the flow rates was obtained by inserting capillaries (*ca.* 40 mm. in length) of varying bores between the reservoirs and the reactor. Further variation in flow rate was obtained by adjusting the pressure head leading to the reservoirs. Both the reactor flask and the reservoirs were submerged in a thermostat held at 37.20 ± 0.05°.

Materials.—The yeast pentose nucleic acid was obtained from Schwarz Laboratories and was used as such. It was analyzed for purines and pyrimidine nucleotides according to the procedure of Kerr, Seraidarian and Wargon.[10] Ion-exchange analysis of the sodium nucleate according to the method described showed that 8% of the optical density units put on the column could be eluted with water. This probably represents the free base and nucleoside content of the sample.[11] Elution with 0.002 *N* hydrochloric acid removed an additional 3% of the optical density units. This material was a mixture of cytidylic and adenylic acids. Elution with 0.003 *N* and 0.005 *N* hydrochloric acid did not yield uridylic or guanylic acids.

Millimoles per gram of nucleic acid

Adenine	Guanine	Uracil	Cytosine	N
0.61	0.65	0.78	0.57	10.5

Kinetic Runs.—Nucleic acid solutions (10.00 g./l.) were made up just prior to use by suspending the acid in distilled water and adding sodium hydroxide to a *p*H value of 6.5. The solution was filtered to remove any particulate matter which causes clogging of the capillaries. The sodium hydroxide was also freshly prepared from carbonate-free alkali. Unless otherwise stated the normality of the sodium hydroxide solutions ranged from 0.690 to 0.700. The reactants were placed in the reservoirs and allowed to reach bath temperature. The reactor cell was filled with a solution composed of 50% of each reactant. Flow was started and the system was allowed to reach a steady state. In general, more reactor-volumes were permitted to traverse the system than was estimated as necessary in order to ensure attainment of a steady state. This was checked several times by analyzing the mixture at various values of traversed volumes. The values of reactor volumes contained in Table I were sufficient to ensure a steady state. Individual and total flow rates were determined by weighing samples (5–10 g.). Flow rates were additive to within less than 1%. At the highest value of total flow rate (7.9 ml./min.), some resistance to flow was indicated since the individual flow rates gave a calculated total flow of about 5% higher than the observed. A further check on the flow rate was realized by observing both the optical density (260 mμ) and *p*H titer of the overflow.

Samples from the over-flow were run into 1.88 *N* hydrochloric acid to quench the reaction. These solutions had a *p*H of about 1. It was shown that standing in the acid solution did not result in any detectable change in the nucleotide content. About 10-g. samples were used for the ion-exchange analyses.

Ion Exchange Analyses.—Ion-exchange analyses were carried out according to the procedure of Cohn[11] by the use of Dowex (Cl^-). The height of resin in the columns was between 6 and 7 cm. with a cross-section of 1.75 $cm.^2$. The samples (from the over-flow) were neutralized with sodium hydroxide to *p*H 6–7 and diluted with water to give a final value of 0.04 *N* sodium chloride. At this salt concentration there was no break through of nucleotides. The size sample was such that about 1000 optical density units (= optical density X volume) were analyzed. The solution (*ca.* 100 ml.) was forced on the column by air-pressure and the columns washed with water to remove free base and nucleosides. Elution of the nucleotides was then started in the prescribed fashion.[11] It was found suitable to use hydrostatic heads which resulted in flow rates of about 1.5 ml./min. In this manner there was little or no overlapping

(1) The author wishes to acknowledge the support of the Atomic Energy Commission (contract AT(30-1)-910) and the National Cancer Institute of the United States Public Health Service.

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

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(8) J. Saldick and L. P. Hammett, *ibid.*, **72**, 283 (1950).

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(10) S. E. Kerr, K. Seraidarian and M. Wargon, *J. Biol. Chem.*, **181**, 767 (1950).

(11) W. Cohn, THIS JOURNAL, **72**, 1473 (1950).

in the elutions patterns. When overlapping did occur the ratios 250/260, 280/260 and 290/260 mμ were used to distinguish the components. Complete separation of the elution bands could be realized if lower flow rates were used. However, guanylic and adenylic acids gave variable results if the analysis required too much time (seven days). This effect was presumably due to bacterial attack. Thus although a certain amount of accuracy was sacrificed (because of overlapping) by carrying out the analysis in a shorter time, the results were more reproducible since bacterial growth was kept at a minimum. It was found that guanylic acid was most susceptible to bacterial attack. Adenylic acid was only slightly attacked and no effects were observable with either cytidylic or uridylic acids. By saturating the eluting solvent with chloroform and placing chloroform in the beakers, the growth could be kept at a minimum. Using these precautions the maximum error of analysis was 5% for cytidylic, adenylic and uridylic acids. In the case of guanylic acid the error was estimated to be about the same, however, in two analyses the values for guanylic acid were completely inconsistent with the other data. For this reason the rate curve for guanylic acid must be viewed with reservation in any quantitative evaluation. There is no doubt, however, of its qualitative character. For any given value of V/u the rate is definitely higher than the rates of the other nucleotides. Since there is very little contribution to the error in the kinetic run itself, the error in the rates is due to the analysis of the nucleotides.

Results and Discussion

The Flow Process.—The ordinary batch method of studying kinetics is characterized by continuously changing concentrations of reactants and products. In a stirred flow reactor the concentrations of all the components of the system maintain constant values after the steady state is reached. To achieve this, reactants in separate reservoirs are allowed to enter the reaction chamber, at a fixed total rate which is equal to the rate of over-flow. During the first stages, the reaction may be considered to obey partially batchwise kinetics; however, as the process continues the concentration of the products reaches a limiting value. This value is determined by the "contact time" ($= V/u$) in the reactor; here V is the volume of the reactor and u is the total flow rate. Thus, after a steady state has been reached, the amount of product is not increased by continuing the flow process. By varying the flow rate u the "contact time" and hence extent of reaction may be altered. Before the steady state is attained the equation $dx/dt = r - (u/V)x$ applies. In the steady state $dx/dt = 0$ and the rate, r of formation of a product x is given by $r = (u/V)x$. The rate, r, is therefore the rate at a fixed concentration of reactants and corresponds to the rate at a particular instant of time.

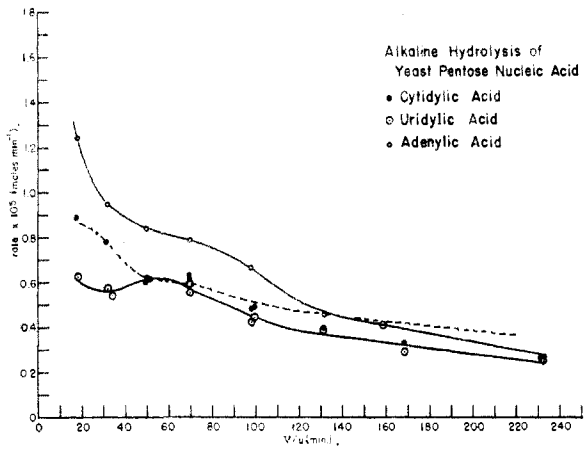


Fig. 1.— – – – – –, theoretical; ——, experimental.

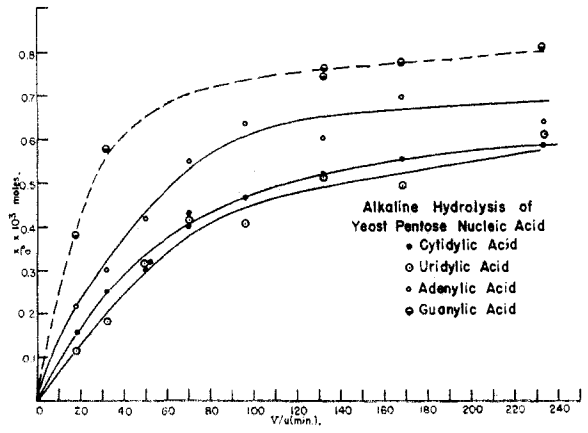


Fig. 2.

In the situation at hand the rate of formation of each nucleotide is $(u/V)(x/a^\circ)$ where u and V have the significance stated above, x is the concentration (in moles per liter) of the particular nucleotide in the reactor in the steady state and a° is the initial concentration of the nucleotide in nucleic acid form. In Fig. 1, the rates $(u/V)\cdot(x/a^\circ)$ are plotted *vs.* V/u. In Fig. 2 x/a° *vs.* V/u is plotted. Although smooth curves have been drawn through the points of Fig. 2, the process may not actually be a continuous one. Examination of some of the points suggests the process to be stepwise. Since the concentration of sodium hydroxide in the reactor was not identical in the various runs, all values of x/a° have been normalized to 0.40 N sodium hydroxide. This procedure is justified since the runs did not differ appreciably from 0.40 N and it was shown that the rates were proportional to the sodium hydroxide concentration for small increments. In Fig. 1 the broken line (cytidylic acid) represents a theoretical curve the construction of which is described below.

Inspection of the curves in Fig. 1 reveals that the rates of formation of the nucleotides are of the same order of magnitude and that they follow the same general pattern. Guanylic acid (Table I) exhibits the highest rate though the exact nature of the curve cannot be given at present for reasons stated in the experimental section. There appears to be no obvious correlation for the relative rates of the various nucleotides. The rate curves are complex in that they possess points of inflection. This indicates that the formation of the nucleotides proceeds through both a consecutive type mechanism and a direct mechanism. Thus if the reaction were of the type

$$A \longrightarrow B \longrightarrow x$$

where A represents the original nucleic acid molecule, B an intermediate and x the nucleotide, the rate of formation of x should be zero at $V/u = 0$. If A were to yield x directly, the rate would be finite at $V/u = 0$. On this basis we have derived an expression for the case of cytidylic acid which describes fairly well the rate as a function of V/u.

TABLE I

u, total flow rate (ml./min.). V, volume of reactor (0.14 liter). τ, reactor volumes which have traversed the system. [NaOH], concn. of sodium hydroxide in the reactor (moles/liter). [N.A.], initial concn. of nucleic acid in the reactor (g./liter). x, concn. of nucleotide in reactor at steady state (moles/liter). η, degree of hydrolysis (x/a^0). r, rate of formation of nucleotide (moles min.$^{-1}$) $= u/V(x/a^0)$ at 0.40 N sodium hydroxide.

					Cytidylic acid			Adenylic acid			Uridylic acid			Guanylic acid		
u	$u/V \times 10^2$	τ	[NaOH]	[N.A.]	$r \times 10^5$	$x \times 10^3$	η	$r \times 10^5$	$x \times 10^3$	η	$r \times 10^5$	$x \times 10^3$	η	$r \times 10^5$	$x \times 10^3$	η
7.90	5.65	6.1	0.417	3.97	0.894	0.372	0.165	1.24	0.550	0.229	0.623	0.353	0.114	2.20	1.05	0.405
4.42	3.15	6.1	.417	3.97	.782	0.588	.261	0.950	0.754	.314	.579	0.590	.192	1.85	1.58	.612
2.80	2.00	4.4	.397	4.21	.605	0.715	.299	.840	1.06	.417	.628	1.02	.312			
2.00	1.43	5.2	.397	4.20	.615	1.02	.429	.787	1.39	.548	.593	1.34	.412			
1.43	1.04	3.6	.403	4.14	.486	1.11	.472	.663	1.61	.642	.420	1.31	.407			
1.06	0.756	2.7	.403	4.14	.393	1.23	.523	.457	1.52	.604	.391	1.68	.522	0.562	2.02	.748
0.83	0.593	2.3	.393	4.30	.329	1.33	.545	.375	1.63	.622	.292	1.61	.482	.427	1.97	.705
0.60	0.428	1.7	.429	3.88	.252	1.39	.632	.293	1.74	.733	.264	1.99	.661	.345	2.18	.863
4.30	3.07	5.5	.198[a]	4.10	.840	0.316	.136	.826	0.330	.133	.765	0.380	.123			

[a] Initial sodium hydroxide concentration was 0.353 N.

It is obvious that a large number of different mechanisms could be written for the hydrolysis of pentose nucleic acid to its mononucleotides. For the most part, however, these may be classified into a few general categories of which we have chosen one particular set to express our data. Doubtless it would be possible to satisfactorily employ slightly different expressions; however, it is felt that the fundamental process is represented by our equation though it undoubtedly is a gross over-simplification of the actual situation.

The observed rate may be expressed by a linear combination of rates of both direct and consecutive reactions

$$A_1 \xrightarrow{k_1} x \quad A_2 \xrightarrow{k_2} x \quad A_3 \xrightarrow{k_3} B_3 \xrightarrow{k_3} C_3 \xrightarrow{k_4} 2x$$
$$+$$
$$1 \qquad 2 \qquad 3 \qquad x$$

A_1, A_2 and A_3 represent nucleic acid molecules which differ from each other in an unknown manner, B_3 and C_3 are intermediates of a polynucleotide nature and x in this case is cytidylic acid. The rate equation for sequence 1 is derived as

$$r_x = k_1 a_1 \tag{1}$$

From

$$a_1^0 = a_1 + x \tag{2}$$

is obtained

$$k_1 a_1 = k_1^0 a - k_1 x \tag{3}$$

which in (1) gives

$$r_x^1 = xu/V = \frac{k_1 a_1^0}{1 + k_1 V/u} \tag{4}$$

The initial concentration of A_1 is given by a_1^0.

The rate equation for sequence 3 is obtained as

$$r_b = b_3 u/V = k_2 a_3 - k_3 b_3 \tag{5}$$

$$r_c = c_3 u/V = k_3 b - k_4 c_3 \tag{6}$$

$$r_x^3 = xu/V = k_3 b_3 + 2k_4 c_3 \tag{7}$$

The material balance is

$$3a_3^0 = 3a_3 + 3b_3 + 2c_3 + x \tag{8}$$

which may be transformed to

$$k_3 a_3 = k_3 a_1^0 - k_3 b_3 - 2/3 k_3 c_3 - 1/3 k_3 x \tag{9}$$

Substitution of (9) and (6) in (5) yields

$$b \left\{ \frac{3(1 + k_4 V/u) + 6k_3 V/u(1 + k_4 V/u) + 2k_3^2 (V/u)^2}{3V/u(1 + k_4 V/u)} \right\} = k\, a_3^0 - 1/3 k_3 x \tag{10}$$

From (10) and (7) we obtain the rate law for sequence 3

$$r_x^3 = \frac{a_3^0 k_3^2 V/u(1 + 3k_4 V/u)}{1 + (2k_3 + k_4)V/u + 2k_3 k_4 (V/u)^2 + k_3^2 (V/u)^2 (1 + k_4 V/u)} \tag{11}$$

Thus, the observed rate is given by

$$r = r^1 + r^2 + r^3 \tag{12}$$

in which it is understood that $a_1^0 + a_2^0 + a_3^0 = a^0$, the initial concentration of the nucleotide groups in the nucleic acid sample.

As was noted above, the reaction mechanism is to be regarded at least as complicated as indicated with the strong reservation that it is probably more complex. Since the values of the k's have been obtained by treating them as parameters it cannot be ascertained which constants apply to the formation of the intermediates. Each k value may be taken as representing an average velocity constant.

TABLE II

CONSTANTS FOR CYTIDYLIC ACID

$a_1^0 = 0.05 \times 10^{-3}$ M	$k_1 = 0.20$ (min.$^{-1}$)
$a_2^0 = 0.70 \times 10^{-3}$	$k_2 = 0.007$
$a_3^0 = 0.25 \times 10^{-3}$	$k_3 = 0.02$
	$k_4 = 0.005$

A batchwise reaction was carried out to determine the rates of liberation of phosphoric acid groups by titration to the appropriate pH value. The rate constant for the reaction

triester phosphate ⟶ diester phosphate is about 3.5×10^{-2} min.$^{-1}$

while that for the reaction

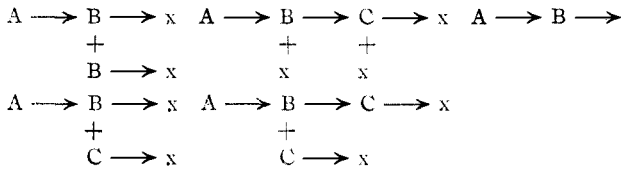
diester phosphate ⟶ monoester phosphate is about 2.9×10^{-2} min.$^{-1}$

These rate constants can only be considered as indicative rather than as representing the actual situation since they were not constant throughout an extended time interval. However, they are of the right order of magnitude.

Another means of following the extent of reaction is through the increase in intensity of absorption at 260 mμ of nucleic acid in sodium hydroxide solution. The total increase amounts to about 18% of the original optical density. The rate constant for this reaction is about 4×10^{-3} min.$^{-1}$. However, k decreased with increasing time. It is not unexpected that the constants for phosphate hydrolysis and the change in the ultraviolet spec-

trum are similar to those for the nucleotide formation.

A few pertinent remarks concerning the process of curve fitting will serve to support our proposed mechanism and lend some validity to the velocity constants so obtained. The expressions given in this paper represent the simplest of a large group which were developed for testing. The following are some of the mechanisms for which expressions were developed.



It was found that no one single equation would adequately express the data. The chief difficulty was that the maximum in the rate curve could not be made to coincide with the observed maximum. In the process of combining rate expressions, the type represented by equation 4 was found to be essential. Combination of equation 4 with the equations from the reaction mechanisms just noted were unsatisfactory.

Thus although the set we have chosen is probably not unique, it is likely that the reaction proceeds in an analogous but more complex manner. Perhaps the most significant feature to arise from the analysis of the rate curve is that in all cases examined both a low and a high velocity constant had to be assumed in order to approach a satisfactory fit. If we are to attach any significance to this in terms of phosphate linkages, it would appear that there are at least two types differing a great deal in their lability toward alkali. The ratios of the rates of formation of the isomeric guanylic acids (*a* and *b*) and adenylic acids (*a* and *b*) were constant and similar throughout the entire range of V/u; these were respectively about 1:1 and 1:1.1. There is a possibility for another type of phosphate linkage such as a phosphoester of the uracil or guanine hydroxyl groups which might be sufficiently different from the sugar phosphates. Data which suggest that such a bond exists were presented previously.[3] It should be emphasized that the constancy of the ratio a/b cannot be construed as conclusive evidence that the intact nucleic acid contains the isomers in this ratio. A reversible alkaline-catalyzed isomerization could also account for the results.

Finally it should be noted that all rate curves indicate a definite rate at $V/u = 0$. This may be taken to mean that a definite amount of the nucleotides are formed directly from the nucleic acid and that all nucleotides may therefore serve as end groups.

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Studies on the Structure of Nucleic Acids. IV. Investigation of Dye Interactions by Partition Analysis[1]

BY LIEBE F. CAVALIERI, ALICE ANGELOS AND M. EARL BALIS

The interaction of both desoxypentose and pentose nucleic acids with rosaniline and trimethyl-(*p*-(*p*-hydroxybenzeneazo)-phenyl)-ammonium chloride has been investigated by the method of partition analysis. The total number of available sites differs for the two dyes. This effect is interpreted in terms of heterogeneity among the sites. Competitive effects of magnesium ions have been investigated both by the method of equilibrium dialysis and partition analysis. Approximate binding constants for the magnesium ion have been calculated. The competitive interaction of bovine serum albumin with desoxypentose and pentose nucleic acids has been examined in the presence of the above-mentioned dyes. There is no evidence of combination between albumin and either nucleic acid.

In previous communications we have presented studies on the interaction of the cationic dye rosaniline with desoxypentose nucleic acid (DNA)[2] and pentose nucleic acid (PNA)[3] based on the method of equilibrium dialysis. In these studies solutions of relatively high ionic strength were used in order to minimize the Donnan effect. Under such circumstances competitive effects may obscure certain aspects of the binding process. Recently[4] a method of partition analysis has been developed in which solutions of much lower ionic strength are used without the complication of the Donnan effect and it is possible, therefore, to study dye interactions under conditions where the competitive interaction is slight. Briefly, the method consists of determining the distribution of an organic dye between an aqueous phase, which contains the nucleic acid, and an organic phase.[5] The quantity of dye bound and the free equilibrium dye concentration are readily calculated and the results are treated as in the dialysis method.[6,7]

In the present paper we report the results of a study of the interaction of both DNA and PNA

(1) The authors wish to acknowledge the support of the Atomic Energy Commission (contract AT(30-1)-910) and of the National Cancer Institute of the United States Public Health Service.

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