formed on a heterogeneous system. There is always danger, therefore, that results derived from such studies may be a reflection of this heterogeneity. This is particularly relevant to experiments carried out at temperatures above 30°. For example, in their investigations on the relative effects of temperature on the conformations of α-chymotrypsin and diisopropylphosphoryl α-chymotrypsin, Havsteen and Hess (1962, 1963) found large changes in the optical rotation of the active enzyme at temperatures above 40°. Since Ca^{2+} was absent it is pertinent to ask whether these changes may have been caused solely by denaturation of the more labile component of α-chymotrypsin.

Since only in the presence of Ca^{2+} does α-chymotrypsin behave as a homogeneous system by the few parameters studied (electrophoretic mobility and stability), it is suggested that Ca^{2+} be present in any investigation of the mechanism of action of this enzyme.

ACKNOWLEDGMENT

We wish to acknowledge the excellent technical assistance of Messrs. E. Lipkin and K. Chu.

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The Ribonuclease-catalyzed Hydrolysis of Uridine-2',3'-phosphate*

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Received June 11, 1964

The values for k_o, K_o, and K_p for the ribonuclease-catalyzed hydrolysis of uridine-2',3'-phosphate at 25° and at various *p*H values are as follows: *p*H 5.0, $k_o = 0.96 \pm 0.15$ sec^{-1}, $K_o = 0.51 \pm 0.35$ mM, $K_p = 0.047 \pm 0.033$ mM; *p*H 6.0, $K_o/k_o = 0.35 \pm 0.11$ mM sec, $K_p = 0.056 \pm 0.018$ mM; *p*H 7.0, $K_o/k_o = 1.45 \pm 0.11$ mM sec, $K_p = 0.47 \pm 0.08$ mM; *p*H 7.5, $k_o = 3.94 \pm 0.08$ sec^{-1}, $K_o = 19.9 \pm 1.2$ mM, $K_p = 2.16 \pm 0.21$ mM; *p*H 8.0, $K_o/k_o = 14.3 \pm 0.4$ mM sec. Inhibitor-free uridine-2',3'-phosphate is hydrolyzed more rapidly than previously reported and at rates comparable to those of cytidine-2',3'-phosphate. Product inhibition is marked at all *p*H values and the ratio K_o/K_p is nearly independent of *p*H and temperature, and equal to values of *ca.* 10. Uridine-2'-phosphate is a more potent inhibitor than uridine-3'-phosphate at low *p*H values. Adenosine-2'-phosphate is a weak inhibitor of ribonuclease. No inhibitory action toward ribonuclease is exhibited at *p*H 5.0 by any of the following compounds: D-ribose; DL-α-glycerophosphate; β-glycerophosphate; D-glucosamine; D-galactose; β-D-glucose; D-gluconic acid; D-fructose; D-fructose-1,6-diphosphate; adenosine; deoxyadenosine; deoxyadenosine-5'-monophosphate; adenosine-3'-phosphate. The temperature dependences of the kinetic parameters at *p*H values 5.0 and 7.0 were studied. The entropy of activation for the decomposition of enzyme-substrate complex to enzyme-product complex is −32 eu/mole at *p*H 5.0 and −29 eu/mole at *p*H 7.0. Uridine-2',3'-phosphate is subject to slow nonenzymic hydrolysis at *p*H 7.0 to yield exclusively uridine-3'-phosphate. Adenosine-2',3'-phosphate is not appreciably hydrolyzed under these conditions.

A detailed study (Herries *et al.*, 1962) of the ribonuclease (RNAase)-catalyzed hydrolysis of cytidine-2',3'-phosphate (C-2',3'-P) has recently been published. The kinetics of this hydrolysis were shown to be consistent with a mechanism involving product inhibition:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_o} EP \overset{K_p}{\rightleftharpoons} E + P$$

where ES and EP are the enzyme-substrate and enzyme-product complexes, respectively, and K_p is the enzyme-product-complex dissociation constant. The kinetics followed the rate law:

$$v = -d(S)/dt = k_o(E)_t(S)/\{(S)(1 - K_o/K_p) + K_o[1 + (S)_i/K_p]\}$$

* Supported by a grant from the National Institutes of Health, U.S. Public Health Service.

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where $(E)_t$ is the formal concentration of enzyme, $(S)_i$ is the initial concentration of substrate, and K_o is the apparent Michaelis constant.[1]

The decrease of k_o at *p*H values below 7 and the increase of K_o at *p*H values above 7 were also observed. Similar results were reported for the hydrolysis of uridine-2′,3′-phosphate (U-2′,3′-P) (Witzel and Barnard, 1962).

We have carried out a similar study of the RNAase-catalyzed hydrolysis of U-2′,3′-P, using a spectrophotometric technique similar to those used for C-2′,3′-P (Crook *et al.*, 1960; Herries, 1960; Herries *et al.*, 1962; Litt, 1961). In addition, we have studied the effects of several potential inhibitors and observed the effects of temperature over the range 15–40° at *p*H 7 and 25–40° at *p*H 5. The activation parameters for the decomposition of enzyme-substrate complex to enzyme-product complex were calculated by use of the transition-state theory of Eyring (1941).

Experimental

Uridine-2′,3′-phosphate.—Uridine-2′,3′-phosphate was prepared by the action of dicyclohexylcarbodiimide on uridine-3′-(2′)-phosphate, according to the procedure of Tener and Khorana (1955) and purified by fractional elution from a cellulose column as described by Brown *et al.* (1952b). The eluted product was apparently free of nucleotide impurities, but was very hygroscopic. A solution of this material was accordingly passed through a column of Dowex-50W × 8 (Ba^{+2} form) resin and lyophilized to a microcrystalline powder. This material, in its purest form, had a ratio of optical density at 280 mμ to that at 260 mμ of 0.197 (Brown *et al.*, 1952a, give a value of 0.195 in 0.01 F formic acid, 0.05 F formate buffer). This ratio is invariant over the *p*H range 5–7. Other substrate samples, prepared in this manner, were less pure, as shown by their higher A_{280}/A_{260} ratios. The question of substrate purity will be considered in more detail under Discussion. Both the ammonium and barium salts were used as substrate after it was demonstrated that the cation, in concentrations of less than 0.005 M, has no effect on the kinetic behavior of the system. This was demonstrated by preparing two stock solutions of identical concentration from the same substrate sample. One was prepared directly from the barium salt while the other was prepared by elution of the barium salt through an ammonium-ion resin. Rates of hydrolysis at various concentrations were identical. The concentrations of the stock solutions were determined spectrophotometrically, using $\epsilon_{258.5} = 9570\ M^{-1}cm^{-1}$ (Brown *et al.*, 1952b).

Uridine-3′-phosphate (U-3′-P).—Stock solutions containing both enzyme and U-3′-P (R_F in isopropanol-water-ammonia = 0.30; $A_{280}/A_{260} = 0.343$ at *p*H 5.0, 0.365 at *p*H 7.0) were prepared by mixing the appropriate amounts of U-2′,3′-P and RNAase in the appropriate buffer solution several hours before use. Complete hydrolysis was shown by observation of the A_{280}/A_{260} ratio.

Uridylic Acid (U-3′-(2′)-P).—Mixtures of U-2′-P and U-3′-P were obtained from two sources, commercial (Calbiochem, Los Angeles, Calif., Na salt) and stock solutions prepared by hydrolysis of U-2′,3′-P at *p*H 3. The commercial material had an A_{280}/A_{260} ratio of 0.348 at *p*H 7.0, while the acidic-hydrolysis product had a ratio of 0.324. This indicates that the acidic-hydrolysis product is richer in U-2′-P than is the commercial material.

Other Potential Inhibitors.—All other potential inhibitors were commercially available materials used without further purification. The following potential inhibitors were obtained from Sigma (St. Louis, Mo.): DL-α-glycerophosphate, disodium–6 H_2O; β-glycerophosphate, disodium–5 H_2O; D-ribose; D-glucosamine-HCl; D-fructose-1,6-diphosphate, barium salt; adenosine-2′-monophosphoric acid; adenosine-3′-monophosphoric acid.

The following potential inhibitors were obtained from Calbiochem: adenosine; deoxyadenosine-H_2O; deoxyadenosine-5′-monophosphoric acid; β-D-glucose. D-fructose, D-gluconic acid, and D-galactose were all Matheson (Cincinnati, O.) reagents.

Chromatography.—All R_F values refer to elution by isopropanol-water-ammonia (70:5:25) on Whatman No. 1 paper.

Buffer Solutions.—Buffer solutions for *p*H values 7.0–8.5 were prepared from Tris ("Sigma 121"; 0.10 F), appropriate amounts of dilute HCl (Baker analyzed) and NaCl (Mallinckrodt, reagent grade) in sufficient quantities to maintain the ionic strength at 0.10 M. Buffer solutions for the *p*H values 5.0–6.0 were prepared from sodium acetate trihydrate (Baker analyzed; 0.10 F) and HCl.

Ribonuclease.—Enzyme stock solutions were prepared on the day of use from ribonuclease A (Worthington, Freehold, N. J.). The concentration was checked by absorbance at 280 mμ, using a molar extinction coefficient of $1.1 \times 10^4\ M^{-1}\ cm^{-1}$ (Worthington Descriptive Manual, 1961).

Spectra.—All spectra were determined using an a-c line-operated Beckman DU spectrophotometer equipped with a photomultiplier attachment. The cell-compartment temperature was maintained at 25.0 ± 0.2° by means of a standard thermospacer set and a Haake ultracirculator. The spectrum of U-2′,3′-P was determined by measurement of the absorbancies of a solution of the barium salt. The spectrum of U-3′-P and the difference spectrum for the hydrolysis reaction were determined after complete hydrolysis of U-2′,3′-P by RNAase.

On the basis of the observed spectra, all kinetic runs were based on following the increase in absorbancy at 280 mμ. The $\Delta\epsilon$ for hydrolysis was determined individually for each kinetic run by measuring the absorbancy of the kinetic test solution versus a solution that was identical except for the absence of RNAase, after 24 hours. A decrease in the difference in absorbancy after several days indicates that the reference cell is subject to slow nonenzymic hydrolysis, but such hydrolysis is negligible during the course of a kinetic run. The change in absorbancy obeyed Beer's law and agreed well with that predicted from the difference spectra as long as the absorbancy of the solution versus water was less than *ca.* 2. When the total absorbancy exceeded this value, ΔA fell significantly below its predicted value. The condition required for constant $\Delta\epsilon$ was maintained by using a 1.0-mm path length for substrate concentrations greater than 1.2 mM. The change in extinction coefficient was not constant over the *p*H range studied, but decreased significantly below *p*H 7. The values for $\Delta\epsilon$ used for each *p*H value are given in Table I.

Kinetic Runs.—The details of a typical kinetic run are as follows: Identical substrate stock solutions were

[1] The symbols k_o and K_o are those introduced by Hein and Niemann (1961) and are not necessarily meant to refer to microscopically discrete reactions between single molecular species. The symbols E, ES, and EP do not necessarily refer to individual molecular species but are meant to indicate all species that could be classified as "free enzyme," "enzyme-substrate complexes," and "enzyme-product complexes," respectively.

TABLE I
DIFFERENCE IN MOLAR EXTINCTION COEFFICIENTS AT 280 mμ BETWEEN URIDINE-3'-PHOSPHATE AND URIDINE-2',3'-PHOSPHATE

pH	$\Delta\epsilon \times 10^{-3}$ M^{-1} cm^{-1} [a]
5.0	1.510
6.0	1.590
7.0	1.670
7.5	1.670
8.0	1.670

[a] Based on $\epsilon_{258.5} = 9570\ M^{-1}\ cm^{-1}$.

prepared by mixing 2.0 ml of a buffer stock solution (0.50 F in Tris or sodium acetate, $\mu = 0.50$ M) of the desired *p*H, the desired volume of substrate, and inhibitor stock solutions, and enough water to bring the total volume to 10.0 ml. The solutions were placed in a thermostat bath of the desired temperature for at least 30 minutes. Water (0.2 ml) was added to one of the solutions, the *p*H was measured, and the mixture was placed in the reference cell. RNAase stock solution (0.2 ml) was mixed rapidly with the other solution and the new solution was placed rapidly in the test cell. Reading was commenced after 50–100 seconds from the time of initial mixing and individual readings were taken every 10–20 seconds. The spectrophotometer was balanced against the reference cell in these intervals and reading was continued until 20% hydrolysis had been observed or until 900 seconds had elapsed. Less frequent readings were taken thereafter.

Calculations.—The presence of severe product inhibition was shown by preliminary experiments. Evaluation of the kinetic parameters was accomplished by a procedure based on the observation that, if the apparent kinetic parameters are determined for a system in which the ratio of the concentration of the competitively inhibiting product to the concentration of substrate is constant,

$$k_{o(app)} = k_o/(1 + \alpha K_o/K_p)$$

and

$$K_{o(app)} = K_o/(1 + \alpha K_o/K_p)$$

where $\alpha = (P)/(S)$ and $K_p = (E)(P)/(EP)$.[2]

The apparent kinetic parameters were determined for various α values by the method of Lineweaver and Burk (1934) using velocities evaluated at various constant percentages of conversion, e.g., 4.76% ($\alpha = 0.050$). A least-squares fit was then made for $1/k_{o(app)}$ versus α. The intercept of the line is equal to $1/k_o$ and the slope to K_o/k_oK_p. Since K_o/k_o is independent of α, it was determined by taking the average value of the intercepts for the Lineweaver-Burk plots. The use of this value allowed evaluation of K_o and K_p. That the decrease in rate with increased conversion is a true example of product inhibition is demonstrated by experiments in which U-3'-P is added to the system (e.g., see Table II, experiments 1 and 2, 9 and 10).

The extrapolation procedure was also used to evaluate kinetic parameters for impure substrate preparations. In these cases the A_{280}/A_{260} ratios were used to determine the initial per cent contamination by U-3'-P. The A_{280}/A_{260} ratio for pure U-2',3'-P was taken as 0.197, the lowest value observed by us. The justification for the view that U-3'-P is the only significant contaminant is given under Discussion. In the absence of a more rapid mixing technique, the extrapolation from data for the contaminated substrate was as precise as direct determination for pure substrate. This is a result of the high degree of curvature produced in the kinetic traces for pure substrate by product inhibition.

[2] These equations are not restricted in application to product inhibition. They can be applied to any system in which the concentration of any contaminating competitive inhibitor is held at a constant ratio to (S).

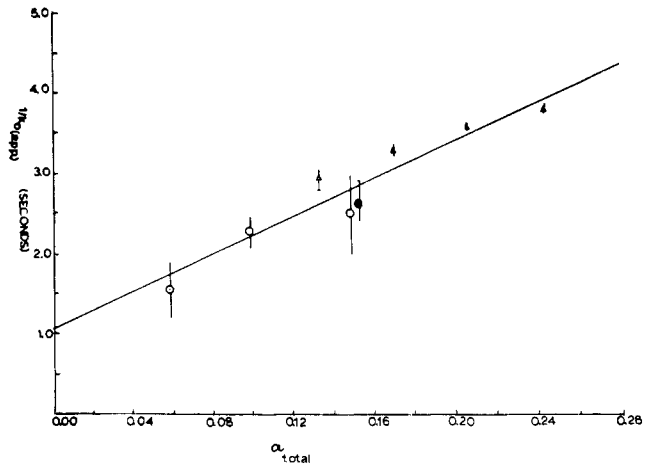

FIG. 1.—Plot of $1/k_0$ versus α_{total} at *p*H 5.0 and 25°. Open circles, NH_4^+ salt, $A_{260}/A_{280} = 0.197$; solid circles, NH_4^+ salt with 0.100 ratio of added U-3'-P; triangles, Ba^{2+} salt, $A_{280}/A_{260} = 0.212$.

Enthalpies of activation were determined by Arrhenius plots using $k_{o(app)}$ values determined for constant α for a single substrate preparation. In this way uncertainties involved in determining the degree of contamination for different substrate preparations could be avoided. The validity of the calculated $\Delta H^{\neq}$ value depends on the validity of our assumption that K_o/K_p is independent of temperature over the range of observation. At many *p*H and temperature values this assumption could not be checked directly because the extrapolated $1/k_o$ value was equal to zero within experimental uncertainty and no reliable K_o value could be calculated. At *p*H 7.0 and at 15.5°, 20°, and 30°, all K_o/K_p values have overlapping, if rather large, uncertainty ranges (8.3 ± 1.2, 11.0 ± 4.7, and 16 ± 11, respectively). The quantity $(K_o/K_pk_o)k_{o(app)}$ for any particular α value also appears to be independent of temperature. This result is consistent with a temperature-independent K_o/K_p ratio.

Free energies and entropies of activation were determined by the Eyring treatment. When extrapolated k_o values could not be determined with sufficient precision, K_o/K_p was taken as 10 and k_o as $3k_{o(app)}$ for $\alpha = 0.200$.

Inhibition constants for inhibitors other than U-3'-P were determined by comparing results in the absence of inhibitor and the results obtained for a system in which $(I)/(S) = \alpha_I$. Rather than use extrapolated data, we compared experiments at the same α_p. The following expression is then valid:

$$k_{o(app)}/k_{o(app)I} = (1 + \alpha_pK_o/K_p + \alpha_IK_o/K_I)/(1 + \alpha_pK_o/K_p)$$

(See footnote *d*, Table II, for the definition of α_p)

K_I was then calculated by assuming $K_o/K_p = 10$.

RESULTS

The apparent kinetic parameters for various *p*H values, α values, and temperatures are shown in Table II. Extrapolated values for $\alpha_{total} = 0$ are given below the data for each *p*H value. If $1/k_o$ is within twice the uncertainty of zero, only the K_o/k_o ratio and K_p are given. Thermodynamic data are given in Table III.

TABLE II
KINETIC PARAMETERS FOR THE RNAASE-CATALYZED HYDROLYSIS OF U-2′,3′-P

Expt	pH	T(°C)	A_{280}/A_{260}[a]	Added Inhibitor	α[b]	α_1[c]	α_P[d]	$k_{o(app)}$[e]	$K_{o(app)}$[f]
1	4.99	25	0.197		0.050		0.050	0.65 ± 0.14	0.38 ± 0.38
	4.99	25	0.197		0.100		0.100	0.44 ± 0.04	0.39 ± 0.15
	4.99	25	0.197		0.150		0.150	0.41 ± 0.09	0.54 ± 0.33
2	4.99	25	0.197	U-3′-P	0.050	0.105	0.155	0.38 ± 0.04	0.23 ± 0.20
	4.99	25	0.197	U-3′-P	0.100	0.110	0.210	0.24 ± 0.01	0.10 ± 0.03
3	4.99	25	0.212		0.034		0.135	0.35 ± 0.01	0.05 ± 0.03
	4.99	25	0.212		0.066		0.171	0.31 ± 0.01	0.04 ± 0.03
	4.99	25	0.212		0.100		0.208	0.28 ± 0.01	0.05 ± 0.01
	4.99	25	0.212		0.133		0.245	0.27 ± 0.01	0.08 ± 0.01
4	4.96	25	0.218		0.000[g]		0.143	0.51 ± 0.02	0.38 ± 0.12
	5.0	25		k_o = 0.96 ± 0.15		K_o = 0.51 ± 0.35		K_p = 0.047 ± 0.033 mM	
5	4.96	30	0.218		0.000[g]		0.143	0.57 ± 0.03	0.36 ± 0.14
6	4.96	35	0.218		0.000[g]		0.143	0.80 ± 0.03	0.62 ± 0.10
7	4.96	40	0.218		0.000[g]		0.143	0.96 ± 0.06	0.99 ± 0.18
8	5.96	25	0.218		0.000[g]		0.143	1.08 ± 0.07	0.57 ± 0.16
9	5.96	25	0.210		0.054		0.143	1.24 ± 0.09	0.45 ± 0.15
	5.96	25	0.210		0.109		0.202	0.80 ± 0.04	0.18 ± 0.08
	5.96	25	0.210		0.164		0.262	0.60 ± 0.02	0.09 ± 0.07
10	5.96	25	0.210	U-3′-P	0.054	0.116	0.258	0.62 ± 0.04	0.27 ± 0.12
	5.96	25	0.210	U-3′-P	0.109	0.124	0.326	0.50 ± 0.02	0.24 ± 0.08
	6.0	25		K_o/k_o = 0.35 ± 0.11 mM/sec			K_p = 0.056 ± 0.018 mM		
11	5.96	25	0.210	U-2′-(3′)-P[h]	0.000	0.114	0.081	0.29 ± 0.02	0.04 ± 0.02
				K_I = *ca.* 0.02 mM					
12	7.00	15.5	0.211		0.033		0.127	1.22 ± 0.04	2.32 ± 0.14
	7.00	15.5	0.211		0.066		0.161	1.04 ± 0.02	2.11 ± 0.10
	7.00	15.5	0.211		0.099		0.198	0.96 ± 0.03	2.00 ± 0.12
	7.00	15.5	0.211		0.167		0.276	0.75 ± 0.03	1.75 ± 0.28
	7.0	15.5		k_o = 2.48 ± 0.15		K_o = 5.3 ± 0.7		K_p = 0.64 ± 0.06 mM	
13	7.00	20	0.211		0.033		0.127	1.19 ± 0.05	1.98 ± 0.16
	7.00	20	0.211		0.066		0.161	1.12 ± 0.03	2.01 ± 0.11
	7.00	20	0.211		0.099		0.198	0.97 ± 0.02	1.83 ± 0.07
	7.00	20	0.211		0.132		0.233	0.85 ± 0.01	1.67 ± 0.05
	7.00	20	0.211		0.167		0.276	0.72 ± 0.02	1.47 ± 0.10
	7.0	20		k_o = 3.15 ± 0.82		K_o = 5.9 ± 1.9		K_p = 0.50 ± 0.19 mM	
14	7.00	25	0.216		0.057		0.192	2.02 ± 0.11	2.64 ± 0.18
	7.00	25	0.216		0.114		0.256	1.87 ± 0.23	2.70 ± 0.40
	7.00	25	0.216		0.172		0.321	1.22 ± 0.02	1.67 ± 0.04
15	7.00	25	0.216	U-3′-P	0.057	0.115	0.307	1.20 ± 0.09	1.89 ± 0.19
	7.00	25	0.216	U-3′-P	0.114	0.120	0.377	0.97 ± 0.17	1.36 ± 0.33
	7.00	25	0.216	U-3′-P	0.172	0.125	0.448	0.80 ± 0.07	1.26 ± 0.14
	7.00	25		K_o/k_o = 1.45 ± 0.11 mM/sec			K_p = 0.47 ± 0.08 mM		
16	7.00	25	0.216	U-2′-(3′)-P[h]	0.057	0.129	0.192	0.85 ± 0.04	1.23 ± 0.07
	7.00	25	0.216	U-2′-(3′)-P[h]	0.114	0.136	0.256	0.79 ± 0.02	1.18 ± 0.05
	7.00	25	0.216	U-2′-(3′)-P[h]	0.172	0.143	0.321	0.62 ± 0.03	0.92 ± 0.07
				K_I = *ca.* 0.6 mM					
17	7.00	25	0.207	A-2′-P	0.033	0.267	0.098	1.21 ± 0.13	1.83 ± 0.31
	7.00	25	0.207	A-2′-P	0.066	0.275	0.132	1.10 ± 0.10	1.70 ± 0.25
	7.00	25		K_I = *ca.* 2 mM					
18	7.00	30	0.211		0.033		0.127	2.31 ± 0.25	3.68 ± 0.37
	7.00	30	0.211		0.066		0.161	2.00 ± 0.19	3.28 ± 0.35
	7.00	30	0.211		0.099		0.198	1.60 ± 0.13	2.58 ± 0.24
	7.00	30	0.211		0.132		0.233	1.42 ± 0.14	2.01 ± 0.23
	7.00	30	0.211		0.167		0.276	1.47 ± 0.13	2.70 ± 0.27
	7.00	30	0.211		0.201		0.310	1.21 ± 0.10	2.10 ± 0.26
	7.0	30		k_o = 7.2 ± 3.6		K_o = 12 ± 6		K_p = 0.75 ± 0.05 mM	
19	7.00	35	0.211		0.033		0.127	4.25 ± 1.00	7.5 ± 2.3
	7.00	35	0.211		0.066		0.161	2.97 ± 0.49	5.4 ± 1.3
	7.00	35	0.211		0.099		0.198	2.40 ± 0.32	4.5 ± 0.9
	7.00	35	0.211		0.132		0.233	1.95 ± 0.22	3.8 ± 0.7
	7.00	35	0.211		0.167		0.276	1.64 ± 0.19	3.3 ± 0.6
	7.00	35	0.211		0.201		0.310	1.53 ± 0.20	3.3 ± 0.7
	7.0	35		K_o/k_o = 1.93 ± 0.12 mM/sec			K_p = 0.76 ± 0.05 mM		

TABLE II (*Continued*)

Expt	pH	T(°C)	A_{280}/A_{260}[a]	Added Inhibitor	α[b]	α_I[c]	αP[d]	$k_{o(app)}$[e]	$K_{o(app)}$[f]
20	7.00	40	0.211		0.033		0.127	3.10 ± 0.21	4.6 ± 0.5
	7.00	40	0.211		0.066		0.161	2.84 ± 0.15	4.5 ± 0.3
	7.00	40	0.211		0.099		0.198	2.35 ± 0.11	3.8 ± 0.3
	7.00	40	0.211		0.132		0.233	1.99 ± 0.13	3.4 ± 0.4
	7.00	40	0.211		0.167		0.276	1.61 ± 0.14	2.7 ± 0.4
	7.00	40	0.211		0.201		0.310	1.28 ± 0.13	2.2 ± 0.4
	7.0	40		K_o/k_o = 1.63 ± 0.08 mM/sec			K_p = 0.59 ± 0.07 mM		
21	7.50	25	0.211		0.022		0.114	1.88 ± 0.09	8.8 ± 0.6
	7.50	25	0.211		0.044		0.138	1.73 ± 0.02	8.6 ± 0.2
	7.50	25	0.211		0.066		0.161	1.59 ± 0.07	8.3 ± 0.4
	7.50	25	0.211		0.088		0.186	1.47 ± 0.04	8.0 ± 0.3
	7.5	25		k_o = 3.94 ± 0.08		K_o = 19.9 ± 1.2		K_p = 2.16 ± 0.21 mM	
22	8.00	25	0.211		0.000		0.084	K_o/k_o = 14.3 ± 0.4 mM/sec	

[a] Determined at pH 7.00 at 25°. [b] (P)/(S) where P is the U-3'-P produced by RNAse-catalyzed hydrolysis of U-2',3'-P. [c] (I)/(S) where I is the added inhibitor. [d] (U-3'-P)/(U-2',3'-P) where the (U-3'-P) is the total from all sources: contamination, enzymatic hydrolysis, and added inhibitor. [e] In sec^{-1}. [f] In mM. [g] Determined by extrapolation of log $(S)/(S)_o$ versus t. [h] Acidic-hydrolysis product.

TABLE III
THERMODYNAMIC PARAMETERS FOR THE PRODUCT-FORMING DECOMPOSITION OF RNAASE-U-2',3'-P COMPLEX

pH	T Range (°C)	$\Delta H^{\neq}$ (kcal/mole)	$\Delta F^{\neq}$ (kcal/mole)[a]	$\Delta S^{\neq}$ (eu/mole)
5.0	25–40	7.8 ± 1.4	17.5 ± 0.1	−32 ± 5
7.0	15.5–40	7.8 ± 1.0	16.8 ± 0.1	−29 ± 3

[a] Determined at 30°.

The following compounds were shown by single kinetic runs at pH 5.0, 25°, $(S)_o$ = 3 − 4 mM and α_I greater than 0.25 to be noninhibitors of RNAase: D-ribose, DL-α-glycerophosphate, β-glycerophosphate, D-glucosamine, D-galactose, β-D-glucose, D-gluconic acid, D-fructose, D-fructose-1,6-diphosphate. The following compounds exhibited no detectable inhibitory action under the same conditions at α_I = 0.12: adenosine, deoxyadenosine, deoxyadenosine-5'-monophosphate, adenosine-3'-phosphate.

DISCUSSION

The values of k_o and K_o appear to be consistently larger at all pH values than those previously reported (Witzel and Barnard, 1962). If K_o/K_p is independent of pH, as it appears to be, the pH dependences of k_o and K_o parallel those previously described. The data for U-2',3'-P may be significantly different from those reported for C-2',3'-P (Herries *et al.*, 1962) in that k_o appears to be less dependent on pH in the range 5–7.

Our method of analyzing the kinetic data by extrapolation is based on the fact that the only important contaminant in our substrate preparations was U-3'-P. That this is so is demonstrated by the following observations:

(1) The only contaminant that could be detected by paper chromatography was material with R_F value 0.30. This corresponds to the R_F value of both U-3'-P and U-2'-P. The degree of contamination indicated by the relative intensities of the contaminant and cyclic phosphate spots was consistent with that calculated from the A_{280}/A_{260} ratio, assuming that U-3'-P is the sole contaminant. However, these data are not sufficiently precise to show the absence of significant amounts of U-2'-P.

(2) The U-2'-P/U-3'-P ratio of the contaminant is much smaller than the ratio in the product of acidic hydrolysis of U-2',3'-P. This can be shown by the following analysis. If $\alpha_I K_o/K_I$ is much greater than 1, then $k_{o(app)} = ca.\ k_o K_I/\alpha_I K_o$. If we then add a known ratio, α_2, of the same contaminant to this substrate preparation, we would predict that

$$k_{o(app)}/k_{o(app)2} = (\alpha_2 + \alpha_1)/\alpha_1 = 1 + \alpha_2/\alpha_1$$

If the contaminant is a weak inhibitor, we would expect even less change in $k_{o(app)}$. This allows us to calculate the maximum value of α_1 in the original substrate preparation:

$$\alpha_{1max} = \alpha_2/(k_{o(app)}/k_{o(app)2} - 1)$$

Reference to the data for experiments 9,10, and 11 shows that the maximum contamination of uridylic acid of U-2'-P/U-3'-P ratio equal to that of the product of acidic hydrolysis of U-2',3'-P is 0.019. This much contamination would cause the A_{280}/A_{260} ratio to be 0.199. The observed ratio was 0.210. Most of the increase in absorbancy at 280 mμ must be caused by contamination by U-3'-P.

(3) Good agreement is obtained between the $k_{o(app)}$ values for uncontaminated substrate after 13.0% conversion (α = 0.150), for uncontaminated substrate to which U-3'-P has been added (α_I = 0.100) after 4.8% conversion (α_{total} = 0.154) and the value for contaminated substrate (α = 0.098 by spectrophotometric data) after 3.3% conversion (α_{total} = 0.135). Furthermore, $1/k_{o\ (app)}$ values for various percentages of conversion for all three substrate preparations, when plotted against α_{total}, can be fitted to a common straight line (Fig. 1).

It is not surprising that the major contaminant is a product of substrate hydrolysis. What is surprising is the apparent specificity of the nonenzymic hydrolysis for the 2'-ester bond of U-2',3'-P. The specificity of hydrolysis under mild conditions was independently demonstrated by the hydrolysis of U-2',3'-P over a period of 10 days in pH 7 buffer at 100° to pure U-3'-P (A_{280}/A_{260} = 0.365). It has been suggested (Witzel and Barnard, 1962) that the acidic and basic hydrolyses of nucleotide cyclic phosphates are good models for the mechanism of RNAase-catalyzed hydrolyses. Purine-nucleotide phosphate esters are hydrolyzed in both acid and base more slowly than the corresponding pyrimidine esters. This has been explained in terms of an interaction between the 2-oxygen on the pyrimidine base and the 2'-oxygen of the ribose portion of the molecule. In our opinion, such an effect should lead to specificity of bond hydrolysis as well as rate enhancement, and such an effect should be most important in the neutral pH range, since the intramolecular catalysis should be in

competition with intermolecular acid catalysis at low *p*H and nucleophilic attack by OH^- on the phosphorous atom at high *p*H. Neither of these intermolecular-catalytic mechanisms should show any marked specificity of bond hydrolysis. The internal mechanism of Witzel and Barnard (1962) should be dependent on *p*H only in so far as the amino group of the cytosine ring is protonated or the hydroxyl group of the uracil ring is dissociated. We would thus expect that the rate of hydrolysis owing to internal catalysis should be constant in the *p*H range 5–9 and that, in this range, hydrolysis should be specific for the 2′-phosphate bond. The purine cyclic phosphates, in which the internal mechanism is not sterically feasible, should not show a *p*H independent and specific hydrolysis in this *p*H range. In a recent experiment we were unable to detect any hydrolysis of adenosine-2′,3′-phosphate after 7 days at *p*H 7.0 and 100°. Further tests of our predictions are now in progress.

The apparent specificity of the nonenzymic hydrolysis of nucleotide cyclic phosphate at neutral *p*H for the base group and the phosphate bond hydrolyzed indicates that this system may be a suitable model for the RNAase-catalyzed hydrolysis. The function of the enzyme could be to constrain the substrate in a conformation proper for internal catalysis and the assistance of the tautomerism of the pyrimidine ring. The proper orientation of the pyrimidine ring might make the entropy of activation for the transformation of enzyme-substrate complex to enzyme-product complex less negative than the entropy of activation for the nonenzymic hydrolysis. The evaluation of the entropy of activation of the nonenzymic hydrolysis is currently in progress. The entropies of activation for k_o at *p*H 5.0 and 7.0 are quite negative, -32 and -29 eu/mole, respectively. This might indicate a change in substrate conformation during decomposition and, in particular, the restriction of rotation required by the Witzel-Barnard (1962) mechanism. However, there are several other equally, if not more, plausible explanations of these values:

(1) The enzyme may bind with the incipient 3′-phosphate group and restrict its freedom of motion as loosening of the 2′-phosphate bond occurs.

(2) Large conformational changes in the enzyme can occur. The work of Richards (1958), Hofmann *et al.* (1963), and Crestfield *et al.* (1963) shows unequivocally that at some stage of the catalytic process the amino and the carboxyl ends of RNAase must be in close proximity. The possibility that this proximity is not required for substrate binding but is required for complex decomposition could easily account for the entropy change.

(3) The tighter binding of one or more water molecules during complex decomposition could contribute significantly to the negative entropy of activation.

It was hoped that the temperature-effect studies at *p*H 5 and 7 would show whether the lower k_o value at *p*H 5 is a result of an entropy or an activation-energy effect. The data are not sufficiently precise to allow this distinction, although the change does appear to be an entropy effect. The entropy effect would be consistent with the view that at *p*H 5 a significant portion of the enzyme-substrate-complex molecules are in a state of protonation that is not optimal for decomposition. The observation that the change in k_o is less than 10-fold in the *p*H region 5–6 may indicate that the protonated complex may still decompose, although by a less favorable mechanistic pathway.

Witzel and Barnard (1962) observed the effects in changes of the pyrimidine group structure and concluded that such changes influence k_o rather than K_o. These observations do not carry over to the effect of replacing a pyrimidine group with a purine group. Both adenosine-2′-phosphate and adenosine-3′-phosphate were tested for their inhibitory action against RNAase. Adenosine-2′-phosphate is a relatively weak inhibitor (K_I = *ca.* 2 mM at *p*H 7.0, 25°) while adenosine-3′-phosphate caused no detectable inhibition. Similar results were found by Davis and Allen (1955). If we may be allowed to extrapolate according to the series $K_{o(-2',3'-)} >> K_{I(-3'-)} \gtrsim K_{I(-2'-)}$ (Herries *et al.*, 1962), we may predict that the failure of adenosine-2′,3′-phosphate to act as a substate of RNAase is due to a high K_o. Ukita *et al.* (1961) report that adenosine-2′,3′-phosphate shows no inhibitory action toward RNAase.

The heterocyclic base group appears to be an absolute necessity for binding of small molecules to the active site of RNAase. A series of sugars and simple phosphate esters had no inhibitory action toward RNAase. Similar results were obtained by Ukita *et al.* (1961). We were unable to study the effect of pyrimidine nucleosides because of their intense absorbance at 280 mμ. Purine nucleotides showed no inhibition toward RNAase. Ukita *et al.* (1961) report very weak inhibition (K_I = 50 mM) by thymidine.

This conclusion concerning small molecules does not apply to macromolecules or polyelectrolytes. There are many reports (e.g., Roth, 1953) concerning the inhibition of RNAase by polyanions such as heparin. In addition, Rosenberg and Zamenhof (1961) report that polyribophosphate is slowly hydrolyzed by RNAase. Polyanions may bind to RNAase at sites of attachment other than those that constitute the active site for small substrates and still block the active site. In the case of polyribophosphate, parts of the molecule may be held sufficiently close to the active site to lead to catalysis.

Acknowledgment

We gratefully acknowledge the valuable assistance given us by Mr. Allen Stock and by the San Fernando Valley State College Computer Laboratory in our calculations.

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Release of Alkaline Phosphatase from Cells of *Escherichia coli* upon Lysozyme Spheroplast Formation*

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Received June 23, 1964

It has been shown that the alkaline phosphatase of *Escherichia coli* is quantitatively released into the medium when cells containing this enzyme are converted to spheroplasts by the action of ethylenediaminetetracetic acid and lysozyme. Other enzymes, such as glutamic acid dehydrogenase, glucose-6-phosphate dehydrogenase, and β-galactosidase, are retained by the spheroplasts. Release of alkaline phosphatase requires dissolution of the cell wall, since it is not observed with spheroplasts prepared by treatment with penicillin or cycloserine. However, the enzyme is not bound to existing cell-wall structure and is released in soluble form when penicillin or cycloserine spheroplasts are lysed by treatment with distilled water. It is suggested that the enzyme lies in a compartment between the cell wall and cell membrane.

In a study of the utilization of phosphate from an organic ester by resting, phosphatase-containing cells of *Escherichia coli* (Malamy, 1963) evidence was obtained for hydrolysis prior to the entry of phosphate into the cell. It was also observed, however, that the phosphate liberated when the ester was hydrolyzed failed to equilibrate completely with inorganic phosphate in the medium, suggesting that the hydrolytic enzyme must be located at or outside of the cell membrane.

In order to test this hypothesis, methods for removing the cell wall from *E. coli* cells without producing significant lysis were explored. In a preliminary report (Malamy and Horecker, 1961) we described the quantitative release of alkaline phosphatase activity into the supernatant solution when spheroplasts were produced with lysozyme and EDTA. We have now investigated spheroplast formation by the action of penicillin or D-cycloserine, and find that such spheroplasts retain most of this enzyme activity.

METHODS

Bacterial Strains.—*E. coli* K12 was obtained from Dr. Werner K. Maas of this department; *E. coli* ML308, which synthesized β-galactosidase constitutively, was provided by Dr. Jacques Monod of the Institut Pasteur. A strain constitutive for alkaline phosphatase synthesis was isolated from *E. coli* K12 by the procedure of Torriani and Rothman (1961).

Media.—"Minimal medium-TEA" was a modification of medium 63 (Cohen and Rickenberg, 1956) in which phosphate buffer was replaced by 0.05 M triethanolamine buffer. The composition per liter was: 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7\,H_2O$, 0.0005 g of $FeSO_4 \cdot 7H_2O$, 0.075 g of KCl, 7.5 g of triethanolamine. The medium was adjusted with HCl to a final *p*H of 7.4. The carbon source was autoclaved separately as a 20% solution and added at the time of inoculation. Phosphate in growth-limiting amounts (*ca.* 10^{-4} M) was added (Echols *et al.*, 1961). "Enriched medium" was prepared by mixing equal volumes of Difco-neopeptone broth with fresh beef infusion.

Materials.—Egg white lysozyme was a product of Nutritional Biochemicals Corp.; glucose-6-phosphate, penicillin, nucleotides, and coenzymes were commercial products; thiomethyl-β-D-galactoside and *o*-nitrophenyl-β-D-galactoside were obtained from Mann Research Corp.; *p*-nitrophenylphosphate was purchased from the Sigma Biochemical Corp. as Sigma 104; Tris was purchased from Sigma Biochemical Corp. as Sigma 121. D-Cycloserine was a gift of Dr. E. Simon of this institution.

Lysozyme Spheroplast Formation.—Cultures were grown overnight at 37° in triethanolamine medium supplemented with a carbon source and 5×10^{-5} M phosphate. The cells were harvested by centrifugation, washed once with the original volume of 0.01 M Tris buffer, *p*H 7.4, and resuspended in a solution of 20% sucrose containing 0.033 M Tris buffer, *p*H 8.0. The suspension was kept at 0° and was treated successively with 0.01 volume of 0.1 M EDTA, *p*H 8.0, and 1 μl of lysozyme suspension (5 mg/ml) per ml of suspension (Repaske, 1958). The suspension was stirred gently, and osmotic fragility was determined at intervals by diluting 0.1-ml aliquots to 1 ml with distilled water and determining the turbidity at either 490 or 600 mμ in the Beckman DU spectrophotometer. When spheroplast formation was complete, as indicated by the absence of further decrease in the turbidity of the diluted sample, the suspension was centrifuged for 15 minutes at 4° in the high-speed attachment of the International PR-2 centrifuge. The supernatant solution was carefully removed and the pellet was washed once with the sucrose-Tris buffer solution. The washed pellet was lysed by adding distilled water.

Penicillin or D-*Cycloserine Spheroplast Formation.*—

* Taken in part from a doctoral dissertation submitted by Michael Malamy to the Graduate School of Arts and Sciences, New York University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Supported by grants from the National Institutes of Health and the National Science Foundation.

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