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The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

3. THE pH-DEPENDENCE OF THE KINETIC PARAMETERS FOR THE HYDROLYSIS OF CYTIDINE 2',3'-PHOSPHATE*

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Only limited data on the variation of the activity of bovine pancreatic ribonuclease with pH have been reported in the literature, presumably because of the difficulty of preparing pure well-defined substrates in quantity. The availability of highly purified cytidine 2',3'-phosphate (Crook, Mathias & Rabin, 1960*a*) together with a convenient method of assay (Crook, Mathias & Rabin, 1960*b*) prompted a thorough investigation of this problem. A preliminary account of this work has been presented (Herries, 1960).

MATERIALS AND METHODS

A number of buffer compositions not used in this paper but referred to in subsequent papers in this series are included. All materials not listed below were AnalaR chemicals.

Water. Glass-distilled water was passed successively

through columns of De-Acidite FF, Zeo-Karb 225 and 215 (mixed) and Bio-Deminolit (The Permutit Co. Ltd., London). All vessels had previously been thoroughly steamed out. Precautions were taken to protect the final effluent from contamination by atmospheric CO₂.

Sodium chloride and potassium chloride. These were AnalaR or Spectrographically Standardized (Johnson, Matthey and Co. Ltd.). They were heated to approximately 400°, and cooled and stored over P₂O₅.

Hydrochloric acid. AnalaR hydrochloric acid (sp.gr. 1.18) was diluted and distilled according to the method of Vogel (1951*a*, p. 228, method B). Solutions of the required dilution were standardized against 2-amino-2-hydroxy-methylpropane-1,3-diol (tris) (Fossum, Markunas & Riddick, 1951).

Carbonate-free sodium hydroxide solution. A saturated aqueous solution of AnalaR sodium hydroxide was diluted under CO₂-free nitrogen and standardized against standard hydrochloric acid. Potentiometric titration under nitrogen showed that less than 0.3% of carbonate, on an equivalent basis, was present.

Formic acid. AnalaR 90% (v/v) solution was used without further purification.

* Parts 1 and 2: Crook, Mathias & Rabin (1960*a*, *b*).

Sodium acetate. AnalaR sodium acetate trihydrate was recrystallized from aqueous ethanol and the dry material fused carefully according to the method of Vogel (1951*b*, p. 192). The anhydrous solid was powdered and kept over P_2O_5 .

Malonic acid. Commercial malonic acid (General Purpose Reagent, British Drug Houses Ltd.) was recrystallized from benzene-ether (1:1, v/v).

Disodium pentane-3,3-dicarboxylate. Diethyl propane-1,1-dicarboxylate (diethyl monoethylmalonate; L. Light and Co. Ltd.) was ethylated by bromoethane in the presence of sodium ethoxide. The resulting diester was saponified with ethanolic KOH, adjusted to pH 1.9 with sulphuric acid, and the acid extracted into ether. The calculated amount of CO_2 -free sodium hydroxide solution was added to the material from the ether extract, and the solution freeze-dried. The salt was recrystallized from aqueous ethanol, and dried *in vacuo* over P_2O_5 . The molecular weight was checked by titration and corresponded to the anhydrous salt.

N-Ethylmorpholine. This was twice redistilled, converted by HCl gas into the hydrochloride, and recrystallized from anhydrous ethanol-acetone (1:2, v/v). The salt is extremely deliquescent.

2-Amino-2-hydroxymethylpropane-1,3-diol (tris). This was purified as described by Crook *et al.* (1960*b*).

Sodium tetraborate decahydrate (borax). The AnalaR material was recrystallized from water at below 55° and stored for brief periods over a solution saturated with respect to sucrose and sodium chloride (Vogel, 1951*a*, p. 232).

Buffer solutions. All buffer solutions were of *I* 0.20, the inert electrolyte being NaCl or KCl. The compositions are given in Table 1.

Ribonuclease. Armour and Co. Ltd. lot no. 21630 was chromatographed according to the method of Hirs, Moore & Stein (1953) and fraction A used throughout. The protein was desalted on Amberlite MB-3 resin, freeze-dried, and stored over P_2O_5 . The molecular weight was taken to be 13683 (Hirs, Moore & Stein, 1956).

Cytidine 2',3'-phosphate. This was prepared according to Crook *et al.* (1960*a*). The sodium salt was prepared by the same method and shown by spectrometric comparison to be the monohydrate.

Cytidine 3'-phosphate. This was prepared from yeast cytidylic acid by the method of Harris, Orr, Roe & Thomas (1953) and converted into the sodium salt by ion-exchange on Amberlite IRC-50. Analysis, by the method of Speakman (1940), of titration data obtained with a Radiometer Automatic Titrator over the pH range 4.4–5.8 gave two pK_a values of 4.3 and 6.25. Comparison of the absorption spectrum with that of the product of enzymic hydrolysis of potassium cytidine 2',3'-phosphate showed the material to be the anhydrous sodium salt.

Measurement of reaction rates. Two techniques have been used for following the reaction.

(1) **Spectrophotometric method.** This was based on that described by Crook *et al.* (1960*b*). A Hilger Uvispek model H. 700.307 or a Cary recording spectrophotometer model 14 was employed with stoppered silica cells of 1 cm. light-path maintained at 25°. The compositions of the buffer solutions are given in Table 1. At each pH the absorption spectra of the substrate and product were measured. A suitable wavelength for any particular substrate concentration was chosen and the validity of Beer's law checked.

(2) **pH-stat method.** This method has been used by other workers (Davis & Allen, 1955; Kalnitsky *et al.* 1959). A Radiometer TTT 1a Titrator and SBR 2b Titrigraph were used to control and record the addition of sodium hydroxide to the reaction mixture. The water-jacketed cell was fitted with a side tube which was filled with saturated KCl and separated from the reaction mixture by a sintered-glass plug. The calomel reference electrode was inserted into the side tube and the semi-micro glass electrode (Radiometer G 222b) into the solution of volume 2 ml. The solution was stirred magnetically and nitrogen gas, previously washed with KOH solution followed by water was bubbled into the reaction mixture. Sodium hydroxide (0.0184*N*) was delivered from an Agla syringe burette which gave a full-scale recording for a volume of 0.4985 ml. With special reduction gearboxes designed and built in this Laboratory and fitted in front of the burette drive, full-scale readings corresponding to one-half and one-quarter of this value could be obtained. The pH of the reaction mixture, before the addition of enzyme, was brought to the desired value by the machine. The actual pH was read with an external

Table 1. *Compositions of buffer solutions used*

The references for pK_a values are: formic acid (Harned & Embree, 1934); acetic acid (Harned & Ehlers, 1933); malonic acid (Hamer, Burton & Acree, 1940); pentane-3,3-dicarboxylic acid (Gane & Ingold, 1931); *N*-ethylmorpholine (Hall, 1956); boric acid (Manov, DeLollis & Acree, 1944).

System	pK at 25°	Moles/l. of soln.	Measured pH at 25°
Formic acid	3.75	0.1	3.44
Sodium hydroxide		0.0437	
Sodium chloride		0.1563	
Formic acid	3.75	0.1	4.00
Sodium hydroxide		0.0711	
Sodium chloride		0.1289	
Sodium acetate	4.756	0.1	4.01
Hydrochloric acid		0.0799	
Sodium chloride		0.1	
Sodium acetate	4.756	0.2	4.49
Hydrochloric acid		0.1114	
(1 vol. diluted to 2 vol. with 0.2 <i>M</i> -NaCl before use)			
Sodium acetate	4.756	0.2	5.01
Hydrochloric acid		0.0568	
(1 vol. diluted to 2 vol. with 0.2 <i>M</i> -NaCl before use)			
Malonic acid	5.70 (pK_2)	0.05	6.25
Sodium hydroxide		0.0962	
Sodium chloride		0.0342	
Disodium pentane-3,3-dicarboxylate	7.29 (pK_2)	0.05	6.56
Hydrochloric acid		0.0341	
Sodium chloride		0.0841	
<i>N</i> -Ethylmorpholine hydrochloride	7.70	0.1	6.90
Sodium hydroxide		0.0099	
Sodium chloride		0.1	
<i>N</i> -Ethylmorpholine hydrochloride	7.70	0.1	7.21
Sodium hydroxide		0.0183	
Sodium chloride		0.1	
Borax	9.234	0.05	9.03
Hydrochloric acid		0.0088	
Sodium chloride		0.1	

meter (Radiometer PHA 620T) calibrated to 0.01 pH. The electrodes were standardized in 0.05M-potassium hydrogen phthalate and 0.05M-borax (Datta & Grzybowski, 1961). The ionic strength in all experiments was adjusted to 0.20 with potassium chloride. The amount of alkali added is a direct measure of the extent of hydrolysis only when the pH of the solution is very much greater than the pK of the secondary phosphoryl dissociation of cytidine 3'-phosphate. A pK of 5.78 at *I* 0.2 was employed for computing the correction factors.

The range of substrate concentrations used at different pH values is shown in Table 2.

RESULTS

The possibility of inhibition by buffer ions was checked by measuring progress curves at fixed enzyme and substrate concentrations. The *I* and pH were maintained constant and the total buffer concentration was varied. None of the buffers used affected the rate measurements.

The results were interpreted in terms of the reaction scheme:

$$K_m = (k_{-1} + k_{+2})/k_{+1}; \quad K_p = k_{+3}/k_{-3}$$

The method of Lineweaver & Burk (1934) was used to determine the operational Michaelis parameters by regression of the following equation:

$$\frac{1}{v} = \frac{K_m}{k_{+3}[E]_0} \cdot \frac{1}{[S]} + \frac{1}{k_{+3}[E]_0}$$

where *v* is the initial reaction velocity for an enzyme concentration $[E]_0$ and substrate concentration $[S]_0$.

Some typical progress curves are shown in Fig. 1. Since difficulties have been experienced in obtaining initial velocities from progress curves such as these (M. A. Litt, personal communication), the methods we have used will be outlined. At pH 4.0 and 4.5 the initial velocities are easily obtained because of the considerable linearity at the start of the reaction. At pH 5.0 and 6.6 it is much more difficult to obtain the initial slope of the progress curves. In such cases it is easier to find the initial slope of a

plot of $\ln([S]_0/[S])$ against time and hence derive the initial velocity (Fig. 2). An example of a Lineweaver-Burk plot in which the initial velocities have been obtained by both methods is shown in Fig. 3. An alternative treatment of the same data, by the method of Foster & Niemann (1953), is given in Fig. 4. This treatment is not satisfactory for determining K_m for ribonuclease because of the acuteness of the angle between the experimental lines and the guide lines (not shown) through the origin, of slope equal to the substrate concentration. The product inhibition constant has, however, been obtained from this type of data by the following method.

The equation of the experimental lines in Fig. 4 is:

$$\frac{[S]_0 - [S]}{t} = - \frac{K_m \left(1 + \frac{[S]_0}{K_p}\right)}{\left(1 - \frac{K_m}{K_p}\right)} \cdot \frac{\ln \left(\frac{[S]_0}{[S]}\right)}{t} + \frac{k_{+3}[E]_0}{\left(1 - \frac{K_m}{K_p}\right)} \quad (1)$$

The slope, *m*, is linearly related to the initial substrate concentration by the equation:

$$m = \frac{K_m}{(K_m - K_p)} \cdot [S]_0 + \frac{K_p K_m}{(K_m - K_p)}$$

A plot of this equation is shown in Fig. 5. K_p was obtained from the slope by using a value of K_m determined by a Lineweaver-Burk plot. At some pH values, K_p and K_i were obtained by plotting $1/v$ against the concentration of inhibitor at constant substrate concentration. The values of the parameters obtained are given in Table 3.

ANALYSIS OF DATA

The basic problem is to derive the dissociation constants of the groups at the catalytic site from the variation with pH of the operationally defined constants in Table 3. The difficulties and limitations inherent in attempting this have been discussed by many authors (Dixon & Webb, 1958; Alberty, 1956; Bruce & Schmir, 1959; Peller & Alberty, 1959).

Table 2. *Range of substrate concentrations used*

pH-stat method			Spectrophotometric method		
pH	Series	Range (10 ⁻⁴ M)	pH	Series	Range (10 ⁻⁴ M)
8.5	XII	154-514	9.0	XIV	1.92- 7.73
8.0	VII	12-300	6.6	V	0.28-13.84
7.5	VIII	94-313	5.5	VI	0.81-17.03
7.0	IX	97-322	5.0	I	0.28- 5.54
6.5	X	16-259	5.0	II	0.55- 6.64
6.0	XI	9.2-88	4.5	III	0.27- 8.10
			4.0	IV	0.26-11.07

Fig. 1. Typical progress curves for the hydrolysis of cytidine 2',3'-phosphate, followed by the spectrophotometric method. ○, pH 5.0; 292 mμ; concn. of substrate 0.55 mM; concn. of enzyme 0.73 μM. △, pH 4.5; 296 mμ; concn. of substrate 0.58 mM; concn. of enzyme 1.5 μM. □, pH 4.0; 298 mμ; concn. of substrate 0.55 mM; concn. of enzyme 5.8 μM. ●, pH 6.6; 292 mμ; concn. of substrate 0.63 mM; concn. of enzyme 0.29 μM. The full lines are initial tangents.

Fig. 2. Typical first-order plots for the hydrolysis of cytidine 2',3'-phosphate, followed by the spectrophotometric method. ○, pH 5.0; △, pH 4.5; □, pH 4.0; ●, pH 6.6. The substrate and enzyme concentrations were as in Fig. 1. The full lines are initial tangents.

Laidler (1958) has devised, by a complete steady-state analysis, the complicated equations shown in Scheme 1.

Our data fit a simplified equation derived by Waley (1953) for a more restricted case:

$$V = \frac{k_{+2}[\text{E}]_0[\text{S}]}{K_m \left(1 + \frac{[\text{H}]}{K_b} + \frac{K_a}{[\text{H}]}\right) + [\text{S}] \left(1 + \frac{[\text{H}]}{K_{b'}} + \frac{K_{a'}}{[\text{H}]}\right)}$$

In some instances, K_a , K_b , $K_{a'}$ and $K_{b'}$ are the approximate acid dissociation constants.

The operationally determined parameters are related to the pH-independent constants, k_{+2} and K_m , by the following equations:

$$k_{+2} = \frac{k_{+2}}{\left(1 + \frac{[\text{H}]}{K_b} + \frac{K_{a'}}{[\text{H}]}\right)} \quad (2)$$

$$K_m = \frac{K_m \left(1 + \frac{[\text{H}]}{K_b} + \frac{K_a}{[\text{H}]}\right)}{\left(1 + \frac{[\text{H}]}{K_{b'}} + \frac{K_{a'}}{[\text{H}]}\right)} \quad (3)$$

$$\frac{k_{+2}}{K_m} = \frac{k_{+2}}{K_m \left(1 + \frac{[\text{H}]}{K_b} + \frac{K_a}{[\text{H}]}\right)} \quad (4)$$

Fig. 3. Typical Lineweaver-Burk plot for the hydrolysis of cytidine 2',3'-phosphate. The concn. of enzyme was 0.73 μM, and the pH 5.0. ○, Initial velocities determined from progress curves; △, initial velocities determined from first-order plots. Several points are omitted at high substrate concentrations for clarity.

The variation of these parameters with pH is shown in Fig. 6. Equations (2) and (4) are both of the form:

$$y = \frac{\bar{y}}{1 + \frac{[H]}{K_b} + \frac{K_a}{[H]}} \quad (5)$$

The separation of the pairs of constants (K_a , K_b ; $K_{a'}$, $K_{b'}$) is insufficient to allow them to be computed independently or by the method of Dixon (1953) and allowance must be made for overlap. The method used is essentially that of Friedenwald & Maengwyn-Davies (1954).

From Fig. 6 the pH corresponding to the maxima of the curves was estimated. This determination was facilitated by utilizing the facts that the curves have an axis of symmetry and the slopes tend to ± 1 in regions remote from the optima. It can be shown that for equations of the type (5),

$$[H]_{\max}^2 = K_a K_b$$

where $[H]_{\max}$ is the H^+ ion concentration corresponding to the pH optimum.

Rearranging eqn. (5) gives

$$\frac{1}{y} = \frac{1}{K_b \bar{y}} \left([H] + \frac{[H]_{\max}^2}{[H]} \right) + \frac{1}{\bar{y}} \quad (6)$$

Fig. 4. Typical Foster-Niemann plot for the hydrolysis of cytidine 2',3'-phosphate (series II). The concn. of enzyme was $0.73 \mu M$, and the pH 5.0. O, Points of intersection of the experimental lines with lines through the origin (not shown) of slope equal to the initial substrate concentration. The straight line of negative slope represents initial conditions and is drawn through the above points of intersection.

Accordingly, $1/k_{+2}$ and K_m/k_{+2} were plotted against $[H] + [H]_{\max}^2/[H]$, and the slopes and intercepts were calculated by the method of least squares. The constants obtained (Table 4) were used to compute k_{+2} and (k_{+2}/K_m) as a function of pH and are compared with the experimental points in Fig. 6.

Fig. 5. The slopes of the experimental lines in the Foster-Niemann plot, shown in Fig. 4, as a function of the initial substrate concentration.

Fig. 6. Variation with pH of the logarithms of the kinetic parameters for the hydrolysis of cytidine 2',3'-phosphate at 25° and $I\ 0.20$. The points are experimental, and the lines are calculated from the data in Table 4. ●, $\log(k_{+2}/K_m)$; ○, pK_m ; Δ, $\log k_{+2}$.

Table 3. *Values of the kinetic parameters*

The kinetic parameters were measured at 25° and at I 0.20; the spectrophotometric method was used for series I–V, XIII and XIV, and the pH-stat method for series VII–XII. The results are given $\pm$ s.d. K_p and K_i are the dissociation constants for competitive inhibition by cytidine 3'-phosphate and cytidine 2'-phosphate respectively.

pH	Series	k_{+2}/K_m (min./M)	k_{+3} (min.)	K_m (M)	K_p (M)	K_i (M)
9.03	XIV	840	—	—	—	—
		± 79				
8.50	XII	8390	300	0.0357	—	—
		± 640	± 25	± 0.0040		
8.00	VII	14900	610	0.041	—	—
		± 1400	± 160	± 0.011		
7.50	VIII	51000	867	0.0170	0.00213	0.000717
		± 4800	± 89	± 0.0024	± 0.00023	± 0.000080
7.00	IX	98200	1088	0.0111	0.000654	(0.000148)*
		± 9300	± 55	± 0.0012	± 0.000071	
6.56	V	160500	—	—	0.000365	—
		± 3400			± 0.000017	
6.50	X	214400	724	0.00338	—	—
		± 8500	± 70	± 0.00035		
6.00	XI	275400	458	0.00166	—	—
		± 8200	± 21	± 0.00009		
5.00	I	163100	59.4	0.000364	0.000088	—
		± 2000	± 8.8	± 0.000054	± 0.000017	
5.00	II	155500	62.5	0.000402	0.0000615	—
		± 2100	± 5.8	± 0.000037	± 0.0000077	
4.50	III	51200	25.2	0.000492	0.000145	—
		± 550	± 5.7	± 0.000112	± 0.000035	
4.00	IV	8104	8.9	0.00109	0.001	—
		± 50	± 2.3	± 0.00028	(= K_m)	
		7940†	9.98†	0.001257†	—	—
		± 630	± 0.38	± 0.000086		
3.44	XIII	(6590)*	—	—	—	—

* Insufficient data to obtain s.d.

† These values were calculated from the equation of the line representing initial conditions in the Foster–Niemann plot.

DISCUSSION

Constants obtained from the pH-dependence of the Michaelis parameters are commonly identified with acid dissociation constants involved in a hypothetical reaction scheme. However, this can only be done with any confidence if the significance of the kinetic parameters is known. The most favourable case for interpretation is when k_{-1} is very much greater than k_{+2} , so that there is equilibrium among all species of the enzyme and the enzyme–substrate complex. There is every reason to suppose that this situation obtains for ribonuclease. Findlay, Mathias & Rabin (1962) have shown classical non-competitive inhibition of the hydrolysis of cytidine 2',3'-phosphate by glycerol and ethylene glycol, and non-competitive inhibition or activation implies equilibrium kinetics (Morales, 1955). The assumptions underlying this proposition are discussed by Bernhard & Gutfreund (1960). A second line of evidence is the similarity of the variation with pH or K_p and K_m as shown in Fig. 7.

Since there is little doubt that K_p is an equilibrium constant, this suggests that K_m is also an equilibrium constant. It is reasonable therefore to write the equations shown in Scheme 2 for ribonuclease.

All the reactions above the broken line in Scheme 2 are at equilibrium and the constants shown are dissociation constants. If the enzyme species EH_2 is represented by $BH \rightleftharpoons AH$, then the minimal scheme

for ionizations from the free enzyme is that shown in Scheme 3. According to Alberty (1956), the

'macroscopic' constants, K_b and K_a , do not differ by more than ± 0.3 pH unit from the 'microscopic' constants, K_β , K_α , $K_{\beta'}$ and $K_{\alpha'}$, a similar series of which occur for ionizations involving the enzyme-substrate complex. It is, however, possible that the pH values are wrongly assigned and the group required in the acid form could well have the lower pK . If so, the assignment is correct for the reverse reaction, where the roles of the catalytic groups are reversed. There is no experimental method which permits a decision to be made between the possibilities.

Scheme 3

The order of magnitude of the determined constants suggests they are associated with imid-

Fig. 7. Variation with pH of the negative logarithms of the Michaelis constant and the dissociation constant of the product-enzyme complex for the hydrolysis of cytidine 2',3'-phosphate at 25° and $I\ 0.20$. The points are experimental and the line for pK_m is calculated; the line for pK_p is of the same shape. O, pK_m ; Δ , pK_p . The experimental points coincide at pH 4.0.

azole groups of histidine residues. Provided that the pK values of the groups do not invert in passing from the free enzyme to the enzyme-substrate complex, binding of the substrate causes both pK values to increase as shown in Table 4. This means that both groups become weaker acids and precludes binding of either of the groups in the base form to the substrate. We suggest that the group required in the base form, which does not interact with the substrate, is required to bind water, and further evidence for this is presented in the papers following.

These conclusions disagree with the published data of Litt (1961), but differ in no significant way from his redetermined constants (M. A. Litt, personal communication).

A noticeable feature of Table 3 is that

$$K_m > K_p > K_i$$

at pH 7.0 and 7.5; this means that the degrees of association of the nucleotides are in the descending order: cytidine 2'-phosphate; cytidine 3'-phosphate; cytidine 2',3'-phosphate. The cyclic phosphate should not really be compared with the other nucleotides because of its charge difference and there are other difficulties in interpreting this type of data (Wigler & Alberty, 1960). The 2'-oxygen atom of cytidine 2'-phosphate is more basic than the 2'-oxygen atom of cytidine 3'-phosphate. The increased binding of cytidine 2'-phosphate as compared with cytidine 3'-phosphate strongly suggests an interaction between the 2'-oxygen atom of the substrate and a positive site on the protein. It is possible that this site is the imidazole group required in the acid form.

SUMMARY

1. The variation of the Michaelis parameters with pH at $I\ 0.2$ and 25° for the hydrolysis of cytidine 2',3'-phosphate catalysed by ribonuclease has been determined. The data are in accordance with the hypothesis that two ionizing groups are required at the active site, one in the base form and the other in the acid form.

2. The determined pK values of the ionizing groups at the active site are: for the free enzyme, 5.22 ± 0.20 and 6.78 ± 0.20 ; for the enzyme-sub-

Table 4. Dissociation constants and other parameters for the variation of the kinetics with pH

All measurements were made at 25° and at $I\ 0.20$.

Free enzyme		Enzyme-substrate complex	
K_b	$(5.97 \pm 2.74) \times 10^{-6}$	$K_{\beta'}$	$(5.04 \pm 1.03) \times 10^{-7}$
K_a	$(1.68 \pm 0.77) \times 10^{-7}$	$K_{\alpha'}$	$(7.90 \pm 1.62) \times 10^{-9}$
pK_b	5.22 ± 0.20	$pK_{\beta'}$	6.30 ± 0.09
pK_a	6.78 ± 0.20	$pK_{\alpha'}$	8.10 ± 0.09
k_{+2}/K_m	$(3.2 \pm 1.5) \times 10^5 \text{ min./M}$	k_{+2}	$1240 \pm 250 \text{ min.}$

strate complex, 6.30 ± 0.09 and 8.10 ± 0.09 . The groups involved are probably histidine residues.

3. Inhibition constants for cytidine 2'-phosphate and cytidine 3'-phosphate are reported. The descending order of affinity of cytidine nucleotides for the enzyme is: 2'-phosphate; 3'-phosphate; 2',3'-phosphate.

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The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

4. THE ACTIVITY IN INERT ORGANIC SOLVENTS AND ALCOHOLS

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Bovine pancreatic ribonuclease is active in high concentrations of organic solvents (Findlay, Mathias & Rabin, 1960). In the presence of methanol, both cytidine 3'-phosphate methyl ester and cytidine 3'-phosphate are formed from cytidine 2',3'-phosphate (Heppel & Whitfeld, 1955). We have investigated the alcoholysis reaction and compared the reactivity of various alcohols. The

effects of inert solvents on these reactions and on the hydrolysis of cytidine 2',3'-phosphate methyl ester have also been investigated.

EXPERIMENTAL

Chromatography. The ascending technique was employed with propan-2-ol-water-aq. ammonia (sp.gr. 0.88) (14:5:1, by vol.) as solvent.