A KINETIC ANALYSIS OF THE pH-DEPENDENCE OF THE ACTION OF BOVINE PANCREATIC RIBONUCLEASE A ON CYTIDINE 2':3'-PHOSPHATE

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The theory of the pH-dependence of enzyme reaction velocities based on the simplest case of two ionising groups in the active site has been developed in part and summarised by Laidler (1958). This treatment analyses the variation of the rate parameters with pH to give the dissociation constants of hypothetical ionising groups on the enzyme and enzyme-substrate complex.

The knowledge of the complete amino acid sequence of ribonuclease (Hirs, Moore & Stein, 1960) and the accumulating evidence for the requirement of certain amino acid residues in the active site prompted a careful investigation of the pH-dependent kinetics of this enzyme with synthetic substrates.

Experimental. Bovine pancreatic ribonuclease (Armour & Co.Ltd., Batch 21630) was chromatographed on IRC-50 resin (Hirs, Moore & Stein, 1953) and fraction A was deionised on a mixed bed resin (Amberlite MB3) and freeze-dried.

Cytidine 2':3'-phosphate was prepared from yeast cytidylic acid by the method of Crook, Mathias & Rabin (1960 a) except that it was converted to the sodium salt monohydrate. Cytidine 3'-phosphate was a gift from Dr. A.P. Mathias and was similarly converted to the disodium salt. The dissociation constants of the latter at 25°C were determined by titration and application of the analysis of Speakman (1940) for overlapping dissociations.

Water and hydrochloric acid were of the purity described by Crook, Mathias & Rabin (1960 b), and sodium hydroxide was prepared CO_2-free from a saturated solution. Buffer components were of Analytical Reagent grade except for disodium pentane 3,3-dicarboxylate which was synthesised from diethyl monoethyl-malonate (L. Light & Co.).

The reactions were followed by spectrophotometric and pH-stat methods. An extension of the assay described by Crook, Mathias & Rabin (1960 b) was used with the following buffer solutions: 0.1 M acetate pH 4.0; 0.1 M formate pH 4.0; 0.1 M acetate pH 4.5; 0.1 M acetate pH 5.0; 0.05 M pentane 3,3-dicarboxylate pH 6.56; all total ionic strength 0.2, made up with NaCl.

A Radiometer TTT 1a automatic titrator and SBR 2b titrigraph controlled and recorded the addition of alkali to the reaction mixture in the region of the secondary phosphate ionisation of cytidine 3'-phosphate. A magnetically stirred, water-jacketed glass cell with saturated KCl bridge built through the side was used to contain the 2 ml. reaction mixture at 25°C. A semimicro glass electrode (Radiometer G222b) was employed. Reaction mixtures were 0.2 ionic strength including nucleotide, made up with KCl.

Kinetic Equations. Product inhibition has been established for this enzyme and the following reaction scheme has been used:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} EP \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E + P$$

The treatment of Foster and Niemann (1953) yields a rate equation which on integration becomes

$$(1 + S_o/K_p) \ln S_o/s + (1/K_m - 1/K_p)(S_o - S) = \frac{k_3 E_o}{K_m}.t$$

where $K_m = (k_2 + k_3)/k_1$; $K_p = k_4/k_5$; S is substrate concentration at time t, S_o, E_o are initial substrate and enzyme concentrations. Graphs of $(S_o - S)/t$ versus $\frac{1}{t} \ln S_o/S$ were found to be linear with slopes a linear function of S_o. From this relationship between slope and S_o, K_p was calculated using the values of k_3 and K_m determined by the method of Lineweaver and Burk (1934) from initial velocities.

The spectrophotometric method enabled runs to be continued for periods of over one hour and such calculations to be made, but in the pH-stat method, product inhibition was measured by the reduction of the initial velocity at constant substrate concentration by varying amounts of cytidine 3'-phosphate.

A theoretical treatment of the pH variation of initial velocities based on two groups on the enzyme, one required in the base form and the other in the acid form for full enzymic activity, yields the following relations (Laidler, 1958):

$$k_3 = \frac{\bar{k}_3}{1 + H/K_b' + K_a'/H}; \quad K_m = \frac{\bar{K}_m(1 + H/K_b + K_a/H)}{(1 + H/K_b' + K_a'/H)} ;$$

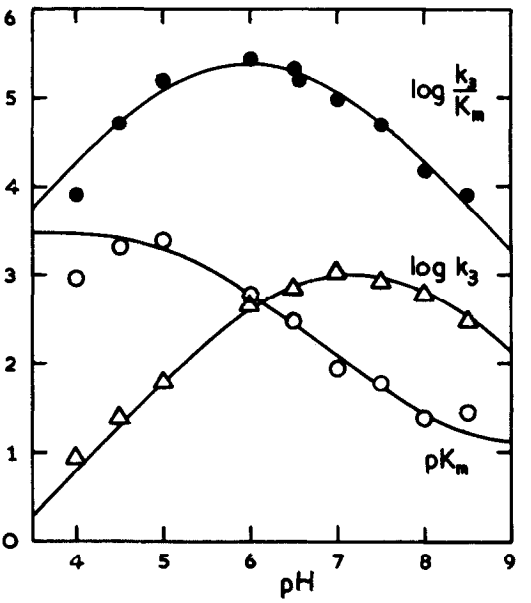
$\frac{k_3}{K_m} = \frac{\bar{k}_3}{\bar{K}_m}(1 + H/K_b + K_a/H)$ where K_a and K_b are the dissociation constants of the groups required in the acid and base forms respectively on the enzyme, and K_a', K_b' are the corresponding dissociations on the enzyme-substrate complex; H is hydrogen ion concentration, and a bar denotes pH-independent constants.

Values of k_3 measured in the pH-stat were corrected for incomplete ionisation of the product resulting in a lowered uptake of alkali. The thermodynamic pK for the secondary phosphate was taken as 6.25 (Ross and Herries, 1960).

Results. Measurements were made at pH values 4.0, 4.5, 5.0, and 6.56 by the spectrophotometric method. There was no difference

in velocities in acetate and formate buffers at pH 4.0. The pH-stat was used at pH values 6.0 to 8.5. All straight line plots were routinely treated by the method of least squares.

The variations of log k_3, pK_m, and log k_3/K_m with pH are shown in the figure.



The pH at the maximum ordinate value in the graphs of log k_3 and log k_3/K_m was taken to be the logarithm of the geometric mean of the relevant pair of dissociation constants. The reciprocal of k_3 was plotted against $(H + K_a'K_b'/H)$ according to the equation

$$1/k_3 = \frac{1}{K_b'\bar{k}_3} (H + K_a'K_b'/H) + 1/\bar{k}_3 .$$

From the resulting straight line, K_b' and $\bar{k}_3$ were determined and hence K_a'. The smooth curve in the figure was then calculated. The ratio k_3/K_m was treated similarly.

The dissociation constants are shown in the Table.

K_b 5.95 x 10^{-6}; pK_b 5.23	K_a 1.68 x 10^{-7}; pK_a 6.77
K_b' 5.04 x 10^{-7}; pK_b' 6.30	K_a' 7.94 x 10^{-9}; pK_a' 8.10
$\bar{k}_3$ 1240 min^{-1}	$\bar{k}_3/\bar{K}_m$ 3.2 x 10^5 $moles^{-1}$ $litre$ min^{-1}

The variation of K_p with pH was found to be similar to that of K_m, but further work is in progress.

Discussion. Equations based on the simplest theory of the effect of pH on enzyme activity have been shown to fit the data. The pK values by this interpretation indicate imidazole groups, and other evidence and details of the mechanistic consequences will be published later. Bruice and Schmir (1959) have warned that such kinetically determined dissociation constants depend on the intricacies of the mechanism, and this reservation should be borne in mind.

The simplified method of Dixon (1953) for determining pK values by fitting lines of integral slope to the experimental curves is unsuitable here because of the closeness of the values; in fact, the pK_m versus pH curve is linear between pH 6 and 7.5 with a slope of -0.67.

Deviation of the experimental values of k_3/K_m and K_m from the calculated curves at pH 4 may be due to protonation of the substrate; this is probably an indirect effect since calculation assuming a pK of 4.28 for the cytosine nitrogen in the 1-position (taken to be the same as the pK of cytidine 3'-phosphate*) show a more extensive effect.

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* Loring et al., 1952; Cavalieri, 1952; Ross and Herries, 1960.

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