The Kinetics of Alkaline Phosphatase*

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SUMMARY

The advantages of an integrated rate equation for the analysis of the reaction kinetics of the alkaline phosphatase of Escherichia coli were demonstrated.

The repressible alkaline phosphatase of Escherichia coli (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) promises to play a role in biochemical genetics comparable to that played by β-galactosidase (1–5). It is also being used increasingly for class experiments. The evaluation of the important characteristic kinetic constants for this enzyme, however, poses certain technical difficulties (6).

The following complications arise. (a) Some buffers, notably Tris, act as phosphate acceptors (7, 8). (b) The enzyme undergoes a marked activation upon incubation with certain buffers (2). (c) The enzymatic activity is sensitive to high ionic strengths. (d) The Michaelis constants (K_m) are often so low that when a substrate concentration of this magnitude is investigated, an appreciable fraction is utilized. This makes it hard to determine the true initial velocity. (e) The products of the reaction are potent competitive inhibitors of the substrate.

The first three sources of error may be readily minimized once they are considered. We wish to point out that the latter two, which reinforce each other, are elegantly handled by the integrated rate equations developed by Niemann and his collaborators (9–11).

One method they suggested for the presentation of experimental data was to plot [(S_0) - (S)]/t against 1/t-ln [(S_0)/(S)]. From such a plot a zero order reaction would yield a horizontal straight line; a first order reaction gives a vertical line cutting the abscissa at a value equivalent to the first order rate constant. Enzymatic reactions in which the products are competitive inhibitors of the substrate yield a series of straight lines with slopes decreasing as the substrate concentration decreases. These lines all appear to emanate from the point V_max/(1 - K_m/K_p) on the y axis. K_m is the substrate Michaelis constant, V_max is the maximum initial velocity, and K_p is the effective product competitive inhibition constant.

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A line through the origin with slope equivalent to the initial substrate concentration intersects the straight line through the experimental data for that particular concentration at a point where the ordinate is the true initial velocity. The true maximum initial velocity (V_max) is the ordinate intercept of the straight line drawn through a series of such intersections. The substrate K_m is the negative slope of this latter line.

The Michaelis Menten equation V_max/V = 1 + K_m/(S) predicts that an enzymatic reaction should appear to be zero order (V - V_max) when the ratio K_m/(S) is small or (S) is large. It also predicts that the reaction should appear to be first order [V = (V_max/K_m) - (S)] when this ratio is large or (S) is small.

EXPERIMENTAL PROCEDURE AND RESULTS

The alkaline phosphatase, which was purchased from Worthington, was dialyzed overnight against distilled water at 4°C. The p-nitrophenyl phosphate was prepared by the method of Bessey and Love (12) and recrystallized (13) before use. The hydrolysis of p-nitrophenyl phosphate was followed at 400 mμ in a Gilford recording spectrophotometer with the cell compartment and reaction mixture maintained at 37°C. It was found that the enzyme was rapidly activated upon dilution into the warm buffer. Reactions were therefore started by the addition of substrate after a 5-min prior incubation of buffer and enzyme. Reactions were followed to completion (absorbance A_e) so that [([S_0] - (S))] was simply proportional to the observed absorbance at time t, (A_t), and the ratio (S_0)/(S) was A_e/(A_e - A_t).

Fig. 1 shows the characteristically anomalous kinetics of the reaction catalyzed by this enzyme at pH 8. For this range of substrate concentrations the reactions appear to be accurately first order but the rate constant (k) is dependent upon the initial substrate concentration. The theoretical initial velocity (V_i = k·S_0) is almost independent of the substrate concentration. The fact that the reactions appear to be first order suggests that the enzyme was far from saturated; the independence of the initial velocity on the substrate concentration suggests the converse.

The same data when plotted in the more sophisticated form suggested by Niemann and collaborators is shown in Fig. 2. From this figure it can be seen that the enzyme was almost saturated and that the first order behavior of the reaction arose from the fact that the Michaelis constant for the substrate is essentially the same as the effective product competitive inhibition constant (K_m ≈ K_p). An even more instructive set of data obtained at pH 7.1 are shown in Fig. 3. Inspection of this figure clearly demonstrates that the reaction tends to become of zero order at low substrate concentrations and of first order at
Fig. 1. The hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase at pH 8.0. All the reaction mixtures contained 0.01 M imidazole buffer at 37°. The reaction was followed at 400 mμ. Substrate was added after a 5 min prior incubation of the enzyme in the buffer. The figures are the initial concentrations in micromolar.

Fig. 2. The data of Fig. 1 plotted in a manner which shows competitive inhibition by the product. The lines through the origin with slope equivalent to the initial substrate concentration cut those through the experimental data at a point where the ordinate is the true initial velocity.

Fig. 3. Data comparable to those in Fig. 2 in which the buffer was replaced with 0.01 M imidazole, pH 7.1.

high substrate concentrations; completely the contrary of the usual dictum.

The data emphasize in particular the dangers of giving significance to the observed order of reaction with enzymes which have a low substrate Michaelis constant and are susceptible to product inhibition.

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REFERENCES
The Kinetics of Alkaline Phosphatase
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