Use of Isotope Competition and Alternative Substrates for Studying the Kinetic Mechanism of Enzyme Action. I. Experiments with Hexokinase and Alcohol Dehydrogenase*

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ABSTRACT: Alternative and isotopic substrates, which function as competitive inhibitors in initial rate experiments under defined conditions, were used to make a choice of mechanism from among a variety of usual two substrate–enzyme types. It is possible to reach a definitive conclusion regarding kinetic mechanisms from only two "isotope competition" experiments, the theory and experimental protocol of which are presented in the paper. Alternative substrates were used to study the mechanism of action of two enzyme systems, liver alcohol dehydrogenase and yeast hexokinase. For the former enzyme, thionicotinamide–NAD was used as alternative substrate, while in the case of hexokinase, D-fructose was the alternative substrate. From these experiments the mechanism of the dehydrogenase was found to be ordered sequential with the nucleotide as the obligatory initial substrate. On the other hand, the mechanism for the phosphotransferase as investigated with alternative and isotopic substrates, appears to be Random Bi Bi.

A number of approaches are currently available for studying the kinetic mechanism of action of enzyme systems which utilize two and three substrates. Some of these methods such as evaluation of Dalziel coefficients (Dalziel, 1957) and the Haldane relationships (Alberty, 1953) are essentially quantitative, while others are basically qualitative. In this latter class are studies of product inhibition as first formulated by Alberty (1958) and later modified by Fromm and Nelson (1962) and Cleland (1963a), isotope exchange (Boyer, 1959), competitive inhibitors (Fromm and Zewe, 1962; Zewe *et al.*, 1964; Fromm, 1964; Fromm, 1967), alternative substrates (Fromm, 1964; Zewe *et al.*, 1964), and alternative products (Fromm and Zewe, 1962; Wratten and Cleland, 1965).

When considering alternative substrates in studying kinetic mechanisms, it was suggested that a choice of mechanism could be made from among a number of two substrate types by evaluating the *summation* of the velocities of the alternative substrate and the substrate in a mixture (Fromm, 1964; Zewe *et al.*, 1964). It has now become clear that a good deal of insight into the kinetic mechanism may be obtained by using the alternative substrate as a "competitive inhibitor" of the substrate and evaluating the inhibitory effect on the other substrate (the one which is not inhibited competitively).

The use of an alternative substrate as a competitive inhibitor was tested with two enzyme systems, which are generally acknowledged to be sequential, liver alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1), and yeast hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1). A number of workers have indicated that the dehydrogenase mechanism is ordered with NAD being the first substrate to add to the enzyme and NADH the last product to dissociate (Theorell and Chance, 1951; Wratten and Cleland, 1963; Dalziel and Dickinson, 1966). On the other hand, there appears to be some doubt concerning the mechanism of yeast hexokinase. Some investigators have proposed an ordered sequential mechanism with glucose binding before ATP (Hammes and Kochavi, 1962a,b; Noat *et al.*, 1968) while others have presented findings which suggest the mechanism may be random (Fromm and Zewe, 1962; Boyer and Silverstein, 1963).

It will be shown in this report that alternative substrates may be used advantageously in studying the kinetic mechanism of enzyme action. The results of our investigation support the idea that the mechanism of yeast hexokinase is Random Bi Bi.

Experimental Procedure

Materials. Crystalline yeast hexokinase (S.A. 200 IU/mg), equine liver alcohol dehydrogenase (S.A. 2.7 IU/mg), and yeast D-glucose 6-phosphate dehydrogenase (S.A. 420 IU/mg) were obtained from Calbiochem. Hexokinase and D-glucose 6-phosphate dehydrogenase were diluted to appropriate concentrations with cold 0.1 M HEPES,[1] pH 7.6, and alcohol dehydrogenase was dialyzed against 0.1 M KP_i, pH 7.15, containing 1 mM ethanol and then diluted to an appropriate concentration with cold 0.1 M KP_i, pH 7.15, containing 1 mM ethanol and 0.5% bovine serum albumin.

Thionicotinamide–NAD, NAD, NADP, and ATP were supplied by Sigma Chemical Co. Uniformly labeled [^{14}C]-glucose was obtained from Schwarz BioResearch and had a specific activity of 250 mCi/mmole. AG 1-X2 anion-exchange resin (200–400 mesh) was a product of Bio-Rad Laboratories. Ethanol was redistilled prior to use.

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[1] Abbreviation used is: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Methods. ALCOHOL DEHYDROGENASE EXPERIMENTS. In studies involving alcohol dehydrogenase the reaction was monitored at 342 nm, the isosbestic point for thionicotinamide–NAD and thionicotinamide-NADH. Initial rate experiments were carried out at 28° in a Cary Model 15 recording spectrophotometer with a thermostatted cell housing, using the 0–0.1 absorbance slide-wire. The reactions were carried out in a volume of 1.0 ml containing 0.10 M KP_i, pH 7.15, ethanol, NAD, and thionicotinamide–NAD in the inhibition studies. The reaction was initiated by addition of the enzyme and the conversion of NAD into NADH was followed continuously at 342 nm. The change in extinction for the formation of NADH at 342 was 6.2×10^6 cm^2 per mole (P-L Circular, 1961). Velocity was expressed as molarity of NADH formed per minute.

HEXOKINASE EXPERIMENTS. The effect of D-fructose on the utilization of D-glucose by hexokinase was studied by following the production of D-glucose 6-phosphate by coupling the reaction to D-glucose 6-phosphate dehydrogenase and measuring the formation of NADPH at 340 nm (Fromm and Zewe, 1962). No formation of NADPH could be detected from fructose in the absence of added glucose.

Initial rate experiments were carried out at 28° in a total volume of 1.0 ml containing 20 mM HEPES, pH 7.6, 2.5 mM free magnesium as $MgCl_2$, 90 mM NADP, D-glucose, $MgATP^{2-}$, and D-fructose at appropriate concentrations. The ATP was 99% chelated and was added as $MgATP^{2-}$ using a stability constant of 100,000 in 20 mM HEPES (Rudolph and Fromm, 1969). Reactions were initiated by addition of enzyme and the conversion of NADP into NADPH at 340 nm was monitored continuously. Velocity was expressed as molarity of NADPH per minute.

The isotope competition experiments were carried out by first varying the D-[^{14}C]glucose in the presence of a constant level of unlabeled glucose at a single $MgATP^{2-}$ concentration. Experiments of this type were repeated using different levels of unlabeled sugar alternative substrate. This experiment should always show that the unlabeled substrate is a competitive inhibitor for the labeled substrate for any reaction. The experiments in which $MgATP^{2-}$ was varied were carried out with a constant amount of D-[^{14}C]glucose in each reaction mixture.

The isotope competition experiments with hexokinase were followed by separating the D-[^{14}C]glucose 6-phosphate formed from D-[^{14}C]glucose by use of AG 1-X2-Cl^- anion exchange columns. The initial rate experiments were carried out at 28° in a total volume of 1.0 ml containing 20 mM HEPES, pH 7.6, 2.5 mM free magnesium as $MgCl_2$, $MgATP^{2-}$, D-glucose, and D-[^{14}C]glucose. The reactions were started with enzyme and samples were assayed at different times to ensure measurement of initial velocities. The reactions were terminated by boiling[2] for 1 min and then 0.7 ml of each reaction mixture was washed onto a 0.8×2.0 cm AG 1-X2 column in the Cl^- form that had been equilibrated with 40 mM D-glucose. The column was then washed with 25 ml of 40 mM D-glucose followed by 25 ml of H_2O. The D-[^{14}C]glucose 6-phosphate was removed by washing the column with five 2-ml additions of 0.1 N HCl. The acid effluent (2 ml) was added to 15 ml of naphthalene–dioxane scintillation fluid and counted in a liquid scintillation spectrophotometer. Velocities are expressed as counts per minute of D-[^{14}C]glucose 6-phosphate formed.

Results

In the case of two-substrate–enzyme systems, there are two basic kinetic mechanisms, Ping-Pong and sequential (Cleland, 1963b). Alberty (1953) has shown how these two classes of mechanisms may be separated by inspection of Lineweaver–Burk (1934) type plots. While there is basically one type of two-substrate Ping-Pong mechanism, there are three types of sequential mechanisms. The Random Bi Bi, ternary complex, and Theorell–Chance mechanisms comprise the latter group.

It is possible by using alternative substrates to differentiate between the random and ordered sequential mechanisms and, in the latter case, to determine the binding order. When considering the Ping-Pong mechanism, it is not possible to make a choice between the first and second substrate by using alternative substrates.

Rate Equations. 1. RAPID-EQUILIBRIUM RANDOM BI BI MECHANISM. In Scheme Ia is shown the Random mechanism in which it is assumed that all steps equilibrate rapidly relative to the interconversion of the ternary complexes.

The case in which steady-state kinetics prevail for this mechanism is not considered in this report; however, all initial rate double-reciprocal plots will be theoretically nonlinear.

SCHEME I

a.

$$E + A \rightleftharpoons EA;\ K_1$$

$$E + B \rightleftharpoons EB;\ K_2$$

$$EA + B \rightleftharpoons EAB;\ K_3$$

$$EB + A \rightleftharpoons EAB;\ K_4$$

$$EAB \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ECD \longrightarrow \text{products} + E$$

b.

$$E + A' \rightleftharpoons EA';\ K_1'$$

$$E + B \rightleftharpoons EB\ K_2$$

$$EA' + B \rightleftharpoons EA'B;\ K_3'$$

$$EB + A' \rightleftharpoons EA'B;\ K_4'$$

$$EA'B \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EC'D \longrightarrow \text{products} + E$$

If the velocity of the reaction in Scheme Ia [$v = k_1$ (EAB)] is measured in the presence of A′, the resulting rate equation

[2] Boiling was found to be an effective and valid method for terminating the hexokinase reaction. Initial velocity was found to prevail for 30 min when reaction mixtures were boiled and then assayed at different time intervals. Furthermore, when enzyme, at the concentration used in these studies, was added to reaction mixtures and brought to boiling or when enzyme was added to a boiling assay solution, no discernable [^{14}C]glucose 6-phosphate was formed from [^{14}C]glucose.

is depicted in eq 1. In this equation E_0, v, and A' represent

$$\frac{k_1E_0}{v} = 1 + \frac{K_4}{A}\left(1 + \frac{A'}{K_4'}\right) + \frac{K_3}{B} + \frac{K_1K_3}{(A)(B)}\left(1 + \frac{A'}{K_1'}\right) \quad (1)$$

total enzyme, initial velocity, and alternative substrate for A, respectively.

Equation 1 shows that A′ will act like a competitive inhibitor for A and a noncompetitive inhibitor for B. It is obvious that an alternative substrate for B, B′, will be competitive for B and noncompetitive relative to A.

2. Ordered bi bi mechanism. a. Scheme IIa depicts the ordered ternary complex mechanism. The basic mechanism is shown in Scheme IIb when an alternative substrate for A, A′, is present along with A.

SCHEME II

a.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EXY \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC + D$$

$$EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + C$$

b.

$$E + A' \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EA'$$

$$EA' + B \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} EXY' \underset{k_6'}{\overset{k_5'}{\rightleftharpoons}} EC' + D$$

$$EC' \underset{k_8'}{\overset{k_7'}{\rightleftharpoons}} E + C'$$

The rate expression for this mechanism when A, A′, and B are present together is

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{A}[1 + X + Y + Z] + \frac{\phi_2}{B} + \frac{\phi_{12}}{(A)(B)}[1 + X + Y + Z] \quad (2)$$

where $\phi_0 = (1/k_5) + (1/k_7)$, $\phi_1 = 1/k_1$, $\phi_2 = (k_4 + k_5)/k_3k_5$, $\phi_{12} = [k_2(k_4 + k_5)/k_1k_3k_5]$,

$$X = \frac{k_1'(A')(k_4' + k_5')}{k_2'(k_4' + k_5') + k_3'k_5'(B)}$$

$$Y = \frac{k_3'(B)(X)}{(k_4' + k_5')}$$

$$Z = \frac{k_3'k_5'(B)(X)}{k_7'(k_4' + k_5')}$$

Equation 2 indicates that A′ will appear to act like a competitive inhibitor for A; however, inhibition relative to B will be parabolic.

b. In the event that an alternative substrate B′ is used along with B, the following will occur.

$$EA + B' \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} EXY'' \underset{k_6'}{\overset{k_5'}{\rightleftharpoons}} EC + D'$$

The modified rate equation will be

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{A} + \frac{\phi_2}{B}\left[1 + \frac{k_3'(B')}{(k_4' + k_5')}\left(1 + \frac{k_5'}{k_7}\right)\right] + \frac{\phi_{12}}{(A)(B)}\left[1 + \frac{k_3'k_5'(B')}{k_2(k_4' + k_5')}\right] \quad (3)$$

Thus while B′ is competitive for B it will appear to act noncompetitively relative to substrate A.

From these considerations, it is clear that a choice can readily be made, at least in theory, between the random and ordered mechanisms. Also from the inhibition patterns, it should be possible to differentiate between the first and second substrates for the latter mechanism.

3. Theorell–Chance mechanism. The form of the rate expression for this mechanism is essentially identical with the mechanism above. Thus a choice cannot be made between the two ordered pathways of enzyme and substrate interaction.

4. Ping-pong mechanism. It has been recognized for some time that Ping-Pong and sequential mechanisms may be distinguished by their primary Lineweaver–Burk (1934) type plots (Alberty, 1953). Parallel line data of the type expected for Ping-Pong pathways are in many cases equivocal and other kinetic approaches are often required to support the initial rate experiments.

The use of alternative substrates as outlined above may be used to test Ping-Pong mechanisms. Scheme IIIa represents a typical Ping-Pong pathway, while Scheme IIIb is an analogous case with an alternative substrate for A, A′.

SCHEME III

a.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E' + C$$

$$E' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EB \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + D$$

b.

$$E + A' \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EA' \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} E'' + C'$$

$$E'' + B \underset{k_6'}{\overset{k_5'}{\rightleftharpoons}} EB' \underset{k_8'}{\overset{k_7'}{\rightleftharpoons}} E + D$$

The rate equation for the Ping-Pong mechanism in which both A and A′ are present is

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{A}\left[1 + \frac{k_1'(A')}{(k_2' + k_3')}\left(1 + \frac{k_3'}{k_7'}\right)\right] + \frac{\phi_2}{B} + \frac{\phi_1}{(A)(B)} \times \frac{k_1'k_3'(k_6' + k_7')(A')}{k_5'k_7'(k_2' + k_3')} \quad (4)$$

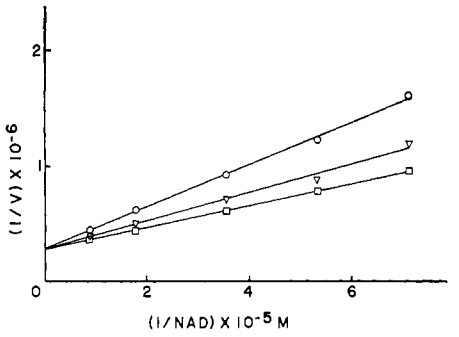

FIGURE 1: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of NAD in the presence and absence of thionicotinamide–NAD. Ethanol was held constant at 3.0 mM and NAD was varied from 1.40 to 11.2 $\times$ 10^{-5} M. Thionicotinamide–NAD concentrations were 0 (□), 6.46 (▽), and 12.9 $\times$ 10^{-5} M (○). Other experimental details are described under Experimental Procedure.

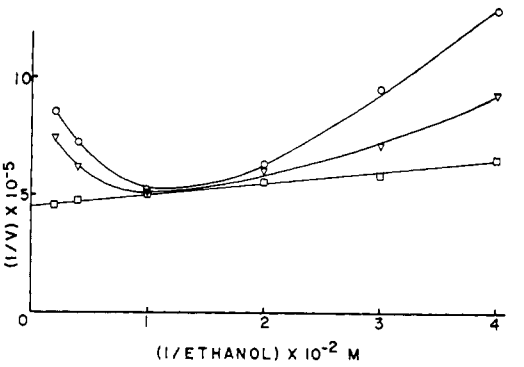

FIGURE 2: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of ethanol in the presence and absence of thionicotinamide–NAD. NAD was held constant at 2.48 $\times$ 10^{-5} M and ethanol was varied from 2.5 to 50 mM. Thionicotinamide–NAD concentrations were 0 (□), 6.46 (▽), and 12.9 $\times$ 10^{-5} M (○). Other experimental details are described under Experimental Procedure.

where $\phi_0 = (1/k_3) + (1/k_7)$, $\phi_1 = (k_2 + k_3)/k_1k_3$, and $\phi_2 = (k_6 + k_7)/k_5k_7$.

If an alternative substrate for B, B′ is used, the pathway

$$E' + B' \underset{k_6'}{\overset{k_5'}{\rightleftharpoons}} EB' \underset{k_8'}{\overset{k_7'}{\rightleftharpoons}} E + D'$$

must be added to Scheme IIIa. The rate equation for the mixture of enzyme, A, B and B′ is

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{A} + \frac{\phi_2}{B}\left[1 + \frac{k_5'(B')}{(k_6' + k_7')}\left(1 + \frac{k_7'}{k_3}\right)\right] + \frac{\phi_1\phi_2k_5'k_7'(B')}{(k_6' + k_7')(A)(B)} \quad (5)$$

Equation 4 predicts that an alternative substrate for A will act competitively relative to A and will be a noncompetitive inhibitor for B. Similarly, B′, a competitive inhibitor for B will function as an noncompetitive inhibitor for A.

If when A′ reacts with E (Scheme IIIb) E′ rather than E″ forms, the resulting rate equation will be of the same form as eq 4.

Equations 1, 4, and 5 show that it is not possible using alternative substrates to differentiate between Ping-Pong and Random mechanisms, however, such experiments would serve to reinforce initial rate experiments of the type proposed by Alberty (1953).

Experiments with Liver Alcohol Dehydrogenase. Thionicotinamide–NAD has been shown to be a substrate for liver alcohol dehydrogenase. In fact, this analog reacts at approximately 3.5 times the rate of NAD (Sund and Theorell, 1963). In the experiments to be described, initial velocities were determined at 342 nm, the isosbestic point for thionicotinamide–NAD and thionicotinamide–NADH. Therefore, in these experiments NAD is the substrate and thionicotinamide–NAD, the alternative substrate.

In Figure 1 are shown initial rate data which indicate that thionicotinamide–NAD is a competitive inhibitor of NAD with alcohol dehydrogenase. Figure 2 depicts results obtained when the concentration of NAD was maintained constant and the concentration of ethanol varied in the presence and absence of thionicotinamide–NAD. The findings of Figure 2 show that thionicotinamide–NAD is a parabolic inhibitor of ethanol. The inhibitor lines are clearly curvilinear and go through a minimum.

The data of Figures 1 and 2 appear to be in harmony with an ordered sequential mechanism for alcohol dehydrogenase as inferred from eq 2. The binding sequence must be nucleotide as first substrate and alcohol as second substrate. The parabolic inhibition pattern is unique for the two ordered sequential mechanisms considered in this report. Certainly, the data obtained for the enzyme agrees with the studies of most investigators regarding the reaction mechanism for the dehydrogenase.

Experiments with Yeast Hexokinase. We have reported in the past that D-mannose acts like a competitive inhibitor of glucose and as a noncompetitive inhibitor of $MgATP^{2-}$ when initial velocity was measured as a function of glucose 6-phosphate production (Fromm and Zewe, 1962; Zewe *et al.*, 1964). Based upon the predicted effects of alternative substrates, these experiments suggest the mechanism for yeast hexokinase is Random Bi Bi. We decided to test this mechanism using D-fructose as an alternative substrate for glucose. Data from the literature indicate that D-fructose reacts 1.8 times more rapidly than does D-glucose in the yeast hexokinase reaction (Crane, 1962).

The data of Figures 3 and 4 show that like D-mannose, D-fructose is a competitive inhibitor of D-glucose and a noncompetitive inhibitor of $MgATP^{2-}$ with the enzyme from yeast. These results exclude both ordered sequential mechanisms for hexokinase; however, they do not eliminate the possibility that the phosphotransferase exhibits a Ping-Pong mechanism. To the best of our knowledge, however, the mass of the kinetic data for this enzyme are at variance with this possibility (Hammes and Kochavi, 1962a,b; Fromm and Zewe, 1962; Noat *et al.*, 1968).

The results of Figures 3 and 4 appear to support the Random Bi Bi mechanism for yeast hexokinase.

Isotope Competition Experiments with Yeast Hexokinase. In many enzymes systems, particularly for those which exhibit a high degree of specificity, obtaining a good alternative substrate may be a difficult undertaking. One way to

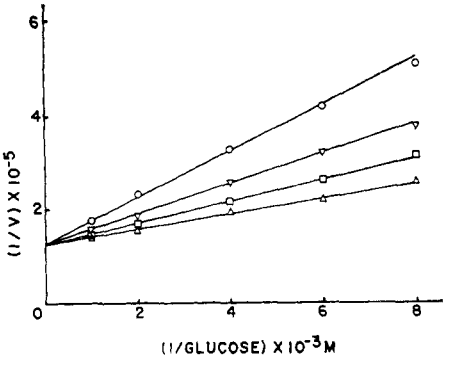

FIGURE 3: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of D-glucose in the presence and absence of D-fructose. $MgATP^{2-}$ was held constant at 0.50 mM and D-glucose was varied from 0.125 to 1.0 mM. D-Fructose concentrations were 0 (△), 1.0 (□), 2.5 (▽), and 5.0 mM (○). Other experimental details are described under Experimental Procedure.

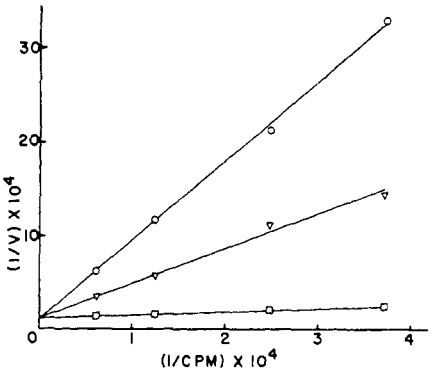

FIGURE 5: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the cpm of D-[^{14}C]glucose in the presence of increasing concentrations of unlabeled D-glucose. $MgATP^{2-}$ was held constant at 1.0 mM and the D-[^{14}C]glucose was varied from 26,900 to 161,500 cpm. Unlabeled D-glucose concentrations were 1.0 (□), 2.5 (▽), 5.0 mM (○). Other experimental details are described under Experimental Procedure.

circumvent this problem is to use a radioactive substrate as the substrate and the nonradioactive compound as the alternative substrate. In experiments of this type, velocity is expressed as a function of labeled product. It is clear that in the presence of nonlabeled substrate, at infinite labeled substrate, the effect of the nonlabeled or alternative substrate will be reversed, *i.e.*, the later compound will appear to be a competitive inhibitor of the labeled compound. Extrapolation to infinite labeled substrate concentration may be achieved from the usual Lineweaver–Burk type graphs. In experiments of this type a good deal of nonradioactive substrate may be present in the labeled substrate. Serial dilution of the isotope solution should give linear double-reciprocal plots; however, care must be exercised to ensure that the reaction mixtures for each series of isotope dilutions contain an identical amount of total (in practical terms, nonlabeled) substrate.

In Figures 5 and 6 are shown data for the hexokinase system where [^{14}C]glucose is taken to be the substrate and [^{12}C]glucose the alternative substrate. It is clear from the graphs that the alternative substrate is a competitive inhibitor of the substrate and a noncompetitive inhibitor of $MgATP^{2-}$. The results are consistent with the data exhibited in Figures 3 and 4 for D-fructose and in the literature for D-mannose (Fromm and Zewe, 1962; Zewe *et al.*, 1964). These experiments demonstrate the value of isotope competition for the purpose described in these investigations. The data are in harmony with the Random Bi Bi mechanism for yeast hexokinase and appear to exclude the ordered sequential mechanism in which the sugar is the obligatory initial substrate.

Discussion

The results of the current investigation suggest that initial rate experiments involving the use of alternative substrates may be a valuable tool for the elucidation of kinetic reaction mechanisms. The rate equations which are presented in this report are rather straightforward, except that it is assumed

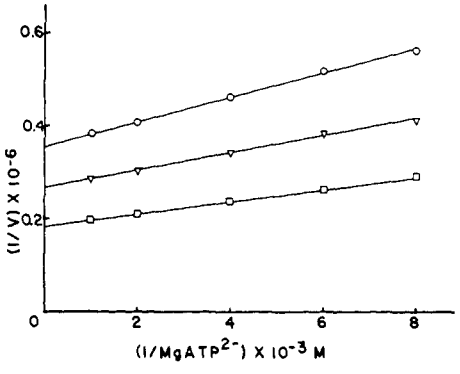

FIGURE 4: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of $MgATP^{2-}$ in the presence and absence of D-fructose. D-Glucose was held constant at 0.20 mM and $MgATP^{2-}$ was varied from 0.125 to 1.0 mM. D-Fructose concentrations were 0 (□), 2.5 (▽), and 5.0 mM (○). Other experimental details are described under Experimental Procedure.

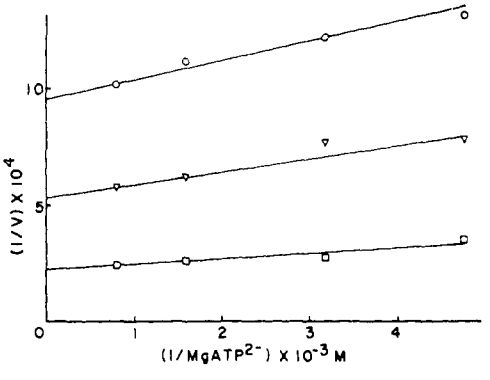

FIGURE 6: Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of $MgATP^{2-}$ in the presence of increasing concentrations of unlabeled D-glucose. D-[^{14}C]Glucose was held constant at 80,750 cpm and $MgATP^{2-}$ was varied from 0.21 to 1.26 mM. Unlabeled D-glucose concentrations were 0.5 (□), 1.5 (▽), 2.5 mM (○). Other experimental details are described under Experimental Procedure.

in the derivations that the substrate and the alternative substrate follow the same reaction pathway.

The employment of isotope competition for the purposes described above appear to increase the usefulness of the equations which predict the kinetic effects of alternative substrates. This would be particularly true in situations where good alternatives substrates are not available.

Experiments with radioactive and nonlabeled substrates appear to have another great virtue. Because the labeled and nonlabeled substrate must react at the same site on the enzyme, *i.e.*, they are competive, it is only necessary to do two kinetic experiments to make a choice of mechanisms from among those cited above. These experiments are of the type shown in Figures 2, 4, and 6 in which the varied substrate is the one that is not competitively inhibited.

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Kinetic Studies on the Mechanism of Insect Acetylcholinesterase*

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ABSTRACT: A purified preparation of acetylcholinesterase from the heads of houseflies was shown to contain a single active enzyme. Studies on transfer of the substrate acetyl group to water or methanol, as well as other evidence, indicated that an acetyl–enzyme intermediate is involved in the hydrolysis mechanism. Formation of this intermediate is probably the rate-limiting step in substrate hydrolysis. Similar maximum velocities for certain substrates having different leaving groups may depend on a common rate-limiting protein conformational change rather than slow reaction of a common intermediate. Substrate inhibition and noncompetitive inhibition were observed with all quaternary ammonium substrates and inhibitors tested and were due to interference with formation of acetyl–enzyme. The enzyme was shown to contain two cation binding sites, one of which attracts the substrate and the other an inhibitor. Binding at the second of these sites causes substrate inhibition and noncompetitive inhibition.

In vertebrate acetylcholinesterase, by contrast, a single anionic site is involved here, and these inhibitions are due to interference with breakdown rather than formation of acetyl–enzyme.

As the result of investigations beginning with those of I. B. Wilson and his collaborators, the mechanism of action of acetylcholinesterase (AChE)[1] from two vertebrate sources, the electric organ of the electric eel and bovine erythrocytes, is now understood in considerable detail (Wilson, 1960; Krupa, 1966a,b). This enzyme is particularly interesting because of its involvement in nerve transmission and because of the

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[1] Abbreviations used are: AChE, acetylcholinesterase; ACh, acetylcholine; ASCh, acetylthiocholine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; ES, enzyme–substrate complex; EA, acetyl–enzyme intermediate.