PART I α-SECONDARY ISOTOPE EFFECTS ON THE REACTION CATALYZED BY FUMARASE

PART II SALT EFFECTS ON FUMARASE

Thesis by

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To My Wife and Parents

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No words can adequately express my thanks for the many sacrifices of my wife and parents during my quarter century of schooling.

ABSTRACT

PART I α-Secondary Isotope Effects on the Reaction Catalyzed by the Enzyme Fumarase

PART II Salt Effects on Fumarase

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PART I

The α-secondary isotope effect of L-malic-2-T-1, 4-C^{14} acid on the reaction catalyzed by fumarase was determined. Evidence was presented which indicated that the reaction proceeded by formation of a carbonium ion in a rate-determining state at C-2 which possesses little sp^2 character.

PART II

The effects of sodium bromide and sodium phosphate at pH 7.3 and 27° were determined. A partial kinetic and mechanistic scheme has been developed to explain these effects.

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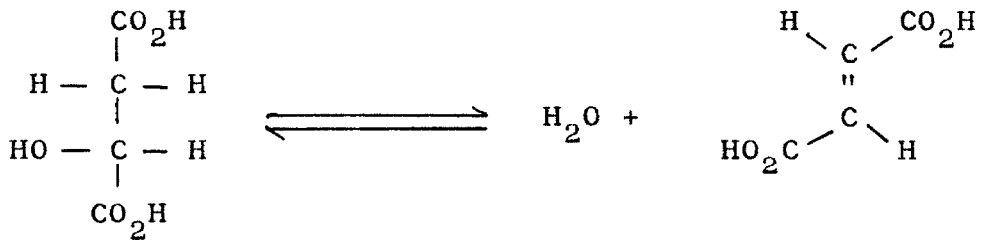
PART I α-SECONDARY ISOTOPE EFFECTS ON THE REACTION CATALYZED BY THE ENZYME FUMARASE

1. INTRODUCTION

The enzyme fumarase catalyzes the interconversion of l-malic acid and fumaric acid.

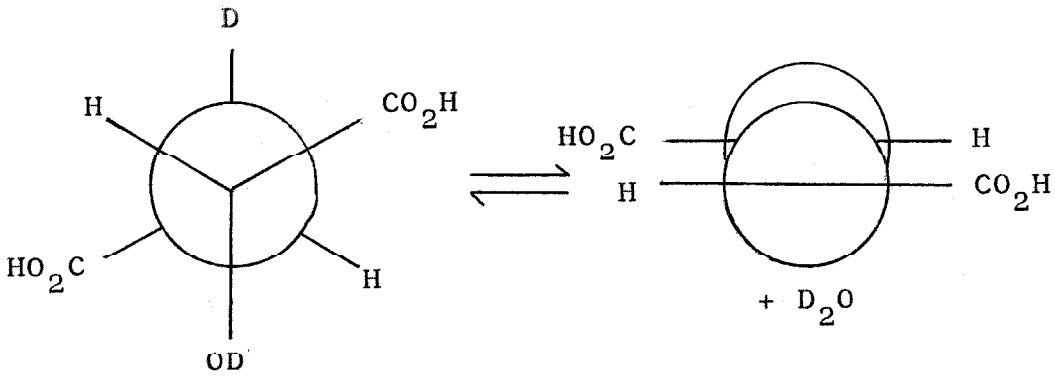


L-malic acid fumaric acid

This reversible reaction has been shown to proceed stereospecifically (1). When dipotassium fumarate was added to a solution of fumarase in deuterium oxide, the resulting l-malic acid contained 0.97 excess atom of deuterium per molecule while the isolated fumarate had incorporated less than $1x10^{-4}$ atom of nonexchangeable deuterium per molecule. This shows that the entering hydrogen atom is added in only one of two possible positions and a hydrogen atom is removed from this identical position in the reverse reaction.

An attempt was made to determine the configuration of the monodeuterio-l-malate by observing the dipolar broadening of the proton magnetic resonance absorption in solid DOOCCH(D)C(OD)HCOOD (31) but this report was later shown to be wrong by stereospecific synthesis of a deuterio-malate compound (2, 3). The 3-deuterio-l-

malate produced by the enzymatic addition of deuterium oxide to fumaric acid has the erythro configuration and has the absolute configuration 2-hydroxy-3-deuterio-succinic-2(R)-3(S) acid. Therefore, the reaction represents the <u>trans</u> addition of the elements of water.



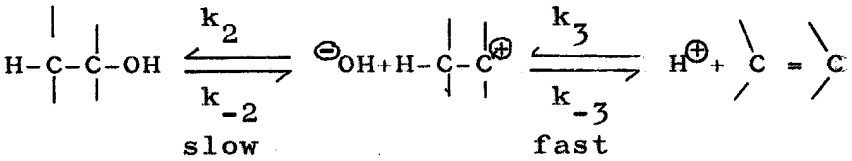
erythro-3-deuterio-
L-malic acid

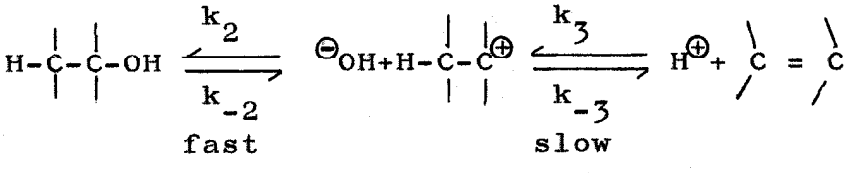
In an attempt to elucidate the mechanism of this reaction, the isotope effect on the rate of dehydration was studied (1, 4). The erythro-3-deuterio-l-malic acid was prepared enzymatically and the primary isotope effect was then determined by comparing the maximum initial velocity of the unlabeled acid with that of the deuterium labeled acid.

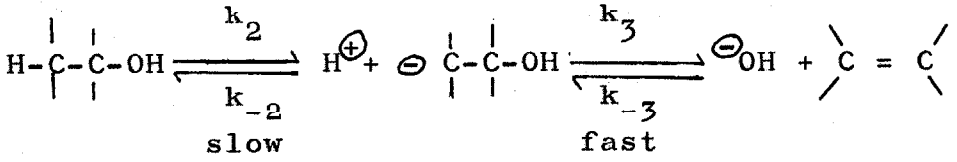
The method of determining the catalytic constant for an enzymatic reaction by measuring the maximum initial velocity has been criticized (5) since the actual asymptotic value of the rate at high substrate concen-

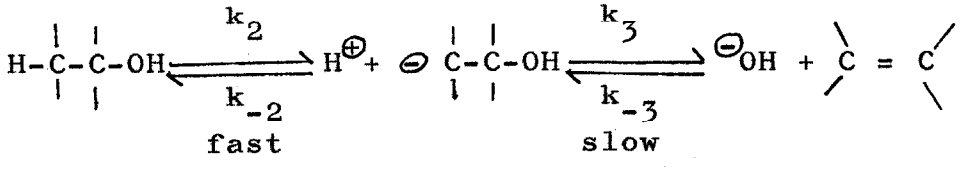
trations is very difficult to determine. Despite the undesirable method used for determining the catalytic constant, a value for the ratio of the maximum velocity for dehydration of the unlabeled acid to the maximum velocity for the dehydration of the labeled acid, V_M^H/V_M^D, of two or greater should have been detectable. In fact this ratio was 0.94 which was equal to unity within the experimental error. In conjunction with the isotope effect experiments, the incorporation of deuterium into l-malate during the dehydration to fumarate by fumarase in deuterium oxide was determined (4). Deuterium was incorporated in l-malate more slowly than fumarate was produced. In fact, the rate of incorporation of deuterium into malate was in quantitative agreement with that expected for the reversal of the dehydration reaction. These authors considered the five mechanisms shown in Table I.

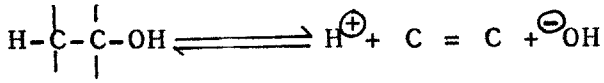
Table I

1. 

2. 

3. 

4. 

5. 

Only those steps involved in the breaking or formation of covalent bonds are shown. The abbreviations C = C, HCCOH, $HCC^{\oplus}$, and $CCOH^{\ominus}$ represent the enzyme-substrate complexes and intermediates in the reaction.

Since isotope effects of two or greater would be expected for mechanisms 2, 3, and 5 (6), the absence of such a large effect would tend to eliminate these mechanisms. The absence of hydrogen exchange would appear to eliminate mechanism 4. The overall mechanism for the interconversion of l-malic acid to fumaric acid was then postulated to be mechanism 1 of Table I and the overall mechanistic picture as:

$$E+M \underset{\text{fast}}{\rightleftharpoons} EM \underset{\text{slow}}{\rightleftharpoons} EX \underset{\text{fast}}{\rightleftharpoons} EF \underset{\text{fast}}{\rightleftharpoons} E+F$$

where

E = fumarase

M = l-malic acid

F = fumaric acid

EM = enzyme-l-malic acid complex

EF = enzyme-fumaric acid complex

EX = enzyme-intermediate complex

However, Dixon and Webb (7) later suggested that the dissociation of fumarate from the enzyme could be the rate-determining step of the reaction in which case no isotope effect would be expected. Determination of the individual rate constants assuming a concerted mechanism (mechanism 5) indicates that the dissociation of the enzyme-fumarate complex is not the slow step if this mechanism applies (12). Unfortunately, limits cannot be set on the values for the rate constants of the other mechanisms with three intermediates. Since the observed isotope effect implicates one of these mechanisms with three intermediates, one cannot rule out the possibility that the enzyme-fumarate dissociation is the slow step although this has been attempted (10).

In apparent contradiction to the isotope effect measured by Alberty (1, 4), Sere (9) found an isotope effect of $V_M^H/V_M^D = 2.1$ in the enzymatic hydration of fumaric acid in a water solution versus that of the hydration in deuterium oxide. This contradiction may arise from the fact that protein hydration will vary with the isotopic composition of the water and that

the conformational details of some proteins may be altered so as to change their catalytic activity. Mechanism 4 is also a possibility. For example, the enzyme might bind the hydrogen it has removed from the malic acid in which case no hydrogen exchange with water will be seen.

This research is directed towards determining a secondary isotope effect in this reaction and towards developing a new method for measuring isotope effects in enzymatic reactions. The value of the secondary isotope effect will tell us something about the reaction mechanism. If a secondary isotope effect is measurable, then the presence of this secondary effect would indicate that a primary isotope effect of two or greater should have been detectable if the reaction proceeds so that hydrogen is removed in the rate-determining step. Detection of a reasonable secondary isotope effect would tend to validate the absence of a primary effect.

2. KINETICS

A. Determination of the Deuterium Isotope Effect

The simplest mechanism that can account for the action of fumarase is given below (4, 10).

$$E+M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EX \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EF \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E+F$$

where

E = enzyme

M = l-malic acid

F = fumaric acid

EM = enzyme--l-malic acid complex

EF = enzyme--fumaric acid complex

EX = enzyme--intermediate complex

Using the steady-state assumption for the change in concentration of the intermediates EM, EX, and EF, equation 1 can be derived for the rate of disappearance of l-malic acid (10).

$$v_{initial} = \frac{-d(M)}{dt} = \frac{\frac{V_M}{K_M}(M) - \frac{V_F}{K_F}(F)}{1 + \frac{(F)}{K_F} + \frac{(M)}{K_M}} \quad (1)$$

Equation 1 will reduce to equation 2 if only the initial velocity of disappearance of l-malic acid is measured.

$$v_{initial} = \frac{V_M(M)}{K_M + (M)} \quad (2)$$

where

$$V_M = \frac{k_2k_3k_4\ (E_o)}{k_2k_3+k_2k_{-3}+k_2k_4+k_{-2}k_4+k_3k_4+k_{-2}k_{-3}} \qquad (3a)$$

$$V_M = k_{cat}(E_o) \quad (3b)$$

$$k_{cat} = V_M/(E_o) \quad (3c)$$

$$K_M = \frac{k_{-1}k_{-2}k_{-3}+k_{-1}k_{-2}k_4+k_{-1}k_3k_4+k_2k_3k_4}{k_1(k_2k_3+k_2k_{-3}+k_2k_4+k_{-2}k_4+k_3k_4+k_{-2}k_{-3})} \qquad (4)$$

(M) = molar concentration of l-malic acid

(F) = molar concentration of fumaric acid

Taking the recipricol of equation 2 gives equation 5.

$$\frac{1}{v_{initial}} = \frac{K_M}{V_M}\frac{1}{(M)} + \frac{1}{V_M} \quad (5)$$

A plot of $1/v_{initial}$ versus $1/(M)$ should give a straight line with the intercept of the $1/v_{initial}$ axis equal to $1/V_M$ and the intercept of the $1/(M)$ axis equal to $1/-K_M$. The velocity of the forward reaction is easily followed by measuring the change in optical density of the reaction solution at 250 mμ (11,12). Fumaric acid has an intense adsorption at 250 mμ while the adsorption by l-malic acid is negligible at this wave length. If deuterated substrates are used in the studies, it is necessary to correct for a change in the extinction coefficients if the deuterium is retained in the fumaric acid.

B. Determination of the Tritium Isotope Effect

This method of determining an isotope effect makes use of l-malic-2-T-1,4-C^{14} acid. Both tritium and carbon-14 are beta particle emitters which allows for their facile detection. Tritium will cause a secondary isotope effect of from 10% to 30% (18). On the other hand, carbon-14 will have little effect on the reaction. Data on secondary carbon isotope effects is rather scanty since the effect is so small and difficult to measure. The conversion of phenyl methyl ketone to a Schiff's base shows a secondary isotope effect of $k_c14/k_c12 = 1.008$ (16). The secondary isotope effect in the decarboxylation of malonić acid is $k_c14/k_c12 = 1.00\pm0.002$ (17). Even primary carbon isotope effects are usually quite small compared with primary hydrogen isotope effects. In the Hoffman elimination of a number of trimethyl-alkyl-ammonium hydroxides, k_c14/k_c12 ranged from 1.03 to 1.07 (18) while the primary deuterium isotope effects often reached a value of eight.

The tritium and carbon-14 are contained in separate acid molecules because l-malic-1,4-c^{14} acid and l-malic-2-T acid were synthesized separately and then mixed. A separate synthesis of each labeled compound is, however, not necessary. Even if tritium is added

to a compound already having carbon-14, the probability of a carbon-14 and a tritium actually occurring in the same molecule is extremely small.

When the doubly labeled substrate is added to the enzyme solution, the following reactions occur simultaneously.

$$E+M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EX \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EF \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E+F$$

$$E+M^T \underset{k_{-1}^T}{\overset{k_1^T}{\rightleftharpoons}} EM^T \underset{k_{-2}^T}{\overset{k_2^T}{\rightleftharpoons}} EX^T \underset{k_{-3}^T}{\overset{k_3^T}{\rightleftharpoons}} EF^T \underset{k_{-4}^T}{\overset{k_4^T}{\rightleftharpoons}} E+F^T$$

$$E+M^{14} \underset{k_{-1}^{14}}{\overset{k_1^{14}}{\rightleftharpoons}} EM^{14} \underset{k_{-2}^{14}}{\overset{k_2^{14}}{\rightleftharpoons}} EX^{14} \underset{k_{-3}^{14}}{\overset{k_3^{14}}{\rightleftharpoons}} EF^{14} \underset{k_{-4}^{14}}{\overset{k_4^{14}}{\rightleftharpoons}} E+F^{14}$$

where

E = enzyme

M = l-malic acid

M^T = l-malic-2-T acid

M^{14} = l-malic-1,4-c^{14} acid

F = fumaric acid

F^T = fumaric-2-T acid

F^{14} = fumaric-1-c^{14} acid

EM, EM^T, EM^{14}
EF, EF^T, EF^{14}
EX, EX^T, EF^{14} various enzyme complexes with the compounds defined above.

The following derivation will be done only for the tritium compound. The expressions for the carbon-14 compounds are strictly analogous with the substitution of pertinent rate constants. Using the steady-state assumption for the change in concentration of the enzyme-substrate complexes, equation 6 can be derived for the rate of disappearance of l-malic-2-T acid

$$v^T = \frac{-d(M^T)}{dt} = k_f^T(M^T) - k_r^T(F^T)\ (E) \quad (6)$$

where

$$k_f^T = \frac{k_1^T k_2^T k_3^T k_4^T}{k_{-1}^T k_{-2}^T k_{-3}^T + k_{-1}^T k_{-2}^T k_4^T + k_2^T k_3^T k_4^T + k_{-1}^T k_3^T k_4^T} \quad (6a)$$

$$k_r^T = \frac{k_{-1}^T k_{-2}^T k_{-3}^T k_{-4}^T}{k_{-1}^T k_{-2}^T k_{-3}^T + k_{-1}^T k_{-2}^T k_4^T + k_2^T k_3^T k_4^T + k_{-1}^T k_3^T k_4^T} \quad (6b)$$

(M^T) = molar concentration of l-malic-2-T acid

(F^T) = molar concentration of fumaric-2-T acid

(E) = molar concentration of enzyme

More intermediates do not change the form of equations 6, 6a, and 6b (28). Equation 6 may be integrated after the substitution of identity "a" is made.

$$(F^T) = (M_0^T) - (M^T) \quad (a)$$

where

(M_0^T) = molar concentration of l-malic-2-T at time t = o.

The integrated form of equation 6 contains a

natural logarithm term which is expanded in a series after substituting the identity b.

$$1 - \frac{(M^T)}{(M_0^T)} = \frac{(F^T)}{(M_0^T)} \qquad (b)$$

The final result of these mathematical manipulations is equation 7.

$$k^T \int_{t=o}^{t=t} (E)\, dt = \frac{(F^T)}{(M_0^T)} \sum_{n=0}^{\infty} \left[\frac{1}{n+1} \frac{(F^T)}{(F_e^T)} \right] \qquad (7)$$

where

(F_e^T) = molar concentration of fumaric-2-T acid at equilibrium.

Dividing equation 7 by the analogous one for the carbon-14 substituted l-malic acid gives equation 8.

$$\frac{k^{14}}{k^T} \frac{\int_{t=o}^{t=t_1} (E)\, dt}{\int_{t=o}^{t=t_1} (E)\, dt} = \frac{(F^{14})}{(F^T)} \frac{(M_0^T)}{(M_0^{14})} \qquad (1 + e)$$

where

$$e = \frac{\sum_{n=1}^{\infty} \left\{ \frac{1}{n+1} \left[\frac{(F^{14})^n}{(F_e^{14})^n} - \frac{(F^T)^n}{(F_e^T)^n} \right] \right\}}{\sum_{n=1}^{\infty} \left\{ \frac{1}{n+1} \frac{(F^T)^n}{(F_e^T)^n} \right\}} \qquad (8)$$

The experiment is performed by removing aliquots of the reaction mixture at predetermined times. These aliquots are then treated to separate the fumaric from the

l-malic acid. The integrated enzyme concentration terms must be equal since they represent the amount of free enzyme in the same solution. Since the aliquots removed from the reaction mixture contain both forms of labeled substrate, the ratio of concentrations reduce to molar ratios. The moles of carbon-14 containing compound and of tritium containing compound are proportional to the decompositions per minute of carbon-14 and tritium respectively. Inspection of equation 11 shows that these proportionality factors will cancel. Thus we are left with ratios of decompositions per minute of carbon-14 and tritium in fumaric acid at some time other than $t = o$ and l-malic acid at time $t = o$. The ratio of summation terms in equation 11 represents the error associated with this method. A small isotope effect will be compensated for by a small error as the difference term of the error will approach zero. The error will always be small since the first term of the denominator is always unity and the first term of the numerator is some fraction less than one-half. The error is least near the beginning of the reaction. If one is measuring a small isotope effect and takes aliquots near the beginning of the reaction the error should be negligible. Assuming that the carbon-14 isotope effect is very small and using the previous

facts, equation 8 reduces to equation 9.

$$\frac{k_f^H}{k_f^T} = \frac{D^F_{C^{14}}}{D^F_T} \frac{D_T^{M_o}}{D^{M_o}_{C^{14}}} \qquad (9)$$

where

$D^F_{C^{14}}$ = decompositions per minute from carbon-14 in fumaric acid.

D^F_T = decompositions per minute from tritium in fumaric acid.

$D^{M_o}_{C^{14}}$ = decompositions per minute from carbon-14 in l-malic acid at t = o.

$D_T^{M_o}$ = decompositions per minute from tritium in l-malic acid at t = o.

Liquid scintillation counters are available which allow the determination of separate activities of tritium and carbon-14 even when the two isotopes are mixed in the same sample. Beta particles emitted by carbon-14 decay are more energetic than those particles emitted by tritium decay. The liquid scintillation counter has two channels of differing energy which register the number of emitted beta particles from the sample. Each channel only registers those beta particles whose energy falls within its lower and upper energy limits. It is necessary to count a carbon-14 standard and then a tritium standard. Both standards are added

directly to the sample. The activities of the standards are determined by taking the difference between the counts per minute before and after adding the standard. The counts per minute for each channel are given by equations 10 and 11.

$$N_1 = D_{C^{14}}\, c_1 + D_T\, h_1 \qquad (10)$$

$$N_2 = D_{C^{14}}\, c_2 + D_T\, h_2 \qquad (11)$$

where

N_1 = cpm recorded in channel 1

N_2 = cpm recorded in channel 2

$D_{C^{14}}$ = dpm of carbon-14 in the sample

D_T = dpm of tritium in the sample

$c_1 = \dfrac{\text{cpm of the carbon-14 standard in channel 1}}{\text{dpm of the carbon-14 standard}}$

$c_2 = \dfrac{\text{cpm of the carbon-14 standard in channel 1}}{\text{dpm of the carbon-14 standard}}$

$h_1 = \dfrac{\text{cpm of the tritium standard in channel 1}}{\text{dpm of the tritium standard}}$

$h_2 = \dfrac{\text{cpm of the tritium standard in channel 2}}{\text{dpm of the tritium standard.}}$

Simple algebraic manipulation of equations 10 and 11 results in equation 12.

$$\frac{D_{C^{14}}}{D_T} = \frac{N_1\, h_2 - N_2\, h_1}{N_2\, c_1 - N_1\, c_2} \qquad (12)$$

Substitution of equation 12 into equation 9 yields equation 13 if the same amount of tritium standard and of carbon-14 standard is used in both the malic acid and the fumaric acid samples. This condition is necessary to make sure that the decompositions per minute from the tritium standard in the malic acid sample will equal the decompositions per minute from the tritium standard in the fumaric acid sample and that the same equality applies for the carbon-14 standard. When this condition holds it is unnecessary to know the absolute activity of the standards since the number of decompositions per minute will cancel.

$$\frac{k^H}{k^T} = \frac{N_1^F h_2' - N_2^F h_1'}{N_2^F c_1' - N_1^F c_2'} \cdot \frac{N_2^{M_o} c_1'' - N_1^{M_o} c_2''}{N_1^{M_o} h_2'' - N_2^{M_o} h_1''} \qquad (13)$$

where

k^H = rate constant for the forward reaction of the unlabeled l-malic acid.

k^T = rate constant for the forward reaction of the tritium labeled l-malic acid.

N_1^F = counts per minute from the labeled fumaric acid in channel 1.

N_2^F = counts per minute from the labeled fumaric acid in channel 2.

$N_1^{M_o}$ = counts per minute from the labeled l-malic acid (t = o) in channel 1.

$N_2^{M_o}$ = counts per minute from the labeled l-malic acid (t = o) in channel 2.

h_1' = counts per minute from the tritium standard for the fumaric acid sample in channel 1.

h_2' = counts per minute from the tritium standard for the fumaric acid sample in channel 2.

h_1'' = counts per minute from the tritium standard for the l-malic acid sample in channel 1.

h_2'' = counts per minute from the tritium standard for the l-malic acid sample in channel 2.

c_1' = counts per minute from the carbon-14 standard for the fumaric acid sample in channel 1.

c_2' = counts per minute from the carbon-14 standard for the fumaric acid sample in channel 2.

c_1'' = counts per minute from the carbon-14 standard for the l-malic acid sample in channel 1.

c_2'' = counts per minute from the carbon-14 standard

for the l-malic acid sample in channel 2.

This method of determining isotope effects on rates in enzymatic reactions should be quite accurate. Probably the greatest source of error in determining the deuterium isotope effect was in the error in knowing the exact substrate concentration and enzyme concentration. This method does not have this problem as none of these concentration terms need to be known. Unfortunately sixteen numbers must be determined to evaluate each ratio of rates. A small error in each number could cause a rather large error in the value of the rate ratio. This disadvantage may, however, be partially circumvented by taking a large number of counts per minutes.

3. Results and Discussion

A. Kinetic Analysis of the Primary Deuterium Isotope Effect

Mechanism 5 of Table I is clearly excluded by the absence of a primary isotope effect for 2-deuterio-2-hydroxy-succinic-2(R)-3(s) acid. This concerted case has only two intermediates.

$$E + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EF \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + F$$

where

E = enzyme

M = l-malic acid

EM = enzyme--l-malic acid complex

EF = enzyme--fumaric acid complex

F = fumaric acid

The interconversion of fumaric acid and malic acid would be a one step abstraction of a water molecule. The steady-state analysis of this mechanism gives an equation of the same form as equation 1 except that V_M is less complex (12)

$$V_M = \frac{k_2 k_3 (E_o)}{k_2 + k_{-2} + k_3} \qquad (14)$$

Alberty (12) has definitely shown that k_3 is larger than $k_2 + k_{-2}$ if this mechanism is followed by the reaction. Therefore equation 14 reduced to equation 15.

$$V_M = k_2 (E_o) \quad (15)$$

The isotope effect which is measured by comparing V_M^D (V_M for the deuterio-l-malic acid) and V_M^H (V_M for the unlabeled (acid) will be given by equation 16.

$$V_M^H/V_M^D = k_2^H/k_2^D \quad (16)$$

Processes which involve the breaking of a carbon-hydrogen bond usually exhibit a hydrogen to deuterium rate constant ratio of from two to eight. Since a ratio of such a large magnitude was not found, the concerted mechanism-mechanism 5--was eliminated.

The remaining mechanisms--1, 2, 3 and 4--all involve three enzyme complexes. Equation 1 has been derived for such a mechanistic picture. The labeling of the rate constants in Table I correspond to the appropriate steps in the mechanism for which equation 1 is derived. Contrary to the discussion by Alberty we will only consider the forward steps (k_2 and k_3) as fast or slow compared with each other and make no assumption about the reverse constants (k_{-2} and k_{-3}).

If the rate constant k_4 is large compared to any rate constant involving a bond breaking or bond formation step, then equation 3c reduces to equation 17. This condition assumes that the dissociation of an enzyme-substrate complex is rapid compared with carbon-

hydrogen on carbon-oxygen bond forming and breaking steps.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \quad (17)$$

For mechanisms 1 and 3 of Table I the rate constant k_3 is considered to be large compared with the rate constant k_2. Under this condition equation 17 reduces to equation 18.

$$k_{cat} = \frac{k_2 k_3}{k_{-2} + k_3} \quad (18)$$

Two possibilities now exist. If the rate constant k_{-2} is large compared to k_3, the equation 18 reduces to equation 19.

$$k_{cat} = \frac{k_2}{k_{-2}} k_3 \quad (19)$$

If the constant k_3 is large compared to k_{-2} then equation 18 reduces to equation 20.

$$k_{cat} = k_2 \quad (20)$$

For mechanisms 2 and 4 of Table I the rate constant k_2 is considered to be large compared to the constant k_3 which allows one to reduce equation 17 to equation 21.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2}} \quad (21)$$

Again two possibilities exist. If the rate constant

k_{-2} is large compared to the rate constant k_2, then equation 21 reduces to equation 22.

$$k_{cat} = \frac{k_2}{k_{-2}} k_3 \quad (22)$$

If the rate constant k_2 is large compared with k_{-2}, then equation 21 reduces to equation 23.

$$k_{cat} = k_3 \quad (23)$$

The case where the rate constant k_2 is larger than k_{-2} has a physical significance which implies an unreasonable energy for the intermediate relative to the enzyme-substrate complex. This assumption indicates that the intermediate is of lower energy than the enzyme--l-malic acid complex. This situation seems unlikely as the intermediates are proposed to be rather unstable carbonium ion or anion species. Of course, interaction of such species with the enzyme could so stabilize these intermediates that they might conceivably represent a minimum on the energy surface of the reaction. In the subsequent analysis, this condition as applied to mechanism 2 is eliminated by the absence of a significant primary isotope effect. However, the experimental evidence available does not exclude it in the case of mechanism 4.

Mechanism 1 will now be considered in view of equations 19 and 20 and the lack of a primary isotope

effect.

If the conditions of equation 19 hold, then the isotope effect will be given by equation 24.

$$\frac{k^H}{k^D} = \frac{k_2^H}{k_2^D}\,\frac{k_{-2}^D}{k_{-2}^H}\,\frac{k_3^H}{k_3^D} = \frac{K_{2eq}^H}{K_{2eq}^D}\,\frac{k_3^H}{k_3^D} \qquad (24)$$

The ratio of K_{2eq}^H/K_{2eq}^D may take on values of from 0.8(20) to 1.15(2). The process represented by the constant k_3 is a carbon-hydrogen bond breaking process and the ratio k_3^H/k_3^D should as a result be at least 2 or greater. Therefore k^H/k^D should be 1.6 or greater. Absence of such a large effect eliminates this mechanistic possibility. If mechanism 1 holds under the conditions of equation 20, then the isotope effect is given by equation 25.

$$\frac{k^H}{k^D} = \frac{k_2^H}{k_2^D} \qquad (25)$$

The rate process represented by k_2 does not involve the rupture of a carbon-hydrogen bond. Any isotope effect exhibited here will be due to the effect of the isotope on the stability of the carbonium ion. Such effects are usually in the range of 10% to 20% (14). This case is thus a distinct possibility.

Mechanism 2 will now be considered in view of equations 22 and 23 and the lack of a primary isotope

effect. If the conditions of equation 22 are valid, then the isotope effect is given by equation 26.

$$\frac{k^H}{k^D} = \frac{k_2^H}{k_{-2}^H}\frac{k_{-2}^D}{k_2^D}\frac{k_3^H}{k_3^D} = \frac{K_{2eq}^H}{K_{2eq}^D}\frac{k_3^H}{k_3^D} \qquad (26)$$

As before K_{2eq}^H/K_{2eq}^D is close to unity and k_3^H/k_3^D is two or greater as a carbon-hydrogen bond is being ruptured. Absence of a large isotope effect excludes this possibility. If the conditions for equation 23 are valid, then the isotope effect for mechanism 2 is given by equation 27.

$$\frac{k^H}{k^D} = \frac{k_3^H}{k_3^D} \qquad (27)$$

The absence of a large primary isotope effect clearly excludes this possibility.

The equations defining the isotope effect for <u>mechanism 3</u> are identical to those for mechanism 1.

$$\frac{k^H}{k^D} = \frac{K_{2eq}^H}{K_{2eq}^D}\frac{k_3^H}{k_3^D} \qquad (24)$$

$$\frac{k^H}{k^D} = \frac{k_2^H}{k_2^D} \qquad (25)$$

If the conditions of equation 24--$k_{-2} > k_3$, $k_3 > k_2$--hold for mechanism 3, then a small isotope effect will be seen. Again the ratio K_{eq}^H/K_{eq}^D is small and k_3^H/k_3^D is

near unity as this step represents a process for identical molecules (the deuterium was removed in the previous step). This mechanism is not excluded by the absence of a primary isotope effect. If the conditions of equation 25--$k_3 > k_2$, $k_3 > k_{-2}$--are valid, then a large isotope effect should be seen as k_2 represents a carbon-hydrogen bond breaking process. Again, as no primary isotope effect is observed, the conditions of equation 25 are excluded as a mechanistic possibility.

The equations defining the isotope effect for mechanism 4 are identical to those of mechanism 2.

$$\frac{k^H}{k^D} = \frac{K^H_{2eq}}{K^D_{2eq}} \frac{k^H_3}{k^D_3} \qquad (26)$$

$$\frac{k^H}{k^D} = \frac{k^H_3}{k^D_3} \qquad (27)$$

If the conditions--$k_{-2} > k_3 > k_{+2}$--for equation 26 are valid, then a small primary isotope effect would be expected since again K^H_{2eq}/K^D_{2eq} will be near unity and k^H_3/k^D_3 will be near unity as no carbon-hydrogen bond is being broken. If the conditions--$k_2 > k_3$, $k_2 > k_{-2}$--for equation 27 are valid, then a very small primary isotope effect near unity will be observed.

Table II lists those mechanisms which are not excluded by the absence of a primary isotope effect.

Also those possibilities in which the improbable condition that k_2 is larger than k_{-2} are omitted from Table II. (see page 22).

Table II

Mechanism	Relation of Rate Constants	Mathematical Expression of the Isotope Effect
1	$k_3 > k_2$, $k_3 > k_{-2}$	k_2^H/k_2^D
3	$k_3 > k_2$, $k_{-2} > k_3$	$(K_{2eq}^H/K_{2eq}^D)(k_3^H/k_3^D)$
4	$k_{-2} > k_3$, $k_{-2} > k_2$	$(K_{2eq}^H/K_{2eq}^D)(k_3^H/k_3^D)$

If steps involved with the dissociation of the enzyme-substrate complexes are rapid compared with those steps involving bond breaking and bond formation and either k_3 or k_{-2} is large, then equation 4 reduces to equation 28.

$$K_M = \frac{k_{-1}}{k_1} \qquad (28)$$

The previously complex constant K_M is now reduced to a simple ratio which may be interpreted as the dissociation constant of the enzyme--l-malic acid complex. The association of l-malic acid with fumarase has been shown to be diffusion controlled (15). It seems likely that the substitution of deuterium for hydrogen would not effect this rate constant, and several mono-, di-, tri-deuterio-l-malic acids have been found to have the same K_M values as their unlabeled analogues (10).

Therefore substitution of one or more hydrogens with deuterium or tritium in malic acid would appear in general not to change the dissociation constant of the acid.

The previous discussion was conducted under the assumption that the dissociation constant for the enzyme--fumaric acid complex was rapid compared to the processes involving covalent bonds. We will now consider the case where the dissociation of the enzyme-substrate complex is slow. If the rate constant k_4 is small compared to those rate constants representing bond formation or bond breaking steps, then equation 3 reduces to equation 29.

$$V_M = \frac{k_2 k_3 k_4}{k_2 k_3 + k_2 k_{-3} + k_{-2} k_{-3}} \qquad (29)$$

and equation 4 reduces to equation 30.

$$K_M = \frac{k_{-1} k_{-2} k_{-3}}{k_1 (k_2 k_3 + k_2 k_{-3} + k_{-2} k_{-3})} \qquad (30)$$

Inspection of equations 29 and 30 show that a large primary isotope effect will never be observed for either kinetic constant. If any one of the terms of the summations in the denominators of equations 29 and 30 is larger than the other two terms, then V_M and K_M will either be the function of the dissociation constants or of equilibrium constants. We have already seen that

the dissociation constants will probably not change on isotopic substitution and that the ratio of equilibrium constants for labeled and unlabeled materials is near to unity. Determination of the secondary isotope effect may be able to eliminate the possibility of k_4 being small. If k_2 k_3 is larger than the sum of the other two terms, then no isotope effect would be expected for V_M^H/V_M^D but a secondary isotope effect would be expected for K_M^H/K_M^D. If k_2 k_{-3} is large, then a small secondary isotope effect would be expected for both the ratios of the maximal velocities and the Michaelis constants. If $k_{-2}k_{-3}$ is the predominate term in the summation $k_2k_3 + k_2k_{-3} + k_{-2}k_{-3}$, then the ratio V_M^H/V_M^D should show a small isotope effect and K_M^H/K_M^D should be equal to unity. We shall return to this question later (see discussion).

B. Secondary Isotope Effect for 2-Deuterio-L-Malic Acid

The compound 2-deuterio-D,L-malic acid was prepared by the reduction of oxaloacetic acid with sodium borodeuteride. The resulting racemic malic acid was resolved by resolution with cinchonine. The 2-deuterio-l-malic acid was determined to be optically pure by melting point and by enzymatic means. The kinetic constants as defined in equation 2 were determined by least squares at pH 7.3, 27°C, and in a 10mM sodium phosphate buffer solution. The substrate concentration

was varied from 3mM to 10mM l-malic acid. Twelve points in all were used in determining the constants for each substrate.

$$K_M^H = 3.9 \pm 0.1$$

$$K_M^D = 3.9 \pm 0.1$$

$$V_M^H/V_M^D = 0.98 \pm 0.10$$

The large error associated with the ratio of the maximum initial velocities is attributable to the error in each of the extinction coefficients. Therefore, this analysis can only put limits on the isotope effect of less than 10%. The error values associated with each number are at the 68% confidence level.

The previous kinetic analysis predicted that the Michaelis constants would be equivalent if the dissociation of the enzyme-substrate complexes were rapid compared with the bond breaking and bond formation steps. Also the Michaelis constants were predicted to be equal under certain conditions if the complex breakdowns were slow compared to steps involving covalent bonds. For 2-deuterio-l-malic acid the Michaelis constants are equal to within the experimental error which supports the theory that the enzyme-substrate dissociation is rapid compared to the processes involving covalent bonds.

C. Tritium Isotope Effect for L-Malic-2-T-1,4-C^{14} Acid

The compound l-malic-2-T-1,4-C^{14} acid was prepared by the reduction of oxaloacetic acid with sodium-boro-hydride-T and resolved as the chinchonine salt. L-malic-1,4-C^{14} acid was prepared by the enzymatic hydration of fumaric-1,4-C^{14} acid. The two labeled acids were then dissolved in ethyl acetate and l-malic-2-T-1,4-C^{14} acid was crystalyzed from the ethyl acetate solution by the addition of ligroin (60-70°). The labeled acid was shown to be pure by melting point, mixed melting point, thin layer chromatography, and column chromatography. The optical purity was determined by melting point and mixed melting point.

The l-malic-2-T-1,4-C^{14} acid was dissolved in a 10mM sodium phosphate buffer solution and the whole titrated to pH 7.3. The final concentration of labeled l-malic acid was 2.2mM. Aliquots were withdrawn from the reaction mixture after addition of enzyme (final concentration $1.8X10^{-8}$mM) and before the reaction reached 10% of equilibrium. The aliquots were quenched by the addition of an equal volume of boiling ethanol. The samples were lyophilized and the acids were separated by thin layer chromotography. The silica gel containing the desired labeled acid was then scrapped into a toluene counting solution containing a suspending agent. A

carbon-14 toluene solution and a tritium toluene solution were used as standards. All samples were counted in a Packard Scintillation Counter. At least 500,000 counts were recorded for each sample. The experiment was done twice with the values from each experiment falling within the experimental error of the other run.

The isotope effect determined by this method and defined in section II-b was

$$k_H/k_T = 1.15 \pm 0.07$$

The error is at the 68% confidence level. The rather large error in the above result probably results from a fault in the experimental procedure. The counting was accomplished by suspending the silica gel which contained the labeled acid in a toluene counting solution. When standards were added to the suspension it was necessary to shake the suspension. Undoubtedly the amount of silica gel adhering to the tops of the vials was not constant. Since the standards were determined by difference, a change in the amount of labeled acid in the suspension would cause an error. The suspension counting method was to be discarded if the acids could be made soluble in toluene and the acids could be eluted from the silica gel. The acids were soluble in

toluene if they were first converted to their "Hyamine" salts by first dissolving the acids in "Hyamine hydroxide" (Packard Instruments). Unfortunately after much effort no satisfactory method for eluting fumaric acid from silica gel was developed.

The rate constants being compared by the determination of this tritium isotope effect are defined by equation 6a. Comparison of equations 3c and 4 with equation 6a, shows that the rate constant derived for the tritium isotope effect is just the ratio of the catalytic constant, k_{cat}, to the Michaelis constant.

$$k_T = \frac{k^T_{cat}}{K^T_M}$$

The isotope effect that was found for the doubly labeled l-malic acid is given by equation 31.

$$\frac{k^H}{k^T} = \frac{K^T_M}{K^H_M} \frac{k^H_{cat}}{k^T_{cat}} \qquad (31)$$

It was shown earlier that the binding constants for the 2-deuterio-l-malic acid and unlabeled acid were equal which supports the assumption that the binding constants for the tritiated l-malic acid and the unlabeled acid are also equal. If the isotopic labeling of one hydrogen has no effect on the Michaelis constants, then equation 31 reduces to equation 32.

$$\frac{k^H}{k^T} = \frac{k^H_{cat}}{k^T_{cat}} \qquad (32)$$

The mathematical expressions for the isotope effects as shown in Table II are valid for the tritium case except that the superscripts designating deuterium should now represent tritium.

The advantages of determining the secondary isotope effect using tritium rather than the deuterium are two. Tritium can be expected to give a much larger isotope effect than deuterium and the detection method for tritium is much more facile and accurate than for deuterium.

D. Discussion

If one assumes that dissociation of the enzyme-fumaric acid complex is rapid compared to the formation or breaking of covalent bonds, then the secondary isotope effects may be interpreted on the basis of the mathematical expressions of Table II.

For mechanism 1 the ratio k_2^H/k_2^T measures the effect which the isotope has on the formation of the carbonium ion. For such an α-secondary isotope effect (effect of an isotope when it is directly bonded to the carbon at which reaction occurs) the ratio k_2^H/k_2^T is a function of the amount of sp^2 character developed in the transition

state (see following discussion) and is invariably greater than unity (14).

For mechanisms 3 and 4 the mathematical expression for the isotope effect in 1-malic-2-T-1,4-C^{14} is given by:

$$\frac{k^4}{k^T} = \frac{K^H_{2eq}}{K^T_{2eq}} \quad \frac{k^H_3}{k^T_3}$$

In this expression the equilibrium constant measures the relative ease of ionization of the hydrogen at C-3 in the 1-malic acid-enzyme complex. Substitution of tritium for hydrogen will tend to stabilize a neighboring anion. For example, acetic-d_3 acid, phenylacetic-α,α-d_2 acid, and benzyl-α,α-d_2-ammonium ion are less acidic by 10-13% than their undeuterated analogs (29). This decrease in acidity on isotopic substitution has been attributed to the fact that C-D bonds are shorter than the C-H bonds. This shorter bond length may cause a greater electron density about the carbon in the deuterated substances which will destabilize adjacent anions. Streitwieser (14) feels that the decrease in acidity may be explained by changes in the vibrational partition functions, but in any case the anion will be destabilized. Tritium will have a greater destabilizing effect than deuterium. As a result K^H_{2eq}/K^T_{2eq} will be greater than

unity. The ratio of rate constants, k_3^H/k_3^T, should also be greater than unity since the major change for this step in mechanisms 3 or 4 is the conversion of a sp^3 to a sp^2 configuration at C-2. Since both the ratio of equilibrium constants and the ratio of rate constants should be larger than unity, the ratio k^H/k^T for mechanisms 3 and 4 should be greater than unity.

Previous discussion indicated that K_M^H/K_M^D could be expected to be unity if the dissociation of the enzyme-substrate complex was rapid relative to any process involving bond breaking or formation. Thus the results of $k^H/k^T = 1.15$ and $K_M^H/K_M^D = 1$ are consistent with these predictions. Moreover, since the secondary isotope effects for tritium are consistent with this kinetic analysis, it seems reasonable that a primary isotope effect would in fact have been observed if the carbon-hydrogen bond between C-3 and the 2-S hydrogen were broken in the rate-determining step.

Of course the possibility of a slow dissociation of the enzyme-fumaric acid complex has been not entirely eliminated by the determination of these isotope effects. The previous kinetic analysis showed that if k_4 is small compared to those rate constants governing the making or breaking of covalent bonds and if $k_{-2}k_{-3}$ is larger than $k_2k_3 + k_2k_{-3}$, then one would expect to observe an isotope

effect for the maximum velocity and no isotope effect on the Michaelis constant which were the results of this study. The association of l-malic acid and fumaric acid with the enzyme proceed at rates which are diffusion controlled (12, 15). Consequently, the dissociation of these species is likely to be rapid compared to bond breaking or making processes and the assumptions above seem reasonable.

Thus we conclude that the secondary isotope effect for tritium determined in these experiments is consistent with either a carbonium ion mechanism (mechanism 1) or a mechanism involving formation of an intermediate anion (mechanism 3 or 4). The isotope effect will give information about the nature of the transition state whichever mechanism is operative.

If the reaction proceeds via a carbonium ion mechanism, then k^H/k^T measures the effect of an α-tritium on the formation of a carbonium ion. Solvolysis of cyclopentyl-1-d tosylate in dry acetic acid containing equivalent amounts of sodium acetate gave a deuterium isotope effect of $k^H/k^D = 1.15$ (14). According to the theory of Streitweiser (14), the kinetic isotope effect of a secondary α-deuterium can be largely ascribed to a decrease in the bending force constant for a C-H bond when a tetrahedral C-H bending vibration present in

the reactant is converted in the transition state leading to the carbonium ion to an out-of-plane bending motion which generally has a lower frequency. Streitweiser further suggested that the out-of-plane C-H vibration in the transition state can be impeded by the proximity of the leaving or entering group; the closer these groups are to the developing carbonium ion, the stiffer will be the out-of-plane C-H bending frequency and the smaller will be the resultant isotope effect. For an isolated carbonium ion k^H/k^D has been calculated to be about 1.4 per α-deuterium atom. In a limiting S_N1 solvolysis, such as the acetolysis of the cyclopentyl-1-d tosylate, the fact that the observed isotope effect $k^H/k^D = 1.15$ is considerably less than the maximum calculated $k^H/k^D = 1.4$ may be attributed to this type of influence by nearby leaving groups. For example, in direct S_N2 displacements, the presence of both entering and leaving groups in the transition state so restricts the out-of-plane vibration of the carbon hydrogen bond that the isotope effect virtually disappears.

For k_H/k_T the calculated maximum isotope effect for isolated carbonium ion in the transition state is 1.6. This calculation was made using the model of Streitweiser (14) for the deuterium isotope effect and the assumption

that $v_H = v_T/1.6$. The number 1.6 was chosen rather than the square-root of three because the reduced mass of the compound is not exactly equal to the mass of tritium and because of possible anharmonicity. An analogous approximation is made in calculating the maximum deuterium isotope effect (14).

The small isotope effect in the dehydration of l-malic acid to fumaric acid for α-secondary deuterium (less than $k_H/k_D = 1.1$) and the low isotope effect for α-secondary tritium ($k_H/k_T = 1.15$) implies that very little sp^2 character has developed in the transition state at C-2. Two explanations can be advanced to account for this result. One possibility is that the hydroxyl group which is lost in forming the carbonium ion may not be appreciably removed in the transition state. An alternative explanation is that the carbonium ion is stabilized by some group furnished by the enzyme which damps the out-of-plane vibrations of the hydrogen at C-2 in the transition state. In either case, the transition state has developed little sp^2 character.

If, on the other hand, the reaction proceeds with preliminary rapid reversible formation of a carbanion the isotope effect is given by $k^H/k^T = (K^H_{2eq}/K^T_{2eq})(k^H_3/k^T_3)$. It was pointed out earlier that the ratio

K^H_{2eq}/K^T_{eq} is likely to be larger than one because tritium will destabilize the anion. The ratio k^H_3/k^T_3 measures the effect of the isotope on the conversion of the anion to the olefin. A somewhat similar process may be involved in the Hofmann elimination of trimethyl-propyl-α-T-ammonium hydroxide which exhibits an isotope effect of $k_H/k_T = 1.10$ (18). This small secondary isotope effect observed in the Hofmann elimination was interpreted as being due to a transition state which has developed little sp^2 character. Consequently, if the enzyme catalyzed reaction proceeds via an anion mechanism, one may predict that there is little sp^2 character at C-2 in the transition state from the anion-enzyme complex to the fumaric acid-enzyme complex.

The isotope effect does not unambiguously distinguish between a carbonium ion and an anion mechanism for the conversion of malic acid to fumaric acid. However, three experiments have been conducted which argue against the possibility of intermediate formation of an anion. The experiments each give negative results and although inconsistent with the anion mechanism they are not conclusive for the reasons given.

If the formation of an anion is rapid and reversible relative to elimination of the hydroxyl group, then deuterium from deuterium oxide solvent might be

incorporated more rapidly into l-malic acid than would be accounted for by hydration of fumaric acid in the reverse reaction. In an experiment to test this question Alberty found that all the deuterium present in the l-malic acid after a short reaction time with fumarase could be accounted for by the hydration of fumaric acid in the presence of the deuterium oxide solvent. There was thus no exchange of hydrogen at C-3 of malic acid without intermediate formation of fumaric acid. On this basis mechanism 4 was rejected in favor of mechanism 1. However, the possibility surely exists that the hydrogen abstracted from the malic acid may be bound to the enzyme and unable to exchange with the solvent as long as the substrate intermediate is bound at the active site of the enzyme. If this possibility exists, rapid reversible formation of the anion could be the first step in the reaction and one would see no isotopic exchange into C-3 of malic acid.

A further possible argument against intermediate formation of an anion comes from the fact that the dianionic forms of the substrates are necessary for the formation of the enzyme substrate complex (21). For example, the mono-and di-methyl esters of fumaric and malic acids are neither substrates nor competitive inhibitors. The absence of inhibition by these esters

indicates that the dianionic forms of the acids are necessary for binding at the active site. Thus, any mechanism which involves formation of intermediate anionic character at C-3 (mechanisms 3 and 4 of Table I) seems unlikely as it would result in an intermediate with three negative charges. Formation of such species may occur but only under rather vigorous conditions. For example, hydrogens at C-3 of malic acid exchange completely after nineteen hours at 90° in 0.5 M sodium deuteroxide (22). This base catalyzed exchange requires the formation of an intermediate anion at C-3 but the conditions for this exchange are much more vigorous than those characterizing the fumarase reaction which occurs at room temperature, and pH 7.3. However, the presence at the active site of the enzyme of positively charged groups may stabilize species with negative charges and, in so doing, facilitate the formation of carbanion character at C-3 so this argument, though persuasive is not conclusive.

A third possible argument against the carbanion mechanism is the finding that DL-β-fluoromalic acid is not a substrate for fumarase (30) although it is a competitive inhibitor and thus probably binds at the active site of the enzyme. The electron withdrawing fluorine at C-3 should make the hydrogen attached to

this carbon more acidic and should thus stabilize an intermediate anion and, as a result, should facilitate a mechanism which involves intermediate formation of an anion at C-3. On the other hand the presence of the electron withdrawing fluorine at C-3 would destabilize a carbonium ion at C-2 and tend to retard the reaction if it proceeds via a carbonium ion mechanism. Thus the failure of DL-β-fluoromalate to be a substrate argues against a mechanism which involves intermediate anion formation. Unfortunately, the stereochemistry of the fluorine atom in the substrate DL-β-fluoromalic acid is not known with certainty and, in fact, the fluorine may occupy the position of the hydrogen which is always removed in the dehydration reaction. As a result, failure to observe reaction of this substrate must be treated with great caution and the stereochemistry of the DL-β-fluoromalate should be determined. Another interesting possibility is the use of fluorofumaric acid as a substrate. The hydration of double bonds in nonenzymatic systems has been extensively studied and results obtained in these systems are useful in the discussion of the enzymatic hydration catalyzed by fumarase.

Taft (23) has presented evidence that the hydration of isobutene in both dilute nitric acid and dilute

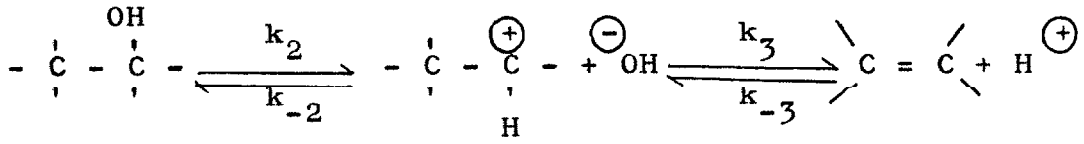
sulfuric acid proceeds through a transition state which contains only isobutene plus a proton that is "free-carbonium ion", the formation of which is the rate-determining step. This mechanism appears to be in contradiction with mechanism 1 which predicts that a water molecule is bound in the rate-determining transition state.

Possibly more applicable to the case of fumarase are the hydrations of trans-crotonaldehyde (24) and β, β-dimethylacrolein (25). These reactions, as well as the acid catalyzed hydration of fumaric acid (26), have been shown to proceed with a water molecule bound in the transition state which is consistent with mechanism 1. However, the role of the enzyme in determining the reaction mechanism is not known, and the enzyme mechanism may not be the same as the solution mechanism.

A similar mechanism has been proposed for the reversible enzymatic hydration of cis-aconitic acid to citric acid and isocitric acid by aconitase (27). This reaction shows the absence of a large primary isotope effect for dehydration although the secondary isotope effects are not determined.

The evidence now available would appear to favor the carbonium ion mechanism-mechanism 1 - for the reversible dehydration of l-malic acid by fumarase. This

mechanism is pictured below:

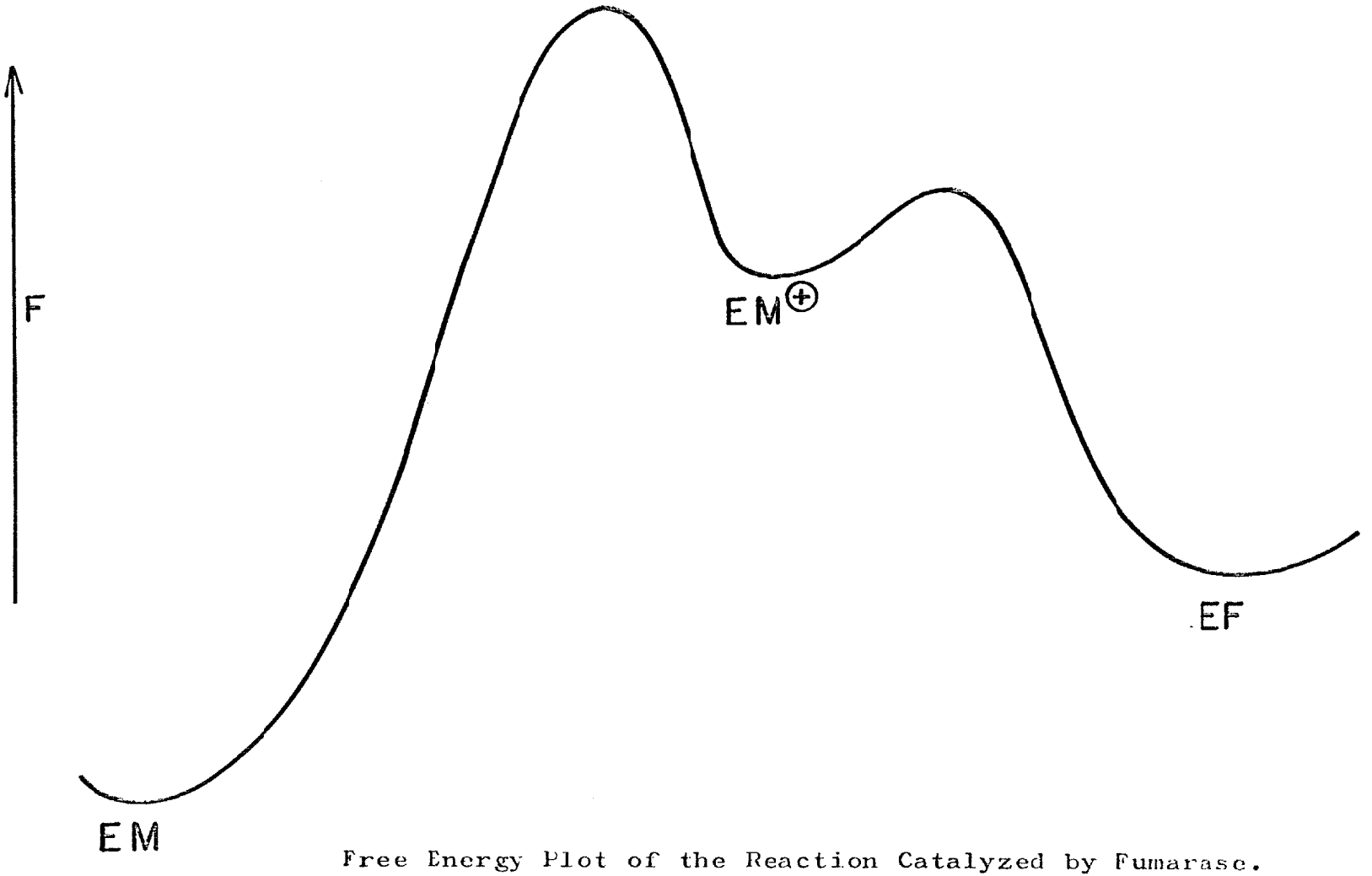


where

$$k_2 < k_3$$

$$k_{-2} < k_3$$

The energy relations of the intermediates are shown in the following diagram. Furthermore, the α-secondary isotope effect indicates that little sp^2 character has developed on the C-2 carbon during the rate-determining transition state.



Free Energy Plot of the Reaction Catalyzed by Fumarase.

4. Experimental

2-Deuterio-D, L-Malic Acid

Oxalacetic acid (5.5g., 41.5 mmoles, Cal-Biochem. Corp.) was dissolved in 50ml. of water and the pH adjusted with cooling to the phenolphthalein end point with sodium hydroxide (1 N). Sodium borodeuteride (0.5g., 48.0 milliequivalents, Alfa Corp.) was added with stirring to the ice-cold solution. After stirring for two days at room temperature, the mixture was neutralized with hydrochloric acid (1 N). The solution was again titrated to the phenolphthalein end point with sodium hydroxide (1 N) and a solution of calcium chloride (11g., 70 mmoles) was added to the stirring solution. After one hour at room temperature, the solution was placed at 0° overnight. The precipitate was filtered and then dissolved in the least amount of hydrochloric acid (1 N). The acidic solution was passed through an ion exchange column (IR-120-H), and the eluate was collected and lyophilized.

Yield: 4.1g. (78%)

M.P.: 132 - 134°

2-Deuterio-L-Malic Acid

The crude product from the previous procedure was dissolved in boiling methanol (40ml.) and cinchonine (9g., equimolar amount) was added to the boiling solu-

tion (32) After sitting at 0° overnight, the precipitate was filtered and recrystallized again from methanol (20ml.).

The crystals were dissolved in the least amount of hot water and concentrated ammonium hydroxide was added until no further precipitate formed. The resulting mixture was filtered and the filtrate passed through an ion exchange column (IR-120-H). The collected eluate was lyophilited, and the resulting 2-deuterio-l-malic acid was recrystallized from ethyl acetate and ligroin (60-70).

Yield: 1.6g. (80%)

M.P.: 101 -102° (corr.)

Optical Parity: Melting point, mixed melting point, enzymatically indistinguishable from l-malic acid.

N. M. R. Spectrum:

l-malic acid (0.5g./0.5ml. of deuterium oxide)

triplet 1.1ppm from water

doublet 3.1ppm from water

2-deuterio-l-malic acid (0.5g./0.5ml. of deuterium oxide)

singlet 3.1ppm from water

less than 1% hydrogen at C-2

Carbon-13 Spectrum

2-deuterio-l-malic acid (0.5g./0.5ml. of deuterium oxide)

Carbon	Chemical Shift from an External Carbon Disulfide Standard	Coupling Constant	Triplet
1	22.8 ppm	JcccH = 3.5	1:2:1
2	131.6 ppm	JcD = 22	1:1:1
3	160.7 ppm	JcH = 132	1:2:1
4	24.4 ppm	JccH = 6.4	1:2:1

Sodium Borohydride-T

Diglyme (1ℓ.), dimethylether of triethylene glycol, was stirred for one hour with calcium hydride (10g.) The mixture was distilled and the fraction boiling at 160.2° was collected. The distillate was then redistilled from lithium aluminum hydride (5g.) under reduced pressure.

Sodium borohydride (5g.) was added to some freshly distilled diglyme (60ml.) in a dry three-necked flask with a stirrer, a nitrogen inlet, and a nitrogen outlet. The mixture was stirred for thirty minutes at 60° until all the solid had dissolved except for a small residue. The stirring was stopped, the solid allowed to settle, and the supernatent liquid forced with nitrogen through a sintered glass funnel into a dry flask. The residual solvent was separated from the solid by centrifugation and added to the bulk of the solution. The solution was left at zero degrees overnight, the solvent decanted, and the crystals put in a vacuum at 60° for four hours (33).

Freshly recrystallized sodium borohydride (5g.) was dissolved in purified diglyme (6ml.) at 50° and sodium borohydride-T (2.5 mc., Nuclear Chicago Corp.) was added. The solution was cooled and the solvent evaporated at 60° under vacuum.

D, L-Malic-2-T Acid

Sodium borohydride-T (0.22g., 0.006 moles) was added as a solid to a colled (0°) solution of oxaloacetic acid(2.112g., 0.016 moles, 1.5-fold excess of reducing agent) in water 20ml. which had been neutralized with sodium hydroxide (1 N) to the phenolphthalein end point. The stirred solution was allowed to warm to room temperature over a period of one hour. After stirring at room temperature for twelve hours, hydrochloric acid (1 N) was added until no more hydrogen was evolved. The resulting solution was titrated with sodium hydroxide (1 N) to the phenolphthalein end point and calcium chloride (5.4g., 0.05 moles) was added. After stirring for one hour, the solution was placed at 0° overnight. The precipitate was filtered and dissolved in hydrochloric acid (1 N). The acidic solution was passed through an ion exchange column, (IR-120-H) collected, and lyophilized.

Yield: 1.65g. (80%)

M.P.: 132 - 133°

L-Malic-2-T Acid

D, L-malic-2-T acid (1.65g., 0.013 moles) was dissolved in boiling methanol (16.5ml.) and cinchonine (3.63g., 0.013 moles) was added. After standing a 0° overnight, the mixture was filtered, and the precipitate was recrystallized from methanol (7ml.). The cinchonine salt was dissolved in 50ml. of hot water, and concentrated ammonium hydroxide (1ml.) was added to the solution. The precipitate was filtered and the filtrate passed through an ion exchange column (IR-120-H). The eluate was then lyophilized, and the residue recrystallized from ethyl acetate-ligroin (60-70).

Yield: 0.630g. (63% overall)

M.P.: 101 - 102° (corr.)

L-Malic-1, 4-C^{14} Acid

Fumaric-1, 4-C^{14} acid (0.232g., 0.002 moles, 9mc) was dissolved in 50 mM sodium phosphate buffer (10ml.) at pH 7.3 at 27°. Sodium carbonate (0.212g., 0.002 moles) was added and, after bubbles ceased to be evolved, fumarase (0.01mg.) was added. After one hour the solution was removed and lyophilized.

Liquid-liquid partition chromatography was used to separate the l-malic acid from fumaric acid (34). Celite was heated at 100° in hydrochloric acid (6 N), filtered, washed with water until the washings were neutral, and

dried at 100° overnight. A 35% n-butanol - 65% chloroform solution was equilibrated with a hydrochloric acid solution (0.5 N) by shaking them together for ten hours. Celite and hydrochloric acid (0.5 N) were mixed in equivalent amounts (w/w) until the solid appeared slightly damp. The celite was suspended in the butanol-chloroform mixture and put on a column in small portions. The column was packed with the aid of a glass rod to remove air bubbles and tamp the celite.

The residue of malic and fumaric acid was dissolved in the least amount of hydrochloric acid solution (0.5 N, 1.5 ml.) possible and then a drop of concentrated hydrochloric acid was added. The solution was added to an equal weight of celite (1.5g.). The solid was then placed on a column (9g. celite) and the acids eluted with the butanol-chloroform mixture at a flow rate of 1 ml. per minute. Ten milliliter fractions were convenient and fumaric acid comes off in the first six fractions while malic acid comes at about the eighteenth fraction.

Yield: 0.10g. (50%)

M.P.: 102.5 - 103.5° (corr)

L-Malic-2-T-1, 4-C^{14} Acid

The compounds l-malic-2-T acid (0.100g., 0.7mmoles)

and l-malic-1, 4-C^{14} acid were mixed and dissolved in hot ethyl acetate (60°). Ligroin (60-70°) was added until the cloud point was reached, and the resulting solution was placed at 60° overnight. The crystals were filtered, washed with cold ethyl acetate-ligroin (50/50), and dried in a vacuum at 40°.

Yield: 0.150g., (75%)

M.P.: 101 - 102° (corr)

Deuterium Isotope Effect

The deuterium isotope effect was determined on a Cary-14 recording spectrophotometer with a thermostated cell holder at pH 7.3, 27°, and 10mM sodium phosphate buffer. A stock enzyme solution was kept at 0° until needed. The enzyme solution (0.3 ml.) was pipetted into a cuvette and allowed to come to temperature. The l-malic acid solution (2.7 ml.) already at the correct temperature was then added. The final enzyme concentration was 1.2×10^{-8} M. The solutions were mixed by capping the cuvette and inverting twice. All initial rate measurements were at 250 mμ. Three readings were taken at each l-malic acid concentration (11mM, 9mM, 6mM, 3mM). The data were analyzed by the method of Lineweaver and Burke (36).

Tritium Isotope Effect

The isotope effect for the reaction catalyzed by fumarase was conducted in sodium phosphate buffer (10mM) at pH 7.3, 27°, and with l-malic-2-T-l, 4-C^{14} acid (3mM). Aliquots were removed so that about 10,000 cpm could be recovered. All aliquots were removed before 10% of the equilibrium value was reached and were quenched by the addition of an equal amount of boiling ethanol. The quenched aliquots were lyophilized, and the resulting residue was spotted on a 0.25 mm or 0.4 mm thick silica gel thin layer plate (20 x 20 cm). The moving phase was ethanol(denatured)/water/25% ammonium hydroxide (100/12/16). The positions of the two acids were visualized by spraying the edge of the plate with a solution of 0.1g. of bromocresol green in 100 ml. of 95% ethanol. The areas containing the l-malic-2-T-l, 4-C^{14} acid and fumaric-2, 3-T-l, 4-C^{14} acid were scrapped into separate counting vials. Into each vial was introduced 20 ml. of counting solution from a stock counting solution containing (35):

150ml. of toluene
5.20g. Cab-o-Sil (Packard Instruments)
0.5g. PPO (Packard Instruments)
0.03g. dimethyl POPOP (Packard Instruments)

The experiment was conducted twice and the acid was purified by column chromotography and recrystallization before the second experiment. Control experiments

showed that the separation was complete and that the l-malic-2-T-l, 4-C^{14} acid contained no radioactive impurity which was present at the fumaric acid portions of the thin layer plate. Toluene-C^{14} and toluene-T were used as standards and a Packard Scintillation Counter was used to monitor the radioactive samples.

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PART II SALT EFFECTS ON FUMARASE

1. INTRODUCTION

The effect of inorganic ions on the activity of fumarase was noticed early in the history of the enzyme (1). The activation by phosphate of enzyme extracts from animal tissues has been shown by several workers (2, 3).

The first relatively complete study of the effect of inorganic anions on crystalline fumarase was performed by Massey (4) who found that the addition of phosphate profoundly alters the activity of the enzyme. With either malate or fumarate the pH-optimum is shifted by phosphate towards the alkaline and there is considerable activation over most of the pH range studied (pH 5-8.5). The maximum activity at pH 6.35 with l-malate as the substrate was reached after the addition of only 5 mM sodium phosphate.

Other polyvalent anions also activate the enzyme. For example, sulfate, arsenite, citrate, borate, phosphate, and arsenate (in an increasing order of effectiveness) activate fumarase with divalent anions activating to a lesser extent than trivalent anions although over the pH range studied only two groups are ionized on the trivalent anions. The greater the amount of activation the further towards the alkaline the pH-optimum is shifted.

Chloride, bromide, thiocyanate, and iodide inhibit fumarase in an increasing order of effectiveness. This inhibition is exhibited in either the absence or the presence of an activating ion and is most pronounced on the acid side of the pH-optimum. The inhibition by thiocyanate was apparently involved in a decrease of the maximum velocity.

Alberty (5) described the effects of phosphate on fumarase and found both an activating and an inhibiting effect by this anion. This author found that the activation by phosphate wasn't complete until the concentration had reached 50 mM when fumarate was the substrate. In experiments with sodium, potassium, and ammonium phosphates the same results were obtained for the kinetic constants which indicates that only the anion has any effect on the activity of the enzyme.

A recent attempt (6) has been made to correlate the inhibition by anions of several enzymes(fumarase included) with an alteration of the tertiary structure of the protein. In general the order of effectiveness of the monovalent anions used in this study on the activity of fumarase paralleled that of the anions investigated by Massey, and this order was about the same for all the enzymes studied.

None of the above mentioned studies have given a

detailed kinetic study of the inhibition of fumarase by monovalent anions. Therefore, this research is directed towards such a kinetic study in order to try to elucidate the mechanism of the effect of anions on fumarase.

2. METHOD

All initial velocity measurements were carried out on a Cary-14 recording spectrophotometer with a thermostated cell holder. In making kinetic measurements it was found advantageous to keep stock solutions of the enzyme in dilute phosphate buffer (10 mM, pH 7.3) at 0°. Preliminary experiments showed that the enzyme solution was stable under these conditions for periods long in excess of the time necessary for a series of initial velocity measurements. A small volume of the enzyme solution (0.3 mM) was pipetted into a 3 ml. cuvette with a 1 ml. optical path and was allowed to come to the proper temperature. The substrate solution (2.7 ml.) at the proper pH, salt concentration (the change in concentration of the substrate solution by dilution with the enzyme solution was taken into account when the substrate solutions were prepared), and temperature was then rapidly added to the enzyme solution. The contents were mixed by capping the cuvette and inverting it twice before placing it into the spectrophotometer.

All the initial velocities, Michaelis constants, and maximum velocities reported here are for the conversion of l-malic acid to fumaric acid at pH 7.3±0.1 and 27.0±0.1°C. The initial rates were determined by following the change in optical density at 250 mμ of

the reaction mixture (5). Fumaric acid displays an intense absorption in this region while that for malic acid is negligible

The maximum velocities and the Michaelis constants were determined by using the method of Lineweaver and Burke (7). This method for determining the kinetic constants of an enzymatic reaction is based on the assumption that the enzyme follows Michaelis-Menten kinetics. In other words the reaction follows a mechanism which yields an equation of the type:

$$V_{initial} = \frac{V_{Max}\ (S)}{K_M + (S)}$$

where

V_{Max} = maximum velocity = $k_{cat}\ (E_o)$

K_M = Michaelis constant

The Michaelis constant and the maximum velocity may be functions of added salt but never a function of the substrate concentration. The method of Lineweaver and Burke involves plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. If the above equation is followed, then a straight line is obtained and the reciprocals of the $1/V_{initial}$ axis and the $-1/(S)$ axis. All constants were determined by graphical methods and four initial

velocities were used to determine a maximum velocity and a Michaelis constant at a particular salt concentration. The substrate concentrations were varied from 0.333 mM to 10.0 mM l-malic acid. The fumarase used in these experiments was obtained from the Cal-Biochem Corp. as was the l-malic acid (mp 103.5-104°).

3. RESULTS

Table I shows the effects of various anions upon the activity of fumarase. In each case the concentration of potassium phosphate was 10 mM, of the monovalent anions was 60 mM, of fumarase was 1.2×10^{-8} M, and of l-malate was 2.2 mM. The pH was adjusted to pH 7.3.

Table I

Salt	Percentage of the Initial Activity
None	100%
KF	100%
KCl	83%
KBr	67%
KSCN	33%
$KClO_4$	55%
$KBrO_3$	91%
Sodium Formate	91%

These results agree qualitatively with those of Massy (4) and Morales (6) although the inhibition by potassium perchlorate was reported to be about equal to that of potassium thiocyanate by the latter author. Morales, however, only investigated the regions where the salt concentrations were 500 mM and greater and where different inhibition mechanisms might be operative.

The effects of these anions upon the catalytic activity of the enzyme do not appear to be due entirely to the change in ionic strength. Although there is a wide range of inhibition by the various ions, the ionic strengths were identical for each determination reported in Table I. If the enzyme inhibition were due to ionic strength effects only, then one would expect the degree of activity loss to be identical for solutions of equal ionic strength and to be little affected by the kind of salt added.

When the reciprocals of the initial velocities for the conversion of l-malic acid to fumaric acid are plotted against the phosphate concentration, a straight line is obtained (Graph I-A). When the same plot is made with sodium fluoride (Graph I-B), sodium chloride (Graph II-B), or sodium bromide (Graph II-A) present in the reaction solution at a concentration of 200 mM, a profound effect on the slope and the shape of these curves is observed. The change in shape of these curves seems to become more pronounced the greater the inhibitory power of the salt on the initial rate (see Table I). The salt concentration above 400 mM does not appear to exert a great influence on the initial rate. For example, when the initial rate was determined for increasing sodium phosphate concentration to 650 mM in the presence

of 200 mM sodium bromide, the initial rate remained constant from 400 mM to 650 mM sodium phosphate while the ionic strength was increasing from 1.4M to 2.15M.

The reciprocal of the initial velocity rather than the initial velocity is plotted against the salt concentration in order to ascertain whether the reciprocals of the experimental initial velocity curves obtained are linear. If the curves are linear then the system is probably exhibiting competitive or non-competitive inhibition which are the two most common kinds of enzyme inactivation (8).

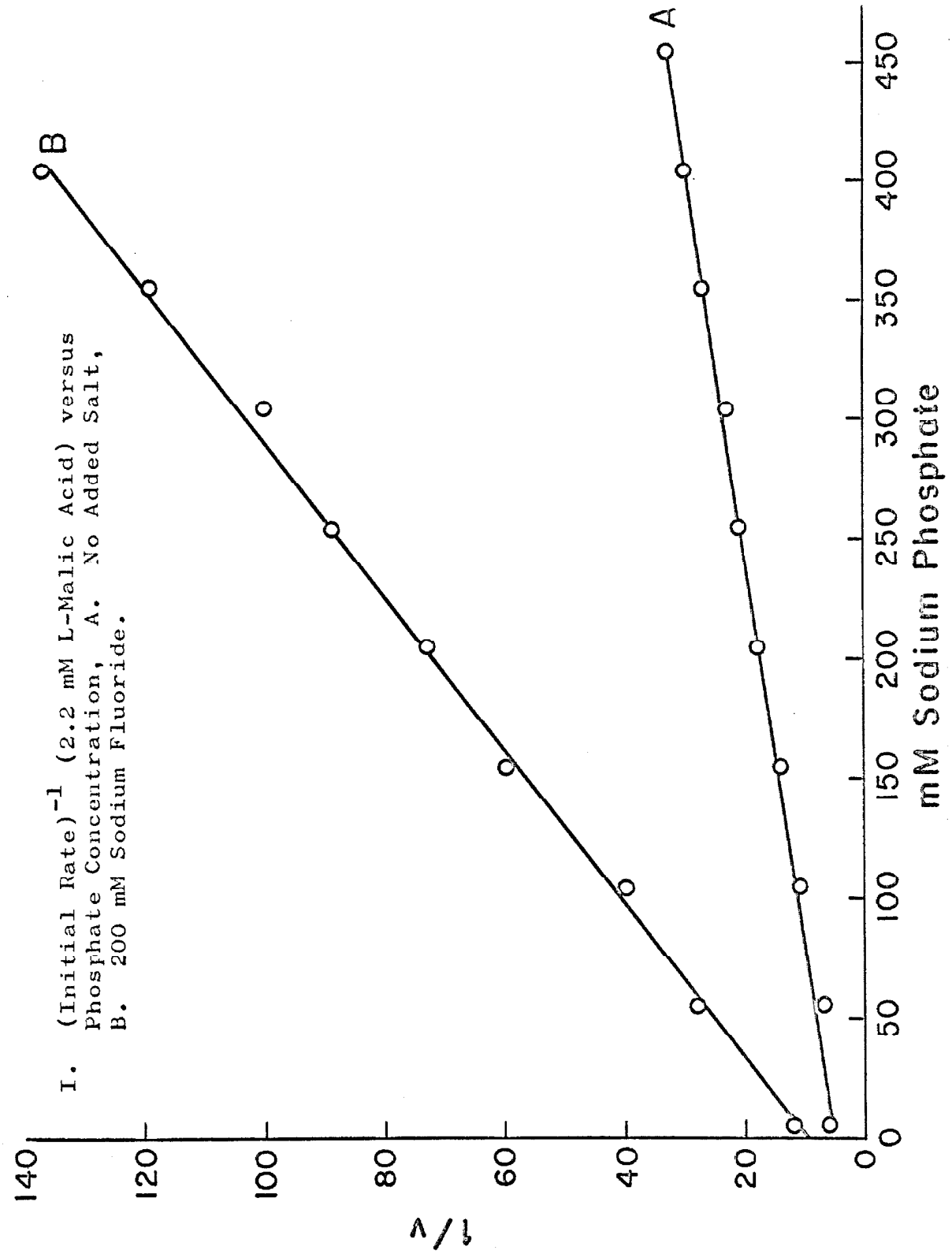
The presence of a competitive inhibitor on the enzyme surface imposes a block which renders the enzyme incapable of combining with the substrate. Likewise, the presence of the substrate on the enzyme makes the enzyme incapable of combining with the inhibitor. The kinetic consequences of competitive inhibition are shown in 1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

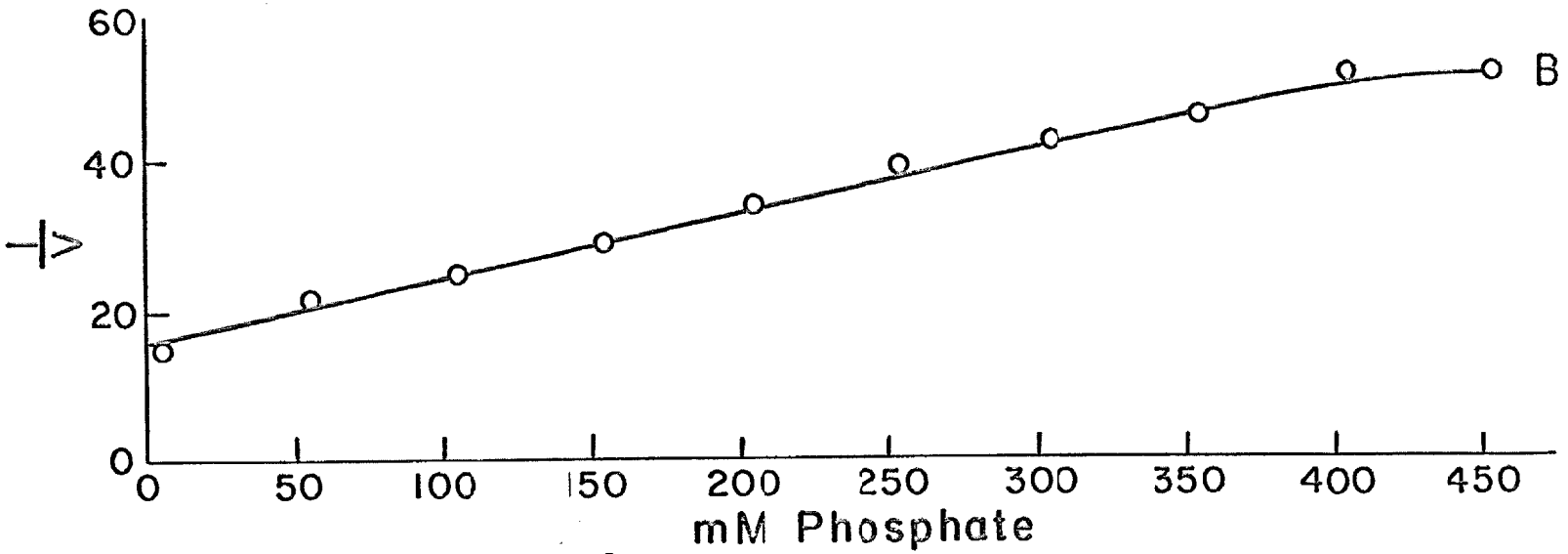
$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI$$

where

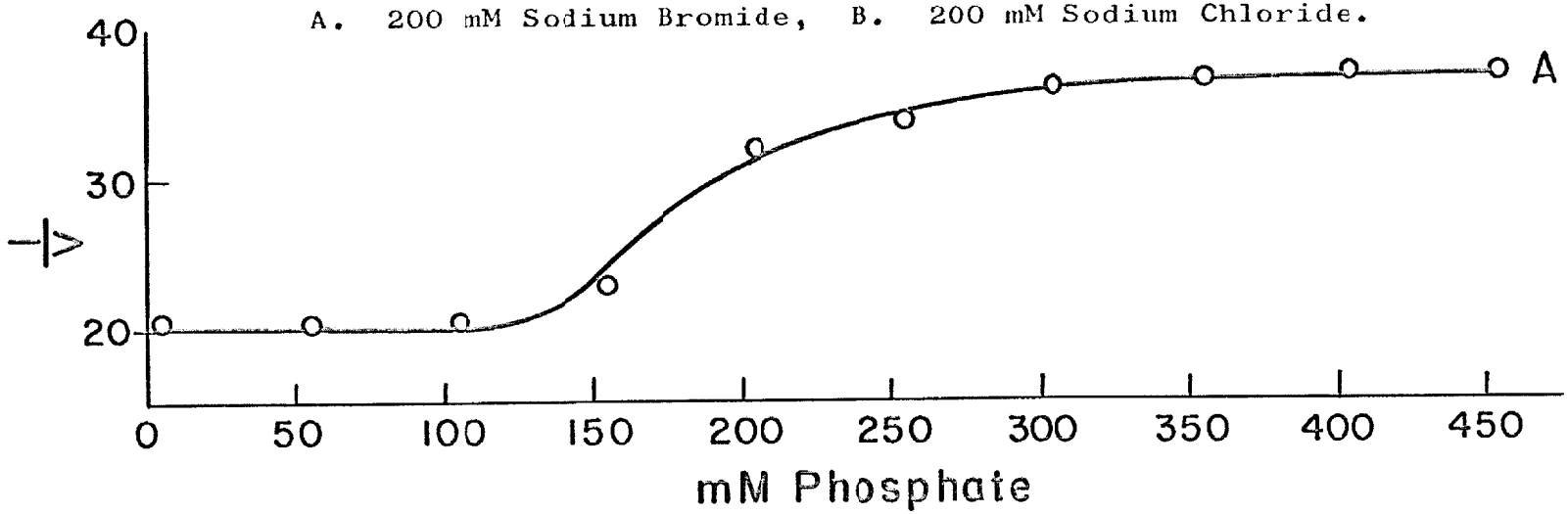
E = enzyme



I. (Initial Rate)$^{-1}$ (2.2 mM L-Malic Acid) versus Phosphate Concentration, A. No Added Salt, B. 200 mM Sodium Fluoride.



II. $(\text{Initial Rate})^{-1}$ (2.2 mM L-Malic Acid) versus Phosphate Concentration
A. 200 mM Sodium Bromide, B. 200 mM Sodium Chloride.



S = substrate

I = inhibitor

P = product

ES = enzyme-substrate complex

EI = enzyme-inhibitor complex

If we assume the usual steady-state conditions, then equation 2 describes this kinetic situation.

$$V_{initial} \frac{V_{Max}\,(S)}{(S) + K_M\left[1 + \frac{(I)}{K_I}\right]} \qquad (2)$$

where

$V_{initial}$ = initial velocity

(S) = initial substrate concentration

(I) = inhibitor concentration

V_{Max} = $k_3\,(E_o)$ = maximum velocity

K_M = k_{-1}/k_1 = Michaelis constant this assumes k_{-1} k_2

K_I = k_{-3}/k_3

Plotting the reciprocal of the initial velocity against the inhibitor concentration should give a straight line since we have transformed equation 2 into a function of the form of $\frac{1}{V_{initial}}$ = a + b(I) which is the form of a straight line.

The presence of a non-competitive inhibitor affects the maximum velocity. The inhibitor reacts equally well

with both the free enzyme and the enzyme-substrate complex and, likewise, the substrate may also react equally well with the free enzyme and the enzyme-inhibitor complex. It is further assumed that the enzyme-substrate-inhibitor complex is not catalytically active. This mechanism is summarized in equation 3.

$$
\begin{aligned}
&E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \\
&E + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \\
&ES + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ESI \\
&EI + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ESI
\end{aligned}
\qquad (3)
$$

where

E = enzyme

S = substrate

I = inhibitor

P = product

EI = enzyme-inhibitor complex

ES = enzyme-substrate complex

ESI = enzyme-substrate-inhibitor complex

The steady-state rate equation for 3 is given by equation 4.

$$V_{initial} = \frac{V_{Max}\ (S)/\ \left[1 + (I)/K_I\right]}{(S) + K_M} \quad (4)$$

where

$V_{initial}$ = initial velocity

(S) = initial substrate concentration

(I) = inhibitor concentration

V_M = $k_3\ E_o$ = maximum velocity

K_M = k_{-2}/k_2 = Michaelis constant if $k_{-2} \gg k_3$

K_I = k_{-1}/k_1

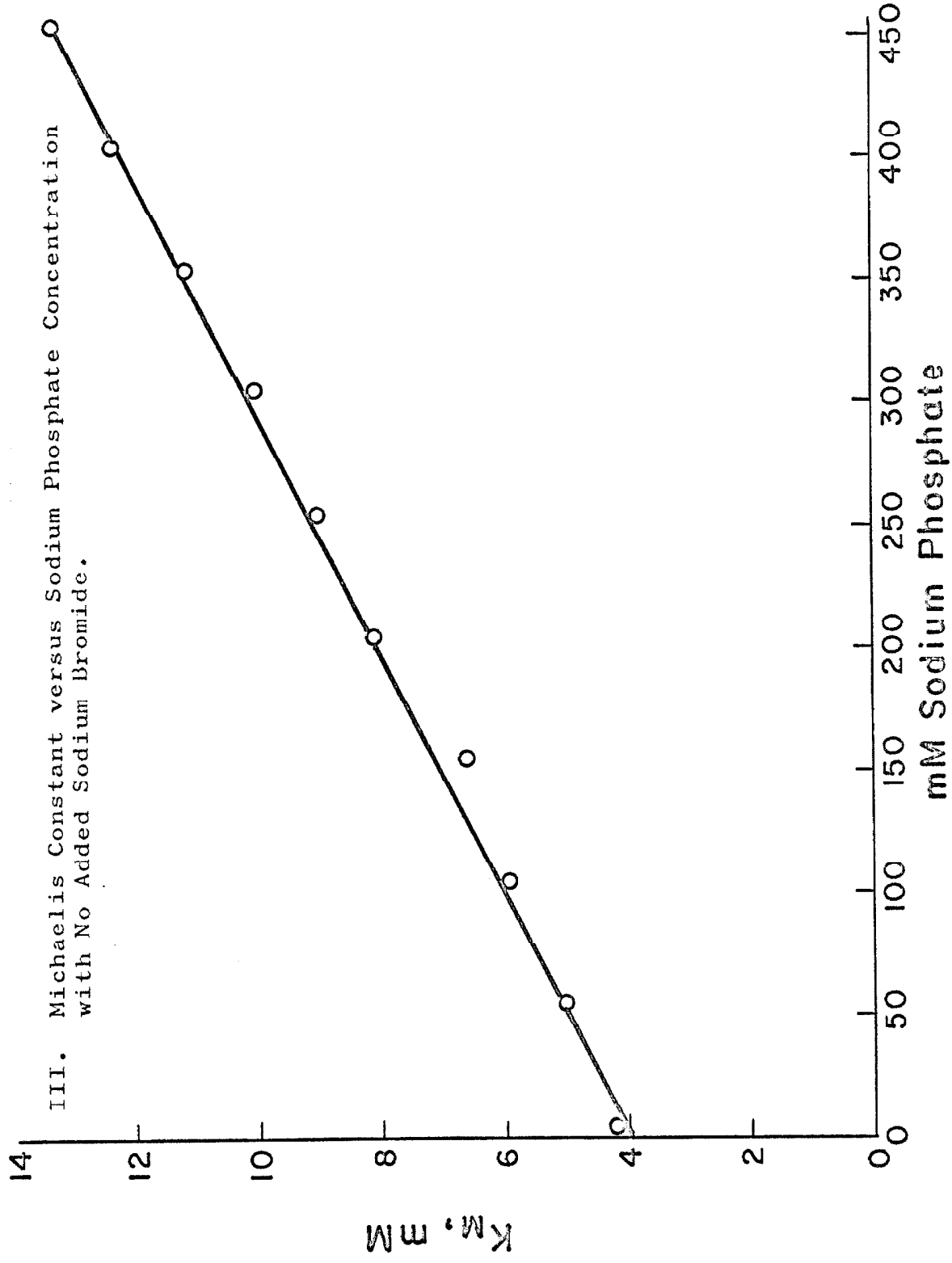
Taking the inverse of equation 4, an equation of the form $\frac{1}{V_{initial}} = a + b\ (I)$ is obtained which will give a straight line when the reciprocal of the initial velocity is plotted against the inhibitor concentration.

The dependence of the reciprocal of the initial velocity on the phosphate concentration in the presence of 200 mM sodium bromide is obviously non-linear (Graph II-A). The effect of sodium bromide on the initial rate was thus chosen for further study because neither competitive nor non-competive inhibition was apparently involved and because the sigmoid character of the curve suggests the possibility of some allosteric behavior by fumarase (14). Since the initial velocity tells nothing of the type of inhibition occurring, the constants K_M and V_M for reaction solutions with varying

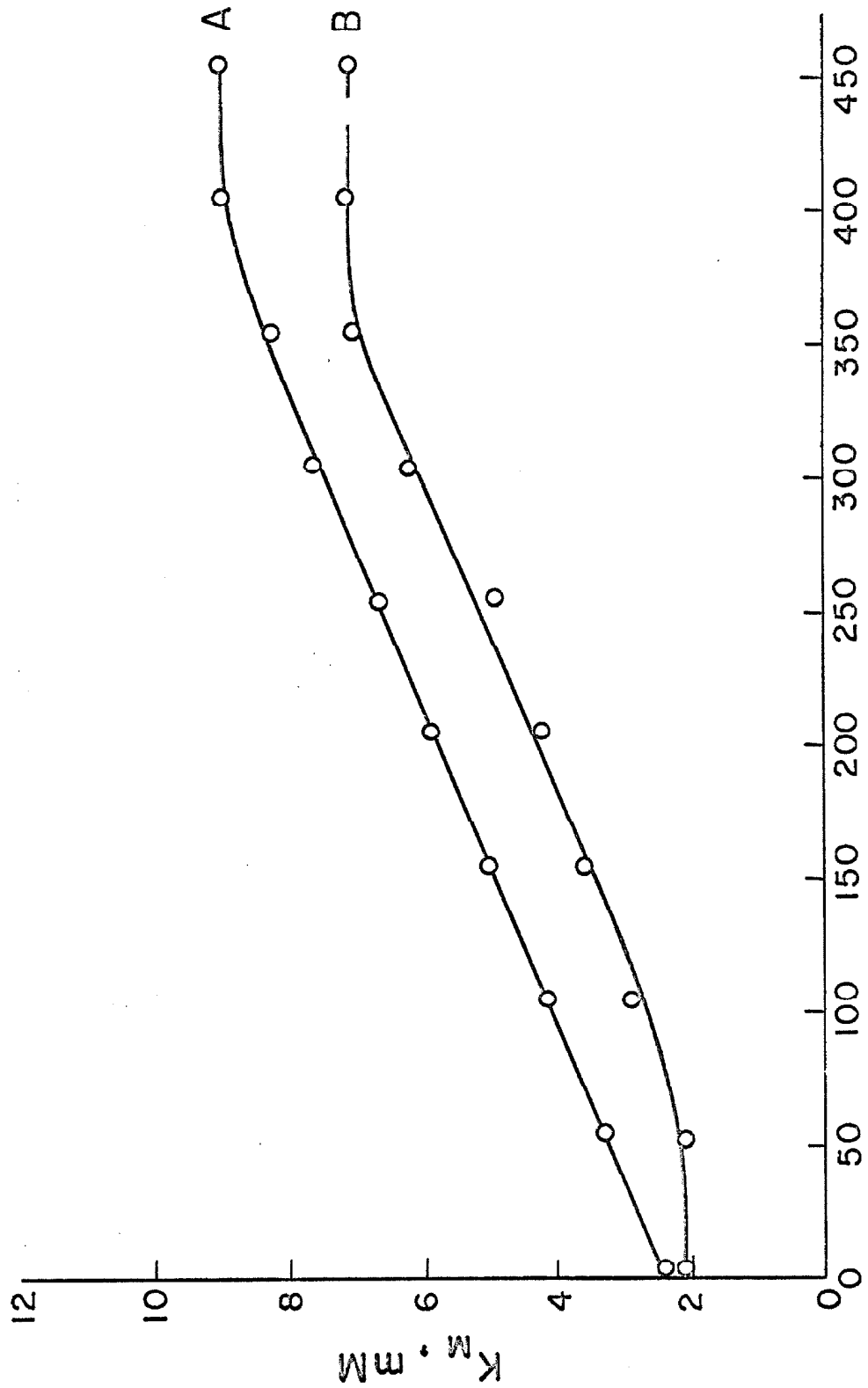
amounts of salt present were determined by the method of Lineweaver and Burke.

When the sodium phosphate concentration was varied at certain fixed sodium bromide concentrations, the Michaelis constant, K_M, varied but the maximum velocity, V_M, remained constant within experimental error. Thus the dependence of the initial velocity on the sodium phosphate concentration is identical to that of the Michaelis constant on the phosphate concentration (Graphs III, IV, and V). In the absence of any sodium bromide the dependence of the Michaelis constant is linear with the sodium phosphate concentration but with increasing amounts of sodium bromide the curves become more sigmoid until 300 mM sodium bromide where the Michaelis constant seems to become independent of increasing monovalent anion concentration. Furthermore, the effect of phosphate is much less marked at high concentrations of bromide. Thus a change of only one millimolar in the Michaelis constant is seen at 300 mM sodium bromide as compared with a change of ten millimolar in the absence of bromide when the phosphate concentration is increased.

When the sodium phosphate concentration was held constant and the sodium bromide concentration was varied, a curious curve was obtained when the reciprocal

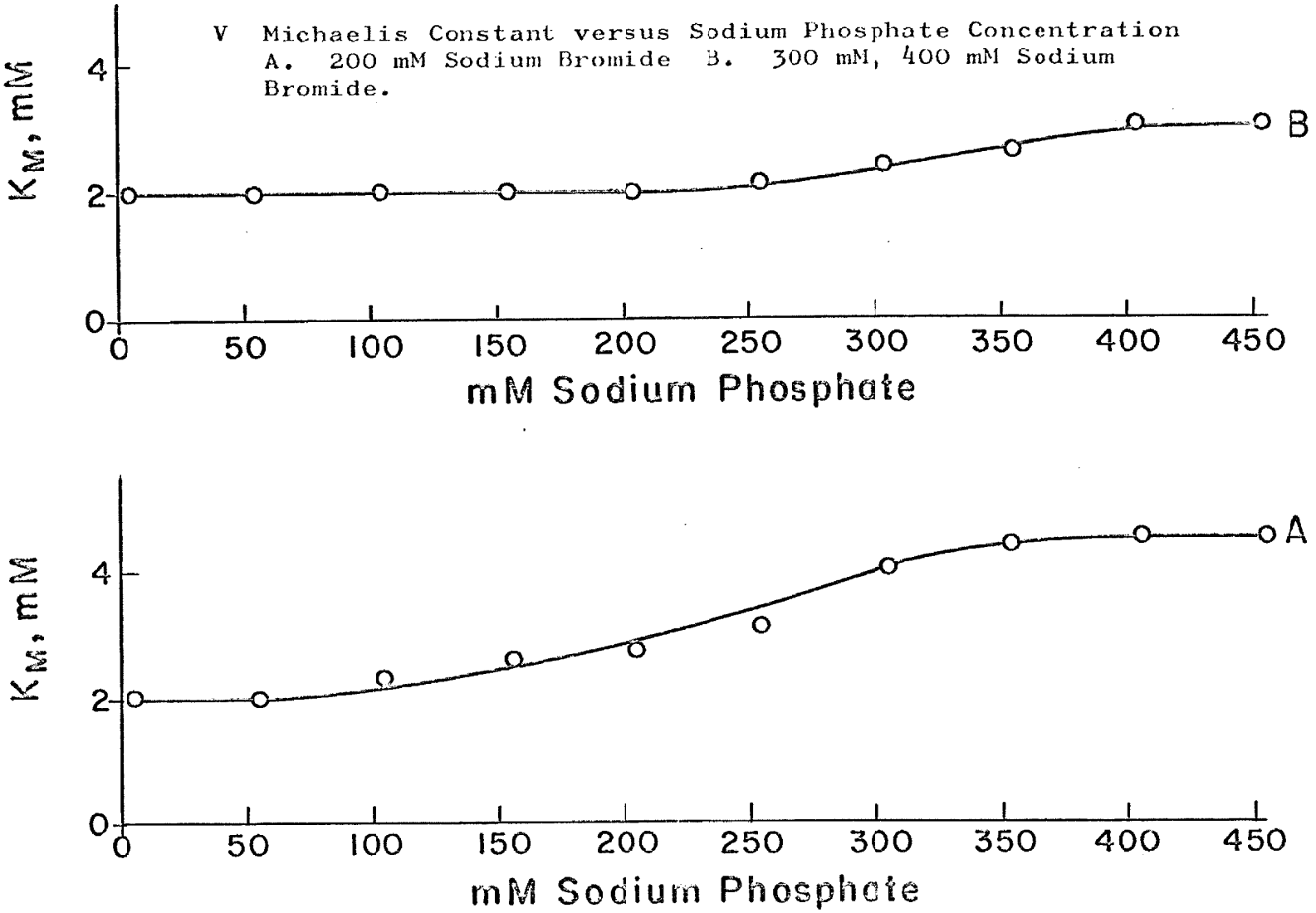


III. Michaelis Constant versus Sodium Phosphate Concentration with No Added Sodium Bromide.



IV Michaelis Constant versus Sodium Phosphate Concentration A. 50 mM Sodium Bromide, B. 100 mM Sodium Bromide.

V Michaelis Constant versus Sodium Phosphate Concentration
A. 200 mM Sodium Bromide B. 300 mM, 400 mM Sodium Bromide.

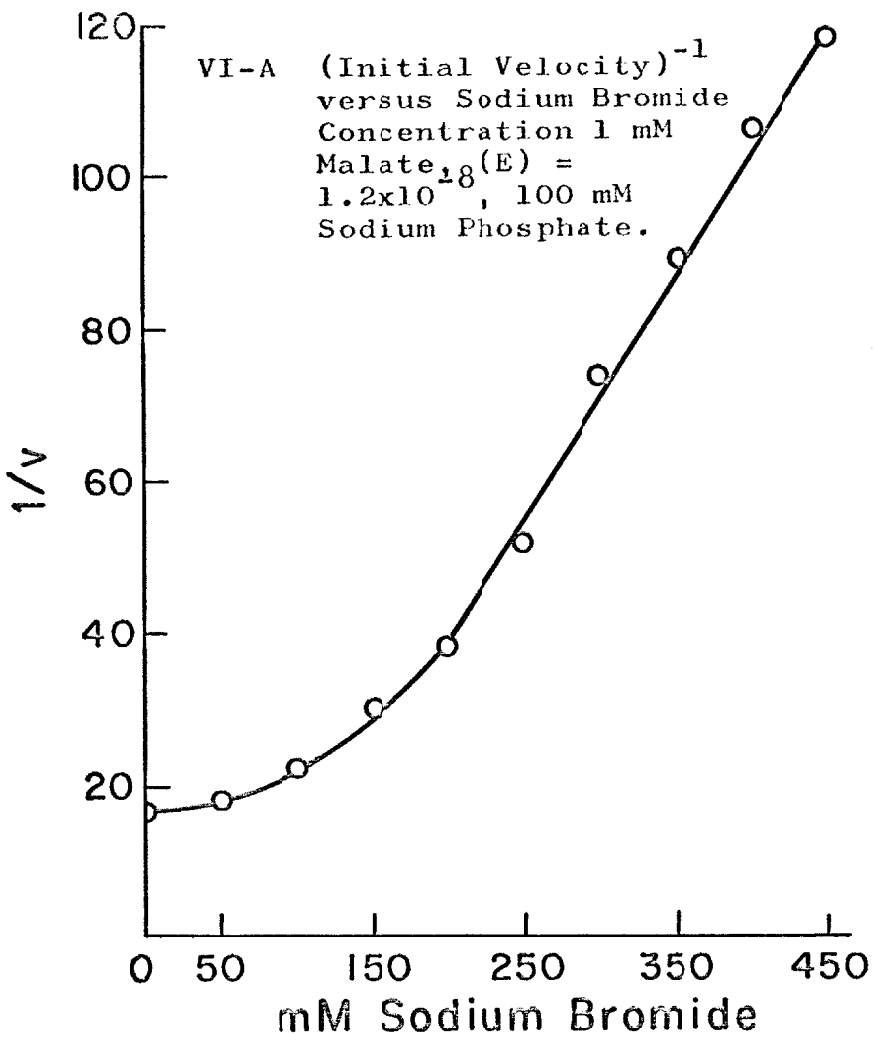


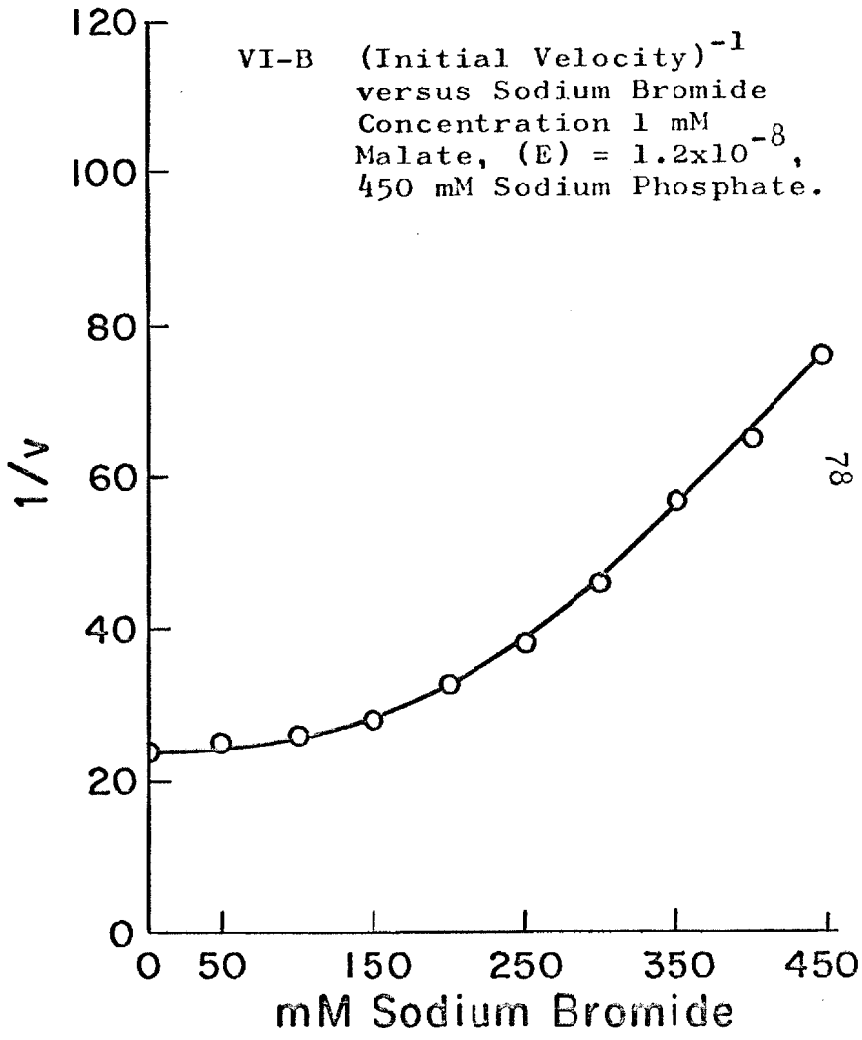
of the initial velocity was plotted against the sodium bromide concentration (Graph VI). Initially the velocity appeared to be independent of the sodium bromide but at higher sodium bromide concentrations the reciprocal of the initial velocity became directly proportional to the concentration of the monovalent anion. The amount of phosphate in the system appeared to have an effect on the curve. For example, the higher the phosphate concentration was taken, the greater the area in which the initial velocity seemed to be independent of the sodium bromide (Graph VI).

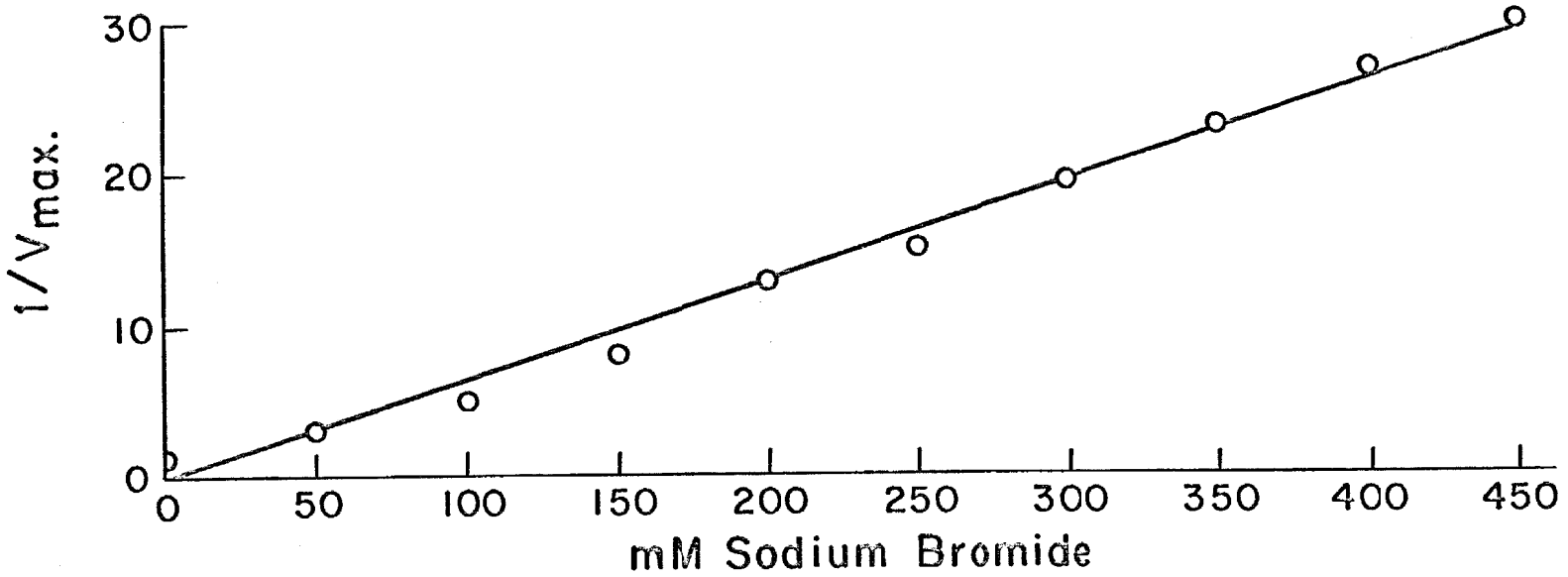
In order to elucidate the origins of this curious kinetic behavior, the Michaelis constants and the maximum velocities were determined for increasing sodium bromide concentrations at fixed concentrations of sodium phosphate. Graphs VII, VIII, IX, X, and XI, illustrate the results of these determinations. Bromide has an effect on V_M. Again the maximum velocity at a given bromide concentration appears to be independent of the amount of phosphate since all $1/V_M$ plots for the various determinations at fixed phosphate concentrations were similar to that shown in Graph VIII where the phosphate concentration is 20 mM. The sodium bromide has an interesting effect on the Michaelis constant. As the sodium bromide concentration increases the Michaelis

constant decreases until a region is reached where K_M seems to become independent of increasing sodium bromide. The amount of bromide necessary to lower the Michaelis constant to this bromide independent region increases with increasing phosphate until 300 mM beyond which increasing phosphate concentration is without effect.

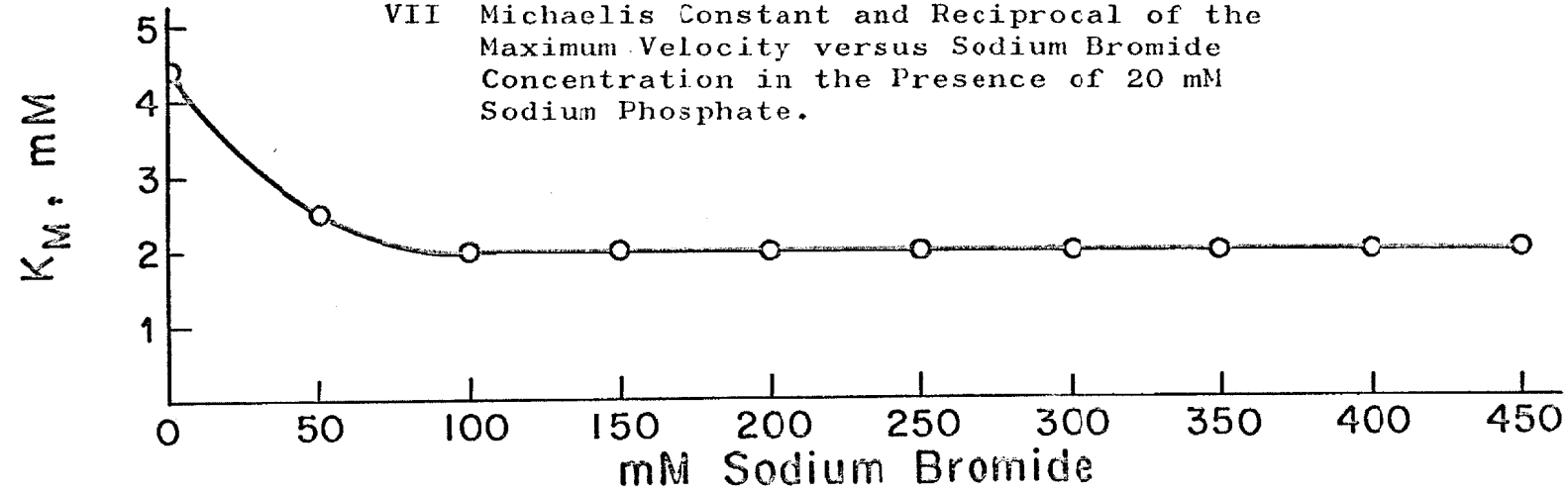
Inspection of Graphs VIII, IX, X, and XI explains the curious curves obtained when the reciprocal of the initial velocity is plotted against sodium bromide concentration at fixed phosphate concentration. The sodium bromide can both activate-by decreasing K_M-and inhibit-by decreasing V_M-fumarase. The initial decrease of the Michaelis constant more or less counterbalances the decrease in the maximum velocity so that the initial velocity appears to be independent of bromide until K_M becomes independent of the salt concentration. At higher phosphate concentrations a wider range of sodium bromide is necessary to decrease the Michaelis constant. Therefore the activation takes place over a larger range of sodium bromide and the counterbalancing of the decrease in V_M takes place over a similar wide range of sodium bromide. Thus the bromide independent region of the initial velocity appears to be greater at higher phosphate concentrations.


VI-A (Initial Velocity)$^{-1}$ versus Sodium Bromide Concentration 1 mM Malate, (E) = 1.2×10^{-8}, 100 mM Sodium Phosphate.
1/v
120
100
80
60
40
20
0
50
150
250
350
450
mM Sodium Bromide

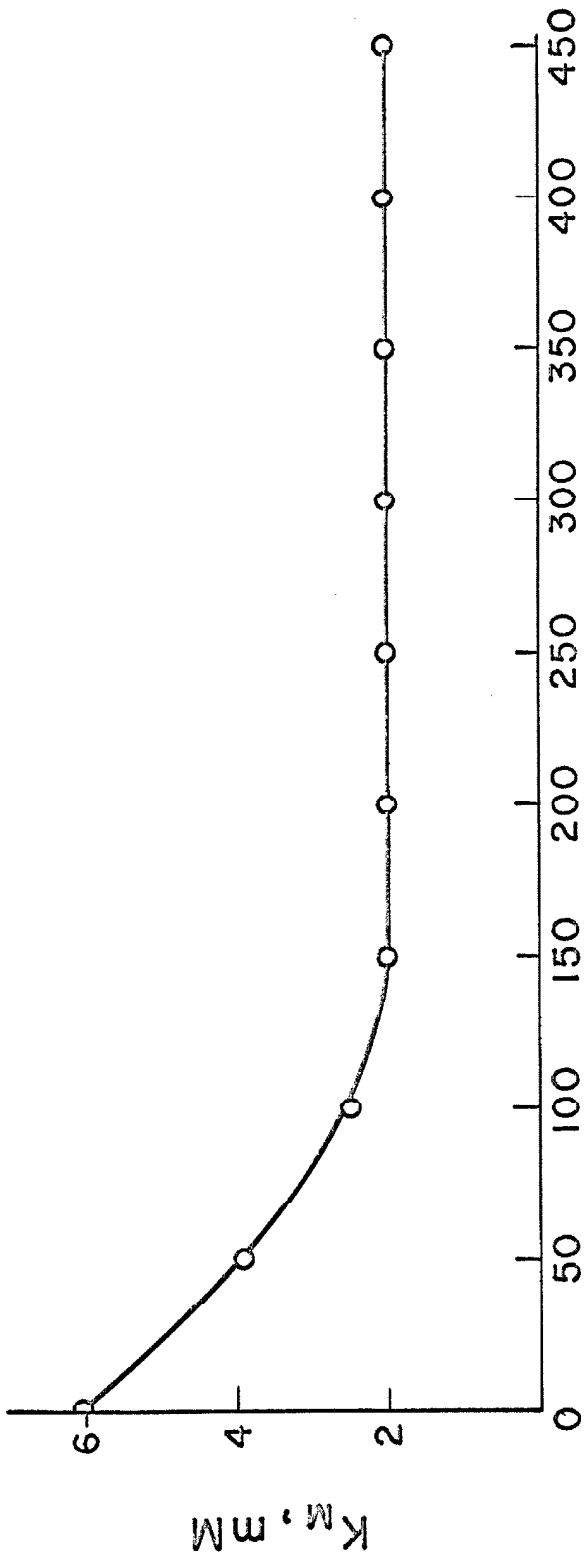

VI-B (Initial Velocity)$^{-1}$ versus Sodium Bromide Concentration 1 mM Malate, (E) = 1.2×10^{-8}, 450 mM Sodium Phosphate.
1/v
120
100
80
60
40
20
0
50
150
250
350
450
mM Sodium Bromide



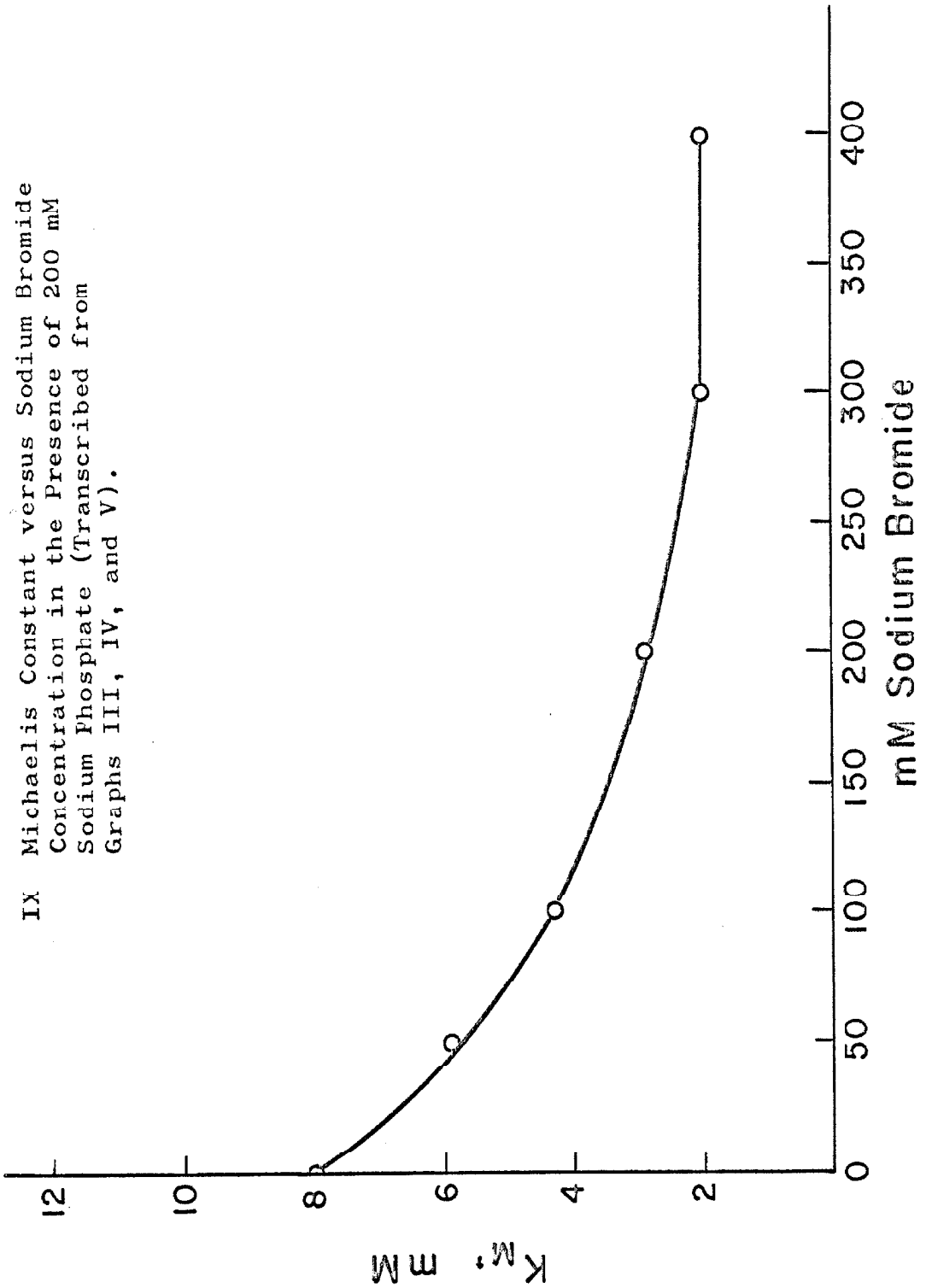
VII Michaelis Constant and Reciprocal of the Maximum Velocity versus Sodium Bromide Concentration in the Presence of 20 mM Sodium Phosphate.



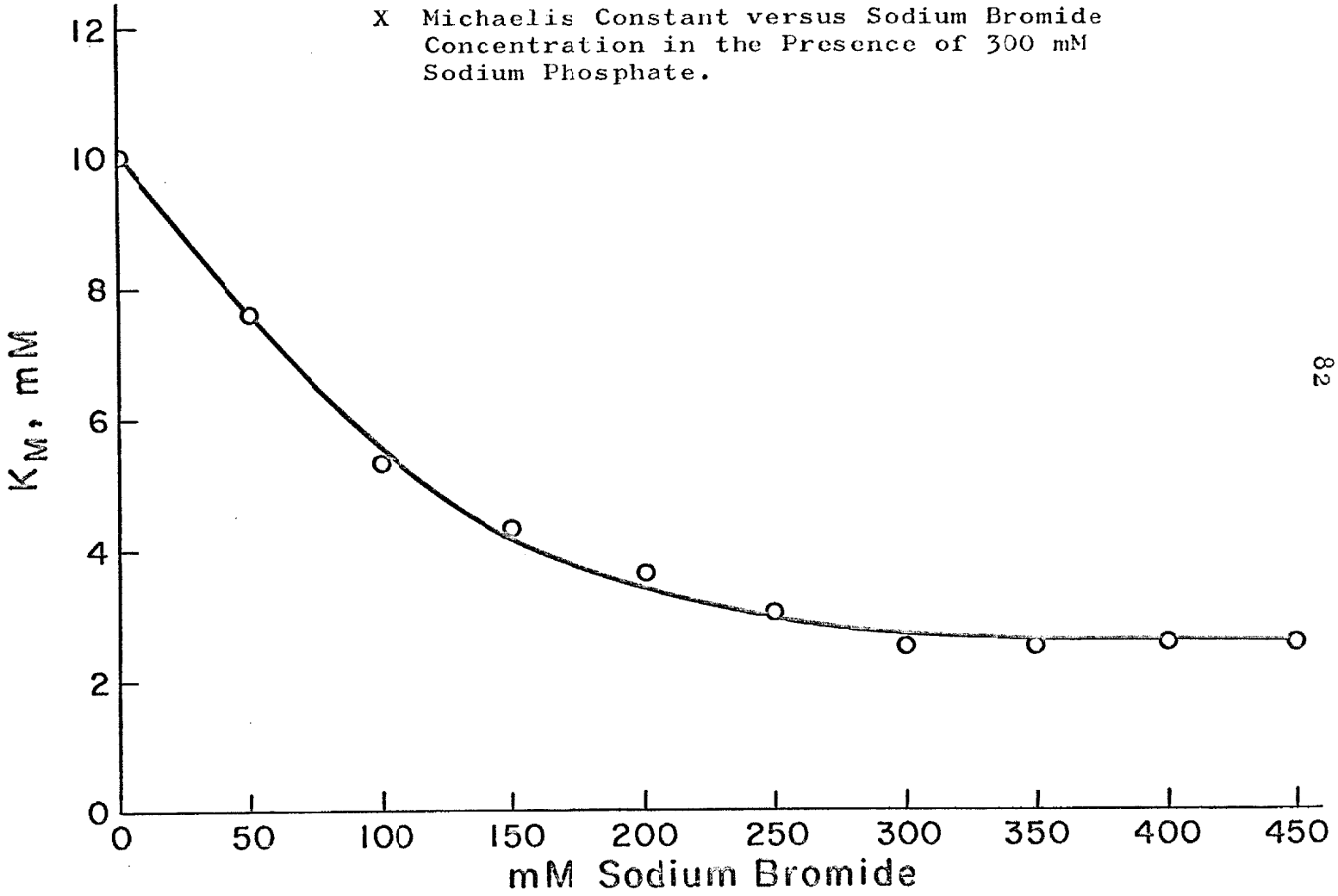
VIII Michaelis Constant and Reciprocal of the Maximum Velocity versus Sodium Bromide Concentration in the Presence of 100 mM Sodium Phosphate.



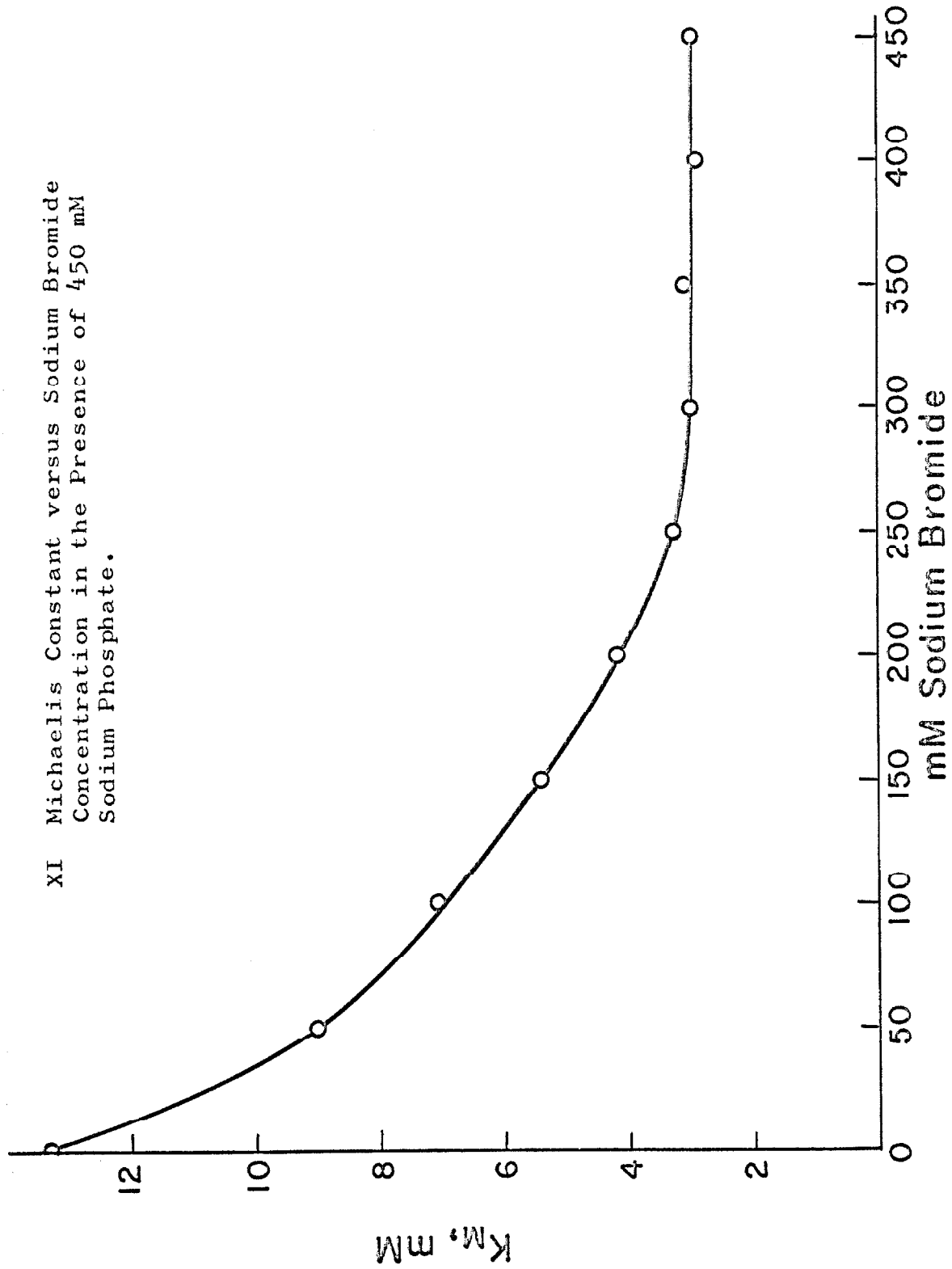
IX Michaelis Constant versus Sodium Bromide Concentration in the Presence of 200 mM Sodium Phosphate (Transcribed from Graphs III, IV, and V).



X Michaelis Constant versus Sodium Bromide Concentration in the Presence of 300 mM Sodium Phosphate.



XI Michaelis Constant versus Sodium Bromide Concentration in the Presence of 450 mM Sodium Phosphate.



4. DISCUSSION

A. Theories for the Inhibition of Fumarase by Anions

Two theories have been advanced in an effort to explain the action of anions on fumarase. These proposals will now be discussed in view of the results found for sodium bromide and sodium phosphate.

Massey (4) attempted to explain the effect of anions on fumarase by proposing that the monovalent anions associate with the acidic groups of the enzyme responsible for catalytic activity while the polyvalent anions associate with the basic groups of the enzyme responsible for catalytic activity. According to the theory of Michaelis and Pechstein (9) the optimal activity of an enzyme is at that pH value at which the maximum fraction of the enzyme molecules have the two groups responsible for catalytic activity in the appropriate state of ionization. Consequently, a change in pK of one of the groups necessary for catalytic action by association with an anion could cause a change in the activity of the enzyme.

In the case of fumarase with both fumarate and l-malate as substrates, only the alkaline branch of the pH-maximum velocity curve is altered by the addition of sodium phosphate which suggests that only the dissociation of a basic group is altered. The effects of

each of the activating ions (sulfate, arsenite, citrate, borate, and arsenate) are similar in this regard to phosphate.

The monvalent anions alter only the acidic side of the pH-maximum velocity curve. Therefore, the monovalent ions appear to affect the dissociation of the acidic group necessary for catalytic activity.

Certain conceptual difficulties arise from this model for enzyme inhibition. First, one wonders why some anions should combine preferentially with basic groups and others with acidic groups although this preferential adsorption at certain sites in the enzyme may be due to differences in site and in charge. Finally, this theory would not take into account the change of the molecular structure of the protein in the presence of neutral salts and inhibitors which was seen in experiments to be described later.

This theory is inconsistent with the data presented in this paper. For instance, Massey would predict that a change in the maximum velocity should occur with either phosphate or bromide but only in the case of bromide was such a change seen. Also this proposal says nothing about the effect of anions on the Michaelis constant which was shown to be altered by both phosphate and bromide. Thus the proposed theory for the inhibition

of fumarase by the association of anions with the catalytic groups of the enzyme appears not to explain fully all the effects of anions on fumarase.

Warren et. al. (6) proposes that the anions simply alter the structure of the enzyme which is reflected by a change in the activity. After comparing the effects of these anions with several other enzymes and seeing a similar catalytic change to that for fumarase, Warren concluded that the disruption of the organized protein structure was of a "general nature" rather than by the binding of the anions at a specific site on the protein. Although the mechanism of disruption by a "general nature" was not discussed, it is presumably connected with the change in ionic strength associated with increasing salt concentration. Such a change in the ionic strength could conceivably cause a change in the helical structure by alteration of hydrogen bonds and thereby an alteration of the protein structure as a whole.

Portions of this proposal explain the effects of bromide and phosphate. The mechanism for the simultaneous change in the Michaelis constant and the maximum velocity on the addition of the sodium bromide can easily be invisioned by a conformational change in the protein structure which would affect the orientation of

groups in the active site necessary for binding and catalytic activity. The change in the Michaelis constant with phosphate could occur by a similar mechanism except now only the active site groups necessary for binding would be reoriented. This would also explain the conformational change of the protein on the addition of salts.

However, if the change in activity of the enzyme by anions is merely due to the effects of an ionic strength change of the medium upon the structure of enzyme, then one would suppose that phosphate and bromide should have similar effects on the activity. Clearly this is not the case since bromide decreases the Michaelis constant and decreases the maximum velocity while phosphate increases the Michaelis constant and has no effect on the maximum velocity. Although a change in the activity of the enzyme may be correlated with an anion induced change in the structure of the protein, the results with phosphate and bromide would seem to suggest a specific binding at a site on the protein rather than a general effect of salts, such as an ionic strength change, to alter the protein conformation.

B. Inhibition of Acetoacetate Decarboxylase by Anions

The modifications of the catalytic properties of

an enzyme by the binding of anions at a specific site on the protein has been suggested for acetoacetate decarboxylase (13). Several similarities exist between these two enzymes. For instance, both enzymes bind a carboxylic acid. The order of effectiveness of monovalent anions for inhibition is the same for both enzymes with thiocyanate, perchlorate, bromide, chloride, bromate, and fluoride in a decreasing order of effectiveness (the polyvalent anions were not investigated for acetoacetate decarboxylase). The concentrations of ions used in the study of acetoacetate decarboxylase were similar to those used in the study of fumarase. As an example the inhibition by potassium chloride in 100 mM potassium phosphate buffer was studied from 0 to 500 mM potassium chloride and $1/V_{Max}$ was consistently a linear function of potassium chloride concentration. Both acetoacetate decarboxylase and fumarase exhibit what appears to be noncompetitive inhibitions, i.e., $1/V_{Max}$ is a linear function of salt concentration. However, the Michaelis constant of fumarase is a function of salt concentration while the Michaelis constant of acetoacetate decarboxylase is not affected by salt.

The following is a brief summary of the conclusions reached for the inhibition of acetoacetate decarboxylase by monovalent anions:

a) Inhibition of acetoacetate decarboxylase by monovalent anions is due to the binding of such anions to a cationic site on the enzyme.

b) Each of the anions investigated inhibits by combining with the same site.

c) The binding of one anion per sensitive site is sufficient to cause the loss of catalytic activity.

Thus we see that the inhibition of enzymes by anions can be treated classically by invoking a specific binding site for the anion on the protein.

C. Conformational Changes of Fumarase in the Presence of Inhibitors.

The neutral salts which inhibit fumarase appear also to effect a change in the molecular structure of the protein. These changes were studied both by fluorescence polarization (10) and by sedimentation (11).

The activity of the enzyme in the presence of neutral salts parallels the reversible changes in ρ_H (relaxation time of the enzyme is determined by fluorescence polarization and is proportional to the size and shape of the molecule); the minimum value of ρ_H is proportional to the maximum activity. The addition of trans-aconitate or ammonium thiocyanate reduces the relaxation time to about one-half the value of the most

active form. Neither small molecule caused a change in the molecular weight.

The fact that trans-aconitate causes a charge in the molecular structure of the enzyme is quite interesting since this trivalent anion has been shown to be a kinetically competitive inhibitor of fumarase (12). Classically competitive inhibitors are thought to bind at the active site of the enzyme thereby blocking the formation of the enzyme-substrate complex. If trans-aconitate did only bind at the active site, then a change in the protein conformation would not be expected. However, if the acid was bound at a site other than the active site and this binding in turn caused a change in the conformation of the active site such that the enzyme-substrate complex could no longer form, then one might anticipate a rearrangement of the tertiary structure of the entire protein and the kinetic consequences would be kinetically indistinguishable from competitive inhibition.

An alteration in the molecular structure of fumarase is to be expected by the binding of thiocyanate since this anion has been shown to be a non-competitive inhibitor (4). Non-competitive inhibitors are thought to bind at a site other than the active site but change

the conformation of the protein so that the enzyme is catalytically inactive but still able to bind the substrate.

The sedimentation coefficent of fumarase is affected by ammonium thiocyanate but not by trans-aconitate. The addition of ammonium thiocyanate to a final concentration of 0.1 M in a solution of 0.5 M phosphate buffer at pH 7.4 caused a decrease in the sedimentation coefficent from S^0_{20} = 9.2S to S^0_{20} = 8.6S. This change in the sedimentation coefficent was ascribed to a conformational change in the protein structure.

Comparison of the studies by sedimentation and by fluorescence polarization on fumarase in the presence of thiocyanate and of trans-aconitate is quite interesting. Both inhibitors cause a change in the relaxation time of the protein but only thiocyanate causes a change in the sedimentation coefficent although all these effects are ascribable to a conformational change. A reasonable explanation for this phenomena may be that both trans-aconitate and thiocyanate cause a change in the tertiary structure of the enzyme; in one case, thiocyanate, the protein structure alters in such a manner as to make the enzyme catalytically inactive while in the other case, trans-aconitate, the enzyme

structure alters differently so that now only the substrate binding is affected while the catalytic constant is unchanged. In any case the competitive inhibitor trans-aconitate might bind at other sites on the enzyme than the active site.

D. The Possibility of an Allosteric Transition in Fumarase

The S-shaped character of the curves of K_M versus sodium phosphate concentration in the presence of sodium bromide suggests that the enzyme is allosteric (14). The assumptions of the allosteric model are outlined:

a) An allosteric protein is a polymeric protein containing a finite number of identical subunits which all occupy equivalent positions.

b) To each ligand able to form a stereo specific complex with a subunit there corresponds one, and only one, site on each identical subunit.

c) The conformation of each subunit is constrained by its association with the other subunits.

d) Two states (at least two) are reversibly accessible to the allosteric protein.

e) The affinity of the ligands toward their sites may be different in the various states of the protein.

Fumarase is able to satisfy points "a" and "b" of these assumptions, since the enzyme contains four identical subunits (17) and probably has four active sites (18). The important part of this model for our considerations is that the protein may exist in two forms which have different binding characteristics towards the ligands. The differential binding of a ligand can shift the equilibrium towards that form which best binds such a ligand. If the two forms also bind the substrate differently, then the Michaelis constant can be changed by shifting the equilibrium by adding ligands. However, since the substrate binds differently to the two protein forms, the substrate itself may also shift this equilibrium and, as a consequence, the Michaelis constant will become a function of the substrate concentration. Therefore, the Lineweaver-Burke equation will be converted to equation 5.

$$\frac{1}{V_{initial}} = \frac{\left[f_{K_M}(S)\right]}{V_M} \frac{1}{(S)} + \frac{1}{V_M} \qquad (5)$$

where

$\left[f_{K_M}(S)\right]$ = the binding constant as a function of the substrate concentration

If a plot of $1/V_{initial}$ versus $1/(S)$ is made, then

a nonlinear line should result because the slope, $\left[f_{K_M}(S)\right]/V_M$, is a function of the substrate concentration which is always changing. Since all Lineweaver-Burke plots (Graphs XII and XIII) were linear, fumarase is assumed not to be an allosteric protein under these conditions and any kinetic scheme derived for the interpretation of the data previously presented probably will follow Michaelis-Menten kinetics.

Two other enzymes have been reported in the literature which exhibit a sigmoid curve when the initial rates are plotted against the concentration of some anion. Glutaminase (15) in the presence of 100 mM sodium chloride exhibits a sigmoid curve when the initial rate is plotted against increasing phosphate concentration. The enzyme muscle glycogen synthetase (16) also shows a sigmoid curve when the initial rate is plotted against potassium chloride concentration in the presence of glucose-6-phosphate. Although both of these enzymes exhibited linear Lineweaver-Burke plots, the sigmoid curves (obtained when $1/V_{initial}$ was plotted against salt concentration)were attributed to an allosteric effect although no kinetic analysis was attempted. Even though this particular effect of anions is not limited to fumarase, and has been attributed in other systems (for example, glutaminase and muscle glycogen synthetase)

to allosteric behavior, the explanation that the enzymes are in fact allosteric is not consistent with linear Lineweaver-Burke plots.

E. Kinetic Model for the Action of Phosphate and Bromide on Fumarase

In trying to determine a mechanism for the inhibition of fumarase by phosphate and bromide, the following assumptions were made:

a) The inhibition was probably not a general salt effect.

b) The binding of anions to specific sites on the protein was probable.

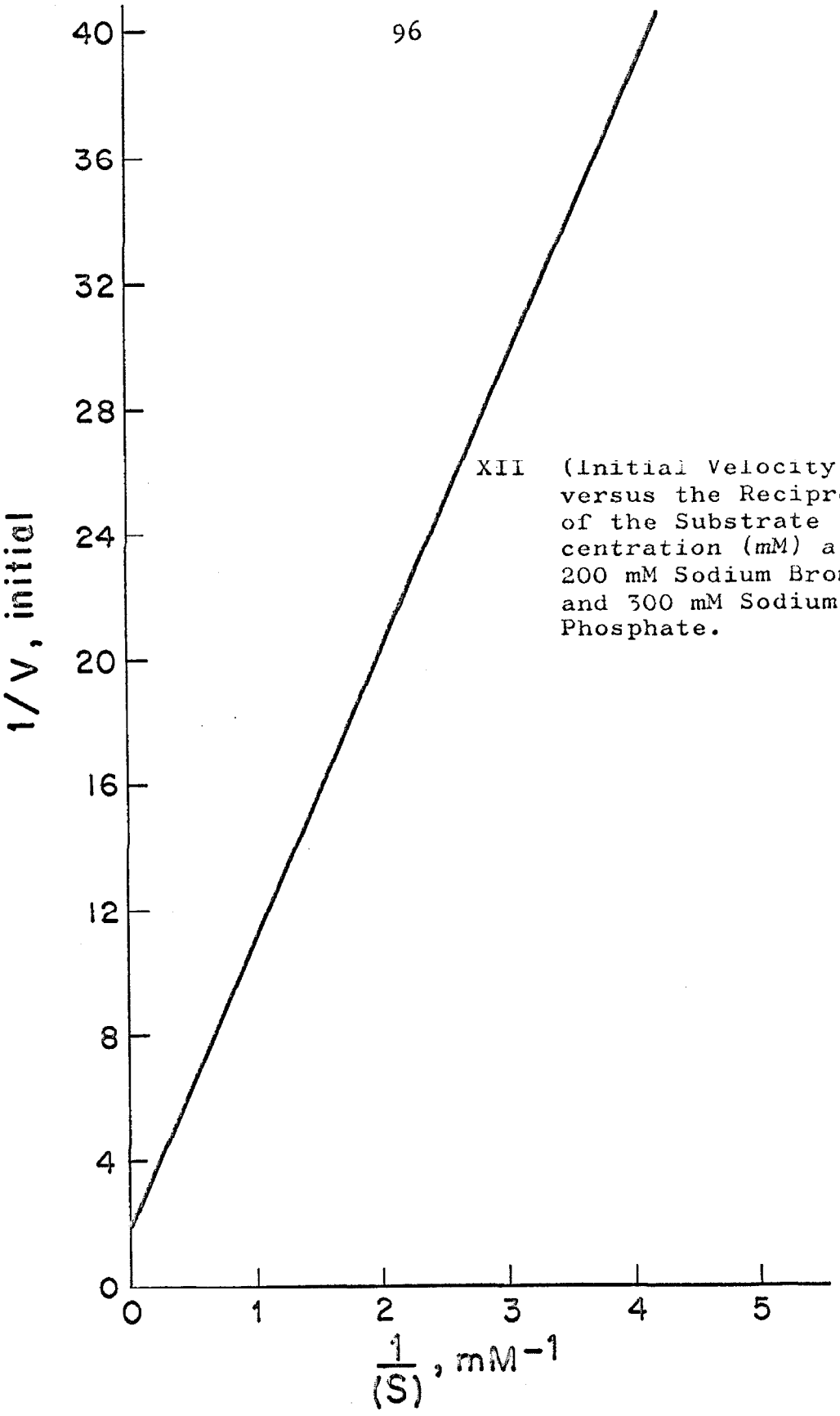
c) Molecules which appear kinetically to be competitive inhibitors are not necessarily bound at the active site.

d) The rate-equation could probably be derived from regular Michaelis-Menten kinetics.

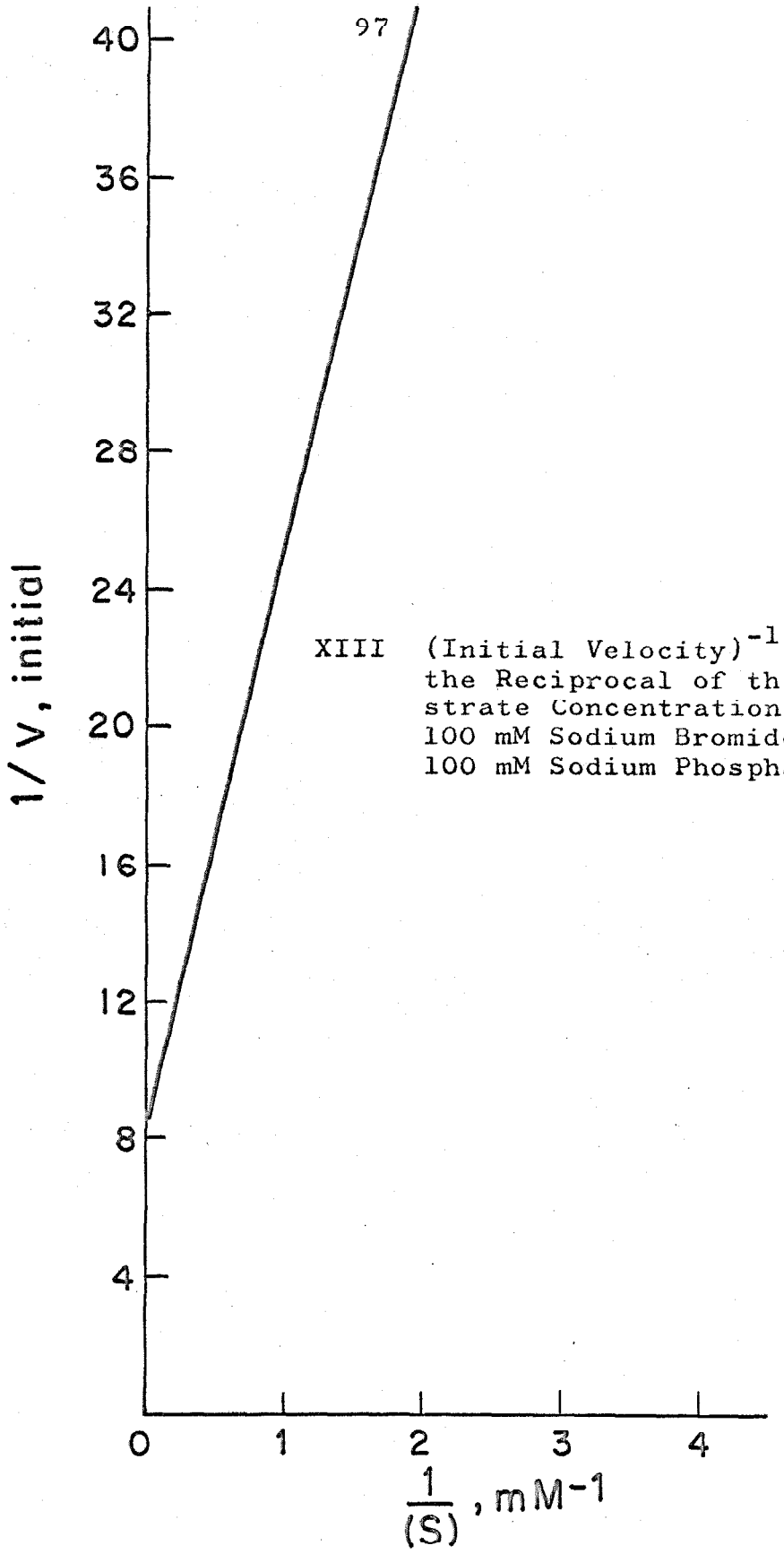
The following mechanism is proposed which follows the above guide lines and which allows the interpretation of at least part of the kinetic data previously presented.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{product}$$

$$Br + E \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} BrE$$



XII (Initial Velocity)$^{-1}$ versus the Reciprocal of the Substrate Concentration (mM) at 200 mM Sodium Bromide and 300 mM Sodium Phosphate.



XIII (Initial Velocity)$^{-1}$ versus the Reciprocal of the Substrate Concentration at 100 mM Sodium Bromide and 100 mM Sodium Phosphate.

$$Br + ES \underset{k'_{-0}}{\overset{k'_0}{\rightleftharpoons}} BrES$$

$$BrE + S \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} BrES$$

$$E + P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} PE$$

where

S = l-malic acid = substrate

E = enzyme

Br = bromide

ES = enzyme-substrate complex

BrE = enzyme-bromide complex

BrES = enzyme-bromide-substrate complex

PE = enzyme-phosphate complex

let

$$K_S = \frac{(E)(S)}{(ES)} = \frac{k_{-1}}{k_1}$$

$$K_{Br} = \frac{(BrE)}{(Br)(E)} = \frac{k_0}{k_{-0}}$$

$$K'_{Br} = \frac{(BrES)}{(ES)(Br)} = \frac{k'_0}{k'_{-0}}$$

$$K'_S = \frac{(BrE)(S)}{(BrES)} = \frac{k'_{-1}}{k'_1}$$

$$K_P = \frac{(PE)}{(P)(E)} = \frac{k_3}{k_{-3}}$$

This mechanism gives the following rate equation assuming a steady-state for all complexes and assuming $k_{-1} \quad k_2$.

$$v_{initial} = \frac{\dfrac{V_{Max}\,(S)}{\left[1 + K'_{Br}(Br)\right]}}{K_S\left[\dfrac{1 + K_{Br}(Br) + K_P(P)}{1 + K'_{Br}\,(Br)}\right] + (S)} \qquad (6)$$

where

$V_{initial}$ = initial velocity.

V_{Max} = $k_3(E_o)$

(S) = initial substrate concentration.

(Br) = bromide concentration.

(P) = phosphate concentrations.

The values of the kinetic constants $1/V_{Max}$ and K_M obtained from the Lineweaver-Burke plots are now defined by equations 7 and 8 respectively.

$$\frac{1}{V_M} = \frac{1}{V_{Max}}\left[1 + K'_{Br}\,(Br)\right] \qquad (7)$$

$$K_M = K_S \cdot \frac{1 + K_{Br}\,(Br) + K_P\,(P)}{1 + K'_{Br}\,(Br)} \qquad (8)$$

The plot of $1/V_M$ versus the sodium bromide concen-

tration should be a straight line with a slope of K'_{Br}/V_{Max} since equation 7 is in the form of y=a+bx. Sodium phosphate should have no effect on the maximum velocity. In fact, the linear dependence of the reciprocal of the maximum velocity on the sodium bromide concentration and the lack of phosphate dependence are seen. Equation 7 gives a correct mathematical definition of the maximum velocity as a function of salt concentration.

Certain portions of the curves of the Michaelis constant, K_M, versus the sodium bromide and sodium phosphate concentrations are explained by equation 8. For example, in the absence of sodium bromide equation 8 reduces to equation 9.

$$K_M = K_S \left[1 + K_P(P)\right] \qquad (9)$$

From equation 9 one again sees the familiar form of the equation of a straight line. Therefore, K_M versus phosphate concentration should be linear and Graph III shows that the dependence is linear in the absence of sodium bromide.

The general shape of the curves obtained when the Michaelis constant is plotted against sodium bromide concentration in the presence of fixed amounts of sodium phosphate is explained by equation 8. Initially, when

the sodium bromide concentration is zero, the Michaelis constant will be defined by equation 9. On the other hand, when the inhibitor concentration is sufficiently large that all the free enzyme is in the form of an EI compound, i.e., $K_{Br}(Br) \gg [1 + K_p(P)]$ and $K'_{Br}(Br) \gg 1$, then equation 8 reduces to equation 10.

$$K_M = \frac{K_S K_{Br}}{K'_{Br}} \qquad (10)$$

Thus at high sodium bromide concentrations the Michaelis constant becomes independent of the concentration of bromide. Physically it appears as if bromide is converting free enzyme into a new enzyme which has a new affinity constant. As the fixed phosphate concentration is increased two things should happen to the K_M versus bromide concentration plots. First the initial value of K_M should increase as can be seen from equation 6, and second the amount of bromide necessary to convert the enzyme to a new form with a new Michaelis constant should increase because the inequality $K_{Br}(Br) \gg [1 + K_p(P)]$ must be satisfied and as the phosphate increases it will take more bromide for this to be true. Equation 8 thus predicts that the plot of the Michaelis constant versus the sodium bromide concentration at fixed phosphate

concentrations should slowly decrease (if $K'_{Br} > K_{Br}$) from some initial K_M value to a region where K_M is independent of sodium bromide, and, furthermore, as the fixed phosphate concentration is increased the initial K_M should increase while higher bromide concentrations will be necessary to attain the region where K_M is independent of sodium bromide. This behavior is in fact what is seen in Graphs VIII, IX, and X.

Unfortunately this mechanistic scheme does not explain all the data presented. For instance, the S-shaped character of the curves of the K_M versus sodium phosphate in the presence of bromide is unexplained. Also, why does the amount of bromide necessary to reach the bromide insensitive region become constant at 300 mM sodium phosphate, and why does the K_M value of this region also change? These questions are unanswered and may become apparent only after more work with other anions or from biophysical studies.

One interesting point of this kinetic scheme is that the constant BrEP never forms. It seems unlikely that the BrE complex can not bind with the phosphate but still bind with the substrate if phosphate were a classical competitive inhibitor. A possible explanation may be that bromide and phosphate occupy the same site on the enzyme for inhibition. This would be suggested

from the results with trans-aconitate and ammonium thiocyanate. Formally phosphate is a competitive inhibitor just as trans-aconitate so the possibility exists that phosphate may act by binding at some other site than the active site and cause a protein structure change.

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Abstract of the Propositions

Proposition I

The use of sulfur-35 labeled ammonium thiocynate is proposed to show that the irreversible dissociation of fumarase in the presence of thiocyanate is due to the reaction of the thiocyanate with certain amino acids in the enzyme.

Proposition II

It is proposed to expose an enzyme system which lactonizes d-camphor to substrate analogues which will undergo reactions characteristic of peracids.

Proposition III

The study of allosteric transitions in proteins by fluorescence depolarization is proposed.

Proposition IV

It is proposed to study the isotope effect of aconitase and fumarase with tritium oxide in order to minimize effects due to protein hydration.

Proposition V

A study of the reaction of imidazole-1-phosphonate with phosphorylated nucleotides is proposed.

PROPOSITION I

It is proposed to use sulfur-35 labeled ammonium thiocyanate to show that the irreversible dissociation of fumarase when treated with thiocyanate is due to reaction of the thiocyanate with certain amino acid residues in the enzyme.

Massey and Johnson (1) have studied the effect of ammonium thiocyanate on the enzyme fumarase. They observed that the sedimentation coefficient of fumarase in phosphate buffer (0.05M, pH 7.4) changes from 9.2S to 8.6S directly upon the addition of thiocyanate, and after two hours the enzyme begins to dissociate into two fractions of equal sedimentation coefficients (5.9S). The dissociation is retarded by the presence of phosphate and is accelerated by higher ammonium thiocyanate concentration and lowered pH. Partial inhibition of the enzyme activity occurs immediately following the addition of thiocyanate and complete inhibition parallels the dissociation into subunits. Dialysis of the dissociated enzyme against phosphate buffer (0.05M, pH 7.4) gave no recovery of enzymatic activity or reassociation despite the fact that dialysis before dissociation readily reversed the partial inhibition.

Massey explains the initial reversible inhibition in terms of ionic effects upon the tertiary structure of

the protein thus affecting its catalytic activity (1,2). He proposes that the dissociation is due to further change in the tertiary structure until splitting into the subunits finally occurs. The subunits undergo an irreversible change which does not allow formation of the active enzyme even after the thiocyanate is removed (1).

Addition of other monovalent anions causes an initial partial inhibition of the enzyme analogous to that of thiocyanate (3); however, in the case of chloride and acetate (4) no dissociation of the enzyme into subunits occurs even after six to eight hours. One would expect dissociation with these anions if ionic interactions were the only cause for irreversible dissociation. Massey's explanation also requires an irreversible change in the tertiary structure of isolated subunits. When fumarase is treated with citric acid (1.0M, pH2.6-9), the enzyme dissociates into subunits (2.9S). However, this dissociation is readily reversed by neutralization (5).

The following alternative explanation is suggested to reconcile these anomalies. In agreement with Massey's hypothesis the initial change is due to ionic factors, but it is proposed that the irreversible dissociation is caused in some manner by chemical reaction of the thiocyanate with the sulfhydryl groups of the cysteines and/or the amino groups of the terminal glycines. The forma-

tion of the "thiocyanated" enzyme could be expected to alter the tertiary structure of the subunits in such a manner that the ionic bonds normally holding the subunits together (7) are functionally changed. Thus we would deduce that chloride and acetate do not cause dissociation because they are incapable of forming compounds with fumarase. The complete inhibition obtained with thiocyanate is irreversible because the fumarase-thiocyanate reaction is not easily reversed.

Cyanate reacts readily with amino acids to form carbamates (8) and particularly rapidly with cysteine to form the S-carbamate (9). Carbamylation of fumarase led to inactivation of the enzyme (16).

The formation of thiohydantoins by the reaction of ammonium thiocyanate with several amino acids has been reported (9,10,11). Potassium thiocyanate forms rhodanine with thioglycolic acid (14) and a thiocarbamate with amino ethyl acetate (13). All these products are quite stable in neutral and acidic solution.

Fumarase has four N-terminal glycine residues and twelve cysteines per molecule (8,9) available for reaction. Thiocarbamates and S-thiocarbamates form more easily in acidic solution (13,14) and the irreversible inhibition of fumarase occurs more rapidly at lower pH (1). When fumarase was titrated with

mercuribenzoate, the enzyme was inactivated to a degree directly proportional to the extent of reaction with the thiol groups (7) which is in accord with the second explanation.

It is proposed to treat fumarase with a solution of sulfur-35 labeled ammonium thiocyanate. Aliquots of the mixture will be removed at predetermined times and dialyzed against phosphate buffer. The amount of dissociation of the enzyme will be compared with the amount of nonexchangeable sulfur-35 activity. If the dissociation and irreversible inhibition are due to purely ionic factors there will be no irreversibly-bound thiocyanate. If the dissociation is caused by reaction of some of the enzyme amino acids with thiocyanate, then the amount of dissociation would be directly proportional to the amount of irreversibly -bound thiocyanate in the subunits.

The thiocyanate products can be hydrolyzed in basic medium (15). Since the irreversible dissociation causes complete inactivation of the enzyme, even a small fraction of the activity of the enzyme obtained on hydrolysis would be powerful evidence supporting the sulfur-35 labeling studies.

References for Proposition I

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PROPOSITION II

It is proposed to expose an enzyme system which lactonizes d-camphor to substrate analogues which will undergo reactions characteristic of peracids.

An enzyme system which converts d-camphor to 1, 2-campholide has recently been isolated (1). The system contains a diphosphopyridine nucleotide dehydrogenase (E_1) which is a flavoprotein and a ketolactonase (E_2) which contains two iron atoms. The proposed sequence of events for the oxidation of the d-camphor by the enzyme system is as follows

$$DPNH + H^+ + FMN \xrightarrow{E_1} DPN^{\oplus} + FMNH_2$$

$$FMNH_2 + E_2 \cdot 2Fe^{+3} \longrightarrow FMN + E_2 \cdot 2Fe^{+2} + 2H^{\oplus}$$

$$E_2 \cdot 2Fe^{+2} \cdot O_2 + \text{d-camphor} + 2H^{\oplus} \longrightarrow E_2 \cdot 2Fe^{+3} + H_2O + \text{1,2-campholide}$$

It is proposed that the mechanism of this enzymatic reaction is similar to that proposed for the Bayer-Villager oxidation of d-camphor by 40% peracetic acid in a sodium acetate buffer (4). The molecular oxygen necessary for the enzymatic reaction is reduced to a peroxide which is either closely associated with the iron atoms or actually forms an enzymatic peracid. The activated peroxide or peracid then attacks the carbonyl of the d-camphor and the lactone is produced

by migration of the most heavily substituted carbon atom. A similar mechanism has been proposed for the enzymatic production of a steroidal lactone for the ketone (5).

In order to test the hypothesis that a peracid or activated peroxide is formed as the active species of the ketolactonase, it is proposed that the enzyme system be exposed to a number of substrate analogues which are able to undergo reactions other than lactonization which are characteristic of peracids. Peracids cause the epoxidation of carbon-carbon double bonds and oxidize primary and tertiary amines (6). In general, amine oxidation and epoxide formation are more facile than the attack on ketones by peracids (6).

The compounds selected for this study are 2-methylene bornane (7,9), bornylene (8), bornylamine (8), neobornylamine (8), bornyldimethylamine (8), and neobornyldimethylamine (8). The stereochemistry of the substrate analogues presents no problems for binding as all the above mentioned compounds may be prepared from d-camphor. Products from the attack of the peracid on most of the above compounds have been previously reported. Epoxidation of bornylene should produce 2,3-epoxybornane (10). Oxidation of the primary amines should produce the 2-nitrobornanes (11) while

oxidation of the tertiary amines will produce bornyldimethylamine N-oxides (8). Formation of the epoxide of 2-methylene bornane may be produced by a process similar for the preparation of camphene oxide (12).

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PROPOSITION III

It is proposed to study allosteric transitions in proteins by fluorescence depolarization.

Allosteric enzymes (1) show large changes in catalytic activity in the presence of small molecules called allosteric effectors. A model (2) for the mechanism of the allosteric phenomenon, based largely on kinetic evidence, has been proposed. The model suggests that the allosteric effectors bind to specific sites on the protein and cause a conformational change in the protein structure. This conformational change in some manner alters the catalytic activity of the enzyme. Obviously evidence supporting or refuting such a conformational change is necessary for determining the validity of the theory. Towards the goal of finding such evidence it is suggested that the fluorescence depolarization of an appropriate allosteric enzyme be studied in the presence of the allosteric effectors of the protein. If there is a conformational change and if this change is proportional to the amount of effector (2), then the amount of fluorescent polarization should be a function of the concentration of the effector.

The theory proposed for the mechanism of allosteric action suggests that the protein exists in several con-

formational states which are all in equilibrium. The allosteric effectors (inhibitor or activator) all possess different affinities for the different states. Addition of any one of the allosteric effectors shifts the equilibrium towards the conformational state for which it has the greatest affinity. Since the different conformational states have differing catalytic activities towards the substrate, the catalytic behavior of the enzyme seems to be altered. The proposed theory thus predicts that the amount or mole fraction of the protein in a particular conformational state will be a function of the concentration of the allosteric effector.

Theoretical treatments of the fluorescence depolarization (3,4) of fluorescent molecules covalently attached to protcins show that the amount of depolarization is proportional to the size and shape of the protein. Weber (4) has shown that

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{x_i a_i}{\frac{1}{p_i} - \frac{1}{3}}}$$

where

$\bar{p}$ = partial polarization observed

a_i = a factor accounting for the change in fluorescence intensity due to the environment of the i^{th} oscillator (5)

X_i = mole fraction of the i^{th} oscillator in the environment characterized by a_i (5)

p_i = partial polarization of the i^{th} oscillator. This factor takes into account the size and shape of the protein.

If one proceeds from this equation for n oscillators bound to each protein molecule, then the previous equation reduces to

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{X_1 \sum_{j=1}^{n} \frac{a_{j,1}}{\frac{1}{p_{j,1}} - \frac{1}{3}} + X_2 \sum_{j=1}^{n} \frac{a_{j,2}}{\frac{1}{p_{j,2}} - \frac{1}{3}} + \ldots + X_m \sum_{j=1}^{n} \frac{a_{j,m}}{\frac{1}{p_{j,m}} - \frac{1}{3}}}$$

where $X_1, X_2, \ldots, X_m$ are the mole fractions of protein conformer; $a_{j,1}, a_{j,2}, \ldots, a_{j,m}$ are the environmental constants for the n oscillators in their respective mole fractions, and $p_{j,1}, p_{j,2}, \ldots, p_{j,m}$ are the partial polarizations of the n oscillators in their respective mole fractions. The summation terms will be constant for constant temperature and viscosity so that

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{X_1 b_1 + X_2 b_2 + \ldots + X_m b_m}$$

where $b_1, b_2, \ldots, b_m$ represent the constant summation terms. As can be seen the observed partial polarization, $\bar{p}$, is a function of the mole fractions of the protein conformers which are in turn functions of the con-

centration of the allosteric effector. Therefore a plot of $\frac{1}{1/\bar{p} - 1/3}$ vs. allosteric effector concentration should give a curve with a slope other than zero unless the factors $b_1, b_2, \ldots, b_m$ are equal (the case of no conformational change).

In the ideal case (2) there are only two conformational states. The previous equation then reduces to

$$\frac{1}{\bar{p}} - \frac{1}{3} = b_2 + X_1 (b_1 - b_2)$$

The equations derived by Monod et. al. (2) for allosteric enzymes show that

$$X = \frac{(1+e)^n}{L(1+ce)^n + (1+e)^n}$$

where

$e = \frac{\text{allosteric effector}}{\text{dissociation constant of the effector to one conformer.}}$

L = equilibrium constant between the two conformers.

c = ratio of the dissociation constants of the effector to both conformers.

n = no. of binding sites where the effector may bind.

Therefore a plot of $\frac{1}{1/\bar{p} - 1/3}$ vs. allosteric effector concentration might be expected to give a sigmoidal curve which is also characteristic of the velocity vs. substrate concentration of many allosteric enzymes. These equations, which appear to predict kinetic data quite well, are only proposed and are derived using the vastly

simplifying assumption that the binding constants for an allosteric effector are all equal for one conformer if there is more than one site.

Aspartate transcarbamylase (ATCase) (6) is one of the most thoroughly investigated and characterized of the proteins exhibiting allosteric behavior. The previous fact plus the advantage of being able to obtain the enzyme in high purity (7) makes this allosteric protein the one of choice for a physical examination.

It has been suggested (8) that ATCase exists in two conformers of differing affinities for the substrate-aspartate. Cytidine triphosphate acts as an allosteric inhibitor by decreasing the affinity of the enzyme for the substrate. Treatment of ATCase with heat, urea, or mercurials splits the protein into monomers which are still catalytically active but which have lost their sensitivity to the allosteric inhibitor. These monomers can be separated into a catalytically active fraction and a cytidine triphosphate binding fraction (9). In the presence of substrate the sedimentation rate of ATCase decreased by 6% (10) and optical rotary dispersion studies on ATCase show a change in the amount of helical structure in the presence of both aspartate and cytidine triphosphate (11). Both these observations

indicate a conformational change of the protein.

It is proposed to conjugate ATCase with some small fluorescent organic molecule such as 1-dimethylaminonaphthalen-5-sulphonyl chloride (12) or fluorescein isothiocyanate (13). Procedures (12, 13) for formation of the conjugate usually call for stirring the fluorescent molecule with the protein for several hours. Since the protein conformers are in equilibrium, a homogeneous population of fluorescent conjugates should be formed. Another alternative would be to form the conjugates with the monomers and recombine these conjugate monomers to the active enzyme (9). Of course, the kinetics must be reexamined after formation of the fluorescent conjugate to see that the enzyme has not lost its catalytic ability or sensitivity to the allosteric effectors. The formation of the fluorescent conjugate may have to be carried out in the presence of substrate analogues and/or allosteric effector if it is found that binding sites are blocked by the added fluorescent molecule. A fluorescent conjugate of some well-known nonallosteric enzyme such as chymotrypsin should be used as a control to see what the effect of change in viscosity due to the addition of the effectors has on the fluorescence polarization of this enzyme. An apparatus described by Johnson and Richards (14) can measure the degree of

polarization of fluorescent radiation to an accuracy of 2% or better which should be adequate to distinguish the difference in conformers of ATCase.

References for Proposition III

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Proposition IV

An objection to determining isotope effects for enzymatic reactions using deuterium oxide as the source of the isotope, as well as the solvent, is that protein hydration will vary with the isotopic concentration of the water. A variation in the hydration of the enzyme may lead to an alteration of the conformational details of the protein which may lead to a change in the catalytic function of the enzyme (1). A possible solution to this problem would be to determine the isotope effect with tritium oxide which would only be present in a minute molar fraction and would have a negligible effect on the protein hydration.

The reactions of several enzymes fumarase (1) and aconitase (2) have recently been studied in deuterium oxide as the solvent. These studies have shown large isotope effects (two and larger) which indicate that a hydrogen is being added in the rate-determining step of the reaction. However, in the study of the reverse reaction with the appropriately labeled substrates, neither fumarase (3) or aconitase (2) exhibited a primary isotope effect.

The theory of microscopic reversibility (4) predicts that if a hydrogen is added in the rate-determining step then the removal of that hydrogen in the reverse reaction

also occurs in the rate-determining step. Therefore, one would expect a large isotope effect (two or greater) for both reactions. A possible objection to the comparison of isotope effects of enzymatic reactions determined in deuterium oxide and in water is that protein hydration may vary with the isotopic concentration of the water. With altered protein hydration the conformational details of some proteins may so be changed as to influence their catalytic activity.

In order to keep the variation in protein hydration to a minimum, it is proposed to measure the isotope effect using tritium oxide as the source of the hydrogen isotope rather than deuterium oxide. Since the amount of tritium oxide in the medium necessary for detection is much less than one percent, the variation in hydration due to the isotopic labeling of the water will be negligible. The isotope effect would then be determined by the Schmidt method (5). For instance, tritium oxide and fumaric-1, 4-C^{14} acid would be the initial reaction mixture for determining the isotope effect for fumarase. The activity of an aliquot of the fumaric-1, 4-C^{14} acid and tritium oxide mixture would be determined and compared with the activity of the l-malic-3-T-1, 4-C^{14} acid produced in the reaction.

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Proposition V

A study of the reaction of imidazole-1-phosphonate with phosphorylated nucleotides is proposed.

Recent studies (1, 2, 3) with phosphorylating systems from mitochondria suggest the possibility that imidazole-1-phosphonate ($\overline{CH:CH.N:CH.N}.PO_3H_2$, PI) may play an important role in oxidative phosphorylation. A recent study (4) showed that phosphorylation of adenosine-5'-monophosphate (AMP) occurred when a ferrochrome solution was oxidized by oxygen in a N, N-dimethylacetamide solution containing imidazole, AMP, and inorganic phosphate. PI was identified as an intermediate in this model reaction for oxidative phosphorylation.

A cursory study of the reaction of PI(as the imidazolium salt), with AMP, ADP (adenosine-5'-diphosphate), and ATP (adenosine-5'-triphosphate) in dimethylformamide has been conducted (5) with the following results:

1) Even in the early stages of the reaction of AMP with PI the major product of the reaction was not ADP but ATP.
2) At equivalent concentrations, the relative initial rates for the reaction of PI with AMP, ADP, and ATP in N, N-dimethylformamide solution at 23° are

$$R_{AMP}:R_{ADP}:R_{ATP} = 1:350:140$$

These results are rather surprising since one would expect the addition of each succeeding phosphate to become more difficult. In fact, phosphoramidic acid ($H_2 \cdot N \cdot PO_3 \cdot H_2$), a similar phosphorylating agent to PI, produced ADP from AMP and ATP from ADP in a step-wise sequence with the formation of ADP from AMP more facile than the production of ATP from ADP (6). Unfortunately, the lack of experimental detail in the rate study does not allow one to have much confidence in the above values. These experimenters apparently followed only the appearance or disappearance of ATP which may not be a good indicator of the amount of reaction since it was only produced in 5% yield (7).

In order to evaluate the above data, it is proposed to redetermine the kinetic constants for the rate of reaction of PI with AMP, ADP, and ATP in a dimethylformamide solution. If the same value for rates is obtained as in the previous experiment, then one might postulate a PI-AMP,-ADP, and-ATP complex which is the active phosphorylating agent via an intramolecular reaction which is more facile for ADP and ATP than AMP. If this postulate is correct then the complex probably forms between the imidazolium ion and the adenine. Therefore, altering the base by substitution of adenine

with other purines and pyrimidines should alter the stability of the complex and, thereby, the rate of the reaction.

Two other questions must also answered about this reaction. First, in acid aqueous solution two PI molecules react to form imidazole-1, 3-diphosphate (DPI) which then hydrolyzed (8). DPI is a much more active phosphorylating agent than PI and, therefore, may be the active phosphoylating agent. This could be checked by comparison of rates of PI to form DPI with the rate of disappearance of AMP, ADP, and ATP and identification of DPI in the reaction medium. Second, both DPI and PI phosphorylate aromatic amine and tertiary amines (8) which are both present in adenine. Therefore, it will be necessary to detect the presence of any N-phosphorylated adenine in the medium.

All nucleotides suggested for use in this proposal may be separated quantitatively on ion-exchange thin layer plates (9). A procedure for the quantitative elution of the nucleosides and the determination by ultraviolet spectrophotometry has been reported. Hopefully the N-phosphorylated compounds can be separated in this manner.

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