KINETIC MODELS FOR ALLOSTERIC ENZYMES: YEAST DIPHOSPHOPYRIDINE NUCLEOTIDE - SPECIFIC ISOCITRIC DEHYDROGENASE

by

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A Thesis

Submitted to

the Faculty of Graduate Studies and Research

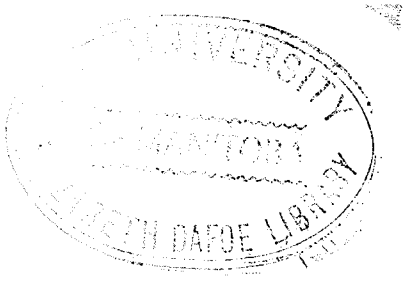
University of Manitoba

In Partial Fulfilment

of the Requirements for the Degree

Master of Science

1965



TO MY FATHER

ACKNOWLEDGEMENT

The writer extends her sincere thanks to Dr. B. D. Sanwal for his patient advice and generous assistance during the course of this work.

ABSTRACT

The DPN specific isocitric dehydrogenase from baker's yeast was purified approximately 30-fold. The enzyme required AMP for maximal activity both at pH 6.5 and at pH 7.8. In the presence of AMP the rate-concentration curves at pH 7.8 were markedly sigmoid when isocitrate was the varied substrate, and hyperbolic when DPN was the varied substrate. Citrate activated the enzyme at pH 7.8 in the presence of AMP and converted the sigmoid rate-concentration curves to rectangular hyperbolas. At pH 6.5, however, citrate brought about activation only in the absence of AMP. Activation and product inhibition studies indicated that the mechanism of isocitric dehydrogenase reaction was ordered in the presence of AMP, but possibly changed to random in its absence.

ABBREVIATIONS

The following abbreviations are used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; AMP, adenosine-5'-monophosphoric acid; ADP, adenosine-5'-diphosphoric acid; IDH, isocitric dehydrogenase; EDTA, ethylenediaminetetracetic acid; Tris, tris (Hydroxymethyl)-aminomethane; DEAE-cellulose, diethylaminoethyl-cellulose.

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INTRODUCTION

Sanwal *et al* (1965) working with *Neurospora* and Atkinson *et al* (1965) using yeast have recently published models for the mechanism of DPN-specific isocitrate dehydrogenase reaction. The two models differ both in the manner of approach to the problem and in certain specific features, notably the mechanisms involved in the interpretation of the sigmoid rate-concentration data characteristic of this enzyme.

The data of Atkinson *et al* (1965) are analyzed on the basis of the Hill equation for the binding of oxygen to hemoglobin (Equation 1)

$$y = \frac{K\,(O_2)^n}{1 + K(O_2)^n} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

(where y = fraction of sites bound by O_2, K is a complex dissociation constant, and n is the number of interacting binding sites). By making certain assumptions (see Historical), Atkinson *et al* (1965) derive the expression (equation 2)

$$\log \left(\frac{v}{V-v} \right) = n \log (S) - \log K \quad \ldots\ldots\ldots\ldots \quad (2)$$

(where v = initial velocity, V = maximal velocity, and S = substrate concentration), which is the equation of a straight line having slope = n. The effect of varying

concentrations of reactants is expressed in terms of fluctuations in the value of n: the term, n, is said to be a measure of the strength of interactions between substrate- and modifier-specific sites. As a result of such analysis, Atkinson et al (1965) conclude that AMP causes isocitrate to bind more readily to the enzyme by lowering the dissociation constant for isocitrate at all sites. Also, the sigmoid nature of initial rate-concentration data is interpreted to be due to a multisite binding of isocitrate on the enzyme surface, such that each successive binding of the substrate lowers the dissociation constants of the remaining unfilled sites ('subunit interaction').

The model of Sanwal et al (1965) is based on the steady-state kinetic theory, which has undergone very sophisticated developments in recent years (Dalziel, 1957; Cleland, 1963a, b, c). According to this model the sigmoid initial velocity plots are interpreted as resulting from a two-site, sequential binding of isocitrate (one allosteric and one catalytic) such that binding at the catalytic site is only possible after binding of the substrate at the allosteric site. Citrate is considered to activate the enzyme by binding at the allosteric site and AMP by ordering the binding of the substrates and the liberation of the products.

In view of the conflicting interpretations proposed for enzymes from two related organisms, it became essential to

examine whether the enzyme from yeast cells was amenable to steady-state approach and if interpretations other than those proposed by Atkinson *et al* (1965) were possible. The following report accordingly deals with the kinetics of NAD-specific isocitrate dehydrogenase from yeast cells, both in the presence and absence of AMP.

HISTORICAL

I. GENERAL

Initial studies of enzyme regulation were concerned with induction and repression of enzymes by their substrates and products, respectively. Repressible and inducible systems were found to be analogous in that initiation of repression (cessation of induction) resulted in a gradual decrease in enzyme activity due to interruption of gene activity and progressive dilution of the enzyme (Monod and Jacob, 1961).

Novick and Szilard (1954), however, showed that repression of tryptophan synthetase by tryptophan resulted in immediate inhibition of activity; therefore a control mechanism other than repression was involved. In 1956, Umbarger working on isoleucine synthesis and Yates and Pardee working on pyrimidine synthesis demonstrated feedback inhibition of the first enzyme of each pathway by the end product of the pathway. A comparable effect was recognized in studies of precursor activation (Sanwal *et al.*, 1963) where precursors of a catabolic or anabolic pathway stimulated the activity of the enzyme which mediated the formation of the end product of that pathway. Distinctive of both types of systems was the fact that the effector (end product or precursor) was not necessarily

related sterically to the substrate of the enzyme it controlled, implying a mechanism different from that of repression.

In several enzymic systems such as phosphoribosyl-ATP-pyrophosphorylase (Martin, 1963), aspartate transcarbamylase (Gerhart and Pardee, 1962) and biosynthetic threonine deaminase (Changeux, 1961) it was found that susceptibility of the enzyme to end product inhibition could be destroyed, without loss of enzyme activity. This led to the suggestion (Monod et al., 1963) that the binding sites for the end products on the enzyme surface were stereospecifically distinct and physically separate from the binding sites of the substrates. Monod et al (1963) coined the term "allosteric" to describe the regulatory enzymes. The substrate receptor was termed the active site; the receptor for the allosteric effector, the allosteric site. Binding of the effector was presumed to cause a reversible alteration in the affinity of the active site for the substrate. Such site interactions were known to occur in the hemoglobin molecule, which became the established model for theories of allosterism (Monod et al., 1963, 1965, Atkinson, et al., 1965).

The biological significance of allosterism (Monod et al., 1963) rests on the susceptibility of allosteric proteins to inhibition and activation by metabolites not immediately involved in the reaction. Since effectors influence the activity of enzyme molecules, allosteric

regulation provides a much more sensitive metabolic control than do induction and repression, which regulate enzyme synthesis and are therefore not fully effective until one or two generations have elapsed (Moyed, 1961).

Several allosteric enzymes (threonine deaminase, aspartate transcarbamylase, etc) not only respond to the pathway-specific precursors or end products, but are also affected (activation or inhibition) by metabolites of the related pathways. As an example, aspartate transcarbamylase (Gerhart and Pardee, 1962) is not only affected by cytidine triphosphate (end product) but also by ATP. The ability of such enzymes to respond to compounds not involved in the pathway may enable them to integrate biosynthesis with the other metabolic pathways. It has been suggested (Sanwal et al., 1963) that precursor activation may serve an important function in ensuring that excessive amounts of the precursor are rapidly metabolized.

II. ISOCITRATE DEHYDROGENASE

Most organisms examined so far have been found to possess two isocitrate dehydrogenases, one specific for TPN and another for DPN. The DPN-specific enzyme, which catalyzes the reaction

$$D_S\text{-isocitrate} + DPN \longleftrightarrow \alpha\text{-ketoglutarate} + CO_2 + DPNH + H^+$$

has been shown to be allosteric and susceptible to activat-

tion by various nucleotides. Thus, the enzyme from guinea pig heart (Plaut and Sung, 1954) and from locust flight muscle (Goebell and Klingenberg, 1964) is activated by ADP. The enzyme from yeast (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963) and Neurospora (Sanwal et al., 1963) is activated by AMP.

The mechanism of activation by nucleotides is not known with certainty. Plaut and his coworkers (Chen et al., 1964) demonstrated that bovine heart isocitrate dehydrogenase undergoes polymerization in the presence of ADP. This aggregation reaction, however, could not be demonstrated for the Neurospora enzyme in the presence of AMP (Sanwal and Stachow, 1965).

In addition to the nucleotide activation, the microbial enzymes are susceptible to stimulation by citrate (Sanwal et al., 1963; Hathaway and Atkinson, 1963). Hathaway and Atkinson (1963) considered that AMP and citrate show "some type of cooperative phenomenon" in the binding of substrates and activators. They suggested the presence of two sites on the enzyme: a reaction site binding isocitrate and citrate, and an activating site binding, isocitrate, AMP, and citrate.

Under optimal reaction conditions, plots of velocity against substrate concentration for isocitrate dehydro-

genase from diverse sources (Klingenberg et al., 1965; Hathaway and Atkinson, 1963; Sanwal et al., 1963) in common with most allosteric proteins (Gerhart and Pardee, 1962; Changeux, 1961, 1962; Monod et al., 1965) are sigmoidal rather than hyperbolic.

Sanwal et al (1963,1964) demonstrated that at its pH optimum of 7.6, IDH from N. crassa yielded sigmoidal rate concentration curves and parabolic double reciprocal plots. Since reciprocal plots of velocity against square of substrate concentration were linear, they suggested that the enzyme had two substrate binding sites, one active and one activating. The reaction was activated by citrate and inhibited by ∝-ketoglutarate (precursor and end product, respectively) at unsaturating concentrations of isocitrate. At pH 6.5 the enzyme followed classical Michaelis-Menten kinetics, and the initial velocity of the reaction was not affected by either citrate or ∝-ketoglutarate, suggesting that the allosteric site became nonfunctional at the lower pH.

III. ALLOSTERIC MODELS

Three models have recently been proposed to account for allosteric interactions. Monod et al (1965) have presented a general model based on considerations of symmetry within the protein molecule. Atkinson et al (1965) and Sanwal et al (1965) have presented specific

models for the mechanism of NAD-specific IDH.

1. The 'subunit interaction' model of Monod is based on the following assumptions:

(a). The allosteric protein is a symmetrical polymer.

(b). The conformation of the associated monomers is constrained in the intact polymer.

(c). Allosteric proteins can exist in two states, differing in conformation and therefore in monomer constraint.

(d). Affinity of receptor sites for ligands is altered in the transition from one state to the other. Substrates and activators preferentially stabilize one form of the protein, inhibitors the other (competition between substrate and inhibitor is explained on this basis).

(e). The sigmoidity of velocity <u>versus</u> substrate curves is due to interactions between subunits of the enzyme, caused by sequential binding of ligands to specific receptor sites.

(f). Each subunit bears only one receptor site of a particular conformation.

The change of state caused by binding of successive ligand molecules is expressed as

$$R_0 \xleftrightarrow{L} T_0$$

The K_{eq} of the transition is termed the allosteric constant (L). In the binding of ligand F,

$$F + Site_R \xleftrightarrow{K_R} F\,S_R$$

$$F + Site_T \xleftrightarrow{K_T} F\,S_T$$

the molecule is assumed to be symmetrical and the binding of ligands independent; K_R and K_T are therefore the same for all sites in a given conformational state.

When n = no. of subunits (homologous sites) the reaction sequence is

$$R_0 \xleftrightarrow{L} T_0$$

$$R_0 + F \xrightarrow{K_R} R_1$$

$$\vdots$$

$$R_{n-1} + F \xrightarrow{K_R} R_n$$

$$T_0 + F \xrightarrow{K_T} T_1$$

$$\vdots$$

$$T_{n-1} + F \xrightarrow{K_T} T_n$$

There are n types of R-F combinations: FR, (2F)R, (3F)R, etc. The number of possible species of each combinations is given by the binomial theorem:

R_0 and R_n are unique

R_1 exists in $\frac{n(n-1)}{2!}$ species

R_n exists in $\frac{n(n-1)(n-2)}{3!}$ species etc.

Therefore:

$$K_{R_1} = n \frac{R_0 F}{R_1}$$

$$K_{R_2} = \frac{n-1}{2} \frac{R_1 F}{R_2} = \frac{n(n-1)}{2!} \frac{R_0 F^2}{K_R R_2}$$

$$K_{R_3} = \frac{n-2}{3} \frac{R_2 F}{R_3} = \frac{n(n-1)(n-2)}{3!} \frac{R_0 F^3}{K_R^2 R_3}$$

etc.

Since $K_{R_1} = K_{R_2} = K_{R_3}$,

$$R_1 = \frac{n R_0 F}{K_R}$$

$$R_2 = \frac{n-1}{2} \frac{R_1 F}{K_R} = \frac{n(n-1)}{2!} \frac{R_0 F^2}{K_R^2}$$

$$\vdots$$

$$R_n = \frac{1}{n} \frac{R_{n-1} F}{K_R} = \frac{1}{n} \frac{R_0 F^n}{K_R^n}$$

By similar reasoning,

$$T_1 = \frac{n T_0 F}{K_T}$$

$$\vdots$$

$$T_n = \frac{1}{n} \frac{T_{n-1} F}{K_T} = \frac{1}{n} \frac{T_0 F^n}{K_T^n}$$

The fraction of the protein in the R state, $\bar{R}$, equals

$$\frac{R_0 + R_1 + \text{---} + R_n}{(R_0 + \text{---} + R_n) + (T_0 + \text{---} + T_n)} \quad \text{.....} \quad (3)$$

The fraction of sites bound by the ligand, $\overline{Y}_F$, equals

$$\frac{(R_1 + 2R_2 + \text{---} + nR_n) + (T_1 + \text{---} + nT_n)}{n\left[(R_0 + \text{---} + R_n) + (T_0 + \text{---} + T_n)\right]} \quad \ldots\ldots\ldots (4)$$

let $\frac{F}{K_R} = \alpha$ and $\frac{K_R}{K_T} = c$

Then equation (3) becomes

$$\overline{R} = \frac{R_0 + R_0 n\alpha + R\frac{n(n-1)}{2!}\alpha^2 + \text{---} + R_0\frac{n!}{n!}\alpha^n}{(R_0 + \text{---} + R_0\alpha^n) + (T_0 + T_0 n\alpha c + \text{---} + T_0\alpha^n c^n)}$$

$$= \frac{R_0\ (1+\alpha)^n}{R_0(1+\alpha)^n + T_0(1+\alpha c)^n}$$

$$= \frac{(1+\alpha)^n}{(1+\alpha)^n + L\ (1+\alpha c)^n} \quad \ldots\ldots\ldots\ldots\ldots (5)$$

The saturation function, $\overline{Y}_F$, equals

$$\frac{(R_0 n\alpha + 2R_0\frac{n(n-1)}{2!}\alpha^2 + \text{---} + nR_0\alpha^n) + (T_0 n\alpha c + \text{---} + nT_0\alpha^n c^n)}{n\left[R_0(1+\alpha)^n + T_0\ (1+\alpha c)^n\right]}$$

$$= \frac{R_0 n\alpha(1+\frac{n-1}{1}\alpha \text{---} + \alpha^{n-1}) + T_0 n\alpha c(1+ \text{---} + \alpha^{n-1}c^{n-1})}{R_0\ n\left[(1+\alpha)^n + L\ (1+\alpha c)^n\right]}$$

$$= \frac{R_0 n\alpha(1+\alpha)^{n-1} + T_0 n\alpha c\ (1+\alpha c)^{n-1}}{R_0 n\left[(1+\alpha)^n + L(1+\alpha c)^n\right]}$$

$$= \frac{\alpha(1+\alpha)^{n-1} + L\alpha c\,(1+\alpha c)^{n-1}}{(1+\alpha)^{n} + L\,(1+\alpha c)^{n}} \qquad \ldots\ldots\ldots\ldots(6)$$

When $c \longrightarrow 0$ (i.e., $K_T \gg K_R$)

$$\overline{Y}_F = \frac{\alpha(1+\alpha)^{n-1}}{(1+\alpha)^{n} + L} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7)$$

When $c = 1$ (i.e., affinity of R and T for F is equal)

and $L \longrightarrow 0$

$$\overline{Y}_F = \frac{\alpha}{1+\alpha} = \frac{F}{K_R+F}$$

which is identical in form to the Michaelis-Menten equation.

By these equations, the model accounts for substrate-substrate interaction, the "degree of cooperativity" being dependent on the values of n, L, and c.

According to the model, substrate-activator and substrate-inhibitor interactions are due to displacements of the equilibrium between the R and T states. When the inhibitor binds preferentially to the T state and the activator to R, the apparent allosteric constant L' is defined as

$$L' = \frac{T_1 + \text{---} + T_n}{R_1 + \text{---} + R_n} = \frac{\sum_0^n T_I}{\sum_0^n R_A}$$

When $\beta = \frac{I}{K_I}$ and $\gamma = \frac{A}{K_A}$, and following the pattern of

reasoning for deriving equations (5) and (6),

$$L' = \frac{nT_0\beta + \frac{n(n-1)}{2!}T_0\beta^2 + \cdots + \frac{1}{n}T_0\beta^n}{nR_0\gamma + \frac{n(n-1)}{2!}R_0\gamma^2 + \cdots + \frac{1}{n}R_0\gamma^n}$$

$$= \frac{T_0}{R_0}\frac{(n\beta + \cdots + \frac{1}{n}\beta^n)}{(n\gamma + \cdots + \frac{1}{n}\gamma^n)}$$

$$= L\frac{(1+\beta)^n}{(1+\gamma)^n}$$

Substituting L' for L in equation (7),

$$\bar{Y}_S = \frac{\alpha(1+\alpha)^{n-1}}{(1+\alpha)^n + L\frac{(1+\beta)^n}{(1+\gamma)^n}}$$

According to this equation, the function of effector is to modify substrate-substrate interaction, causing changes in the shape of the substrate saturation curve. The activator "abolishes the cooperativity of the substrate"; the curve becomes hyperbolic. The inhibitor has the opposite effect.

While this model accounts for cooperative interactions between the same (homotropic) and different (heterotropic) ligands, and offers an explanation for desensitization and dissociation, no attempt has been made to eliminate other possible models (see Discussion). Furthermore, since the constants in equation (6) are thermodynamic equilibrium constants and involve no rate expression,

their usefulness is restricted to physico-chemical studies: they cannot validly be applied to kinetic data.

2. Atkinson, Hathaway, and Smith (1965) determine the order of the IDH reaction for each of the reactants (isocitrate, DPN, Mg^{++}, AMP) from the equation

$$K = \frac{(E)\ (S)^n}{(ES_n)}$$

where E = free enzyme

S = substrate

S_n = the complex of n S molecules per E mol.

$K = K_1\ K_2$ --- K_n,

the product of the n dissociation constants of the separate binding steps. It is assumed

(a) that the reaction $ES_{n-1} + S \longrightarrow ES_n$ is the only rate-limiting step

(b) that the concentrations of all ES complexes involving less than n molecules of S are negligibly small (or, in rapid equilibrium). This is shown to be possible mathematically when the binding of S is "sufficiently cooperative", i.e., when the values of the dissociation constants decrease for each successive S molecule bound.

Under the above conditions,

$v = K\ (ES_n)$

$$V_{max} = k \left(E_T \right)$$

where k = rate constant

v = reaction rate

V_{max} = rate at S saturation

E_T = total enzyme

Since $E = E_T - ES_n$

$$= \frac{V_{max} - v}{k},$$

$$kE = V_{max} - v$$

$$\therefore \frac{(E\ S_n)}{(E)} = \frac{v}{V_{max}-v} = \frac{(S)^n}{K}$$

$$\therefore \log \frac{v}{V_{max}-v} = n \log (S) - \log K \quad \text{(equation 2)}$$

Plots of $\log \frac{v}{V-v}$ against $\log (S)$ yield straight lines of slope = n. The slope of the lines is considered to be a function both of the number of interacting sites per molecule and of the "strength" of the interactions.

Calculations of "interaction factors", measuring the dependence of n on the strength of site interactions, are based on the assumptions:

(a) Binding of one substrate molecule facilitates binding of others.

(b) The intrinsic dissociation constants for

isocitrate from all sites are equal.

(c) Binding at any site has the same effect on all unbound sites.

(d) The rate of reaction of each complex is proportional to the number of substrate molecules bound.

The lines approach n=1 at low substrate concentrations. As the interaction factor is increased, n approaches a value numerically equal to the number of substrate sites per molecule.

Since n, the order of the reaction, is found to be 3.9 for isocitrate and 1.7 - 2.0 when DPN, Mg^{++}, or AMP is varied, Atkinson et al., (1965) conclude that the IDH molecule probably has two catalytic sites, binding isocitrate, DPN, and Mg^{++}, two regulatory sites binding isocitrate, and two regulatory sites binding AMP.

In calculating the overall kinetic order of the reaction, the assumptions of equation (2) are presumed to hold. Since n = 4,

$$v_i = k\ (E\ S_4\ A_2\ D_2\ M_2)$$

$$= \frac{k\ E\ S^4\ A^2\ D^2\ M^2}{K}$$

where K = the overall dissociation constant

S = isocitrate

A = AMP

D = DPN

M = Mg^{++}

As predicted by this equation, eleventh order kinetics are obtained in experiments on dilute systems.

The model, based on calculations from initial velocity data, is as follows:

(a) The results of interactions between substrate and effectors are attributed to modified dissociation constants.

(b) Binding of isocitrate at any site decreases the dissociation constant for isocitrate at other sites by a factor of 20.

(c) Binding of DPN at a catalytic site decreases the dissociation constant for isocitrate by a factor of 20 at that site only; i.e., DPN interactions are indirect, mediated by the effect on isocitrate binding.

(d) Binding of AMP at a regulatory site decreases the dissociation constant for isocitrate at all four sites by a factor of 5. AMP has no effect on isocitrate interactions.

(e) Citrate can bind at any isocitrate site, is a competitive inhibitor of isocitrate at catalytic sites, and has the same effect on other parameters as does isocitrate.

Predictions from this model of the effect on initial velocity of varying isocitrate, DPN, AMP, and citrate

correspond to experimental results. However, as the authors themselves state, some of the assumptions on which the model is based seem unlikely, e.g., the assumption that both catalytic and regulatory sites have the same dissociation constant for isocitrate. A more serious fault is the questionable validity of equation (2) as it is applied to mechanisms about which few facts are known (see Discussion).

3. The model of Sanwal et al., (1965) for IDH of N. crassa is based on the steady-state kinetic theory, as developed by Reiner (1959) and Cleland (1963 a,b,c). The use of the steady-state method of analysis is advantageous for the following reasons:

(a) The full rate equation can be expressed in terms of measurable kinetic constants.

(b) From the rate function, equations for initial velocity and product inhibition can be derived relatively easily.

(c) The nature of the mechanism (ordered or random, pingpong or sequential, presence or absence of dead-end complexes) can be determined directly by inspection of product inhibition data and of changes in initial velocity patterns with varying substrate levels.

Using the steady-state approach, Sanwal et al., (1965, and unpublished) distinguish between two types of allosteric

effects, both of which are based on the assumption that there are two separate substrate binding sites on the enzyme surface, one allosteric (not releasing products) and another catalytic. The two types of allosteric effects are:

(a) Partial allosterism: Here it is assumed that the enzyme-catalytic site-substrate complex (ES) is capable of liberating products, i.e., ES complex is active. The enzyme becomes more active, however, if another molecule of substrate also is bound at the allosteric site (ES'S complex). The velocity in such a case is the sum of activity of ES and ES'S forms,

$$v = V_1 \left(\frac{A}{Ka_1 + A}\right)\left(\frac{K_1}{K_1 + A}\right) + \left(\frac{V_2 A^2}{[Ka_2+A][K_1+A]}\right)$$

(where V_1 and Ka_1 are for ES form and V_2 and Ka_2 are for ES'S form. K_1 is dissociation constant from the allosteric site, A = substrate). This equation reduces to, in the standard reciprocal form,

$$\frac{1}{v} = \frac{1 + (K_1+Ka_1+Ka_2)\left(\frac{1}{A}\right) + (K_1Ka_1+K_1Ka_2+Ka_1Ka_2)\left(\frac{1}{A}\right)^2 + K_1Ka_1Ka_2\left(\frac{1}{A}\right)^3}{V_2 + (V_1K_1+V_2Ka_1)\left(\frac{1}{A}\right) + V_1K_1Ka_2\left(\frac{1}{B}\right)^2} \quad \ldots\ (8)$$

Equation (8) is a 3/2 function, the curves of which have an asymptote. This kind of curve can either be

concave up (if $V_2 > V_1$, and $Ka_1 > Ka_2$) or concave down near the vertical axis of the double reciprocal plots. That this sort of allosteric effect can occur in reality is evident in the recent kinetic work of Gest et al., (1965) on the allosteric homoserine dehydrogenase of Rhodospirillium.

(b) Total allosterism: Here it is assumed that the ES complex has no activity unless the substrate is bound at the allosteric site (ES'S complex) i.e., binding of the substrate at the allosteric site is necessary for the binding of the substrate at the catalytic site and for release of the products. For such a mechanism, the initial velocity equation is given by,

$$v = V\left(\frac{A}{Ka + A}\right)\left(\frac{A}{K_1 + A}\right)$$

or, in the standard reciprocal form,

$$\frac{1}{v} = \frac{K_a K_1}{V}\left(\frac{1}{A}\right)^2 + \left(\frac{K_1 + K_a}{V}\right)\left(\frac{1}{A}\right) + \frac{1}{V} \quad \ldots\ldots\ldots\ldots (9)$$

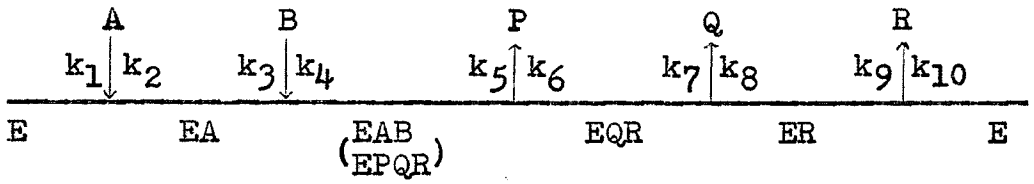
which is, $y = ax^2 + bx + c$

In accordance with the mechanisms proposed by Sanwal et al., (1965), the effect of an allosteric activator would be to change the non-linear double reciprocal plots, predicted by equations (8) and (9), to straight lines (equation 10),

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

provided, either, that the activator can bind at the allosteric site, or, by binding at some other specific site brings about the same conformational changes of the catalytic site as are normally brought about by the substrate binding at the allosteric site.

In the case of IDH from Neurospora specifically, it has been shown (Sanwal et al., 1963, 1964) that allosteric effects do not occur in the presence of AMP at pH 6.4 or, in the presence of AMP and citrate, at pH 7.6. Competitive inhibition by fixed levels of DPNH when DPN is varied under both of the above conditions indicates an ordered binding of substrates and release of DPNH. Product inhibition studies using CO_2 and α-ketoglutarate show that the reaction in the presence of AMP is probably



(Ordered Bi Ter)

(where A is DPN, B is isocitrate, P is CO_2, Q is α-ketoglutarate, and R is DPNH).

When DPN is varied at pH 6.4 and 7.6, the reciprocal plots are linear, indicating that DPN binds at only one site. Plots where isocitrate is varied at pH 6.4

and at pH 7.6 in the presence of citrate are also linear: binding must obviously occur at the allosteric site.

In the absence of citrate at pH 7.6 (when the allosteric site is functional) double reciprocal plots for isocitrate are curved, indicating that isocitrate is bound at two sites. Since only one molecule of isocitrate binds when the allosteric site is occupied by citrate, the second isocitrate site must be the allosteric site.

An equation of the type

$$v = \frac{AB^2+AB^3+ABM+AB^2M}{\text{const.} +A+AM+AB+B+M+ABM+AB^2 +B^2+BM+AB^2M+AB^3+B^3+B^2M}$$

(where A = NAD; B = isocitrate; M = modifier) derived with the assumption of total allosteric effect, is consistent with experimental data in that

1. isocitrate plots are predicted to be $\frac{3}{1}$ functions
2. NAD plots are linear
3. citrate plots are linear, replots of K_{app} being $\frac{3}{2}$ functions of isocitrate.

MATERIALS AND METHODS

I. SOURCE OF ENZYME

Diphosphopyridine nucleotide-specific IDH was extracted from baker's yeast (Fleischmann Company). A lyophilized powder was prepared from the commercial product and stored below $0^{\circ}C$ prior to extraction.

II. EXTRACTION AND PURIFICATION

All purification steps were carried out between 0° and $5^{\circ}C.$, in the presence of 10^{-4} M EDTA.

The powder was suspended, usually 20 gm at a time, in 8 times its volume of 0.1 M $NaHCO_3$. After 1.1/2 hours continuous stirring, the suspension was centrifuged in an RC-2 Sorvall centrifuge at 12,000 r.p.m. for 15 minutes.

The crude extract was purified essentially by the method of Kornberg and Pricer (1951). Alcohol, $MnCl_2$, and heat precipitations were also attempted but were unsuccessful.

The procedure was as follows:

Step 1. Solid ammonium sulfate was added to the extract slowly, with continuous stirring, to a level of 240 mg/ml. After further stirring for 20 minutes to ensure complete precipitation, the suspension was centrifuged at 12,000 r.p.m. for 15 minutes, and the precipitate discarded.

Step 2. Sixty-five mg $(NH_4)_2 SO_4$ per ml was added slowly to the supernatant from Step 1. Following stirring and centrifugation, as in Step (1), the supernatant was discarded.

Step 3. The precipitate from step 2 was resuspended in distilled water (1/3 the volume of original crude extract). An equal volume of 0.1 M acetate buffer, pH 5.5 was added, followed by $(NH_4)_2SO_4$ to a level of 260 mg/ml. The suspension was stirred for 20 minutes and centrifuged at 12,000 r.p.m. for 15 minutes.

Step 4. The precipitate was resuspended in 1/10 the original volume of 0.02 M $NaHCO_3$. To this suspension were added, 3 volumes of distilled water and 1 volume of alumina C γ (40 mg/ml). The mixture was stirred for 10-15 minutes, then centrifuged at 10,000 r.p.m. for 10 minutes.

Step 5. The alumina C γ with adsorbed protein was washed twice with 8-10 volumes of 0.02 M K_2HPO_4 buffer, pH 7.2, and then eluted with 10 ml 0.1 M K_2HPO_4 buffer, pH 7.6. The enzyme in this eluate was purified approximately 30 fold.

Results of a typical purification are given in Table 1.

TABLE I

Purification of DPN Specific IDH from Baker's Yeast

Fraction	Activity units/ml	Total Activity	Protein mg/ml	Specific Activity	Enzyme Recovery
crude	324	66,000	20.4	15.9	
1. sup.	168	36,100	15	11.2	55%
2. ppte.	280	28,000	9.5	29.5	42%
3. ppte.	330	20,400	7.0	47.1	31%
alumina CY eluate	1,200	12,000	2.3	522.0	18%
			purification = X33		

III. CHEMICALS

The following chemicals were used in routine assays:

DL - isocitric acid, trisodium salt, Type 1:
Sigma Chemical Company.

Adenosine-5'-monophosphoric acid, from yeast, sodium salt, Type II:
Sigma Chemical Company.

β-diphosphopyridine nucleotide, from yeast, Grade IV:
Sigma Chemical Company

Reduced β-diphosphopyridine nucleotide, from yeast, disodium salt, Grade III.
Sigma Chemical Company

For experiments on DPNH inhibition, commercial DPNH was purified by chromatography on DEAE-cellulose columns essentially according to the procedure of Silverstein and Boyer (1964).

IV. ENZYME ASSAY

Isocitrate dehydrogenase was assayed by following the rate of DPN reduction at 340 mu. on a Model 2000 Gilford Recording Spectrophotometer. All reaction mixtures contained 3.3 mM $MgCl_2$ and, when AMP was used, 0.67 mM AMP, in addition to the other components. Approximately 80 ug of the purified enzyme was used in each assay. Concentrations of other reactants are given in the legends

to the figures. The standard assay mixture contained:

3.3 mM $MgCl_2$

0.67 mM AMP

0.5 mM DPN

0.67 mM isocitrate

0.1 M Tris or phosphate buffer

The total volume of the reaction mixture was 3.0 ml. Potassium phosphate buffer was used at pH 6.5 and in most of the experiments at pH 7.8. Tris-acetate buffer, pH 7.8, was used in the experiments on DPNH inhibition.

Reactions were run in cuvettes of 10 mm light path, at temperatures of 24° - 26°C.

The unit of activity is defined as the amount of IDH which causes an increase in optical density at 340 mu of 0.01 per minute. Specific activity is expressed as units of activity per milligram protein. The method of Lowry et al., (1951) was used for the determination of protein.

RESULTS

I. STABILITY OF THE ENZYME

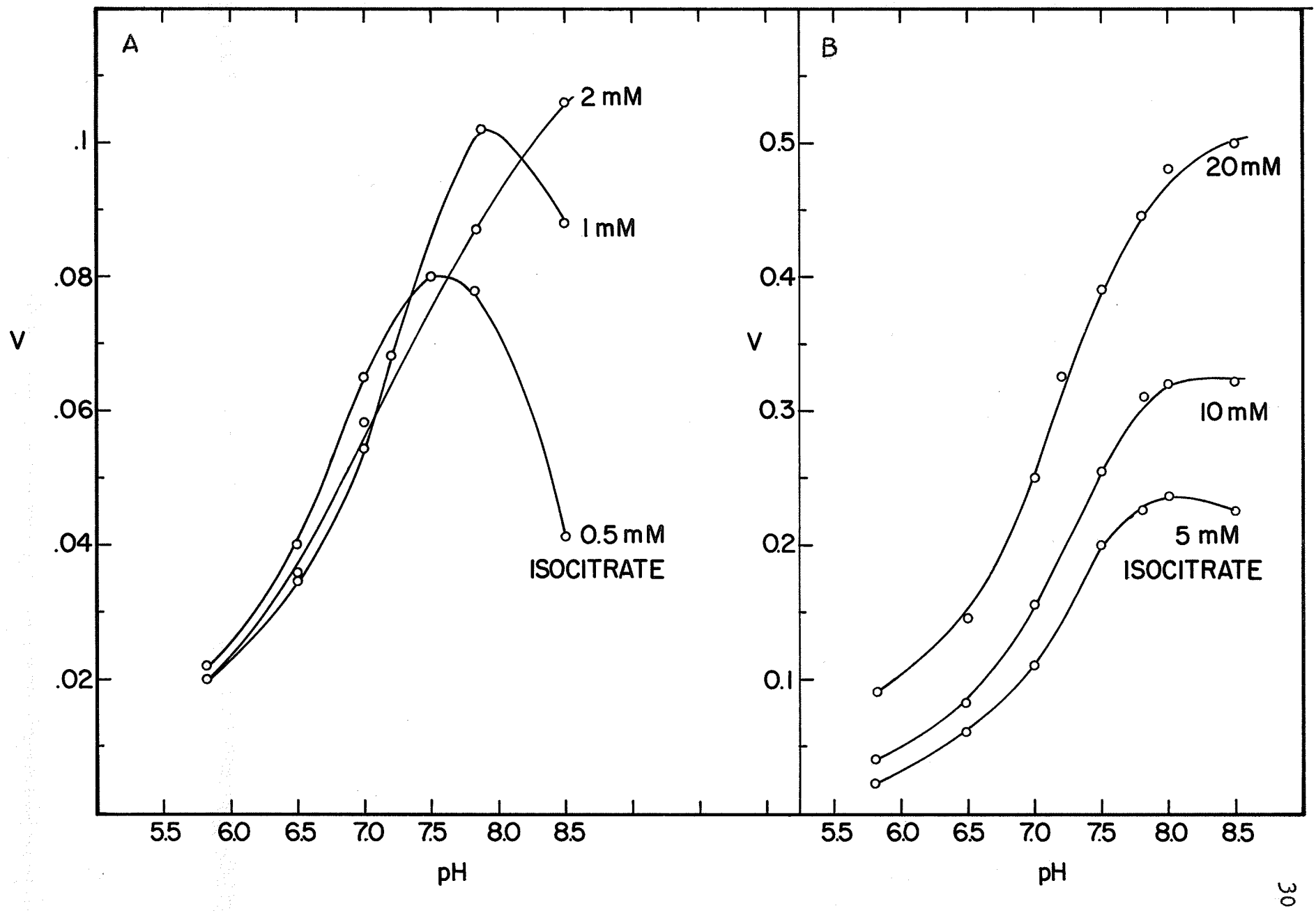
The purified enzyme preparations were stable for 48 hours at 0°C; activity of older preparations was lost very rapidly on thawing. The enzyme was equally stable when stored at pH 7.5 and at pH 6.5.

II. EFFECT OF pH

The pH optimum for reaction under standard conditions was 7.8. This optimum varied, both in the presence and in the absence of AMP, with changing concentrations of isocitrate (Figure 1), shifting toward alkaline pH as isocitrate concentration increased.

The pH profiles given in Figure 1 are important for understanding the reaction mechanism. Since the pH optimum shift occurs both in the presence and absence of AMP, it is clear that this is an effect associated only with isocitrate binding. Obviously also, the dissociation of isocitrate with changing pH could not bring about these effects, because all three pK values of isocitrate are below 5.6. The only reasonable explanation seems to be that binding of isocitrate to the enzyme requires protonation at the active sites.

FIGURE 1. Variation in pH optimum with isocitrate concentration in presence (a) and in absence (b) of AMP. Final concentrations of DPN: (a) 0.5 mM, (b) 2.0 mM.


A
B
2 mM
1 mM
0.5 mM
ISOCITRATE
20 mM
10 mM
5 mM
ISOCITRATE
V
pH
.1
.08
.06
.04
.02
0.5
0.4
0.3
0.2
0.1
5.5
6.0
6.5
7.0
7.5
8.0
8.5

III. INITIAL VELOCITY ANALYSIS AT pH 6.5

The results were similar to those obtained for IDH from Neurospora (Sanwal et al., 1965). The reaction followed normal Michaelis-Menten kinetics at pH 6.5 in the presence of AMP. When DPN was varied against isocitrate as the fixed changing substrate, both the double reciprocal plots (Fig. 2) and the replots of slopes and intercepts (Fig.3) were linear. These data fit the initial velocity equation obeyed by sequential mechanisms,

$$\frac{1}{v} = \left(\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 \right)\frac{1}{V} \quad \ldots\ldots\ldots\ldots (11)$$

(where A and B are DPN and isocitrate, respectively; K_{ia} is the dissociation constant, of A; K_a and K_b are Michaelis constants of A and B, respectively). Kinetic constants determined from the replots were:

K_a	0.15 mM
K_{ia}	2.64 mM
K_b	1.43 mM

When isocitrate was the variable and DPN the fixed changing substrate, the reciprocal plots were also linear (Fig.4).

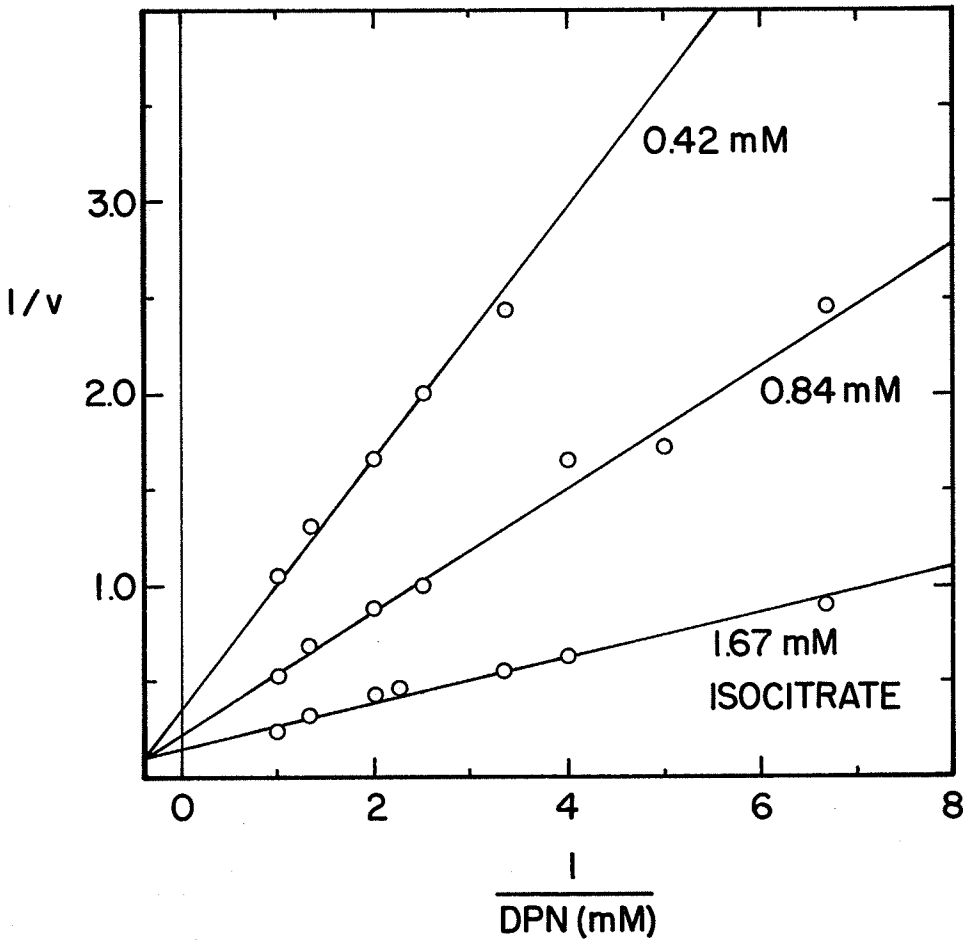


FIGURE 2. Double reciprocal plots of velocity versus varying DPN concentrations at pH 6.5 in the presence of AMP.

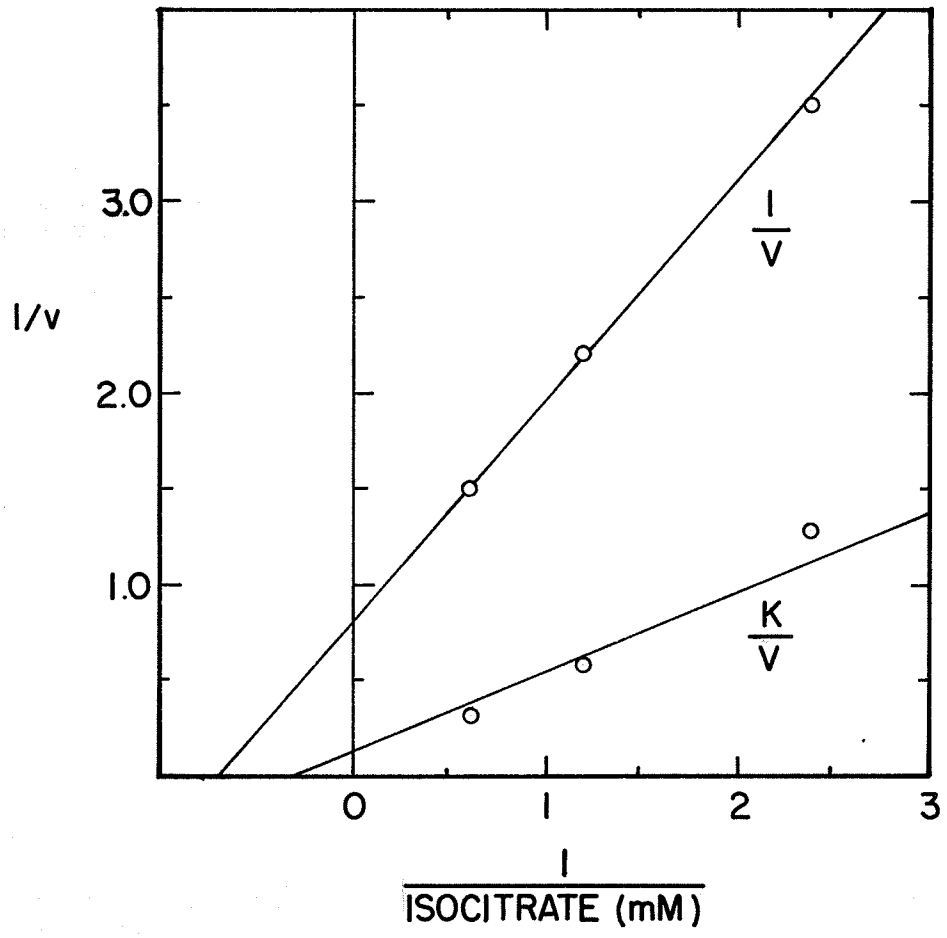


FIGURE 3. Replots of slopes and intercepts from Figure 2.

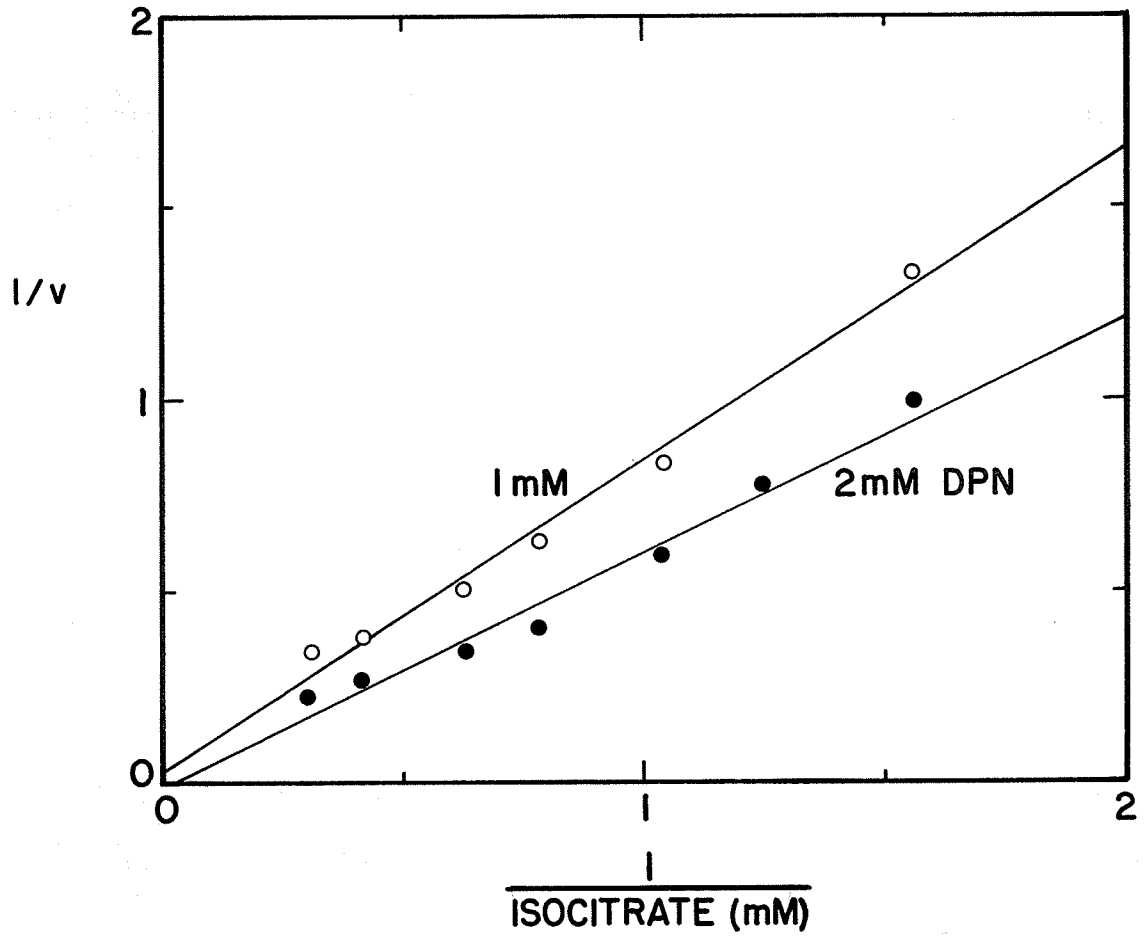


FIGURE 4. Double reciprocal plots of velocity versus varying isocitrate concentrations at pH 6.5 in the presence of AMP.

IV. INITIAL VELOCITY ANALYSIS AT pH 7.8

(a) In presence of AMP

Varying isocitrate in the standard assay at different levels of DPN (Fig.5) yielded curved double reciprocal plots, indicating binding at more than one site. Fits of these curves to a function of the type (equation 12)

$$v = \frac{VB^2}{a + bB + B^2} \quad \ldots\ldots\ldots (12)$$

using iterative procedures yielded significant fits, but the value of the sigmoid constant, b, was always negative. DPN plots (Fig.6) on the other hand, remained linear, suggesting that DPN binds only once, as at pH 6.5.

DPNH showed competitive inhibition (equation 13)

$$v = \frac{VA}{K_a(1+ \frac{DPNH}{K_i}) + A} \quad \ldots\ldots\ldots (13)$$

when DPN was the varied substrate (Fig.7). Replots of slopes gave K_{DPNH} = 0.03 mM. Since the effect was competitive, DPN and DPNH must bind to the same form of the enzyme: the reaction must therefore be ordered, with DPN possibly binding prior to isocitrate.

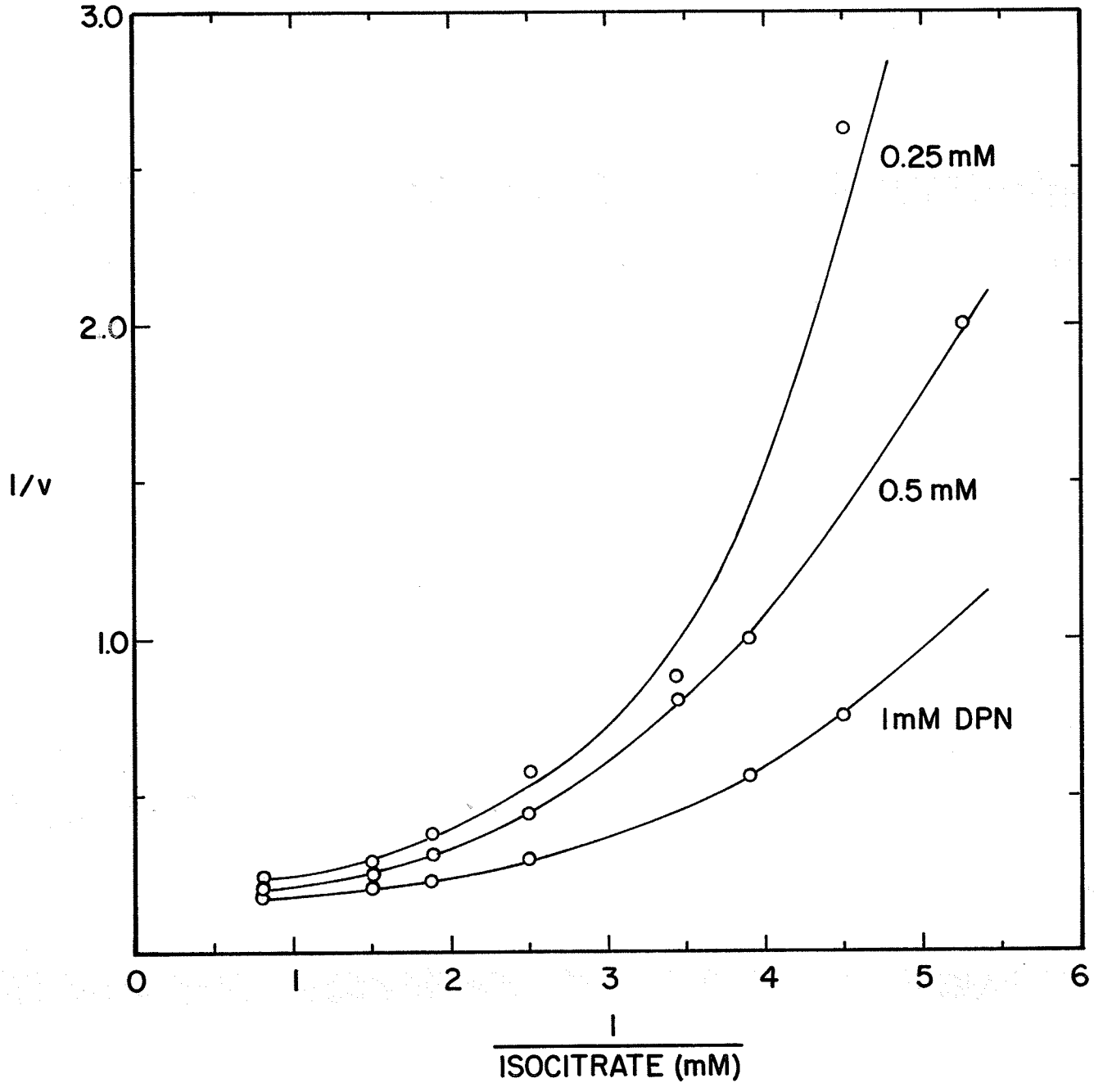


FIGURE 5. Double reciprocal plots of velocity versus varying isocitrate concentrations at pH 7.8 in the presence of AMP.

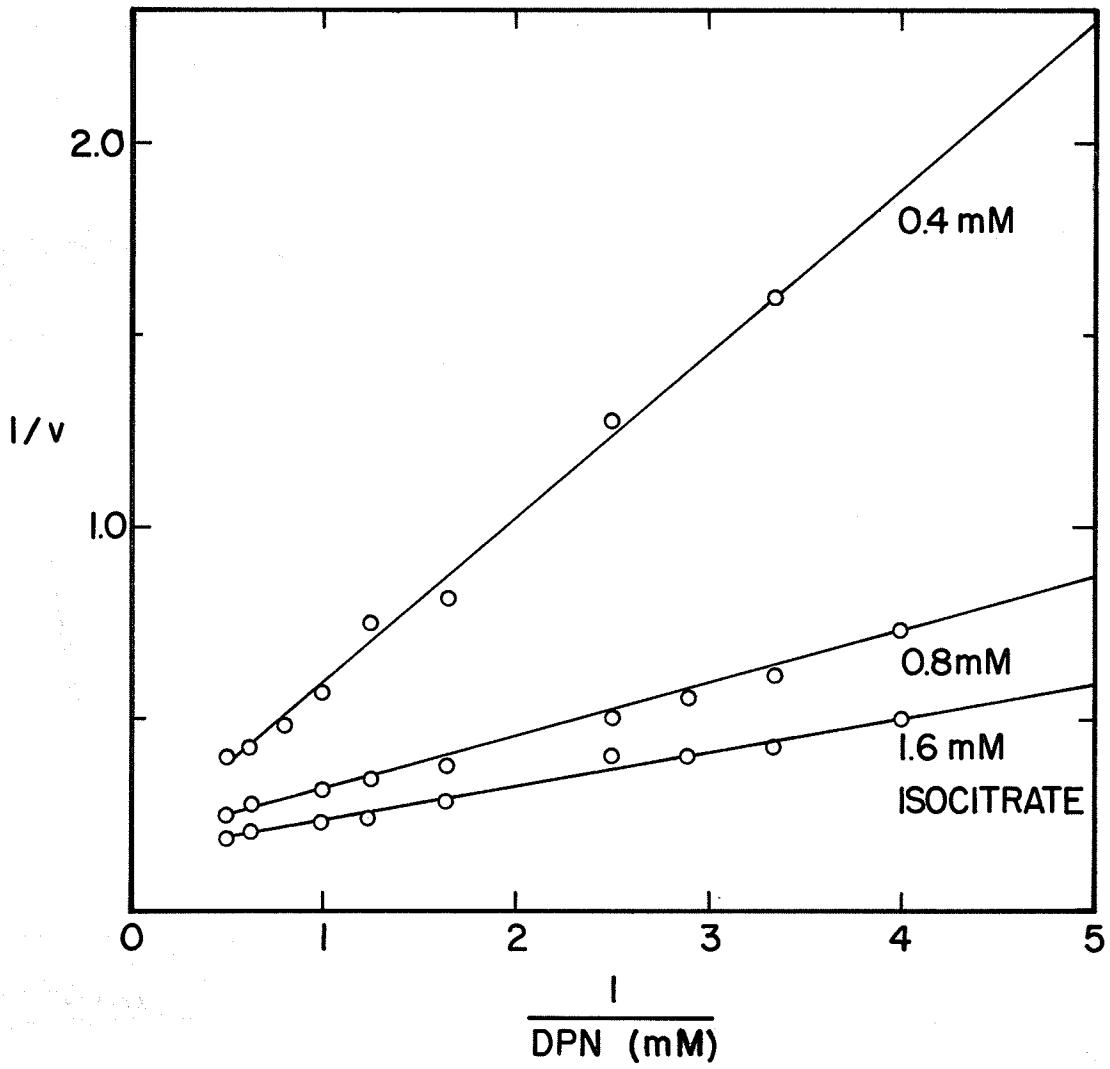


FIGURE 6. Double reciprocal plots of velocity versus varying DPN concentrations at pH 7.8 in the presence of AMP.

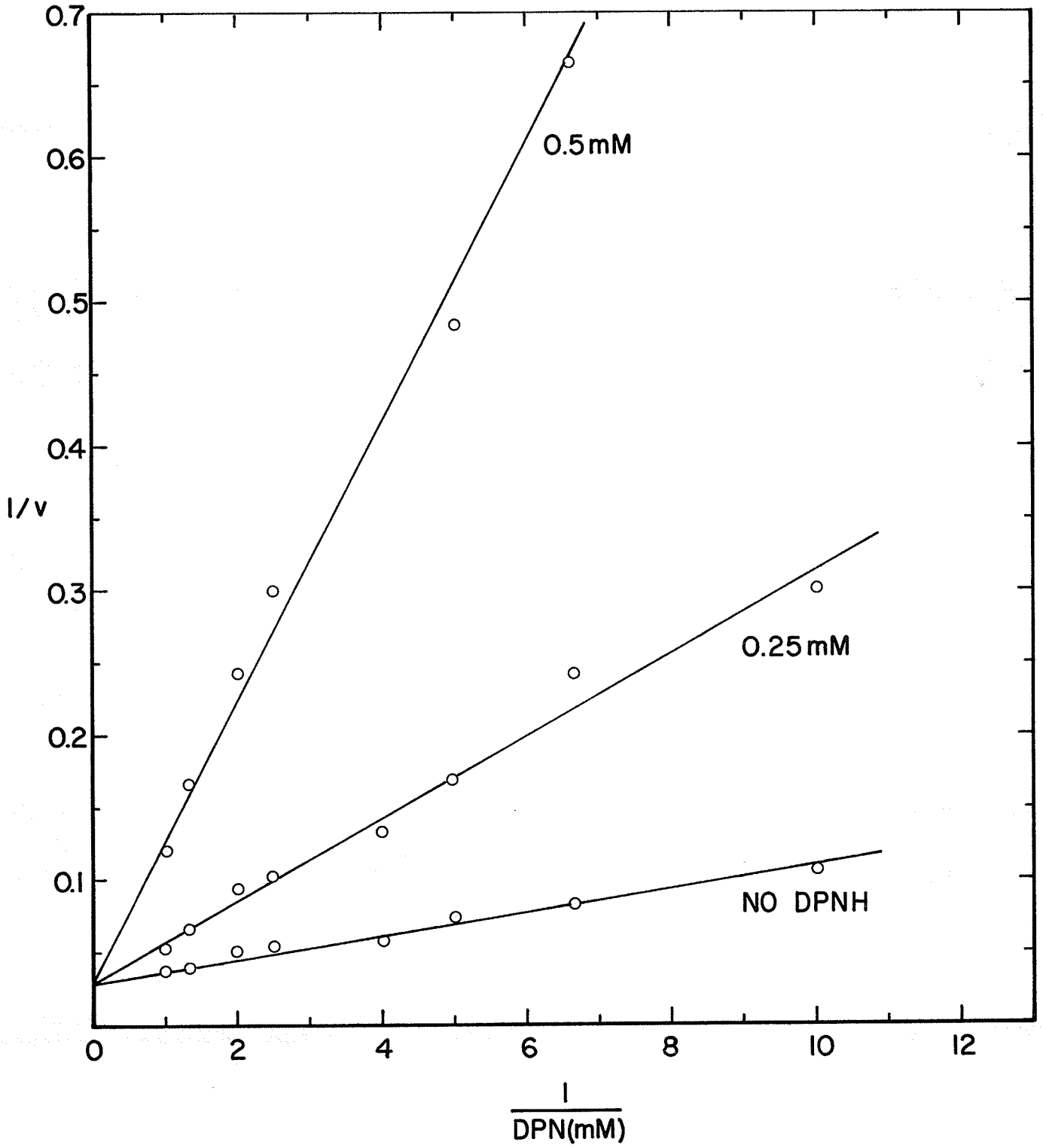


FIGURE 7. Product inhibition by DPNH at pH 7.8 in the presence of AMP, with DPN varied and isocitrate constant (3 mM).

(b) In absence of AMP

When DPN was varied against isocitrate, log-log plots (equation 2) (Fig.8) yielded values of n varying from 3.6 at the lowest concentration of isocitrate to 1.2 at the highest. Similar plots where isocitrate was varied showed changes in the value of n with increasing concentrations of DPN (Fig.9). When the initial velocity data obtained with DPN was plotted in the standard double reciprocal form (Fig.10), the curves obtained showed no relationship to equation (13) at non-saturating concentrations of isocitrate. However, with saturation by isocitrate (40 mM, Fig.10), the plot was perfectly linear. As discussed later, such behaviour is indicative of a random mechanism, where the binding of substrates becomes diffusion-limited. Since alternate pathways are set up in a random reaction, and both A and B can bind to the free enzyme form, the inhibition by products is always non-competitive. When DPNH was used as a product inhibitor and DPN was varied, non-competitive inhibition was obtained (Fig.11) in the absence of AMP. This may be contrasted with competitive inhibition given by DPNH in the presence of AMP (Fig.7).

(c) Citrate activation

In agreement with previous work of Sanwal et al.,

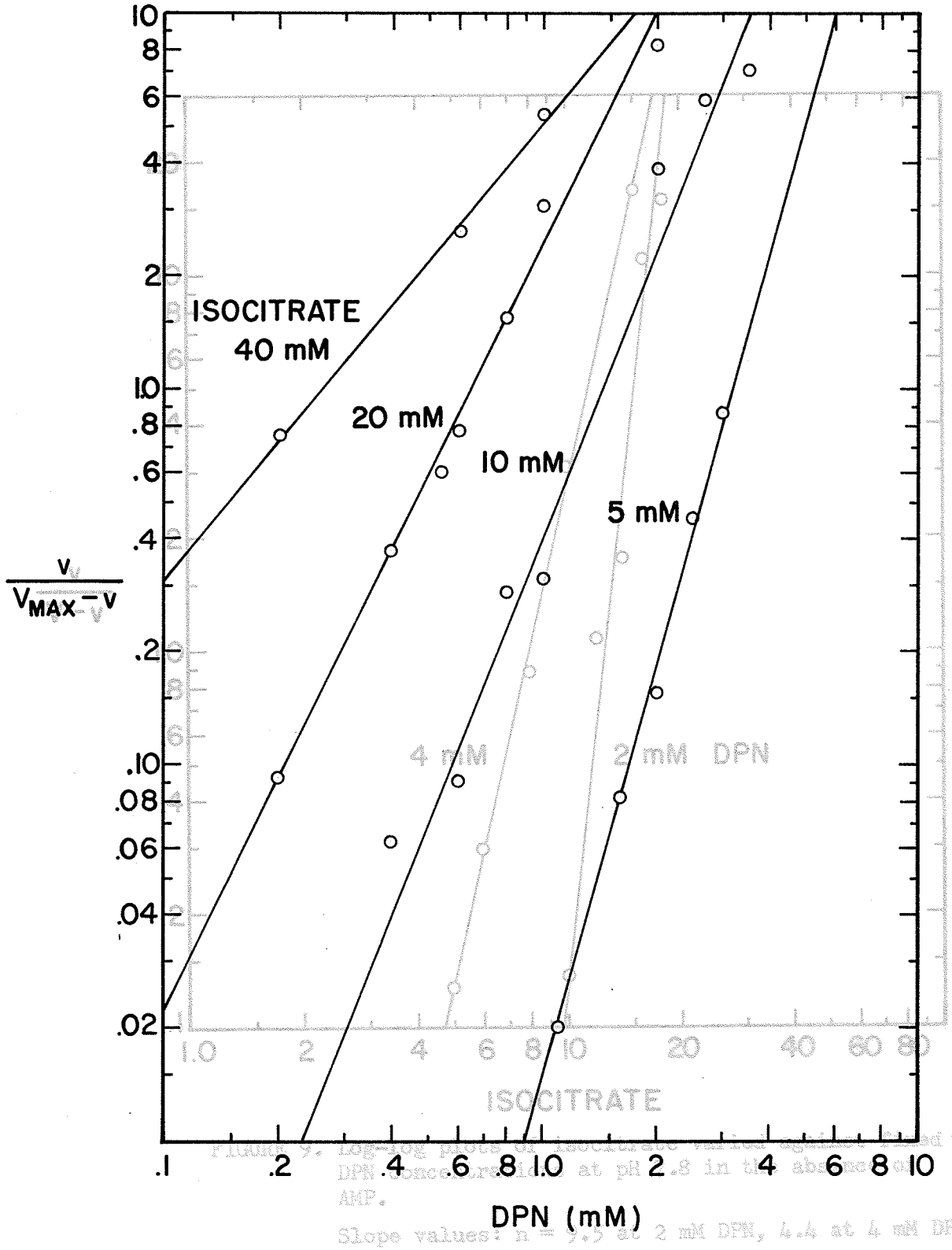


FIGURE 8. Log-log plots of DPN varied against fixed isocitrate concentrations at pH 7.8 in the absence of AMP.

Slope values : n = 3.6 at 5 mM isocitrate, 2.5 at 10 mM, 2.0 at 20 mM, 1.2 at 40 mM.

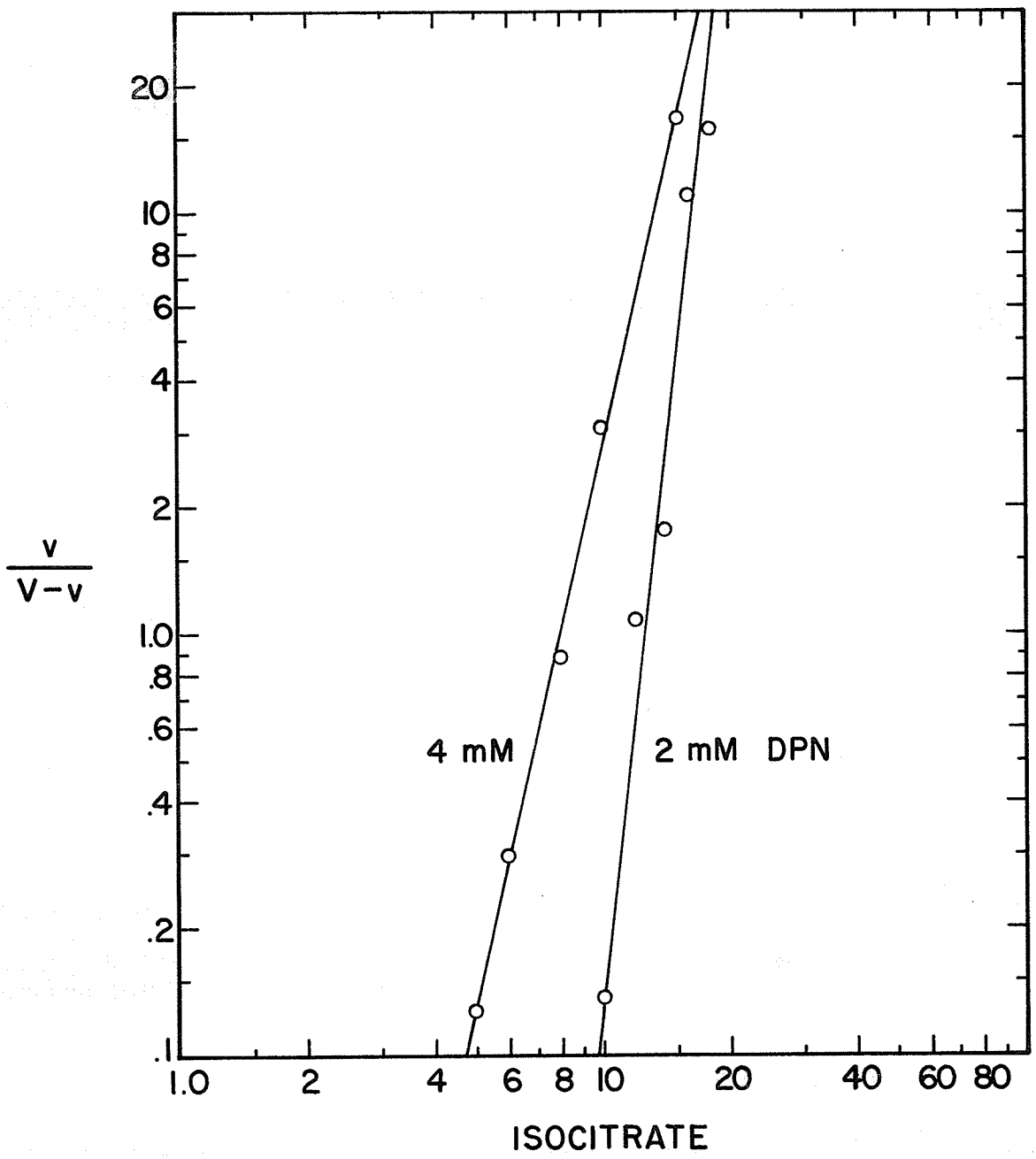


FIGURE 9. Log-log plots of isocitrate varied against fixed DPN concentrations at pH 7.8 in the absence of AMP.

Slope values: n = 9.5 at 2 mM DPN, 4.4 at 4 mM DPN.

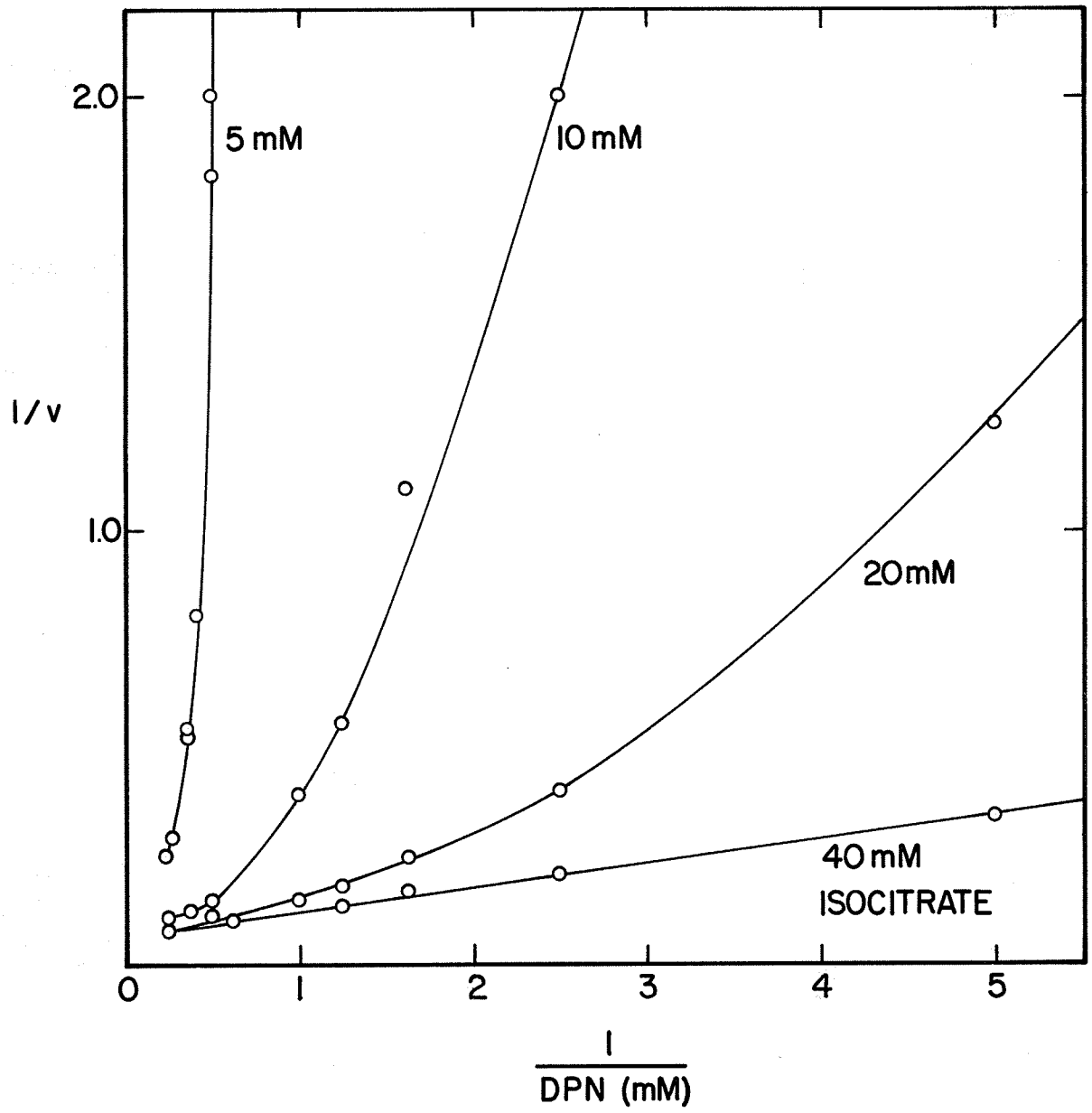


FIGURE 10. Double reciprocal plots of velocity versus varying DPN concentrations at pH 7.8 in the absence of AMP.

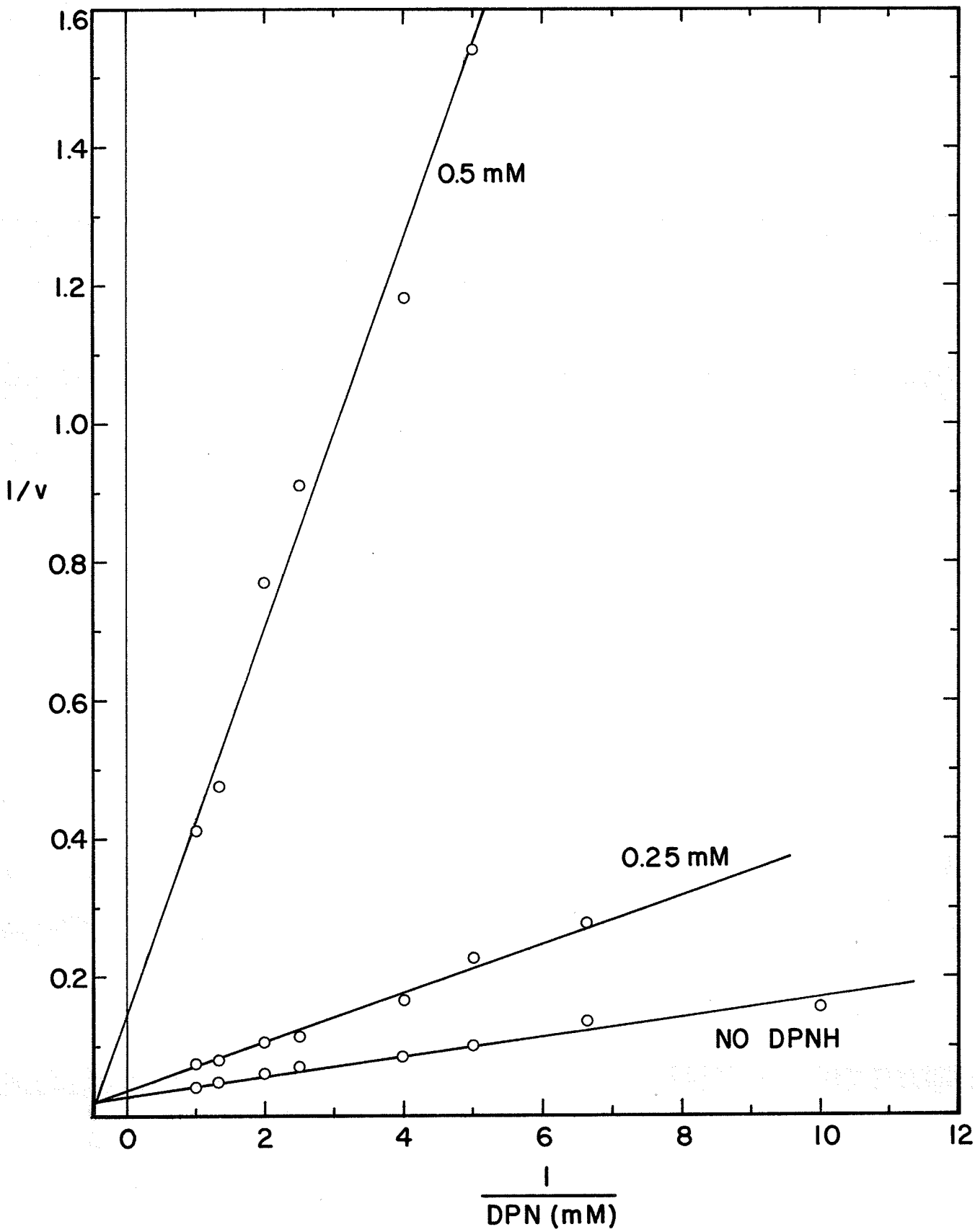


FIGURE 11. Product inhibition by DPNH at pH 7.8 in the absence of AMP, with DPN varied and isocitrate constant (3 mM).

(1963), low levels of citrate were found to activate the reaction at pH 7.8 with unsaturating concentrations of isocitrate (Fig.12). Citrate was found not to serve as a substrate of the enzyme. At pH 6.5 (in the absence of allosteric effects) citrate had no effect on the reaction velocity (Fig.12). When isocitrate was varied in the presence of citrate at pH 7.8, with added AMP, the reciprocal plots became linear (Fig.13), and conformed to the Michaelis-Menten equation (14).

$$v = \frac{VB}{K + B} \quad \ldots\ldots\ldots\ldots\ldots (14)$$

This would seem to indicate that in the presence of citrate, only one molecule of isocitrate binds on the enzyme surface.

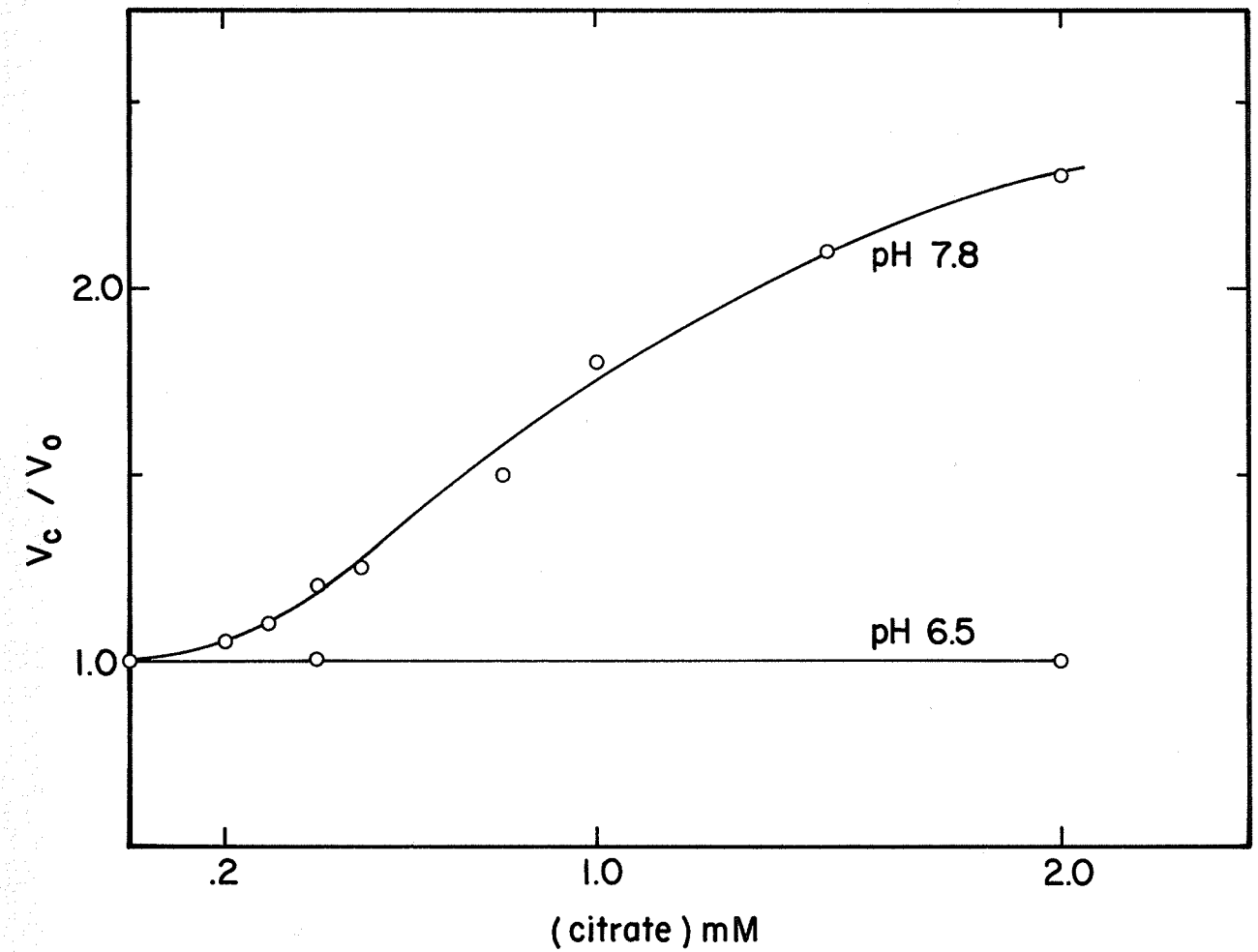


FIGURE 12. Effect of citrate on reaction velocity in the presence of AMP. Final concentration of DPN, 0.5 mM; of isocitrate, 1.0 mM.
V_C, velocity in the presence of citrate;

V_O, velocity in the absence of citrate.

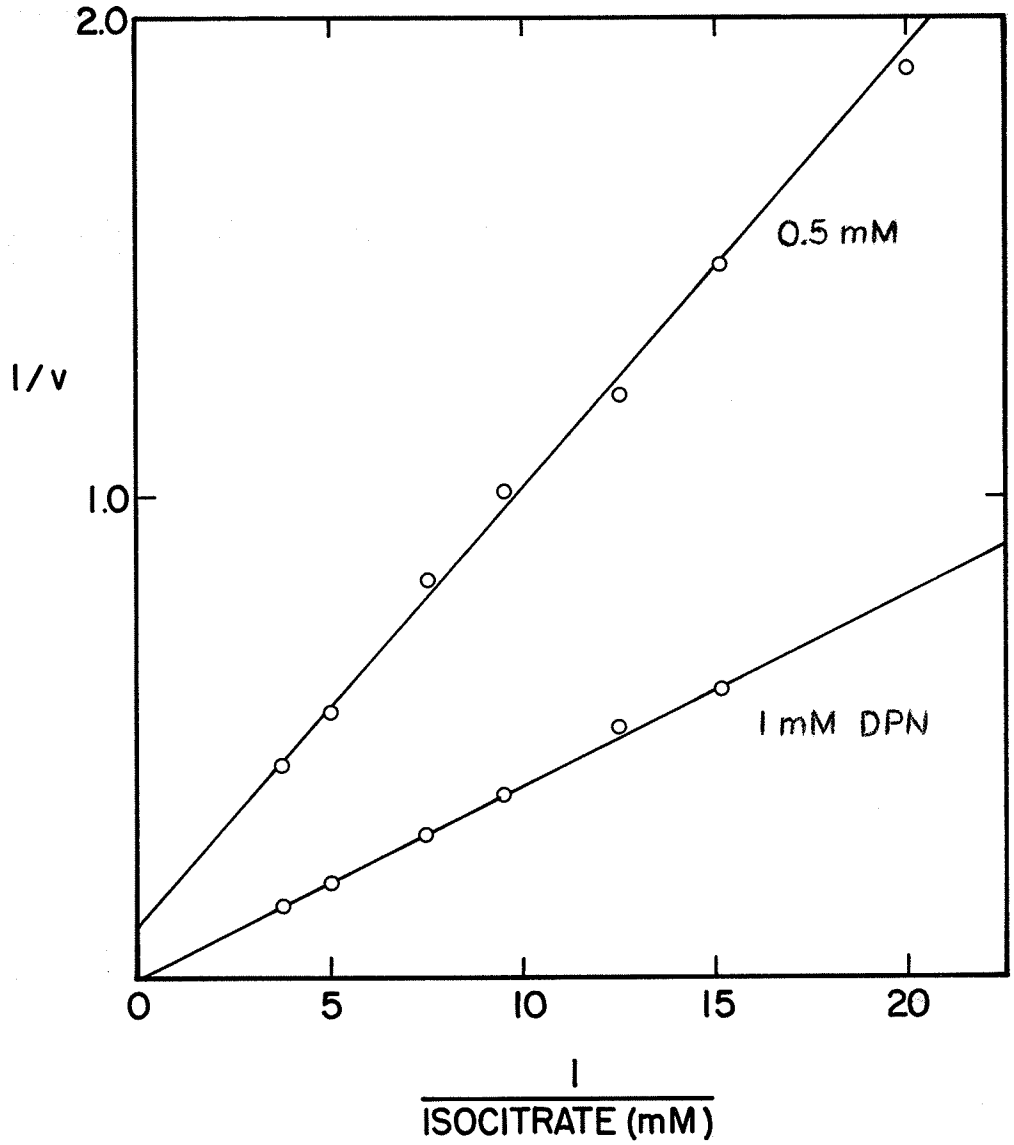


FIGURE 13. Double reciprocal plots of velocity versus isocitrate concentration in the presence of citrate (0.6 mM) and AMP, pH 7.8.

DISCUSSION

Recent studies of the kinetics of allosteric enzymes (Gerhart and Pardee, 1964; Monod *et al.*, 1965; Atkinson *et al.*, 1965) have embodied the concept of "subunit interactions" - i.e., interactions between identical binding sites on the same enzyme molecule. Such interactions are considered to cause the deviations from the normal Michaelis-Menten kinetics - S-shaped plots of velocity vs. substrate concentration and curved double reciprocal plots - which are characteristic of many allosteric enzymes.

Initial velocity is generally expressed by the equation

$$v = \frac{V\,(S)^n}{K + S_n}$$

which, on the assumptions that ES_n is the only rate-limiting step and that concentrations of all other complexes are negligible, yields

$$\log \frac{v}{V-v} = n \log (S) - \log K \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The term *n* has been interpreted as a function of the number of interacting sites and of the strength of the interactions (see Historical).

Inspection of equation 2 shows that the significance of *n* depends on the truth of the two assumptions, which must therefore be justified experimentally before any

valid meaning can be assigned to n. Ideally, such justification should come from physico-chemical binding studies of the enzyme. The usual findings from kinetic studies have been that the value of n decreases in the presence of activators and increases in the presence of inhibitors (Atkinson et al., 1965, Gerhart and Pardee 1964). These results are taken to indicate that activators decrease subunit interactions, while inhibitors increase them. However, no attempt has been made to exclude the operation of other mechanisms, such as alternate reaction sequences or partial inhibitions, which can lead to similar results, i.e., curved reciprocal plots and linear log-log plots. Nor are the assumptions used in deriving equation 2 applicable to bi- or multireactant mechanisms, of which many allosteric reactions are examples.

According to the hypothesis of site interactions, the nonlinearity of double reciprocal plots where DPN is varied against isocitrate in the absence of AMP should be interpreted in terms of interactions between two DPN-specific sites. When the data are replotted in log-log form, n changes from a value of approximately 3 at low isocitrate concentrations to 1 at higher concentrations. It is evident that the above interpretation is valid in these cases, only if binding of isocitrate can be said to

cause total loss of interactions between sites occupied by DPN, which is indeed hard to visualize.

If the subunit interaction hypothesis is rejected the data obtained here should be explained on some other reasonable basis. It is suggested that in the presence of AMP, the reaction mechanism is sequential and ordered, but changes to random in its absence. In the following account this alternate hypothesis is examined in detail:

1. Evidence for an ordered mechanism in the presence of AMP:

It is clear from Figures 2, 3 and 4 that at pH 6.5 the double reciprocal plots as well as the replots are linear. Were there alternate pathways, non-linearity of the double reciprocal plots or the replots should be very much evident. Also, product inhibition by DPNH is linear competitive (Fig.7). These data suggest a rate equation of the type (equation a) derived by King and Altman's method,

$$v = \frac{V_1\left(AB - \frac{PQR}{K_{eq}}\right)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_bK_qP}{K_pK_{iq}} + \frac{K_{ia}K_bR}{K_{ir}} + \frac{K_bAP}{K_{ip}} + \frac{K_{ia}K_bK_rPQ}{K_pK_{iq}K_{ir}} + \frac{K_aBR}{K_{ir}} + \frac{K_{ia}K_bQR}{K_{iq}K_{ir}} + \frac{K_{ia}K_bK_qPR}{K_pK_{iq}K_{ir}} + \frac{ABP}{K_{ip}} + \frac{K_{ia}K_bPQR}{K_pK_{iq}K_{ir}} + \frac{K_rK_bAPQ}{K_pK_{iq}K_{ir}}}$$

$$+ \frac{V_1(AB-\frac{PQR}{K_{eq}})}{\frac{ABQ}{K_{iq}} + \frac{K_{ia}K_bBQR}{K_{ib}K_{iq}K_{ir}} + \frac{ABPQ}{K_{ip}K_{iq}} + \frac{K_{ia}K_bBPQR}{K_pK_{ib}K_{iq}K_{ir}}} \quad \text{.....(a)}$$

(where A, B, R are DPN, isocitrate, and DPNH; P and Q are the remaining two products (CO_2 and -ketoglutarate), K_a, K_b, K_p, K_q, K_r are Michaelis constants of A, B, P, Q, R and K_{ia}, K_{ib}, K_{ip}, K_{iq} and K_{ir} are inhibition constants).

In initial velocity studies, when product concentration is zero, this equation simplifies to equation (b),

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad \text{.....(b)}$$

and, as seen before, indeed fits the data (Figs. 2,3, and 4). When DPNH is present, equation (a) reduces to equation (c) in the double reciprocal form,

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{R}{K_{ir}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \quad \text{.....(c)}$$

This equation predicts linear competitive inhibition by DPNH when DPN is the varied substrate. It is thus amply clear that, at least at pH 6.5, the reaction mechanism is ordered. At pH 7.8, DPN gives linear double reciprocal plots, but the plots when isocitrate is varied are markedly curved and show no relationship to equation (b). This apparent anomaly is still consistent with an ordered mechanism provided it is assumed that isocitrate binds twice

on the enzyme surface (total allosterism, see Historical), and a minor alternate pathway exists involving dissociation of isocitrate from the allosteric site in the central ternary complex (shown by dotted line in Figure 14).

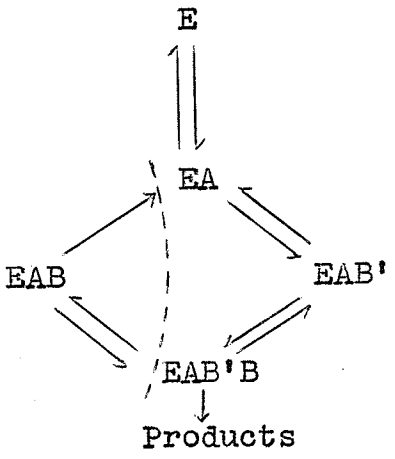


Figure 14.

(E is free enzyme; A is DPN; B' is isocitrate at the allosteric site, B is isocitrate at the catalytic site).

Using King and Altman's method (King and Altman, 1956) on this mechanism (assuming saturation by A) yields a rate equation of the type,

$$\frac{v}{E_t} = \frac{aB^2 + bB^3}{\text{constant} + cB + dB^2 + eB^3} \quad \ldots\ldots\ldots\ldots (15)$$

(where a, b --- e are combinations of rate constants)

which in the reciprocal form is a 3/1 function. It has already been mentioned that curves obtained at pH 7.8 when isocitrate is the varied substrate give significant fits to equation (12) (Fig.5), but the value of some constants is negative. This can only be if the curves given in Fig.5 are indeed 3/1 functions.

From the above it is clear that in the presence of AMP the reaction is ordered and there is an allosteric site for isocitrate operative at pH 7.8 but non-functional at pH 6.5. This interpretation is supported by the kinetics of the enzyme in the presence of citrate. If citrate were capable of binding at the allosteric site only, the curved plots at pH 7.8 should become linear, which they do (Fig.13). Further, when the allosteric site is inoperative at pH 6.5, citrate should not activate the enzyme, which is our experimental finding.

2. Evidence for a random mechanism in the absence of AMP:

It is seen from Fig.10 that the double reciprocal plots at pH 7.8 for DPN, linear in the presence of AMP, became non-linear in its absence. Saturation by isocitrate makes these plots linear: this suggests randomness of the reaction. If there were one binding site each for DPN and isocitrate, and the mechanism were random (Fig.15) the initial velocity would be given by equation (16).

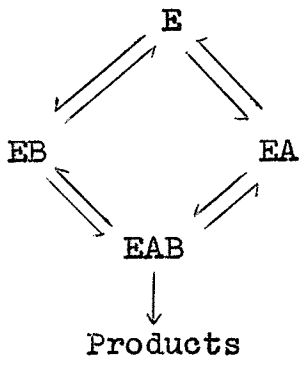


Figure 15.

$$v = \frac{k_9 E_t \left[k_5 k_7 (k_1 A + k_3 B)AB + (k_1 k_4 k_5 + k_2 k_3 k_7)AB\right]}{\text{constant} + aA^2B + bAB^2 + cA^2 + dB^2 + eAB + fA + gB} \quad ..(16)$$

where, a, b --- g are combinations of constants. This equation in the reciprocal form is a 2/1 function,

$$\frac{1}{v} = \frac{a + b(1/X) + c(1/X)^2}{1 + d(1/X)}$$

(where X is substrate concentration)

Since there are square terms in A and B in the numerator of equation (16), the double reciprocal plots are expected to be non-linear. When B (=isocitrate) is saturating, equation (16) reduces to,

$$v = \frac{k_9 E_t A}{(k_8+k_9)/k_7+A}$$

i.e., the Michaelis-Menten form. However, when curves given in Figure 10 are fitted to a 2/1 function, insignificant fits are obtained. It is apparent from log-log plots (Fig.8) that the n value is approximately 3. It can readily be shown that the value of n obtained from equation (2) gives a maximum for the power to which substrate concentration occurs in the rate equation derived by steady state methods. It is at once clear that the rate equation for the curves of Figure 10 must have A^3 terms in the numerator. This is possible if one assumes that in the absence of AMP at pH 6.5 the allosteric binding site for isocitrate becomes operative (possibly due to a conformational change), and DPN can bind both before and after allosteric binding of isocitrate, as given in Figure 16.

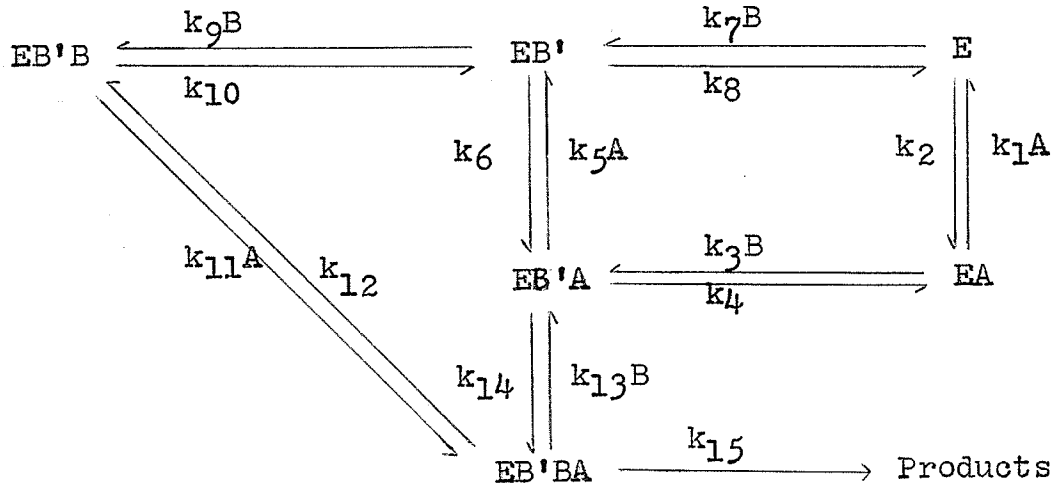


Figure 16.

The initial velocity equation for this mechanism is,

$$v = \frac{aAB^2 + bAB^3 + cAB^4 + dA^2B^2 + eA^2B^3}{\text{constant} + fB + gA + hAB + iB^2 + jB^3 + kB^4 + lAB^2 + mAB^3 + nAB^4 + oA^2 + pA^2B + qA^2B^2 + rA^2B^3}$$

This equation has cube powers in A and fourth powers in B. This is what one obtains from the log-log plots.

Inhibition by DPN in the absence of AMP at pH 6.5 is non-competitive, which further supports the idea that the isocitrate dehydrogenase reaction is random in the absence of AMP.

In conclusion, it is evident that certain data obtained from yeast IDH cannot logically be explained by the model of Atkinson et al., (1965) for the yeast IDH reaction - i.e., by the hypothesis of subunit interactions. The data presented here are however readily interpretable in terms of the model proposed by Sanwal et al., (1965) for the reaction of the Neurospora enzyme.

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