Effect of Adenylic Acid on the Regulatory Nicotinamide–Adenine Dinucleotide Specific Isocitrate Dehydrogenase*

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ABSTRACT: The mechanism of activation of isocitrate dehydrogenase by adenylic acid (AMP) has been studied at pH 6.5 using product inhibition and initial velocity data. In the presence of AMP the reaction mechanism appears ordered, with nicotinamide–adenine dinucleotide (NAD) binding first or preferentially to the free enzyme. In the absence of AMP, however, the double reciprocal plots for both NAD and isocitrate (linear in the presence of AMP) become markedly curved. Saturation by isocitrate has the effect of converting the nonlinear NAD plots to straight lines, while saturation by NAD does not lead to the same result for the double reciprocal plots of isocitrate, *i.e.*, they remain curved. When the enzyme is saturated with citrate, however, the double reciprocal plots for both substrates at unsaturating fixed concentrations of each other are 2/1 functions. These plots become linear with approaching saturation by the fixed changing substrate. It is concluded that in the absence of AMP the mechanism of the reaction becomes Random (*i.e.*, the steps for the addition of substrates to the enzyme become partially rate limiting). Attempts have been made to show that nonlinear double reciprocal plots are possibly not due to the binding of the substrate at multiple sites on the enzyme surface and an "interaction" between these sites.

A few serious attempts have been made to understand the kinetic basis of allosteric effects since the early enzymological work of Umbarger (1956). With a few exceptions (Sanwal *et al.*, 1964, 1965; Worcel *et al.*, 1965) the kinetic analysis of allosteric enzymes has been based, by and large, on that of hemoglobin, *i.e.*, on the assumption that the S-shaped plots of velocity *vs.* substrate concentration are due to the interaction of identical binding sites on the enzyme polymer ("subunit interaction").[1] This model slightly modified to suit particular situations has been applied to aspartate transcarbamylase (Gerhart and Pardee, 1964), threonine deaminase (Changeux, 1964), deoxycytidylate deaminase (Scarano *et al.*, 1963), phosphofructokinase (Vinuela *et al.*, 1963; Atkinson *et al.*, 1965a), and a few others. In general, it has been suggested that the initial velocity for an allosteric enzyme exhibiting the so-called substrate–substrate interactions (yielding nonlinear double reciprocal plots) is given by the equation

$$v = \frac{VS^n}{K + S^n} \qquad (1)$$

which by suitable manipulation yields (Monod *et al.*, 1963; Atkinson *et al.*, 1965b),

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

Thus a plot of log $(v/(V - v))$ against log of concentration of S is a straight line, the slope of which is equal to n. Despite how n is interpreted, embodied in eq 2 are the assumptions that more than one molecule of substrate binds on the enzyme surface, and that the only rate-limiting step is the breakdown of ES_n complex, other equilibria (*e.g.*, ES_1, ES_2, ... etc.) being adjusted rapidly.

It is well known, however, that nonlinear double reciprocal plots such as are commonly obtained with allosteric enzymes (Monod *et al.*, 1965) can result from a variety of causes (Reiner, 1959; Cleland, 1963a,b,c; Frieden, 1964; Maeba and Sanwal, 1966), yet plotted on the basis of eq 2 may yield very nearly straight lines. It was, therefore, considered desirable to test the validity of the assumptions embodied in eq 2 by using the allosteric nicotinamide–adenine dinucleotide (NAD[2]) dependent isocitrate dehydrogenase of *Neurospora* (Sanwal *et al.*, 1963, 1964; Sanwal and Stachow, 1965), for which we have already presented a comprehensive kinetic model (Sanwal *et al.*, 1965). We will show in the following report that while the reaction sequence seems ordered in the presence of adenylic acid (AMP), with one substrate showing a totally allo-

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[1] The term "subunit interaction," as used here, refers to the interaction between *identical* binding sites present on identical subunits. Interactions between regulatory subunits and catalytic subunits, such as occur in the case of aspartate transcarbamylase (Gerhart and Schachman, 1965), are not included in this definition.

[2] Abbreviations used in this work: AMP, adenylic acid; NAD, nicotinamide–adenine dinucleotide; $NADH_2$, reduced NAD.

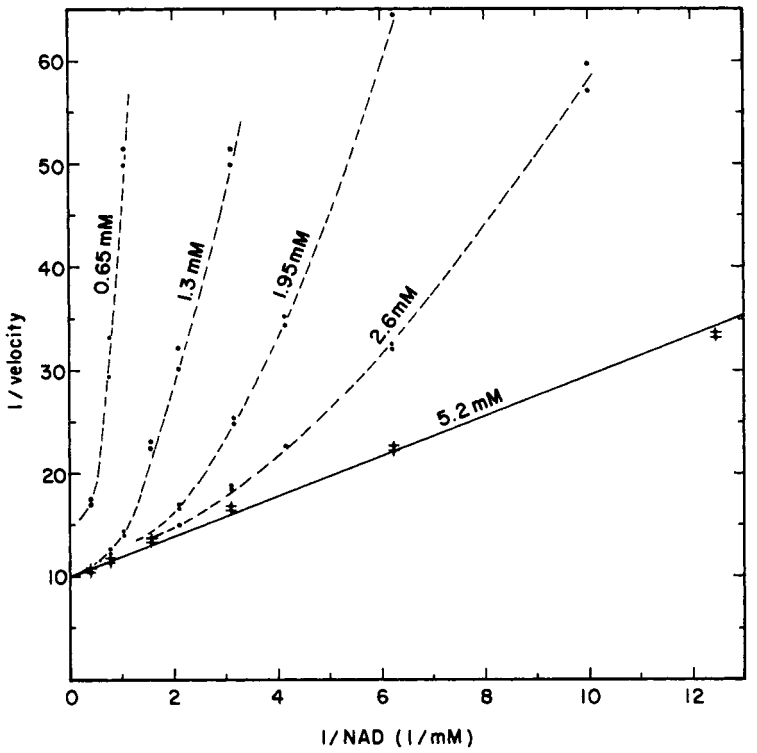


FIGURE 1: Double reciprocal plots of 1/velocity *vs.* 1/NAD at several fixed concentrations of isocitrate in the absence of AMP. Phosphate buffer, 0.2 M, pH 6.5, was used in all experiments. The solid line has been drawn from fit to eq 3. The dashed lines are fitted by eye.

steric effect, the mechanism of the reaction perhaps becomes Random in the absence of AMP (*i.e.*, the steps for the addition of NAD and the allosteric substrate, isocitrate, to the enzyme become partially rate limiting), and this leads to departures from normality in the plots of the initial velocity data.

Experimental Section

Reagents. The NAD-specific isocitrate dehydrogenase used here was purified from lyophilized cells of *Neurospora crassa* by alcohol precipitation (Sanwal *et al.*, 1964) and chromatography on DEAE-cellulose column (Sanwal and Stachow, 1965). Since repeated thawing and freezing affect the kinetic parameters associated with the activation of the enzyme by AMP, the preparations were stored at −20° in 5-ml lots and thawed as required.

Synthetic potassium *threo*-D_sL_s-isocitrate[3] was generously donated by Dr. H. B. Vickery (Connecticut Agricultural Experiment Station). Disodium salt of $NADH_2$, NAD, and sodium salt of adenosine 5-phosphate were purchased from Sigma Chemical Co.

Assay Procedure. Enzyme assays and kinetic measurements were performed at 24–25° in a final volume of 3 ml as described earlier (Sanwal *et al.*, 1965). The solutions of $NADH_2$, NAD, and AMP were prepared just before use. The enzyme was checked periodically for any denaturation during experiments by using a standard reaction mixture which contained 4.9 μmoles of isocitrate, 1.0 μmole of adenosine 5-phosphate, 120 μmoles of Tris, pH 7.6, 9.0 μmoles of $MgSO_4$, and 0.75 μmole of NAD in 3 ml total volume. Assays at pH 7.6 were made in 0.2 M Tris–acetate and at pH 6.5 in 0.2 M potassium phosphate buffer. The concentration of adenylic acid when used was 0.533 mM. All reaction mixtures also contained 3.3 mM $MgSO_4$.

For all kinetic studies, a Gilford Model 2000 optical density converter connected to a Beckman DU monochromator and a self-balancing recorder with a multiple-chart drive was used. Small velocity changes were recorded using the full-scale sensitivity of the recorder between the optical densities of 0.1 and 0.2.

Analysis of the Kinetic Data. All kinetic data were processed according to Cleland (1963d) using an IBM 1620 digital computer, as already described (Sanwal *et al.*, 1965). Fits were made to eq 3 when visual examination of the plots of data in the double reciprocal form indicated that they were straight lines

$$v = \frac{VS}{K + S} \qquad (3)$$

to eq 4 when the plots were parabolic

[3] The nomenclature is that proposed by Vickery (1962).

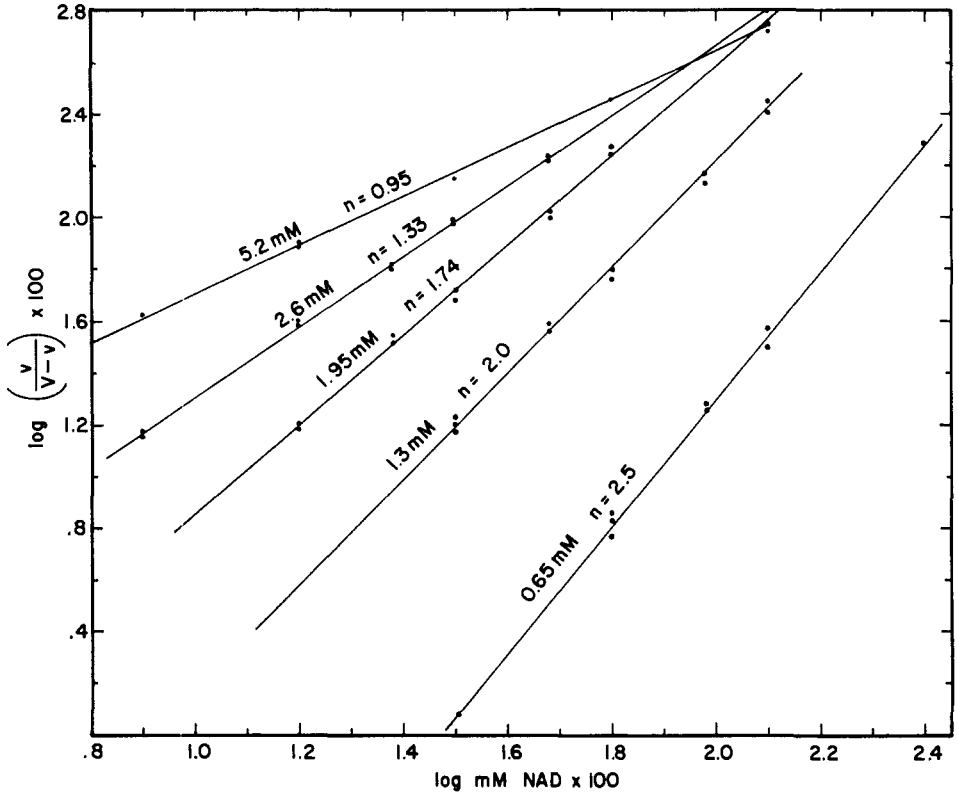


FIGURE 2: Data from Figure 1 drawn in the form of log–log plots (eq 2). The lines have been fitted by eye.

$$v = \frac{VS^2}{a + bS + S^2} \tag{4}$$

and to eq 5 when they appeared to be 2/1 functions[4]

$$v = \frac{V(S^2 + cS)}{a + bS + S^2} \tag{5}$$

The computer programs gave the value of various kinetic constants together with their standard errors and weighting factors, which were used for replot analyses. Product inhibition experiments were analyzed as described elsewhere (Sanwal *et al.*, 1965).

Results

Mechanism of the Reaction in the Presence of Adenylate. In an earlier study (Sanwal *et al.*, 1965) it was shown by steady-state kinetic treatment that *in the presence* of saturating concentrations of adenylic acid the mechanism of isocitrate dehydrogenase was consistent with an ordered reaction, with NAD possibly binding first, followed by isocitrate. At pH 7.5, isocitrate was also shown to bind at an allosteric site, while at pH 6.5, owing to the linearity of the double reciprocal plots, it was conjectured that binding of isocitrate occurred only at the active site. Support was lent to this conjecture by the finding that citrate which activated the reaction by binding at the allosteric site at pH 7.5, and restoring normal kinetics, did not activate the reaction at pH 6.5, when presumably the allosteric site was "inactive."

In the present study these conclusions were confirmed by initial velocity and product inhibition data obtained at pH 6.5. The Michaelis constants, K_a and K_b (for NAD and isocitrate, respectively), could be evaluated as $K_a = 0.22$ mM and $K_b = 0.17$ mM, which show excellent agreement with the constants obtained in our earlier work (Sanwal *et al.*, 1965).

Initial Velocity Studies in the Absence of AMP. It has been shown earlier (Sanwal *et al.*, 1964) that AMP only activates the enzymic reaction, but there is no absolute requirement for it. The V_{max} is entirely unaffected in its presence. However, double reciprocal plots, when NAD is varied against isocitrate, become markedly nonlinear (Figure 1) in the absence of AMP at pH 6.5. If the data given in Figure 1 are plotted on the basis of eq 2, it can readily be seen that at low isocitrate concentrations the value of n is 2.5 (Figure 2), which seems to indicate a limiting value of 3. This value changes to about 1 when isocitrate concentration is increased. Also, from Figure

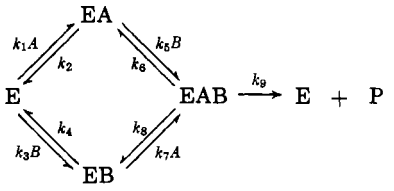
[4] This refers to the highest power of substrate concentration that occurs in the numerator and denominator of the rate equation when written in a double reciprocal form. The curves given by this function are either concave up or concave down near the vertical axis, but become linear when $1/s$ approaches infinity. The curves plot as $y = (a + bx + cx^2)/(1 + dx)$.

1 it can be seen that when isocitrate is nearly saturating (5.2 mM, which is about 30 × K_b) the reciprocal plot is linear and yields a K_a value of 0.21 ± 0.01, which is in excellent agreement with the value obtained in the presence of AMP. Since the value of n changes from approximately 3 to 1, it is clear that eq 2 does not describe the situation, unless one assumes that binding of one substrate leads to a complete loss of interactions between sites occupied by the other substrate. If the curves given in Figure 1 were parabolas, one could perhaps surmise that in the absence of adenylate NAD formed two successive complexes with the enzyme in the sequence

$$E \rightleftharpoons E\text{–}NAD \rightleftharpoons E\text{–}NAD\text{–}isocitrate \rightleftharpoons E\text{–}NAD\text{–}isocitrate\text{–}NAD \longrightarrow products$$

In such a case saturation by isocitrate would give a linear double reciprocal plot. However, these curves when fitted to eq 4 give very large standard errors of the constants, and, therefore, do not appear to be parabolas.

A more likely alternative seems to be that, in the absence of AMP, the mechanism of the reaction becomes Random, *i.e.*, both NAD and isocitrate are able to bind to the free enzyme form and that these addition steps become partially rate limiting. If at pH 6.5 there are only two sites on the enzyme surface, one specific for NAD (= A) and another for isocitrate (= B; mechanism I), steady-state derivation of the initial velocity equation, using King and Altman's method (1956), yields eq 6



MECHANISM I

$$v = \frac{k_9E_tk_5k_7(k_1A + k_3B)AB + (k_1k_4k_5 + k_2k_3k_7)AB}{[\text{constant} + aA^2B + bAB^2 + cA^2 + dB^2 + cAB + fA + gB]} \quad (6)$$

where a, b ..... g are combinations of constants. When one substrate concentration is held constant and the other is varied this equation is of the general form (5), or in the double reciprocal form (eq 7)

$$\frac{1}{v} = \frac{a + b\left(\frac{1}{s}\right) + c\left(\frac{1}{s}\right)^2}{d + e\left(\frac{1}{s}\right)} \quad (7)$$

When A is saturating, eq (6) reduces to

$$v = \frac{k_9E_tB}{(k_6 + k_9)/k_5 + B} \quad (8)$$

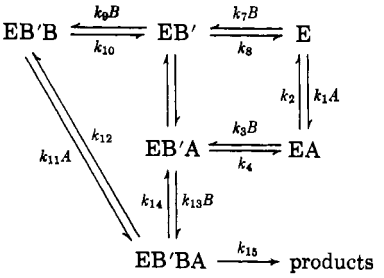
and when B is saturating,

$$v = \frac{k_9E_tA}{(k_8 + k_9)/k_7 + A} \quad (9)$$

Both eq 8 and 9 conform to Michaelis–Menten formulation (eq 3) and will plot as straight lines in double reciprocal form.

When the curves shown in Figure 1 are fitted to a 2/1 function (eq 5) insignificant fits are obtained, which suggests that the mechanism of reaction may be more complex. The complexity of these curves led to the suspicion that at pH 6.5 in the absence of AMP, in addition to randomness, the allosteric binding site of isocitrate may perhaps be exposed. If this were true, one would not only be dealing with an initial velocity equation more complex than (6), but it is also expected that the double reciprocal plots when isocitrate (= B) is varied will never assume a linear form in the presence of saturating concentrations of NAD.

Since the value of n in eq 2 gives a *minimum* for the power to which substrate concentration occurs in the rate equation, a mechanism which gives A^3 terms in the rate equation is indicated (Figure 2). If one assumes that total allosterism (no activity unless isocitrate were bound at the allosteric site; Sanwal *et al.*, 1965) is still exhibited by isocitrate in the absence of AMP, and further that NAD can bind before as well as after allosteric binding of isocitrate (Mechanism II)



B′ = binding of B at the allosteric site

MECHANISM II

the numerator of the rate equation will have terms containing the fourth power of B and third power of A in addition to other terms, and curves obtained when NAD is the variable substrate will not normally correspond to eq 5 although saturation by isocitrate will still yield plots conforming to eq 3. The curves given by Mechanism II are impossible to fit, but regardless of how random the mechanism, the plots for isocitrate will always be curved (predicted to correspond to eq 4 from Mechanism II) when NAD is saturating. This can be seen from Figure 3. The curve obtained in the presence of 5.0 mM NAD (which is a nearly saturating value, approximately 25K_a) shows very significant fits to eq 4, but the value of the constant b is negative, which means that the curve is very likely to be a 3/1 (or more complex function) defined by an equation of the type (10)

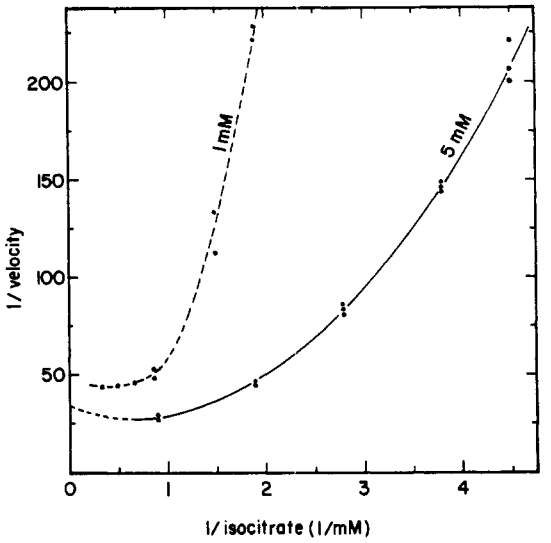


FIGURE 3: Double reciprocal plots of 1/velocity *vs.* 1/isocitrate at two fixed levels of NAD in the absence of AMP. The buffer was 0.2 M phosphate, pH 6.5. The solid line (lower) has been drawn from fits to eq 4, dotted part being extrapolated parts of the curve. The dashed line is fitted by eye.

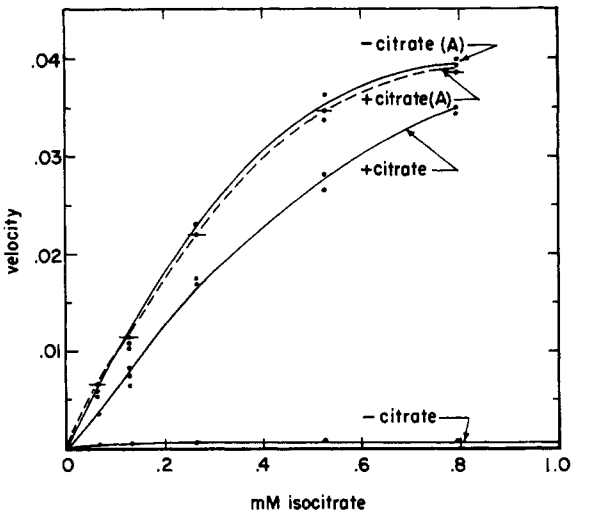
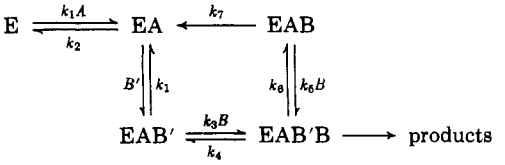


FIGURE 4: The activation of isocitrate dehydrogenase by citrate in the presence and absence of AMP at pH 6.5. When used, AMP concentration was 0.533 mM and citrate concentration was 1.5 mM. The NAD concentration was kept constant (0.8 mM unsaturating). (A) refers to assay in the presence of AMP.

$$v = \frac{B^2 + B^3}{\text{constant} + B + B^2 + B^3} \qquad (10)$$

This is the equation one obtains (see Sanwal *et al.*, 1965) for Mechanism III



E = free enzyme, A is NAD, and B′ is allosteric binding of isocitrate

MECHANISM III

The occurrence of the branch EAB′B $\rightleftharpoons$ EAB $\rightarrow$ EA in Mechanism III was earlier (Sanwal *et al.*, 1965) considered to be likely on the basis of experiments done at pH 7.6 in the presence of AMP, and if this holds also at pH 6.5 in the absence of AMP, one would expect the curves to be 3:1 or more complex functions in the presence of nearly saturating concentrations of NAD. It may be mentioned that curves for isocitrate obtained below a fixed NAD concentration of less than 2.0 mM show insignificant fits to eq 4 (Figure 3), and this is likely if the reaction mechanism is Random in the absence of AMP.

When the data obtained with isocitrate as the variable substrate at various fixed changing levels of NAD are plotted by means of eq 2, the value of n which is approximately 3.7 at low levels of NAD approaches 2.8 with near saturation by NAD. Again assumptions embodied in eq 2 are not applicable here, unless one argues that NAD reduces interactions between isocitrate binding sites.

Activation of the Reaction by Citrate in the Absence of AMP. If in the absence of AMP at pH 6.5 the allosteric site is indeed operational (as suggested by data showing n values >2 for isocitrate), citrate should activate the reaction in the absence of AMP, because we have clearly demonstrated earlier that citrate can bind at the allosteric site (Sanwal *et al.*, 1965). This is indeed the case, and can be seen from a study of Figure 4. It will also be noted that activation of the reaction by citrate does not occur if AMP is present at pH 6.5.

The finding that citrate activates the reaction in the absence of AMP is very important, not only because it constitutes evidence that isocitrate combines at a regulatory site instead of causing site interactions on a tetramer but also because citrate activation can now be used as a potent tool to decide whether the reaction indeed becomes Random in the absence of AMP.

If the allosteric site is saturated with citrate so that most of the enzyme is present in the form of enzyme–citrate complex, and the concentration of either substrate is varied at fixed levels of the other, the data should conform to eq 5 provided that the reaction is Random and the concentration of the nonvaried substrate is not saturating. When NAD is used as the variable substrate and isocitrate as the nonvaried substrate in the presence of nearly saturating and noninhibitory concentrations of citrate (6.6 mM), the double reciprocal plot at 0.166 mM isocitrate (which is the approximate value for K_b) is concave up near the vertical axis (Figure 5) and gives a good fit to eq 5. In ac-

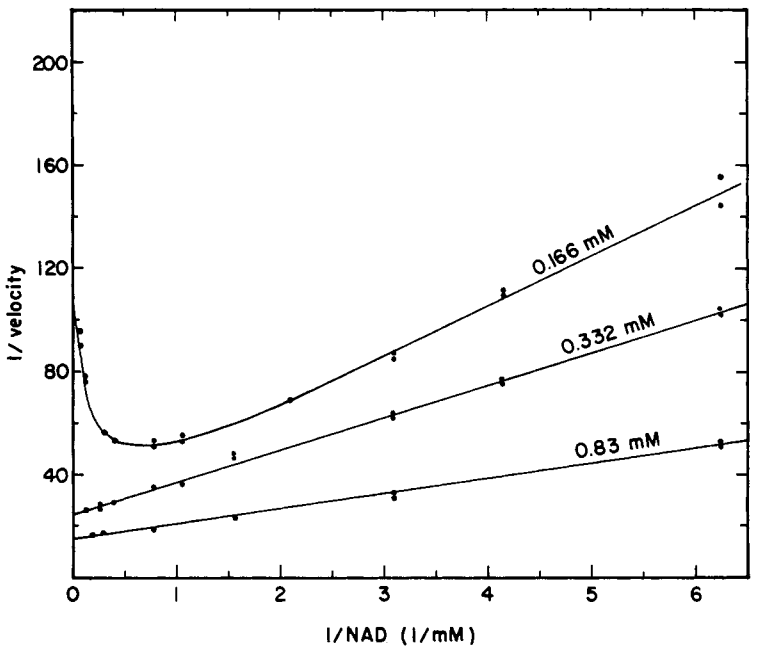


FIGURE 5: Double reciprocal plots of 1/velocity *vs.* 1/NAD at several fixed levels of isocitrate in the absence of AMP. All cuvets contained 6.6 mM citrate and 0.2 M phosphate buffer, pH 6.5. The uppermost line (0.166 mM isocitrate) has been drawn from fits to eq 5 and the lower two lines from fits to eq 3.

cordance with either Mechanism I or II the lines become straight at higher concentrations of isocitrate (Figure 5). This observation also rules out the possibility that curvature may be due to substrate inhibition. Nearly identical results with those shown in Figure 5 are also obtained when citrate is used at a concentration of 2.2 mM.

When isocitrate is used as the variable substrate and NAD as the fixed changing substrate in the presence of constant high levels of citrate (6.6 mM), the curve (Figure 6) obtained at 0.16 mM NAD (slightly less than K_a value) fits eq 5. Although the standard errors of the fitted constants are in the range 20–30%, these fits still yield the lowest residual least square compared with fits to eq 4. Fits to eq 3 are insignificant. At higher concentrations of NAD the double reciprocal plots become perfectly straight (Figure 6). This observation is again consistent with a Random mechanism.

Product Inhibition in the Absence of AMP. A powerful tool for the study of reaction mechanisms is the determination of product inhibition patterns (Cleland, 1963b). If, as has been suggested above, the reaction mechanism becomes random in the absence of AMP, the inhibition pattern should be different compared to that in the presence of AMP. Specifically, since terms corresponding to product concentrations exist both in the numerator and denominator of the rate equation for a Random reaction (Cleland, 1963a,b), inhibition by any of the products when the enzyme is saturated by one of the substrates and the other one is varied is expected to be hyperbolic noncompetitive (eq 11)

$$v = \frac{VS}{\frac{K(1 + I/K_{in_1})}{(1 + I/K_{id})} + \frac{S(1 + I/K_{in_2})}{(1 + I/K_{id})}} \qquad (11)$$

where K_{in} and K_{id} are numerator and denominator inhibition constants, respectively.

Figure 7 shows the results of an experiment using $NADH_2$ as product inhibitor at pH 6.5 (in the absence of AMP) under conditions where the enzyme was saturated with isocitrate and NAD was the variable substrate. The inhibition is clearly noncompetitive, and this result may be contrasted with that obtained in the presence of AMP, when the inhibition is competitive (Sanwal *et al.*, 1965). The replot of intercepts from Figure 7 appears hyperbolic, although the fitted constants give large standard errors. This experiment lends further support to our theory that in the absence of AMP the reaction is Random.

Activation by AMP. Since reasonable evidence has been accumulated above regarding randomness of the reaction, the question arises whether activation by AMP is also random. This question, however, is difficult to answer definitely, but indications that AMP may bring about random activation come from initial velocity studies. It is expected that, when AMP is varied at several fixed levels of one of the substrates, keeping the second substrate constant, the double reciprocal plots will be nonlinear, but will eventually become linear with saturation by the changing fixed substrate. That such is the case can be seen from Figure 8. When

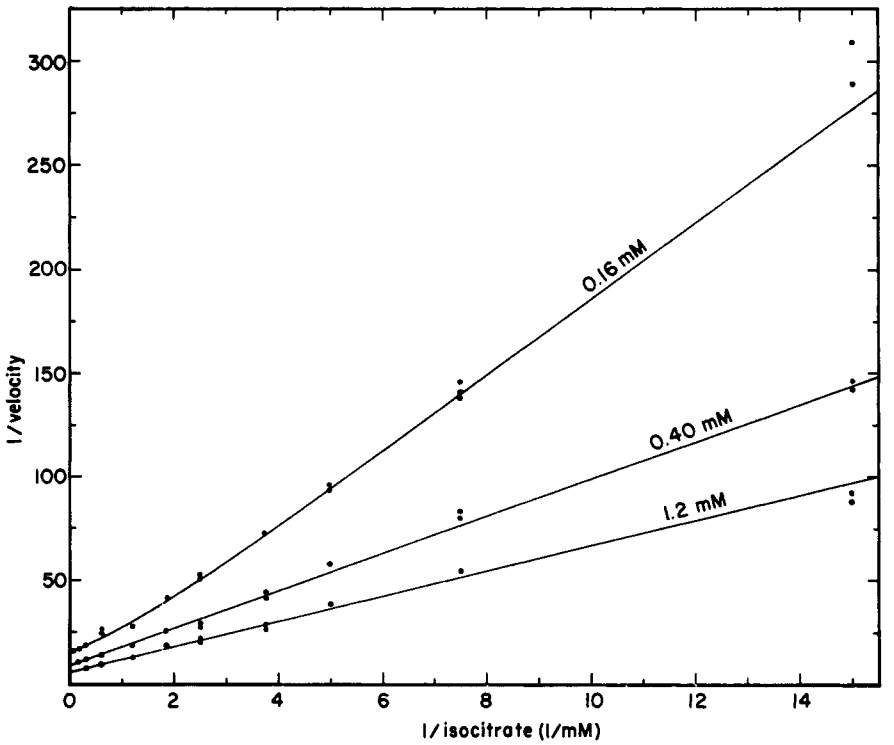


FIGURE 6: Double reciprocal plots of 1/velocity *vs.* 1/isocitrate at several fixed levels of NAD in the absence of AMP. All assay mixtures, pH 6.5, contained 6.6 mM citrate. The uppermost line has been drawn from fits to eq 5 and the lower two lines from fits to eq 3.

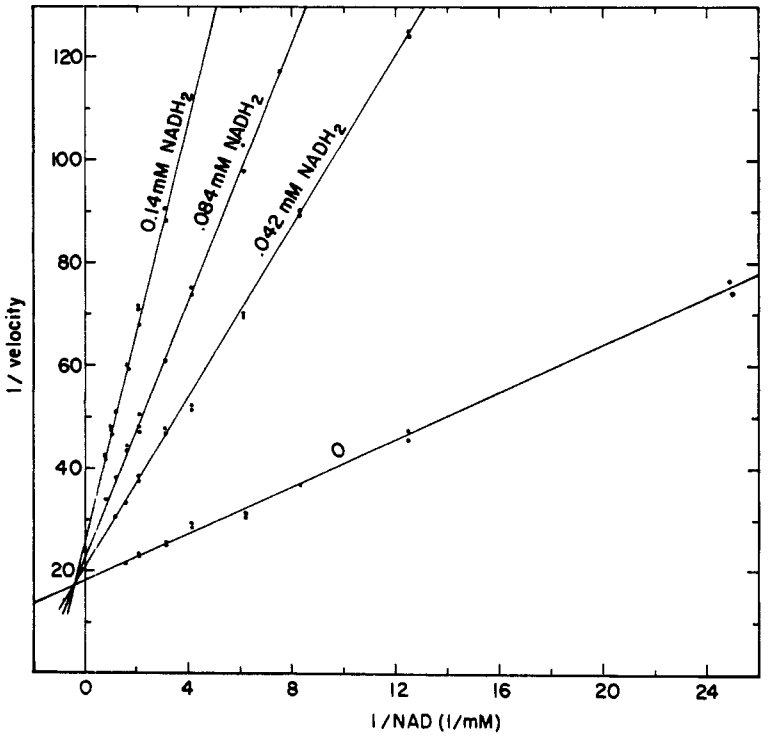


FIGURE 7: Product inhibition of isocitrate dehydrogenase at pH 6.5 by $NADH_2$ (in the absence of AMP) with NAD as the variable substrate and a constant high concentration of isocitrate (13.2 mM). Lines have been drawn from fits to eq 3.

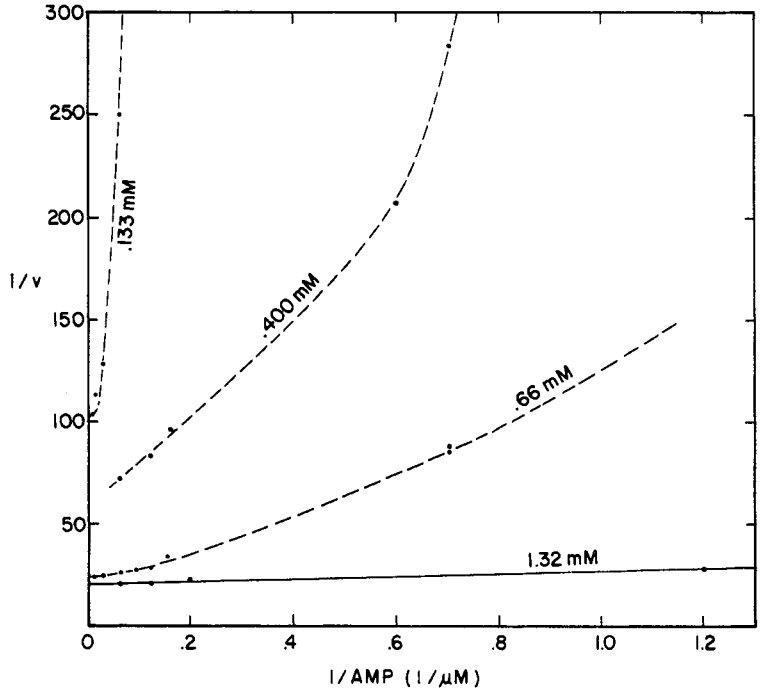


FIGURE 8: Double reciprocal plots of 1/velocity *vs.* 1/AMP at pH 6.5 at several fixed concentrations of isocitrate. The NAD concentration throughout was 2 mM. The lowermost line has been drawn from fits to eq 3. The dashed lines are fitted by eye.

these curves are plotted according to eq 2 the value of n which at lowest isocitrate concentration (0.133 mM) is 2.1 changes to approximately 1 at concentrations of isocitrate higher than 0.66 mM. Nearly similar results are obtained when the experiment is repeated at pH 7.6.

Discussion

The striking fact that emerged from our earlier observations (Sanwal *et al.*, 1964, 1965) was that the reaction mechanism appeared to be ordered, or at least NAD bound preferentially to the free enzyme form in the presence of AMP. This was demonstrated by both the initial velocity as well as product inhibition studies. However, in the absence of AMP there is very reasonable evidence that the mechanism becomes Random, *i.e.*, both NAD and isocitrate can add to the free enzyme form, and these addition steps become partially rate limiting. As a result of this the double reciprocal plots for NAD become nonlinear. We have found no evidence suggesting that more than one molecule of NAD binds on the enzyme surface.

Since the reaction mechanism possibly changes from Random to Ordered in the presence of AMP, it is quite probable that the conformational state in which the enzyme exists in the absence of AMP is different than in its presence. That the *rate* of binding of a ligand to a single protein in each of its two conformational states can be quite different has already been demonstrated by Antonini *et al.* (1963) for hemoglobin. Also, Ullmann *et al.* (1964) have given convincing evidence that AMP causes conformational changes by binding to phosphorylase *b*. It is thus conceivable that the NAD site and the allosteric site for isocitrate are both exposed in the particular conformational state that the enzyme assumes in the absence of AMP and the binding of these substrates is independent of each other and diffusion limited, while in the AMP-activated conformational state only the NAD site is exposed and the rate of binding of the coenzyme is rapid. In the latter state (with AMP) binding of NAD is perhaps necessary to "unfold" the allosteric isocitrate site (thereby ordering the sequence of addition of substrates) by a further conformational change in a manner implicit in the "induced fit" hypothesis of Koshland (1963). In a previous publication we have shown (Sanwal *et al.*, 1965) that total allosterism (where there is no catalytic activity unless the allosteric site is also filled) with regard to isocitrate is probably exhibited by the enzyme at pH 7.6 in the presence of AMP. However, at the lower pH value (6.5) the allosteric binding site for isocitrate is not recognizable kinetically in the presence of AMP. This could be either due to a conformational change induced by the combined presence of low pH and AMP, or due to a lowering of the dissociation constant for isocitrate from the allosteric site to such an extent that the allosteric binding is not recognizable, at least within the sensitivity limits of our assay procedures.

This brings us to the question that was raised earlier, *viz.*, the validity of the assumptions embodied in eq 2 and proof of "subunit interactions" in enzymes by log–log plotting procedures. For a bireactant mecha-

nism with A and B as substrates (with B binding more than once on the enzyme surface in the reaction sequence), eq 2 is valid provided that only enzyme forms E, EA, EB_n, and EAB_n are present and the reaction follows a Rapid Equilibrium Random mechanism. However, if binding of A and B is partially rate limiting (Random mechanism), as has been shown here, the initial velocity data plotted by log–log procedures may yield nearly straight lines but the conclusions drawn from the data will obviously be erroneous.

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References

Antonini, E., Wyman, J., Moretti, R., and Rossi-Fanelli, A. (1963), *Biochim. Biophys. Acta 71*, 124.

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965a), *Biochem. Biophys. Res. Commun. 18*, 1.

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965b), *J. Biol. Chem. 240*, 2682.

Changeux, J. P. (1964), *Brookhaven Symp. Biol. 17*, 232.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 173.

Cleland, W. W. (1963c), *Biochim. Biophys. Acta 67*, 188.

Cleland, W. W. (1963d), *Nature 198*, 463.

Frieden, C. (1964), *J. Biol. Chem. 239*, 3522.

Gerhart, J. C., and Pardee, A. B. (1964), *Federation Proc. 23*, 727.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

King, E. L., and Altman, C. (1956), *J. Phys. Chem. 60*, 1375.

Koshland, D. E., Jr. (1963), *Cold Spring Harbour Symp. Quant. Biol. 28*, 473.

Maeba, P., and Sanwal, B. D. (1966), *Biochemistry 5*, 525.

Monod, J., Changeux, J. P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12*, 88.

Reiner, J. M. (1959), Behaviour of Enzyme Systems, Minneapolis, Minn., Burgess.

Sanwal, B. D., and Stachow, C. S. (1965), *Biochim. Biophys. Acta 96*, 28.

Sanwal, B. D., Stachow, C. S., and Cook, R. A. (1965), *Biochemistry 4*, 410.

Sanwal, B. D., Zink, M. W., and Stachow, C. S. (1963), *Biochem. Biophys. Res. Commun. 12*, 510.

Sanwal, B. D., Zink, M. W., and Stachow, C. S. (1964), *J. Biol. Chem. 239*, 1597.

Scarano, E., Geraci, G., Polzella, A., and Campanile, E. (1963), *J. Biol. Chem. 238*, PC1556.

Ullman, A., Vagelos, P. R., and Monod, J. (1964), *Biochem. Biophys. Res. Commun. 17*, 86.

Umbarger, H. E. (1956), *Science 123*, 848.

Vickery, H. B. (1962), *J. Biol. Chem. 237*, 1739.

Vinuela, E., Salas, M. L., and Sols, A. (1963), *Biochem. Biophys. Res. Commun. 12*, 140.

Worcel, A., Goldman, D. S., and Cleland, W. W. (1965), *J. Biol. Chem. 240*, 3399.