A Kinetic Model for the Mechanism of Allosteric Activation of Nicotinamide–Adenine Dinucleotide–specific Isocitric Dehydrogenase*

B. D. Sanwal, C. S. Stachow, and R. A. Cook

ABSTRACT: Kinetic studies of nicotinamide–adenine dinucleotide–specific isocitric dehydrogenase of *Neurospora* show that the reaction mechanism is most probably ordered, with nicotinamide–adenine dinucleotide binding first followed by isocitrate. The release of products then occurs in the order: CO_2, α-ketoglutarate, and reduced nicotinamide–adenine dinucleotide. It is shown that citrate activates the enzyme by binding at an allosteric site which is also capable of accommodating isocitrate.

The sigmoidal plots of velocity versus isocitrate are interpreted as resulting from a twosite sequential binding of the substrate such that no binding occurs at the active site unless one molecule of isocitrate has been bound at the allosteric site (total allosteric effect).

The existence of a class of enzymes which serves a regulatory function *in vivo* has been recognized as a result of studies concerned with feedback inhibition of biosynthetic pathways in bacteria (Umbarger, 1956; Yates and Pardee, 1956; Gerhart and Pardee, 1962, 1964; Changeux, 1963; Martin, 1963). Monod *et al.* (1963) coined the term "allosteric proteins" to describe such enzymes. Essentially, these are characterized by the presence of an allosteric site which normally binds molecules (effectors or modifiers) that are totally unrelated chemically to the substrate. Unrelatedness of the substrate and the allosteric effector or modifier (Monod *et al.*, 1963) is, however, not a sufficient condition for identifying any protein as allosteric, or the site where they are bound as an allosteric site. Kinetically, for any enzyme to be called allosteric, two criteria must be met, and these are: (1) Combination of a modifier at a site other than the active one should alter the kinetic properties of the active center for the substrate. (2) For at least one modifier, an enzyme-substrate-modifier complex should be discernible (demonstrating that combination of the modifier is not at the active site). The allosteric effect may be considered partial when there is some activity without the modifier attached at the allosteric site (thus resulting in alternate reaction pathways), or the effect may be considered total when there is no activity without the modifier attached at the allosteric site. A distinction between partial and total allosteric effect is possible as a result of the sophistication introduced by Cleland (1963a,b,c,d) in a series of excellent papers on the kinetic and statistical analysis of the usual rate-concentration and product-inhibition data. It is obvious that a complete understanding of the kinetics of partial and total allosteric effects is of utmost importance in the interpretation of the role of such sites in the regulation of enzyme activity.

In our earlier studies (Sanwal *et al.*, 1963, 1964) it was demonstrated that the NAD-specific isocitric dehydrogenase of *Neurospora crassa* is specifically activated by citrate and inhibited by α-ketoglutarate. This, coupled with the observation that isocitrate gave sigmoidal curves in the plots of velocity against concentration instead of the usual hyperbolic ones, led to the surmise that this protein possibly had an allosteric site on which isocitrate, citrate, or α-ketoglutarate could be adsorbed, and such allosteric effects were probably total. The activation of the enzyme by citrate was considered (Sanwal *et al.*, 1963) to be an important metabolic control mechanism; and to distinguish this phenomenon from cases of nonspecific activation of enzymes by cations, anions, or other metabolites, the term "precursor activation" was applied to this and other systems where the activator is the first compound (precursor) in a chain of reactions leading to the formation of a substance which is generated by the activated reaction. One case of this kind is the well-known activation of glycogen synthetase from diverse sources (Traut and Lipmann, 1963) by glucose 6-phosphate. However, before the significance of precursor activation is assessed, the kinetic parameters of this activation must be studied.

We will show in the following report that the activation of NAD-specific isocitric dehydrogenase is brought about by citrate by binding at an allosteric site of the enzyme, that the enzyme probably has a compulsory binding order for the various reactants, and that the enzyme possibly has no activity without citrate or isocitrate bound at the allosteric site (total allosterism).

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It will also become clear that for regulatory enzymes with two or more substrates or products (like threonine deaminase [Umbarger, 1956; Changeux, 1963] and aspartate transcarbamylase [Gerhart and Pardee, 1962]) kinetically meaningful interpretations of allosterism can be obtained only by applying techniques and theory developed for bi- or multireactant enzymic mechanisms. Understanding of the overall mechanism of allosterism will, however, have to await physical chemical studies of the enzymes.

Experimental

Reagents. Chromatographically pure, synthetic potassium *threo*-D_sL_s-isocitrate[1] was a generous gift of Dr. H. B. Vickery (Connecticut Agricultural Experiment Station). Disodium salt of $NADH_2$, NAD, sodium salt of adenosine 5-phosphate, and NADP-specific isocitric dehydrogenase were purchased from the Sigma Chemical Co. To obtain *threo*-L_s-isocitric acid, the D_s isomer in the D_sL_s salt was destroyed by NADP-specific isocitric dehydrogenase, and the L_s enantiomer was concentrated by chromatography on Dowex 1 (formate) columns according to the procedure of Palmer (1955). *threo*-L_s-Isocitrate prepared in this manner was found to be neither a substrate nor an inhibitor of NAD-specific isocitric dehydrogenase reported here.

Purification of the Enzyme. The NAD-specific isocitric dehydrogenase was purified from lyophilized cells of *Neurospora crassa* strain STA 4 by alcohol precipitation (Sanwal *et al.*, 1964) and chromatography on DEAE-cellulose column (Sanwal and Stachow, 1965). In all experiments reported here, an enzyme preparation with a specific activity of 50,000–60,000 was used (one unit is defined as an increase of absorbancy of 0.001 at 340 mμ/min per mg protein). This level of specific activity represented an approximate 500- to 700-fold purification of the enzyme over that of crude extracts. The preparation was free of the following enzymes tested: condensing enzyme, aconitase, NADP-specific isocitric dehydrogenase, NAD- and NADP-specific glutamic dehydrogenase, α-ketoglutaric acid dehydrogenase complex, $NADH_2$ and $NADPH_2$ oxidase activity, and isocitritase.

Apparatus. For all kinetic studies, a Gilford Model 2000 optical density converter connected to a Beckman DU monochromator and a 25-cm self-balancing Servo recorder with a multiple-chart drive was used. At low substrate concentrations, the full-scale sensitivity of the recorder was selected between 0.1 and 0.2 absorbance unit. Small absorbance changes in the presence of $NADH_2$ or α-ketoglutarate as inhibitor of the enzymic reaction were recorded after neutralizing the original absorbance.

Kinetic Measurements. All reagents (except buffer) including enzyme solutions were maintained at 0°. Solutions of $NADH_2$ and AMP were prepared just before use. Before assay, the filled cuvets were prewarmed to 24–25°. The reaction was started by adding a properly diluted sample of the enzyme. The final volume in each cuvet was 3.0 ml. The velocity of the reaction was measured in silica cuvets of 10 mm light path by recording the change in absorbancy at 340 mμ. The recorder curves were extrapolated to zero time and slopes were taken as initial velocities. The reaction was linear for at least 4 minutes. All experiments were carried out at *p*H 7.6 in 0.2 M Tris-acetate buffer or at *p*H 6.5 in 0.2 M potassium phosphate buffer. The enzyme was checked periodically for any denaturation during experiments by using a standard reaction mixture which contained 4.9 μmoles isocitrate, 1.0 μmole adenosine 5-phosphate, 120 μmoles Tris, *p*H 7.6, 9.0 μmoles $MgSO_4$, and 0.75 μmole NAD in 3 ml total volume.

Analysis of the Kinetic Data. All kinetic data were processed according to Cleland (1963d) using an IBM 1620 digital computer.[2] The nomenclature of reaction mechanisms and description of kinetic constants used here is also that proposed by Cleland (1963a). After preliminary plots were made of the data in the reciprocal form (plots of $1/v$ versus $1/s$), iterative least-square fits were made to equation (1) when the reciprocal plots were straight lines,

$$v = \frac{VS}{K + S} \tag{1}$$

to equation (2) when they were parabolas,

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

and in some cases to equation (3) when it appeared that they might be a cubic function

$$v = \frac{VS^3}{a + bS + cS^2 + S^3} \tag{3}$$

Replots of slopes or intercepts obtained from this analysis were made against inhibitor concentration to determine the nature of the inhibition, or against the reciprocal of the concentration of the nonvaried substrate in initial velocity studies. The replots were fitted, when appropriate (Cleland, 1963d), to a straight line, $y = ax + b$, or a parabola, $y = a + bx + cx^2$, using weighting factors supplied by the first fits to equations (1) or (2). Appropriate fits were then made, wherever possible, to an overall rate equation describing the observed type of inhibition, or the proper initial velocity pattern. In particular, data were fitted in the case of linear competitive inhibition to equation (4),

[1] The subscript "s" indicates that the configurational prefix refers to the lowest numbered asymmetric carbon atom.

[2] The FORTRAN programs for all analyses reported in this paper were generously provided to us by Dr. W. W. Cleland, University of Wisconsin.

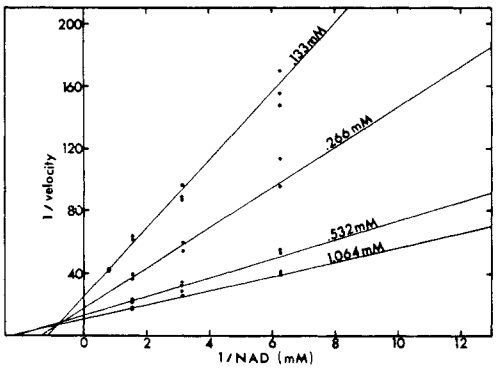


FIGURE 1: Double-reciprocal plots of 1/velocity versus 1/NAD at several fixed concentrations of isocitrate. Enzyme concentration was 100 units per cuvet. Experiments performed in 0.2 M phosphate buffer, *p*H 6.5. Lines have been drawn from fits to equation (1).

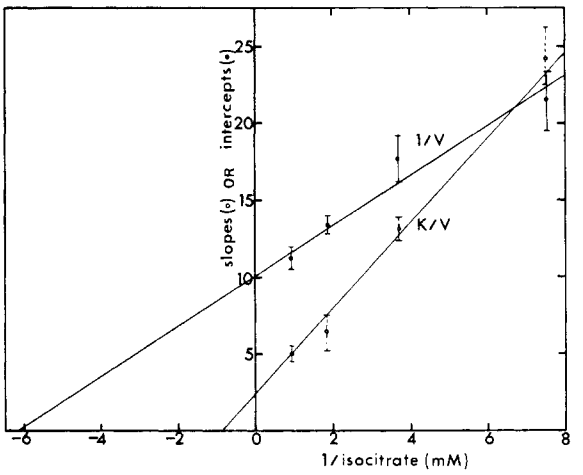


FIGURE 2: Replots of slopes and intercepts from Figure 1 versus reciprocal of isocitrate concentrations.

$$v = \frac{VS}{K(1 + I/K_i) + S} \tag{4}$$

in the case of linear noncompetitive inhibition to equation (5),

$$v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii})} \tag{5}$$

in the case of linear uncompetitive inhibition to equation (6),

$$v = \frac{VS}{K + S(1 + 1/K_i)} \tag{6}$$

and, finally, in the case of *S*-parabolic *I*-linear noncompetitive inhibition to equation (7),

$$v = \frac{VS}{K(1 + I/K_{i1} + I^2/K_{i2}) + S(1 + I/K_{ii})} \tag{7}$$

The values of inhibition constants were obtained together with standard errors of their estimates from overall fits to these equations.

It was shown earlier (Sanwal *et al.*, 1964) that NAD-specific isocitric dehydrogenase requires AMP for maximal activity at all *p*H values. All experiments reported here were therefore conducted with nearly saturating but noninhibitory concentrations of AMP (0.533 mM, which is 20 times the Michaelis constant for AMP, Sanwal *et al.*, 1964). Also, since preliminary experiments demonstrated that only *threo*-D_s-isocitrate (an authentic sample of which was supplied by Dr. Vickery) and not the L_s isomer serves as a substrate for the enzyme, all experiments were performed with potassium *threo*-D_sL_s-isocitrate. As has already been mentioned, L_s-isocitrate does not inhibit enzyme activity in the concentration range 0.2–1.0 mM. In all of the experiments reported here, the concentrations given for isocitrate are for *threo*-D_sL_s-isocitrate. The kinetic constants for the *threo*-D_s form are thus half of those reported here. All reaction mixtures also contained 3.3 mM (final) $MgSO_4$.

Results

Initial Velocity Analysis at pH 6.5. It was shown in an earlier communication (Sanwal *et al.*, 1963, 1964) that allosteric effects exhibited by NAD-specific isocitric dehydrogenase at its *p*H optimum (7.6) are absent at *p*H 6.5 (in conformity with similar findings in other allosteric proteins: Gerhart and Pardee, 1964; Changeux, 1963). For a kinetic analysis of allosteric effects at *p*H 7.6, therefore, it was essential to investigate the nature of the enzymic reaction mechanism at a *p*H value where allosteric effects were absent.

When NAD was used as a variable substrate at several fixed concentrations of isocitrate the double-reciprocal plots (Figure 1) were linear. Replots of values for slopes and intercepts obtained from fits to equation (1) against the reciprocals of the concentrations of isocitrate were likewise linear (Figure 2). With isocitrate as the variable, and NAD as the changing fixed substrate, the double-reciprocal plots as well as replots of $1/V$ and K/V were also linear. Data given in Figure 1 correspond to equation (8):

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{8}$$

(where A and B are substrate concentrations, K_a and K_b are Michaelis constants of A and B, respectively, and K_{ia} is the dissociation constant for A). As has already been shown by Cleland (1963a), equation (8) is obeyed by several sequential mechanisms (such as Rapid Equilibrium Random or Ordered mechanisms

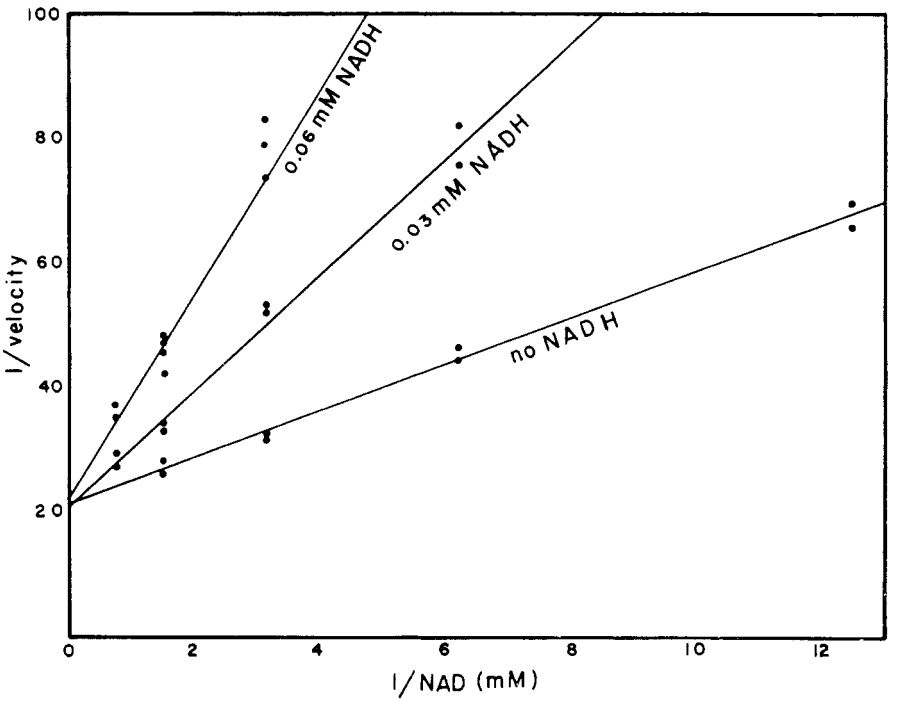


FIGURE 3: Product inhibition of isocitric dehydrogenase by $NADH_2$ with NAD as the variable substrate and a constant high concentration of isocitrate (6.6 mM). Lines have been drawn from fits to equation (1). Phosphate buffer, 0.2 M, *p*H 6.5, was used in all experiments. Concentration of enzyme in each cuvet = 50 units.

[8]), and it is also impossible to tell *A* and *B* apart from initial velocity analysis alone.

Product Inhibition Studies at pH 6.5. To distinguish between various possible sequential mechanisms and to determine the order of binding of the substrates and release of products, inhibition studies were made using various products as inhibitors. With NAD as the varied substrate, $NADH_2$ gave competitive inhibition (Figure 3). Slopes of these plots were found to be a linear function of the concentration of $NADH_2$. When isocitrate was used as the variable substrate and $NADH_2$ as inhibitor, linear double-reciprocal plots showing noncompetitive inhibition were obtained (Figure 4). The inhibition was nearly abolished by using saturating levels of NAD in the reaction mixtures. Replots of intercepts or slopes versus $NADH_2$ concentrations were linear (Figure 5). Since $NADH_2$ shows competitive inhibition with NAD and noncompetitive with isocitrate, it is reasonable to assume that both NAD and $NADH_2$ bind to the free enzyme form. With a very highly purified enzyme preparation, we were also able to demonstrate (unpublished observations) this directly by a quenching of fluorescence of enzyme solution at 340 mμ (activation wavelength, 290 mμ) by $NADH_2$.

Using α-ketoglutarate as product inhibitor and NAD as the variable substrate in the presence of high concentrations of isocitrate (about 18 times its K_m), the inhibition was found to be uncompetitive (Figure 6). A replot of the intercepts against inhibitor concentrations was linear. When a similar experiment was performed with isocitrate as the variable substrate, the inhibition was found to be noncompetitive (Figure 7). A replot of intercepts was linear, as was apparently that of slopes (Figure 8), but it seems possible (owing to the rather large standard errors) that in reality the inhibition by α-ketoglutarate is *S*-parabolic *I*-linear noncompetitive. This is borne out by the inconsistency in the calculation of K_{iq} (α-ketoglutarate inhibition constant) from Figures 6 and 7. The K_{iq} value predicted from Figure 6 is 80 mM but from Figure 8 this value comes out to be 139 mM. This suggests that α-ketoglutarate may combine with the enzyme-NAD complex in a dead-end manner as well as combining as a product inhibitor.

When $KHCO_3$ was used as an inhibitor and NAD was varied in the presence of nonsaturating concentrations of isocitrate (Figure 9), the inhibition was found to be noncompetitive. Replots of intercepts and slopes against inhibitor concentrations were also seemingly linear (Figure 10). When $KHCO_3$ was used as an inhibitor and isocitrate was the varied substrate (Figure 11), the inhibition was again found to be noncompetitive. Replots of intercepts and slopes (Figure 12) were linear, although in the case of slopes parabolic inhibition could not be completely ruled out (owing to the fact that there were only three points and their standard error was not small enough). However, as with α-ketoglutarate, it appears that $KHCO_3$ combines as a product inhibitor and also in a dead-end manner with the

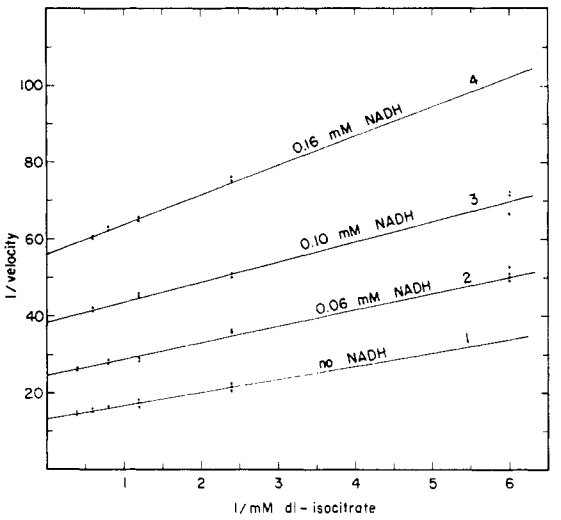


FIGURE 4: Product inhibition of isocitric dehydrogenase by $NADH_2$ with isocitrate as the variable substrate at a constant unsaturating concentration of NAD (0.2 mM). Lines have been drawn from fits to equation (1). Enzyme concentration was 125 units per cuvet. Phosphate buffer, 0.2 M, *p*H 6.5, was used in all experiments.

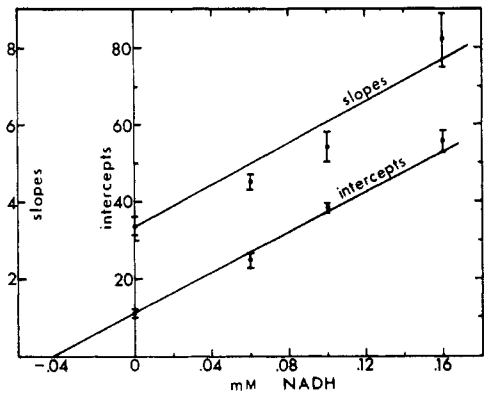


FIGURE 5: Replots of slopes and intercepts from Figure 4 versus $NADH_2$ concentrations.

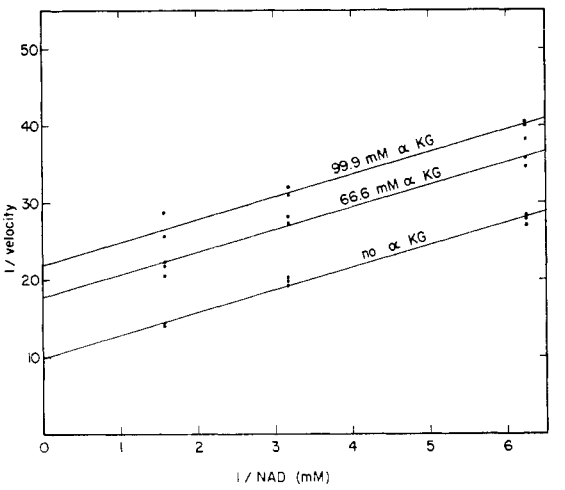


FIGURE 6. Product inhibition of isocitric dehydrogenase by α-ketoglutarate with NAD as the variable substrate and a fixed concentration of isocitrate (4.065 mM). Lines have been drawn from an overall fit to equation (6). Enzyme concentration was 125 units per cuvet. Experiments performed with 0.2 M phosphate buffer, *p*H 6.5.

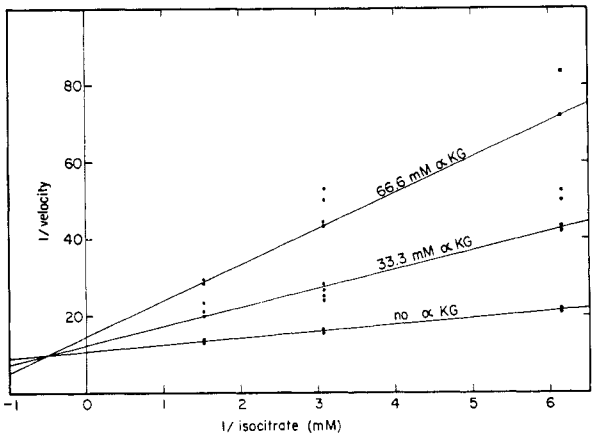
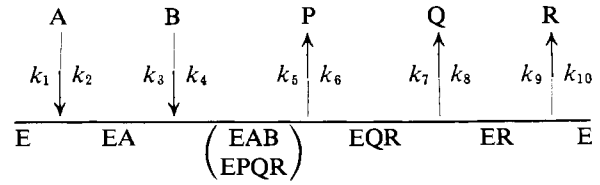


FIGURE 7: Product inhibition of isocitric dehydrogenase by α-ketoglutarate with isocitrate as the variable substrate and a constant concentration of NAD (0.8 mM). Lines have been drawn from an overall fit to equation (5). Enzyme concentration in each cuvet = 125 units. Phosphate buffer, 0.2 M, *p*H 6.5, was used in all experiments.

enzyme-NAD complex. This surmise is, again, based on the quantitative reasoning that the slope-inhibition constant (isocitrate varied, Figure 11) is predicted to be 140–150 mM on the basis of the K_i values when NAD is varied, but it is actually lower (98 mM). This suggests that data represented in Figure 11 may actually be *S*-parabolic *I*-linear noncompetitive inhibition.

Reaction Sequence in the Absence of Allosteric Effects. Initial velocity studies at *p*H 6.5 (Figures 1 and 2) show that the reaction obeys equation (8) which indicates that both NAD and isocitrate must bind to the enzyme before the products are released. This rules out a Ping-Pong kind of mechanism because, as has been shown by Cleland (1963a,b), in such a mechanism the $K_{ia}K_b$ term is missing from the denominator of equation (8). The product-inhibition data indicate that the simplest mechanism compatible with it is an ordered addition of substrates and liberation of products (ordered Bi Ter) as shown in equation (9)



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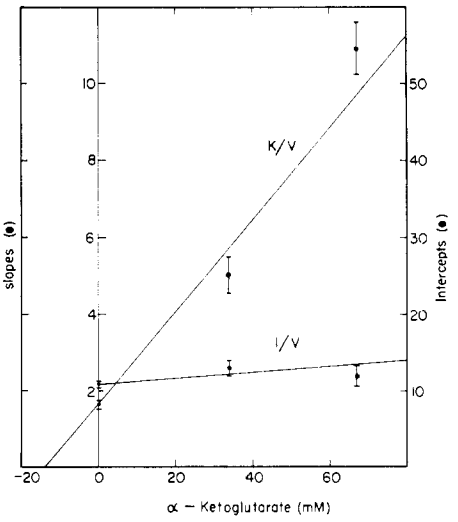


FIGURE 8: Replots of slopes and intercepts from Figure 7 against concentrations of α-ketoglutarate.

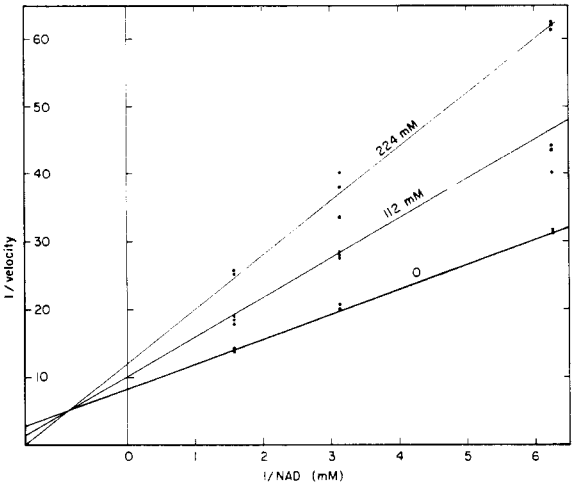


FIGURE 9: Product inhibition of isocitric dehydrogenase at *p*H 6.5 by HCO_3^- with NAD as the variable substrate and a constant concentration of isocitrate (0.4878 mM, unsaturating). Lines are drawn from an overall fit to equation (5). Enzyme concentration was 170 units per cuvet. Phosphate buffer was used.

(where A is NAD, B is isocitrate, P is CO_2, Q is α-ketoglutarate, and R is NADH). The steady-state rate equation for this mechanism can be derived according to Cleland's method (1963a) in the form (10):

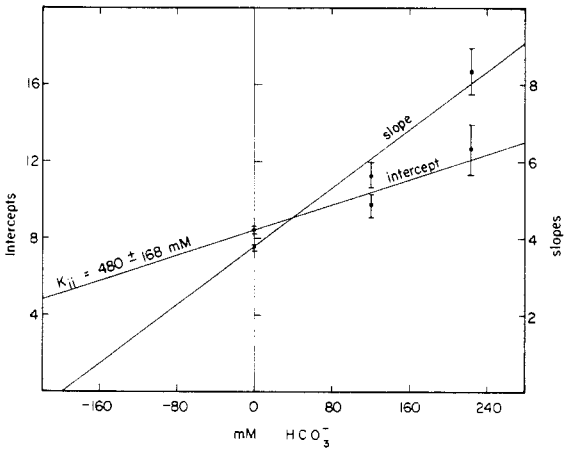


FIGURE 10: Replots of slopes and intercepts from Figure 9 versus concentrations of HCO_3^-.

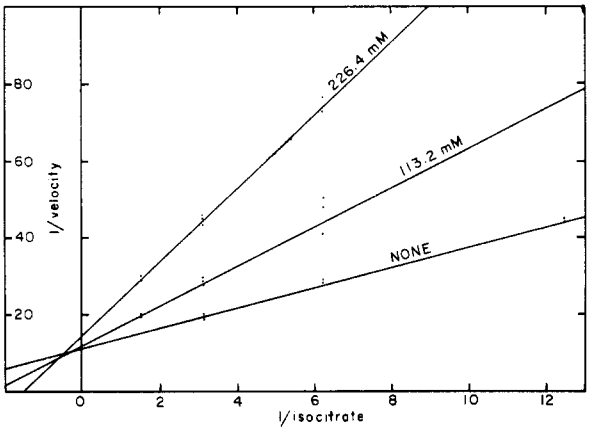


FIGURE 11: Product inhibition of isocitric dehydrogenase by HCO_3^- with isocitrate as the variable substrate and a fixed concentration of NAD (0.8 mM). Individual lines are drawn from fits to equation (1). Enzyme concentration was 125 units per cuvet. Phosphate buffer, 0.2 M, *p*H 6.5, was used in all experiments.

Equation (10) is very useful because it can be used to predict product-inhibition patterns and to distinguish the ordered mechanism from Theorell-Chance type and certain Rapid Equilibrium Random mechanisms (where A and B both can bind the free enzyme but the rate-limiting step is the interconversion of the central complexes). The presence of ABPQ and BPQR terms in the denominator of equation (10) combined with the

$$v = \frac{V_1\left(\mathrm{AB} - \frac{\mathrm{PQR}}{K_{eq}}\right)}{\left[K_{ia}K_b + K_b\mathrm{A} + K_a\mathrm{B} + \mathrm{AB} + \frac{K_{ia}K_bK_q\mathrm{P}}{K_pK_{iq}} + \frac{K_{ia}K_b\mathrm{R}}{K_{ir}} + \frac{K_b\mathrm{AP}}{K_{ip}} + \frac{K_{ia}K_bK_r\mathrm{PQ}}{K_pK_{iq}K_{ir}} + \frac{K_a\mathrm{BR}}{K_{ir}} + \frac{K_{ia}K_b\mathrm{QR}}{K_{iq}K_{ir}} + \frac{K_{ia}K_bK_q\mathrm{PR}}{K_pK_{iq}K_{ir}} + \frac{\mathrm{ABP}}{K_{ip}} + \frac{K_{ia}K_b\mathrm{PQR}}{K_pK_{iq}K_{ir}} + \frac{K_rK_b\mathrm{APQ}}{K_pK_{iq}K_{ir}} + \frac{\mathrm{ABQ}}{K_{iq}} + \frac{K_{ia}K_b\mathrm{BQR}}{K_{ib}K_{iq}K_{ir}} + \frac{\mathrm{ABPQ}}{K_{ip}K_{iq}} + \frac{K_{ia}K_b\mathrm{BPQR}}{K_pK_{ib}K_{iq}K_{ir}}\right]} \quad (10)$$

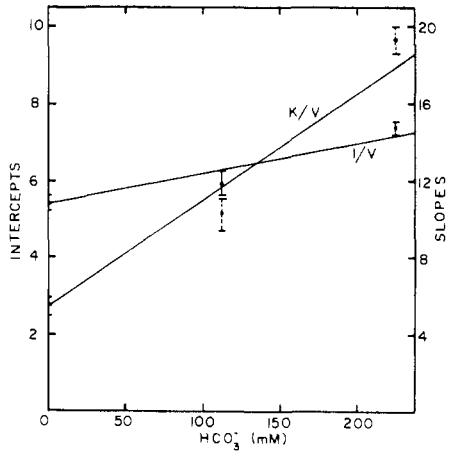


FIGURE 12: Replots of slopes and intercepts from Figure 11 versus concentrations of HCO_3^-.

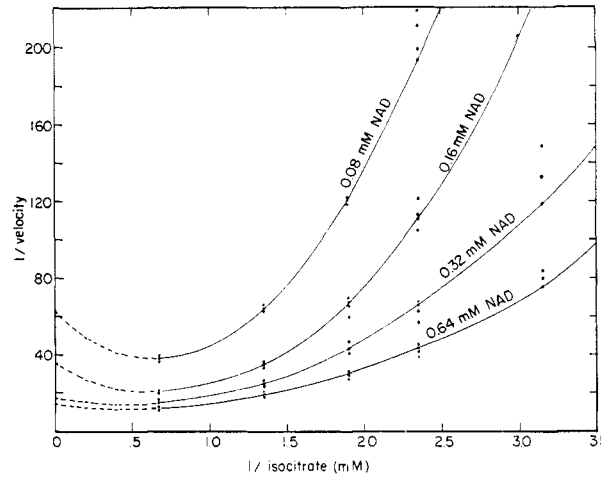


FIGURE 13: Double-reciprocal plots of 1/velocity versus 1/isocitrate at several fixed concentrations of NAD. Enzyme concentration was 100 units per cuvet. The lines have been drawn from fits to equation (2), dotted lines being the extrapolated parts of the curves. Tris-acetate buffer, 0.2 M, *p*H 7.6, was used in all experiments.

fact that mechanisms without central complexes are unlikely (Wratten and Cleland, 1963) rules out a Theorell-Chance type of reaction mechanism. Similarly, the Rapid Equilibrium Random mechanism (without dead-end complexes) lacks APQ, BQR, ABPQ, and BPQR terms in the denominator of equation (10). However, a Rapid Equilibrium Random mechanism with some dead-end complexes gives a rate equation similar to (10), but this can be eliminated from the results of product-inhibition studies with bicarbonate and α-ketoglutarate presented earlier. Further, a Random mechanism also can be ruled out on the basis of product-inhibition data which show that slopes and intercepts of double-reciprocal plots are in general linear functions of inhibitor concentrations, and when they are not they can be explained easily on the basis that the inhibitors act both as product and as dead-end inhibitors. In all likelihood, therefore, the isocitric dehydrogenase reaction is ordered Bi Ter. The value of some kinetic constants at *p*H 6.5 is shown in Table I.

TABLE I: Some Kinetic Constants of Isocitric Dehydrogenase.

Constant	*p*H 6.5 (mM)	*p*H 7.6[a] (mM)
K_a	0.265 ± 0.068	0.327 ± 0.073
K_b	0.16 ± 0.04	0.154 ± 0.03
K_{ia}	0.712 ± 0.15	0.552 ± 0.159
K_{ip}	370 ± 130[b]	
K_{iq}	80 ± 5[b]	
K_{ir}	0.017 ± 0.003	0.02 ± 0.001

[a] In the presence of 2.2 mM citrate. [b] The validity of these figures is in doubt owing to the problems discussed in the text.

Initial Velocity and Production-Inhibition Analysis at pH 7.6. It was shown in our earlier work (Sanwal *et al.*, 1963, 1964) that allosteric effects are exhibited by isocitric dehydrogenase at *p*H 7.6 (in common with other allosteric enzymes at their *p*H optima: Gerhart and Pardee, 1964; Changeux, 1963). When isocitrate was varied against several fixed concentrations of NAD, the double-reciprocal plots were curved (Figure 13). These curves were fitted to equation (2) and significant fits were obtained, but produced negative coefficients, which would be impossible if these curves were parabolas. According to the theory of multireactant enzyme kinetics developed by Cleland (1963a,c,d), parabolic curves would be obtained for an ordered mechanism without alternate pathways if the varied substrate participated twice in the reaction sequence and if the points of addition of varied substrate were connected by reversible steps. However, data represented in Figure 13 are still consistent with the idea that isocitrate binds at the allosteric site (since, as shown earlier [Sanwal *et al.*, 1964], it is operative at *p*H 7.6) as well as the active site. A three-place addition of the substrate during reaction sequence (assuming two allosteric sites) is inconsistent with the data because of the insignificant fits obtained to equation (3).

In contrast to the curved plots obtained with isocitrate, when NAD was varied against several fixed concentrations of isocitrate the double-reciprocal plots were all linear (Figure 14), suggesting that NAD perhaps binds only once during the reaction sequence. Replots of slopes and intercepts from Figure 14 against the reciprocals of isocitrate concentrations, as expected, were curved and were not analyzed further.

Reduced NAD when used as a product inhibitor

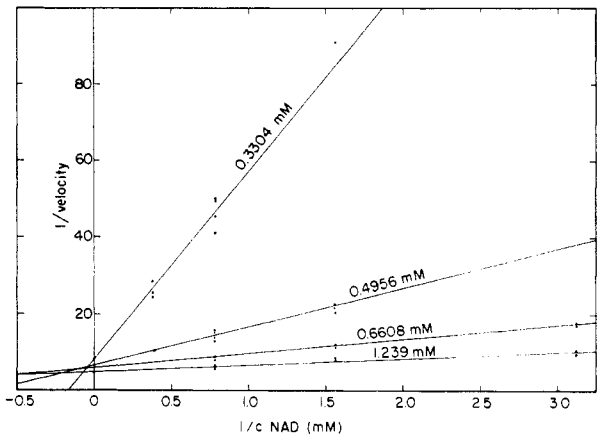


FIGURE 14: Double-reciprocal plots of 1/velocity versus 1/NAD at several fixed concentrations of isocitrate. Individual lines are drawn from fits to equation (1). Enzyme concentration was 100 units per cuvet. All experiments were performed in 0.2 M Tris-acetate buffer, *p*H 7.6.

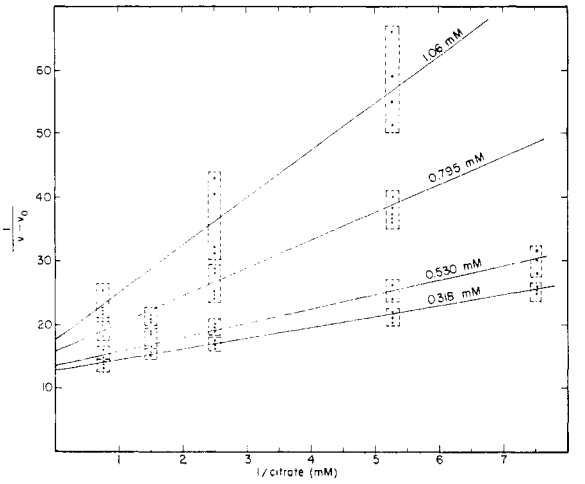


FIGURE 15: The effect of citrate on the velocity of enzymic reaction at *p*H 7.6. Here v = velocity in the presence of citrate and v_0 = velocity in the absence of citrate. Lines shown are fitted to equation (1). Figures above the lines refer to the concentration of isocitrate. All reaction mixtures contained 0.8 mM NAD. Enzyme concentration was about 250 units per cuvet. Tris-acetate buffer (0.2 M) was used throughout.

gave competitive inhibition against NAD, as at *p*H 6.5. Replots of slopes against $NADH_2$ were also linear, giving a K_{ir} value ($NADH_2$ inhibition constant) of 0.02 mM ± 0.008, which is similar to that obtained at *p*H 6.5 or that obtained in the presence of an allosteric activator (see below). It is very likely that, as at *p*H 6.5, NAD and $NADH_2$ both bind to the free enzyme form at *p*H 7.6.

Reaction Kinetics in the Presence of Allosteric Modifier. The formulation of a kinetic model for allosteric effects is made easier if certain parameters of this effect can be clearly defined in quantitative terms. Specifically, it is useful to know (in order to distinguish between partial and total allosterism) whether the various kinetic constants change at all in the presence of an allosteric effector.

We had earlier (Sanwal *et al.*, 1963) described the activation of isocitric dehydrogenase by citrate, and recently we have found *erythro*-L$_s$-isocitrate as an equally effective activator (Sanwal and Stachow, 1965). The effects of both of these activators are almost similar, viz., in their presence the curved double-reciprocal plots (as in Figure 13) become linear, the activators do not serve as substrates for the enzyme, and, finally, the activators are effective only at *p*H 7.6 (when the enzyme shows allosteric effects) but do not affect enzyme activity at *p*H 6.5 (when, presumably, the allosteric site is not operative).

If one assumes a total allosteric mechanism (no activity without substrate or modifier binding at the allosteric site) for isocitric dehydrogenase, then in the presence of modifier (M), substrate (B), and coenzyme A), when citrate is varied at several levels of isocitrate equation (11) should hold (where v_o = initial velocity in the absence and v = velocity in the presence of modifier, and K_1 and K_2 are dissociation constants of B and M, respectively, from the allosteric site). This

$$\left(\frac{1}{v - v_o}\right) = \left(1 + \frac{B}{K_1}\right)\left(\frac{K_{ia}K_b}{AB} + \frac{K_b}{B} + \frac{K_a}{A} + 1\right) \times \left(\frac{K_2}{V}\left(1 + \frac{B}{K_1}\right)\frac{1}{M} + \frac{1}{V}\right) \quad (11)$$

equation predicts that double-reciprocal plots of $(1/[v - v_o])$ versus $(1/M)$ will be linear and the apparent Michaelis constants (slope/intercept) will be a linear function of B. When an experiment of this kind is performed, it is found (Figure 15) that the reciprocal plots are indeed linear, but replots of K_{app} against concentration of isocitrate are curved (Figure 16). It is thus quite clear that the simple relationship given by equation (11) does not hold. The dissociation constant of isocitrate from the allosteric site cannot be determined in an unambiguous manner owing to the curvature of the K_{app} replot. The data show that the dissociation constant is a function of isocitrate concentration, and it becomes lower as the isocitrate concentration rises. In other words, combination at the active site influences combination at the allosteric site. However, the fact that the double-reciprocal plots are straight (Figure 15) shows that there is a combination of only one molecule of citrate on the allosteric site. If more than one combined, the reciprocal plots would be parabolas or curves of a more complex nature.

To determine whether kinetic constants or product-inhibition patterns change at all owing to allosteric binding, two experiments were performed at *p*H 7.6 in the presence of high (2 mM) concentrations of citrate (to saturate the allosteric site). In one experiment

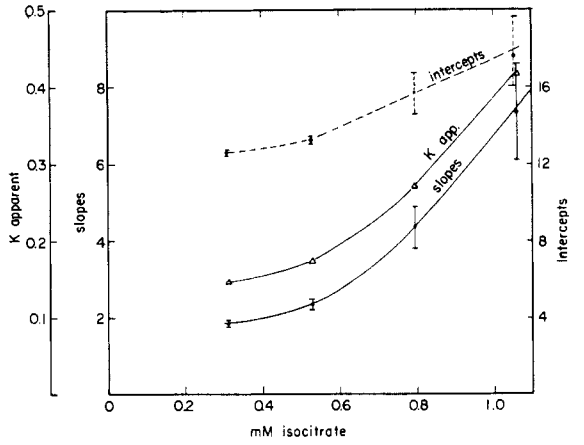


FIGURE 16: Replots of slopes, intercepts, and apparent Michaelis constants (K_{app}) from Figure 14 versus concentrations of isocitrate.

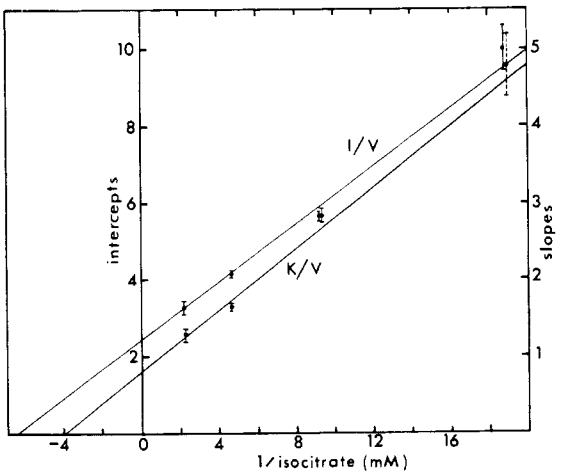


FIGURE 18. Replots of intercepts and slopes from Figure 16 versus the reciprocals of isocitrate concentrations.

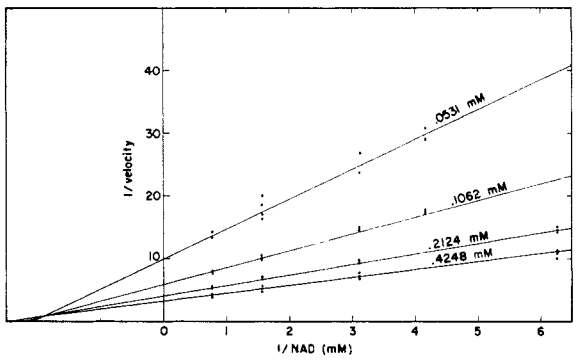


FIGURE 17: Double-reciprocal plots of 1/velocity versus 1/NAD at several fixed concentrations of isocitrate in the presence of a constant high concentration (2.2 mM) of citrate. Each cuvet contained about 400 units of enzyme. The lines have been drawn from an overall fit to equation (8). All experiments performed in Tris-acetate buffer, 0.2 M, *p*H 7.6.

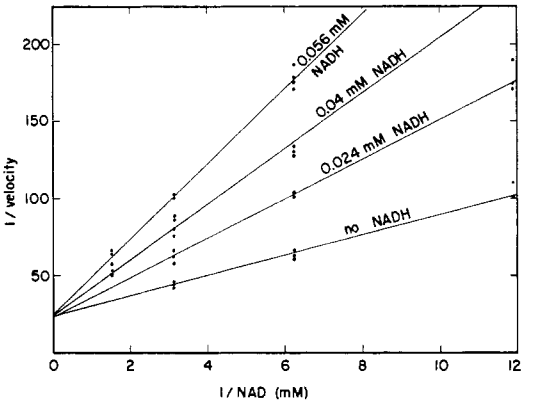


FIGURE 19: Product inhibition of isocitric dehydrogenase by $NADH_2$ at *p*H 7.6 in the presence of a constant high concentration of citrate (2.2 mM). NAD was used as the variable substrate and isocitrate was kept constant (2 mM). Lines are drawn from an overall fit to equation (4). Enzyme (60 units) was used in each cuvet. Tris-acetate, 0.2 M, was used in all experiments.

NAD was used as the variable and isocitrate as the changing fixed substrate (Figure 17). Replots of slopes and intercepts against the reciprocal of isocitrate concentrations (Figure 18) yielded straight lines (compare Figure 14), which means that when the allosteric site is saturated only one molecule of isocitrate combines with the enzyme. In the second experiment, NAD was varied against several fixed concentrations of $NADH_2$ as a product inhibitor. As expected, competitive inhibition was obtained (Figure 19). The replot of slopes against $NADH_2$ was also linear. The various kinetic constants which could be evaluated from this data are given in Table I. It will be seen that the kinetic constants are similar at *p*H 6.5 and at *p*H 7.6 in the presence of the allosteric activator.

Discussion

The simple model that emerges as a result of the experiments reported here is that in the absence of allosteric effects (at *p*H 6.5 or saturation with a modifier at *p*H 7.6) the mechanism of isocitric dehydrogenase reaction is ordered, with A (= NAD) adding first, followed by B (= isocitrate). The release of products then occurs in the order: CO_2 (P), α-ketoglutarate (Q), and $NADH_2$ (R). In addition, dead-end complexes are very likely formed by P and Q combining with enzyme-NAD complexes.

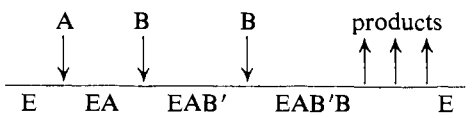
It is clear from our data that NAD binds at only one site on the enzyme surface both in the presence and the absence of allosteric effects. Binding at places other than the active site would be recognizable kinetically

by a curvature of the reciprocal plots when NAD was the varied substrate (Figures 1, 14, and 17) or the curvature of the replots of slopes or intercepts against the reciprocal of NAD when isocitrate was the varied substrate. Cleland (1963a) has already shown that reciprocal plots for substrates, activators, or inhibitors, which bind at places other than the active site, or those resulting from some Random mechanisms, are 2/1 functions, such as

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

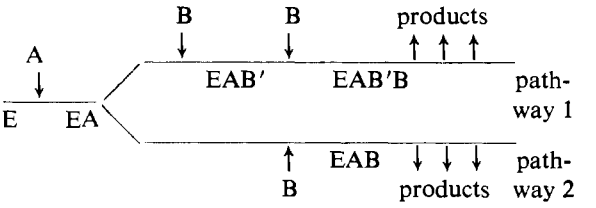
(where a, b, c, d, and e are functions of other reactant concentrations), or more complex curves. The same argument applies to the binding of isocitrate in the absence of allosteric effects at *p*H 6.5. It is nearly certain that isocitrate binds only at the active site when the allosteric site is inactive (*p*H 6.5) or is saturated by the modifier (*p*H 7.6). Similarly, citrate binds only at one place when the allosteric site is operative at *p*H 7.6. This is evidenced by the linearity of the reciprocal plots shown in Figure 15. The binding of citrate must be only at the allosteric site because, as was shown in our earlier work (Sanwal *et al.*, 1963), citrate shows no effect on the velocity of the reaction at *p*H 6.5 (when presumably the allosteric site is inoperative).

The foregoing analysis leaves us with the interpretation of double-reciprocal plots which result when the allosteric site is operational (at *p*H 7.6) and isocitrate is the varied substrate (such nonlinear double-reciprocal plots seem to be a characteristic of many regulatory enzymes from diverse sources: Gerhart and Pardee, 1964; Changeux, 1963; Vinuela *et al.*, 1963; Scarano *et al.*, 1963; Maley and Maley, 1963; Passonneau and Lowry, 1963). Since we have shown that the reaction mechanism is ordered, the curvature of the reciprocal plots (Figure 13) suggests that isocitrate binds at this *p*H at two different sites and that there is a reversible connection between the points of binding of the substrate in the reaction pathway. It is obvious that one of these sites must be the active site, and it is very probable that the other site is an allosteric site. This is clearly borne out by the results presented in Figure 18, where in the presence of high levels of citrate at *p*H 7.6 (and near saturation of the allosteric site) only one molecule of isocitrate binds to the enzyme (binding of more than one molecule would result in the curvature of the replot). The question now arises whether the binding of two molecules of isocitrate, one at the allosteric and another at the active site, is a prerequisite for the release of products (Total Allosterism, Mechanism 12



(12)

(where B′ represents binding of B at the allosteric site and B represents binding at the active isocitrate site) or, there is activity with isocitrate bound only at the active site, but more activity with another molecule of isocitrate bound also at the allosteric site (Partial Allosterism, Mechanism 13)



(13)

Assuming that the conversion of EA to the EAB′ form is slow compared with other catalytic steps in the reaction mechanisms (12) and (13), W. W. Cleland (unpublished) has recently derived rate equations for these mechanisms. The form of equation for mechanism (12) is obtained by multiplying the proper initial velocity equation by the factor (B/[K_1 + B]), where K_1 is the allosteric dissociation constant for isocitrate, and equals:

$$\frac{(EA)(B')}{(EAB')}$$

In terms of K_m and V_{max}, this is (14):

$$v = V\left(\frac{B}{K_b + B}\right)\left(\frac{B}{K_1 + B}\right) \qquad (14)$$

or, in the standard reciprocal form (15):

$$\frac{1}{v} = \frac{K_b K_1}{V}\left(\frac{1}{B}\right)^2 + \left(\frac{K_1 + K_b}{V}\right)\left(\frac{1}{B}\right) + \frac{1}{V} \qquad (15)$$

which is an equation for a parabola.

For mechanism (13), the rate equation is the sum of activities of EA and EAB′ forms (16):

$$v = V_1\left(\frac{B}{K_{b1} + B}\right)\left(\frac{K_1}{K_1 + B}\right) + \left(\frac{V_2 B^2}{(K_{b2} + B)(K_1 + B)}\right) \qquad (16)$$

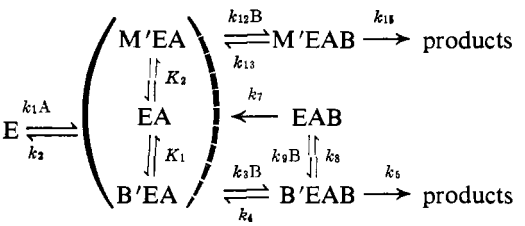
(where V_1 and K_{b1} are maximum velocity and Michaelis constant for the EA form, and V_2 and K_{b2} are constants for the EAB′ form).

Equation (16) when written in a reciprocal form is a 3/2 function, the curves of which, unlike a parabola, have an asymptote, and the curve tends to be concave up if $V_2 > V_1$ and $K_{b1} > K_{b2}$. Both equations (15) and (16) reduce to the classical Michaelis-Menton equation (1) when the allosteric site is unoperational (at *p*H 6.5) or is saturated with a modifier (at *p*H 7.6). On the basis of equation (16), mechanism (13) can be ruled

out since the curves obtained when isocitrate is the varied substrate at *p*H 7.6 (Figure 13) show no relationship to equation (16). The curves shown in Figure 13 are also not parabolas because, despite the fact that when the data are fitted to equation (2), very significant fits are obtained, but the value of at least one coefficient is negative. These curves must then be 3/1 or more complex functions, and clearly mechanisms other than those given by equation (15) and (16) are operative. The data from Figures 13 and 16 suggest a rate equation of the type:

$$v = \frac{AB^2 + AB^3 + ABM + AB^2M}{\text{constant} + A + AM + AB + B + M + ABM + AB^2 + B^2 + BM + AB^2M + AB^3 + B^3 + B^2M} \quad (17)$$

(A and B have the usual meaning, and M is the modifier). Equation (17) is derived with the assumption that the enzyme exhibits total allosterism (no activity without the substrate or a modifier first binding at the allosteric site); the allosteric combinations do not affect absorption or release of A; and the mechanism of reaction is:



[where E = free enzyme, B = isocitrate, M = citrate, B′ and M′ denote binding at the allosteric site, K_1 = (EA)B′/(B′EA), and K_2 = (EA)M′/(M′EA)].

The distribution equations for the steady state are:

$$\frac{d(EA + M'EA + B'EA)}{dt} = k_1A(E) - k_2(EA + M'EA + B'EA) + k_7(EAB) + k_4(B'EAB) + k_{13}(M'EAB) - k_{12}B(M'EA) - k_3B(B'EA) = 0$$

$$\frac{d(EAB)}{dt} = k_8(B'EAB) - (k_7 + k_9B)(EAB) = 0$$

$$\frac{d(B'EAB)}{dt} = k_3B(B'EA) + k_9B(EAB) - (k_4 + k_5 + k_8)(B'EAB) = 0$$

$$\frac{d(M'EAB)}{dt} = k_{12}B(M'EA) - (k_{13} + k_{15})(M'EAB) = 0$$

also, E + (EA + B′EA + M′EA) + EAB + B′EAB + M′EAB = E_t. This mechanism is consistent with the data of Figures 13 and 16 in that the reciprocal plots (at *p*H 7.6) when isocitrate is varied are expected to be 3/1 functions while plots of NAD as the varied substrate are linear. Similarly, when citrate is varied at several levels of isocitrate (*p*H 7.6) the plots of $(1/[v - v_0])$ (Figure 15) against (1/citrate) are expected to be linear and the replot (Figure 16) of K_{app} is expected to be a 3/2 function of isocitrate.

A study of Table I shows that the various kinetic constants of isocitric dehydrogenase do not change either with *p*H (within the limits of change of 1 *p*H unit) or, in the presence of the allosteric modifier, citrate (consistent with the mechanism proposed before). Although no detailed kinetic study is available, threonine deaminase (Changeux, 1963) and aspartate transcarbamylase (Gerhart and Pardee, 1964), both allosteric enzymes, are similarly affected by a change of *p*H. The reasons for this apparent "loss" of the allosteric site are not known, but may be connected with conformational changes induced by a change in the hydrogen ion concentration. Indeed, it is known from work with aspartate transcarbamylase (Gerhart and Pardee, 1962, 1964) and other regulatory proteins (Changeux, 1963; Martin, 1963) that the allosteric site is "fragile" and can be altered or disrupted by mild treatment with heat and reaction with thiol group reagents or urea. However, "fragility" seems to be neither a characteristic nor a sufficient condition to identify modifier sites as allosteric sites. Martin (1963), for instance, has shown that the regulatory enzyme, phosphoribosyl-ATP-pyrophosphorylase, still binds histidine (an allosteric inhibitor) after treatment to disrupt the allosteric site. In the case of isocitric dehydrogenase, however, kinetic studies show that, at *p*H 6.5, isocitrate is adsorbed only at the active site. Kinetically important binding at sites other than the active one would be recognized by a curvature of double-reciprocal plots in the form of parabolas, 2/1 or 3/2 functions.

Our earlier work (Sanwal *et al.*, 1963) has shown that citrate activates the enzyme at *p*H 7.6, but has no effect at *p*H 6.5. *erythro*-L_s-Isocitrate behaves in a similar manner (Sanwal and Stachow, 1965). This fact strongly supports the hypothesis that the allosteric site is capable of accommodating isocitrate and citrate and possibly also *erythro*-L_s-isocitrate. The nature of the interaction of these compounds with the allosteric site is of extreme

importance in understanding the mechanism of control of this enzyme, and a kinetic and physicochemical study of this interaction has been left as an object of future study. However, the fact that total allosterism is most probably exhibited by this enzyme makes for a greater precision of control of its activity by an allosteric modifier.

We have refrained so far from mentioning the nature or mode of interaction of AMP with the enzyme, but evidence indicates that in sucrose density-gradient centrifugations neither AMP alone (tested at an enzyme concentration of 0.5 mg/ml and 10^{-3} M AMP; unpublished observations), nor mixtures of AMP and citrate, nor mixtures of AMP, NAD, Mg^{2+}, and citrate cause any significant variations of the sedimentation velocity of the enzyme (Sanwal and Stachow, 1965). Thus, at least with the small amounts of enzyme employed in density-gradient centrifugations (0.5 mg/ml), "associative" changes, such as have been shown to occur in the case of NAD-specific isocitric dehydrogenase of bovine heart in the presence of ADP (Chen *et al.*, 1964), do not occur with the *Neurospora* enzyme. However, discrete conformational changes (within the framework of Koshland's theory of induced fit [Koshland, 1964]), in response to the binding of AMP at its site or the binding of citrate and isocitrate at the allosteric site, may occur. Indeed, it is difficult to visualize why it should be necessary for the substrate or modifier to bind at the allosteric site (total allosterism) before a second molecule of substrate can bind at the active site unless it be that allosteric binding is necessary for the "unfolding" of the active site by some sort of a conformational change. These problems are not amenable to kinetic approach, and an attempt is being made to answer these questions by the use of physicochemical methods.

In conclusion, it may be mentioned that the kinetic model proposed here, while it possibly will require expansion and revision as a result of physicochemical studies of homogeneous preparations of the enzyme and kinetic studies of AMP activation, constitutes the simplest possible model which fits most of the data reported here, and may, perhaps, also be applicable to the kinetic data obtained with other regulatory enzymes (Gerhart and Pardee, 1962, 1964; Changeux, 1963; Vinuela *et al.*, 1963; Scarano *et al.*, 1963; Maley and Maley, 1963; Passonneau and Lowry, 1963; Hathaway and Atkinson, 1963).

Acknowledgment

The authors are greatly indebted to Dr. W. W. Cleland, University of Wisconsin, for stimulating discussions with Dr. Sanwal, and for suggesting numerous experiments. Much of the data given here were also kindly analyzed independently by him. Some equations dealing with allosteric kinetics used in this paper are the unpublished results of Dr. Cleland, and his allowing us their use before publication is also deeply appreciated.

Reference

Changeux, J. P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Chen, R. F., Brown, D. M., and Plaut, G. W. E. (1964), *Biochemistry 3*, 552.

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.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

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

Hathaway, J. A., and Atkinson, D. E. (1963), *J. Biol. Chem. 238*, 2875.

Koshland, D. E., Jr. (1964), *Federation Proc. 23*, 719.

Maley, G. F., and Maley, F. (1963), *Biochim. Biophys. Acta 68*, 293.

Martin, R. G. (1963), *J. Biol. Chem. 238*, 257.

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

Palmer, J. K. (1955), *Conn. Agr. Expt. Sta., New Haven, Bull. 589*.

Passonneau, J. V., and Lowry, O. H. (1963), *Biochem. Biophys. Res. Commun. 13*, 372.

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

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.

Traut, R. R., and Lipmann, F. (1963), *J. Biol. Chem. 238*, 1213.

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

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

Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.

Yates, R. A., and Pardee, A. B. (1956), *J. Biol. Chem. 221*, 757.