BBA 65531

ANION AND SUBSTRATE INHIBITION AND KINETIC BEHAVIOR OF NAD+-SPECIFIC ISOCITRATE DEHYDROGENASE FROM BAKER'S YEAST

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(Received August 4th, 1966)

SUMMARY

1. The NAD+-specific isocitrate dehydrogenase (L-isocitrate:NAD+ oxidoreductase (decarboxylating), EC 1.1.1.41) from baker's yeast is inhibited competitively by certain anions. AMP counteracts this inhibition.

2. The enzyme is also inhibited by the substrate isocitrate. This inhibition appears to be due to the removal of the cofactor Mg^{2+} by the formation of an isocitrate complex.

3. Kinetic experiments, at pH 7.6, have shown that the order of the reaction with respect to isocitrate is decreased by AMP from about 3 to 1.5.

4. The order of the reaction with respect to Mg^{2+} is increased from 1 to 2 by the addition of KCl, and restored to 1 by AMP.

5. It is suggested that the addition of isocitrate and Mg^{2+} to the enzyme may be random, and the possibility is discussed that the occurrence of a random mechanism for the addition of the reactants may explain the kinetic behavior of the enzyme.

INTRODUCTION

The NAD+-specific isocitrate dehydrogenase (L-isocitrate:NAD+ oxidoreductase (decarboxylating), EC 1.1.1.41) from baker's yeast has been found to be activated by AMP[1,2]. The enzyme does not obey the Michaelis–Menten kinetics[3]. Similar findings have been reported for the enzyme from *Neurospora crassa*[1–6]. The anomalous kinetics of the isocitrate dehydrogenase from baker's yeast have been explained[3] by assuming the existence on the enzyme of several interacting isocitrate-binding sites; several sites were also assumed for the binding of each NAD+, Mg^{2+}, and AMP.

Previous investigations[7,8] have shown that the isocitrate dehydrogenase from baker's yeast is inhibited by some salts, and that AMP counteracts this inhibition; it

has been suggested that the assumption of several interacting isocitrate-binding sites is unnecessary to explain the behavior of the enzyme.

In the present research, it has been shown that the inhibition by salts appears to be due to the anions. The substrate, isocitrate, also appears to inhibit the enzyme. The kinetic behavior of the enzyme with respect to isocitrate and Mg^{2+} was also investigated. All the experiments were carried out at pH 7.6, where the peculiar behavior of the isocitrate dehydrogenase was found to be more evident[3,4].

METHODS

Enzyme

The enzymic preparation used was the "Cγ eluate" purified about 40-fold from baker's yeast essentially according to KORNBERG AND PRICER[1]. Fresh yeast was disrupted with Alcoa A 305. 30 g of Alcoa were used for 10 g of yeast, and the paste was extracted with 50 ml of 0.1 M $NaHCO_3$. Precipitation with $(NH_4)_2SO_4$ and adsorption on aluminum hydroxide gel Cγ were carried out as described by KORNBERG AND PRICER[1]. The enzyme was eluted from alumina with 13 ml of 0.1 M phosphate buffer (pH 7.6). All the procedures were carried out at 0–3°. In this rather concentrated preparation (the protein content was about 0.8 mg/ml) the enzyme appeared to be more stable than in the preparation made by the above authors: the activity decreased about 10% after 24 h at 0°.

Assay

The enzymic activity was determined by measuring the rate of NAD^+ reduction at 366 mμ with an Eppendorf Photometer, in 10-mm light path cuvettes. The temperature was maintained at 22°. The final volume of the reaction mixtures was 3 ml. All the experiments were carried out at pH 7.6; Tris–HCl or Tris–acetic acid buffers (33 mM in Tris base) were used. The detailed composition of the reaction mixtures is described in the individual experiments. The reaction was started by adding 0.04 ml of "Cγ eluate", and the increase in absorbance at 366 mμ was followed by readings at 15-sec intervals. As already reported[1], the enzymic activity declines rather rapidly with time. The difference between the readings at 30 sec and 90 sec after the addition of the enzyme was taken as the initial activity. The results are expressed in activity units, one unit corresponding to an increase in absorbance of 0.100/min per cuvette. The activity of our enzymic preparations was 0.35–0.45 units under standard conditions, that is in the presence of 3 mM isocitrate, 3.3 mM $MgSO_4$, 0.33 mM NAD^+ and 0.17 mM AMP. Blanks without substrate were routinely run. All the reaction rates are expressed in activity units. The reported concentrations are always final concentrations. In the figures, the concentrations are molar.

Chemicals

The trisodium salt of DL-isocitric acid was purchased from Nutritional Biochemicals Corporation. The solutions of isocitrate were brought to pH 7.6 with dilute acetic acid. The concentrations given are always those of a single isomer, when not otherwise specified. NAD^+ and AMP were purchased from Boehringer und Soehne.

The results of kinetic experiments were plotted according to the logarithmic plot proposed by MONOD, CHANGEUX AND JACOB[9] for the treatment of the kinetic data obtained with allosteric enzymes, when the double reciprocal plots are not linear. The same method was used by ATKINSON, HATHAWAY AND SMITH[3] for isocitrate dehydrogenase from baker's yeast. The logarithmic plot was applied[3] on the assumption that, for an enzyme having n interacting sites for the binding of the substrate, the following equation holds:

$$v = \frac{V\,S^n}{K + S^n} \tag{1}$$

where v is the initial velocity, V is the maximum velocity, and S the concentration of the substrate. Eqn. 1 can be transformed into

$$\log \frac{v}{V - v} = n \log S - \log K \tag{2}$$

According to this interpretation, the slope n of the straight line, obtained by plotting $\log [v/(V-v)]$ against $\log S$, would correspond to the number of sites if the interactions are strong. Otherwise, the determined value of n would be a minimal estimate of the number of sites. If no interaction occurs between the substrate-binding sites, n will be 1, and the reaction will follow normal Michaelis–Menten kinetics.

However, the determined value of n could merely correspond to a minimal estimate of the power to which the concentration of substrate occurs in a rate equation, also different from Eqn. 1 (*cf.* ref. 6). Thus, n could mean the minimal number of steps involving the addition of the substrate to the enzyme, in a random reaction mechanism. With this reservation, the logarithmic plot can often be considered as a simple and useful method for the treatment of kinetic data, since it can give straight lines, whereas the double reciprocal plots are curved. This fact, casual as it may be[6], means that the apparent order of the reaction does not change when the concentration of the substrate is varied in the range giving measurable enzymic activities. In these conditions, the logarithmic plot gives the apparent order of the reaction, and it makes possible an easy evaluation of the modifications of the apparent order under different experimental conditions. The logarithmic plot can also be applied to the kinetics with respect to other participants in an enzymic reaction[3].

By applying the logarithmic plot to our results, we met with some difficulty in the estimation of the maximal velocity, depending on the inhibitory effects observed with the enzyme and, most important, on an activating effect, probably connected with the value of the ionic strength, which can be exhibited by high concentrations of isocitrate (see RESULTS). *E.g.*, the presence of Tris–HCl buffer (pH 7.6), 33 mM in Tris base, gives an ionic strength of 0.027 corresponding to the HCl concentration; the addition of 6 mM DL-isocitrate already contributes 0.036 to the ionic strength. This contribution cannot be minimized by increasing the concentration of the buffer or by adding a salt, owing to the inhibitory effect of the anions. Therefore, the estimation of the maximum velocity is best carried out in the presence of AMP, when the maximum velocity can be determined at lower substrate concentrations. In the absence of AMP, it is possible to choose an exceedingly high value for the maximum velocity. The logarithmic plot will then yield curves concave downward, apparently showing a decrease in the slope with increasing isocitrate concentrations. The lower part of the curve, however, is still a straight line, and its slope is not changed.

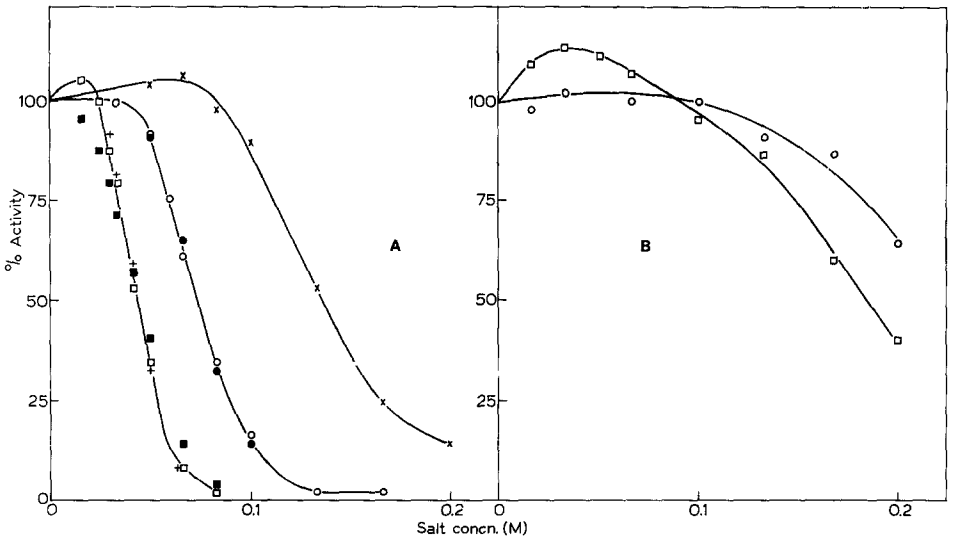


Fig. 1. Effects of salts on the activity of isocitrate dehydrogenase, in absence of AMP (A) and in presence of 0.17 mM AMP (B). All the reaction mixtures contained in a final volume of 3 ml: 33 mM Tris–HCl buffer (pH 7.6), 1 mM isocitrate, 0.33 mM NAD^+, 3.3 mM $MgSO_4$, and 0.04 ml of "$C\gamma$ eluate". The following salts were added: NaCl, ○; KCl, ●; Na_2SO_4, □; $(NH_4)_2SO_4$, ■; sodium phosphate mixture at pH 7.6, +; sodium acetate, ×. The activities are expressed in per cent of the activity without addition of salt.

In the present work, the kinetics with respect to isocitrate and Mg^{2+} have been studied at pH 7.6 and in the presence of 0.33 mM NAD^+, a concentration that is not saturating. All the kinetic experiments have been carried out many times, with enzymic preparations showing different activities.

RESULTS

Anion inhibition

Previous investigations[7,8] have shown that the isocitrate dehydrogenase from baker's yeast is inhibited by buffers or added salts, and that AMP appears to counteract this inhibition. The present results seem to indicate that the salt inhibition depends more specifically on the anion. As shown in Fig. 1A, KCl and NaCl gave similar inhibitions. Furthermore, Na_2SO_4, $(NH_4)_2SO_4$ and a mixture of sodium phosphates at pH 7.6 containing a prevalence of the anion HPO_4^{2-}, all inhibited the enzyme in a similar fashion. The degree of inhibition appears to be unrelated to the calculated values of the ionic strength. It thus seems possible that the inhibition depends on the charge of the inorganic anions, and that the bivalent anions are more inhibitory than the monovalent ones. However, the acetate ion inhibited less than the inorganic anions. As shown in Fig. 1B, AMP counteracted the inhibition by Cl^- and SO_4^{2-}. In the experiments of Fig. 1, the buffer and $MgSO_4$ also provided some Cl^- and SO_4^{2-}.

When the kinetic behavior of the enzyme with respect to Mg^{2+} is studied, variable concentrations of an inhibitory anion are brought into the reaction mixtures. Under some conditions this inhibitory effect can be very strong at high concentrations of the magnesium salt, and can simulate an activator inhibition. Fig. 2 (Curve 3)

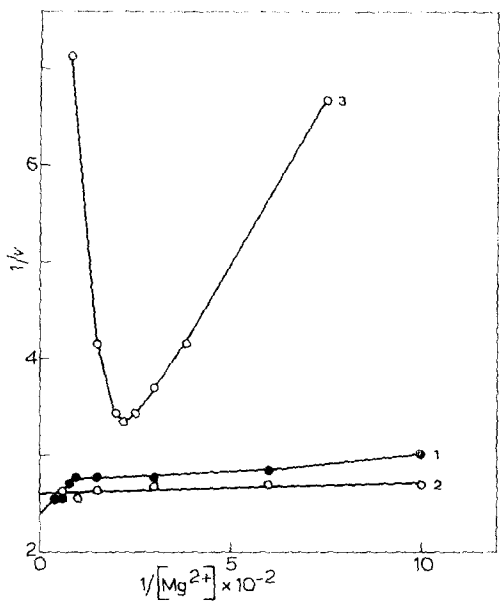


Fig. 2. Double reciprocal plot of reaction rate against Mg^{2+} ($MgSO_4$) concentration. All the reaction mixtures contained: 33 mM Tris–HCl buffer (pH 7.6), 0.33 mM NAD^+, 2 mM isocitrate, and 0.04 ml of "C_γ eluate". Curve 1, no addition; Curve 2, 0.13 M KCl and 0.17 mM AMP; Curve 3, 0.13 M KCl.

shows this effect, which can be explained as due to SO_4^{2-}, since it was removed by AMP (Fig. 2, Curve 2), and was also obtained, in separate experiments, when the magnesium salt was partially replaced by a corresponding concentration of Na_2SO_4.

Substrate inhibition

The results presented in Fig. 3A, in the form of the double reciprocal plots, show that isocitrate dehydrogenase was inhibited by isocitrate, if the concentration of Mg^{2+} was suitably low. The substrate inhibition was not apparent when the concentration of Mg^{2+} was 3.3 mM, in absence or in presence of KCl (Curves 1 and 2); it was evident in presence of 0.17 mM Mg^{2+} (Curve 3); and it was very strong in presence of 0.033 mM Mg^{2+} (Curve 4). The substrate inhibition can therefore be explained by the removal of Mg^{2+} *via* the formation of an isocitrate complex. The formation constant for the complex of DL-isocitrate with Mg^{2+} has been measured[10]: log K_f was found to be 2.24. According to this value, in Curve 4 of Fig. 3A, the free Mg^{2+} concentration fell from 0.033 mM to 0.002 mM for the first point at the left, corresponding to the highest DL-isocitrate concentration used (97.33 mM). The free Mg^{2+} concentration fell from 0.17 mM to 0.01 mM for the corresponding point of Curve 3.

The possibility that the isocitrate complex is the true substrate and that free isocitrate inhibits by competing with the complex for the enzyme was considered. However, the results seem not to support this possibility: *e.g.*, the same activity was observed with 0.5 mM DL-isocitrate, a concentration less than saturating, and both 3.3 mM and 0.67 mM Mg^{2+}, when the concentrations of the DL-complex were 0.18 mM and 0.05 mM, respectively.

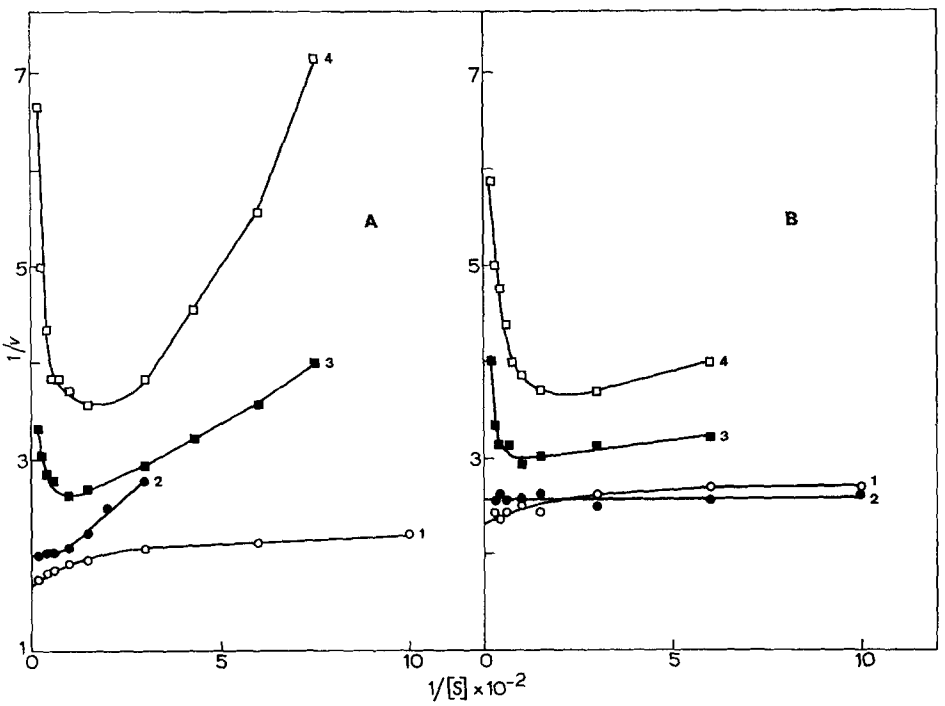


Fig. 3. Double reciprocal plot of reaction rate against isocitrate concentration, in absence of AMP (A) and in presence of 0.83 mM AMP (B). The reaction mixtures contained: 33 mM Tris–HCl buffer (pH 7.6), 0.33 mM NAD^+, and 0.04 ml of "C_γ eluate". Curves 1A and 1B, 3.3 mM Mg^{2+}; Curves 2A and 2B, 3.3 mM Mg^{2+} and 0.13 M KCl; Curves 3A and 3B, 0.17 mM Mg^{2+}; Curves 4A and 4B, 0.033 mM Mg^{2+}. $MgSO_4$ was used.

Comparison of the curves of Fig. 3B (experiments in presence of 0.83 mM AMP) with the corresponding curves of Fig. 3A, shows that the presence of AMP had no effect on the substrate inhibition.

Activation by ionic strength

According to the results reported in Figs. 3A and 3B (Curves 1), isocitrate exhibited an activating instead of an inhibiting effect, in presence of a high Mg^{2+} concentration. This effect, although complicated by the occurrence of anion inhibition, appeared to be unspecifically related to the ionic strength, for the following reasons. (a) The substrate activation was not evident in presence of 0.13 M KCl (Curves 2 of Figs. 3A and 3B). (b) A similar activating effect (Figs. 1A and 1B) was obtained, particularly in presence of AMP, with some salts, such as Na_2SO_4, giving higher ionic strengths, before reaching an inhibitory concentration. $MgSO_4$ also showed this effect (Fig. 2, Curve 1), but not in presence of 0.13 M KCl (Fig. 2, Curve 2).

Order of the reaction with respect to isocitrate

The experiments on the kinetics of isocitrate dehydrogenase with respect to isocitrate, reported in Fig. 4, were carried out in presence of 33 mM Tris–HCl buffer (pH 7.6), 3.3 mM $MgSO_4$, and 0.33 mM NAD^+. In the absence of other additions

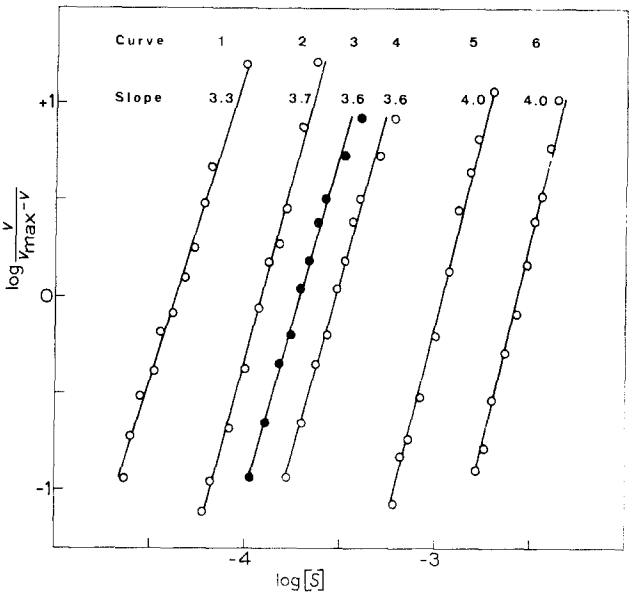


Fig. 4. Logarithmic plot of reaction rate against isocitrate concentration. All the reaction mixtures contained: 33 mM Tris–HCl buffer (pH 7.6), 0.33 mM NAD^+, 3.3 mM $MgSO_4$, and 0.04 ml of "C_γ eluate". Curve 4, no addition; Curve 1, 1.67 mM AMP; Curve 5, 0.067 M KCl; Curve 6, 0.13 M KCl; Curve 2, 0.13 M KCl and 1.67 mM AMP. In Curve 3 the data of Curve 4 are plotted against the concentrations of free isocitrate.

(Curve 4), a slope of 3.6 was obtained, whereas the addition of 1.67 mM AMP (Curve 1) resulted in a displacement of the curve towards lower substrate concentrations, with only a slight decrease in the slope to 3.3. These results are essentially in agreement with those obtained by ATKINSON, HATHAWAY AND SMITH[3] under comparable conditions. No change was observed by these authors for the slope in presence of AMP.

Curves 5 and 6 in Fig. 4 show that the addition of two different concentrations of KCl displaced the kinetic curve towards higher substrate concentrations without any marked change in the slope. The maximum velocity appeared not to be appreciably modified by the addition of KCl, when the increase in the ionic strength was considered (see also Fig. 3), thus indicating a competitive inhibition. The addition of AMP together with KCl again displaced the kinetic curve towards the lower substrate concentrations, without change in the slope (Fig. 4, Curve 2). Furthermore, no change in the slope was obtained when the calculated concentrations of free isocitrate were plotted instead of those of total isocitrate (Curve 3).

The results were similar when the Cl^- and SO_4^{2-} anions of the buffer and of the magnesium salt were replaced by acetate.

When the isocitrate concentration was varied in presence of a lower Mg^{2+} concentration (0.17 mM), the other conditions remaining unchanged, different results were obtained (Fig. 5). In absence of AMP (Curve 1) the observed slope was 2.7, and the addition of increasing concentrations of AMP decreased the slope to a minimal value of 1.5 (Curves 2, 3, 4), the maximal velocity remaining almost unchanged. Similar results were obtained in the presence of 1 mM or 2 mM NAD^+.

It must be considered that the maximal velocity in presence of 0.17 mM Mg^{2+}

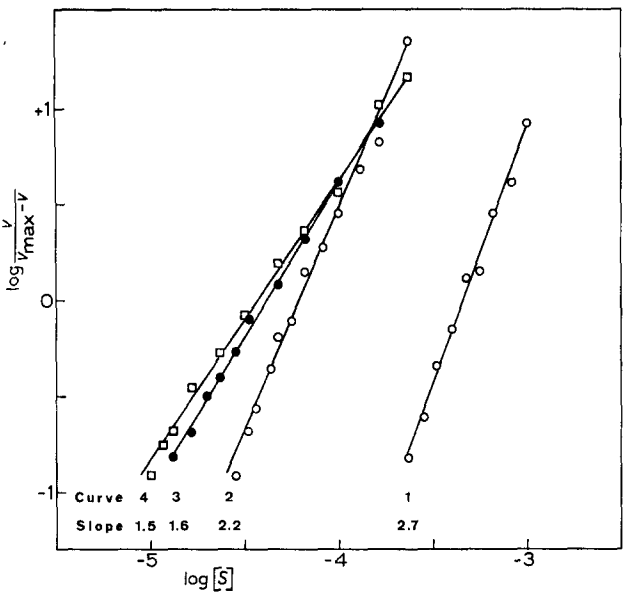


Fig. 5. Logarithmic plot of reaction rate against isocitrate concentration. All the reaction mixtures contained: 33 mM Tris–acetate buffer (pH 7.6), 0.33 mM NAD^+, 0.17 mM magnesium acetate, and 0.04 ml of "C_γ eluate". Curve 1, no addition; Curve 2, 0.17 mM AMP; Curve 3, 0.83 mM AMP; Curve 4, 1.67 mM AMP.

was only 10% lower than the maximal velocity in presence of 3.3 mM Mg^{2+}. Therefore, the lower concentration also appears almost saturating. A slope of about 3 was also obtained in presence of 0.33 mM Mg^{2+}.

It is therefore difficult to explain the further increase in the apparent order of the reaction with respect to isocitrate, in presence of 3.3 mM Mg^{2+}. It seems possible that this high Mg^{2+} concentration produces some inactivation of the enzyme, particularly at low substrate concentrations. Inhibitory effects have been observed with other metal ions used as activators. Inhibition by Mn^{2+} has been reported[1,2]. Zn^{2+} also appears to be inhibitory at a moderate concentration, particularly when the isocitrate concentration is low[8]. However, experiments carried out to test this for Mg^{2+} failed to give conclusive results. When the enzyme was incubated for a few minutes in the reaction mixture and the reaction was started by the addition of isocitrate, the activity was notably lower (in comparison with the normal procedure, according to which the enzyme was the last addition) if the isocitrate were added in low concentration, but almost unchanged with a high isocitrate concentration. However, it appeared uncertain whether the decrease in the activity depended essentially on the Mg^{2+} concentration.

Results at Mg^{2+} concentrations lower than 0.17 mM are difficult to analyze, since high isocitrate concentrations must be used in these conditions, and the Mg^{2+} concentration itself becomes a variable quantity, due to the formation of the isocitrate complex.

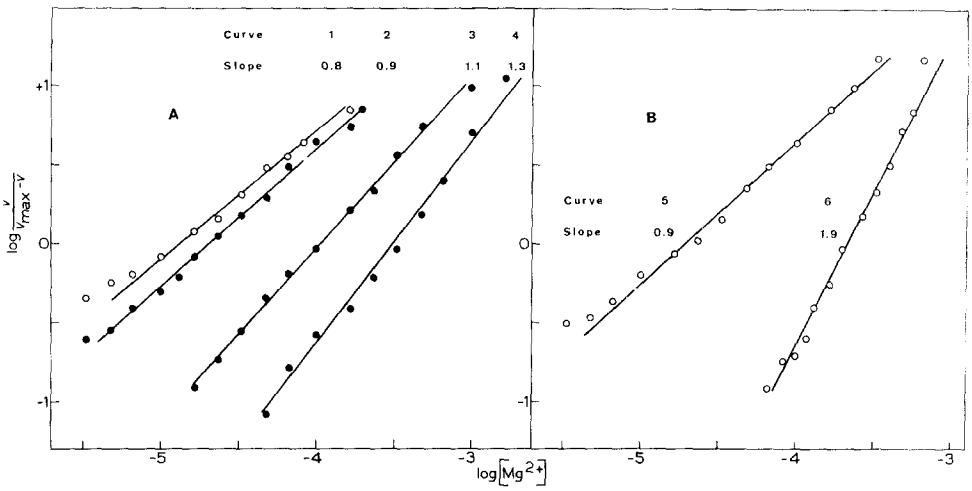


Fig. 6. Logarithmic plot of reaction rate against Mg^{2+} (magnesium acetate) concentration. All the reaction mixtures contained: 33 mM Tris–acetate buffer (pH 7.6), 0.33 mM NAD^+, and 0.04 ml of "C_γ eluate". A. Curve 1, 6.67 mM isocitrate; Curve 2, 2 mM isocitrate; Curve 3, 0.67 mM isocitrate; Curve 4, 0.27 mM isocitrate. B. Curve 5, 6.67 mM isocitrate, 0.13 M KCl and 1.67 mM AMP; Curve 6, 6.67 mM isocitrate and 0.13 M KCl.

Order of the reaction with respect to Mg^{2+}

The experiments on the kinetics of isocitrate dehydrogenase with respect to Mg^{2+}, reported in Fig. 6A, showed that the slopes of the curves obtained at four fixed isocitrate concentrations, all gave values around 1, although a slight increase was observed as the isocitrate concentration decreased. A value of 1.3 was obtained in presence of 0.27 mM isocitrate, which was the lowest concentration tested. This concentration could not be lowered any further, in order to avoid its becoming a

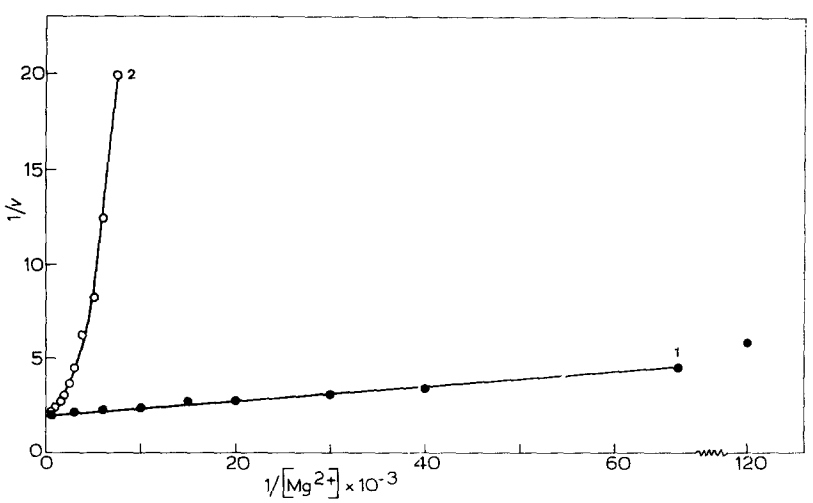


Fig. 7. Double reciprocal plot of reaction rate against Mg^{2+} ($MgSO_4$) concentration. All the reaction mixtures contained: 33 mM Tris–HCl buffer (pH 7.6), 0.33 mM NAD^+, 6.67 mM isocitrate, and 0.04 ml of "C_γ eluate". Curve 1, no addition; Curve 2, 0.13 M KCl.

variable quantity *via* the formation of the complex with Mg^{2+}. Maximum velocities were: 0.32 activity units for the Curves 1, 2, 3 of Fig. 6A and 0.24 for Curve 4. The values lower than 1, obtained in the presence of the higher isocitrate concentrations tested, can be explained by the reaction mixtures probably containing a certain amount of activating ion impurities. Blanks run under these conditions, without added Mg^{2+}, actually gave measurable activities.

Essentially the same results were obtained when the kinetic data were plotted against the calculated concentrations of free Mg^{2+}.

These results are difficult to reconcile with those reported by ATKINSON, HATHAWAY AND SMITH[3]: these authors, in experiments in presence of 1.7 mM isocitrate, obtained a slope of 2, by plotting the data against the free Mg^{2+} concentration calculated on the basis of the formation constant of the citrate complex with Mg^{2+}.

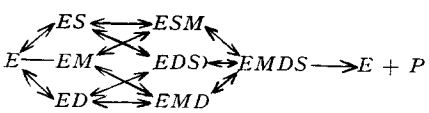
Fig. 6B shows that, in presence of 6.67 mM isocitrate, the addition of 0.13 M KCl increased the slope to about 2 (*cf.* Curve 1 of Fig. 6A and Curve 5 of Fig. 6B). When AMP was also added, the slope returned to the normal value of about 1 (Curve 6 of Fig. 6B). As shown in Fig. 7, the effect of KCl on the kinetics with respect to Mg^{2+} is also made evident by the use of the double reciprocal plot. The maximum velocity appeared not to be greatly affected by KCl.

GENERAL DISCUSSION

The peculiar kinetic behavior of the NAD+-specific isocitrate dehydrogenase is particularly evident at pH 7.6, whereas it appears to be less complicated at pH 6.5. (refs. 2–6). The present researches have been carried out at pH 7.6.

ATKINSON, HATHAWAY AND SMITH[3] have suggested that, at pH 7.6, the enzyme from baker's yeast can bind at least 4 molecules of isocitrate, with an interaction factor of about 20 between the binding sites of the enzyme: *i.e.*, the binding of one molecule of isocitrate at one site should increase the affinity for the substrate of each other site by a factor of about 20. This assumption could explain the value of about 4, found for the slope of the straight line obtained by the logarithmic plot of the kinetic data with respect to isocitrate. The above authors also observed that AMP displaces the kinetic curve towards lower substrate concentrations without affecting the slope; therefore, AMP should increase the affinity of the four isocitrate-binding sites by a similar factor, without affecting the degree of interaction between these sites. ATKINSON, HATHAWAY AND SMITH[3] have also assumed the existence of at least two sites for the binding of each NAD+, Mg^{2+} and AMP.

We shall discuss here the possibility that the kinetic behavior of the enzyme can be explained by the occurrence of a random reaction mechanism. Considering only the forward reaction, and ignoring the order of release of the products of the reaction, a completely random mechanism for the addition of the reactants (isocitrate, NAD+, Mg^{2+}) to the enzyme would be the following:



(Mechanism I)

where, E is the enzyme, S is isocitrate, D is NAD+, M is the metal ion activator, and P the products.

In the hypothesis that all the steps are reversible, the steady-state rate equation for the above mechanism will be very complicated, but it can be stated that the concentration of each reactant must occur both in the numerator and in the denominator of the usual form of this equation with powers up to 4. The actual order of the reaction with respect to each reactant will be that of the prevalent terms of the equation, depending on what the rate-determining steps are, under particular experimental conditions. It can be anticipated that the apparent order could be decreased to a value of 1 with respect to a reactant, by increasing the concentrations of the other reactants in order to bring to equilibrium some rate-determining steps[11].

On the other hand, for each step involving the addition of isocitrate, *e.g.*, the first one, side reactions could occur such as:

$$\begin{array}{ccc} E\text{-}AMP & \rightleftharpoons & (E\text{-}AMP\text{-}S) \\ \uparrow\downarrow & & \uparrow\downarrow \\ E & \rightleftharpoons & ES \\ \uparrow\downarrow & & \uparrow\downarrow \\ E\text{-}A & \rightleftharpoons & (E\text{-}A\text{-}S) \end{array} \qquad \text{(Mechanism II)}$$

where A is an inhibitory anion. For the sake of simplicity, it may be assumed that E-AMP-S and E-A-S react similarly to ES in the subsequent steps of the mechanism, or rapidly give rise to ES by releasing AMP or A. On the other hand, the formation of E-AMP could result in an enhanced affinity for isocitrate, whereas the formation of E-A could have the opposite effect. It has been shown in the present work that anions such as chloride competitively inhibit the enzyme, and that AMP removes this inhibition without affecting the maximum velocity. Thus, an increase in the concentration of an inhibitory anion would be equivalent to a decrease in the concentration of isocitrate, whereas the addition of AMP would correspond to an opposite change (*cf.* ref. 3), with respect to their kinetic effects.

Some results reported in the present work seem to indicate a random mechanism for the addition of isocitrate and Mg^{2+} to the enzyme. Evidence in support of this possibility is the occurrence of substrate inhibition, which seems to be due to the removal of the metal ion by the formation of an isocitrate complex. Our results show that this inhibition gives rise to a decrease in the activity when the isocitrate concentration is increased, if the Mg^{2+} concentration is suitably low. It has been pointed out by WEBB[12] that this behavior occurs only if the addition of the substrate and of the activator to the enzyme is random. Furthermore, the kinetic results show that the apparent order of the reaction with respect to Mg^{2+} is 1, in presence of 2 mM isocitrate. If a random mechanism occurs for the addition of isocitrate and Mg^{2+} to the enzyme, it should be possible to observe an increase in the order of the reaction by decreasing the fixed isocitrate concentration. The results show that the order is enhanced to 1.3 in presence of 0.27 mM isocitrate. The possibility of a further increase in the order of the reaction cannot be demonstrated experimentally in a simple way, because a further decrease in the isocitrate concentration would result in making this concentration a variable also, *via* the formation of the complex with Mg^{2+}. However, the observed increase in the order of the reaction to about 2 in presence of KCl, and the fact that AMP further decreases this order to 1 could be considered indirect evidence in this respect, as previously discussed.

In a recent work, SANWAL AND COOK[6] proposed a random addition of isocitrate and NAD^+ to isocitrate dehydrogenase from *N. crassa*; at pH 6.5, the apparent order

of the reaction with respect to NAD^+ was found to vary from 2.5 to 0.95 when the concentration of isocitrate was increased. On the other hand, ATKINSON, HATHAWAY AND SMITH[3] reported that AMP decreases the order with respect to NAD^+ in the case of the enzyme from baker's yeast. SANWAL AND COOK[6] have also shown that the order with respect to AMP can be brought to 1 by increasing the concentration of isocitrate.

A completely random mechanism could thus occur for the NAD^+-specific isocitrate dehydrogenase.

As to the kinetics with respect to isocitrate, it appears that the apparent order cannot be decreased much below 3 by merely increasing the concentrations of the other reactants (*cf.* ref. 6). However we have observed that in the presence of AMP the order of the reaction with respect to isocitrate can be decreased to 1.5. This result was obtained only when the concentration of Mg^{2+}, although almost saturating, was not too high. Perhaps inactivation effects can occur (see RESULTS), and the observed value of 2.7, in presence of 0.17 mM Mg^{2+}, could also be too high. It is interesting to note that, with 0.083 mM Zn^{2+} used as activator instead of Mg^{2+}, at pH 7.6, we observed an apparent order of about 2 in the absence of AMP, and of 1 in its presence[13]. Such behavior could be explained according to Mechanism II. In the at pH 7.6, we observed an apparent order of 2 in the absence of AMP, and of 1 in its presence[13]. Such behavior could be explained according to Mechanism II. In the absence of AMP, if the enzyme is predominantly in the form *E-A*, whereas most of the reaction occurs *via* $E \rightarrow ES$, this step being the rate-determining one in the overall Mechanism I, the order of the reaction could be 2 (*cf.* KING[14]), in presence of saturating concentrations of the other reactants. The addition of AMP could bring *E* predominantly into the form *E-AMP*, which would be rapidly transformed into *E-AMP-S* the rate-determining step in the overall Mechanism I possibly being the final release of the product. Thus, the apparent order will decrease to 1.

In the case of the enzyme from *N. crassa*, the kinetics with respect to isocitrate appears to be normalized by the addition of AMP at pH 6.5, but not at pH 7.6 (see refs. 5, 6).

Many points in this discussion are tentative, but it appears possible to suggest an alternative to the hypothesis that the NAD^+-specific isocitrate dehydrogenase from baker's yeast has several binding sites for the reactants[3]. The complex behavior of this enzyme could also be explained on the basis of a random kinetic mechanism, whatever the way in which AMP (and inhibitory anions) affects the affinity of the enzyme for isocitrate

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