The Effect of Adenylic Acid on Yeast Nicotinamide Adenine Dinucleotide Isocitrate Dehydrogenase, a Possible Metabolic Control Mechanism*  

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The nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase of yeast was reported by Kornberg and Priére (1) to have an absolute requirement for adenylic acid and not to catalyze the reverse reaction under conditions suitable for such catalysis by the nicotinamide adenine dinucleotide phosphate-specific enzyme. Similar results have been obtained for the nicotinamide adenine dinucleotide enzyme of Aspergillus (2), but variable slight stimulation by adenosine 5'-phosphate, rather than an absolute requirement, has been reported for the enzyme from animal sources (3). In contrast, the nicotinamide adenine dinucleotide-specific enzymes of Acetobacter aceti (4) and of pea seedlings (5) are apparently not affected by adenosine 5'-phosphate although reversibility was not demonstrated in either case.

In studies on the NAD-specific isocitrate dehydrogenase of Acetobacter peroxydans we were unable to demonstrate any effect of adenylic acid and the enzyme-catalyzed reaction was found to be freely reversible. Because of the difference between those properties and those of the yeast enzyme, we reinvestigated the latter enzyme. Adenylic acid was found not to be required for enzymic activity but to affect very markedly the rate of the reaction under some conditions. These properties are described in this paper along with the conditions under which reversal of the reaction may be observed. Partial purification and some properties of the enzyme from Acetobacter peroxydans are also reported.

EXPERIMENTAL PROCEDURE

Source of Enzyme—Acetobacter peroxydans, ATCC 838, was grown in 12- to 15-liter cultures in 20-liter bottles with vigorous stirring and aeration in the medium previously described (6) with ethanol as carbon source. Cells were harvested in a Sharples continuous flow centrifuge. The wet cell paste was concentrated by freezing, crushing, and centrifugation at 60,000 × g for 25 minutes. Solid (NH₄)₂SO₄ was added with constant stirring to the supernatant fraction in the proportion of 0.22 g per ml. After 15 minutes the mixture was centrifuged and the precipitate discarded. The supernatant fraction was brought to approximately 50% saturation by the addition of 0.15 g of (NH₄)₂SO₄ per ml. The suspension was centrifuged after 15 minutes and the pellet was dissolved in 0.05 M phosphate, pH 7.4 (3 ml for each g of the wet cell paste taken initially).

Commercial bakers’ yeast (The Fleischmann Company) was broken either with a Sagers press or, after suspension in about half its volume of pH 7.4 phosphate, in a French press (American Instrument Company, Inc.).

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Assay—The concentration of NADH was followed spectrophotometrically at 340 mp. One unit of activity corresponds to reduction of 1 μmole of NAD⁺ per minute. Compositions of the reaction mixtures are specified in each figure legend. D.L-Isocitric acid was used, but all amounts or concentrations given are those of a single isomer. Protein was estimated by the biuret method or spectrophotometrically.

RESULTS

Enzyme from Acetobacter peroxydans

When assayed spectrophotometrically, crude A. peroxydans preparations appear to contain a NADP-specific isocitrate dehydrogenase, but not the corresponding NAD enzyme. However, when the same preparations are assayed manometrically with isocitrate as substrate, O₂ uptake is stimulated by the addition of NAD but not of NADP. This apparent discrepancy results from the presence of two isocitrate dehydrogenases, one specific for NAD and the other for NADP. Because of the high NADH oxidase activity in these preparations, the NAD dehydrogenase is not observed spectrophotometrically unless the oxidase system is removed by centrifugation or poisoned with cyanide; manometric experiments do not reveal the NADP enzyme because this organism lacks NADPH oxidase and transhydrogenase activities (8). The two dehydrogenases are readily separable. The NAD enzyme was partially purified and some of its catalytic properties were determined.

Purification—All preparations were kept below 5° except as noted. A broken cell suspension from the sonic disintegrator (or the broken cell paste from the press after dilution with about 5 times its volume of 0.05 M phosphate, pH 7.4) was centrifuged at 60,000 × g for 25 minutes. Solid (NH₄)₂SO₄ was added with constant stirring to the supernatant fraction in the proportion of 0.22 g per ml. After 15 minutes the mixture was centrifuged and the precipitate discarded. The supernatant fraction was brought to approximately 50% saturation by the addition of 0.15 g of (NH₄)₂SO₄ per ml. The suspension was centrifuged after 15 minutes and the pellet was dissolved in 0.05 M phosphate, pH 7.4 (3 ml for each g of the wet cell paste taken initially).

Six volumes of alumina C (4 mg per ml) were added per volume of the preparation. After 20 minutes the alumina was centrifuged and discarded. The supernatant fraction was concentrated by freezing, crushing, and slow melting. The activity was quantitatively recovered in the liquid phase when about 30% of the ice had melted. The resulting solution was held at...
Table I

Purification of NAD isocitrate dehydrogenase
from Acetobacter peroxidans

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
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<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td>units/mg protein</td>
<td>%</td>
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<tr>
<td>Extract, 60,000 X g</td>
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<tr>
<td>Partial melt</td>
<td>27</td>
<td>155</td>
<td>29</td>
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<td>Heated</td>
<td>27</td>
<td>146</td>
<td>14</td>
<td>10.4</td>
<td>34</td>
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</tbody>
</table>

Fig. 1 (left). Rate as a function of substrate concentration for the reaction catalyzed by NAD isocitrate dehydrogenase of Acetobacter peroxidans. X, Reaction mixture contained in 3 ml: 100 μmoles of Tris, pH 8.0; 10 μmoles of MgCl₂; 1 μmole of NAD⁺; isocitrate as indicated; and 0.024 unit of enzyme (specific activity, 1.1). O, Same, plus 0.17 mM AMP.

Fig. 2 (right). Reversal of the reaction catalyzed by the NAD isocitrate dehydrogenase of Acetobacter peroxidans. The reaction mixture initially contained in 3 ml: 100 μmoles of Tris, pH 8.0; 10 μmoles of MgCl₂; 0.2 μmole of NADH; 0.12 μmole of isocitrate; and 0.54 unit of enzyme (specific activity, 10.4). Additions: 0.1 ml of 0.16 M α-ketoglutarate and 0.3 ml of 0.1 M NaHCO₃ at 12 minutes; 0.1 ml of 20 mM isocitrate at 24 minutes. Readings are corrected for dilution and for absorption by α-ketoglutarate.

Fig. 3 (left). Rate as a function of pH for the reverse reaction catalyzed by NAD isocitrate dehydrogenase. The reaction mixture contained in 3 ml: 25 μmoles of cacodylate or 100 μmoles of Tris at the indicated values of pH; 10 μmoles of MgCl₂; 0.20 μmole of NADH; 16 μmoles of α-ketoglutarate; 30 μmoles of NaHCO₃; and, with the yeast enzyme only, 0.5 μmole of AMP. A, Acetobacter peroxidans enzyme, 0.5 unit (specific activity, 10.4 in Tris); Y, yeast enzyme, 0.14 unit (specific activity, 1.7).

Fig. 4 (right). Interaction of isocitrate and AMP concentrations in determining the rate of the reaction catalyzed by yeast NAD isocitrate dehydrogenase. Reaction mixtures contained in 3 ml: 100 μmoles of Tris, pH 7.5; 10 μmoles of MgCl₂; 1 μmole of NAD⁺; isocitrate at the concentrations indicated; 0.035 unit of enzyme (specific activity, 0.6). C, No AMP; B, 0.05 μmole of AMP; A, 0.5 μmole of AMP.

55° for 10 minutes after which it was centrifuged and the pellet discarded. The suspension contained about 30% of the original activity at a purification ratio of approximately 50-fold. Limited attempts at further purification by column chromatography were unsuccessful. A typical preparation is summarized in Table I. At this level of purification the enzyme retains nearly full activity after several months at -20°.

Properties—At all levels of purification obtained the Acetobacter enzyme is unaffected by AMP (Fig. 1). After we observed that the effect of AMP on the yeast enzyme depends on the concentration of isocitrate (reported in this paper), the Acetobacter enzyme was retested at concentrations of isocitrate down to that giving a barely detectable rate of reaction, but still no AMP effect was observed. Other adenine derivatives tested (ADP, ATP, 2',3'-AMP, 3',5'-AMP, 3',5'-cyclic AMP) were similarly without effect. The reciprocal plot of Fig. 1 shows that the reaction follows classical Michaelis kinetics; the value of Kₘ derived from this plot is 0.34 mM. The metal ion requirement could be met by Mn⁺⁺ or Mg⁺⁺ and to a lesser extent by Co⁺⁺ or Zn⁺⁺, in that order. Although Mn⁺⁺ was effective at the lowest concentration, Mg⁺⁺ was routinely used in assays because of inhibition by Mn⁺⁺ at higher concentrations. The rate of the reaction is nearly independent of pH between 7.5 and 8.5 and falls to about 70% of maximum at 7.0 and 9.0. At pH 8 the reaction is inhibited about 50% by phosphate at 40 mM and prevented completely by pyrophosphate at the same concentration. Oxaloacetate is neither decarboxylated nor reduced by this enzyme.

The reaction is readily reversible under the conditions of the standard assay; that is, in Tris buffer at pH 8 (Fig. 2). The optimal pH for the reverse reaction is however considerably lower (Fig. 3). The rate in the cacodylate buffer used for this pH range is less than half of that in Tris at the point of overlap.

Purification of Yeast Enzyme

Most of the experiments reported in this paper utilized preparations purified 50- to 60-fold essentially by the method of Kornberg and Pricer (1). A modified procedure was later developed for which the data of Table II (40% yield of 400-fold purified enzyme) are typical. Frozen crude preparations could be stored for days with little loss of activity but the preparations in column eluates was largely lost on overnight storage at either 4°C or 0°C.

Table II

Purification of NAD isocitrate dehydrogenase from bakers' yeast

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
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<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td>units/mg protein</td>
<td>%</td>
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<tr>
<td>Crude</td>
<td>640</td>
<td>357</td>
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<td>(100)</td>
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<td>1,710</td>
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<td>121</td>
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<tr>
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<td>27</td>
<td>432</td>
<td>562</td>
<td>0.77</td>
<td>121</td>
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<tr>
<td>Acetone precipitate</td>
<td>4.5</td>
<td>701</td>
<td>102</td>
<td>0.75</td>
<td>57</td>
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<td>Sphadex eluate</td>
<td>7.2</td>
<td>96</td>
<td>63</td>
<td>1.4</td>
<td>65</td>
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<td>DEAE-cellulose eluate</td>
<td>4.8</td>
<td>281</td>
<td>2.1</td>
<td>13</td>
<td>44</td>
</tr>
</tbody>
</table>

* Values after 5 days storage at -20°.
† A 10-ml sample of the preceding fraction (specific activity, 0.48) was used.
‡ A 3.5-ml sample of the preceding fraction was used.
bakers' yeast was suspended in 4 volumes of 0.1 M NaHCO₃ (pH unadjusted) and stirred for 20 minutes. The suspension was centrifuged for 50 minutes at 90,000 × g and the pellet was discarded. The crude preparation was made to contain 0.05 M MnCl₂ by the addition of 1 M MnCl₂, stirred for 15 minutes, and centrifuged for 25 minutes at 10,000 × g. The pellet was discarded. Assays of the crude preparation are unreliable and this step often led to apparent increases in total activity.

**MnCl₂ Fractionation**—After disruption in a Sagers press, bakers' yeast was suspended in 4 volumes of 0.1 M NaHCO₃ (pH unadjusted) and stirred for 20 minutes. The suspension was centrifuged for 50 minutes at 90,000 × g and the pellet was discarded. The crude preparation was made to contain 0.05 M MnCl₂ by the addition of 1 M MnCl₂, stirred for 15 minutes, and centrifuged for 25 minutes at 10,000 × g. The pellet was discarded. The supernatant solution was brought to an acetone concentration of 36%, the temperature being held at -10° or below. The mixture was immediately cooled to -20° and centrifuged for 5 minutes at 10,000 × g and the pellet was discarded. The supernatant solution was brought to an acetone concentration of 42% and centrifuged as before. The protein was eluted with 0.01 M phosphate, pH 7.0, to a volume 0.04 that of the crude preparation.

**Acetone Fractionation**—Acetone at -70° was added slowly, with stirring, to the (NH₄)₂SO₄ fraction to a concentration of 36%, the temperature being held at -10° or below. The mixture was immediately cooled to -20° and centrifuged for 5 minutes at 10,000 × g at -20°. The pellet was discarded. The supernatant solution was brought to an acetone concentration of 42% and centrifuged as before. The pellet was resuspended in 0.02 M phosphate, pH 7.0, to a volume 0.02 that of the crude preparation.

**Sephadex Treatment**—The solution was placed on a column of G-25 coarse Sephadex (Pharmacia Fine Chemicals, Inc.) which had been equilibrated with 0.01 M phosphate, pH 7.5. The protein was eluted with 0.01 M phosphate, pH 7.5, at room temperature, and recovered by following the yellow-brown color of the protein through the column, collecting 1 or 2 ml of solution on each side of the colored band.

**DEAE-cellulose Fractionation**—Diethylaminoethyl cellulose (Brown Company) was treated with 0.02 M NaOH and washed with water. It was then packed and equilibrated with 0.01 M phosphate, pH 7.5, and finally with the same buffer containing 0.25% dextran 500 (Pharmacia). Approximately 30 mg of protein were placed on a column, 15 cm × 1 cm². The protein was washed with 0.02 M phosphate, pH 7.0, containing 0.25% dextran. The recovery was approximately 70% in this step with about 10-fold purification. No significant amount of activity was recovered when dextran was not used.

**Properties of Yeast Enzyme**

**AMP Effect**—Preliminary experiments with AMP included in the reaction mixtures gave a Kₐ for isocitrate of 0.1 mM, in agreement with that reported by Kornberg and Pricer (1). Accordingly, to assure that the reaction would be zero order in substrate, assays were routinely run at an isocitrate concentration of 0.7 mM (occasionally 0.3 mM). Under these conditions the AMP effect reported by Kornberg and Pricer could not be reproduced. The reaction went well in the absence of AMP and addition of this nucleotide produced only slight and somewhat variable stimulation. This situation was independent of the degree of purification or previous history of the preparation and was unchanged by procedures that might remove loosely bound nucleotide (dialysis, Sephadex treatment, or partial heat inactivation). The discrepancy was ultimately explained by finding that the AMP effect is a function of isocitrate concentration (Fig. 4). At low levels of isocitrate the reaction does not proceed detectably in the absence of AMP, which thus appears as an absolute requirement; however, at substrate concentrations in the range of 0.7 mM or higher, the reaction rate approaches maximal velocity without added nucleotide. Michaelis kinetics is obviously not followed when the reaction proceeds in the absence of AMP; hence the concept of a Michaelis constant is not applicable. The rate of reaction has been determined as a function of isocitrate concentration in the absence of AMP under a number of conditions (different stages of purification of the enzyme, different levels of enzyme activity, Mn²⁺ or Zn²⁺ replacing Mg²⁺ as the activating cation) and in each case a curve nearly identical with the corresponding curve of Fig. 4 was obtained. Little or no reaction was observed below an isocitrate concentration of 0.2 mM and half-maximal velocity was reached in different experiments at 0.4 to 0.6 mM. (As reported above, the Kₐ of isocitrate in the presence of AMP is 0.1 mM.) These values change however when the concentration of NAD⁺ is varied. The results of such an experiment are given in Fig. 5A. Doubling the standard NAD⁺ level of 1 μmole in a 3-ml assay volume increases the rate markedly at all low concentrations of isocitrate on the apparent Kₐ for Mg²⁺ of yeast NAD isocitrate dehydrogenase in the absence of AMP. Reaction mixtures contained in 3 ml: 100 μmoles of Tris, pH 7.5; 10 μmoles of MgCl₂; 0.042 unit of enzyme (specific activity, 0.6); isocitrate and NAD⁺ as indicated. In A, figures identifying the curves indicate the number of micromoles of NAD; in B, the number of micromoles of isocitrate.

![Fig. 5 (left)]. Interaction of isocitrate and NAD⁺ concentrations in determining the rate of the reaction catalyzed by yeast NAD isocitrate dehydrogenase in the absence of AMP. Reaction mixtures contained in 3 ml: 100 μmoles of Tris, pH 7.5; 10 μmoles of MgCl₂; 0.042 unit of enzyme (specific activity, 0.6); isocitrate and NAD⁺ as indicated. In A, figures identifying the curves indicate the number of micromoles of NAD; in B, the number of micromoles of isocitrate.

![Fig. 6 (right)]. Effect of the concentration of AMP and of isocitrate on the apparent Kₐ for Mg²⁺ of yeast NAD isocitrate dehydrogenase. Reaction mixtures contained in 3 ml: 100 μmoles of Tris, pH 7.5; 0.042 unit of enzyme (specific activity, 0.6); AMP and isocitrate as noted; and appropriate levels of Mg²⁺. Values of Kₐ were estimated from the usual reciprocal velocity-substrate plots where possible. When AMP is lacking or at very low concentration such plots do not apply (see text); in such cases the values plotted are [Mg²⁺] at which v = Vmax/2. A, effect of varying AMP concentration in the presence of 1 or 4 μmoles of isocitrate; B, effect of varying isocitrate concentration in the presence of 0 or 0.54 μmole of AMP.
of activity which was found by chromatography on Dowex 1 to result from contamination with AMP. ATP at a concentration of 1 mM did not inhibit the reaction when tested either at high isocitrate concentration (0.7 mM) in the absence of AMP or at low isocitrate concentration at which AMP is required for activation (0.17 mM isocitrate, 0.17 mM AMP).

**Cation Requirement**—As with the Acetobacter enzyme Mn++ was the most effective cation tested but was somewhat inhibitory at high concentrations. Mg++ was a close second in effectiveness and was not inhibitory at any concentration tested. Zn++ was nearly half as effective as Mn++ whereas Co++ showed very little activity. Under all conditions tested the requirement for Mg++ or other cation is absolute, but the level at which the cation is effective is a function of concentrations of isocitrate and AMP. As shown in Fig. 6, the apparent K for Mg++ decreases with an increase in the concentration of either isocitrate or AMP which, of course, is opposite in direction to the effect that might be expected if a cation were sequestered by excess substrate or nucleotide.

**pH Response**—The effect of pH on the rate of the reaction depends strikingly on the concentration of isocitrate and on the presence or absence of AMP (Fig. 7). It will be noted: (a) that the addition of AMP or an increase in isocitrate concentration leads to identical results; (b) that under the conditions of this experiment either "high isocitrate" or AMP enhances the reaction rate markedly in the range from pH 7.5 to 9, but has no effect at pH 6.5 or below; and that as a consequence (c) the value of pH observed to be optimal moves upward on the addition of AMP or the increase of isocitrate concentration. Results b and c follow at least in part from a much greater apparent affinity of the enzyme for isocitrate at pH 6.5 than at 7.5. The AMP effect is not abolished at pH 6.5, but only moved to lower isocitrate concentrations (compare Fig. 8 with Fig. 4). In effect, the lower of the two isocitrate concentrations used in the experiment of Fig. 7 (80 μM) is "low" (requiring activation by AMP) at pH 6.5, but "high" (giving maximal rate in the absence of AMP) at pH 6.5. These relationships are summarized in the following comparison of concentrations of isocitrate required for half-maximal velocity under standard assay conditions.

With no AMP: pH 7.5, 500 μM; pH 6.5, 15 μM
With 0.2 mM AMP: pH 7.5, 100 μM; pH 6.5, 8 μM

As noted previously, such values for reactions run in the absence of AMP cannot be termed Michaelis constants because of the peculiar dependence of rate on substrate concentration.

**Reversal**—Unlike the *Acetobacter* enzyme, the dehydrogenase from yeast does not catalyze isocitrate production from α-ketoglutarate at an appreciable rate under the conditions usually used for assay of the forward reaction (1). Because of the low pH optimum for reversal with the *Acetobacter* enzyme, reversal with the yeast enzyme was attempted at pH 7.5. Under these conditions the reverse reaction proceeds, but very slowly in comparison with the forward reaction, so that a higher enzyme concentration is needed if a convenient rate is to be obtained. As shown in Fig. 9A, the reverse reaction requires both AMP and bicarbonate. A false reversal (oxidation of NADH dependent on α-ketoglutarate but independent of both AMP and bicarbonate) may be observed in crude preparations, presumably owing to the action of glutamic dehydrogenase. Fig. 9B shows that the reaction may be reversed after equilibrium has been established.

![Graph](image-url)
attained in the forward direction and that in this case also bicarbonate is required.

The rate of the reverse reaction as a function of pH is shown in Fig. 3. Other experiments indicated that for the yeast enzyme Tris and cacodylate buffers are approximately equivalent. It will be noted that the rate is negligible at pH 6.5 and pH 7.0. Since the lag was not eliminated by prior incubation of the enzyme with reaction components, singly or in any combination, stimulation by a product seemed a possible explanation. The addition of isocitrate was found to abolish the lag completely (Fig. 10) although it decreased the reaction rate at all but the lowest levels tested.

Effects of Citrate—In a system fully activated by AMP, citrate is a competitive inhibitor for isocitrate (Fig. 11). However, in the absence of AMP, citrate mimics “high isocitrate.”

![Figure 10](image1.png)

**Fig. 10.** Effect of isocitrate on the reverse reaction catalyzed by yeast NAD isocitrate dehydrogenase. Reaction mixture contained in 3 ml: 25 μmoles of cacodylate, pH 7.0; 10 μmoles of MgCl₂; 0.11 μmole of NAD⁺; 16 μmoles of α-ketoglutarate; 30 μmoles of NaHCO₃; 0.11 μmole of AMP; 0.065 unit of enzyme (specific activity, 4.9); and isocitrate as labeled in micromoles per cuvette.

![Figure 11](image2.png)

**Fig. 11.** Competitive inhibition by citrate of the reaction catalyzed by yeast NAD isocitrate dehydrogenase. Reaction mixtures contained in 3 ml: 100 μmoles of Tris, pH 7.5; 10 μmoles of MgCl₂; 5.0 μmoles of AMP; 1 μmole of NAD⁺; 0.065 unit of enzyme (specific activity, 4.9); isocitrate and citrate as indicated. Figures along curves indicate citrate present in micromoles.

The reverse reaction proceeds linearly, or nearly so, from zero time at pH 5.5 or 6.0, but a lag is usually noticeable at pH 6.5 and pH 7.0. Since the lag was not eliminated by prior incubation of the enzyme with reaction components, singly or in any combination, stimulation by a product seemed a possible explanation. The addition of isocitrate was found to abolish the lag completely (Fig. 10) although it decreased the reaction rate at all but the lowest levels tested.

![Figure 12A](image3.png)

**Fig. 12A.** Activation by citrate of forward reaction catalyzed by yeast NAD isocitrate dehydrogenase. Control contained in 3 ml: 100 μmoles of Tris, pH 7.5; 10 μmoles of MgCl₂; 1 μmole of NAD⁺; 0.25 μmole of isocitrate; and 0.022 unit of enzyme (specific activity, 4.9). Upper curve, same plus 5 μmoles of AMP. B. Activation by citrate of reverse reaction catalyzed by yeast NAD isocitrate dehydrogenase. Reaction mixture contained in 3 ml: 25 μmoles of cacodylate, pH 6.5; 10 μmoles of MgCl₂; 16 μmoles of α-ketoglutarate; 0.26 μmole of NADH; 30 μmoles of NaHCO₃; 0.11 unit of enzyme (specific activity, 4.9), and citrate as indicated in micromoles per cuvette.

The rate of the reverse reaction as a function of pH is shown in Fig. 3. Other experiments indicated that for the yeast enzyme Tris and cacodylate buffers are approximately equivalent. It will be noted that the rate is negligible at pH 6.5 and pH 7.0. Since the lag was not eliminated by prior incubation of the enzyme with reaction components, singly or in any combination, stimulation by a product seemed a possible explanation. The addition of isocitrate was found to abolish the lag completely (Fig. 10) although it decreased the reaction rate at all but the lowest levels tested.

Effects of Citrate—In a system fully activated by AMP, citrate is a competitive inhibitor for isocitrate (Fig. 11). However, in the absence of AMP, citrate mimics “high isocitrate.”

![Figure 12B](image4.png)

**Fig. 12B.** Shows that the reverse reaction likewise is activated by citrate at low concentrations, but that higher concentrations inhibit.

**DISCUSSION**

Our results with the yeast enzyme confirm those reported by Kornberg and Pricer (1) in all cases where direct comparison is possible. The paradox inherent in the contrast between the apparent nonreversibility of the reaction catalyzed by NAD isocitrate dehydrogenase and the easy reversibility of the corresponding reaction catalyzed by the NADP enzyme (since the oxidation potentials for the two pyridine nucleotides are nearly identical, the equilibrium constants for the two isocitrate dehydrogenases must be equal) has however been resolved. The reverse reaction proceeds slowly, but may be observed by increasing the concentration of the enzyme, especially at a pH lower than that optimal for the forward reaction. Also the AMP requirement, which appeared to be absolute in the earlier work (1), has been found to exist only at low substrate concentrations. The standard assay of Kornberg and Pricer contained isocitrate at 0.17 mM, which is approximately the threshold concentration for reaction in the absence of AMP (Fig. 4).

The kinetics of the reaction catalyzed by the yeast enzyme seems unusually complicated in terms of interactions between substrates and activators. In general an increase in the concentration of any component of the reaction appears to decrease the quantity of one or more other components required for half-saturation. Thus, less isocitrate is needed when AMP is present; less NAD when the isocitrate concentration is high and vice versa; less magnesium when the concentration of either isocitrate or AMP is increased. Such results evidently indicate some type of cooperative phenomenon in the binding of substrates and activators to the enzyme.

These results cannot be satisfactorily explained at present,
and it appears that a stable, highly purified preparation may well be a prerequisite of their elucidation. The available results seem to suggest the presence on the enzyme of two effectively distinct sites, the reaction site at which the actual catalysis occurs and an activating site which must be occupied by isocitrate or AMP in order that the reaction site will be active. Because of the high specificity for AMP, it seems necessary to assume that the two activators must bind at distinct subsites. According to this hypothesis, Curve A of Fig. 4 represents the kinetics of the reaction site when the activating site is saturated with AMP, and the corresponding $K_M$ of 0.1 mm should be the intrinsic Michaelis constant for the reaction site. In the absence of AMP (Curve C, Fig. 4) the activating site must be occupied by isocitrate, and the approximately 0.5 mm concentration required may be taken as a rough approximation of the dissociation constant for isocitrate at this site. The hypothesis of a separate activating site is helpful also in connection with the enhancement of the reverse reaction by isocitrate, a product of this reaction (Fig. 10). The inhibition caused by isocitrate at higher concentrations presumably results from its competition with $\alpha$-ketoglutarate for the reaction site. Finally, this hypothesis helps to explain the dual effect of citrate, which appears to inhibit competitively by binding at the reaction site (Fig. 11) but to enhance both the forward and reverse reactions under appropriate conditions by attachment at the activating site (Fig. 12). If indeed an activating site exists, it seems probable that its role relates to the binding of divalent cation, since in increase in the concentration of either isocitrate or AMP markedly decreases the effective Michaelis constant for Mg$^{++}$ (Fig. 6).

The term "activation site" is used in a very broad sense; activation by AMP, citrate, or isocitrate may, of course, be effected through structural changes in the enzyme protein, aggregation of subunits, or other unknown mechanisms.

The high specificity of the AMP effect suggests that we are dealing with an intrinsic property of the enzyme, rather than an artifact or coincidence; thus it seems plausible to suspect a regulatory role for the interaction. An increase in AMP concentration has the effect of decreasing the concentration of isocitrate needed to saturate the dehydrogenase. Since the concentration of AMP will in general vary inversely with that of ATP, the observed effect may amount to a negative feedback control of the reaction site at which the actual catalysis occurs; however, the role of AMP may be a prerequisite of their elucidation.
In the presence of AMP the reaction follows normal Michaelis kinetics, with the previously reported $K_m$ of about 0.1 mM isocitrate. In the absence of AMP, essentially no reaction occurs at isocitrate concentrations below 0.2 mM, and half-maximal velocity is reached at about 0.5 mM. An increase in the concentration of either isocitrate or AMP decreases the apparent $K_m$ of Mg++. Increasing the concentration of either isocitrate or NAD$^+$ decreases the level of the other required for half-maximal velocity. Citrate inhibits competitively, but can also partially replace isocitrate as an activator of both the forward and reverse reactions. These results evidently reflect a strong interaction between the reaction components with respect to enzyme binding; they can be partially rationalized by the hypothesis of distinct reaction and activation sites.

It is suggested that the AMP effect may provide regulation of the citric acid cycle in response to metabolic demand for adenosine triphosphate.

REFERENCES

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