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Nicotinamide–Adenine Dinucleotide-Specific Isocitrate Dehydrogenase from Pea Mitochondria

PURIFICATION AND PROPERTIES

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1. A method of stabilizing the enzyme by using glycerol is described. 2. A purification procedure is presented giving a higher purification than previously described. 3. Data showing substrate activation and activation by citrate are presented. 4. Kinetic constants for NAD^+, NADH and certain bivalent metal ions are given. 5. Pronounced inhibitory buffer effects are described. 6. A brief comparison between the NAD-specific isocitrate dehydrogenase from peas and that from other sources is made.

NAD-specific isocitrate dehydrogenase of higher plants [*threo*-D_S-isocitrate–NAD oxidoreductase (decarboxylating), EC 1.1.1.41] was first isolated and partially purified from pea mitochondria by Davies (1955). Under the conditions of isolation the enzyme was exceedingly unstable and characterization in a precise manner was therefore difficult.

Studies on NAD-specific isocitrate dehydrogenase from *Neurospora crassa* (Sanwal, Zink & Stachow, 1964; Sanwal & Stachow, 1965) and from yeast (Hathaway & Atkinson, 1963) have shown that the enzyme has kinetic properties consistent with a regulatory function. In both cases AMP and citrate have been shown to activate the enzyme, although the proposed mechanisms and the conclusions drawn from the experimental data differ. Co-operative substrate activation has also been demonstrated with the enzyme from both sources.

The NAD-specific isocitrate dehydrogenase from heart, described by Chen & Plaut (1963), does not show co-operative binding of substrate but does exhibit activation and stabilization by ADP.

Criticism of work on the NAD-specific isocitrate dehydrogenase from baker's yeast has been made by Cennamo, Montecuccoli & Bonaretti (1965). The enzyme was found to be sensitive to changes in concentration of tris–hydrochloric acid and potassium phosphate buffers and for this reason the validity of the studies *in vitro* was questioned.

It was considered important for the understanding of the regulation of the tricarboxylic acid cycle in plants to characterize the enzyme more precisely and to establish any regulatory properties that it may possess. The present paper reports a method of stabilizing the enzyme that has permitted its purification and study.

MATERIALS AND METHODS

Peas. Pisum sativum, var. Alaska, were soaked overnight in running tap water and then planted thickly in trays of well moistened vermiculite. After incubation for 10 days in a dark room at 25°, the shoots were approx. 10 cm. long and were harvested.

DEAE-cellulose. This was prepared for use by consecutive washings with 0·5N-NaOH, 0·5N-HCl and 0·5N-NaOH, followed by copious quantities of distilled water, and finally with 0·2M-$NaHCO_3$ until the pH of the effluent reached 7·8. Before use, the DEAE-cellulose was equilibrated with a solution containing $NaHCO_3$ (0·05M) and glycerol (5M) and packed into a column, under pressure provided by a small hand pump, to give a column 13 cm. × 1·5 cm. The hand pump was used to produce a flow rate of approx. 60 ml./hr.

Buffers. All buffers were prepared with double-glass-distilled water. 2-(*N*-2-Hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid (Hepes) (Good *et al.* 1966) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. The buffer was dissolved in water at 25° and adjusted to the required pH with freshly prepared NaOH.

Tris was obtained under the name of Trizma from Sigma Chemical Co., St Louis, Mo., U.S.A. It was dissolved in water at 25° and adjusted to the required pH with A.R. HCl.

Bio-Gel P-10. Beads were soaked overnight in either a solution containing $NaHCO_3$ (0·05M) and glycerol (5M) or a solution of Hepes, pH 7·6 (0·05M), and glycerol (5M), depending on the fractionating column for which they were being prepared.

Alumina C_γ. The alumina C_γ was obtained from Sigma Chemical Co. and contained 8% of solids.

Other materials. Trisodium *threo*-D_sL_s-isocitrate [the nomenclature according to Vickery (1962) is followed throughout], NAD^+, NADH and *p*-hydroxymercuribenzoate were all purchased from Sigma Chemical Co.

Glycerol and all inorganic chemicals were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex.

Protein measurements. Protein was determined throughout by the optical method of Warburg & Christian (1942).

Enzyme assays. The standard assay was carried out at pH 7·6 by measurement of ΔE_{340} with 0·05 M-Hepes as the buffer. The assay mixture contained trisodium *threo*-D_sL_s-isocitrate (4 mM; 2 mM with respect to *threo*-D_s-isocitrate), $MnSO_4$ (1 mM), NAD^+ (0·66 mM) and buffer to give a final volume of 3·0 ml. The reaction was started by the addition of 0·1 ml. of enzyme solution suitably diluted to give an activity of 7·5–30 units/ml. (the unit of enzyme activity is defined below).

Under these conditions of assay, the relation between reaction rate and enzyme concentration was linear.

Enzyme assays were carried out with a Unicam SP. 800 spectrophotometer coupled to a Honeywell strip chart recorder. With the maximum expansion available with this instrument, a full-scale deflexion equivalent to 0·1 *E* unit was possible. All assays were carried out at 25° with a thermostatically controlled cell housing.

Definition of unit of enzyme activity. One unit of enzyme activity is defined as the production of 10 mμmoles of NADH/min. This is equivalent to an increase in E_{340} of 0·0273/min., the extinction coefficient being taken to be $6{\cdot}22 \times 10^3$ cm.^{2}mole^{-1}. Specific activity is defined as the units of enzyme activity/mg. of protein.

Enzyme purification. The shoots (300 g.) were ground in 50 g. quantities each with 50 ml. of a solution of 0·5 M-sucrose in 0·1 M-potassium phosphate buffer, pH 7·6, in a pestle and mortar. The ground tissue was strained through nylon gauze and gently squeezed, giving a final volume of filtrate of approx. 600 ml. All operations were at 0°.

The extract was centrifuged for 5 min. at 1000*g* in a Sorvall RC–2B centrifuge (GSA head). The supernatant was centrifuged for a further 15 min. at 20000*g* to give a firm pellet, which has been shown by electron microscopy to be predominantly mitochondrial. The supernatant contained approx. 95% of the NADP-specific isocitrate dehydrogenase activity, but there was no detectable NAD-specific isocitrate dehydrogenase activity. The supernatant was therefore discarded. Washing of the pellet did not yield significant increases in the initial specific activity and this was consequently omitted.

The pellets were resuspended in a solution containing $NaHCO_3$ (0·1 M) and glycerol (5 M) to give a final volume of about 15 ml. A French press cooled to 0° was used to disintegrate the mitochondria. A pressure of 3000 lb./in.^{2} was applied with a hydraulic press and the extract collected in a cooled vessel and centrifuged at 100000*g* for 20 min. in an MSE Superspeed centrifuge (10 × 10 ml. head). The clear supernatant had a volume of approx. 20 ml. and is termed the crude extract.

A solution containing $NaHCO_3$ (0·1 M) and glycerol (5 M) was added to the crude extract to give a protein concentration of 10 mg./ml. Then 1 g. of alumina C_γ was added/100 mg. of protein present, with the aim of adsorbing 80–90% of the original activity, and the gel thoroughly dispersed. The suspension was centrifuged at 5000*g* in a bench centrifuge for 5 min., and the supernatant assayed to establish the extent of the adsorption. A 6 ml. volume of solution containing $NaHCO_3$ (0·05 M), glycerol (5 M) and trisodium citrate (0·2 M) was added to the pellet of alumina C_γ, and the gel dispersed thoroughly and left to equilibrate for 15 min. Again the suspension was centrifuged at 5000*g* for 5 min. The supernatant was retained and the pellet subjected to identical treatment with a further 3 ml. of elution medium.

To remove citrate, the combined fractions were run through a desalting column (30 cm. × 2 cm.) of Bio-Gel P-10 that had been equilibrated with a solution containing $NaHCO_3$ (0·05 M) and glycerol (5 M). The active fractions were collected.

The Bio-Gel P-10 eluate was pumped on to the column of DEAE-cellulose by using the small hand pump and the column washed with 20 ml. of a solution containing $NaHCO_3$ (0·1 M), glycerol (5 M) and trisodium citrate (0·04 M). This operation removed most of the remaining pigmented material and entirely separated the NADP-specific isocitrate dehydrogenase from the column. The column was then washed with a solution containing $NaHCO_3$ (0·1 M), glycerol (5 M) and trisodium citrate (0·06 M) and the eluate collected in 1 ml. fractions. This treatment removed the NAD-specific isocitrate dehydrogenase from the column. It was important to work quickly during this step as evidence suggested that the enzyme was unstable while on the column. The active fractions were combined (usually 3–4 ml.) and passed through a further desalting column (15 cm. × 2 cm.) of Bio-Gel P-10 equilibrated with a solution containing Hepes, pH 7·6 (0·05 M), and glycerol (5 M). The eluate was collected in 1 ml. fractions and the active ones were combined and stored at −15°.

The results of a typical run are shown in Table 1. Higher purifications are sometimes obtained by using the same procedure.

The preparation described above is entirely free of the following enzymes: NADP-specific isocitrate dehydro-

Table 1. *Summary of purification of NAD-specific isocitrate dehydrogenase*

Fraction	Total units of activity	Protein (mg./ml.)	Specific activity	Yield (%)	Purification
Crude extract	1100	17·4	3·0	100	0
First alumina C_γ eluate	578	4·3	22·5	52	7·5
Second alumina C_γ eluate	130	3·1	14·0	12·0	4·7
DEAE-cellulose fractions (0·06 M-citrate)					
Fraction 24	23	0·24	96	2·0	32
Fraction 25	47	0·26	180	4·5	60
Fraction 26	33	0·24	138	3·0	46
Fraction 27	20	0·15	133	2·0	44
Combined fractions 24–27	123	0·90	137	11·5	46

Table 2. *Shortened purification scheme with DEAE-cellulose*

Fraction	Total units of activity	Protein (mg./ml.)	Specific activity	Yield (%)	Purification
Crude extract	222	11·4	5·2	100	0
0·06 M-Citrate					
Fraction 3	17·8	0·38	18·8	8·0	3·60
Fraction 4	91·0	0·55	66·2	41·0	12·7
Fraction 5	23·5	0·3	31·5	10·5	6·0
Combined fractions 4 and 5	114·5	0·43	49·0	51·5	9·4
Bio-Gel P-10					
Fraction (*a*)	45·5			20·5	
Fraction (*b*)	22·2			10·0	
Fraction (*c*)	10·5			4·8	
Combined fractions (*a*) and (*b*)	67·7	0·21	64·5	30·5	12·4

genase; NAD-specific glutamate dehydrogenase; aconitase; malate dehydrogenase.

A quicker procedure is to omit the alumina C_γ step and to use only cellulose fractionation followed by desalting on Bio-Gel P-10. This gives a lower purification but a much improved yield. Some of the experiments were done with the enzyme prepared in this way. A typical run with this shortened purification is shown in Table 2.

RESULTS

Glycerol stabilization. A crude extract of the enzyme was prepared in 0·1 M-sodium hydrogen carbonate. Its preparation was identical with that described in the Materials and Methods section except that the glycerol was absent. To equal volumes of this preparation various volumes of a solution containing sodium hydrogen carbonate (0·1 M) and glycerol (10 M) were added. The samples were adjusted to identical protein concentrations with 0·1 M-sodium bicarbonate, to give a series of glycerol concentrations in the range 0–6·0 M, and then stored at 0°. The activities of the samples were assayed at saturating conditions over a period of 72 hr. The results are shown in Fig. 1.

To ensure that the effects were entirely due to the effect of glycerol on the stability of the enzyme, rather than to an activation effect in the assay system, a solution of Hepes, pH 7·6 (0·05 M), and glycerol (5 M) was added in varying volumes to the assay system containing an unstabilized crude extract. The results are shown in Fig. 2.

The two experiments show clearly that even low concentrations of glycerol aid the stability of the enzyme and that this cannot be explained by the apparent slight activation effect in the assay system.

Effect of metal ions. There was found to be an absolute requirement for certain bivalent metal ions: Mn^{2+}, Mg^{2+} and Zn^{2+} all activate the enzyme, though Zn^{2+} appears to inhibit at higher concentrations. The metal ion concentrations required for half maximum velocity are: Mn^{2+}, 18 μM; Mg^{2+}, 57 μM; Zn^{2+}, 50 μM. These results are shown in Figs. 3(*a*), 3(*b*) and 3(*c*).

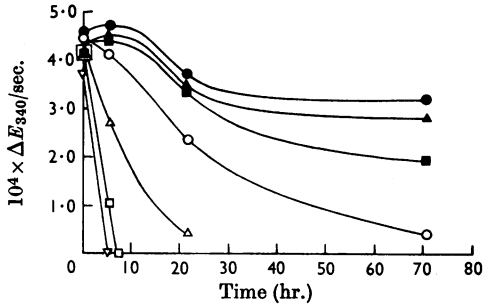

Fig. 1. Stabilization of NAD-specific isocitrate dehydrogenase by various concentrations of glycerol: ▽, none; □, 1 M; △, 2 M; ○, 3 M; ■, 4 M; ▲, 5 M; ●, 6 M.

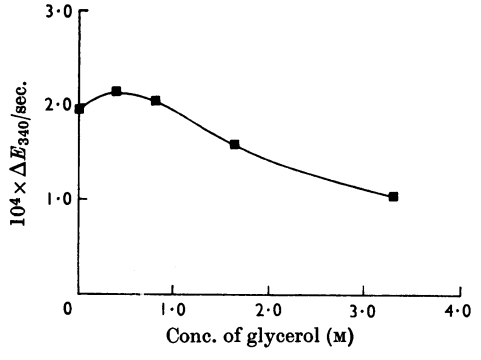

Fig. 2. Effect of glycerol on the standard assay system.

Reaction with substrate. Both the crude and partially purified enzyme give sigmoid plots when

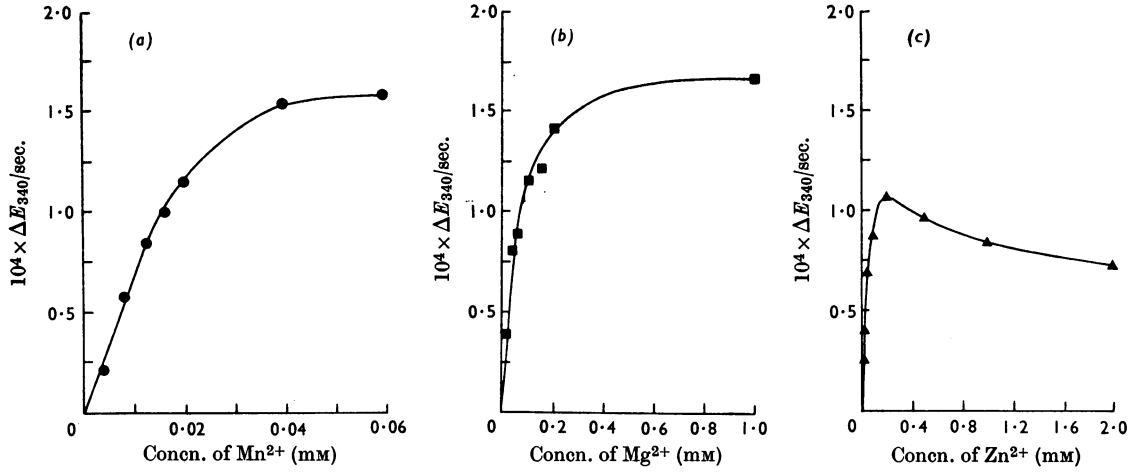

Fig. 3. Activation of NAD-specific isocitrate dehydrogenase by certain bivalent metal ions: (*a*) ●, Mn^{2+}; (*b*) ■, Mg^{2+}; (*c*) ▲, Zn^{2+}.

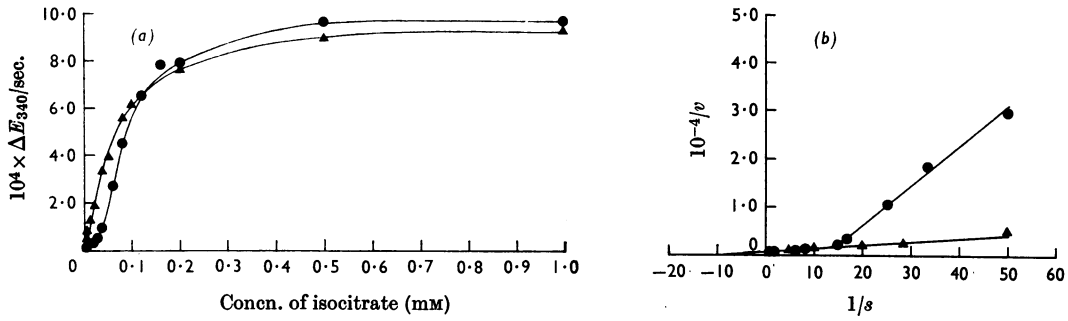

Fig. 4. (*a*) ●, Relation between initial rate (*v*) and substrate concentration (*s*) with a crude extract, the assay being carried out in 0·05 M-Hepes, pH 7·6; ▲, effect of 1 mM-citrate with otherwise identical conditions of assay. (*b*) Reciprocal plots of the results from (*a*): ●, no citrate; ▲, 1 mM-citrate.

initial rates are plotted against substrate concentration. Low concentrations of citrate have been found to activate the enzyme. With 1 mM-citrate the sigmoid plot is entirely absent and 'normal' kinetics are now observed. At higher concentrations of isocitrate the citrate behaves as a competitive inhibitor. The results of an experiment with a crude extract that demonstrate these effects are shown in Figs. 4(*a*) and 4(*b*). (Only the concentration of *threo*-D_S-isocitrate is given although *threo*-D_SL_S-isocitrate was used.) In all experiments where the crude extract has been used for characterization of the enzyme, it has previously been run through a column of Bio-Gel P-10 equilibrated with a solution containing Hepes, pH 7·6 (0·05 M), and glycerol (5 M), to remove small molecules.

NAD^+ requirement. The plot of initial rate against NAD^+ concentration obeys 'normal' Michaelis–Menten kinetics in both the crude and the purified preparations with saturating concentrations of the other components. The Michaelis constant, K_m, calculated from the results with the purified preparation is $2{\cdot}2 \times 10^{-4}$ M.

Inhibitory effects of NADH. When the crude extract is used in the presence of low concentrations of NADH, the plot of initial rate against NAD^+ concentration becomes sigmoid. However, the purified preparation shows no such effect, but does demonstrate simple competitive inhibition. The inhibition constant, K_i, calculated from this experiment is $1{\cdot}9 \times 10^{-4}$ M. A comparison of the effects with the crude and the purified preparations is shown in Figs. 5(*a*) and 5(*b*).

Optimum pH. The pH optimum for the reaction

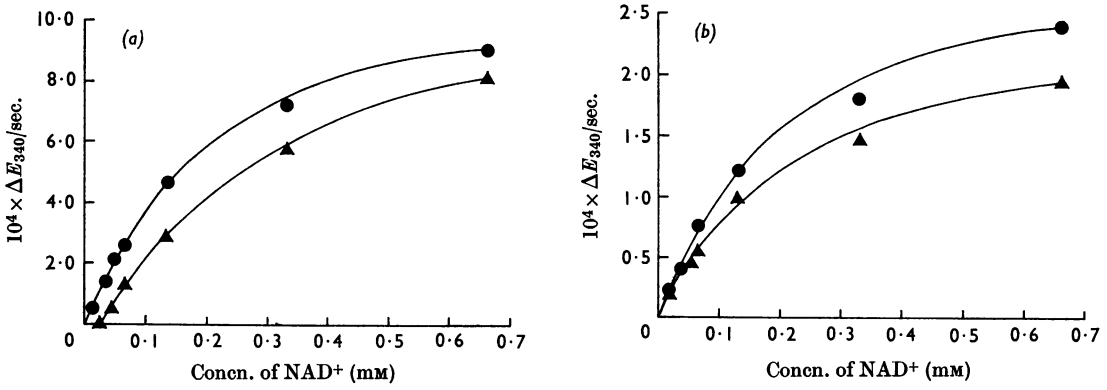

Fig. 5. (*a*) Effect of NAD+ concentration on the rate of reaction in the presence and absence of NADH with a crude extract: ●, no NADH; ▲, 0·073 mM-NADH. (*b*) Effect of NAD+ concentration on the rate of reaction in the presence and absence of NADH with a partially purified preparation: ●, no NADH; ▲, 0·073 mM-NADH.

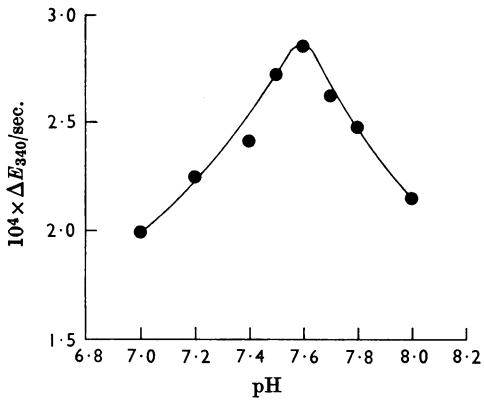

Fig. 6. Effect of pH on enzymic activity with 0·05 M-Hepes.

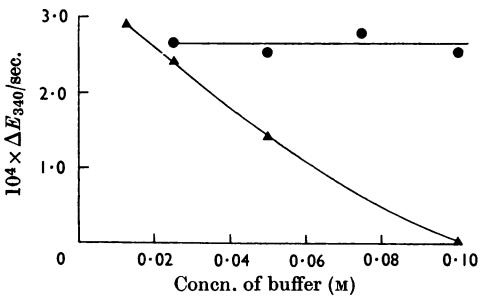

Fig. 7. Comparison of Hepes (sodium salt) with tris–HCl when assay buffer concentrations are changed at non-saturating substrate concentrations. Both buffers were at pH 7·6, NAD+ (0·66 mM), Mn^{2+} (1 mM) and isocitrate (0·1 mM). The partially purified enzyme was used. ●, Hepes (sodium salt); ▲, tris–HCl.

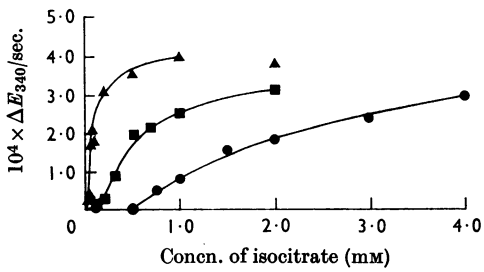

Fig. 8. Effect of change of concentration of tris–HCl buffer, pH 7·6, in the assay on the plot of initial rate against substrate concentration. ▲, 0·05 M-Tris–HCl; ■, 0·125 M-tris–HCl; ●, 0·2 M-tris–HCl.

with the standard assay system described in the Materials and Methods section was 7·6 in 0·05 M-Hepes (Fig. 6).

Buffer effects. Significant buffer effects could be demonstrated with tris–hydrochloric acid buffer. The extent of the inhibition in the presence of non-saturating concentrations of isocitrate (0·1 mM) and the comparison with Hepes (sodium salt) under identical conditions is shown in Fig. 7. The effect on the sigmoid nature of the plots of rate against substrate concentration is shown in Fig. 8.

Further experiments enabled these effects to be more clearly defined. With 0·05 M-Hepes, pH 7·6, a series of initial rates with various substrate concentrations were obtained in the presence of 0·1 M-sodium fluoride, 0·1 M-sodium chloride, 0·1 M-sodium bromide and 0·1 M-potassium iodide. The results shown in Fig. 9(*a*) demonstrate that the larger the anion the more sigmoid the plot. Acetate, nitrate, bicarbonate and chlorate were all found to produce similar effects. Reciprocal plots of these results

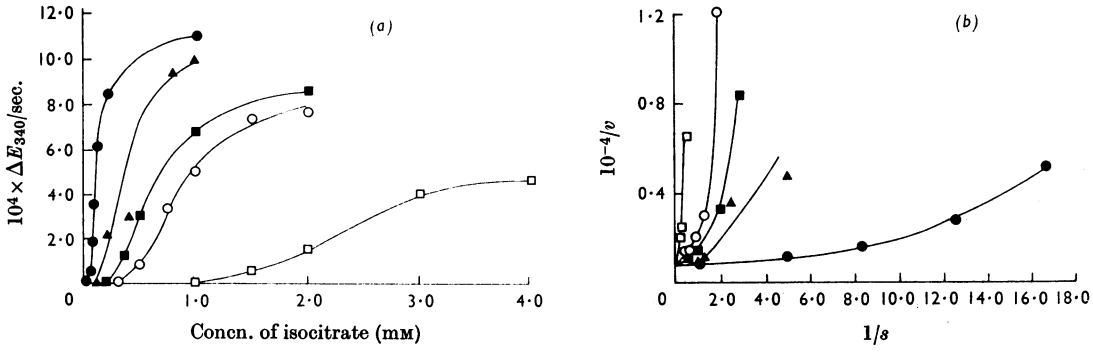

Fig. 9. (*a*) Effect of the halogens on the plot of initial rate (v) against substrate concentration (s) when present in the assay at a concentration of 0·1 M. All assays were carried out in 0·05 M-Hepes, pH 7·6. ●, No halogens; ▲, NaF; ■, NaCl; ○, NaBr; □, KI. (*b*) Reciprocal plots of the results shown in (*a*): ●, no halogens; ▲, NaF; ■, NaCl; ○, NaBr; □, KI.

suggest a competitive type of inhibition Fig. 9(*b*). However, the gross curvature of the plots argues against applying such a simple explanation to the effect. Sulphate, succinate and phosphate were also investigated for similar characteristics, but no inhibition could be demonstrated at the concentrations used. It is obvious that all the inhibitors are univalent anions.

AMP, ADP and ATP requirement. Stimulation of activity was not observed by any of the nucleotides with either the crude or the purified preparations when they were present at a concentration of 1 mM.

Cysteine requirement and the effects of thiol inhibitors. When the enzyme was prepared in the manner described above no cysteine requirement could be demonstrated. The presence of 1 mM-iodoacetate in the assay system had no apparent effect on the reaction rate at saturating conditions. However, *p*-hydroxymercuribenzoate, present in the assay at a concentration of 0·066 mM, caused 40% inhibition. Neither substrate nor coenzyme protection could be demonstrated.

Reversibility of the reaction. Attempts to reverse the reaction were made at a range of pH values between 6·0 and 8·5. The conditions of assay were: α-oxoglutarate (5 mM), NADH (0·08 mM), sodium hydrogen carbonate (2 mM), manganese sulphate (1 mM) and buffer to 3·0 ml. A 0·1 ml. volume of purified enzyme was used (sufficient to give 4·0 units of activity in the forward direction).

Reversal of the reaction could not be demonstrated by using the highest expansion available on the recorder. Doubling the concentrations of α-oxoglutarate and sodium hydrogen carbonate at pH 7·6 was also unsuccessful in reversing the reaction. The possibility of activation by citrate was investigated, but reversal of the reaction was not observed when 1 mM-citrate was present.

DISCUSSION

The results show that the enzyme responds to changes in substrate concentration in a similar manner to the NAD-specific isocitrate dehydrogenase from *Neurospora crassa* and from yeast. The activation by citrate is also a characteristic shared by each enzyme. The control of the reaction differs most markedly, however, in the response to nucleotides. It appears that a possible control over the enzyme from peas is exerted by the concentrations of reduced and oxidized coenzyme.

The buffer effects that have been described are similar to those observed by Cennamo *et al.* (1965) with the enzyme from yeast. In view of these observations, some scepticism is inevitably aroused with regard to precise data for the NAD-specific isocitrate dehydrogenase isolated from other sources in which tris–hydrochloric acid buffer has been used.

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