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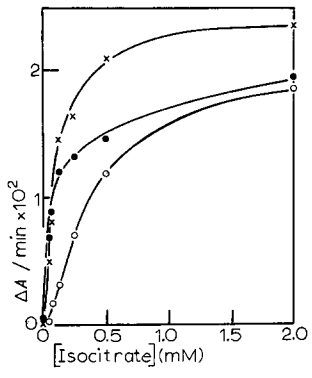
NAD-specific isocitrate dehydrogenase from a plant source. Sigmoid kinetics and enzyme stability

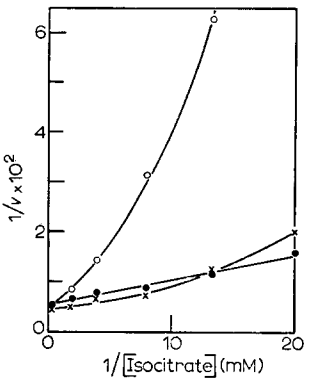
Sigmoidal plots of initial velocity *versus* substrate concentration are thought to be typical of regulatory enzymes and may indicate cooperative binding[1,2], although other explanations for this type of behaviour have been suggested[3–5]. Sigmoid kinetics have been demonstrated for the NAD-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate:NAD oxidoreductase (decarboxylating), EC 1.1.1.41) and this enzyme isolated from plants has properties similar to the enzyme from yeast and animal sources[6–8]. In the presence of citrate the enzyme shows normal kinetics and citrate will reverse the effect of anions[8] which increase the sigmoidicity at high concentrations[7–9]. During an investigation of the plant isocitrate dehydrogenase it was found that the rate response to increasing substrate concentration becomes more sigmoid in the presence of sodium dodecyl sulphate. Although this is typical of a negative effector, in this case it is probably due to enzyme inactivation and not to normal reversible kinetics. It is important therefore to show in other cases that an increase in the sigmoidal nature of the rate response to increasing substrate concentration in the presence of an apparent negative effector is a reversible phenomenon and is not due to enzyme inactivation.

NAD-specific isocitrate dehydrogenase was prepared from one-week-old etiolated pea seedlings. The preparation of an acetone powder of the mitochondrial fraction was as described previously[8] except for the omission of sand during homogenisation. The acetone powder was suspended by means of a Teflon homogeniser in 6 M methanol in 50 mM *N*-tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid (TES) buffer (pH 7.4) at a concentration of 10 mg per ml. The enzyme from peas is stabilised by 6 M methanol at 0° (ref. 10). The suspension was centrifuged at 105 000 $\times g$ for 30 min and the supernatant used as the source of enzyme. The assay contained 1.0 mM NAD^+, 1.0 mM $MnSO_4$ and 50 mM TES buffer (pH 7.4) in a total volume of 2.4 ml. The isocitrate used was *threo*-D_SL_S-isocitrate and the concentrations quoted are the total of both isomers. All biochemicals were obtained from the Sigma Chemical Co. The reaction was started by the addition of 0.1 ml of the enzyme preparation. Sodium dodecyl sulphate and other additions were placed in the assay mixture before the addition of enzyme. The reaction was followed by measuring the absorbance at 340 nm in a Unicam SP 800 recording spectrophotometer with an external recorder.

The effect of sodium dodecyl sulphate at a concentration of 0.14 mM on the rate *versus* substrate plot is shown in Fig. 1. It causes some lowering of V but the effect on the reaction rate is greatest at low substrate concentrations. This is typical of negative effectors and very similar to the effect of anions[6,8]. In the presence of citrate the inhibition by the detergent at low substrate concentrations is reversed but there is still a depression of V. A double reciprocal plot of velocity against substrate is curved for the control and this becomes more pronounced in the presence of the detergent (Fig. 2). In the presence of citrate a straight line is obtained even in the presence of the detergent. This data suggest that sodium dodecyl sulphate is a negative

Abbreviation: TES buffer, *N*-tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid.





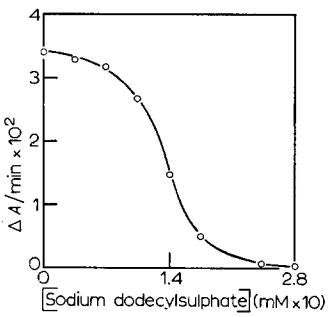


Fig. 1. The effect of isocitrate concentration on the rate of isocitrate dehydrogenase. × with no additions; ○, in the presence of 0.14 mM sodium dodecyl sulphate; ●, in the presence of 0.14 mM sodium dodecyl sulphate and 1.0 mM citrate.

Fig. 2. A double reciprocal plot of rate against isocitrate concentration. ×, with no additions; ○, in the presence of 0.14 mM sodium dodecyl sulphate; ●, in the presence of 0.14 mM sodium dodecyl sulphate and 1.0 mM citrate.

Fig. 3. The effect of sodium dodecyl sulphate concentration on the rate of NAD specific isocitrate dehydrogenase in the presence of 0.25 mM isocitrate.

effector, the effect of which can be reversed by the positive effector citrate. The concentration of detergent is important since there is a sigmoidal response when rate is plotted against its concentration (Fig. 3). Complete inhibition is obtained at 0.28 mM sodium dodecyl sulphate in the presence of 0.25 mM isocitrate.

It was found, however, that if the rate of reaction was measured for 4 min in the presence of 0.05 mM isocitrate and 0.14 mM sodium dodecyl sulphate the rate could not subsequently be increased by raising the isocitrate concentration to 2.0 mM (Table I). This concentration of isocitrate saturates the enzyme in the absence of the

TABLE I

The assay mixture is described in the text. The rate was determined for approx. 4 min in the presence of the initial substrate concentration shown above. In the samples in which this was less than 2.0 mM, 0.2 ml of buffer containing 25 mM isocitrate was added to give a final isocitrate concentration of approx. 2.0 mM. In the reaction with an initial isocitrate concentration of 2.0 mM, 0.2 ml of buffer was added. The final rate is the rate following increase of the substrate concentration to 2.0 mM. The final rate of all the samples might be expected to be lower than the 2.0 mM initial rate because of the dilution of the enzyme.

Additions	*Initial rate (ΔA/min) × 10²*			*Final rate (ΔA/min) × 10²*
$[S]_i$* *(mM)*:	*0.05*	*0.25*	*2.0*	
0	0.65	—	—	1.97
0.14 mM sodium dodecyl sulphate	0.10	—	—	0.15
50 mM NaCl	0.10	—	—	1.98
0	—	2.67	—	2.32
0.14 mM sodium dodecyl sulphate	—	1.10	—	0.82
0	—	—	3.01	2.59

* Initial substrate concentration.

detergent (Fig. 1). This did not apply when the detergent was omitted or if the enzyme had been inhibited by the addition of 50 mM NaCl (Table I). The rate of reaction was linear with time over the period of the assay. This was also the case when single assays were performed containing 0.25 mM isocitrate and 0.14 mM sodium dodecyl sulphate and the reading of absorbance started after 10 sec.

To explain this data it is suggested that the detergent combines with the enzyme extremely rapidly to form an inactive complex, the formation of which may be prevented when substrate is bound to the enzyme. As the substrate concentration is lowered from that required to give V more free enzyme is available for reaction with the detergent. The sigmoid nature of the rate response to substrate in this case may be due, therefore, to different amounts of active enzyme in the assay at different substrate concentrations. Since this is a crude preparation there are other proteins in the solution which could also complex sodium dodecyl sulphate so that the free from will only exist in solution for a short time. After the initial free enzyme has been inactivated the concentration of detergent will be so low that any further enzyme released from the enzyme–substrate complex will not be inactivated and linear rates are obtained. It has been suggested that citrate can bind to a regulator site of the enzyme which can also bind isocitrate[8]. Citrate and isocitrate stabilize the enzyme[10] and it is probable that they act in a similar manner to protect against inactivation by the detergent.

It has been shown in this case that the increase in sigmoidicity of the substrate saturation curve in the presence of sodium dodecyl sulphate is not directly related to the regulatory properties of the enzyme, but is probably due to an inactivation of the enzyme. It is important, therefore, to determine that the action of suspected negative effectors can be reversed by increasing the substrate concentration and is not an artifact caused by enzyme inactivation, especially when crude enzyme preparations are used.

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