BBA 12169

# ACTIVATION OF ISOCITRATE LYASE AND TRIOSEPHOSPHATE DEHYDROGENASE IN *AZOTOBACTER VINELANDII* EXTRACTS

I. R. KENNEDY AND M. J. DILWORTH*

*Institute of Agriculture, University of Western Australia, Nedlands (Australia)*

(Received June 19th, 1962)

## SUMMARY

Isocitrate lyase is formed in *Azotobacter vinelandii* during growth on acetate.

Reported requirements of isocitrate lyase for $Mg^{2+}$ and cysteine have been confirmed, but EDTA could replace cysteine for activation, and did so more efficiently. This obviates the serious interference by cysteine in glyoxylic acid measurement.

Isocitrate lyase has a half-maximum velocity at $8.6 \cdot 10^{-5}$ M threo-Ds-isocitrate and is not inhibited by high DL-isocitrate concentration (up to 8 mM).

Triosephosphate dehydrogenase has been identified in ammonium sulphate fractions of crude extracts of Azotobacter. This enzyme is also activated by EDTA more efficiently than by cysteine.

It is concluded that these activations by cysteine and EDTA are the result of binding of inhibitory heavy metals in the assay medium. The possible nature of such metals is discussed.

## INTRODUCTION

During studies of oxygen toxicity on enzymes concerned in oxidative pathways in *Azotobacter vinelandii*, suitable conditions for assay of isocitrate lyase (Ls-isocitrate glyoxylate lyase EC 4.1.3.1) and triosephosphate dehydrogenase (D-glyceraldehyde 3-phosphate : NAD oxidoreductase, EC 1.2.1.12) were sought.

Isocitrate lyase catalyses the reversible cleavage of isocitric acid to glyoxylic acid and succinic acid, and has been demonstrated previously in extracts of acetate-grown *A. vinelandii* and *A. agile*[1]. Requirements for a divalent metal ion and thiol compounds for maximal activity have been reported[2-4]; SMITH AND GUNSALUS[3] found that glutathione was slightly less active than equimolar cysteine in meeting the latter requirement. A similar thiol requirement for activation of triosephosphate dehydrogenase from mammalian systems has been reported[5,6], though SEGAL AND BOYER[7] showed that muscle enzyme could be activated successfully by EDTA in place of cysteine.

* Present address: Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford (Great Britain).

Because thiol compounds would complicate studies with gaseous oxygen, these findings have been examined for Azotobacter enzymes. Serious disadvantages in the use of cysteine for isocitrate lyase activation have been demonstrated.

## METHODS

### *Bacteriological*

For preparation of extracts containing isocitrate lyase, a strain of *Azotobacter vinelandii* designated S was used[8]. *A. vinelandii* O was used for preparation of triosephosphate dehydrogenase. Cultures were preserved by vacuum-drying[9] and carried on M6 agar medium[10] for broth inoculation. Larger quantities of cells for preparation of cell-free extracts were grown on acetate medium, of the following composition, per l: Na acetate, 20 g; $KH_2PO_4$, 0.15 g; $Na_2HPO_4 \cdot 12\ H_2O$, 0.15 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $CaSO_4 \cdot 2\ H_2O$, 0.05 g; $Na_2MoO_4$, 0.003 g; EDTA iron complex, 0.015 g; NaCl, 0.1 g; $CuSO_4 \cdot 5\ H_2O$, 0.4 mg; $MnSO_4 \cdot 4\ H_2O$, 2.0 mg; $ZnSO_4 \cdot 7\ H_2O$, 0.8 mg; $H_3BO_3$, 2.4 mg; with the extract from autoclaving 12.5 g of peeled potato in 25 g of water for 40 min at 5 lb/in$^2$. Sterilisation was by heat, with the phosphates sterilised separately to prevent precipitation. Inocula were 5% volumes of culture grown on nitrogen-deficient sucrose medium (M22) (see ref. 8). With acetate as carbon source, alkali was produced and the pH was checked periodically during growth and returned to neutral by the addition of sterile HCl.

Cultures were grown in 20-l Pyrex jars under forced aeration at controlled temperature. Cells were harvested after 36 h growth by centrifugation with a Servall model SS-1, with continuous-flow attachment, and stored at −20°.

Extracts containing triosephosphate dehydrogenase were obtained from cells grown on M22.

### *Preparation of cell-free extracts*

Cell-free extracts were obtained by alumina grinding of frozen cells[11]. The ground cell material was suspended in 0.015 M phosphate buffer (pH 7.0) at a rate corresponding to 1 g wet weight of cells per 20 ml of buffer, and centrifuged first for 10 min at 4000 × *g* to remove alumina particles and cell debris, and then for 10 min at 20 000 × *g*. The supernatant was used for enzyme assay and contained approx. 3 mg of protein/ml. These operations were carried out at or below 3°.

### *Reagents*

Distilled water used throughout was deionised on Bio-Deminrolit resin (Permutit Co.) to a specific conductance of 0.1 μmho/cm. The alumina used for preparation of cell-free extracts was Aloxite-600 (The Carborundum Co., Manchester), which had been washed with 0.01 M EDTA (pH 8.0), rinsed several times with deionised water, and dried at 80°.

Biochemicals were obtained from the following sources: crystalline lysozyme and oxaloacetic acid, from Nutritional Biochemicals Corporation; NAD and NADP from Sigma Chemical Co.; $Na_3$-threo-D$_S$L$_S$-isocitrate from Fluka Chemische Fabrik; glyoxylic acid monohydrate, from Light and Co.; and glyceraldehyde 1-bromide 3-phosphate dioxane oxonium salt, from California Foundation for Biochemical Research.

*Analytical*

Isocitrate lyase activity was determined by measurement of glyoxylic acid formation by the method of McFADDEN AND HOWES[12], which involves colorimetry of the 1,5-diphenyl-formazan carboxylic acid formed on oxidation of glyoxylic acid phenylhydrazone with $K_3Fe(CN)_6$. To ensure reproducibility of results, time of heating was carefully standardised at 5 min. A reference sample of glyoxylic acid monohydrate was 99.5 % pure by titrimetric assay[12].

DL-Isocitric acid was 90% pure, as shown by NADP reduction with a crude preparation of isocitric dehydrogenase from acetate-grown *A. vinelandii*. NADP reduction was followed at 340 mμ in a Beckman-DU spectrophotometer, with cell compartment at 30°. The procedure followed that of KORNBERG[13], with 0.06 M Tris buffer (pH 7.5) replacing phosphate.

In the initial experiments, the assay system for isocitrate lyase was similar to that described by SMITH AND GUNSALUS[2], carried out in Thunberg tubes, or in flasks attached to the manometers of a Warburg respirometer. The final volume was 3.0 ml, with nitrogen as the gas phase throughout. Reaction was started by addition of isocitrate from the side arm after temperature equilibration at 30°, and stopped with 0.2 ml of 80% (w/v) trichloroacetic acid. After protein was removed by centrifugation, an aliquot of the supernatant was analysed for glyoxylic acid. Controls to which substrate was added after trichloroacetic acid addition contained negligible amounts of glyoxylate.

Triosephosphate dehydrogenase was assayed by observation of NAD reduction at 340 mμ. The assay system follows the procedure of KREBS[6], which uses cysteine as activator. The purity of commercial DL-glyceraldehyde 3-phosphate 1-bromide dioxane oxonium salt was determined with Azotobacter enzyme; it assayed at 67%.

Triosephosphate dehydrogenase was prepared from 6 g wet weight of sucrose-grown cells, which were ground for 20 min with 15 g of alumina. The ground material was taken up in 48 ml of 0.03 M pyrophosphate buffer (pH 8.7), containing 2.5 ml of 0.01 M nicotinamide, and centrifuged for 12 min at 7000 × *g*. Enzyme was partially purified from the supernatant by ammonium sulphate fractionation, most activity being precipitated between 0.6 and 0.8 saturation. The enzyme was stable for at least 3 months at −20°. Further purification was not undertaken, since activity could be satisfactorily determined without observable reoxidation of $NADH_2$.

Protein was measured by the method of LOWRY *et al.*[14], with crystalline lysozyme as standard.

*Chromatography*

Keto acids were converted into their 2,4-dinitrophenylhydrazones[2] and separated by ascending chromatography on Whatman No. 4 paper. Solvents were butanol–ethanol–water (70:10:20)[15] and isopropanol–ammonium hydroxide–water (100:5:10)[16]. For colour development of spots, dried papers were dipped in 2% NaOH in 90% ethanol. Markers were prepared from pure glyoxylic, pyruvic, oxaloacetic and α-ketoglutaric acids[16].

## RESULTS

### *Isocitrate lyase*

Glyoxylic acid formation was verified by paper chromatography of the keto acid produced. $R_F$ values of the derivative from the reaction mixture and its colour reaction with ethanolic NaOH were the same as those of authentic glyoxylic acid 2,4-dinitrophenylhydrazone.

The spectrum of the unknown dinitrophenylhydrazone confirmed the identity of the keto acid. It showed a maximum at 450 mμ in aqueous alkali, while the ratio of the absorbancies at 490 mμ and 540 mμ was 1.84. The spectra of the pyruvic and α-ketoglutaric acid derivatives show a plateau between these wavelengths, with a ratio approximating unity[2], while authentic glyoxylic acid dinitrophenylhydrazone has a ratio of 1.9.

The semi-specific analytical method of McFADDEN AND HOWES[12] offers further evidence for glyoxylic acid formation, as it is specific for aldehydes[17]. The spectrum of the derivative formed from authentic glyoxylic acid was the same as that formed from the reaction product, showing a maximum at 520 mμ.

The reverse reaction of formation of isocitric acid from glyoxylic acid and succinic acid was readily demonstrated[2], though chromatography failed to detect succinic acid after the isocitrate lyase reaction. It seems reasonable to conclude that succinic acid was formed during reaction and further metabolised by the unpurified extract.

Isocitrate lyase activity of unpurified extracts was comparable to that reported previously for similar extracts of acetate-grown *A. vinelandii* and *A. agile*[1] and of *Pseudomonas aeruginosa*[2]. The highest activity obtained was 10 μmoles of glyoxylic acid produced per mg of protein per h, though activity declined slightly with storage. After 6 months at −20° in 0.015 M phosphate buffer (pH 7.0) cell-free extracts retained 75% of their initial activity. Sucrose-grown cells showed negligible isocitrate lyase activity.

### *Activation with cysteine and EDTA*

The activation of Azotobacter isocitrate lyase by a range of cysteine or EDTA concentrations is shown in Fig. 1. Cysteine is less effective than EDTA, both in

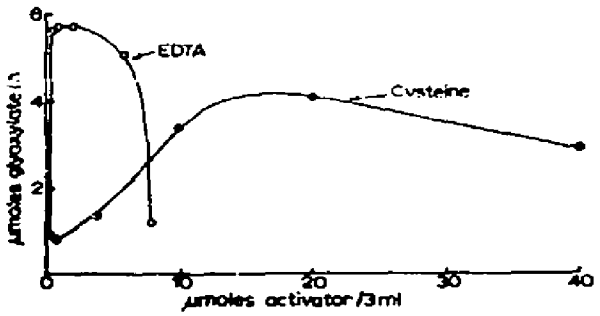


Fig. 1. The effect of cysteine and EDTA on isocitrate lyase activity of *A. vinelandii* S extracts. Each Thunberg tube contained (in μmoles/3.0 ml final volume): Tris buffer (pH 7.0) 200; $MgCl_2$, 6; EDTA or neutralised cysteine hydrochloride, as indicated; $Na_3$-DL-isocitrate, in side-arm, 20; plus Azotobacter extract, 1.0 mg. Reaction was for 25 min at 30° after 5 min preincubation.

terms of concentration required, and in maximum activity reached. The rapid decrease in lyase activity above 2 mM EDTA is apparently due to inhibition caused by chelation of $Mg^{2+}$.

To establish the concentration of $Mg^{2+}$ required to maintain maximum enzyme activity in the presence of 0.67 mM EDTA (which was ample for activation), a saturation curve for $Mg^{2+}$ was determined.

Since isocitrate lyase activity was only slightly below maximum in the presence of equimolar EDTA and $Mg^{2+}$, sufficient $Mg^{2+}$ in subsequent enzyme assays was ensured by use of a 3-fold excess. As can be seen from Fig. 2, this did not interfere with the activating influence of EDTA.

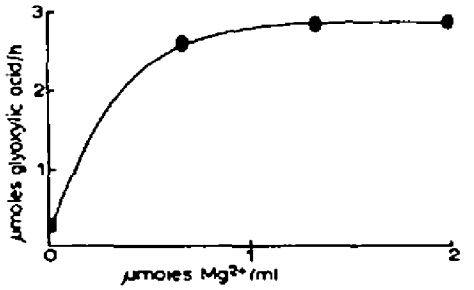


Fig. 2. Isocitrate lyase activation by $Mg^{2+}$. Thunberg tubes contained reagents described under Fig. 1, except: *A. vinelandii* extract, 0.5 mg; EDTA, 2 μmoles; $MgCl_2$, total as indicated, in final volume of 3.0 ml. Reaction was for 15 min at 30° after 5 min preincubation.

Addition of cysteine to extract maximally activated with EDTA did not result in increased activity; in fact, lower activity was recorded. Activation by EDTA was apparently rapid, for tubes were incubated for only 5 min before reaction was started. Incubation of extract with EDTA in the presence of $Mg^{2+}$ in Tris buffer for periods up to 6 h before starting reaction did not result in any significant change in activity. The pattern of activation of Azotobacter isocitrate lyase did not change with time, EDTA still being superior to cysteine in activation after 6-months' storage at −20°. The mechanism of both EDTA and cysteine activation was apparently the same.

When protein concentration was decreased from 1.0 mg/3 ml to 0.12 mg/3 ml and EDTA concentration reduced to 0.067 mM, isocitrate lyase activity was only 74% of that obtained with 0.67 mM EDTA. This indicates that EDTA activation was dependent on its final concentration in the assay medium, and not on a stoichiometric relationship with the amount of protein.

*Effect of pH*

The effect of pH on isocitrate lyase activity is shown in Fig. 3. Maximum activity occurred between pH 7.6 and 8.0 in 0.066 M Tris buffer. This corresponds closely to the optimum of pH 7.7 observed with an extract from *Pseudomonas indigofera*[18].

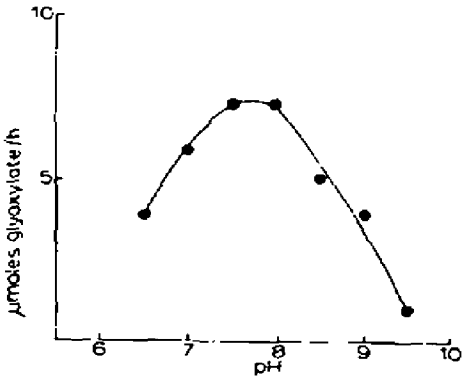


Fig. 3. pH dependence of isocitrate lyase. Thunberg tubes contained (in μmoles/3.0 ml final volume): Tris buffer, 200; $MgCl_2$, 6; EDTA, 2; $Na_3$-DL-isocitrate, 20; plus *A. vinelandii* extract, 1.0 mg. Reaction was for 10 min at 30° after 5 min preincubation.

*Protein concentration*

When sufficient EDTA or cysteine was added to achieve maximum activity of isocitrate lyase, glyoxylic acid formation with varying levels of Azotobacter extract was as shown in Fig. 4.

The plot for the series with EDTA activation is quite linear and, unlike that with cysteine, passes through the origin on extrapolation. At low protein concen-

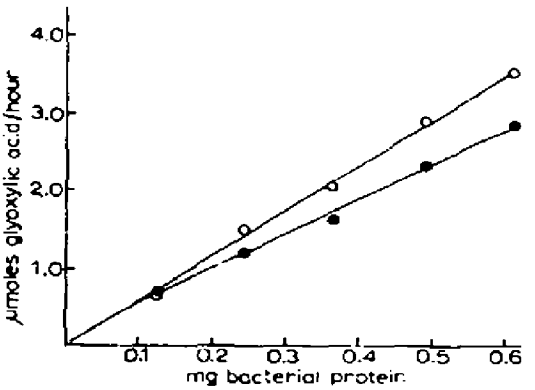


Fig. 4. Isocitrate lyase activity with increasing concentration of Azotobacter extract. Thunberg tubes contained (in μmoles/3.0 ml final volume): Tris buffer (pH 7.8), 200; $MgCl_2$, 6; $Na_3$-DL-isocitrate, in side-arm, 15; ○—○, EDTA 2; ●—●, cysteine hydrochloride, 15. Reaction was continued for 12 min at 30° after 15 min preincubation with *A. vinelandii* extract added as indicated.

trations, cysteine has occasionally proved slightly superior to EDTA (see Table II), suggesting that under these conditions it exerts some undefined activation. In comparison to the EDTA effect at higher protein levels (Fig. 4), such an effect appears to be very slight.

*Glyoxylate reaction with thiols*

The depression of isocitrate lyase activity with higher levels of cysteine (Fig. 1) was unlikely to be caused by inhibition consequent upon $Mg^{2+}$ binding, as occurred with EDTA, for cysteine has only low affinity for $Mg^{2+}$ (log $K$ less than 4)[19]. In addition, the lower activity observed during activation with EDTA plus cysteine, rather than EDTA alone, suggested some interference by cysteine. Reaction between cysteine and glyoxylic acid is now well substantiated[20,21], and we have also observed rapid reaction between glyoxylic acid and cysteine at neutral pH.

Glyoxylic acid could not be detected after a 1.5 mM solution was incubated for 30 min at 30° with a 16 mM solution of cysteine in 67 mM Tris buffer (pH 7.8) when the analytical method of FRIEDEMANN AND HAUGEN[22] was used (without solvent extraction). With the method of MCFADDEN AND HOWES[12], the recovery of glyoxylic acid with heating time in phenylhydrazine hydrochloride at pH 1 was as shown in Fig. 5. The control series also shown in Fig. 5 contained no cysteine.

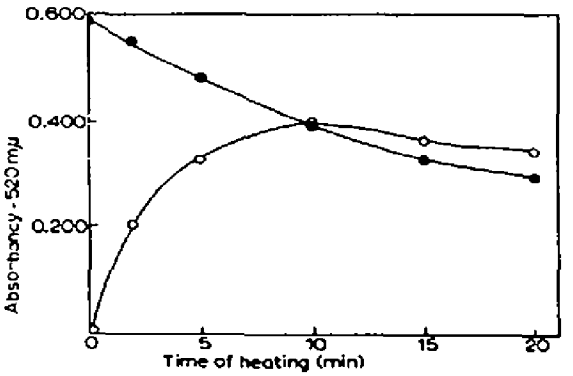


Fig. 5. Glyoxylic acid recovery from cysteine complex with heating time. Glyoxylate was incubated at 30° for 30 min in solution containing (in μmoles/6.0 ml): Tris buffer (pH 7.8) 1000; EDTA, 4; cysteine hydrochloride, 100; glyoxylic acid, 9. A control contained no cysteine. Colour produced was as shown when 0.165 ml samples were analysed with phenylhydrazine hydrochloride[12] by heating for variable time at 95°. ○--○, plus cysteine; ●—●, control.

Greatest colour development was obtained where heating was omitted and the sample incubated with phenylhydrazine hydrochloride at room temperature for 20 min. This emphasizes the need for careful standardisation of heating time, or its complete omission, in agreement with the conclusions of KRAMER *et al.*[17] for a method using essentially the same principle. Though it is obvious that heating releases glyoxylic acid from its complex with cysteine, the rate of decomposition of glyoxylic acid phenylhydrazone to yield a product which is not oxidised to the formazan makes this method impracticable for the determination of glyoxylic acid after incubation with cysteine.

Percentage loss of 1 mM glyoxylic acid incubated with the same range of cysteine concentrations as used for activation of isocitrate lyase (Fig. 1) is shown in Table I. These results suggest that cysteine, at sufficiently high concentrations, is as complete an activator as EDTA, and that discrepancies between the two result from interaction between glyoxylic acid and cysteine.

TABLE I

EFFECT OF CYSTEINE CONCENTRATION ON ASSAY OF GLYOXYLIC ACID WITH PHENYLHYDRAZINE HYDROCHLORIDE

Conditions of assay: 1 mM glyoxylic acid was incubated at 30° with cysteine for 20 min in 200 mM Tris buffer (pH 7.8); 2 mM $MgCl_2$; 0.67 mM EDTA, final volume 3.0 ml. Glyoxylate in 0.24-ml samples was assayed immediately[12] with 5-min heating time.

| Cysteine-HCl (μmoles/3.0 ml) | $A_{324\ m\mu}$* | Decrease (%) |
|---|---|---|
| 0 | 0.536 | 0 |
| 5 | 0.488 | 9 |
| 10 | 0.464 | 13 |
| 15 | 0.411 | 23 |
| 20 | 0.360 | 33 |
| 30 | 0.333 | 38 |
| 40 | 0.339 | 37 |

When glutathione was tested for interaction with glyoxylic acid in the systems described above, no interfering effect could be found. Glyoxylic acid recovery with 2,4-dinitrophenylhydrazine[22] or phenylhydrazine[12] was the same as that in control mixtures to which no glutathione had been added. Glutathione has been reported to react with carbonyls[23]; however, any compound formed here must have been easily dissociable.

*Activity with time*

Spectrophotometric assay methods for isocitrate lyase dependent on the formation of compounds of glyoxylic acid with semicarbazide (absorbing at 252 mμ, see ref. 24), and with phenyl-hydrazine hydrochloride (absorbing at 324 mμ, see ref. 25), show short lag periods before significant changes in absorbancy occur, probably due to slow reaction between glyoxylate and the reagents.

With Azotobacter enzyme, and with direct chemical assay of glyoxylic acid formation, the time course of isocitrate lyase reaction was as shown in Fig. 6. The line of best fit, by the method of least-squares, passes almost directly through the origin.

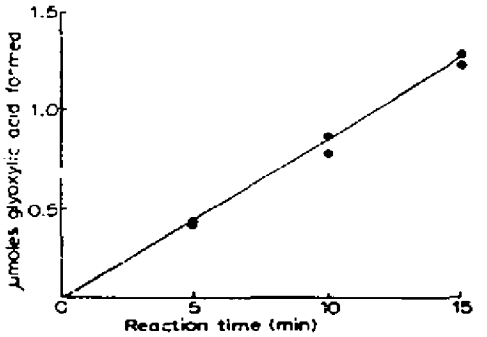


Fig. 6. Linearity of isocitrate lyase reaction with time. Warburg flasks contained (in μmoles/3.0 ml final vol.): Tris buffer (pH 7.6) 200; $MgCl_2$, 6; EDTA, 2; $Na_2$-DL-isocitrate (side-arm), 30; plus *A. vinelandii* extract, 0.78 mg. Reaction was started after 30 min preincubation at 30° and stopped at the times indicated.

*The effect of substrate concentration*

The substrate of *P. indigofera* isocitrate lyase has been reported as D$_s$(+)-isocitrate[18], though other workers[2,3] considered the L$_s$(+)-form as the active isomer for isocitrate lyase from other microbes. DL-Isocitric acid was exactly half as active as the single isomer for isocitrate lyase, indicating that only one form was metabolised and confirming that the inactive form produced no inhibition[3,18]. In this paper we have followed the nomenclature of VICKERY[28], who recently suggested an unequivocal system to resolve the present confused position. The Enzyme Commission has recommended that isocitrate lyase (formerly isocitritase) be known systematically as L$_s$-isocitrate glyoxylate lyase. This should be superseded by threo-D$_s$-isocitrate glyoxylate lyase to agree with the nomenclature of VICKERY[28] for the natural isocitric acids.

The reaction rate curve for saturation of *A. vinelandii* isocitrate lyase with threo-D$_s$-isocitrate is shown in Fig. 7, and conforms with MICHAELIS–MENTEN

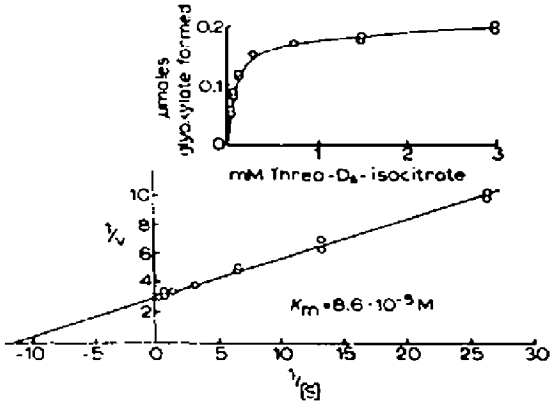


Fig. 7. Evaluation of $K_m$ for isocitrate lyase. Thunberg tubes contained (in µmoles/6.0 ml final volume): Tris buffer (pH 7.6) 400; $MgCl_2$, 12; EDTA, 4; threo-D$_s$-isocitrate (as $Na_3$-DL-form in side-arm), as indicated; plus *A. vinelandii* extract, 0.15 mg protein. Tubes were preincubated at 30° for 10 min and the reaction stopped at 7 min by addition of 0.4 ml of 80% trichloroacetic acid. Results shown are single analyses on duplicated levels of substrate.

kinetics. A LINEWEAVER–BURK[27] double reciprocal plot of the rate of glyoxylic acid formation *versus* the threo-D$_s$-isocitrate concentration was linear, and extrapolated to a $K_m$ of $8.6 \cdot 10^{-5}$ M. This lies within the range of $K_m$ values previously reported of $4.5 \cdot 10^{-4}$ M (see ref. 2) and $5 \cdot 10^{-5}$ M (see ref. 28). Purified yeast enzyme required $1.2 \cdot 10^{-3}$ M "L$_s$(+)-isocitrate" for half-maximum activity[2].

A report that isocitrate lyase from *P. indigofera* was inhibited by concentrations of "D$_s$(+)-isocitrate" greater than $8 \cdot 10^{-4}$ M, or of DL-isocitrate of $1.6 \cdot 10^{-3}$ M (see ref. 18), was not in agreement with the present results. Because it was at first suspected that this inhibition was the result of thiol reaction with glyoxylic acid at higher concentrations of isocitrate, and hence of glyoxylate, rates of reaction with either cysteine or EDTA as activators were followed. At low protein concentration (40 µg/ml) with low glyoxylic acid production, activation by cysteine and EDTA was closely similar. As shown in Table II, no inhibition was observed at concentra-

TABLE II

EFFECT OF DL-ISOCITRATE CONCENTRATION ON ISOCITRATE LYASE ACTIVITY IN *A. vinelandii* EXTRACT

Conditions of assay were as described in Fig. 7 except: *A. vinelandii* extract, 0.24 mg protein; reaction was for 12 min in 6.0-ml final volume.

| DL-Isocitrate (μmoles/ml) | Glyoxylic acid formed (μmole/6 ml) | |
|---|---|---|
| | 1.3 mM cysteine | 0.67 mM EDTA |
| 0.10 | 0.091 | 0.091 |
| 0.30 | 0.153 | 0.168 |
| 1.50 | 0.240 | 0.204 |
| 3.00 | 0.242 | 0.216 |
| 5.00 | 0.242 | 0.226 |
| 8.00 | 0.246 | 0.228 |

tions of DL-isocitrate up to $8 \cdot 10^{-3}$ M. Clearly the inhibition observed[18] is not due to a reaction of this type, especially as glutathione was used for activation. It therefore appears that substrate inhibition is not a general property of isocitrate lyase.

*Triosephosphate dehydrogenase*

The presence of this enzyme in *A. vinelandii* has been inferred from indirect evidence by MORTENSON, HAMILTON AND WILSON[29]. Although direct reduction of NAD did not occur in an extract with added fructose diphosphate and aldolase

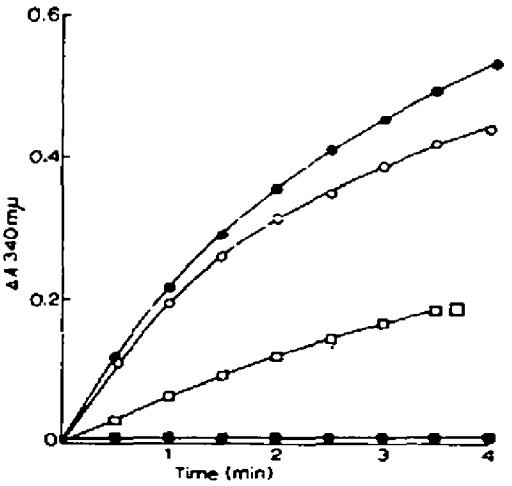


Fig. 8. The effect of cysteine and EDTA on NAD reduction by triosephosphate dehydrogenase from *A. vinelandii*. Each cuvette contained (in μmoles/0.8 ml final volume): sodium pyrophosphate (pH 8.5) 18; sodium arsenate (pH 8.5) 4.2; DL-glyceraldehyde 3-phosphate, 0.38; NAD, 0.25; enzyme, 0.23 mg protein. Reaction was initiated by the addition of mixed glyceraldehyde 3-phosphate/arsenate reagent. Temperature 30°. ●—●, with 0.8 μmole EDTA (pH 8.5); ○--○, with 3.5 μmoles cysteine hydrochloride (pH 8.5); ■—■, with 0.25 μmole NADP replacing NAD and 0.8 μmole EDTA (pH 8.5); □—□, no additions.

(EC 4.1.2.7), $NADH_2$ oxidation could be demonstrated in such systems. Substantial NAD reduction has been achieved with partially purified enzyme from *A. vinelandii*. As shown in Fig. 8, 1 mM EDTA was slightly better for activation of NAD reduction by glyceraldehyde 3-phosphate and Azotobacter extract than 3.5 mM cysteine. SEGAL AND BOYER[7] had noted a similar superiority of EDTA over cysteine for activation of triosephosphate dehydrogenase from muscle. Pyrophosphate gave greater activity than did Tris of the same pH and molarity (22.5 mM) and maximum activity was obtained at pH 9.0. Unlike the preparation of SEGAL AND BOYER[7], Tris buffer did not allow a longer period of linear absorbancy change. $NADP^+$ was not reduced in the system.

The glyceraldehyde 3-phosphate concentration for half-maximum reaction velocity was estimated[27] at $5.3 \cdot 10^{-5}$ M at pH 9.0 and 30°.

An attempt was made to activate the enzyme with cysteine, and then to remove the excess by dialysis. 2 ml of enzyme (4.6 mg of protein) were treated with 0.05 ml of 0.3 M cysteine hydrochloride at pH 7.2 for 2 h at 0° in a Thunberg tube under nitrogen. The enzyme was quickly transferred to dialysis tubing (EDTA washed) and dialysed with stirring against 1 l of deionised water in an anaerobic Filmer jar filled with nitrogen and containing an oxygen absorbent. After 18 h dialysis, the enzyme was removed and assayed without cysteine. No increase in activity above the untreated enzyme could be demonstrated, although the dialysed enzyme could be reactivated by cysteine to an activity identical with that found after the initial cysteine activation.

This result indicates that there is a requirement for complexing reagent in the medium during enzyme action, and points to the possibility that the activation was concerned with a protective effect of cysteine against other components of the reaction mixture.

## DISCUSSION

In agreement with previous findings (see KORNBERG[30] for review), isocitrate lyase has again proved adaptive to growth on acetate. During growth on sucrose, which does not require net synthesis of dicarboxylic acids, no isocitrate lyase activity was detected.

The finding that EDTA is an efficient activator of isocitrate lyase assumes special significance because of the serious interference in assay by cysteine. Reactions of the type occurring between glyoxylic acid and cysteine are now well documented. The ease of reaction between cysteine and ketones at neutral pH and room temperature has been noted[31]. Glyoxylic acid and cysteine yield a substance which only reacts with phenylhydrazine at 100°, and forms a thiazolidine quite different from the normal hydrazone formed in the absence of cysteine[20], thus explaining incomplete recoveries of glyoxylate. The seriousness of the reaction between glyoxylic acid and cysteine has recently been related to the assay of isocitrate lyase[21].

The formation of a cysteine–glyoxylate complex may be considered to produce two results: (a) an apparent lowering of activity dependent on recovery of glyoxylate, which will be a function of the time of enzyme assay used, and the degree of hydrolysis in the analytical method (a satisfactory method for hydrolysis of the complex should obviate difficulties caused by its formation[21]); (b) a lowering of activity depending on the extent of removal of cysteine by glyoxylate. During the course of

enzymic reaction, a continuously decreasing cysteine concentration may be expected to make the determination of absolute activity difficult.

It is therefore inadvisable to use cysteine for isocitrate lyase activation. Glutathione is apparently a suitable substitute, but EDTA has the advantage of not being autoxidisable, and should therefore allow aerobic assay of isocitrate lyase.

The implicit assumption in cysteine and glutathione activation of isocitrate lyase has been a specific effect of their –SH group on the enzyme molecule. By contrast, EDTA does not possess such a group. It would therefore appear that explanations involving cysteine in reduction of enzymic –SH groups are improbable in comparison with one involving binding of heavy-metal ions from the enzyme. The non-additivity of the cysteine and EDTA effects is supporting evidence for the similarity of their action.

Stimulation by metal-chelating substances has been shown for a wide range of enzymes. DPNase (EC 3.8.2.1) is activated by 2,2'-dipyridyl[32], $K^+$-activated aldehyde dehydrogenase (EC 1.2.1.5) by thiols, histidine and EDTA[33], and *cis*-aconitic decarboxylase (EC 4.1.1.6) by cysteine, EDTA and 8-hydroxyquinoline[34]. Chelation of inhibitory metals has been suggested as the mechanism of activation by EDTA of muscle triosephosphate dehydrogenase (EC 1.2.1.12)[7], heart-muscle oxidase preparations[35] and phosphoglucomutase (EC 2.7.5.1)[36,37].

Purified yeast isocitrate lyase is inhibited by low concentrations of $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$, while substantial inhibition of isocitrate lyase occurred in $10^{-3}$ M *p*-chloromercuribenzoate, and this inhibition was 80% reversed by excess cysteine[3]. This points to the presence of an essential –SH group in the yeast enzyme, and it is therefore likely that activation of Azotobacter enzyme by cysteine and EDTA is the result of chelation of inhibitory heavy metals bound to it, perhaps to an –SH group. The possibility of other metal-binding sites remains; MILSTEIN[37] presents evidence that $Zn^{2+}$ inhibition of phosphoglucomutase is not due to the formation of a mercaptide and suggests alternative binding sites—amino, imidazolyl, carboxyl and phosphate.

The concentration of cysteine required to activate isocitrate lyase in Azotobacter extracts is condiderably greater than that of EDTA. Allowing for interaction of glyoxylic acid with cysteine, the concentration of the thiol required is at least 3 mM, while with EDTA, 0.16 mM produces almost maximum activity. If a metal ion is responsible for the inactive form of isocitrate lyase, it appears that cysteine is less efficient in binding this metal.

From the stability constants for complexes between various metal ions and either cysteine or EDTA[19], calculations were made of the ratio of free to bound metal for a range of ions, making allowance for formation of 1 : 2 chelates between some metals and cysteine. For 0.16 mM ligand (the EDTA concentration giving maximum activity, see Fig. 1) and a constant metal-ion level, the equilibrium concentration of free $Co^{2+}$, $Fe^{2+}$, $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ would be greater with cysteine than with EDTA, while the reverse would be true for $Fe^{3+}$ and $Hg^{2+}$. It is therefore unlikely that the latter ions are responsible for the inactive form of isocitrate lyase. Of the former, $Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$ and $Co^{2+}$ have been shown to activate isocitrate lyase[2,3,38], making it unlikely that they are concerned in its inactivation. Although a stability constant for the $Cd^{2+}$–cysteine complex is not available, it is

probably similar to that for the $Cd^{2+}$–glutathione complex, in which a lower proportion of metal is bound than in the $Cd^{2+}$–EDTA complex.

If these idealised chemical data are applicable to the system used for isocitrate lyase assay, then, of the cases available for comparison, the ions most likely to cause inactivation are $Zn^{2+}$, $Ni^{2+}$, $Pb^{2+}$ and $Cd^{2+}$. It is possible that $Cu^{2+}$ is also involved. Although departures from ideality are obvious (in terms of pH, temperature, ionic strength and other ions), the differences in ratios of free to bound metal for EDTA and cysteine are so large that some assessment of the relative advantages of these compounds is possible.

A variation in response can be expected during activation by different chelating substances, the activating efficiency of which will be a function of the type and quantities of the contaminants present, and not necessarily a constant property of the enzyme. It is therefore possible that the superiority of EDTA over cysteine will not always be observed, although in the majority of cases considered above, EDTA removal of metals should be more complete.

## ACKNOWLEDGEMENTS

Grateful acknowledgment is made to Dr. C. A. PARKER for his continuous encouragement and to Professor E. J. UNDERWOOD, Director of the Institute, for his support. Acknowledgement for financial assistance is made to the Commonwealth Scientific and Industrial Research Organization for studentships, and to the University of Western Australia for research grants.

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