Phytochemistry, 1980, Vol. 19, pp. 769–774. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0501-0769 $02.00/0

ISOLATION AND PROPERTIES OF ISOCITRATE LYASE FROM *LUPINUS* SEED

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(*Revised received* 30 *July* 1979)

Key Word Index—*Lupinus*; Leguminosae; isolation; isocitrate lyase; seed germination; glyoxylate; oxalate; glycolate; malonate; enzyme SH.

Abstract—Isocitrate lyase (threo-D$_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1) was purified from cotyledons of *Lupinus* seedlings. The final preparation showed two bands after polyacrylamide-gel electrophoresis. The optimum pH using phosphate, Tris or imidazole buffer was at pH 7.5; with triethanolamine (TRA) it was at pH 7. The enzyme required Mg^{2+} for maximal activity, and *N*-ethylmaleimide (NEM) inactivated the enzyme. Activity was increased by incubation with the reducing agents, glutathione (GSH), acetylcysteine (acetylcys), dithionite ($Na_2S_2O_4$), thioglycolate (TG) or 1,4-dithioerythritol (DTE). $Na_2S_2O_4$ and DTE were the most active among the tested substances and DTE prevented much of the inactivation by NEM. The apparent K_m value for isocitrate was *ca* 1 mM in phosphate buffer at pH 6.8 or 7.5 but was substantially lower (0.1–0.2 mM) using Tris, TRA or imidazole buffers. Glyoxylate, oxalate and malonate were competitive inhibitors of the enzyme. Synthase activity of the enzyme (i.e. formation of isocitrate from succinate and glyoxylate) was demonstrated. The K_m values for glyoxylate and succinate were 0.05 and 0.2 mM, respectively. The addition of glyoxylate to the culture medium in which *Lupinus* seeds germinate resulted in a reduced development of isocitrate lyase activity during germination.

INTRODUCTION

Isocitrate lyase is one of the two key enzymes involved in the glyoxylate cycle [1] and is of particular phylogenetic interest because of its distribution in nature. The purification of isocitrate lyase from different sources permits us to compare physico-chemical properties of the enzyme at the molecular level. The enzyme has been obtained in a homogeneous form only from bacteria [2, 3], algae [4], a nematode [5], *Neurospora* [6] and the higher plant *Linum usitatissimum* [7]. Other attempts at purification have been reported from *Ricinus communis* [8], yeast [9] and *Pinus pinea* [10]. In this paper we describe the purification and properties of isocitrate lyase from cotyledons of *Lupinus* cultivars.‡ Some preliminary data about the *in vivo* effect of glyoxylate on isocitrate lyase activity during the germination of *Lupinus* seeds are also reported.

RESULTS AND DISCUSSION

Purification

In the process of purification (Table 1) specific activity was increased 100-fold. During storage at −30°, activity of the purified enzyme fell as shown in Fig. 1. In the absence of the added DTE, activity was lost almost completely in 11 days but with 0.25 mM DTE present it was more stable. The UV absorption spectrum was determined after dialysis of the enzyme for 24 hr against water (*ca* 8 l.). The spectrum had a maximum at *ca* 280 nm and the absorbancy ratio, E_{280}/E_{260} nm, was 1.8; therefore our preparation was probably free of nucleic acid.

Electrophoretic behaviour

The purified enzyme preparation gave two bands and a small amount of contaminating protein.

Sephadex G-200 chromatography

The elution of isocitrate lyase activity from Sephadex G-200 indicated the presence of two activity peaks. Proteic contaminants were absent. Apparent MWs of the two components were *ca* 170 000 and 145 000.

Optimum pH and Mg^{2+} effect

Activity was measured at different pH values and with different buffers. Optimum pH for activity was 7.5 with 80 mM phosphate, 80 mM Tris and 25 mM imidazole; with 80 mM TRA, highest activity was at pH 7. Magnesium was necessary for the enzyme activity, highest activity occurring with 5 mM $MgCl_2$; this concentration was included in all assays.

Effect of reducing agents

The effect of some reducing agents (GSH, DTE, TG, acetylcysteine and $Na_2S_2O_4$) at various concentrations on the enzymatic activity was determined. The

‡ A preliminary report was presented at the 10th FEBS Meeting in Paris. See (1975) Vanni, P., Vincenzini, M. T. and Vincieri, F., abstr. 910. See also ref. [15].

Table 1. Isocitrate lyase purification

Fractions	Protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Volume (ml)
1. Crude extract	11700	21	100	540
2. Acetone fractionation	5600	21	46.0	390
3. 40% $(NH_4)_2SO_4$ precipitate	139	290	15.5	9
4. Sephadex G-150 conc. pool	3.2	2200	2.8	15
5. 60% $(NH_4)_2SO_4$ precipitate	1.9	2400	1.8	4
6. 60% $(NH_4)_2SO_4$ precipitate after dialysis	1.9	1830	1.4	4

Enzyme activity is expressed as nmol of glyoxylate formed/min. Reaction mixtures for enzyme assays always contained 8 mM DTE.

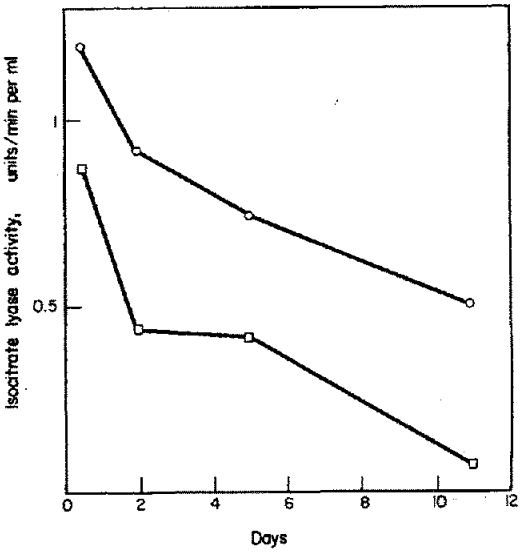

Fig. 1. Change of activity of purified isocitrate lyase with time of storage at −30°. Enzyme stored with 0.25 mM DTE (○—○), enzyme without DTE (□—□).

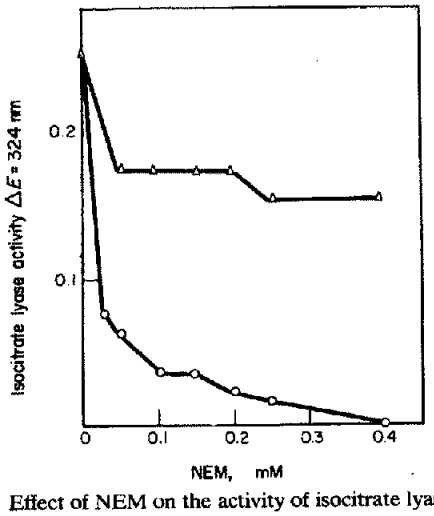

Fig. 2. Effect of NEM on the activity of isocitrate lyase in the absence (○—○) and presence of 8 mM DTE (△—△).

enzymatic activity reached a maximum with 1 mM acetylcysteine, 1 mM $Na_2S_2O_4$, 2 mM TG, 8 mM DTE. Dithionite and DTE were the most effective in increasing activity. Fig. 2 shows the effect of NEM on the activity of isocitrate lyase and the positive effect of 8 mM DTE. In the presence of 0.4 mM NEM enzyme activity was negligible but with 8 mM DTE added to the reaction mixtures, the inactivating effect of NEM was much less.

Michaelis constants

The apparent K_m values for isocitrate at different pH and with different buffers were determined. The K_m values were obtained by the Lineweaver and Woolf–Augustinsson–Hofstee plot [11]. With phosphate buffers of pH 6.8 and 7.5 the apparent K_m was always *ca* 1 mM. This was the highest value obtained. In TRA buffer at pH 7, the K_m was 0.11 mM and with Tris at pH 7.5 the value was *ca* 0.2 mM. With imidazole it was 0.12 mM.

Effect of some metabolites

Because isocitrate lyase is subject to feed-back inhibition by several metabolites, we studied the effect of glycolate, oxalate and malonate, 3 substances that are functional and structural analogues to glyoxylate, on the enzyme activity. All 3 metabolites competitively inhibited the isocitrate lyase activity; the inhibition was linearly competitive. The Hunter and Downs plot [12] (Fig. 3) shows the inhibition of isocitrate lyase activity at one concentration of all the 3 metabolites. The K_i values for glycolate, oxalate and malonate were 0.6, 0.012 and 3 mM, respectively, oxalate being the most effective competitive inhibitor.

Reversibility of the reaction

It is well known [13, 14] that isocitrate lyase from various sources can catalyse the condensation reaction of glyoxylate and succinate. We studied this with our *Lupinus* enzyme using different buffers at various pH values. Only with imidazole at pH 7 did we find a satisfactory agreement [15] between the activities of the condensation and cleavage reaction. The K_m for isocitrate, glyoxylate and succinate in 25 mM imidazole buffer was 0.12, 0.05 and 0.2 mM, respectively.

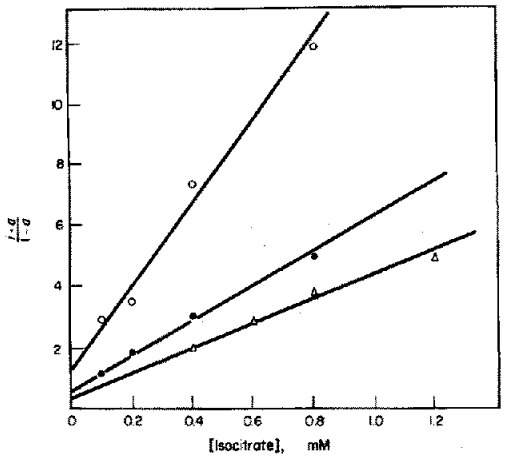

Fig. 3. Inhibition of purified *Lupinus* isocitrate lyase by 0.02 mM oxalate, 3mM glycolate and 25 mM malonate as a function of isocitrate concentration (Hunter and Downs method). O—O, Oxalate (ordinate values must be multiplied by 10^{-3}); △—△, malonate; (ordinate values must be multiplied by 10); ●—●, glycolate.

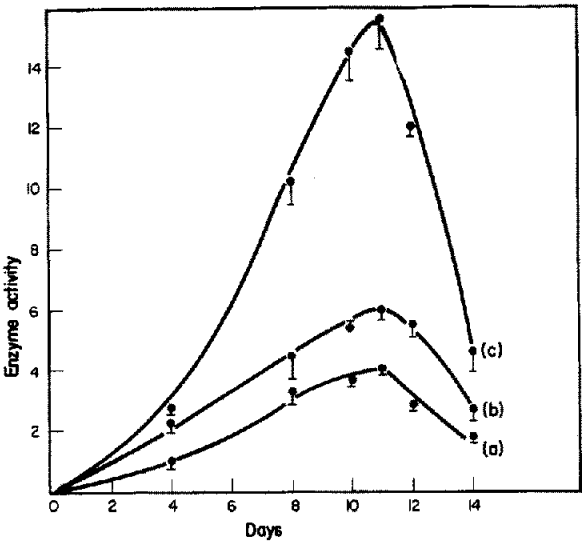

Fig. 4. Development of isocitrate lyase activity during germination. 60 or 30 mM glyoxylate was added to the culture medium from the commencement of germination (zero point). Enzyme activity is expressed as μmol of substrate transformed per min per mg of protein. The points in the figure are averages of three individual determinations ±S.E. a = 60 mM glyoxylate, b = 30 mM glyoxylate, c = controls.

In vivo *effect of glyoxylate*

In a previous paper [16] we reported the effect of glyoxylate added to the culture medium of germinating *Pinus pinea* seeds. We repeated the same experiment with *Lupinus* seeds: germination was followed over 15 days and the amounts of isocitrate lyase were measured. The results (Fig. 4) clearly show that added glyoxylate results in the development of less isocitrate lyase activity. The action of glyoxylate appears to be selective for isocitrate lyase. None of the other enzymes tested were significantly affected by the addition of glyoxylate (Table 2). It must be noted that 60 mM glyoxylate produces a moderate reduction in the speed of the seed germination.

The enzyme was extracted from cotyledons of germinating *Lupinus* seeds on the 11th day of germination. The specific activity of our enzyme was increased *ca* 100-fold; the recovery was very low, *ca* 2%, lower than the 16% of John and Syrett [4], the 11–14% of McFadden *et al.* [2, 7] and 9% of Reiss and Rothstein [5]. Polyacrylamide gel electrophoresis demonstrated the presence of two protein bands relative to the peak of the maximum activity from the Sephadex G-150 chromatography. On the other hand, chromatography on Sephadex G-200 showed that the collected fractions had two peaks of isocitrate lyase. We wish to emphasize that by chromatographic analysis on Sephadex G-150 we obtained 4 or 5 peaks of activity. Possibly *Lupinus* isocitrate lyase consists of a number of isoenzymes, as is reported for isocitrate lyase from *Turbatrix aceti* and *Linum*, and we have probably isolated only the two major components of this family of isoenzymes. Determination of the pH optimum demonstrated that the optimum was at pH 7.5 in

Table 2.

Enzymes	Days of germination 4	11	12	14
Isocitrate dehydrogenase (c)	85.5	35.8	34.6	29.0
Isocitrate dehydrogenase (c) +30 mM G	78.0	33.5	32.7	28.0
Isocitrate dehydrogenase (c) +60 mM G	81.0	40.0	39.4	30.0
Glucose-6-phosphate dehydrogenase (c)	13.6	16.5	28.0	27.0
Glucose-6-phosphate dehydrogenase (c) +30 mM G	12.0	18.0	26.5	26.0
Glucose-6-phosphate dehydrogenase (c) +60 mM G	8.2	20.0	27.0	20.0
6-Phosphogluconic dehydrogenase (c)	54.0	36.0	27.0	23.0
6-Phosphogluconic dehydrogenase (c) +30 mM G	49.0	24.0	22.0	19.0
6-Phosphogluconic dehydrogenase (c) +60 mM G	45.0	25.0	28.5	22.5
Malic dehydrogenase (c)	3.0	4.0	2.5	2.0
Malic dehydrogenase (c) +30 mM G	5.0	5.5	2.0	2.0
Malic dehydrogenase (c) +60 mM G	8.0	5.5	2.2	2.2

Activities of representative enzymes of some metabolic pathways in controls and in seeds germinated in the presence of 30 and 60 mM glyoxylate. The enzymatic activities are expressed as μmol of substrate transformed per min per mg of protein. (c) = controls, G = glyoxylate.

potassium phosphate, Tris and imidazole buffers as it was for castor bean enzyme [8] or *Linum* enzyme [7]. On the other hand, our optimum pH is quite similar to that of the *Chlorella, Linum, Pseudomonas indigofera* or *Nematode* enzyme [4, 5, 7]. With TRA, the optimum was at pH 7. A stimulatory effect of Mg^{2+} was also evident with *Lupinus* enzyme.

The *Lupinus* enzyme was very unstable (Fig. 1), and reducing agents were necessary to preserve tha activity. John and Syrett [4] showed that dithiothreitol (DTT) was more effective than GSH; for the *Lupinus* enzyme DTE and $Na_2S_2O_4$ were *ca* 4 times more active than GSH. 8 mM DTE protected the enzyme from NEM, a strong inactivator of isocitrate lyase activity.

The K_m values obtained for isocitrate lyase depend on the methods used and also on the buffers employed; a continuous spectrophotometric assay is preferable [17]. Apparent K_m values in potassium phosphate at pH 6.8 or 7.5 was always *ca* 1 mM in agreement with that (0.82 mM) of McFadden *et al.* for *Pseudomonas indigofera* enzyme [2], and with Olson's values for the yeast enzyme [9]. The K_m values in TRA at pH 7 and in Tris at pH 7.5 were very similar, between 0.1 and 0.2 mM and are in good agreement with the data presented by Ashworth and Kornberg for *Escherichia coli* ($K_m = 0.18$ mM), determined by the continuous spectrophotometric method [18]. These values are *ca* 10% of those obtained in phosphate buffer but are still 10 times higher than the values determined by John and Syrett for *Clorella* [4], by McFadden *et al.* for *Pseudomonas indigofera* [13] and by Syrett and John [17]. However these values are difficult to compare because of the different methods and conditions used (Table 3). However the K_m values obtained with higher plant enzymes (*Ricinus communis, Linum usitatissimum* and *Lupinus* cultivars) are in good agreement; they range from 0.1 to 0.3 mM. Our inhibition studies demonstrated that glycolate, oxalate and malonate are competitive inhibitors of isocitrate lyase. The K_i values of oxalate were very low, as reported by other authors. With our preparation, although the principal function of isocitrate lyase would appear to be that of splitting isocitrate, the reverse reaction was also catalysed; the K_m values for glyoxylate and succinate (0.05 and 0.2 mM) were in quite good agreement with the values of McFadden *et al.* (0.05 and 0.5 mM) for *Pseudomonas indigofera* enzyme. Finally, glyoxylate, a competitive inhibitor of the enzyme [14] (see also the effect of oxalate and glycolate in Fig. 3) caused a clear decrease of isocitrate lyase activity during the germination of *Lupinus* seeds as it did with *Pinus pinea* [16]: therefore some regulatory role of these C_2 compounds may be assumed.

EXPERIMENTAL

Chemicals. DL-Isocitrate was prepared by saponification with KOH according to ref. [8] from the lactone (allofree)

Table 3. Comparison of isocitrate lyase from various sources

Year	Species	Fold purification	Assay method	pH optimum	Temp.	Activating and stabilizing compounds	K_m (M) isocitrate	MW
1957 [27]	*Pseudomonas aeruginosa*	30	disc.	8–8.5 Tris	30	Mg^{2+} GSH Co^{2+} Mn^{2+} Fe^{2+} Cysteine	4.5×10^{-4} Tris	—
1959 [9]	Yeast	70	cont.	6.0 phosphate	25	Mg^{2+} GSH Co^{2+} Mn^{2+} Fe^{2+} Cysteine	1.2×10^{-3} phosphate	—
1959 [8]	*Ricinus communis*	18	disc. cont.	7.5 phosphate	30	Mg^{2+} GSH Mn^{2+} Co^{2+} Fe^{2+}	3.2×10^{-4} phosphate	—
1965 [2]	*Pseudomonas indigofera*	14	disc.	7.7 Tris	30	Mg^{2+} GSH Co^{2+} Mn^{2+} EDTA	8.2×10^{-4} Tris	222 000
1967 [4]	*Chlorella pyrenoidosa*	14	disc. cont. for K_m	7.5 TRA	30	MG^{2+} GSH EDTA DTT	2.3×10^{-5} TRA	170 000
1972 [10]	*Pinus pinea*	34	cont.	7.8 Tris	30	—	1.4×10^{-5} phosphate	—
1974 [5]	*Turbatrix aceti*	30	cont.	7.6 Tris	25	Mg^{2+} Cysteine	6.6×10^{-4} Tris	480 000
1975 [6]	Neurospora	80	disc.	6.8 Tris	30	Mg^{2+} DTT	3.3×10^{-3}	265 000
1977 [7]	*Linum usitatissimum*	111	disc.	7.5 Tris	30	Mg^{2+} Cysteine	2.8×10^{-4} Tris	264 000
1978 [3]	Thermophilic *Bacillus*	14	cont.	8.0 Tris imidazole	30	Mg^{2+} 2-mercapto-ethanol	2.0×10^{-5} imidazole	180 000
1975* 1979 [15]	*Lupinus* cultivars	101	cont.	7.5 Tris	30	Mg^{2+} GSH DTE	1.1×10^{-4} Tris	170 000 145 000

* See text for reference on preliminary report presented at 10th FEBS Meeting in Paris.

Sigma; glyoxylic acid and α-chymotrypsinogen-A (bovine pancreas) were from Sigma. Isocitrate dehydrogenase, β-fructosidase (yeast) and NADP were from Boehringer; thioglycollate from BDH and *N,N'*-methylenbisacrylamide from Serva. Sephadex G-150 and G-200 were from Pharmacia. All other products were obtained from Merck.

Germination of seeds. *Lupinus* seeds were germinated in darkness at room temp. in Petri dishes on paper dampened with H_2O. The seedlings did not form chlorophyll under these conditions. The experimental period was from September to May. Cotyledons were collected for enzyme extraction 11 days after sowing, when isocitrate lyase showed maximal activity [19]. For the expts on the effect of glyoxylate on the activity of isocitrate lyase during germination, seeds were germinated in the presence of glyoxylate (30 or 60 mM) with the pH medium constant at *ca* 7. After the desired period of germination the seedlings were collected and enzyme activities and protein contents determined [19].

Enzyme assays. Isocitrate lyase activity was assayed at 30° by a continuous optical method [20]. The reaction mixture, slightly modified [21], contained the following in a final vol. of 2.5 ml: 80 mM triethanolamine (TRA), pH 7; 5 mM $MgCl_2$; 8 mM DTE; 4 mM phenylhydrazine; 4 mM isocitrate. For the expts on the pH optimum we also used 80 mM Tris, 80 mM phosphate and 25 mM imidazole buffers. In the condensation reaction (reverse reaction) the amount of isocitrate formed was measured spectrophotometrically by observing the continuous reduction of NADP added to the reaction mixture with an excess of isocitric dehydrogenase (IDH); the reaction mixture at 30° contained, in a final vol. of 2.5 ml, 25 mM imidazole, pH 7; 5 mM $MgCl_2$; 0.5 mM NADP; 0.16 U/ml IDH; 0.8 mM succinate and 0.8 mM glyoxylate. The reaction was usually initiated by the addition of glyoxylate or succinate. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconic dehydrogenase (EC 1.1.1.44), malic dehydrogenase (EC 1.1.1.37) and isocitrate dehydrogenase (EC 1.1.1.42) were determined by continuous optical assay based on the *A* change of pyridine nucleotide coenzymes at 366 nm according to ref. [22].

The protein content of the individual fraction was determined by a modified Biuret method [23, 24]. Proteins in the column fractions and in the final step of purification were determined by measuring E_{260} and E_{280}. From these readings protein content was calculated using the formula of ref. [25].

Preparation of Sephadex columns. For the purification procedure we used TEM buffers, pH 7.7, containing 100 mM Tris, 2 mM EDTA, 1 mM KCl, 10 mM 2-mercaptoethanol. For determining MW by column chromatography, we used 0.05 M Tris buffer, pH 7.7, containing 100 mM KCl, 2 mM EDTA, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Sephadex G-150 and G-200 were prepared according to the manufacturer's instructions. Sephadex G-150 was suspended in TEM buffer before being packed into a 110 × 3 cm column. Sephadex G-200 was packed into a 50 × 2.5 cm column. Each column was run with a constant pressure head of eluting buffers. The columns were eluted for 24 hr with buffers before application of the protein samples. Fractions (*ca* 3.6 ml from Sephadex G-150, *ca* 2.2 ml from Sephadex G-200) were collected every 10 min. Sephadex G-200 column was calibrated with 3 proteins of known MW (β-fructosidase, ovalbumin and α-chimotripsinogen-A); the mixture contained blue dextran.

Electrophoresis. Enzyme samples (40–80 μg of protein) containing glycerol were electrophoresed on 7.4% acrylamide gels. The gels were either stained for protein with Coomassie blue and the bands located by scanning at 620 nm (after destaining with a mixture of HOAc, MeOH, H_2O (1:5:4)) or gels were scanned directly at 280 nm using a Gilford spectrophotometer. Polyacrylamide gel disc electrophoresis was conducted for 3–4 hr using a constant current of 4 mA/tube in 5 mM Tris–glycine buffer, pH 8.9.

Purification of isocitrate lyase. Step 1: Cotyledons (1 cotyledon = *ca* 0.7 g) were homogenized with medium (1.5 × wt of cotyledons) containing 0.1 M TEM, pH 7.7, and Tween 80 (1% v/v) [26] in a Ultra Turrax apparatus for 1 min. The homogenate was filtered through cheesecloth and centrifuged at 10 000 g for 30 min. The insoluble fraction was discarded and a dicalite filter-aid was added to the fluid supernatant (10% w/v). The soln was filtered through a Buchner funnel and the filtrate centrifuged at 10 000 g for 30 min. The supernatant thus obtained was the crude extract and called the first fraction. *Step* 2. Me_2CO (4 vols.) precooled to −20° was added to the first fraction (1 vol.). The ppt. was collected and subsequently redissolved in 0.1 M TEM buffer in a vol. equal to the vol. of crude extract used. This soln was dialysed with continuous stirring for 12 hr against 0.1 M TEM buffer. The dialysate was centrifuged at 10 000 g for 1 hr; the supernatant from this step constituted the second fraction. *Step* 3. Solid $(NH_4)_2SO_4$ was stirred into the second fraction to give a final concn of 40% satn. The soln thus obtained was left for 15 min at 0°. The ppt. was collected by centrifugation (10 000 g for 0.5 hr) and redissolved in *ca* 10–15 ml TEM buffer. This fraction was called the third fraction. *Step* 4. The third fraction was applied to column of Sephadex G-150 and the proteins were eluted with 0.1 M TEM buffer, pH 7.7. Fractions (3.6 ml) were collected and assayed for isocitrate lyase activity; their E_{260} and E_{280} values were also determined; the peak of enzyme activity occurred in fractions 121–131. These fractions were pooled and 1 mM EDTA, 5 mM $MgCl_2$ and 0.25 mM DTE added to them. This pool represented the fourth fraction. *Step* 5. Solid $(NH_4)_2SO_4$ was added to the fourth fraction to give 60% satn. After standing for 15 min at 0° the ppt. was collected by centrifugation at 40 000 g for 30 min and redissolved in 3–5 ml TEM buffer. This constituted the fifty fraction. *Step* 6. Residual $(NH_4)_2SO_4$ was removed by dialysis for 30 min against 1 l. TEM buffer. DTE was added to the dialysed soln to a final concn of 0.25 mM. This soln represented the sixth fraction and contained the purified enzyme; it was stored at −30°.

Acknowledgements—This research was supported by grants from Italian C.N.R. and M.P.I. Thanks are due to Professor Vincenzo Baccari for fruitful discussion and critical reading of the manuscript. The collaboration of Mr. G. Pinzauti, Mr. G. Giovannini and Miss A. Degli Innocenti is gratefully acknowledged. We are also grateful to the Gruppo di Ricerca Cap. XI/b, Sostanze Naturali e di Sintesi di Interesse Biochimico University of Florence for skilful technical assistance. Finally we must thank Professor Syrett for his helpful and interesting criticisms of the manuscript.

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