The Purification and Properties of Isocitrate Lyase from *Chlorella*

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1. Isocitrate lyase (*threo*-D₅-isocitrate glyoxylate-lyase, EC 4.1.3.1) has been purified from acetate-adapted cells of *Chlorella pyrenoidosa*. 2. The final preparation was homogeneous by the criteria of sedimentation, diffusion and polyacrylamide-gel electrophoresis. 3. The sedimentation coefficient ($S_{20,w}$) was 9.04×10^{-13} sec. and the diffusion coefficient ($D_{20,w}$) 4.62×10^{-7} cm.²/sec.; from these values the molecular weight of the enzyme was calculated to be 170 000 and its Stokes radius to be 4.63×10^{-7} cm. 4. The elution of the enzyme from Sephadex G-100 was studied and estimates of molecular weight and Stokes radius were obtained from the elution data. 5. The turnover number of the enzyme was 5950 moles of glyoxylate formed/min./mole of enzyme at 30°. 6. With *threo*-D₅(+)-isocitrate as substrate, the K_m of the enzyme was 0.023 mM.

In common with many other micro-organisms, several strains of the green alga *Chlorella*, when forced to use acetate or a precursor of acetate as their sole carbon source for growth, contain the enzyme isocitrate lyase (Syrett, Merrett & Bocks, 1963; Harrop & Kornberg, 1963, 1966). In *Chlorella pyrenoidosa*, after adaptation to growth on acetate, relatively large amounts of this enzyme are formed and the enzyme protein amounts to as much as 10% of the total soluble protein or almost 1% of the dry weight of the cell (John & Syrett, 1965). During our work on the formation and disappearance of isocitrate lyase in *C. pyrenoidosa* it became desirable to purify the enzyme as much as possible. The starting point for the purification was the method described by Shiio, Shiio & McFadden (1965) for isocitrate lyase from *Pseudomonas indigofera*. However, it soon became clear that this method would have to be considerably modified before it would be successful with the *C. pyrenoidosa* enzyme. After some preliminary work we obtained a purified enzyme that showed only a single protein component on ultracentrifugation or polyacrylamide-gel electrophoresis. The preparation and some properties of this enzyme are described below.

MATERIALS AND METHODS

Chemicals. L₄(+)-Isocitric acid (i.e. *threo*-D₅(+)-isocitric acid; Vickery, 1962), as the monopotassium salt, was obtained from Sigma (London) Chemical Co. Ltd., London, S.W. 6; Sephadex G-25, G-100 and G-200 and blue dextran were from Pharmacia, Uppsala, Sweden; DEAE-cellulose (Whatman DE11) was from H. Reeve Angel and Co. Ltd., London, E.C. 4; dithiothreitol (Cleland, 1964) was from Calbiochem Ltd., London W. 1; crystalline bovine

serum albumin and crystalline bovine γ -globulin were from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Growth of alga. *C. pyrenoidosa* (Cambridge Culture Collection strain 211/8p) was grown in a medium containing (per l. of glass-distilled water) 0.75 g. of NH₄NO₃, 0.4 g. of MgSO₄·7H₂O, 10 mg. of ferric iron as ferric EDTA complex, 1 ml. of Arnon's (1938) A5 trace-element solution, 5.80 g. of K₂HPO₄ and 4.53 g. of KH₂PO₄. The phosphates were autoclaved separately and added to a sterile solution of the remaining salts when cold. The pH of the medium after mixing was 6.7. The cultures were illuminated with daylight-fluorescent tubes giving a light-intensity of 28 000 ergs/cm.²/sec. at the surface of the cultures, and aerated vigorously with air containing 0.5% of CO₂. After 5 days' growth, when the cell density had reached about 1 mg. dry wt./ml., the cultures were adapted to acetate by the addition of sterile sodium acetate solution, to give a final concentration of 0.2%, and aeration for 24 hr. in darkness. Sufficient acetate to give another 0.1% was then added and aeration in darkness continued for a further 12–14 hr., after which the cells were harvested. For the final purification of the enzyme four 5 l. cultures were grown and adapted in this way. The cells were harvested by centrifugation, washed, suspended in 300 ml. of cold 0.1 M-phosphate buffer, pH 6.0, and broken by passage through a French pressure cell at 4°. The suspension emerging from the pressure cell was diluted with additional buffer and centrifuged at 100 000 g for 1½ hr. at 4°. The supernatant was then treated as described below, all steps being carried out in a cold-room at 4°.

Enzyme assay. Isocitrate lyase activity was determined by measuring the quantity of glyoxylate formed during 10 min. incubation at 30°. The incubation mixture contained the following in a final volume of 1 ml.: 100 μ moles of triethanolamine hydrochloride-NaOH buffer, pH 7.6; 5 μ moles of MgCl₂; 10 μ moles of GSH (adjusted to pH 7.6). Sodium isocitrate (20 μ moles) was added to start the reaction, and the reaction was stopped by the addition of 0.2 ml. of 20% (w/v) trichloroacetic acid. DNP-hydrazine

(0.3 ml. of a 0.1% solution in 2N-HCl) was added, followed 30 min. later by 2.5 ml. of 4.0% (w/v) NaOH; after centrifugation, the resultant E_{445} due to glyoxylate DNP-hydrazone was determined. Glyoxylate standards were run simultaneously.

For the determination of the K_m of isocitrate lyase a continuous spectrophotometric assay was used (Kornberg, 1965); the substrate was $\text{I}_4(+)$ -isocitrate.

The assay for ribose 5-phosphate carboxylase activity was based on that of Peterkofsky & Racker (1961) for ribulose diphosphate carboxylase. The assay mixture contained, in 1 ml.: 100 μ moles of tris-HCl buffer, pH 7.8; 4 μ moles of GSH; 0.8 mg. of bovine plasma albumin; 8 μ moles of MgCl_2 ; 20 μ moles of KHCO_3 ; 3 μ C of $\text{Na}_2^{14}\text{CO}_3$; 10 μ moles of ribose 5-phosphate; 10 μ moles of ATP. After incubation with cell extract for 10 min. at 30°, the reaction was stopped by the addition of 1 ml. of 50% (v/v) acetic acid in ethanol. After standing overnight at 4°, the tubes were centrifuged to remove the precipitate that had formed and 0.05 ml. portions of the supernatant were evaporated to dryness and counted for the estimation of non-volatile ^{14}C .

Protein determination. Protein in column fractions was determined approximately by measuring E_{260} and E_{280} . From these readings protein content was calculated after a correction for absorption due to nucleic acids (Layne, 1957). Protein was also determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin standards.

Buffer. Most of the purification was carried out with the proteins dissolved in TEM buffer, which contained 1 mM-EDTA and 1 mM-mercaptoethanol (final concns.) in 0.014 M-tris-HCl buffer, pH 7.7.

Preparation of DEAE-cellulose and Sephadex columns. DEAE-cellulose was pretreated according to the instructions of the manufacturer. It was equilibrated overnight in 0.1 M-tris-HCl buffer, pH 7.7, and then suspended in TEM buffer before being packed into a 230 mm. $\times$ 13 mm. column. Proteins were eluted with a solution of NaCl in TEM buffer, the chloride concentration being increased from 0.04 M to 0.20 M during the elution. Fractions (11 ml.) were collected by drop-counting.

Sephadex G-100 and G-200 were prepared according to the manufacturer's instructions. Dry powder (12 g.) was stirred for 7 days with 500 ml. of TEM buffer in a stoppered flask. This quantity formed a 430 mm. $\times$ 25 mm. column when Sephadex G-100 was used, or a 340 mm. $\times$ 41 mm. column when Sephadex G-200 was used. The column outlet was plugged with acid-washed glass wool, and enough Sephadex G-25 to give a layer 1 cm. deep was placed in the column as a support for the Sephadex G-100 or G-200. Each column was run with a constant pressure head of the eluting TEM buffer; the Sephadex G-200 column was run with reversed flow to avoid packing the gel. The column was eluted for 24 hr. with TEM buffer before application of the protein samples. Elution of the proteins took place overnight. The flow rates remained constant and, with Sephadex G-100 columns, fractions (about 3.5 ml.) were collected every 30 min. With Sephadex G-200, 10 ml. fractions were collected every 20 min.

Dialysis. Dialysis was carried out in Visking tubing, previously boiled twice in 10 mM-disodium EDTA, pH 7.0, and once in distilled water. Glassware was cleaned in a mixture of conc. HNO_3 and conc. H_2SO_4 ; chromic acid was not used. For the removal of $(\text{NH}_4)_2\text{SO}_4$ from enzyme

preparations, 10 ml. samples were dialysed overnight at 4° against TEM buffer that completely filled a 2 l. flask; the flask was closed by a piece of polythene sheet and the buffer stirred magnetically.

Electrophoresis. Polyacrylamide-gel disk electrophoresis was carried out by the methods of Ornstein & Davis (1961) and Racusen & Calvanico (1964).

Ultracentrifugation. Examination of the purified enzyme by ultracentrifugation and calculation of its sedimentation coefficient and diffusion were kindly done by Dr M. S. Rosemeyer, Department of Biochemistry, University College London. The purified enzyme used had been stored at -40° for 1 month. It was diluted to 2 mg. of protein/ml. by the addition of TEM buffer and sufficient 1.5 M-KCl to give a final concentration of 0.15 M-KCl. Sedimentation was carried out at 59780 rev./min. at 10° in a Spinco model E centrifuge fitted with an RTIC unit. The diffusion coefficient was determined at 12590 rev./min. by using a synthetic-boundary cell; the duration of the determination was 66 min. The area of the peak remained constant during this time, indicating the homogeneity of the protein sample. $D_{20,w}$ was calculated by relating the area:height of peak ratio to time (Schachman, 1957).

RESULTS

Purification of isocitrate lyase

Ammonium sulphate precipitation. The bulk of the isocitrate lyase activity precipitated at between 45% and 54% saturation with ammonium sulphate. When cells were broken in tris or phosphate buffer at pH 7.6 and ammonium sulphate precipitation was carried out at this pH, the precipitated proteins contained a major fraction that was not separated from isocitrate lyase during subsequent elutions from Sephadex or DEAE-cellulose columns. This contaminating protein was a ribose 5-phosphate carboxylase enzyme complex, i.e. it contained enzymes catalysing the conversion of ribose 5-phosphate into ribulose diphosphate, as well as ribulose diphosphate carboxylase. In this it resembled purified fraction I protein from green leaves (van Noort & Wildman, 1964). By carrying out the ammonium sulphate precipitation at pH 6.0 much better separation of the isocitrate lyase and ribose 5-phosphate carboxylase activities was achieved (Table 1).

Use of Sephadex columns. Columns of both Sephadex G-100 and Sephadex G-200 were used in preliminary experiments. When low-molarity TEM buffers were used to elute proteins, the elution peak of isocitrate lyase from Sephadex G-200 was very diffuse. Consequently Sephadex G-100 was used for the preparative work although it is in theory less suitable for molecules as large as those of isocitrate lyase.

Outline of final purification procedure. All stages were carried out at 4°. Cells from 20 l. of acetate-adapted cultures were suspended in 300 ml. of ice-cold 0.1 M-phosphate buffer, pH 6.0, and broken

Table 1. Separation of ribose 5-phosphate carboxylase and isocitrate lyase enzyme activities by ammonium sulphate fractionation

Acetate-adapted cells were suspended in 0.1 M-potassium phosphate buffers, pH 6.0 or 7.0, or in 0.1 M-tris-HCl buffer, pH 8.5. After the cells had been broken in a French pressure cell, the suspensions were centrifuged at 100000g for 60 min. at 4°. The supernatants were adjusted to 8mg. of protein/ml. and (NH₄)₂SO₄ fractions taken between the percentage saturations shown, by adding solid (NH₄)₂SO₄. The precipitated protein was dissolved in the same volume of 0.1 M-triethanolamine hydrochloride-NaOH buffer, pH 7.6, as the original extract, and assayed for enzyme activities. The activities are expressed as a percentage of the enzyme activities/unit vol. of the original extract, i.e. they show the percentage of the total enzyme in the original extracts recovered in each fraction.

pH	(NH ₄) ₂ SO ₄ fraction (% saturation)	Enzyme	Recovery of activity (%)		
			40-45	45-54	54-61
6.0	Ribose 5-phosphate carb-oxylase	Isocitrate lyase	13	0	0
		Isocitrate lyase	43	40	1
7.0	Ribose 5-phosphate carb-oxylase	Isocitrate lyase	47	2	0
		Isocitrate lyase	20	48	34
8.0	Ribose 5-phosphate carb-oxylase	Isocitrate lyase	3	41	48
		Isocitrate lyase	21	50	7

Fig. 1. Elution of isocitrate lyase activity from Sephadex G-100 during the preparation of purified enzyme. Elution was with 0.014 M-TEM buffer, pH 7.7. ----, Nucleic acid determined from E_{260} ; ○, total protein; ●, isocitrate lyase activity. The fractions marked P were pooled for further purification. For details and for definition of TEM buffer, see the text.

in a French pressure cell. After centrifugation at 100000g for 1½ hr., the supernatant was decanted and sufficient solid ammonium sulphate added, with stirring, to give 45% saturation (26.35g./

Fig. 2. Elution of isocitrate lyase activity from DEAE-cellulose during the preparation of purified enzyme. Elution was with NaCl dissolved in 0.014 M-TEM buffer, pH 7.7. ----, Nucleic acid determined from E_{260} ; ○, total protein; ●, isocitrate lyase activity; —, NaCl concentration during the elution. For details and for definition of TEM buffer, see the text.

100ml. of supernatant). After the solution had stood for 30 min. the precipitate was removed by centrifugation and discarded. To the supernatant, now 1.07 times its initial volume, was added enough ammonium sulphate to give 52% saturation (4.19g./100ml. of supernatant). After the solution had stood for 30 min. the precipitate was spun down and dissolved in 5 ml. of TEM buffer. This solution was applied to a column (430mm. × 25mm.) of Sephadex G-100 and the proteins were eluted overnight with TEM buffer. Fractions (3.5 ml.) were collected and assayed for isocitrate lyase activity; their E_{260} and E_{280} values were also determined (Fig. 1). The fractions marked P in Fig. 1 were pooled and applied to a column (230mm. × 13mm.) of DEAE-cellulose. This column was washed with 200 ml. of TEM buffer, and protein was eluted by a linear sodium chloride gradient increasing from 0.04 M to 0.2 M in 800 ml. of TEM buffer. The column was run overnight and 11 ml. fractions were collected and assayed. Fig. 2 shows the good correspondence between enzyme activity and protein content of the later fractions; nucleic acids were removed in the early fractions. To the pooled fractions with high enzyme activity (162 ml.) were added 0.1 vol. of 1.0 M-tris-hydrochloric acid buffer, pH 7.5, and solid ammonium sulphate to give 60% saturation (36.9g./100ml.). After the solution had stood for 45 min. in an ice bath the precipitate was obtained by centrifugation at 10000g for 15 min. It was dissolved in 10 ml. of TEM buffer, and residual ammonium sulphate was removed by dialysis overnight against TEM buffer. The purified enzyme was stored at -40° after the addition of dithiothreitol to a final concentration of 0.25 mM.

Table 2. *Progress of isocitrate lyase purification*Enzyme activity is expressed as μ moles of glyoxylate formed/min.

Fraction	Volume (ml.)	Protein		Enzyme activity			Recovery of activity (%)
		(total mg.)	(mg./ml.)	(units/ml.)	(total units)	(units/mg. of protein)	
100 000g soluble supernatant	203	2190	10.8	27.2	5520	2.52	100
(NH ₄) ₂ SO ₄ ppt. at pH 6.0	6.6	202	30.6	345	2270	11.3	41
Pooled eluates from Sephadex G-100	13.5	95	7.0	125	1690	17.8	31
Pooled eluates from DEAE-cellulose	162	36	0.22	6.4	1040	28.7	19
(NH ₄) ₂ SO ₄ ppt. at pH 7.5	10	25.8	2.58	91	908	35.0	16
After dialysis	12.1	24.8	2.05	72	870	35.0	16

Fig. 3. Sedimentation peak obtained with the purified enzyme after centrifugation at 59780 rev./min. in the Spinco model E centrifuge for 48 min.

The progress of the purification is shown in Table 2.

Physical properties of purified isocitrate lyase

Sedimentation and diffusion coefficients. Sedimentation in an ultracentrifuge showed only a single protein peak with a $S_{20,w}$ value of 9.04×10^{-13} sec. (Fig. 3). The diffusion coefficient, $D_{20,w}$, was 4.62×10^{-7} cm.²/sec.

Molecular weight, Stokes radius and frictional ratio. From these $S_{20,w}$ and $D_{20,w}$ values, assuming a partial specific volume of 0.725 ml./g. (Martin & Ames, 1961), the molecular weight of the protein was calculated to be 170 000 (Schachman, 1957). The Stokes radius was calculated to be 4.63×10^{-7} cm. The Stokes radius of a spherical protein

Fig. 4. Elution, from a column of Sephadex G-100 (430 mm. $\times$ 25 mm.), of a mixture containing blue dextran (3 mg.), bovine γ -globulin (6 mg.), bovine serum albumin (6 mg.) and purified isocitrate lyase. Elution was with 0.014 M-TEM buffer, pH 7.7. ----, Blue dextran determined from E_{630} ; $\circ$, standard protein determined from E_{280} ; $\bullet$, isocitrate lyase activity ($V_e - V_0 = 10.0$ ml.). The first protein peak ($V_e - V_0 = 1.1$ ml.) is due to γ -globulin, the second peak ($V_e - V_0 = 18.0$ ml.) to serum albumin. The volumes of the peak maxima were determined by the method of Porath (1963). $V_1 - V_0 = 139$ ml., $V_2 - V_0 = 146$ ml. In estimating the concentration of standard proteins from E_{280} , corrections were made for the amounts of isocitrate lyase and blue dextran present. For details and for definition of TEM buffer, see the text; for definition of the symbols V_0 , V_e , V_1 and V_2 , see Gelotte (1960).

molecule with this molecular weight is 3.7×10^{-7} cm. The calculations therefore indicate that the isocitrate lyase molecule is not spherical but somewhat elongated, with a frictional ratio, f/f_0 , of 1.25.

Behaviour during Sephadex-gel filtration. The filtration behaviour of purified isocitrate lyase was compared with that of two proteins of known molecular weight and Stokes radius, bovine serum albumin (mol.wt. 65 000, Stokes radius 3.5×10^{-7} cm.; Creeth, 1952) and bovine γ -globulin (mol.wt. 175 000, Stokes radius 5.3×10^{-7} cm.;

Fig. 5. Polyacrylamide-gel electrophoresis of purified isocitrate lyase: (a) by the Ornstein & Davis (1961) procedure; (b) by the Racusen & Calvanico (1964) procedure. Proteins were stained with Naphthalene Black. There is a secondary band above the major protein band in (b).

Deutsch & Hess, 1948). The mixture also contained a high-molecular-weight blue dextran that was completely excluded by the gel particles. Fig. 4 shows the elution pattern.

Electrophoretic behaviour. The purified enzyme preparation gave only a single band with R_f 0.35 when run on 7.5% polyacrylamide gels prepared by the standard Ornstein & Davis (1961) method. However, when an ammonia-glycine buffer as used by Racusen & Calvanico (1964) was substituted for the tris-glycine buffer used by Ornstein & Davis (1961), the stained protein band on the gels was distinctly split, and showed a subsidiary band above the main one (Fig. 5). Attempts to produce double-banding in the Ornstein & Davis (1961) method by first preincubating the enzyme with 20 mM-GSH, 10 mM-GSSG or 5 mM-dithiothreitol were unsuccessful.

Absorption spectrum. The absorption spectrum of the purified enzyme had a peak at 280 m μ . The E_{280}/E_{260} ratio was 2.05, which indicated the absence of nucleic acid contamination.

Kinetic properties of purified isocitrate lyase

Effect of pH. This is shown in Fig. 6. In 0.1 M-phosphate buffers the optimum pH was 7.25, but in 0.1 M-triethanolamine hydrochloride-sodium hydroxide buffers the optimum pH was 7.6.

Fig. 6. Effect of pH on the activity of purified isocitrate lyase. $\circ$, 0.1 M-Potassium phosphate buffers; $\bullet$, 0.1 M-triethanolamine hydrochloride-NaOH buffers. The assay was carried out as described in the Materials and Methods section; the assay mixture contained 5 mM-Mg $^{2+}$.

Table 3. Effect of Mg $^{2+}$ concentration on enzyme activity

The assay system used was as described in the Materials and Methods section with triethanolamine hydrochloride-NaOH buffer, pH 7.6.

Final concn. of added Mg $^{2+}$ (mM)	Enzyme activity (% of maximum)
0.0	0
0.1	53
1.0	94
5.0	100
10.0	78

Effect of Mg $^{2+}$. Enzyme activity was depressed at Mg $^{2+}$ concentrations below 1 mM or above 5 mM (Table 3). The effect of pH was investigated at 5 mM-Mg $^{2+}$, and this concentration was used for subsequent assays.

Effect of GSH and dithiothreitol. The presence of 10 mM-GSH was required for maximum activity (Fig. 7). At GSH concentrations below 4 mM the addition of 1 mM-EDTA increased enzyme activity, but at higher GSH concentrations the addition of EDTA had no effect. Dithiothreitol (Cleland, 1964) was more effective than GSH, almost maximum activity being reached at a concentration of 2 mM-dithiothreitol in the absence of EDTA, and at 1 mM in its presence (Fig. 7).

Michaelis constant and molecular activity. Isocitrate lyase cleaves only one isomer of isocitric acid (Smith & Gunsalus, 1957). This isomer used to be termed L $_s$ (+)-isocitric acid, but following the suggestion of Vickery (1962) it is now called threo-D $_s$ (+)-isocitric acid. The relationship between enzyme activity and concentration of this substrate is shown in Fig. 8 as a Lineweaver-Burk plot. From this, the K_m is 0.023 mM. At maximum enzyme

Fig. 7. Effect of GSH or dithiothreitol on the activity of purified isocitrate lyase (stored for 4 days at 4°). ●, With GSH; ○, with GSH and 1 mM-EDTA; ▲, with dithiothreitol; △, with dithiothreitol and 1 mM-EDTA.

Fig. 8. Lineweaver-Burk plot showing the effect of $L_x(+)$ -isocitrate concentration on the activity of isocitrate lyase. The assays were carried out at pH 6.8 by a continuous spectrophotometric method (Kornberg, 1965). The initial rate, v , is expressed as μ moles of glyoxylate produced/min.

activity, 1 μ g. of purified enzyme catalysed the formation of 35 μ moles of glyoxylate/min. at 30° (Table 2). This corresponds to a molecular activity of 5950 mol. of substrate attacked/min./mol. of enzyme.

DISCUSSION

Our procedure for the purification of *C. pyrenoidosa* isocitrate lyase differs from that of Shiio *et al.* (1965) for a *Pseudomonas* enzyme in that we carried out ammonium sulphate precipitation at pH 6.0 and removed nucleic acids by elution of the enzyme from a column of Sephadex G-100 instead of by protamine sulphate precipitation. The enzyme protein finally obtained appeared to be homogeneous from sedimentation and diffusion analysis. It gave only a single band on polyacrylamide-gel electrophoresis with tris-glycine buffers. A secondary band was, however, observed

when electrophoresis was carried out in an ammonia-glycine buffer. This could be a contaminating protein but was more probably a secondary band formed from a homogeneous protein during electrophoresis (Cann, 1966; McKenzie & Sawyer, 1966).

Our final enzyme preparation was obtained after only 14-fold purification; if this was pure isocitrate lyase it follows that this enzyme must have been 7% of the total protein in the 100 000 g supernatant. It is remarkable that this value agrees exactly with the amount of isocitrate lyase protein in the high-speed supernatant fraction from acetate-grown cells of *P. indigofera* (Shiio *et al.* 1965), in spite of the fact that in *Chlorella*, but not in *Pseudomonas*, the ribose 5-phosphate carboxylase complex contributes much to the total soluble protein.

Calculations from the sedimentation and diffusion data give a molecular weight of 170 000, a Stokes radius of 4.63×10^{-7} cm. and a frictional ratio of 1.25 for purified *C. pyrenoidosa* isocitrate lyase.

Andrews (1964, 1965) has attempted to relate the order of elution of proteins from Sephadex columns to their molecular weights, whereas others (Porath, 1963; Laurent & Killander, 1964; Ackers, 1964; Siegel & Monty, 1966) have related the order of elution to the Stokes radii of the protein molecules. Fig. 4 shows the elution of purified isocitrate lyase, bovine serum albumin and bovine γ -globulin from a column of Sephadex G-100. From the data in this Figure, K_d values (Gelotte, 1960) can be calculated. They are 0.0079 for γ -globulin, 0.072 for isocitrate lyase and 0.130 for serum albumin. The K_{av} values used by Laurent & Killander (1964) differ slightly from the K_d values. They are 0.0075 for γ -globulin, 0.069 for isocitrate lyase and 0.124 for serum albumin. Clearly, isocitrate lyase was eluted much later than γ -globulin, yet the estimate of the molecular weight of isocitrate lyase from the sedimentation data is 170 000, almost identical with that of γ -globulin. Andrews (1964) showed that several globular proteins obeyed the relationship established by Porath (1963) for the gel-filtration behaviour of dextrans, namely that $(K_d)^{0.33} \propto (\text{mol. wt.})^{0.5}$. Application of this relationship, with the K_d values given above, gives a molecular weight for isocitrate lyase of about 90 000. However, as Andrews (1965) points out, considerable deviations occur when the protein molecules are not globular. Siegel & Monty (1966) have very clearly shown that the order of elution from Sephadex columns is a function of the Stokes radius of the protein molecules rather than of molecular weight. Similar proposals have been made by Porath (1963), Laurent & Killander (1964) and Ackers (1964), who all give equations by which the Stokes radius of an unknown molecule can be calculated from its K_d or K_{av} value when it is eluted from a Sephadex

column together with known standard proteins. Although all three equations are different, Siegel & Monty (1966) found that all fitted their data reasonably well. When these equations are applied to the K_d values calculated from Fig. 4, all three equations give a Stokes radius for isocitrate lyase of between 4.0×10^{-7} and 4.2×10^{-7} cm.

Our results therefore confirm that the elution of isocitrate lyase from a column of Sephadex G-100 is determined by the Stokes radius of its molecules rather than by its molecular weight. The Stokes radius estimated from the elution data is somewhat lower than that calculated from the diffusion coefficient. It is likely that the estimate from Sephadex G-100 gel filtration is too low, for calculations based on the value of K_d (0.26) obtained by elution from Sephadex G-200 columns, on which standard proteins were not, however, run simultaneously, suggest a value of about 4.8×10^{-7} cm. for the Stokes radius.

These parameters can be compared with those of the purified isocitrate lyase of *P. indigofera* (Shiio *et al.* 1965) and those of the isocitrate lyase partially purified by Harrop & Kornberg (1966) from a strain of *Chlorella vulgaris*. The molecular weight of the *P. indigofera* enzyme is 222000 and its Stokes radius 5.55×10^{-7} cm. Its frictional ratio was calculated to be 1.39. Thus it is larger and somewhat more asymmetric than the molecule of the *C. pyrenoidosa* enzyme. The turnover number of the *Pseudomonas* enzyme is about 7500 moles of glyoxylate formed/min./mole of enzyme at 30° at pH 7.7 compared with a value of about 6000 for the *C. pyrenoidosa* enzyme. Harrop & Kornberg (1966) estimate the molecular weight of partially purified isocitrate lyase from *Chlorella vulgaris* (Brannon no. 1 strain) to be 120000, but this value was calculated from the elution behaviour of the enzyme from a Sephadex G-100 column and, as discussed above, is likely to be too small.

C. pyrenoidosa isocitrate lyase was activated by Mg^{2+} , a concentration of 1.0–5.0 mM giving maximum activity. In this respect the enzyme is similar to that from other micro-organisms (Smith & Gunsalus, 1957; Shiio *et al.* 1965) and from higher plants (Carpenter & Beevers, 1959). The optimum pH of the enzyme was about pH 7.5, which agrees with that of the enzyme from other sources. The lower value of the pH optimum in phosphate buffers compared with triethanolamine buffers may have resulted from the increasing combination of phosphate with Mg^{2+} at higher pH values, so that insufficient free Mg^{2+} was available for enzyme activation. Olson (1959) and McFadden & Howes (1962) showed that phosphate buffers inhibit isocitrate lyase activity. For these reasons, triethanolamine buffers were used in kinetic experiments.

Purified *C. pyrenoidosa* enzyme required the addition of a thiol compound, 10 mM-GSH or 2.0 mM-dithiothreitol, for maximum activity. With lower concentrations of added thiol compound, the addition of 1 mM-EDTA stimulated activity slightly, but did not by itself give full activation. Addition of a thiol compound is generally necessary for full isocitrate lyase activity in cell-free preparations (Smith & Gunsalus, 1957; Carpenter & Beevers, 1959; Olson, 1959; Haigh, 1963; Ashworth & Kornberg, 1963). Kennedy & Dilworth (1963) reported that EDTA would replace thiol compounds in promoting full activity of isocitrate lyase from *Azotobacter vinelandii*. Shiio *et al.* (1965) made similar observations with purified isocitrate lyase from *P. indigofera* and showed that, although freshly purified enzyme could be activated by EDTA alone, added thiol compound was needed for full activity of aged enzyme preparations. They suggested that added thiol compounds had two functions, one of which was the removal of inhibitory heavy-metal ions; for this EDTA could replace thiol compounds. The other function, for which EDTA could not replace thiol compounds, was the generation of the thiol groups essential for the active enzyme, and they suggested that in freshly prepared enzyme these groups were fully active. With the *C. pyrenoidosa* enzyme we found a progressive requirement during preparation for more GSH for full activation, but freshly purified enzyme could never be fully activated by EDTA alone.

The K_m value of 0.023 mM for *C. pyrenoidosa* isocitrate lyase is in agreement with the K_m values recorded for the bacteria *Escherichia coli*, *Achromobacter* and *Micrococcus denitrificans* (Ashworth & Kornberg, 1963), which are between 0.018 and 0.05 mM. Harrop & Kornberg (1966) give a value of about 0.05 mM for the K_m of isocitrate lyase from *C. vulgaris* (Brannon no. 1 strain), but this enzyme did not show normal Michaelis-Menten kinetics in that there was evidence of a co-operative effect between isocitrate molecules. In contrast, the enzyme from our strain showed normal kinetics (Fig. 8).

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