Isocitrate Lyase from the Free-Living Nematode, *Turbatrix aceti:* Purification and Properties†

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ABSTRACT: Isocitrate lyase (D$_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1) has been purified from the free-living nematode *Turbatrix aceti*. The purified enzyme preparation consists of five isozymes which separate clearly on polyacrylamide gels. A method for the direct assay of activity on the gel is described. Molecular weight determination by gel filtration on Bio-Gel A-15m gave a value of 480,000 for the isozyme mixture. Electrophoresis in the presence of sodium dodecyl sulfate resulted in the appearance of a single sharp protein band having a molecular weight of 123,000. Thus, the enzyme appears to consist of four subunits. The isozyme mixture requires Mg^{2+} and cysteine for maximal activity and has a pH optimum of 7.6 in both phosphate and Tris buffers. *p*-Hydroxymercuribenzoate at 1.6×10^{-5} M completely inhibits enzyme activity. Malonate, tartronate, and tartrate inhibited the enzyme competitively. The K_m of the enzyme for D$_s$(+)-isocitrate was 6.6×10^{-4} M and the turnover number was 48,000 mol of glyoxylate formed per min per mol of enzyme at 25°. The properties of isocitrate lyase from *T. aceti* are compared with those of the enzyme from various unicellular organisms.

Evidence for the presence of "inactive" enzymes in old nematodes was reported by Gershon and Gershon (1970). They showed that as the free-living nematode, *Turbatrix aceti*, aged, the specific activity of isocitrate lyase decreased. The authors concluded that the enzyme in old organisms consisted of two molecular species: totally active and totally inactive. Both forms reacted with the antibody prepared from the active (young) enzyme. Though this work was performed with crude homogenates, similar conclusions were drawn from studies with purified mouse liver aldolase (Gershon and Gershon, 1973a) and mouse muscle aldolase (Gershon and Gershon, 1973b). Studies of aldolase in homogenates of *T. aceti* yielded further evidence for this phenomenon (Zeelon *et al.*, 1973).

In preparation for the investigation of the structure and mechanism of the formation of "inactive" enzyme, studies on the active (young) isocitrate lyase have been carried out. This paper reports on the purification and enzymic properties of isocitrate lyase from "young" *T. aceti*.

The enzyme isocitrate lyase is of particular phylogenetic interest because of its distribution in nature. It is one of the two enzymes involved in the glyoxylate cycle (along with malate synthetase). The cycle is found in bacteria, algae, protozoa, fungi, and plants, but has not been found in mammals. In fact, the first unequivocal report of isocitrate lyase in a multicellular animal was made by Rothstein and Mayoh (1964), who found the enzyme in the free-living nematode, *Caenorhabditis briggsae*. Subsequently, the enzyme was found in other free-living nematodes (Rothstein and Mayoh, 1965) as well as the companion enzyme of the glyoxylate cycle, malate synthetase (Rothstein and Mayoh, 1966). Recently, isocitrate lyase has also been found in two parasitic worms (Pritchard and Schofield, 1969; Barrett *et al.*, 1970). The limited distribution of the enzyme in nature makes it of particular interest to compare the properties of the nematode (animal) enzyme with those reported for yeast (Olson, 1961), *Neurospora* (Sjogren and Romano, 1967; Johanson *et al.*, 1972), bacteria (Shiio *et al.*, 1965; McFadden *et al.*, 1968), and algae (John and Syrett, 1967).

Materials and Methods

Growth Conditions. T. aceti was grown axenically at 30° in 50 ml of basal medium containing 4% acetic acid (Rothstein and Cook, 1966), 500 μg/ml of myoglobin (Hieb *et al.*, 1970), and 50 μg/ml of sterols (Hieb and Rothstein, 1968). By first dissolving the myoglobin in water and then adding it to the medium, a clear solution results (Hieb and Rothstein, 1973). After 14 days, the worms were harvested by filtration on Whatman No. 1 filter paper in a Buchner funnel. The organisms were washed several times with 0.1 M Tris buffer (pH 7.5) and resuspended in a small amount of the buffer. The worms were either used immediately or stored at −20°.

Enzyme Assay. The reaction mixture consisted of 1.1 ml of 0.1 N Tris-HCl (pH 7.5) which contained: 5.5 μmol of $MgCl_2$, 2.2 μmol of cysteine-HCl (Sigma Chemical Co.), 3.6 μmol of phenylhydrazine-HCl (Aldrich Chemical Co.), and 10 μmol of isocitric acid (Sigma). To this, 0.05 ml of the enzyme solution was added and the rate of glyoxylate–phenylhydrazone formation was measured at 324 nm using a Hitachi Perkin-Elmer Model 139 spectrophotometer equipped with a Sargent recorder. The enzyme reaction was carried out at 25° for 4 min. A variable initial lag period was observed, depending upon the enzyme activity. One unit of enzyme activity is, therefore, defined as a change of 0.1 absorbance unit at 324 nm between the 3rd and the 4th min, and is equivalent to 0.25 μmol of glyoxylate formed per min. Protein was determined by the method of Lowry *et al.* (1951).

Purification of Isocitrate Lyase. A suspension of worms containing $1.5–2.5 \times 10^5$ worms in about 40 ml of buffer was homogenized using a cold French pressure cell at 1800 psi. Two volumes of 0.1 M Tris buffer (pH 7.5) were added and the crude homogenate was centrifuged at 25,000*g* for 15 min at 4°. The supernatant was decanted through two layers of cheesecloth to remove fatty materials and was recentrifuged at 105,000*g* for 60 min. The microsomal pellet was discarded.

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TABLE I: Purification of Isocitrate Lyase from *T. aceti*.

Fraction	Vol (ml)	Protein mg/ml	Protein Total (mg)	Total Units	Act. (Units/mg of Protein)	Recov (%)	Fold Purifcn
Crude extract	96	3.6	346	4600	13	100	
33–55% $(NH_4)_2SO_4$ precipitate after dialysis	12.5	10	125	3100	25	68	1.9
DEAE-cellulose (concentrated pool)	2	3	6	1240	207	27	15
Bio-Gel A-15m (I) (concentrated pool)	1.5	1.3	2	630	310	14	23
Bio-Gel A-15m (II) (concentrated pool)	1	0.99	0.99	400	404	9	30

The supernatant, referred to as crude extract, was adjusted to 33% saturation with ammonium sulfate by adding 0.196 g of solid $(NH_4)_2SO_4$/ml of solution, and the precipitate was removed 30 min later by centrifugation at 12,000*g* for 10 min. The precipitate contained only traces of enzyme activity. Additional ammonium sulfate (0.142 g/ml) was added to the supernatant to achieve 55% saturation. The precipitate was removed as before and was resuspended in 10 ml of 0.05 M Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (TEM buffer). Dialysis against TEM (700 ml) was conducted for 5 hr with a single change of buffer. The dialysate was placed on a 2.5 × 15 cm column containing 40 g of DEAE-cellulose (Whatman DE-52) which had been equilibrated with TEM buffer. The column was eluted with 75 ml of TEM buffer containing 0.05 M NaCl, followed by linear gradient elution from 0.05 to 0.175 M NaCl in TEM buffer. Each reservoir contained 225 ml of solution. Elution was conducted at a rate of 34 ml/hr. The fractions (2 ml) were assayed for enzyme activity. The active fractions were pooled and the enzyme was precipitated with $(NH_4)_2SO_4$ (55% saturation). The precipitate was redissolved in 1–2 ml of TEM buffer and placed on a column (1 × 85 cm) packed with Bio-Gel A-15m (Agarose, 200–400 mesh) which had been equilibrated with TEM buffer. Fractions of 1 ml were collected at a flow rate of 3 ml/hr. The fractions showing enzyme activity were pooled, concentrated with $(NH_4)_2SO_4$ as before and rechromatographed on the Agarose column. All procedures were carried out at 4°. The final enzyme preparation consists of the pooled concentrated fractions dissolved in TEM buffer containing 20% glycerol (TEMG). The enzyme preparation was stored at −20°.

Gel Electrophoresis. The enzyme samples (40–130 μg of protein), containing glycerol, were placed on top of 6% acrylamide gel. Electrophoresis was conducted for 3–4 hr, using a constant current of 4 mA/tube in Tris–glycine buffer (pH 8.3). The gels were stained for protein with Naphthol Blue Black (Eastman Kodak Co.) (0.2% dye in 7% acetic acid) or were assayed for enzyme activity (see below). Proteins were located by scanning at 620 nm after destaining with 3% acetic acid.

Location of Enzyme Activity on Gels. Location of the enzyme activity was achieved by incubating the gel, after electrophoresis, in a test tube containing the enzyme assay mixture. After 30–60 min at room temperature, the gel was removed from the test tube and scanned at 324 nm using a linear transport apparatus Model 2410-S, attached to a Gilford 2400-S spectrophotometer. A control gel was incubated in the assay mixture less isocitric acid. The aperture plate used for the scanning was 0.05 × 2.36 mm.

The activity determination does not interfere with the staining for protein, so that the same gel can be used for both location of activity and protein staining.

Molecular Weight of Isocitrate Lyase. The molecular weight of the enzyme was determined by gel filtration on Bio-Gel A-15m using as standards: β-galactosidase (*Escherichia coli*), glucose oxidase (*Aspergillus niger*) and alcohol dehydrogenase (horse liver) having molecular weights of 520,000, 150,000, and 80,000, respectively.

Sodium Dodecyl Sulfate Gel Electrophoresis. Molecular weight determination of the subunits was made by the method of Weber and Osborn (1969). The electrophoretic mobilities of the subunits and the standards were examined at both 6 and 10% gel concentrations.

Inhibitors. Stock solutions of malonate, tartronate, and tartrate were prepared in 0.1 M Tris-HCl and the pH was adjusted to 7.5. Final concentration of each acid was 0.2 mM in the reaction mixture which contained varying amounts of D$_s$(+)-isocitric acid. The effects of these acids on the activity and the K_m of the enzyme were compared.

Inhibition by *p*-hydroxymercuribenzoate (PHMB,[1] Sigma) was investigated by preincubation of the enzyme for 5 min at room temperature with varying amounts of the inhibitor in the preincubation mixture. The enzyme reaction was initiated by adding DL-isocitric acid and phenylhydrazine-HCl. In one set of experiments, cysteine (final concentration 2.2 mM) was added to the reaction mixture either together with or after preincubation with PHMB. In another set of experiments, cysteine was omitted from both the reaction mixture and the preincubation mixture.

Production of Antiserum to Purified Isozyme Mixture of Isocitrate Lyase. Two rabbits recived in the foot pads multiple injections of 0.5 mg of pure isocitrate lyase in complete Freund's adjuvant (Difco Laboratories). The procedure was repeated 1 and 2 weeks later and the rabbits were bled 2 and 4 weeks after the first injection. Normal serum was obtained by preimmunization bleeding of each rabbit.

Immunodiffusion. The immunodiffusion experiment was performed in a 1% Agarose medium prepared in physiological saline containing phosphate buffer (0.01 M; pH 7.2). Plates were incubated for 24 hr at room temperature.

Results

When isocitrate lyase from *T. aceti* was purified as outlined in Table I, the specific activities of the crude extracts and the

[1] Abbreviation used is: PHMB, *p*-hydroxymercuribenzoate.

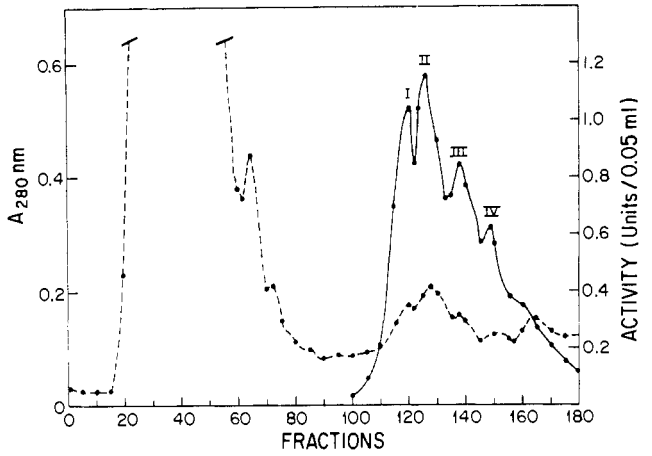

FIGURE 1: Chromatography of isocitrate lyase and DEAE-cellulose. Elution took place with increasing concentration of NaCl dissolved in 0.05 M TEM at pH 7.6: (●- - -●) A_{280nm}; (●—●) enzyme activity. Fractions 112–154 were pooled for further purification (see text).

pure enzyme preparations were consistent from preparation to preparation. The specific activities of the crude extracts ranged from 9 to 13 units per mg of protein and those of the final preparations ranged from 360 to 420 units per mg of protein. The yield of the pure enzyme ranged from about 0.3 to 0.5% by weight of the total protein in the crude extract. Purification ranged from 28- to 35-fold. The pure enzyme is moderately stable in storage at −20°. An activity loss of 25–30% was observed after 4 weeks.

Isozymes. Four distinct isozymes were eluted from the DEAE-cellulose column (Figure 1). They all appear to have identical or closely similar molecular weights as judged by the elution pattern of the Agarose columns (Figures 2 and 3). After electrophoresis on a 6% polyacrylamide gel, five clear bands resulted. It was assumed that isozyme I (Figure 1) was resolved into two isozymes, Ia and Ib (Figure 4). The protein bands correspond exactly to the bands representing enzyme activity.

Determining enzyme activity on the gel required frequent monitoring. Overincubation of the gel in the enzyme reaction mixture resulted in poor resolution due to diffusion of the reaction products throughout the gel (see activity shoulder of isozyme Ia in Figure 4). Therefore, the gel should be scanned at short time intervals and returned to the reaction mixture if necessary. This method for enzyme location on gels should be applicable to other enzymes where a visible color staining method is not readily available and where the reaction products can be directly measured by absorption.

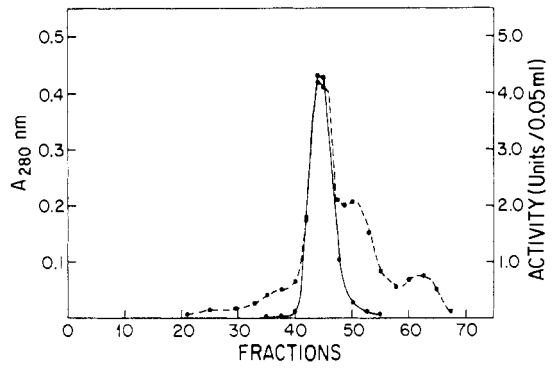

FIGURE 2: Elution pattern from Bio-Gel A-15m (I): (●- - -●) A_{280nm}; (●—●) enzyme activity. Fractions 42–47 were pooled for further purification (see text).

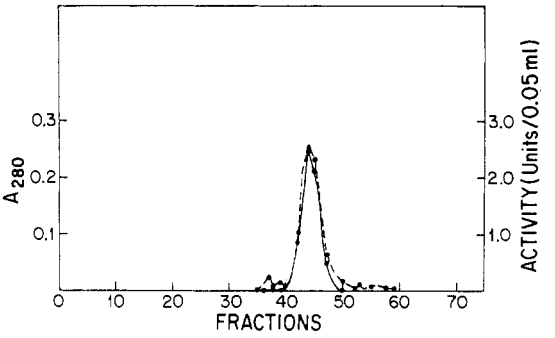

FIGURE 3: Elution pattern from Bio-Gel A-15m (II): (●- - -●) A_{280nm}; (●—●) enzyme activity. Fractions 42–47 were pooled and concentrated with $(NH_4)_2SO_4$ and the precipitate was dissolved in TEMG. For details, see text.

Molecular Weight Determination. The molecular weight of the isozymes is 480,000 as determined by gel filtration on an Agarose column. Gel electrophoresis (10% gel) in the presence of sodium dodecyl sulfate showed a single sharp protein band and a small amount of contaminating protein (Figure 5). From the electrophoretic mobility of this band in relation to that of the standard proteins, the molecular weight of the subunits was calculated to be 123,000. Similar results were obtained when 6% gel was used.

pH Optimum. Isocitrate lyase has a pH optimum of approximately 7.6 in sodium phosphate buffer. The pH optimum was the same in Tris buffer except that in this case, the enzyme exhibited a somewhat higher level of activity.

Reagents Requirement. Isocitrate lyase requires Mg^{2+} and a sulfhydryl compound for maximal activity. In fact, the magnesium is absolutely essential for enzyme activity; omission of this metal from the reaction mixture resulted in total loss of activity. Omission of cysteine resulted in an activity loss of

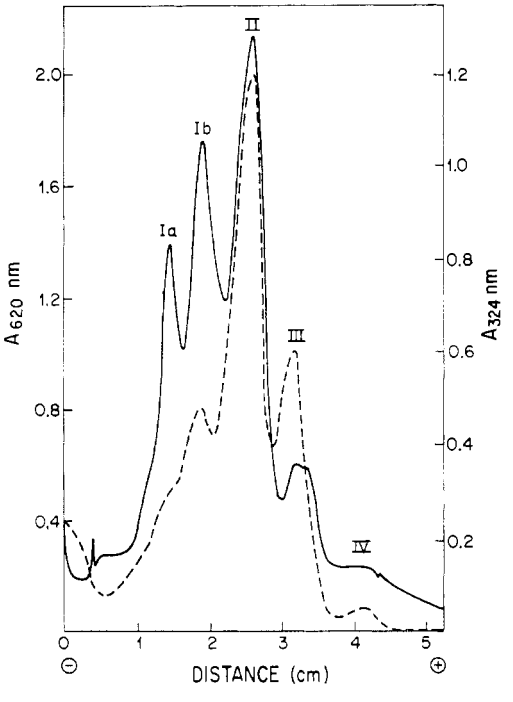

FIGURE 4: Scan showing location of proteins and enzyme activities of a purified enzyme preparation of polyacrylamide gel. Enzyme samples were run on duplicate gels. One was stained for protein and the other assayed for enzyme activity (see text). The gels were scanned at a rate of 1 cm/min: (—) protein (A_{620nm}); (- - - -) enzyme activity (A_{324nm}).

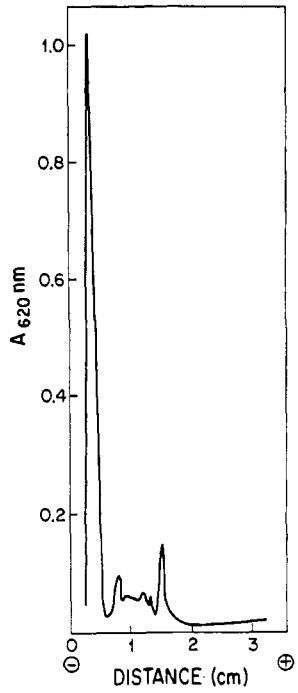

FIGURE 5: Scanning pattern of isocitrate lyase subunits on sodium dodecyl sulfate gel. After destaining, the gel was scanned at 620 nm at a rate of 1 cm/min.

about 50%. The K_m values of isocitrate lyase for D$_s$(+)-L-isocitrate, $MgCl_2$ and cysteine were 6.6×10^{-4} M, 2.5×10^{-4} M, and 1.0×10^{-5} M, respectively.

Inhibitors. Malonic acid, tartronic acid, and tartaric acids competitively inhibited isocitrate lyase (Figure 6). Malonate and tartronate were more effective inhibitors than the four-carbon acid, tartrate.

From Figure 7, it can be seen that *p*-hydroxymercuribenzoate at 1.6×10^{-5} M reduced enzyme activity by 96% when cysteine was omitted from the reaction mixture. The addition of cysteine, even after preincubation of the enzyme with PHMB, reduced the inactivation to less than 40%. When the enzyme was preincubated with cysteine, addition of PHMB had no inhibitory effect.

Immunodiffusion. From Figure 8, it is clear that the isolated isocitrate lyase exhibits a high degree of purity. Antibody prepared from pure enzyme yields only a single identity line when tested against a crude extract from the nematodes. Thus, there are no other antigenic proteins from the homogenate present in the purified enzyme.

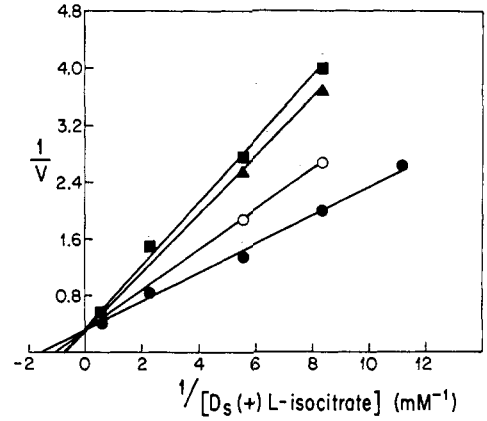

FIGURE 6: Lineweaver–Burk plots showing the effect of the competitive inhibitors tartronic acid, malonic acid, and tartaric acid on the activity and K_m of isocitrate lyase isozyme. The final concentration of each acid in the reaction mixture was 0.2 mM. In the control experiment, 0.1 M Tris-HCl (pH 7.5) was substituted for the inhibitors: (■) tartronate, $K_{m,app} = 1.43 \times 10^{-3}$ M; (▲) malonate, $K_{m,app} = 1.25 \times 10^{-3}$ M; (○) tartrate, $K_{m,app} = 9.1 \times 10^{-4}$ M; (●), control (no inhibitor), $K_m = 6.66 \times 10^{-4}$ M.

Discussion

Table II compares several properties of isocitrate lyase from various sources. The turnover number for isocitrate lyase from *T. aceti* is much higher than that for the *Chlorella*, yeast, or the *Pseudomonas* enzyme. This value should be considered as an average since there are differences in the specific activity of the five isozymes shown in Figure 4.

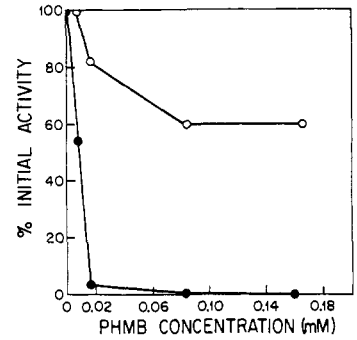

FIGURE 7: Effect of *p*-hydroxymercuribenzoate (PHMB) on the activity of isocitrate lyase isozymes and the protective effect of cysteine: (●) cysteine omitted from the reaction mixture and the preincubation mixture; (○) cysteine added to the reaction mixture after preincubation.

TABLE II: Comparison of Isocitrate Lyases from Various Sources.[a]

	Mol Wt	Subunits	Isozymes	Turnover No.[b]	pH Optimum (Tris Buffer)	K_m (mM) (Isocitrate)
T. aceti	480,000	4	5	48,000	7.6	0.66
Chlorella pyrenoidosa	170,000	NR[c]	NR[c]	5,950	7.6	0.023
Pseudomonas indigofera	222,000 (206,000)	4	NR[c]	7,300	7.7	0.82
Neurospora crassa	265,000	4	2	NR[c]	6.8	3.3[d] / 1.85
Yeast				1,000[e]		1.2

[a] See text for references. [b] Moles of glyoxylate per minute per mole of enzyme. [c] None reported. [d] Depending on growth conditions. [e] The reported value is 500 for 100,000 g of enzyme. Assuming a molecular weight of 200,000, the turnover number would be 1000.

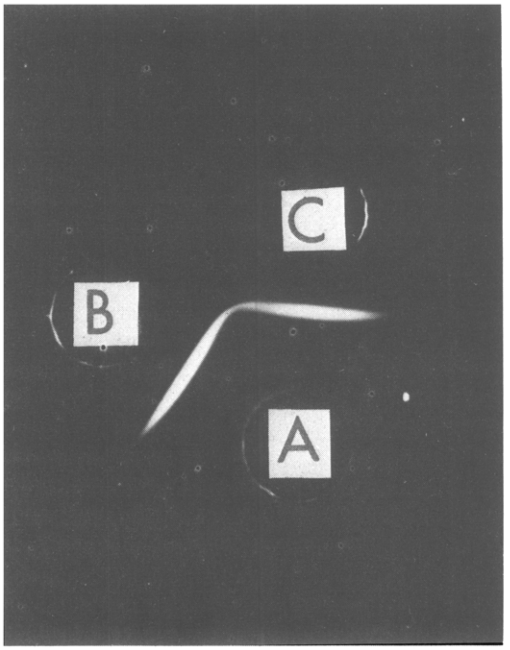

FIGURE 8: Immunodiffusion of antiserum prepared from pure isocitrate lyase tested against pure enzyme and crude extract: A, antiserum; B, crude extract; C, pure enzyme.

Isocitrate lyase from *T. aceti* required Mg^{2+} and a sulfhydryl compound (cysteine) for maximal activity, was competitively inhibited by malonate and had an optimal activity at pH 7.6. Thus, it appears that the requirements for the catalytic activity of isocitrate lyase from *T. aceti* are similar to those of the enzyme derived from other sources. In addition, the *Neurospora*, *Pseudomonas*, and nematode enzymes all consist of four subunits.

On the other hand, isocitrate lyase from *T. aceti* possesses several distinctly different properties from the other isocitrate lyases. While two isozymes were reported in *Neurospora*, and none in the other organisms, five distinct enzyme species were found in the nematode. Moreover, the isozymes all have molecular weights of 480,000, a figure which is more than twice that reported for the enzymes from *Pseudomonas* and *Chlorella* and nearly twice the size of that given for the *Neurospora* enzyme. Thus, the physical structure of the nematode enzyme is unique among the isocitrate lyases so far reported. The separation of five isozymes, each consisting of four subunits having identical molecular weights of 123,000, implies differences in the charge of the subunits. Presumably, there are two different chains, on the model of lactate dehydrogenase. Further work is required to isolate the individual isozymes, to study their properties and to characterize the subunits.

Gershon and Gershon (1970) have shown that the specific activity of isocitrate lyase decreased as *T. aceti* aged. Their study was performed on crude homogenates. Using pure preparations of isocitrate lyase from young and old *T. aceti*, we have observed a similar decrease (approximately 50% at half-life span) in the specific activity of the enzyme. As will be reported in a separate communication, Michaelis constant, molecular weight, charge, and antigenicity were the same for the enzymes from old and young organisms. Thus far, our results support the concept of "inactive enzymes." Apparently, part of the enzyme in old organisms is altered, either by a sequence change or by a postsynthetic modification of the protein. The purification and study of the properties of the "young" enzyme reported here have provided us with the necessary baseline from which to determine the nature of this change.

Acknowledgment

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References

Barrett, J., Ward, C. W., and Fairbairn, D. (1970), *Comp. Biochem. Physiol. 35*, 577.

Gershon, H., and Gershon, D. (1970), *Nature (London) 227*, 1214.

Gershon, H., and Gershon, D. (1973a), *Proc. Nat. Acad. Sci. U. S. 70*, 909.

Gershon, H., and Gershon, D. (1973b), *Mech. Age Develop. 2*, 33.

Hieb, W. F., and Rothstein, M. (1968), *Science 166*, 777.

Hieb, W. F., and Rothstein, M. (1973), private communication.

Hieb, W. F., Stokstad, L. R., and Rothstein, M. (1970), *Science 168*, 143.

Johanson, R. A., Hill, J. M., and McFadden, B. A. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 475.

John, P. C. L., and Syrett, P. J. (1967), *Biochem. J. 105*, 409.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

McFadden, B. A., Rao, G. R., Cohen, A. L., and Roche, T. E. (1968), *Biochemistry 7*, 3574.

Olson, J. A. (1961), *Enzymes, 2nd Ed., 5*, 387.

Pritchard, R. K., and Schofield, P. J. (1969), *Comp. Biochem. Physiol. 29*, 581.

Rothstein, M., and Cook, E. (1966), *Comp. Biochem. Physiol. 17*, 683.

Rothstein, M., and Mayoh, H. (1964), *Arch. Biochem. Biophys. 108*, 134.

Rothstein, M., and Mayoh, H. (1965), *Comp. Biochem. Biophys. 16*, 361.

Rothstein, M., and Mayoh, H. (1966), *Comp. Biochem. Physiol. 17*, 1181.

Shiio, I., Shiio, T., and McFadden, B. A. (1965), *Biochim. Biophys. Acta 96*, 114.

Sjogren, R. E., and Romano, A. H. (1967), *J. Bacteriol. 93*, 1638.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Zeelon, P., Gershon, H., and Gershon, D. (1973), *Biochemistry 12*, 1743.