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# Two Distinct Isocitrate Lyases from a *Pseudomonas* Species

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The isocitrate lyases of acetate- and methylamine-grown *Pseudomonas* MA (Shaw strain) were studied. They were shown to be different by a variety of physical criteria including chromatographic elution patterns, heat inactivation kinetics, pH variation of $K_m$ values, and migration on polyacrylamide gels. The implications and significance of the existence of two enzymes in relation to the role of isocitrate lyase in methylamine utilization is discussed.

*Pseudomonas* MA is able to grow on methylamine as its sole source of carbon, nitrogen, and energy. The pathway of carbon assimilation in this organism (Fig. 1) was recently elucidated, (1; E. Bellion, Fed. Proc. **21**:3522, 1972). Two key enzymes in this pathway were identified as isocitrate lyase (*threo*-$D_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1.) and an adenosine 5'-triphosphate-malate lyase system (12), subsequently shown to consist of two activities, a malate thiokinase (malate-coenzyme A ligase [adenosine 5'-triphosphate], EC 6.2.1.X) and a malyl-coenzyme A lyase (malyl-coenzyme A glyoxylate lyase, EC 4.1.3.X) (11). This pathway was found, by later work in other laboratories, to be operative in *Hyphomicrobium* X (9), in *Bacterium* 5H2 (3), and in *Pseudomonas aminovorans* and *Pseudomonas* MS (14). The finding of this pathway revealed a new role for isocitrate lyase, since this was the first report of its involvement in one-carbon metabolism. Prior to this time, isocitrate lyase was known to be utilized by organisms growing with acetate or compounds capable of degradation to acetate alone, for example, fatty acids (13). *Pseudomonas* MA belongs to the group of organisms that are capable of growth on acetate, as well as on $C_1$ compounds, also with the involvement of isocitrate lyase. An investigation of the properties of the isocitrate lyase from both methylamine- and acetate-grown cells was undertaken, and this manuscript presents the results of these studies. (A preliminary report of this work was presented at the 1st International Symposium on $C_1$ Metabolism held in Tokyo, Japan, September 1974.)

## MATERIALS AND METHODS

**Maintenance and growth of organism.** *Pseudomonas* MA (Shaw strain) (18) was grown in mineral salts solution with methylamine as sole source of carbon and nitrogen or with acetate and ammonium chloride as sole sources of carbon and nitrogen, respectively (1). The liquid cultures were grown at 30 C with either shaking or forced aeration. Growth was monitored with a Bausch & Lomb Spectronic 20, using a wavelength of 560 nm.

Cells were harvested at an absorbance reading of 1.2, at which time the maximum specific activity of isocitrate lyase occurs (E. Bellion and Y. S. Kim, unpublished observations). They were washed in 0.02 M phosphate buffer containing 1 mM ethylenediaminetetraacetate, and cell extracts were prepared immediately.

**Preparation of cell extracts.** Cells were resuspended in 0.02 M phosphate buffer containing 1 mM ethylenediaminetetraacetate, pH 7.5, at a ratio of 1 g (wet weight) of cells to 3 ml of buffer. They were disrupted by passing the suspension through a French pressure cell at 10,000 lb/in$^2$ at 4 C. After treatment with deoxyribonuclease (deoxyribonucleate oligonucleotide hydrolase, EC 3.1.4.5) the exudate was centrifuged at 15,000 $\times$ *g* for 15 min, and the pellet was discarded.

**Assay of protein.** Protein was assayed in crude extracts by the biuret procedure; in more dilute solutions the Folin-Ciocalteu method as described by Lowry et al. (15) was employed. Bovine serum albumin was used in the preparation of both standard curves

**Enzyme assay.** Isocitrate lyase (*threo*-$D_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1.) was assayed by the continuous spectrophotometric method as described by Dixon and Kornberg (6).

For routine assays for purposes such as detection from columns, DL-isocitrate was employed, whereas for assays used in the determination of kinetic constants *threo*-$D_s$-(+)-isocitrate was used.

**Partial purification procedures. (i) Heat treatment.** Magnesium chloride was added to previously prepared cell extract to give a final $Mg^{2+}$ concentration of 0.06 M. The extract was immersed in a water bath maintained at 50 C and heated for 5 min with continuous shaking. It was then immediately withdrawn and cooled to 5 C in an ice-salt bath. The denatured protein was removed by centrifugation, and samples of the supernatant fluid were assayed for protein and isocitrate lyase activity.

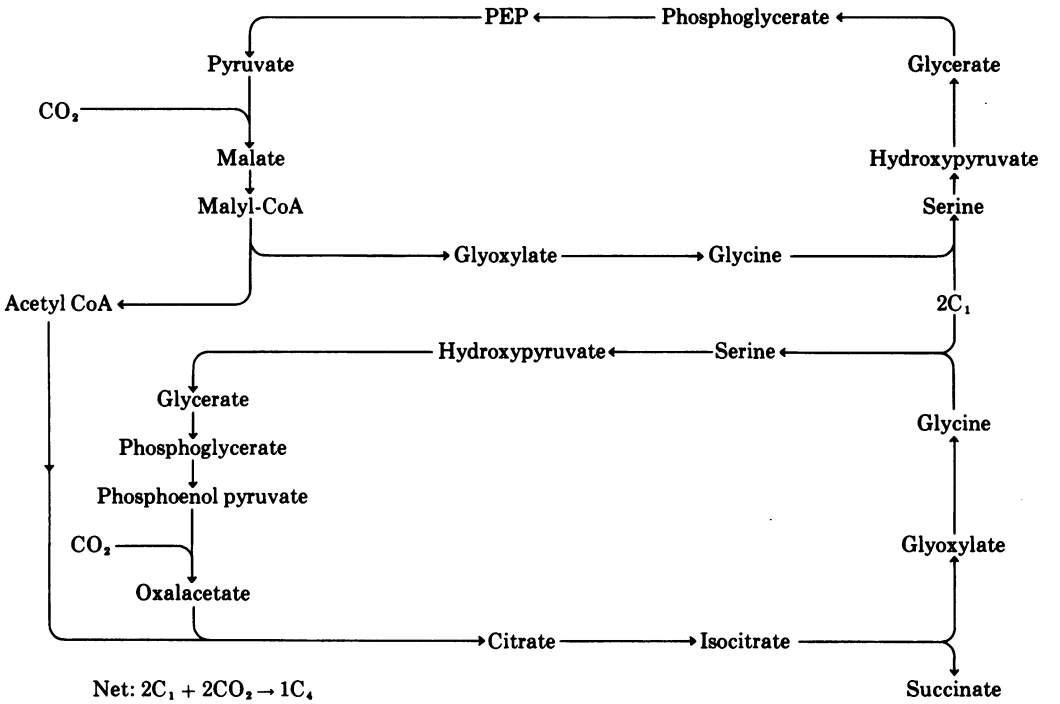


FIG. 1. *Pathway for methylamine assimilation in Pseudomonas MA.*

**(ii) Ammonium sulfate precipitation.** The protein concentration of the above supernatant fluid was adjusted to 10 mg/ml, and the extract was brought to 55% saturation with respect to ammonium sulfate by the slow addition of the required quantity of solid enzyme-grade ammonium sulfate to a constantly stirred solution held at 4 C. The resultant precipitate was removed by centrifugation, and the supernatant fluid was brought to 75% saturation by the same procedure. The precipitate, which contained most of the activity, was obtained by centrifugation and redissolved in 0.02 M phosphate buffer, pH 7.5, containing 1 mM ethylenediaminetetraacetate and 1 mM 2-mercaptoethanol (referred to as MEP buffer). The solution was dialyzed against MEP buffer.

**Gel filtration.** Gel filtration was performed with columns of Sephadex G-200 and Sephadex G-150 having bed dimensions of 1.7 by 72 cm that had been equilibrated for 24 h with MEP buffer, and 1-ml fractions were collected. Isocitrate lyase was located in the fractions by assaying each tube for activity.

**DEAE-cellulose chromatography.** Diethylaminoethyl (DEAE)-cellulose chromatography was carried out with protein solutions at pH 7.5 using DEAE-cellulose columns (bed dimensions, 1.7 by 14 cm) which had previously been equilibrated with 100 ml of 0.02 M MEP buffer (pH 7.2). The columns were eluted with MEP buffer containing 0.1 M NaCl, followed either by a gradient of 0.1 to 0.3 M NaCl in MEP buffer or by 0.3 M NaCl in MEP buffer alone. This gradient was not applied until the protein concentration in the previous eluent had reached a stable minimum. Protein concentration in the eluent was measured continuously using an LKB Uvicord II ultraviolet absorptiometer with wavelength at 280 nm. Fractions (7 ml) were collected and assayed for isocitrate lyase activity.

**Detection of isocitrate lyase on polyacrylamide gels.** Electrophoresis was conducted according to the method described by Davis (5). Electrophoretic experiments were carried out at 4 C at pH 8.3 with a current of 2 to 3 mA/tube. Isocitrate lyase activity was detected on polyacrylamide gels by using the fuchsin dye method of Reeves and Volk (17).

## RESULTS

**Elution of enzymes from DEAE-cellulose columns.** During preliminary purification procedures it was observed that isocitrate lyase activity obtained from acetate-grown cells was eluted from DEAE-cellulose in the 0 to 0.1 M NaCl fraction, whereas that from methylamine-grown cells was eluted only in the 0.2 to 0.3 M NaCl fraction. The elution profiles from these experiments are shown in Fig. 2a and b. To rule out any anomalous effect, a mixture comprised of acetate- and methylamine-grown extracts was applied to the column. It was eluted first with 0.1 M NaCl until the protein concentration in the eluent had reached a minimum. Then 0.3 M NaCl was applied and the elution was continued. The results of this procedure are shown in Fig. 2c. It is apparent that two

fractions of activity were obtained, one corresponding to the acetate-grown isocitrate lyase and the other corresponding to the methylamine-grown isocitrate lyase. The possibility remained that the activity observed in the 0.3 M NaCl fraction was simply residual enzyme on the column "tightened up" and eluted as one component by the 0.3 M NaCl. This was checked by applying to the column a mixture of methylamine and acetate extract such that there was a 4:1 ratio of methylamine-grown isocitrate lyase to acetate-grown isocitrate lyase. The column was eluted as before with both 0.1 and 0.3 M NaCl. Greater than 92% of the total activity applied was recovered, of which 18.2% was found in the 0.1 M NaCl fraction and 81.8% was found in the 0.3 M NaCl fraction. This result further substantiated the hypothesis that the two enzymes were nonidentical.

**Kinetics of thermal denaturation.** The rates of thermal denaturation of the two enzymes were examined. For this, crude extracts containing identical protein concentrations (10 mg/ml) were incubated at either 45 or 50 C in the

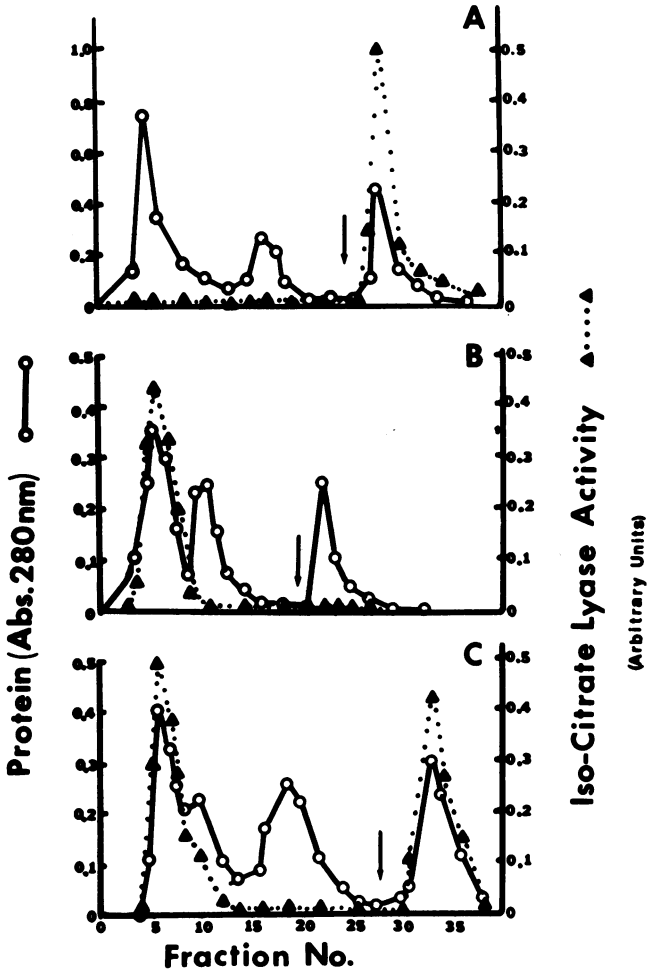


FIG. 2. *Results of DEAE-cellulose chromatography of isocitrate lyase obtained from both methylamine- and acetate-grown Pseudomonas MA. (A) Extract obtained from methylamine-grown cells, containing 53 mg of protein (8 U of enzyme), was applied to the column (1.7 by 14 cm) (B) Extract obtained from acetate-grown cells containing 32 mg of protein (6 U of enzyme) was applied to the column. (C) A mixture of extracts obtained from both methylamine- and acetate-grown cells containing a total of 76 mg of protein (8 U of acetate enzyme, 9.5 U of methylamine enzyme) was applied to the column. The extracts used had been partially purified through the ammonium sulfate precipitation stage as outlined in Materials and Methods; 7-ml fractions were collected and each was assayed for activity. Symbols: O, absorbance at 280 nm; ▲, isocitrate lyase activity. Elution of the columns with 0.3 M NaCl was commenced at the points indicated by the arrows.*

presence of varying amounts of $Mg^{2+}$. Samples were withdrawn at timed intervals and immediately chilled in ice water. Residual isocitrate lyase activity in the samples was assayed after removal of precipitated protein by centrifugation. Plots of logarithms of percentage activity remaining against time are given in Fig. 3. The two enzymes have different heat stabilities at all conditions employed, the greatest difference occurring at 50 C and 6 mM $Mg^{2+}$, where the acetate enzyme has a half-life of 3 min compared to 11 min for the methylamine enzyme. Also apparent is the differing response to $Mg^{2+}$ concentration. The acetate enzyme is stabilized more by increasing $Mg^{2+}$ concentration than is the methylamine enzyme.

The possibility that there was a denaturing or hydrolyzing enzyme in the acetate extract but not in the methylamine extract was eliminated by the following experiment. A mixture of acetate extract (0.33 U) with methylamine extract (0.32 U) was prepared in a total volume of 2 ml. This mixture was incubated at 45 C for 4 min, without the addition of $Mg^{2+}$. Simultaneously, 0.33 U of methylamine extract and 0.42 U of acetate extract were heated in separate tubes. After this time all of the acetate enzyme should have been inactive, compared to only 50% of the methylamine enzyme; that is, 25% of the total original activity in the mixture tube should have been remaining. The results of this experiment (Table 1) indicate that the loss of activity is due to thermal denaturation alone.

**Polyacrylamide gel electrophoresis.** Figure 4 shows the results of polyacrylamide gel electrophoresis of the acetate and methylamine enzymes when run separately and as a mixture. Isocitrate lyase activity was located on the gels using the fuchsin dye method. Under the conditions employed, the acetate enzyme migrates 2.6 cm whereas the methylamine enzyme migrates 1.1 cm. A mixture of the two extracts applied simultaneously results in the appearance of two bands of activity.

**Kinetic constants.** The Michaelis-Menten constants for isocitrate with each enzyme were obtained at three pH values from double reciprocal plots of the kinetic data. Figure 5 shows the results obtained at pH 6.8 and 7.2. Although at pH 7.2 the values are essentially identical, the $K_m$ values diverge at the lower pH values. A summary of the $K_m$ values obtained at the pH values employed is presented in Table 2. The intercepts were obtained from a computer-generated, least-squares fit of the data points. Both enzymes exhibited typical Michaelis-Menten kinetics.

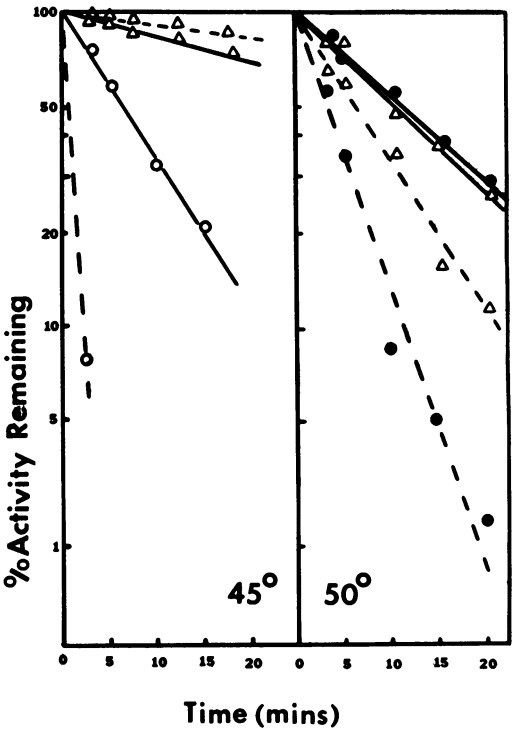


FIG. 3. *Kinetics of thermal inactivation of isocitrate lyase from both methylamine- and acetate-grown Pseudomonas MA. A 2.5-ml amount of crude extract containing 25 mg of protein (3 to 5 U of enzyme) was placed in water baths at either 45 or 50 C with the indicated concentrations of $Mg^{2+}$. After various time periods samples were withdrawn, chilled, centrifuged to remove precipitated protein, and assayed for residual isocitrate lyase activity. Symbols: -----, extract from acetate-grown Pseudomonas MA; ——, extract from methylamine-grown Pseudomonas MA. O, no $Mg^{2+}$; ●, 6 mM $Mg^{2+}$; △, 12 mM $Mg^{2+}$.*

**Molecular weights.** Values for molecular weights were obtained by gel filtration methods using Sephadex G-200 and G-150. Both enzymes have molecular weights in the region of 150,000 to 170,000 on the G-200 column (Fig. 6). Higher and less reliable values were obtained when a G-150 column was employed. The molecular weights obtained from this column were between 170,000 to 185,000.

## DISCUSSION

The results described in this communication show conclusively that *Pseudomonas* MA is able to elaborate two distinct isocitrate lyase enzymes. One of these enzymes is produced by the cells during growth with acetate and the other during growth with methylamine. The nonidentity was established by a variety of

TABLE 1. *Thermal inactivation of isocitrate lyase from both methylamine and acetate-grown Pseudomonas MA*[a]

| Origin of extract | Initial U | U recovered | % Recovered |
|---|---|---|---|
| Methylamine | 0.33 | 0.165 | 50 |
| Acetate | 0.42 | 0 | 0 |
| Methylamine and acetate | 0.33, 0.32 } 0.65 | 0.18 | 28 |

[a] Crude cell-free extracts containing 25 mg of protein were heated at 45 C with no addition of $Mg^{2+}$ for 4 min. They were then rapidly removed, chilled, and centrifuged to remove precipitated protein, and the supernatants were assayed for residual isocitrate lyase activity.

experiments involving separation methods, heat stabilities, and kinetic constants.

Thus, they are eluted from DEAE-cellulose at very different salt concentrations, indicating that they possess differing net charges at the pH utilized, a fact further substantiated by the observation that they have different rates of migration on polyacrylamide gels. They exhibit substantially different heat stabilities, the methylamine enzyme being more stable than the acetate in low $Mg^{2+}$ concentration and the acetate enzyme being protected from thermal denaturation to a greater degree by an increased $Mg^{2+}$ concentration.

The two enzymes also show different response to pH changes with respect to their $K_m$ values. Thus the $K_m$ values for the methylamine enzyme dropped more drastically than those of the acetate enzyme over the range employed (7.2 to 6.8). Although the differences in $K_m$ values at each pH value are not substantial, they are reproducible. These facts together with the differing pH responses serve to further substantiate the nonidentity of the two enzymes. Likewise, the difference between molecular weights alone would not normally be sufficient evidence for nonidentity, the difference being less than 10%. However, we feel that this difference is real, since on both Sephadex G-150 and Sephadex G-200 the methylamine enzyme was the smaller of the two. More accurate determination of the molecular weights will have to await the purification of both enzymes to homogeneity and measurement of molecular weight by ultracentrifugation methods. McFadden et al. (16) reported a molecular weight of 206,000 for isocitrate lyase from butyrate-grown *Pseudomonas indigofera*, obtained by sedimentation methods. This group also reports kinetic properties markedly different from those reported here. It is not unlikely that the two enzymes studied in this report are both different from that studied by McFadden and his colleagues.

Having established that the two enzymes are different, we are left with two major questions to answer: in what way are they different, and why do the bacteria synthesize two different enzymes with the same function?

With reference to the first question, it is clear from the molecular weight determinations that one enzyme is not simply a dimer or other such aggregate of the other. The obviously different net charges on the enzymes tend rather to indicate completely different proteins with different amino acid compositions and sequence; however, the results do not eliminate the possibility that both enzymes are the product of a single structural gene, with one having undergone postsynthesis modification. It will be possible to answer these questions only when both

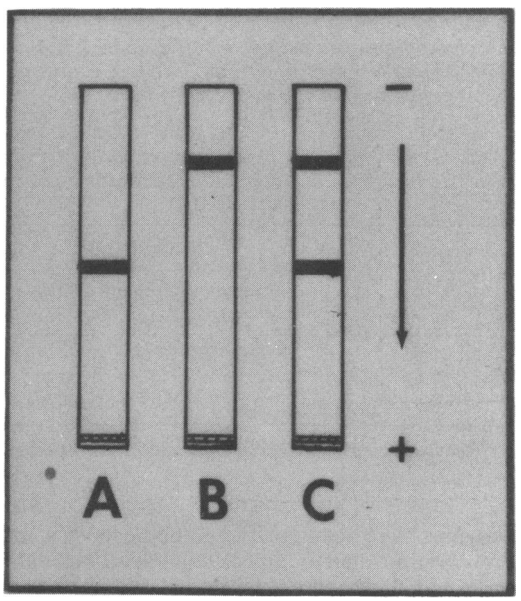


FIG. 4. *Migration of isocitrate lyase obtained from methylamine- and acetate-grown Pseudomonas MA on polyacrylamide gels. (A) Gel containing acetate-grown extract, with 0.3 mg of protein (0.1 to 0.5 U). (B) Gel containing methylamine-grown cell extract, with 0.3 mg of protein (0.1 to 0.5 U). (C) Gel containing a mixture of methylamine- and acetate-grown cell extracts with 0.3 mg of protein total. Electrophoresis and staining of the gels for enzyme activity were performed as described in Materials and Methods. The presence of isocitrate lyase activity is shown by the appearance of a red band in the gel after 30 to 45 min of incubation in the staining mixture. Symbols: zzz, bromothymol blue; ▬, isocitrate lyase.*

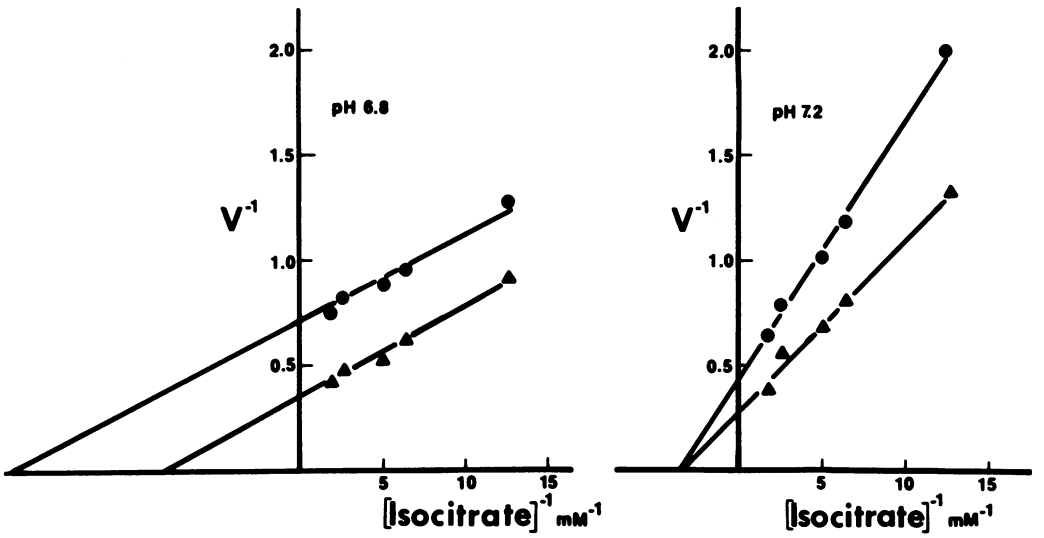


FIG. 5. *Comparisons of kinetics of isocitrate lyase from methylamine- and acetate-grown Pseudomonas MA at pH 6.8 and 7.2. Reaction mixtures contained: partially purified extract (ammonium sulfate precipitates, 0.1 to 0.2 mg of protein), threo* D*, (+)-isocitrate as indicated, 10 mM* $Mg^{2+}$*, 10 mM glutathione, 100 mM 2-(N-morpholino)-propane sulfonate buffer, and 6.7 mM phenyl hydrazine in a final volume of 1 ml. The reactions were started by addition of isocitrate. Rates are expressed in micromoles of glyoxylate formed per minute. Symbols:* ●*, methylamine-grown;* ▲*, acetate-grown.*

TABLE 2. *pH variation of* $K_m$ *values for isocitrate lyases derived from acetate- and methylamine-grown Pseudomonas MA*[a]

| pH | $K_m$ (mM) | |
|---|---|---|
| | Acetate-grown | Methylamine-grown |
| 7.2 | 0.28 | 0.28 |
| 7.05 | 0.16 | 0.11 |
| 6.8 | 0.13 | 0.06 |

[a] Reaction conditions were those described for Fig. 5.

enzymes have been purified to homogeneity and their amino acid compositions have been determined.

With reference to the second question, it is tempting to conclude that the cell has two completely different enzyme systems for the utilization of acetate and of methylamine, under different control. Although chemically the reaction catalyzed by the isocitrate lyases is the same, biochemically the reasons for it are different. In methylamine growth the glyoxylate produced is used for glycine synthesis, whereas in acetate growth it is used for malate synthesis. Clearly these functions are completely separate entities. It is of interest in this connection to note that malate synthase, the enzyme for the subsequent reaction in acetate growth, is severely repressed in methylamine growth (1). This is not surprising in view of the presence during methylamine growth of the adenosine 5'-triphosphate-malate lyase system which accomplishes the reverse reaction of malate synthase. If acetate isocitrate lyase is coordinately repressed along with malate synthase, then the need for a second type of isocitrate lyase during methylamine growth is apparent. In succinate growth malate synthase is not repressed, unlike isocitrate lyase. This finding would tend to rule out coordinate repression of the two enzymes, although the acetate isocitrate lyase could also be separately controlled. Additionally, the malate synthase present in succinate-grown cells may be a different enzyme from that found in acetate-grown cells. It has been found that *Escherichia coli* elaborates two malate synthases, one during acetate growth, where it functions in an anaplerotic role, and the other during glycolate growth functioning in a respiratory role (7). The genetic redundancy exhibited by *Pseudomonas* MA in producing two isocitrate lyases is presumably the price it has to pay for more selective and specific control over the induction of its enzymes.

The capability of *Pseudomonas* MA to produce two isocitrate lyases adds to the list of known examples of the occurrence of isofunctional enzymes. Most examples of this phenomenon relate to two enzymes catalyzing the same

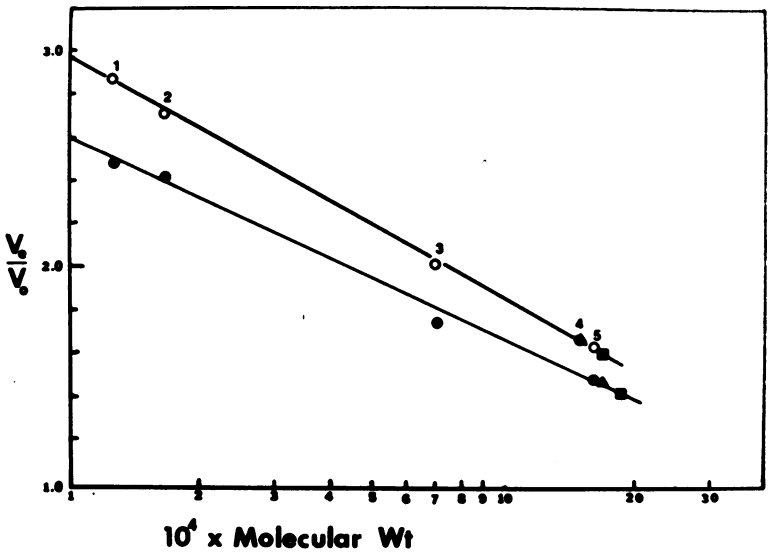


FIG. 6. *Chromatography of isocitrate lyase from methylamine- and acetate-grown Pseudomonas MA on Sephadex columns. Extracts, partially purified by ammonium sulfate precipitation and containing 4 to 6 mg of protein (0.5 to 1.5 U of enzyme), were applied to columns of both Sephadex G-150 and G-200 (1.7 by 73 cm) which had been precalibrated with marker proteins. The markers used and their molecular weights were: (1) cytochrome c, 12,400; (2) myoglobin, 16,900; (3) bovine serum albumin, 70,000; (4) aldolase, 149,000; (5) gamma-globulin, 160,000. Thyroglobulin, 670,000, and blue dextran were eluted in the void volumes. Extracts from methylamine- and acetate-grown cells were applied in separate experiments. Symbols: ○, Sephadex G-200; ●, Sephadex G-150; ▲, point of emergence of isocitrate lyase derived from methylamine-grown cells; ■, point of emergence of isocitrate lyase derived from acetate-grown cells. $V_e$ Elution volume of protein; $V_o$ void volume of column.*

chemical reaction in the same direction but for different purposes, e.g., the biosynthetic and degradative dihydro-orotate reductases described by Taylor et al. (20) and the similar examples cited by Dagley and Chapman (4). Also, Harder and Quayle (10) postulated that *Pseudomonas* AM1 possessed two different serine hydroxymethyltransferases, one for general metabolic purposes, e.g., for biosynthesis of glycine, and the other for use in the $C_1$ assimilation pathway of this organism. An example of the occurrence of isofunctional enzymes acting in the same direction and for the same purpose was given by Cánovas and Stanier (2). They found, in *Moraxella calcoacetica*, that the enzyme which attacks β-ketoadipate *enol*-lactone, a hydrolase, and the following enzyme, a CoA transferase, were different depending on whether the cells were grown on benzoate or *m*-hydroxybenzoate. Interestingly, they also reported that in *Pseudomonas putida*, performing similar aromatic catabolism, this was not the case, the same enzymes being used irrespective of the carbon and energy source. The presence of two isocitrate lyase isozymes has been shown in *Neurospora crassa* (8, 19). Both enzymes were present in approximately equal amounts during glucose growth, but one was induced to a much greater extent during acetate growth. Although these results are different from ours, there are several similarities in the properties of the enzymes. In the earlier *Neurospora* study (19) it was found that the acetate isocitrate lyase was eluted first from DEAE-cellulose and also was the less heat stable of the two enzymes. The authors questioned the function of the isocitrate lyase found during glucose growth. They suggested that perhaps its function was a biosynthetic one, providing glyoxylate for use in glycine biosynthesis. In the case of *Pseudomonas* MA, the acetate enzyme is serving in an anaplerotic capacity during operation of the glyoxylate cycle, whereas the methylamine enzyme could be considered as serving a biosynthetic function to bring about the synthesis of succinate, via glycine, from the $C_1$ compound. This would also provide an explanation for the occurrence of two distinct enzymes.

On the basis of the results in *Neurospora* and those reported in this paper, it is perhaps possible to assign to isocitrate lyase the alternative role of that of a participant in glycine biosynthesis. Experiments to determine if the low level of isocitrate lyase found in succinate-

and glucose-grown *Pseudomonas* MA is that corresponding to the acetate or methylamine type are currently underway in our laboratory.

Although, so far, the organisms that utilize isocitrate lyase for $C_1$ metabolism also grow on $C_2$ compounds with the participation of isocitrate lyase, the finding that in *Pseudomonas* MA the two enzymes are not identical leads to the conclusion that organisms may exist that can grow on $C_1$ compounds, using isocitrate lyase, but are unable to grow on $C_2$ compounds.

## ACKNOWLEDGMENTS

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