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RAPID PURIFICATION OF PIG HEART FUMARASE BY GENERAL LIGAND CHROMATOGRAPHY

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A rapid method for the purification of fumarase from pig heart muscle has been developed using general ligand chromatography with adenosine triphosphate as ligand. Fumarase exhibited distinctive elution patterns from several types of nucleotide–agarose matrices, which may prove of value in distinguishing putative isozymic forms. Fumarase purified from both soluble and particulate fractions of cardiac tissue appeared to be identical in terms of subunit molecular weight, electrophoretic mobility on cellulose acetate, substrate kinetics, and inactivation by several inhibitors. When fresh cardiac tissue was suspended in sucrose medium, both fumarase and citrate synthase were released from the mitochondria to about the same extent (10%). However, fumarase release was increased approximately three-fold, while the release of citrate synthase increased only slightly, when tissue which had been frozen and thawed was suspended in sucrose medium.

INTRODUCTION

Fumarase is a Krebs cycle enzyme (E.C. 4.2.1.2.) which has been reported to exist both in the mitochondria and in the cytosol of liver (1) and brain tissue (2). Several laboratories have reported that pig heart fumarase exists in multiple forms as detected by gel electrophoresis and ion-exchange chromatography (3–5). It is not known whether these forms represent mitochondrial and cytosolic isozymes, since there are no reports of a direct comparison of fumarase isolated from these cellular compartments.

Most of the procedures for the purification of fumarase involve homogenization of pig heart muscle followed by precipitation of the enzyme at low pH (6,7), followed by a series of ammonium sulfate precipitations (6–8). Final purification is achieved by repeated recrystallization of the enzyme over a 1–2 week period from ammonium sulfate solutions. A purification method for pig heart and pig liver fumarase using pyromellitic acid as an affinity ligand has been reported recently (9). L-Malate was used to elute fumarase along with 6–7 other proteins. Final purification was achieved by crystallization by dialysis against ammonium sulfate solutions.

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During a series of experiments originally designed to compare pig heart mitochondrial fumarase and the putative cytosolic fumarase, we have developed a rapid method of purification using general ligand chromatography with ATP-agarose. We chose ATP as a ligand based upon reports that it was an inhibitor for fumarase (10). In this method, there is no need for a crystallization step following the affinity chromatography. In addition, the enzyme exhibits characteristic elution patterns from several types of nucleotide-agarose matrices which may prove of value in distinguishing isozymic forms.

MATERIALS AND METHODS

Whole pig hearts were provided by Owens Country Sausage, Inc. Commercial pig heart fumarase was obtained from Boehringer Mannheim. Reagents for acrylamide gel electrophoresis were obtained from Eastman; coenzyme A, acetyl phosphate, acetate kinase, and the affinity column matrix materials were obtained from P.L. Biochemicals. Type 2 agarose-hexanenucleotide has the nucleotide moiety bound to the agarose through the adenine N^6 amino group using a six-carbon spacer. Type 4 agarose-hexanenucleotide has had the nucleotide ribose moiety oxidized and then reacted with adipic acid hydrazide, the agarose binding the nucleotide moiety through the ribose hydroxyl groups. Acetyl-coenzyme A was prepared from acetic anhydride and coenzyme A by the method of Simon and Shemin (11).

Fumarase activity was measured by the method of Hill and Bradshaw (12), lactate dehydrogenase activity was measured by the method of Stolzenbach (13), and citrate synthase activity by the method of Srere et al. (14). All enzyme units are expressed as μmoles substrate converted per min at 25°C. Specific activities are reported as units/mg protein. Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard. The molar extinction of fumarate was considered to be 1.45 mM^{-1} cm^{-1} at 250 nm (16).

The Lineweaver and Burk method was used to determine K_m values (17). The rate of fumarase reaction was determined for six substrate concentrations ranging from 0.2 to 1.0 mM fumarate, and 0.5 to 20.0 mM L-malate. The best double reciprocal line was determined by linear regression analysis of the data.

Sodium dodecyl sulfate (SDS) disk gel electrophoresis was performed using the method of Laemmli (18). Two-dimensional gel electrophoresis was carried out according to the method of O'Farrell (19) as modified by Henslee (20). Cellulose acetate electrophoresis was performed on a Beckman Microzone apparatus, using the citrate-phosphate buffer of Tolley and

Craig (21) at pH 8.0. Samples were run at 100V for 1.5 h. Fumarase activity was stained with a solution containing 0.4 M fumarate, 5 mM NAD, 0.3 mg/ml nitroblue tetrazolium, 0.013 mg/ml phenazine methosulfate, 0.05 mg/ml of malate dehydrogenase (Boehringer Mannheim 720 u/mg) in 0.1 M Tris-Cl, pH 7.5.

Purification

Hearts were placed in crushed ice immediately after excision from freshly slaughtered animals and were transported immediately to the laboratory. Left ventricular muscle was cleaned of connective tissue, cut into small cubes and frozen until used. The following steps were performed at room temperature since fumarase has been reported to be cold labile (3,4).

Thawed pig heart muscle was passed through a meat grinder and weighed. To extract cytosolic constituents, 5 ml of 0.25 M sucrose, adjusted to pH 7 as necessary with a 10 N NaOH solution, was added per gram of tissue. The suspension was centrifuged at 20,000 *g* for 10 min, the supernatant solution being designated herein as the "cytosol-containing" fraction and the pellet as the "mitochondria-containing" fraction. The 20,000-*g* supernatant solution was recentrifuged for an additional hour at 100,000 *g*. Virtually all (96–100%) of the extracted fumarase activity remained in the 100,000 *g* supernatant solution.

The cytosol-containing fraction was brought to 50% saturation with solid ammonium sulfate,[1] stirred 15 min, and centrifuged as above. The supernatant solution then was brought to 65% saturation with ammonium sulfate, stirred 30 min, and centrifuged at 20,000 *g* for 20 min. The pellet was resuspended in a volume of 10 mM Tris-Cl, pH 8.1, equal to 1/60 the volume of the 65% ammonium sulfate supernatant solution. The suspension then was brought to 35% saturation with ammonium sulfate. The amount of ammonium sulfate in the resuspended pellet was estimated by assuming the volume increase above the volume of buffer added to be contributed by 65% saturated ammonium sulfate (12). The suspension was stirred and centrifuged as above, and the supernatant solution brought to 55% saturation with ammonium sulfate.

The original mitochondria-containing pellet was homogenized in a Waring blender in 3 ml of 0.05 M Na-barbital buffer, pH 8.3, per gram of original ground muscle. Homogenization was carried out for four 30-sec bursts with the temperature maintained between 20 and 25°C. The homogenate was centrifuged as above, the supernatant solution made to 40% saturation with solid ammonium sulfate, and centrifuged again. The supernatant solution was then taken to 65% saturation with ammonium sulfate,

[1]Ammonium sulfate concentrations were calculated assuming 0°C.

centrifuged, and the pellet resuspended in a volume of 10 mM Tris-Cl, pH 8.1, equal to 1/20 the volume of the 65% ammonium sulfate supernatant solution. The suspension was brought to 30% saturation with ammonium sulfate, centrifuged, and the supernatant solution brought to 55% saturation with ammonium sulfate.

The supernatant volumes were measured after centrifugation of the 55% ammonium sulfate pellets obtained from both the cytosol-containing and mitochondria-containing fractions. Each pellet was then resuspended in 3/4 of this volume, using 10 mM Tris-Cl, pH 8.1. The suspensions were brought to 45% saturation with ammonium sulfate and centrifuged. Each supernatant solution was brought to 60% saturation with ammonium sulfate, centrifuged, and the supernatant volumes were measured. Each resulting pellet was dissolved in 1/8 of this supernatant volume, using 10 mM Tris-Cl, pH 8.1.

The two solutions were each passed through a Sephadex G-25 desalting column which had been equilibrated in 10 mM Tris-Cl, pH 8.1. The most active fractions eluted from this column were pooled and passed through an affinity column of type 4 ATP-agarose which had been equilibrated with the same buffer. After the column was washed with 15 mM Tris-Cl, pH 8.1, the fumarase was specifically eluted with 5 mM Na-fumarate, pH 7.5. Fractions from the affinity column were collected directly into tubes containing a volume of saturated ammonium sulfate such that the final fractions in the tubes were approximately 10% saturated with ammonium sulfate. The column was washed with 2 M NaCl following elution, and the ligand was regenerated as necessary with a solution of acetyl phosphate, acetate kinase, and $MgCl_2$, using the procedure of Lindberg and Mosbach (22).

RESULTS AND DISCUSSION

The efficiency of cytosol extraction in 0.25 M sucrose, pH 7.6, was determined by monitoring lactate dehydrogenase activity (as a marker for cytosol) and citrate synthase (as a marker for mitochondrial matrix) in whole heart homogenate, sucrose supernatant solution, and sucrose pellet homogenate. It is expected that release of any cytosolic fumarase from the cell would parallel that of lactate dehydrogenase since their molecular weights are similar; 194,000 (23) and 142,000 (24), respectively. It was found that 1.5 h of stirring in a fivefold volume of sucrose solution was sufficient to release about 90% of the cytosolic lactate dehydrogenase of the cells with only about 5% of the mitochondrial citrate synthase released (Table 1). The activity of fumarase did not decrease even after 2.5 h of sucrose treatment at

TABLE 1. **Effect of Duration of Stirring on Release of Cytosolic and Mitochondrial Enzymes**[a]

Time (h)	0.5	1.5	2.5
Activity (units/ml) of:			
Citrate synthase in cytosolic fraction	60	50	50
Citrate synthase in mitochondria-containing pellet	1140	893	857
Lactate dehydrogenase in cytosolic fraction	2960	3010	3180
Lactate dehydrogenase in mitochondria-containing pellet	774	336	416
% Cytosol marker in mitochondria-containing pellet	21	10	11
% Mitochondrial matrix marker in cytosolic fraction	5	5	6

[a]Three 20-g portions of ground pig heart tissue were stirred at room temperature with 100 ml of 0.25 M sucrose, pH 7.6. One portion was taken at each time indicated, centrifuged at 20,000 g for 10 min, and the volume of supernatant solution ("cytosol-containing") measured. The pellet ("mitochondria-containing") was homogenized in 80 ml of 0.05 M Na-barbital, pH 8.3, for two 30-sec bursts. The homogenate was then centrifuged at 20,000 g for 10 min, and the volume of supernatant solution measured. Enzyme activities in the supernatant solutions were assayed as described in the text.

room temperature. It was expected that the release of fumarase from the mitochondria would not be significantly greater than the release of citrate synthase [mol. wt. 100,000 (25)] under these conditions of sucrose treatment. In studies performed with mitochondria made permeable by toluene treatment, leakage of fumarase from the mitochondria was actually less than leakage of citrate synthase (26).

A number of affinity materials were examined for possible use in the purification of fumarase. Figure 1 shows the fumarase elution patterns of cytosolic and mitochondrial preparations passed through 1-ml columns of type 4 ATP-agarose, type 4 ADP-agarose, type 4 AMP-agarose, and type 2 AMP-agarose. The behavior of fumarase isolated from the cytosol-containing fraction and the mitochondria-containing pellet is very similar on each of the five affinity columns. However, the elution patterns differ considerably from ligand to ligand. Adenosine triphosphate has been reported to be a strong inhibitor of pig heart fumarase, ADP has been reported to be a much poorer inhibitor, and AMP has been reported not to inhibit the enzyme (10). The strength of fumarase binding to the type 4 nucleotide-agaroses followed this inhibitory pattern. However, type 2 AMP-agarose was found also to bind fumarase. On the basis of these preliminary experiments (Fig. 1), type 4 ATP-agarose was chosen for routine purification of pig heart fumarase.

The result of a typical purification procedure is shown in Table 2. Total yield of enzyme from the ATP-agarose column was approximately 100%, but since only the fractions with the highest activities were pooled, the overall yield is diminished. However, this step is much faster than the

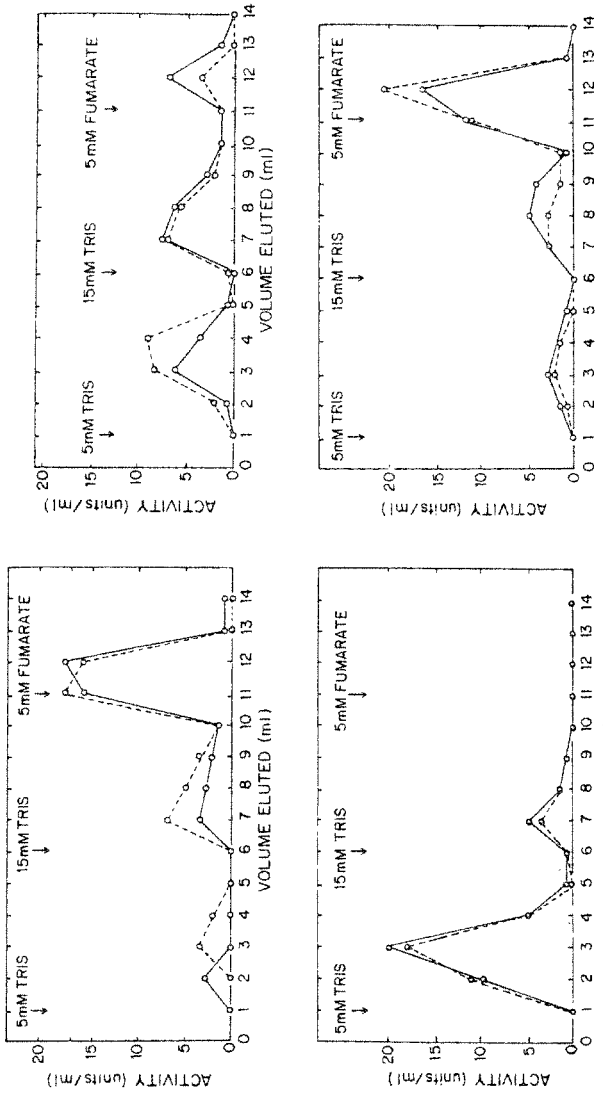

FIG. 1. Elution of "mitochondrial" and "cytosolic" fumarase from nucleotide-agarose columns. One-milliliter samples (approximately 50 fumarase units) of homogenized muscle were dialyzed for 3 h against 5 mM Tris-Cl, pH 8.1, and then were passed through 1-ml columns of nucleotide-agarose, which had been equilibrated with the same buffer. One-milliliter samples were collected and assayed for fumarase activity as described in the text. The eluting buffers used are indicated at the arrows. O——O, fumarase from mitochondrial-containing fraction; O- - - -O, fumarase from cytosol-containing fraction. Column materials were (a, upper left) type 4 ATP-agarose, (b, upper right) type 4 ADP-agarose, (c, lower left) type 4 AMP-agarose, (d, lower right) type 2 AMP-agarose.

TABLE 2. **Purification of Pig Heart Fumarase**[a]

Step and fraction	Volume (ml)	Activity (U/ml)	Total activity ($U \times 10^{-3}$)	Protein (mg/ml)	Specific activity (U/mg)	% Yield	Purif.
Cytosol-containing fraction							
Sucrose supernatant solution	895	1.4	1.25	3.3	0.4	100	1
50–65% $(NH_4)_2SO_4$ precipitate	22	54.4	1.20	25.5	2.1	96	5
35–55% $(NH_4)_2SO_4$ precipitate	17	67.4	1.14	22.8	3.0	91	7
45–60% $(NH_4)_2SO_4$ precipitate	5	197.6	0.99	37.3	5.3	80	13
Pooled fractions of Sephadex G-25	7	134.6	0.94	—	—	75	—
Pooled fractions of ATP-agarose	4	160.7	0.64	1.3	119.9	51	300
Mitochondria-containing pellet							
Homogenate supernatant solution	400	3.9	1.56	11.4	0.3	100	1
40–65% $(NH_4)_2SO_4$ precipitate	26	65.2	1.67	31.5	2.1	107	7
30–55% $(NH_4)_2SO_4$ precipitate	21	73.3	1.54	19.7	3.7	99	12
45–60% $(NH_4)_2SO_4$ precipitate	6	237.9	1.59	45.4	5.2	101	17
Pooled fractions of Sephadex G-25	8	162.8	1.30	—	—	83	—
Pooled fractions of ATP-agarose	4	208.0	0.82	1.6	130.0	52	433

[a]Data shown for 185 g of ground pig heart tissue.

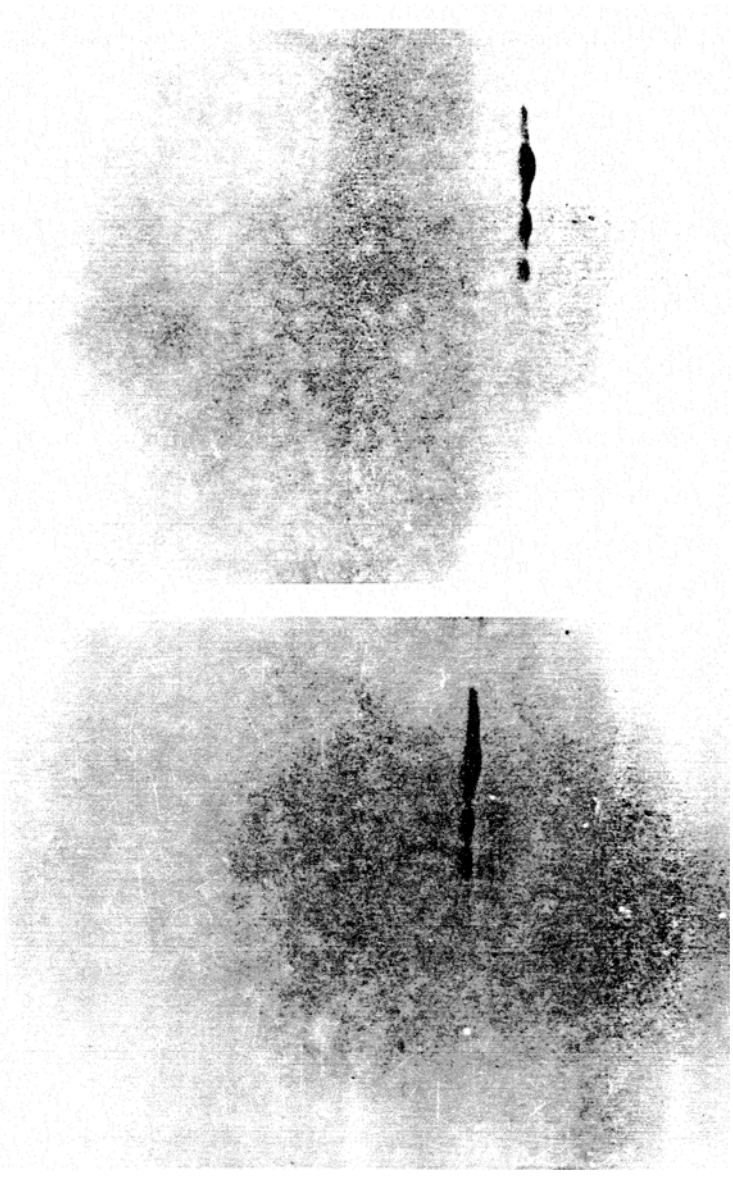

FIG. 2. Two-dimensional gel electrophoresis of fumarase from mitochondria-containing (a, left) and cytosol-containing (b, right) fractions. Isoelectric focusing was performed in the first dimension (left to right in the figure) with an ampholine composition of 1.3%, pH 5–8; 0.6%, pH 9–11; and 0.1%, pH 3.5–10. The second dimension (top to bottom in the figure) was performed on a 8–13% exponential acrylamide gradient slab gel. The pH increases left to right.

multiple recrystallizations from ammonium sulfate solution used by others. Fumarase from both the cytosol-containing fraction and the mitochondria-containing pellet behaved similarly throughout the procedure, with both preparations having a final yield of about 50% and a specific activity of about 120–130 units/mg. This corresponds to a specific activity of about 30,000 "enzyme units"/mg calculated by the method of Hill and Bradshaw (12).

Electrophoresis of both pure preparations on 5% acrylamide gels showed similar broad protein regions with multiple overlapping protein bands. Two-dimensional gel electrophoresis was performed to further assess this heterogeneity. Figure 2 shows that both preparations of fumarase exhibited similar electrophoretic heterogeneity, with each component band exhibiting the same sub-unit molecular weight. Other workers have reported that multiple pig heart fumarase fractions separated by ion-exchange chromatography have identical molecular weights by sucrose density centrifugation (3). One-dimensional SDS gel electrophoresis with marker proteins showed a sub-unit molecular weight of 52,600 for each preparation (Fig. 3). This compares with the value of 48,500 reported by other workers (3,23).

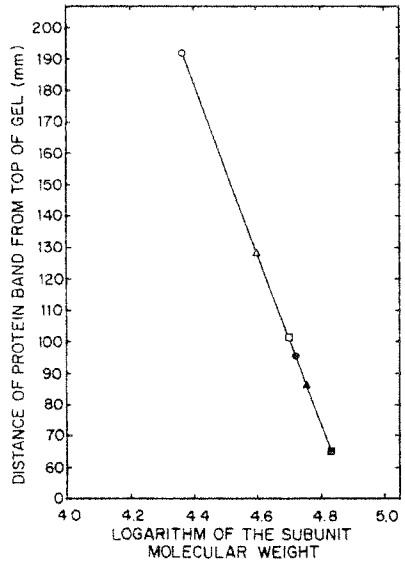

FIG. 3. Subunit molecular weight of fumarase. SDS acrylamide gel electrophoresis was performed as referenced in the text, using 10% acrylamide gel and the following proteins (subunit mol. wt.): ○, myokinase (23,000); △, aldolase (40,000); □, citrate synthase (50,000); ●, "cytosolic" fumarase; ▲, glutamate dehydrogenase (57,000); ■, bovine serum albumin (68,000). Commercial fumarase migrated with an R_f identical to those preparations from mitochondria-containing and cytosol-containing fractions (not shown).

FIG. 4. Cellulose acetate electrophoresis of "mitochondrial" and "cytosolic" fumarase. Experimental details are given in the text. (a, left) Wells from left to right contained fumarase purified from cytosol-containing fraction, fumarase purified from mitochondria-containing fraction, fumarase from both fractions mixed 1 : 1, fumarase from unfractionated cells, and fresh low-speed supernatant fraction from crude pig heart homogenate. (b, right) Wells from left to right contained fresh low-speed supernatant fraction from crude pig heart homogenate, corresponding high speed supernatant fraction, low speed supernatant fraction diluted 1 : 1 buffer, and a 1 : 1 mixture of fumarase purified from the cytosol-containing and mitochondria-containing fractions.

Fumarase purified from the cytosol-containing fraction and the mitochondria-containing fraction, and fumarase which had been purified as described above but without the initial sucrose step, all gave similar activity-stain patterns on cellulose acetate electrophoresis (Fig. 4a). A supernatant solution obtained from a low speed centrifugation of freshly homogenized pig heart tissue showed an activity band with a lower R_f value (Fig. 4a), but the R_f increased to that of the purified enzyme when the solution was recentrifuged at a higher speed or was diluted with buffer (Fig. 4b). Commercially obtained fumarase also migrated with the same Rf as the purified enzymes (data not shown). The kinetics of the purified preparations were examined and compared with those of a commercial preparation of pig heart fumarase. With the reaction catalyzed in either direction, the K_m's of the substrates for fumarase were the same regardless of the enzyme source (Table 3). The effects of several known inhibitors were also examined (Table 4). Fumarase purified from both the cytosol-containing fraction and the mitochondria-containing pellet were similarly inhibited under the conditions examined.

The similarity of the physical and kinetic properties of fumarase from the cytosol- and mitochondria-containing fractions prompted us to examine more closely the release of the enzyme from the mitochondria. In additional experiments we have found that the amount of fumarase released into the cytosol-containing fraction from fresh pig heart tissue is only about 10% of the total fumarase, compared to the 30–40% found in the cytosol-containing fraction prepared from frozen pig heart tissue. At the same time, no difference in the release of LDH (a cytosol marker), creatine kinase (a mitochondrial intermembrane space marker), or citrate synthase (a mitochondrial matrix marker) was observed between extracts of fresh or frozen

TABLE 3. **K_m Values of Purified Pig Heart Fumarase**

Source	K_m L-malate → fumarate[a] (mM)	K_m Fumarate → L-malate (mM)
Cytosolic	2.6 ($R = 0.999$)	0.57 ($R = 0.999$)
Mitochondrial	2.3 ($R = 0.997$)	0.49 ($R = 0.995$)
Commercial[b]	2.1	0.67
Literature[c]	3.0	0.74

[a]The enzymes were dialyzed against 33.3 mM potassium phosphate buffer, pH 7.5. Substrate solutions were adjusted to pH 7.5. Rates were measured at 25°C. The K_m values were determined as described in the text. Coefficients of correlation (R) for the best-fit regression line are in parentheses.
[b]Boehringer Mannheim.
[c]Ref. 16.

pig heart tissue. These results could be explained either by preferential binding of a cytosolic fumarase to an insoluble structure in the fresh tissue extract or by preferential binding of both creatine kinase and citrate synthase to insoluble components in the frozen tissue extract. At present, therefore, we can come to no conclusion about the putative existence of a cytosolic fumarase in pig heart.

In any case, the fumarase purified from both fractions of the extracted frozen tissues proved to be identical enzymes in terms of size, electrophoretic mobility, and kinetic properties. It should be noted that the results of Beeckmans and Kanarek (9) on the pure fumarase from total extracts of pig heart and pig liver showed the existence of only one enzyme form.

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