PURIFICATION AND CHARACTERIZATION OF ALDOLASE FROM *AMBYSTOMA TIGRINUM*

THESIS

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By

Dorothy J. Woolever, B. S.
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The muscle aldolase from Ambystoma tigrinum has been purified 73-fold to a final specific activity of 13.2 units per mg. The purified enzyme appeared to be homogenous by ultracentrifugation and electrophoretic criteria.

A molecular weight of 159,000 ± 1000 was determined by gel filtration on Sephadex G-200 and high speed sedimentation equilibrium ultracentrifugation. The enzyme migrated identically with rabbit muscle aldolase when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and is apparently a tetramer of nearly identical subunits of approximately 40,000 MW.

The catalytic constants of the salamander enzyme were similar to those reported for other muscle aldolases with the exception of the unusually low Fru-P_2/FlP ratio.

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INTRODUCTION

Aldolase was first defined in 1934, by Meyerhof and Lohman, as an activity that catalyzes the aldyl cleavage condensation reactions involving dihydroxyacetone phosphate (DHAP) and a variety of aldehydes (Meyerhof and Lohman, 1934). Using muscle and yeast extracts, they noted that three reactions were catalyzed with the highest efficiencies:

(1) DHAP + D-glyceraldehyde 3-P ⟶ Fructose 1,6-bisphosphate

(2) DHAP + D-glyceraldehyde ⟶ Fructose-1-phosphate

(3) DHAP + D-erythrose 4-P ⟶ Sedoheptulose-7-phosphate

They further observed that DHAP was the favored product in reaction (1) and concluded that the total conversion was the result of the concerted action of two independent catalysts (i.e., Fructose 1,6-bisphosphate ⟶ DHAP + glyceraldehyde 3-P, and glyceraldehyde 3-P ⟶ DHAP) (Meyerhof et al., 1936). In 1940, Herbert and coworkers extensively purified aldolase from rabbit muscle (free of triosephosphate isomerase activity) and clearly established aldolase as a distinct activity (Herbert et al., 1940).

The first indication of the existence of different types of aldolase came from the work of Warburg and Christian. They crystallized aldolase from rat muscle and from

yeast and noted that, although the muscle enzyme was unaffected by chelating agents, the yeast enzyme was strongly inhibited by such agents. The inhibition was reversible upon the addition of certain divalent metal cations (Warburg and Christian, 1943). As work on various aldolases progressed, two distinct types of the enzyme emerged, denoted Class I and Class II aldolases (Rutter et al., 1968). Class I aldolases are found predominantly in "higher organisms". i.e., animals, plants, protozoa, and algae. They are tetramers with molecular weights from 120,000 to 160,000 (Kawahara and Tanford, 1966; Fluri et al., 1967; Sia and Horecker, 1968; Alarcon et al., 1971). Class I aldolases are less sensitive to sulfhydryl-binding reagents than are the Class II enzymes, exhibit a broad pH optimum, and form a Schiff base intermediate with their substrate (Grazi and Horecker, 1962).

Class II aldolases, on the other hand, are found in bacteria, fungi and blue-green algae. These enzymes are dimers with molecular weights of 70,000 to 80,000, they require Zn^{2+} or other divalent metal ions for catalysis and therefore are inhibited by chelating agents. The Class II enzymes exhibit a sharp pH optimum, are stimulated by potassium or ammonium ions, and are readily inhibited by reagents which react with sulfhydryls (Rutter et al., 1968).

Isozymes of Class I aldolases have been found in vertebrate tissues (Penhoet et al., 1966; Masters, 1967; Rutter

et al., 1968; Lebherz and Rutter, 1969; and Kochman et al., 1971) and the Class I aldolases have been subdivided into isozyme types A, B, and C. The three isozymes of the Class I enzymes are distinguished, primarily, by their electrophoretic properties, kinetic properties, and tissue origin. Isozyme A is sometimes designated as the muscle enzyme; isozyme B is found predominantly in liver and kidney (Rajkumar, 1967), and isozyme C occurs predominantly in neural tissues (Penhoet et al., 1966). Five membered, A-B and A-C sets have been detected in mammalian tissues (Rutter et al., 1968; Penhoet et al., 1966; and Lee, 1974). B-C hybrids have been found in early embryonic chick liver and yolk sac membranes (Lebherz et al., 1972). Transitions from A to B or from A to C aldolases have been noted in developing fetal tissues (Baron, 1966; Burton, 1974). Apparently a "reverse transition" occurs in hepatomas in which the aldolase activity has been chemically, physically, and kinetically identified as isozyme A (muscle type) rather than the isozyme B of normal liver (Brox et al., 1968; Gracy, 1969).

Aldolase has been isolated and characterized from numerous organisms including rabbit (Taylor et al., 1948), boa constrictor (Schwartz and Horecker, 1966), chicken (Herskovits et al., 1967; Marquardt, 1969), pig (Anderson et al., 1969), beef (Anderson et al., 1969), rat (Ikehara et al., 1970), lobster (Guha et al., 1971), frog (Ting

et al., 1971), shark (Caban and Hass, 1971), codfish (Lai et al., 1971), Ascaris suum (Dedman et al., 1972; Kochman and Kwiatkowska, 1972), sturgeon (Anderson, 1972), human heart (Allen et al., 1972) and others. These muscle aldolases appear to be homologous proteins (derived from a common ancestral gene) in that they exhibit many similarities in amino acid composition, functional properties, and active site sequences. All of these enzymes are tetramers in which the amino terminus is proline and the carboxyl terminus is tyrosine. All of these enzymes form a Schiff base between the carbonyl of the substrate and the ε-amino group of a lysine in the active site. Catalytic properties are similar, with the Michaelis constants for Fru-P_2 ranging from 1-3 x 10^{-6} to 6 x 10^{-5} M. FruP_2/F-1P, V_{max} ratios range from 40-50:1 for the rabbit enzyme to 75:1 for the codfish enzyme. Although the total amino acid compositions vary from species to species, it is now apparent that the amino acids involved in catalysis (those of the active site) are highly conserved (Ting et al., 1971).

The homologies that are present in congruent sequences of aldolases from different species are reminiscent of those observed in the cytochromes c for which the amino acid sequences of some 37 species have now been determined (Dayhoff, 1972). The comparative studies on the amino acid sequences of the cytochromes c have not only provided

information on the probable roles that some of the amino acids play in the function and the conformation of the protein, but also have afforded insight into the trends in protein evolution (Smith, 1966; Dickerson and Geis, 1969). The aldolases present an analgous system for study. It was, therefore, the purpose of this investigation to purify the muscle aldolase from the urodelea amphibian, Ambystoma tigrinum, and to determine the physicochemical and the catalytic properties of the enzyme.

MATERIALS AND METHODS

Materials

Reduced nicotinamide adenine dinucleotide (NADH), fructose-1-phosphate, a mixture of α-glycerophosphate dehydrogenase (specific activity 130 units/mg) and triose phosphate isomerase (specific activity 1080 units/mg), and fructose 1,6-bisphosphate (Fru-P_2), purchased from Sigma. Rabbit muscle aldolase, pig heart malic dehydrogenase, horse heart cytochrome C, pig heart fumarase, blue dextran, ovalbumin, and cellulose phosphate (coarse, exchange capacity, 0.94 meq/mg) were also products of Sigma. Sephadex G-200 was obtained from Pharmacia. "Sequenal grade" guanidinium chloride was a product of Pierce Chemical Company. Carrier ampholines were products of LKB. Electrophoresis grade acrylamide, Bis-acrylamide, TEMED, ammonium persulfate, sodium dodecyl sulfate and coomassie brilliant blue were purchased from Bio-Rad Laboratories. All other chemicals used were of the highest purity commercially available.

Methods

Enzyme Assay

Aldolase was assayed spectrophotometrically by monitoring the oxidation of NADH at 340 nm. The method used was a modification of that of Blostein and Rutter (1963).

Routine assays were run at room temperature in a 3.0 ml volume. The reaction mixture contained 0.75 micromoles of NADH, 3.0 micromoles of Fru-P_2, 10 micrograms each of triose phosphate isomerase and α-glycerophosphate dehydrogenase, and 130 micromoles of triethanolamine buffer at pH 7.8. Assays were run at two different enzyme concentrations and the initial velocity values were averaged.

Kinetic studies were carried out in 1.0 ml cuvettes at 30°C with the recorder scale expanded to 0.1 A. The reaction mixture contained 50 micromoles of triethanolamine, 0.15 mg of NADH, and 10 micrograms each of triose phosphate isomerase and α-glycerophosphate dehydrogeanse. The range of substrate concentrations for Fru-P_2 was 1×10^{-4} to 1×10^{-6} M. Fructose-1-phosphate concentrations ranged from 1×10^{-3} to 1×10^{-1} M. Most experiments were repeated three to four times and the initial velocities were averaged. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reaction of one micromole of substrate per minute under initial velocity conditions at 30°C. The specific activity is expressed as units of enzyme per milligram of protein.

Protein Determination

Protein concentration in fractions eluted from columns was estimated spectrophotometrically from their absorbance at 280 nm. Protein in crude preparations was determined by the Lowry method (Lowry et al., 1951). Protein concentration

of the purified enzyme was determined spectrophotometrically using the extinction coefficient ($\varepsilon_{280}^{1\%}$) for Ambystoma tigrinum aldolase of 12.0 (vide supra).

Ion Exchange Chromatography

Cellulose phosphate was prepared according to the procedure of Peterson and coworkers (1962), and equilibrated with 10 mM triethanolamine, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.8 (hereafter referred to as TEA buffer). The columns were packed at room temperature at an adjusted flow rate of 1.5 to 2.0 ml per minute. Columns were placed in a coldbox at 4°C and washed with several void volumes of TEA buffer). The columns were packed at room temperature at an adjusted flow rate of 1.5 to 2.0 ml per minute. Columns were placed in a coldbox at 4°C and washed with several void volumes of TEA buffer at a flow rate of 1.0 ml per minute. Eluant was checked for proper pH before use. All chromatography was done at 4°C and constant flow rates were maintained by hydrostatic pressure.

Gel Filtration

Sephadex G-200 (particle size 40-120 microns) was swollen and equilibrized in several changes of 50 mM sodium phosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.2. A 2.5 x 35 cm column was packed under 10 cm of hydrostatic pressure. The column was washed with buffer at 4°C until the bed height stabilized and a constant flow rate of 6 ml

per hour was obtained. The void volume (V_o) and elution volume (V_e) were determined with blue dextran and standard proteins, respectively. All samples were made 20% in sucrose (w/v). The elution volumes were correlated with molecular weights (Andrews, 1964).

Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the procedure of Maizel (1971). Gels were run at pH 10.0.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were conducted by the method of Fairbanks et al, (1971) with the following modifications: the electrophoresis buffer was diluted 1:1, 5 mM 2-mercaptoethanol was used in all buffers, and the sample was boiled for one minute before dialysis.

Ultracentrifugation

Sedimentation velocity and sedimentation equilibrium experiments were conducted in a Beckman-Spinco Model E analytical ultracentrifuge equipped with RTIC temperature and electronic speed control. Samples were dialysed extensively in 0.025 M Tris, pH 8.0, 0.1 M NaCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA. Schlieren patterns and Rayleigh interference fringes were measured with a Nikon model 6-C microcomparator equipped with digital encoders. Sedimentation equilibrium studies were conducted by the meniscus

depletion method of Yphantis (1964) and Van Holde (1967). Densities and viscosities of buffers were calculated as described by Kawahara and Tanford (1966).

Amino Acid Analysis

One milligram samples were dialysed extensively against deionized water. Samples were made 6.0 N with respect to hydrochloric acid and hydrolyzed for 24 and 48 hours in sealed, evacuated tubes at 110°C (Moore and Stein, 1963). Norleucine was added as an internal standard prior to hydrolysis. Analyses were performed on a Beckman model 120 C automatic amino acid analyzer (Spackman et al., 1958). Cysteine was determined by titration with 5,5'-dithiobis (2-nitrobenzoic acid): 750 microliters of 20 mM triethanolamine, pH 8.5, 10 mM EDTA; and 200 micrograms of DTNB in 1 M Tris-HCL, pH 8.0, were added to 0.1 mg of enzyme. The reaction was monitored for an increase in absorbance at 412 nm for 30 minutes. After 30 minutes, 50 microliters of 10% SDS was added and the reaction was monitored to completion (Ellman, 1959; Habeeb, 1972).

Isoelectric Focusing

Isoelectric focusing was carried out according to Haglund (1970). A final concentration of 2% ampholines, pH 7-10, and 0.5% ampholines, pH 5-8, was present in the sucrose density gradient. Electrofocusing was conducted at 4°C for 84 hours. Fractions of 1.0 ml were collected

at a constant flow rate of 0.5 ml per minute, maintained by a peristaltic pump. The pH of each fraction was measured immediately at 4°C and subsequently each fraction was assayed for aldolase activity.

RESULTS

Isolation of Aldolase from Ambystoma tigrinum

Step I - Crude Extract

Three salamanders were decapitated, skinned, the muscle was removed, and placed in cold "homogenizing buffer" (20 mM triethanolamine, 0.1 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.8). All further procedures were carried out at 4°C. The muscle (77 g) was blotted dry, weighed and homogenized in three volumes of buffer in a Waring blendor. The homogenate was centrifuged for one hour at 35,000 rpm, and the supernatant was filtered through glass wool to remove lipid. This fraction is referred to as the crude extract.

Step II - Batch

The "crude extract" was immediately mixed with an excess of phosphocellulose which had been previously equilibrated with TEA buffer. The mixture was stirred for thirty minutes at 4°C, and then filtered. The phosphocellulose was washed with fresh buffer until the eluant was protein-free. The phosphocellulose cake was resuspended in TEA buffer containing 2.5 mM Fru-P_2, and again stirred for thirty minutes at 4°C. After filtering, the solution was dialysed against TEA buffer which had been saturated with ammonium sulfate, pH 8.0. The precipitated protein was

sedimented by centrifugation, resuspended in 10 ml of TEA buffer, and dialyzed against three-one liter changes of TEA buffer.

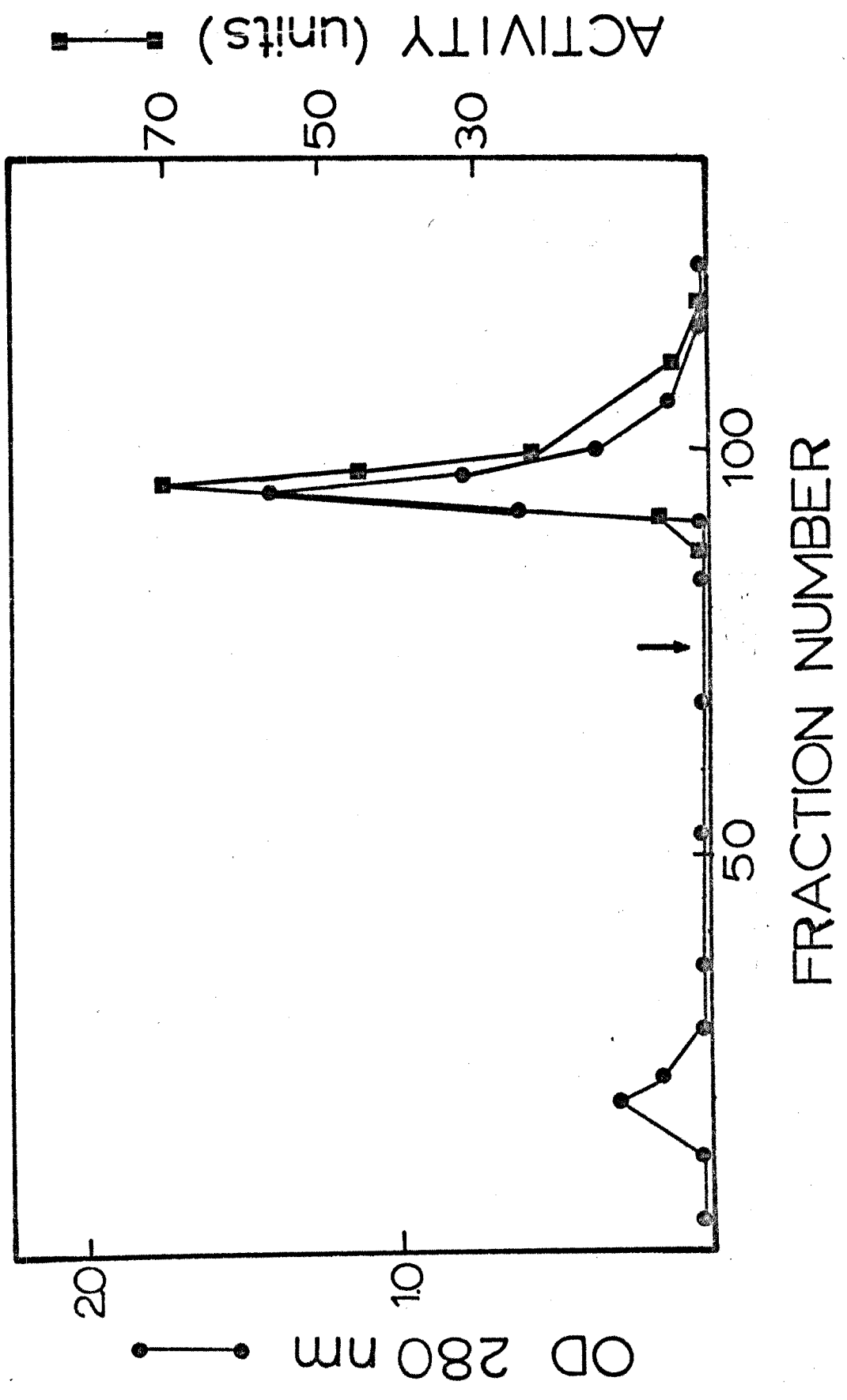
Step III - Cellulose Phosphate Chromatography with Substrate Elution

The dialyzed protein solution was applied to a 2.5 x 25 cm cellulose phosphate column which had been previously equilibrated with TEA buffer. The flow rate was maintained at 0.5 ml a minute and 5.0 ml fractions were collected. The column was washed with buffer until the eluant gave an absorbance at 280 nm of 0.02 or less. Substrate elution was accomplished by the application of 2.5 mM Fru-P_2 in TEA buffer to the column. Protein and activity were eluted simultaneously in fractions 92-101 (Fig. 1). The fractions were pooled and concentrated by ammonium sulfate precipitation. The aldolase isolated by this procedure was found to be homogeneous by ultracentrifugation analysis (vide infra). However, both polyacrylamide gel electrophoresis and SDS gel electrophoresis showed a minor band of protein which migrated slightly anodal to the aldolase. The enzyme preparation was submitted to isoelectric focusing, in an effort to remove the contaminating protein.

Step IV - Isoelectric Focusing

Isoelectric focusing was conducted by the method of Haglund (1970). The 0.5% ampholines, pH 5-8, were included

Fig. 1. Column chromatography of Ambystoma tigrinum muscle aldolase on cellulose phosphate. The dialyzed protein solution was applied to a cellulose phosphate column (2.5 x 25 cm). The column was washed with TEA buffer until an absorbance of 0.02 or less was obtained. The flow rate was maintained at 0.5 ml a minute and 5.0 ml fractions were collected. The enzyme was eluted with a solution of 2.5 mM Fru-P_2 in TEA^+ buffer. Arrow = application of 2.5 mM Fru-P_2.


ACTIVITY (units)
70
50
30
OD 280 nm
20
10
50
100
FRACTION NUMBER

to provide a buffer zone at the anode to prevent enzyme degradation. As depicted in Fig. 2 and 3 two peaks of activity were observed, one at pH 9.85, and the other at pH 9.90. Seventy-one percent recovery was obtained upon pooling fractions 20-32. At this stage of the purification, densitometer tracings of both polyacrylamide gels and SDS polyacrylamide gels showed the minor component to be eight percent of the total protein. The contaminant was determined (by enzymatic assay) to be glyceraldehyde-3-phosphate dehydrogenase. Isoelectric focusing was repeated, in a further attempt to remove this contaminant. Because the isoelectric points of glyceraldehyde-3-phosphate dehydrogenase and aldolase are similar (the pI of rabbit muscle G-3-PDH is 8.0-8.5, Susor et al., 1969), only fractions 22-26 were pooled, resulting in only a 23% recovery. However, the resulting enzyme preparation was determined to be free of contamination by SDS polyacrylamide gel electrophoresis (Fig. 4).

Table I shows the purification procedure. The final specific activity of 13.2 is similar to that of both the frog muscle aldolase, for which the specific activity is 11.5 (Ting et al., 1971), and the rabbit muscle enzyme, for which the reported value is 12 (Lai et al., 1965).

An extinction coefficient ($\varepsilon_{280}^{1\%}$) of 12.0 was determined for the muscle aldolase of Ambystoma tigrinum. A similar

Fig. 2. Isoelectric focusing of Ambystoma tigrinum muscle aldolase. The dialyzed enzyme (2-3 mg) was applied to a sucrose density gradient containing 2% ampholines, pH 7-10, and 0.5% ampholines, pH 5-8. Isoelectric focusing was performed at 4°C in the presence of 10 mM dithiothreitol. A voltage of 500 V was applied over a period of 84 hours. Fractions of 1.0 ml were collected at a constant flow rate of 0.5 ml per minute.

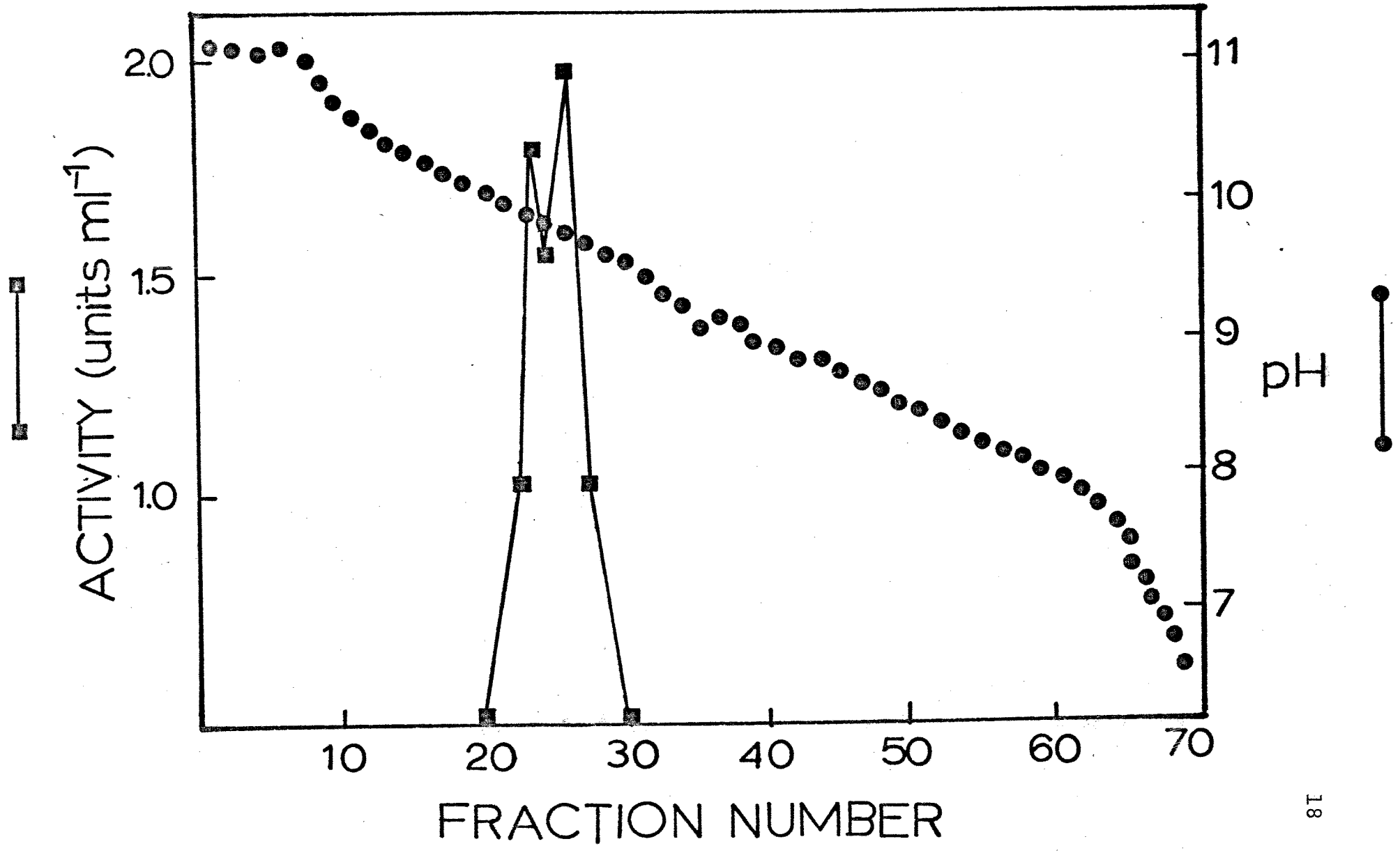

ACTIVITY (units ml^{-1})
2.0
1.5
1.0
11
10
9
8
7
pH
10
20
30
40
50
60
70
FRACTION NUMBER

Fig. 3. Isoelectric focusing of Ambystoma tigrinum muscle aldolase. The dialyzed enzyme (8 mg) was applied to a sucrose density gradient containing 2% ampholines, pH 7-10, and 0.5% ampholines, pH 5-8. Isoelectric focusing was performed at 4°C in the presence of 10 mM dithiothreitol. A voltage of 500 V was applied over a period of 84 hours. Fractions of 1.0 ml were collected at a constant flow rate of 0.5 ml per minute.

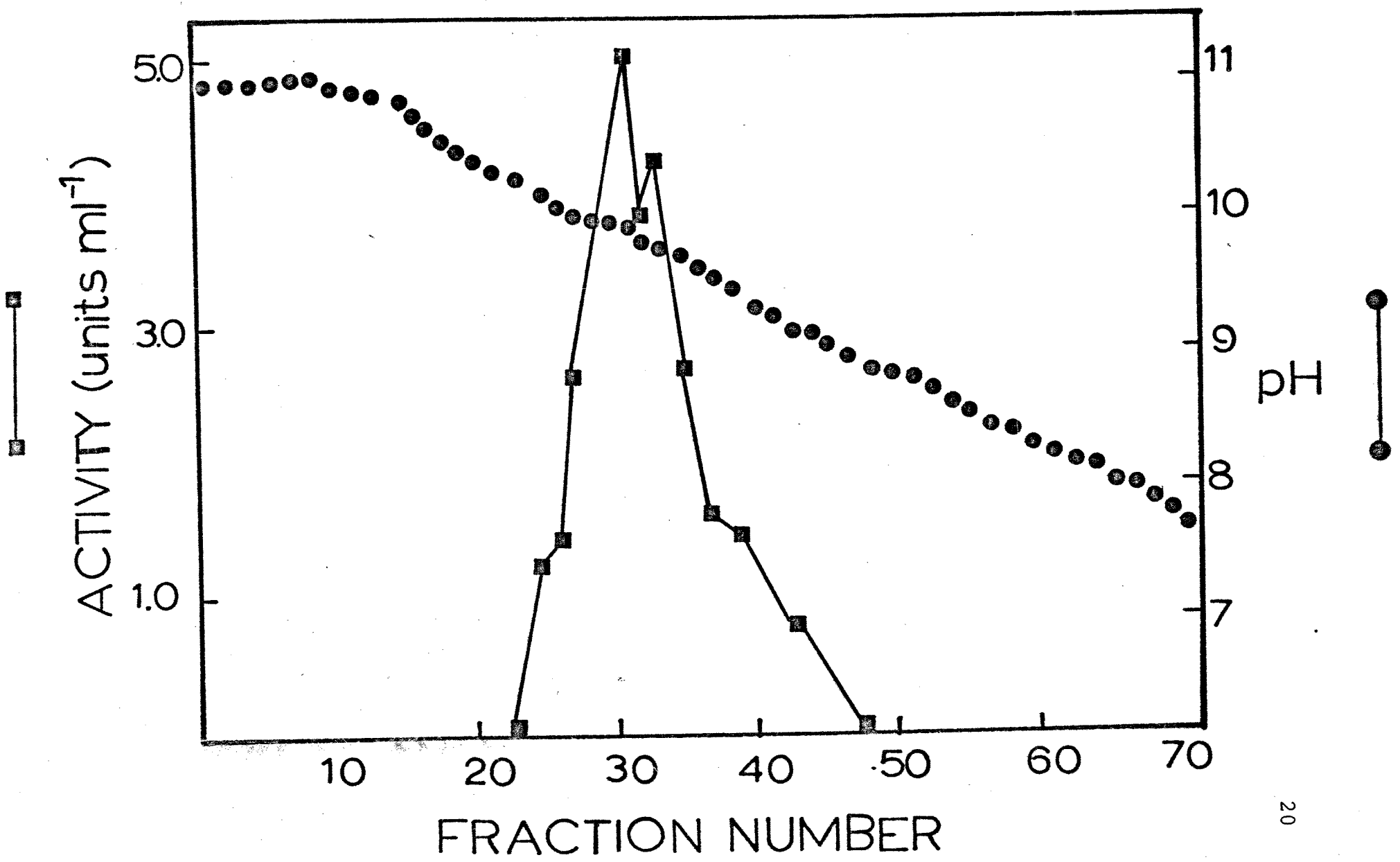

ACTIVITY (units ml^{-1})
5.0
3.0
1.0
11
10
9
8
7
pH
10
20
30
40
50
60
70
FRACTION NUMBER

Fig. 4. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of Ambystoma tigrinum muscle aldolase. Purified enzyme (25-50 micrograms) was applied to each gel. The gel on the left contains the enzyme before isoelectric focusing and the gel on the right contains the enzyme after isoelectric focusing.

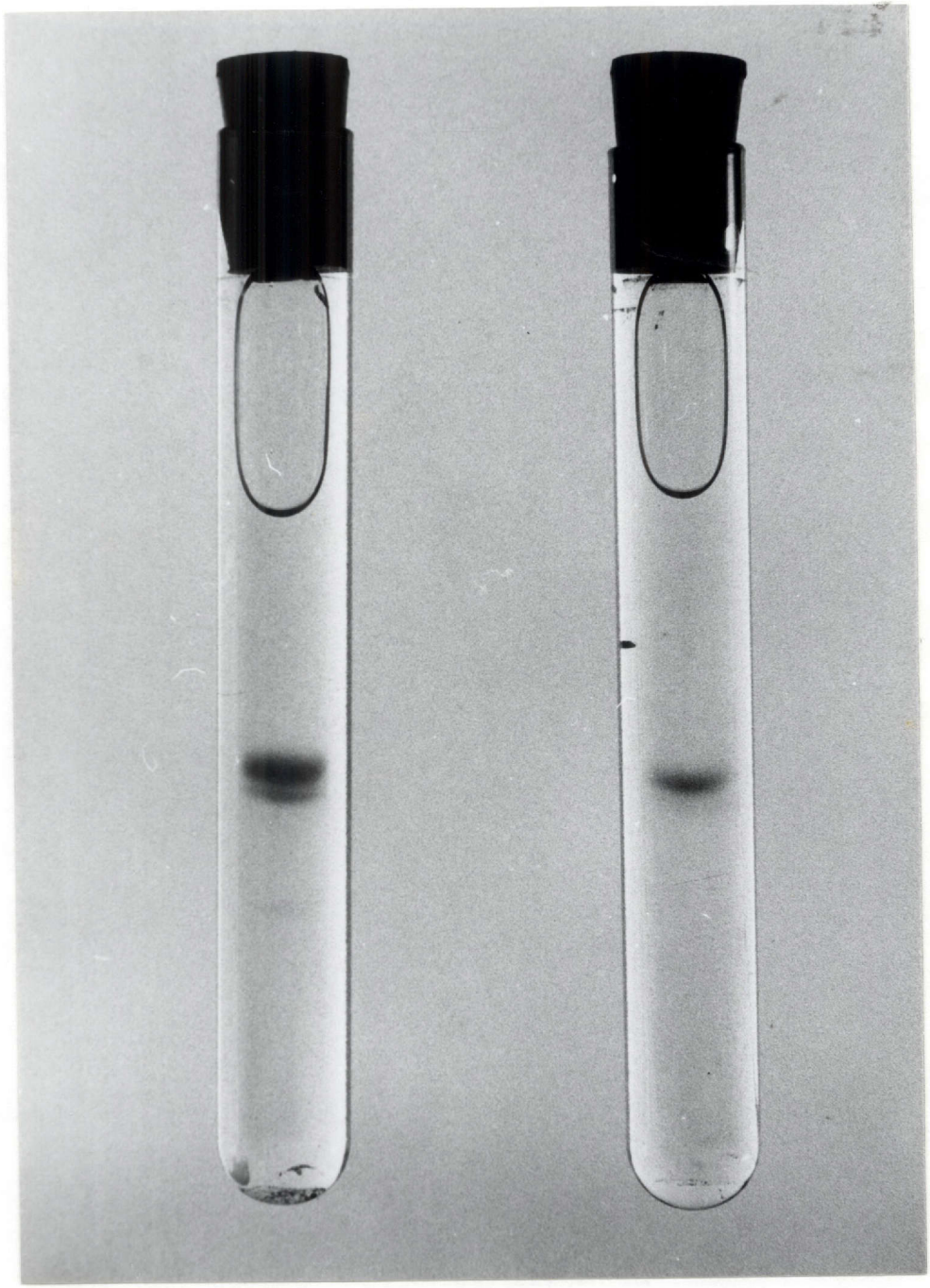


TABLE I

PURIFICATION OF *AMBYSTOMA TIGRINUM* MUSCLE ALDOLASE

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification	Recovery %
Crude Extract	5671.0	1012	0.18	---	100
Batch Eluate	270	868	3.20	17.8	86
Phosphocellulose Column	40.8	527	12.9	71.6	52
Isoelectric Focusing	18.0	237.6	13.2	73.3	23.5

value of 11.2 has been obtained for the lobster muscle enzyme (Guha et al., 1971).

The enzyme is stable for several months when stored in ammonium sulfate at 4°C.

Ultracentrifugation

The following studies were conducted to ascertain the purity of the enzyme preparation. Upon centrifugation at 60,000 rpm, the enzyme sedimented as a single, symmetrical boundary throughout the cell (Fig. 5). Analysis of the sedimentation velocity data yielded a s_{20w} of 7.8×10^{-13} (5 mg/ml). The salamander aldolase was further subjected to sedimentation equilibrium centrifugation. A linear distribution of ln C *vs.* r^2 (Fig. 6) indicated that the protein solution was homogeneous, and a weight average molecular weight, $\bar{M}_w$, of 159,000 daltons was calculated. Finally, the enzyme was applied to a calibrated Sephadex G-200 column, from which it eluted as a single peak. By correlating the elution volume of the salamander aldolase with the elution volumes of known proteins, a molecular weight of approximately 160,000 was obtained for the salamander enzyme (see Fig. 7). Although the ultracentrifugation studies suggested a homogeneous protein solution, a more rigorous test of purity is polyacrylamide gel electrophoresis. Therefore, the protein was submitted to electrophoretic analysis.

Fig. 5. Sedimentation velocity patterns of Ambystoma tigrinum muscle aldolase. The purified enzyme (3.7 mg ml^{-1}) was dialyzed against 25 mM Tris-Cl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 M NaCl, pH 8.0.

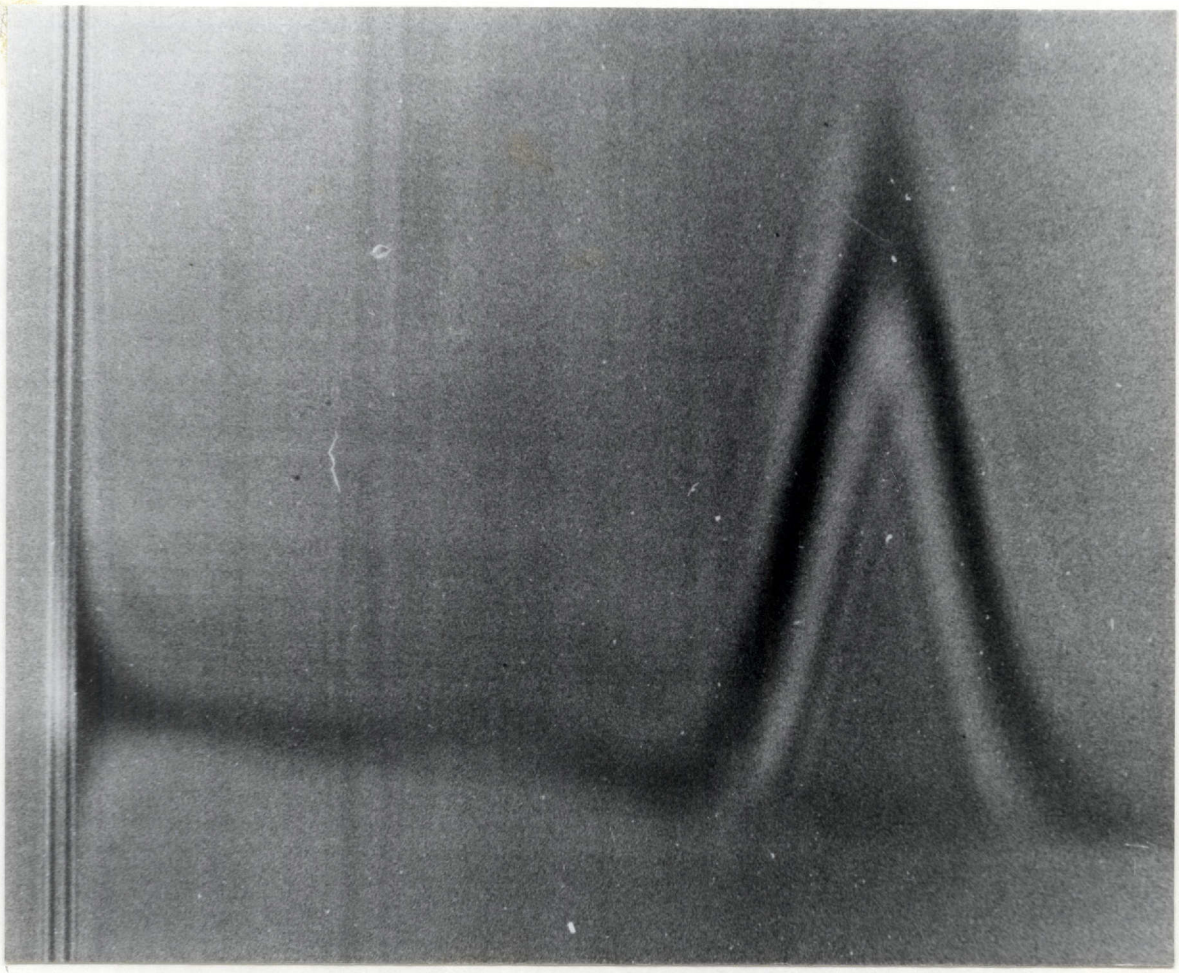


Fig. 6. Molecular weight determination of Ambystoma tigrinum muscle aldolase by sedimentation equilibrium ultracentrifugation. The protein (0.5 mg ml^{-1}) was dialyzed against 25 mM Tris-Cl, 0.1 M NaCl, 5 mM 2-mercaptoethanol, pH 8.0. Sedimentation equilibrium was attained at 18,000 rpm in 24 hr at 19 C. Logarithm of fringe displacement was plotted as ordinate against the square of the distance (centimeter) from the center of rotation as abcissa.

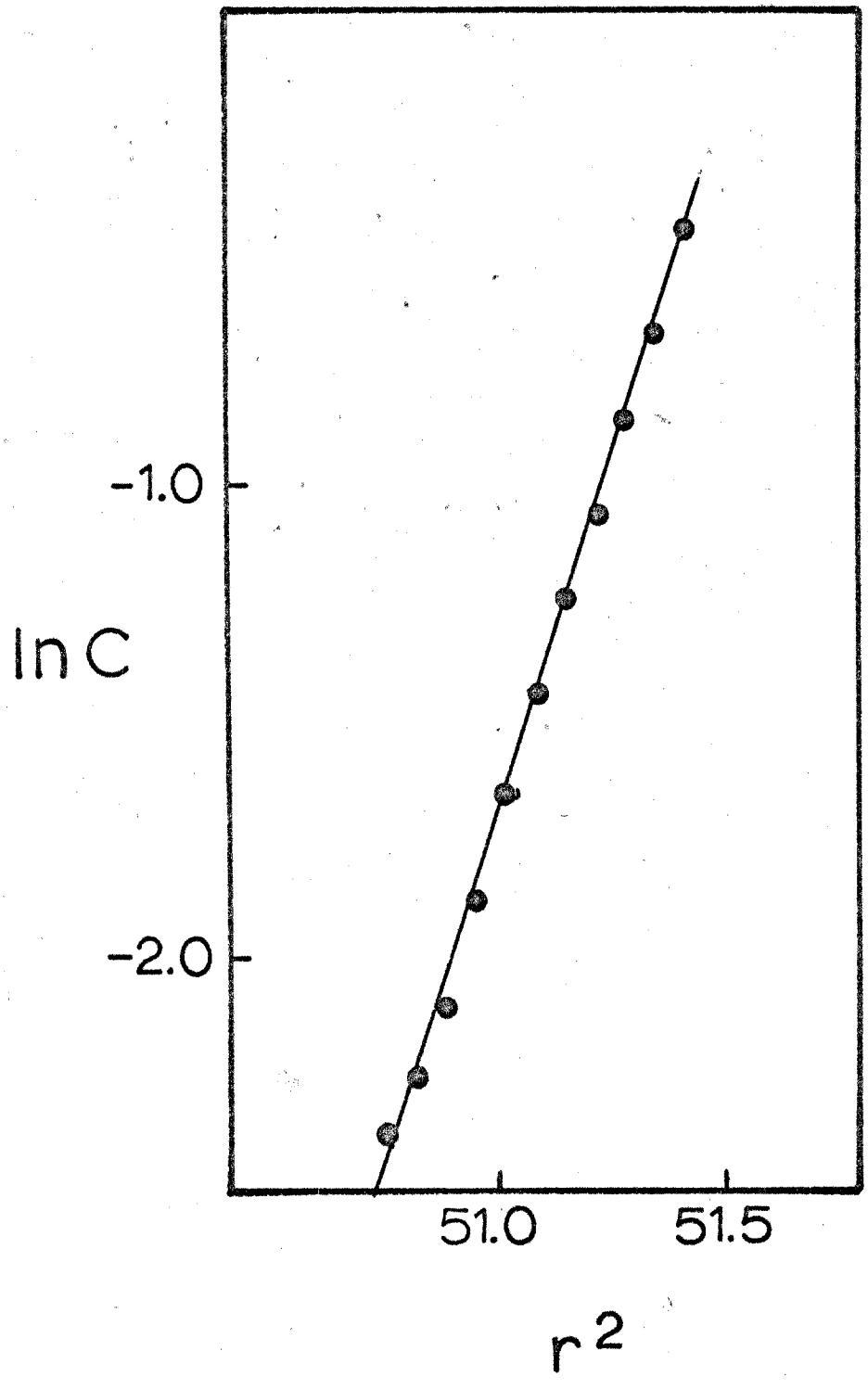
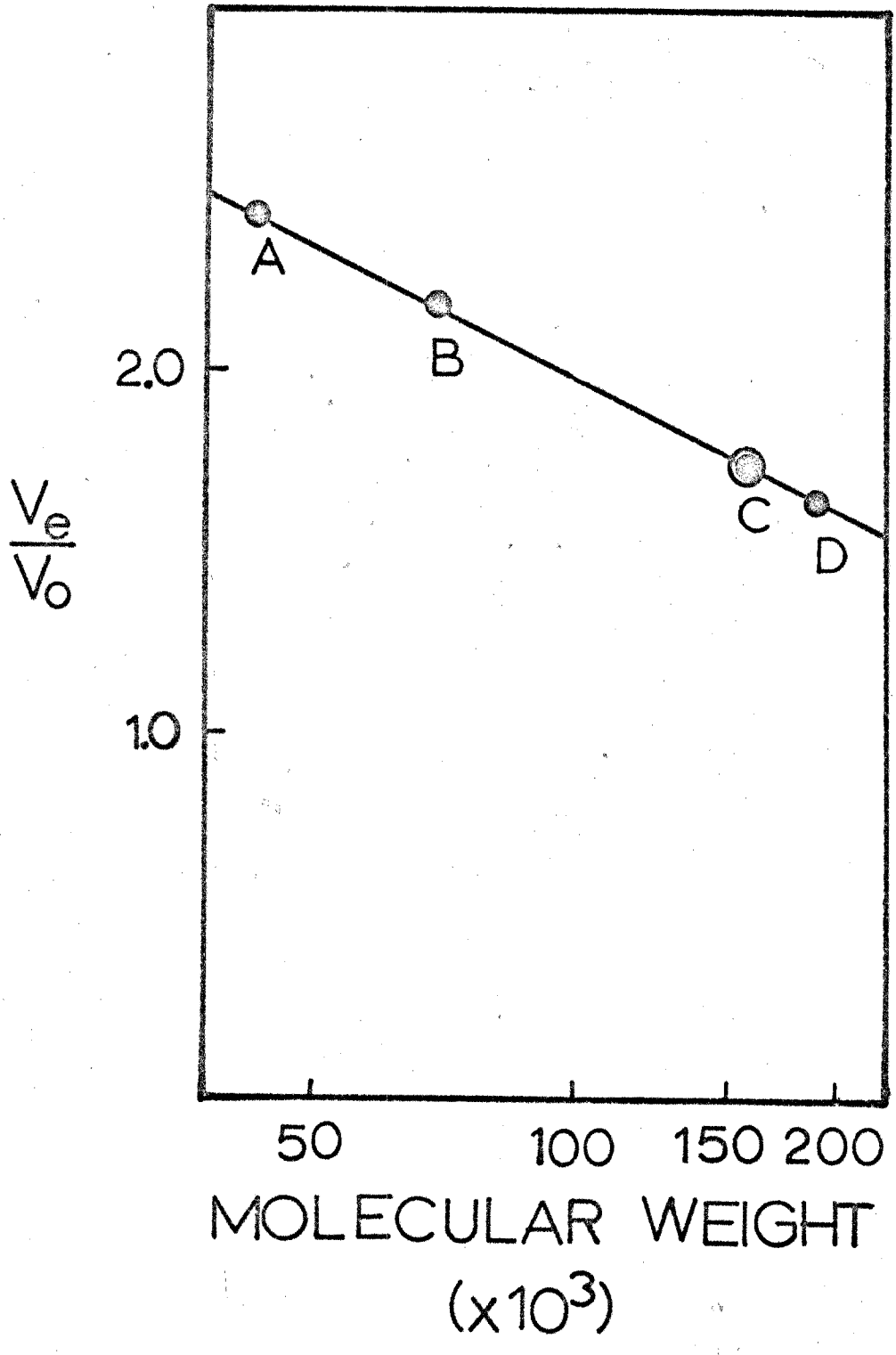

-1.0
-2.0
ln C
51.0
51.5
r 2

Fig. 7. Determination of molecular weight of salamander aldolase by gel filtration. The ordinate represents the ratio of V_e (elution volume) to V_o (void volume) and the abscissa, the molecular weight plotted on a log scale. Ovalbumin, MW 45,000 is represented by A, malate dehydrogenase, MW 70,000 is represented by B, rabbit muscle aldolase, MW 158,000, is represented by C, and salamander aldolase is shown as an open circle. Fumarase, MW 190,000, is represented by D.


A
B
C
D
2.0
1.0
$\frac{V_e}{V_o}$
50
100
150
200
MOLECULAR WEIGHT
(x10^3)

Electrophoresis

Polyacrylamide disc gel electrophoresis was performed according to the method of Maizel (1971). A single protein band was observed for the focused salamander aldolase. Sodium dodecyl sulfate gel electrophoresis was performed as described in methods. A single protein band was present, and the salamander enzyme migrated identically with rabbit muscle aldolase (Fig. 8). An approximate subunit molecular weight of 40,000 is in good agreement with the subunit molecular weight of 41,000 obtained for frog muscle aldolase (Ting et al., 1971). Because the molecular weight of the native enzyme is 159,000, the salamander enzyme is apparently a tetramer of nearly identical subunits.

Amino Acid Composition

The amino acid composition of salamander aldolase, calculated on the basis of a molecular weight of 158,000, is shown in Table II. The amino acid compositions of frog muscle aldolase and of rabbit muscle aldolase are presented for comparison. Although the overall compositions of the salamander muscle enzyme, the frog muscle enzyme and the rabbit muscle enzyme are similar, there are some notable homologies shared only by the amphibian enzymes. The single methionine residue of the frog enzyme is unique among the muscle aldolases studied to date (Ting et al., 1971). The salamander enzyme has 1.6 methionine residues. Both the

Fig. 8. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of Ambystoma tigrinum muscle aldolase and rabbit muscle aldolase. The gel on the left is the rabbit muscle aldolase and the gel on the right is the salamander muscle aldolase.

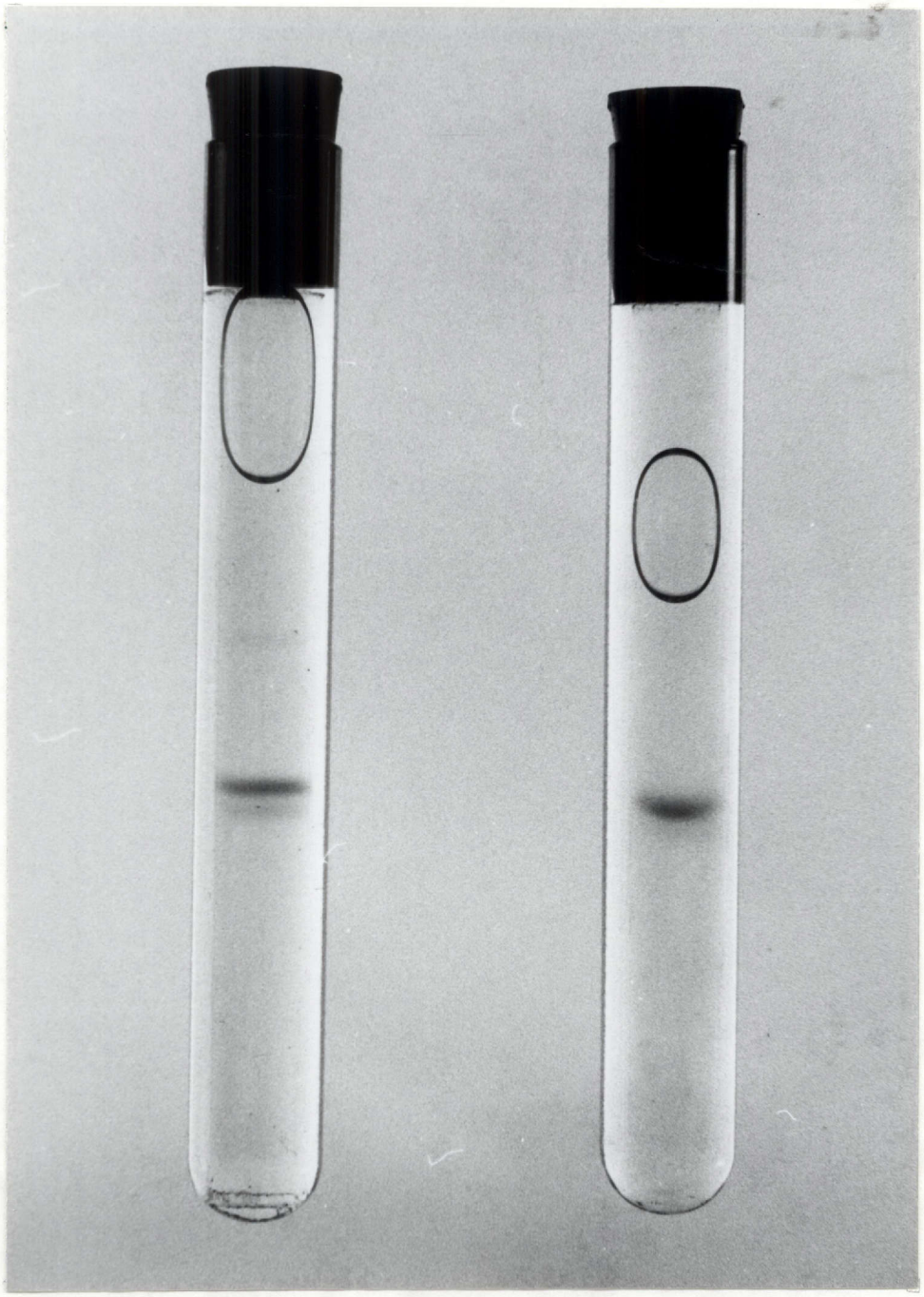


TABLE II

A COMPARISON OF THE AMINO ACID COMPOSITIONS OF SALAMANDER MUSCLE ALDOLASE, FROG MUSCLE ALDOLASE, AND RABBIT MUSCLE ALDOLASE

RESIDUE	SALAMANDER MUSCLE	FROG MUSCLE	RABBIT MUSCLE
Lys	27.9	30.4	25
His	11.3	11.3	11
Arg	14.7	13.9	15
Asp	43.7	30	25
Thr	15.2	25.9	22
Ser	5.2	20.3	20
Glu	42.6	40.2	40
Pro	18.2	16.5	19
Gly	26.3	29.1	30
Ala	64.1	41.2	41
Cys	6.8	8.1	8
Val	29.4	22	21
Met	1.6	1.1	3
Ile	15.6	15.1	19
Leu	33.7	32.1	19
Tyr	6.5	11.7	12
Phe	10.5	9.2	7
Trp	--	--	3

salamander and the frog enzymes have a significantly higher leucine content than does the rabbit enzyme. Additionally, lysine and phenylalanine content is slightly elevated in the amphibian enzymes. The high alanine and aspartic acid contents of the salamander enzyme are also noteworthy. A composition coefficient (CC) of 0.883 was obtained for salamander muscle versus frog muscle enzyme. The CC for salamander enzyme versus rabbit muscle enzyme is 0.85 and for the salamander versus the human heart enzyme the CC is 0.824. These values support the contention that the CC generally decreases with the decrease in phylogenetic similarity (Dedman et al., 1974).

Kinetics

Kinetic studies were performed on the purified enzyme as described in the section on Methods. The Michaelis constant for Fru-P_2 is 1.25×10^{-5} M and for F-1-P is 5.7×10^{-3} M at 30°C (see Figs. 9 & 10). The Fru-P_2/F-1-P ratio, calculated from V_{max} values, is 9.4. Although the K_m for Fru-P_2 is typical of the muscle aldolases, the K_m for F-1-P is lower for the salamander muscle aldolase than the K_ms reported for other muscle aldolases for this substrate. Table III lists the kinetic constants for some representative muscle aldolases. The salamander, the horseshoe crab, and the pigeon muscle aldolases exhibit the lower K_m for F-1-P. The Fru-P_2/F-1-P ratio of 9.4 is also unusual for a muscle aldolase. The crab

Fig. 9. Lineweaver-Burk plot for salamander aldolase with fructose 1,6-bisphosphate as substrate. Three micrograms of enzyme with a specific activity of 12-13 was assayed at 30° at each substrate concentration. The ordinate is the reciprocal initial velocity in units/mg. The abscissa is the reciprocal substrate concentration in micromoles.

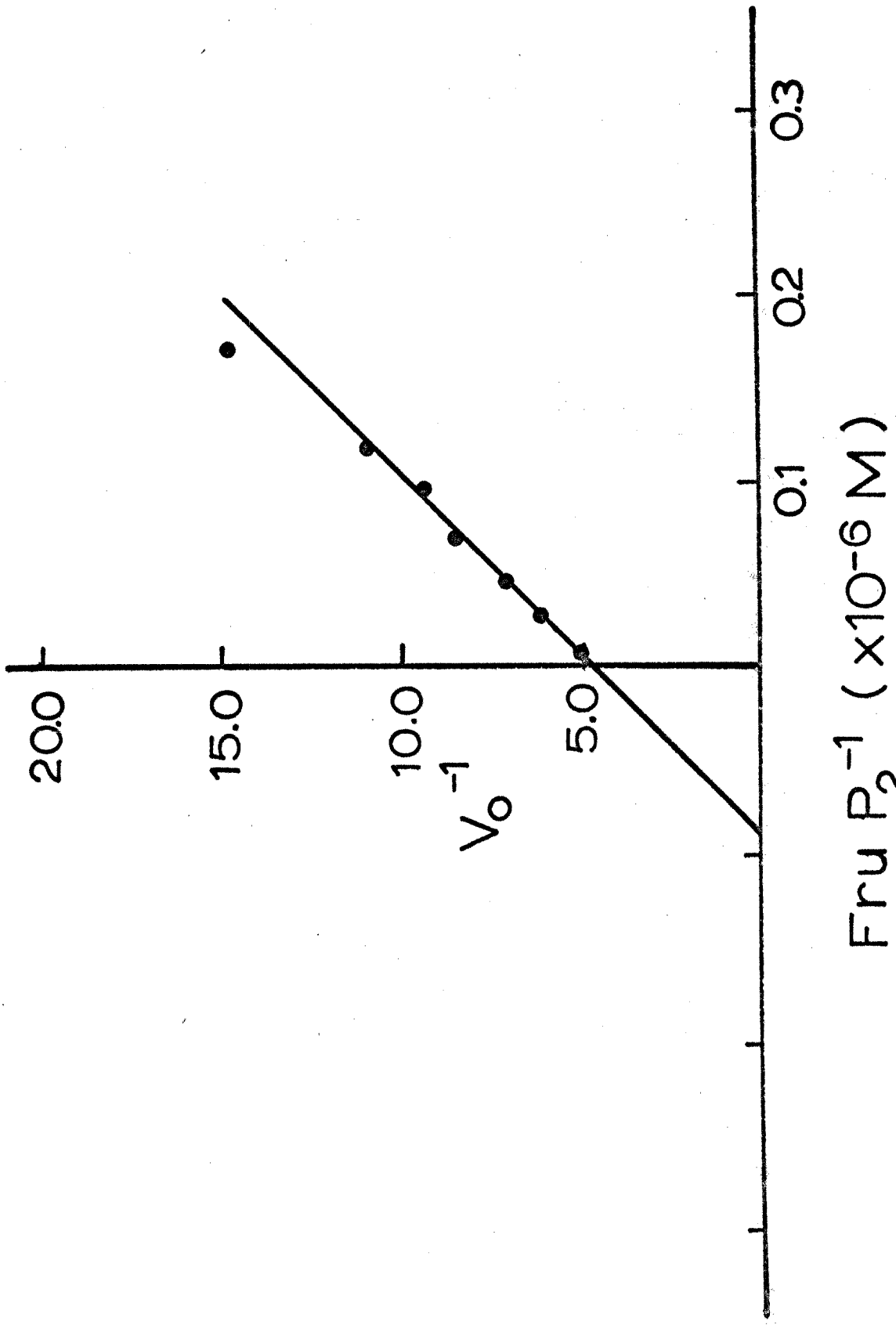

20.0
15.0
10.0
5.0
V_o^{-1}
0.1
0.2
0.3
Fru P_2^{-1} (x10-6 M)

Fig. 10. Lineweaver-Burk plot for salamander aldolase with fructose-1-phosphate as substrate. Seven micrograms of enzyme of specific activity of 12-13 was assayed at 30° at each substrate concentration. The ordinate is the reciprocal initial velocity in units/mg. The abscissa is the reciprocal substrate concentration in millimoles.

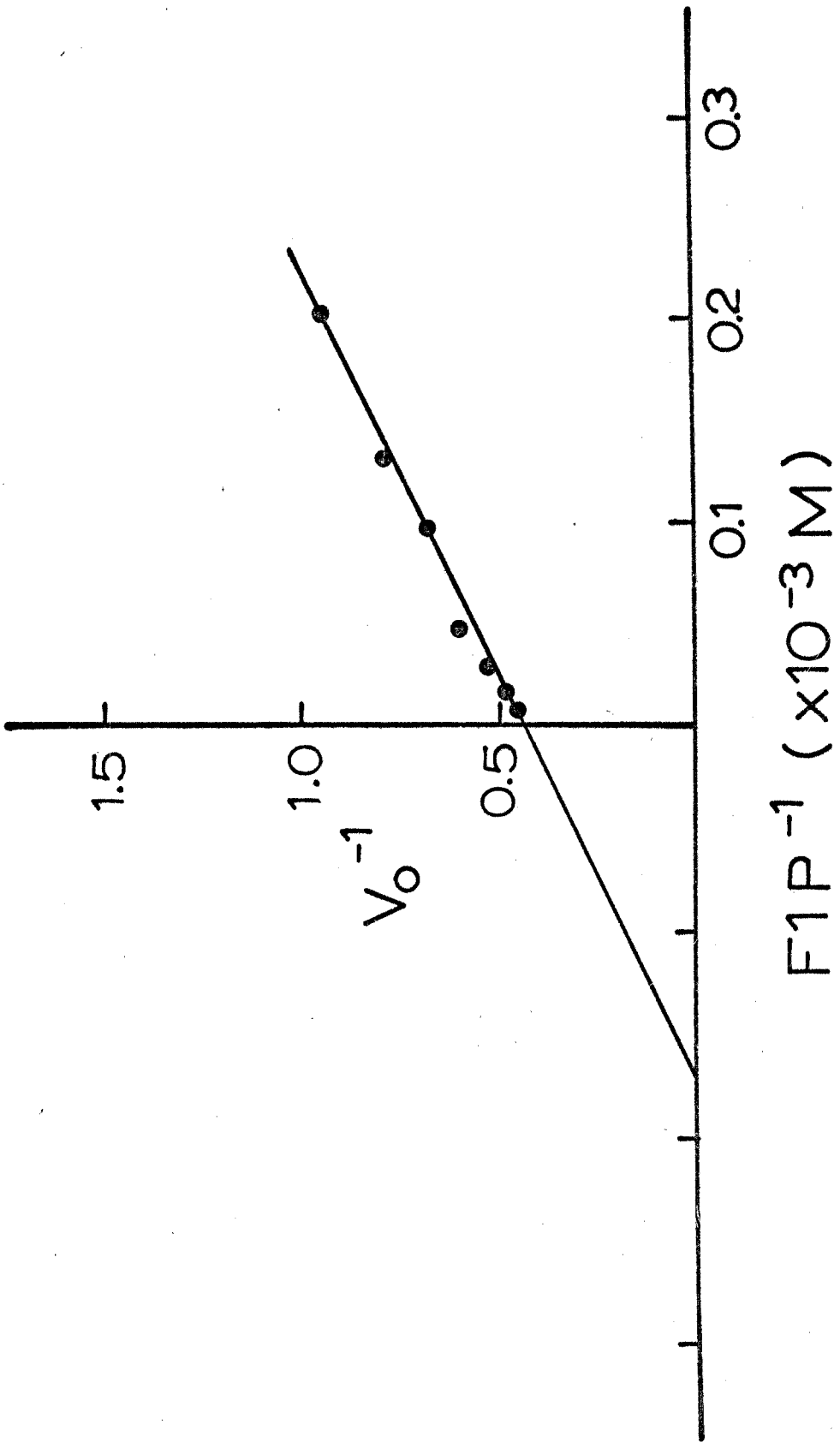

1.5
1.0
0.5
V_o^{-1}
0.1
0.2
0.3
$F1P^{-1}$ ($\times 10^{-3}$ M)

TABLE III

A COMPARISON OF THE KINETIC PROPERTIES OF MUSCLE ALDOLASES

(M)	Salamander	Horseshoe Crab[a]	Codfish[b]	Lobster[c]	Pigeon[d]	Rabbit[e]
$Fru\text{-}P_2$ K_m	1.25×10^{-5}	1.7×10^{-5}	3×10^{-5}	1×10^{-5}	0.5×10^{-6}	6×10^{-5}
FlP K_m	5.7×10^{-3}	2.5×10^{-3}	3.8×10^{-2}	1.25×10^{-2}	1.1×10^{-3}	1×10^{-2}
$Fru\text{-}P_2$ to FlP ratio	9.4	30	75	200	20	50

a Suzuki et al., 1975
b Lai et al., 1971
c Guha et al., 1971
d Gibbons et al., 1972
e Rutter, 1964

and the pigeon enzymes, similarly, exhibit ratios that are lower than those determined for other type A aldolases.

DISCUSSION

In the present study, the muscle aldolase from Ambystoma tigrinum had been purified approximately 73-fold and was found to be homogeneous by ultracentrifugation and electrophoretic criteria. The molecular weight of 159,000 and the tetrameric subunit structure are consistent with that of other muscle aldolases (Sia and Horecker, 1968). Although there are certain obvious differences in the amino acid compositions of the rabbit enzyme and the salamander enzyme, the overall amino acid content has been remarkably conserved from species to species. The K_m for Fru-P_2 of 1.25×10^{-5} M is similar to the values that have been determined for other muscle aldolases (Rutter et al., 1969; Marquardt, 1969; Gracy et al., 1970). The K_m of salamander aldolase for F-1-P, 5.7×10^{-3} M, is lower than the Michaelis constants usually reported for this substrate for muscle aldolases (Rutter, 1964; Guha et al., 1971; Lai et al., 1971). However, Edwards and Gibbons (1972) report the K_m values for F-1-P for the rabbit, pigeon, and sturgeon muscle enzymes to be 1.3×10^{-3} M, 1.1×10^{-3} M, and 0.9×10^{-3} M respectively. The K_m obtained for the salamander enzyme is in good agreement with these values. The Fru-P_2/F-1-P ratio of 9.4 is also low for a muscle aldolase. This ratio may vary from 40-50 for the rabbit muscle enzyme (Rutter, 1964;

Ikehara et al., 1969), to 200 for the lobster enzyme (Guha et al., 1971). More recently, Edwards and Gibbons (1972), and Lebherz and Rutter (1972), have determined a ratio of 25 for the rabbit muscle enzyme. Lebherz has noted that the Fru-P_2/F-1-P ratios reported for muscle enzymes are erroneously high. He argues that the high concentration of F-1-P needed to saturate some muscle aldolases (0.1M) may impair enzymatic activity. He further maintains that Fru-P_2/F-1-P ratios should be calculated only from maximal velocities for each substrate. When maximal velocities are used to calculate the Fru-P_2/F-1-P ratio for the rabbit muscle aldolase, the ratio falls from 50 to 25 (Lebherz and Rutter, 1972). The Fru-P_2/F-1-P ratios for the sturgeon and pigeon muscle aldolases are 13 and 20 respectively (Edwards and Gibbons, 1972). Although the Fru-P_2/F-1-P ratio of 9.4, calculated from V_{max} values for the salamander enzyme, is lower than the ratios reported for most muscle enzymes, it is probably not atypical.

Isoelectric focusing of the salamander enzyme (2-3 mg) yielded two peaks of activity, one at pH 9.85, and the other at pH 9.90. A subsequent experiment, in which more enzyme was focused (8 mg), exhibited the same two peaks with shoulders. These observations are consistent with those of Susor et al. (1973) who resolved crystalline rabbit muscle aldolase into five peaks of activity with isoelectric focusing. From a series of experiments, Susor and coworkers

concluded that peak I contained a single type of subunit, a, and peak V also contained a single type of subunit, a'. Peak III presumably contained equal amounts of the two subunits, and peaks II and IV were the a_3a' and aa'_3 hybrids. He surmises that rabbit muscle aldolase is a random combination of a subunits rather than the obligatory $\alpha_2\beta_2$ structure that Lai has suggested. Lai and coworkers have shown that the only structural difference between the α and the β chains of rabbit muscle aldolase occurs in the carboxyl terminal hexapeptide. The third amino acid from the C-terminal end is asparagine in the α subunit and aspartic acid in the β subunit (Lai et al., 1974). They suggest that the asparagine residue is deamidated in vivo and that the extent of deamidation is dependent upon the age of the animal (Lai et al., 1969). Although the actual, in vivo, subunit composition of the native enzyme cannot be deduced from the preceding studies, the data are consistent with the presence of two slightly dissimilar subunits. Whether the differences in the subunits are representative of in vivo structure or are artifacts of purification has not been entirely resolved. Both deamidation and proteolysis are known to produce heterogeneity in other systems. In these studies on the salamander enzyme, peak II was the predominant peak in fresh enzyme and peak I was the predominant peak in "aged" enzyme (enzyme which had been stored in ammonium sulfate at 4°C for two months). This observation

would lend support to probable structural modifications occurring during purification procedures. The two major peaks could represent a population of native enzyme and one of dissociated enzyme/subunits. A satisfactory explanation for this phenomenon will have to await further studies.

Although the similarities of the muscle aldolases are well substantiated, the significance of the notable differences in amino acid composition has yet to be explained. For example, the methionine content may vary from species to species. The value is 1 for the frog (Ting et al., 1971) and 1.6 for the salamander, but is 8 for the codfish muscle enzyme (Lai et al., 1971). The salamander exhibits an unusually high number of alanine and asparagine/aspartic acid residues. These subtle, but potentially significant changes in primary structure would certainly affect the tertiary structural characteristics of the enzyme. The differences might represent temperature or pH stability adaptations. The temperature optima for the muscle aldolases from trout and antarctic fishes are lower than those of the rabbit, carp, or tuna (Nagahisa et al., 1971). Certainly, the aldolase of diving vertebrates must endure abnormally acidic pH's during long dives. The blood of the red-eared turtle is over 100 mM in lactate and blood pH drops from 7.9 to 6.8 during a two week dive (Storey and Hochachka, 1974). However, the significance of the variation in amino acid composition from species to species

can neither be discerned nor appreciated until a more thorough understanding of the three dimensional structure of the enzyme is attained. Concomitant with this knowledge will come a better understanding of the roles that the amino acids play in catalytic activity and enzyme stability.

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