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Characteristics of Arginase from the Terebellid Polychaete *Pista pacifica* Berkeley

Karen Laurel O'Malley
Portland State University

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AN ABSTRACT OF THE THESIS OF Karen Laurel O'Malley for the Master of Science in Biology presented September 27, 1973.

Title: Characteristics of Arginase from the Terebellid Polychaete
Pista pacifica Berkeley.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Leonard Simpson, Chairman

Robert C. Terwilliger

Joan Loehr

Arginase has been found to occur in the tentacles, gut, and body wall of Pista pacifica Berkeley. Partially purified arginase from the intestine has a molecular weight of 200,000, a K_m of about 155 mM, an arginase/canavanase ratio of 22, a pH optimum of 10.5, and a temperature optimum of 60°C. In addition, P. pacifica arginase is competitively inhibited by ornithine but is not inhibited by high arginine concentrations, nor by sulfhydryl reagents. The enzyme is not stimulated by exogenous manganese and breaks down into an active subunit under harsh treatment. The subunit has a K_m of about 118 mM and is also unaffected by exogenous manganese.

Polychaete arginase shows most of the properties characteristic of arginases from other animal and plant species. However, none of the characteristics observed to date can be correlated with a particular mode of nitrotelism.

CHARACTERISTICS OF ARGINASE FROM THE TEREHELLID

POLYCHAETE Pista pacifica Berkeley

by

KAREN LAUREL O'MALLEY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1973

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
Karen Laurel O'Malley presented September 27, 1973.

Leonard Simpson

Robert C. Terwilliger

Joann Loehr

APPROVED:

Earl Fisher, Jr., Head, Department of Biology

David T. Clark, Dean of Graduate Studies and Research

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INTRODUCTION

The terebellid polychaete Pista pacifica is commonly found in sandy mud flats of relatively protected areas such as bays or estuaries. These terebellid worms are easily located by the top of their tube, an elaborately shaped hood which lies on the surface of the mud, presumably protecting the animal from predation or clogging of the tube by debris. The tubes, composed of mucous, sand and mud, extend vertically 70 - 80 cm into the substrate and are closed at the end by a small plug of sand with holes resembling a button. The worm comes to the top of the tube to feed by extending its long white tentacles out over the surface mud. Nutrients are sent back to the mouth by means of a ciliated groove along each tentacle. Defecation is accomplished by reversing positions in the tube so that the anus is projected outside. Other inhabitants of the tube include the scale worm Halosydna brevisetosa and large colonies of the bryozoan Tritecella elongata which live on the inner tube surfaces and on the body wall of Pista itself.

Evidence is presented in this paper for the presence of an arginase in the tentacles, gut tissues, and body wall of Pista pacifica. L-arginase (L-arginine ureohydrolase. E.C. 3.5.3.1.) catalyzes the hydrolysis of L-arginine into L-ornithine and urea. This enzyme once thought to be present only in ureotelic species (Clementi, 1937) now appears to be widespread throughout the animal kingdom. Characterization data have been reported for an oligochaete annelid (Bishop & Campbell, 1965; Reddy & Campbell, 1968), a crayfish (Hartenstein, 1971), a mollusc (Camp-

bell, 1966), insects (Reddy & Campbell, 1969a), amphibians (Soberón et al., 1967; Carlisky & Sadnik, 1972), reptiles and birds (Mora et al., 1965; Brown, 1966), and mammals (Greenberg, 1960; Hirsch-Kolb et al., 1970).

Several research groups have reported significant differences in arginases from ureotelic, uricotelic, and ammonotelic organisms with respect to antigenic properties, molecular weights, Michaelis constants, substrate inhibition, and stability during dialysis (Mora et al., 1965, 1966; Soru, 1965; Brown, 1966; Soberón, et al., 1967; Reddy & Campbell, 1968, 1969a; Rossi & Grazi, 1969; Middlehoven, 1969; Hirsch-Kolb et al., 1970). However, more recent studies (Reddy & Campbell, 1970; Hartenstein, 1971) contradict the hypothesis of a distinction between a species' mode of nitrogen excretion and its arginase characteristics. In view of this apparent dichotomy the present study was undertaken to characterize the arginase of a probable ammonotele, Pista pacifica. Characterization data reported for this worm include molecular weight, Michaelis constant, specificity, activation by sulfhydryl compounds and the presence of an active subunit.

METHODS AND MATERIALS

Enzyme Source and Assay

Pista pacifica Berkeley was collected from the mudflats near Charleston, Oregon. Only freshly collected specimens were used in tissue preparations.

Preliminary tissue homogenates were prepared in either cetyltrimethylammoniumbromide for the localization of activity or in 50 mM Tris-chloride buffer, pH 7.5, containing 100 mM KCl and 50 mM MnCl₂ for the enzyme purification.

Unless otherwise stated the principle assay medium used at 25°C contained 0.85 M arginine, pH 9.5; 5 mM MnCl₂; 0.5 M sodium glycinate buffer, pH 9.5; and a sample of the enzyme in a final volume of 1 ml. The assay was stopped after ten minutes with 5 ml of 0.5 M HClO₄, and urea determined according to Archibald (1945). The colorimetric determination of urea is based on the red color formed when urea is heated in an acid solution with isonitrosopropiophenone. One enzyme unit was defined as the amount of enzyme that produces one umole of urea per minute at 25°C. Specific activity was expressed in enzyme units per mg protein. Protein was determined by the method of Lowry et al., (1951) as modified by Linn & Lehmann (1965). Bovine serum albumin was used as a standard.

The possibility that the worm was producing urea was tested by using Nessler's Reagent as described by Hult (1969), by the method of Ternberg & Hershey (1964), and by the method of Fawcett & Scott (1960). The tests for possible urea production included samples of fluid from

the tube itself and from animals placed in a small amount of sterile sea water for several days. In all cases urea was not detected. From these preliminary tests it appears that Pista pacifica is primarily an ammonotele.

Molecular Weight

An estimate of the molecular weight of Pista pacifica arginase was made on Sephadex G-200. The eluting solution was 100 mM KCl - 50 mM Tris-HCl - 50 mM MnCl₂, pH 7.5. With a 20 cm pressure head, the column (2x150 cm) had a flow rate of 15 ml/hr at 10°C. Blue dextran (2,000,000 molecular weight), bovine catalase (232,000 molecular weight), bovine serum albumin (68,000 molecular weight), ovalbumin (43,000 molecular weight), α chymotrypsinogen A (25,700 molecular weight) and sperm whale metmyoglobin (17,200 molecular weight) were used as standards. Fractions were assayed for arginase activity.

Polyacrylamide Gel Electrophoresis

Running and stacking gels were prepared as in Davis (1964) with the exception that both Tris buffer solutions were altered to pH 9.5 and pH 7.5 respectively. Samples were dissolved in large pore solution and photochemically polymerized on top of the stacking gel. Electrophoresis was performed in Tris-glycine buffer at pH 9.2. Gels were stained with Amido Schwartz, and destained with 7% acetic acid according to Davis (1964).

Sterile Sea Water

To eliminate the possibility of bacterial contamination, fresh

worms were placed in filtered, autoclaved sea water containing streptomycin sulfate (1 mg/ml) and penicillin (0.3 mg/ml). Each worm was maintained in a liter of water which was replaced twice a day with fresh, sterile sea water.

Reagents

L-arginine (free base), L-ornithine, streptomycin sulfate, penicillin-G, bovine serum albumin, chymotrypsinogen A, bovine catalase, sperm whale metmyoglobin, dithiothreitol, γ -guanidinobutyric acid, p-hydroxymercuribenzoate, L-canavanine, D-arginine, agmatine sulfate, β -guanidinopropionic acid, 2-mercaptoethanol, argininic acid, L-cysteine, Sephadex G-200-120, and DEAE-cellulose were purchased from Sigma Chem. Co.; phenol reagent from Fisher; and blue dextran from Pharmacia.

RESULTS

Purification of *Pista pacifica* Arginase

The scheme outlined below represents steps described in the purification of rat liver arginase (Schimke, 1962) and rabbit liver arginase (Vielle-Breitburd & Orth, 1972) adapted for the purification of *Pista pacifica* arginase. The purification procedure is summarized in Table I and illustrated in Figures 1 and 2. Unless otherwise stated all procedures were carried out in the cold.

Extraction. Several worms were cut open in cold sea water and the intestine quickly removed, weighed and homogenized in a ground glass homogenizer with 3 volumes of 50 mM Tris-HCl buffer containing 100 mM KCl and 50 mM MnCl₂, pH 7.5. The homogenate was centrifuged for 15 min at 13,000 x g in a Sorvall RC2-B refrigerated centrifuge and the pellet was discarded.

Heat Treatment. The supernatant fluid containing the enzyme activity was incubated at 60°C for 20 min, cooled and centrifuged for 10 min at 13,000 x g. The pellet was discarded.

Ethanol Fractionation. Three volumes of absolute ethanol containing 50 mM MnCl₂ at -10°C were added to the supernatant. The resulting precipitate was removed by centrifugation for 10 min at 15,000 x g at -10°C. The pellet was then resuspended in a small amount of 0.01 M Tris-HCl buffer, pH 9.0 and dialyzed over night against the same buffer.

DEAE-cellulose Chromatography. The dialyzed solution was placed on a DEAE-cellulose column (2x10 cm) equilibrated with 0.01 M Tris-HCl,

pH 9.0. The column was then washed with two void volumes of the equilibration buffer. Enzyme elution was performed with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. The activity was eluted at a narrow range of molarity (approximately 0.2 M) in a single broad peak. No additional arginase activity was eluted by washing the column with the same buffer containing 1 M NaCl. The active fractions were pooled and lyophilized (Figure 1).

Chromatography on Sephadex G-200. Lyophilized enzyme was redissolved in 2 ml of 0.01 M Tris-HCl, pH 9.0, and chromatographed on Sephadex G-200. The column (2x130 cm) was previously equilibrated with the same buffer. The enzyme eluted as a single symmetrical peak. The active fractions were pooled and represented the primary enzyme source (Figure 2). In the following text it will be referred to as G-200 enzyme.

TABLE I
PURIFICATION OF Pista pacifica ARGINASE

Procedure	Vol ml	Total activity units	Total protein mg	Yield %	Specific activity units/mg
Extract	10	25	310.2	100	0.082
Extract supernatant	9	23.6	21.87	94	1.07
Heat	8.5	17.34	7.73	69	2.24
ETOH	2	16.5	1.56	66	10.57
DEAE*	5	16	0.47	64	34.64
G-200	20	15.9	0.168	63	94.8

* After lyophilization and redissolution.

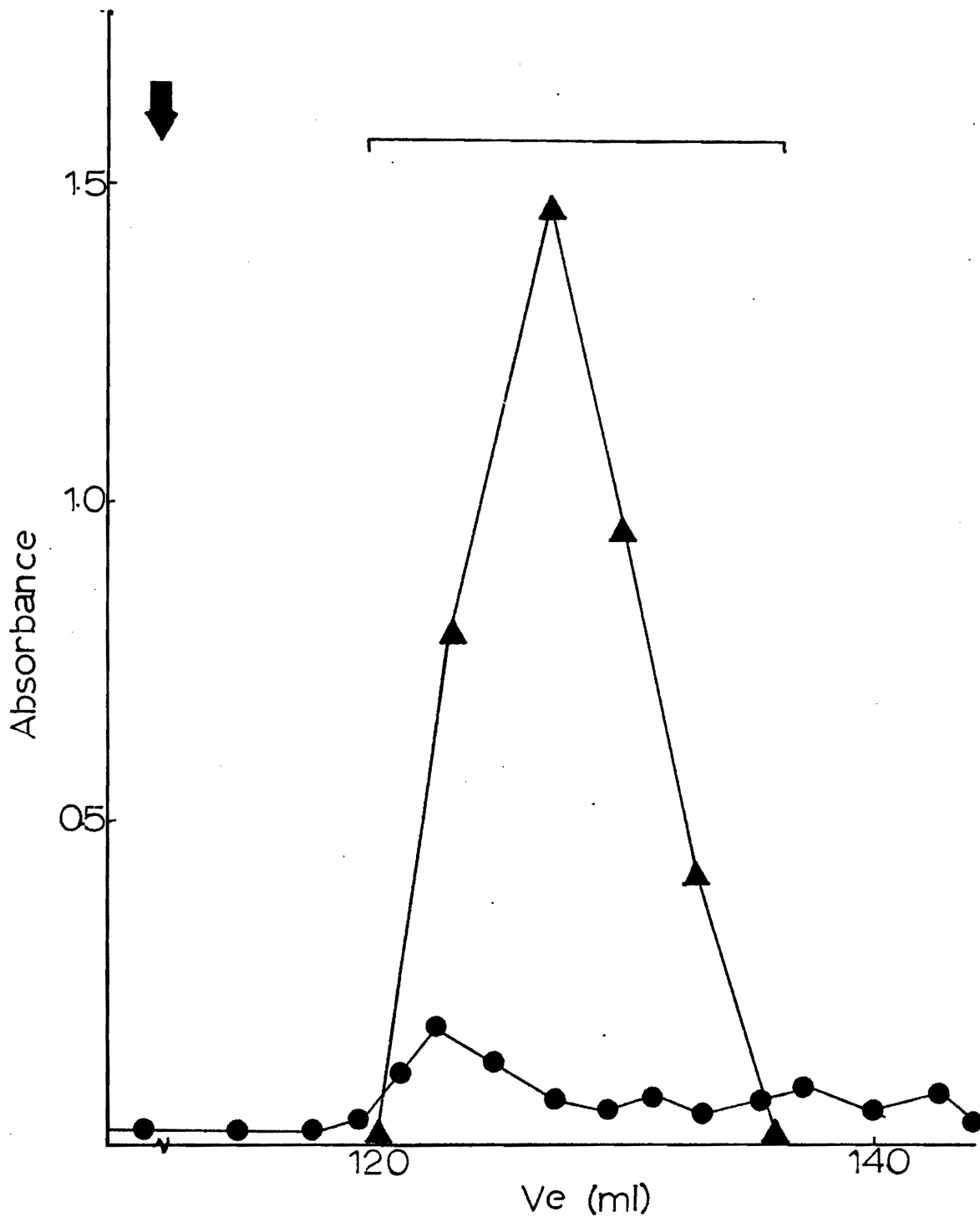


Figure 1. DEAE-cellulose chromatographic fractionation of *P. pacifica* arginase after ethanol fractionation. The active fractions were pooled as indicated by the bar and lyophilized. The arrow indicates the start of the salt gradient. ●'s represent protein concentration measured at 280 nm; ▲'s represent arginase activity determined colorimetrically by absorbance at 540 nm.

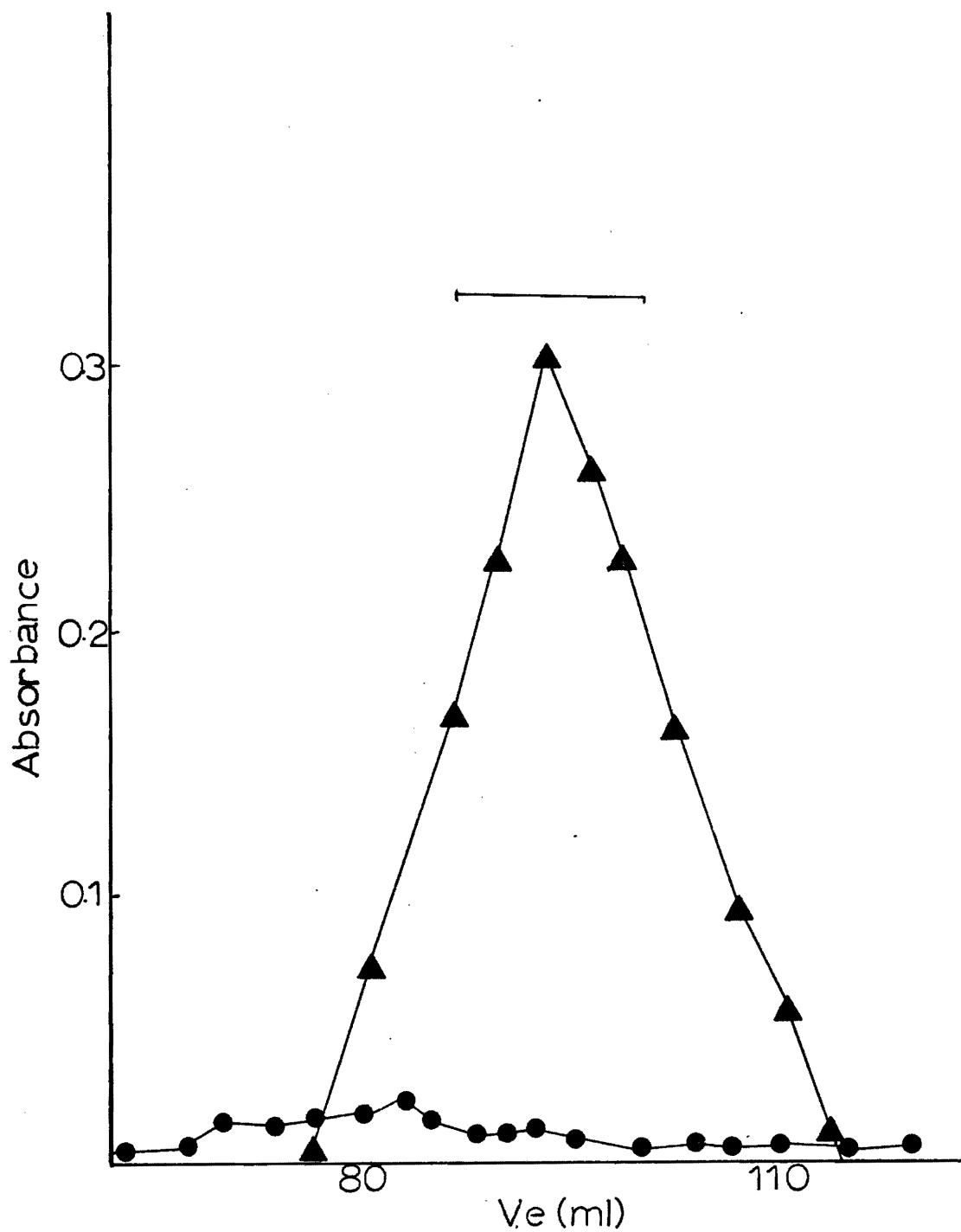


Figure 2. Sephadex G-200 gel filtration of polychaete arginase after DEAE-cellulose chromatography. The active fractions were pooled as indicated by the bar. ●'s represent protein concentration measured at 280 nm. ▲'s represent arginase activity determined colorimetrically by absorbance at 540 nm.

The specific activity of the enzyme after chromatography on Sephadex G-200 was about 1000-fold greater than the specific activity of the crude extract. If the G-200 enzyme is lyophilized and electrophoresed on polyacrylamide gels at pH 9.2, 8 bands of varying intensities will appear in the gels. Whether these bands represent impurities or breakdown products of the enzyme itself has not yet been determined.

Distribution of Arginase

Arginase was present in the tentacles, body wall and gut tissues of P. pacifica (Table II). Whether the activity in the tentacles and the intestine could be attributed to bacterial origin cannot be said though worms maintained in sterile sea water for 72 hours still exhibited arginase activity (Table III). The intestine was consistently the richest source of arginase, particularly in terms of total units.

TABLE II

DISTRIBUTION OF ARGINASE IN THE TISSUES OF Pista pacifica

worm	tentacles	esophagus	anterior stomach	posterior stomach	intestine	body wall
1	216*	79.6	0.22	0.25	67.9	0.91
2	22.7	0.5	0.16	0.24	17.7	0.57
3	120.0	5.5	0.90	0.40	12.6	0.68
4	1.1	11.4	0.36	0.10	18.6	0.58
5	1.5	1.7	0.70	0.24	95.8	0.90
6	66.0	1.8	0.50	0.28	82.0	0.90

* umoles urea/min/mg protein x 1000

Worms were dissected and the tissues removed, weighed and homogenized in 9 vol of CTAB. 0.2 mls homogenate were added to the assay medium.

TABLE III
 DISTRIBUTION OF ARGINASE IN THE TISSUES OF
Pista pacifica MAINTAINED IN STERILE
 SEA WATER FOR 72 HOURS

worm	tentacles	esophagus	anterior stomach	posterior stomach	intestine	body wall
1*	83.0**	0	0.27	3.58	38.6	1.47
2	59	0.43	0.10	1.32	13.5	0.97
3	6.3	0	0.20	1.82	34.2	0.97
4	12.6	0.33	0.27	1.84	20.2	1.16
5	1.7	0	0.2	0.69	22.8	0.97

* control

** umoles urea/min/mg protein x 1000

Worms were dissected and the tissues removed, weighed and homogenized in 9 vol of CTAB. 0.2 ml of homogenate were added to the principle assay medium.

pH Optimum

A pH optimum of 10.5 was observed in four different experiments. The results of one experiment are shown in Figure 3. Although the optimal pH was 10.5 assays were conducted at pH 9.5 in this study so that results could be compared with reported data (Hirsch-Kolb et al., 1970).

Effect of Temperature

The effect of temperature on P. pacifica arginase is shown in Figure 4. Figure 5 shows a plot of log V versus 1/T for temperatures between 273 - 333°K. From this graph the energy of activation (E) was calculated according to: slope = -E/4.576. Energy of activation was found to be 8.09 kcal/mole.

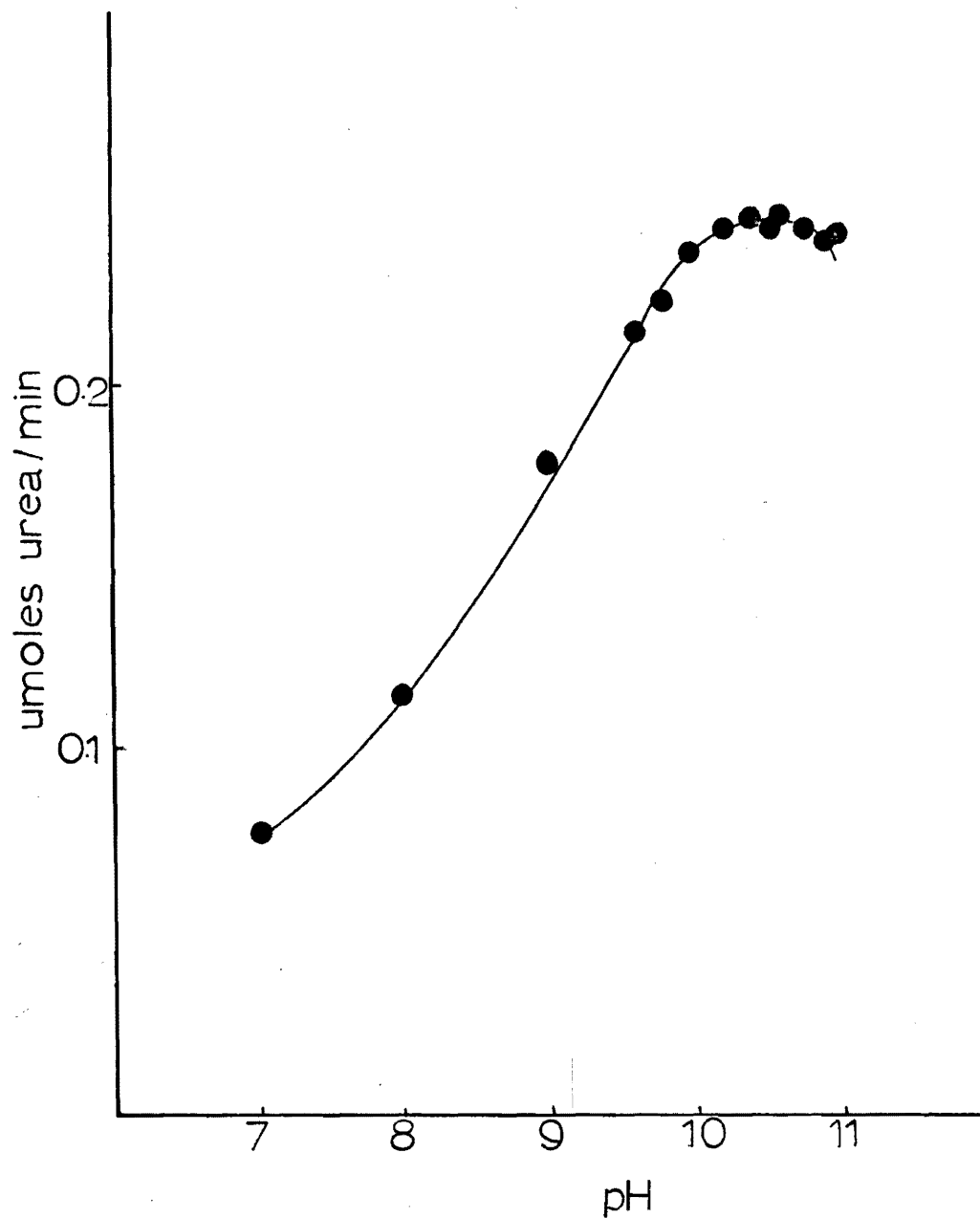


Figure 3. pH optimum of *Pista pacifica* arginase. 50 mM sodium glycinate was used as a buffer between pH 9 and 11; 50 mM Tris-HCl was used below pH 9. Two-tenths ml enzyme (1 mg/ml) was used in the principle assay medium that was adjusted to the indicated pH's.

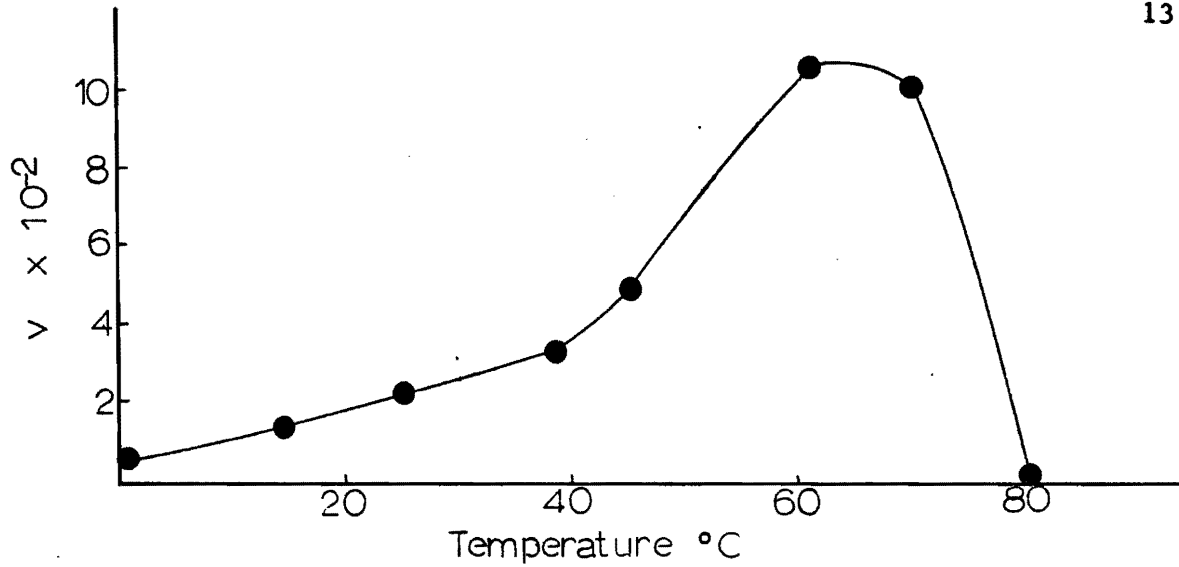


Figure 4. The effect of temperature on *P. pacifica* arginase. A plot of temperature versus initial velocity. Two-tenths ml enzyme (1 mg/ml) was used in the assay medium equilibrated to the appropriate temperatures.

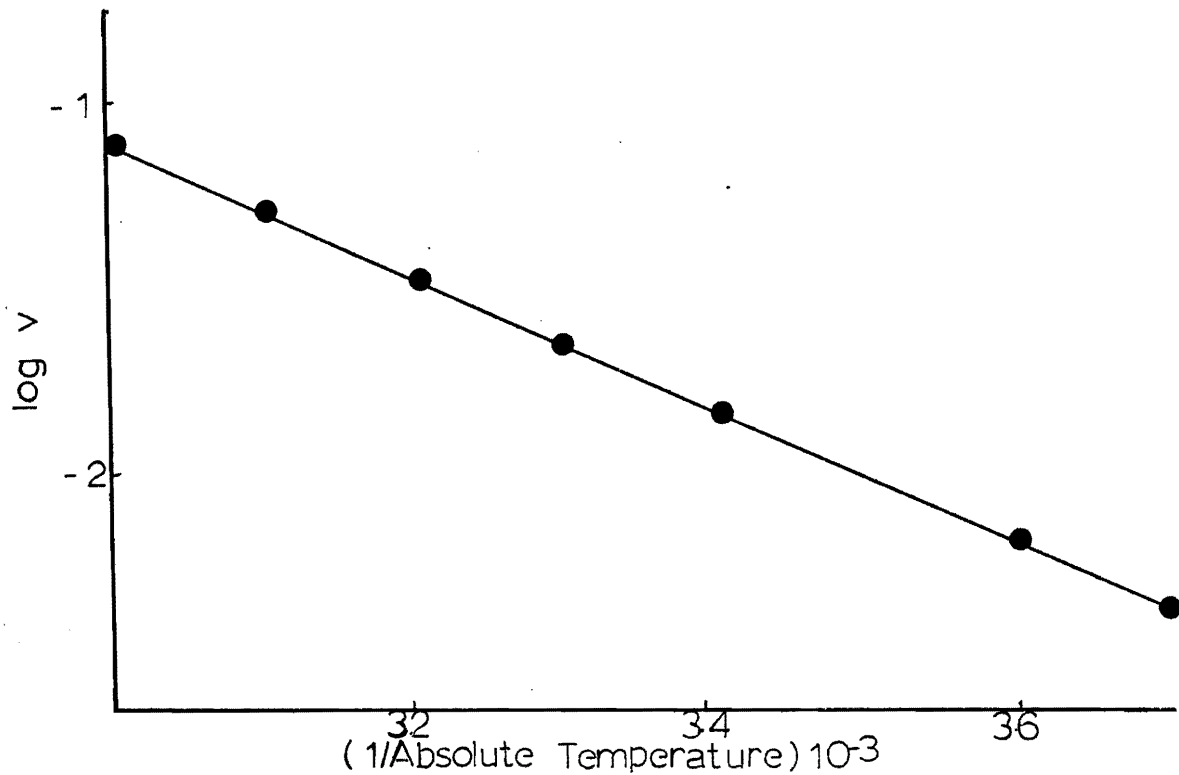


Figure 5. Plot of log V versus 1/T from which the energy of activation was obtained. Two-tenths ml G-200 enzyme (1 mg/ml) was used in the assay medium equilibrated to the appropriate temperatures.

Substrate Kinetics

The apparent Michaelis-Menten constant for L-arginine was 155 - 160 mM in several experiments. As shown in Figure 6 there was no indication of inhibition by high substrate concentrations. The substrate concentration used for conducting assays on P. pacifica arginase was 0.85 M. At this concentration 95 - 100% of the calculated maximum velocity (V_m) was obtained.

On the same graph L-ornithine (100 mM) appears to be a competitive inhibitor. Hill plots for both enzyme and enzyme + inhibitor at the concentrations shown yield n values of 1.034 and 1.040 respectively, where n equals the slope of $\log(V_m - V_0)/V_0$ versus $\log S$ (Figure 7).

Effect of Mn^{2+} Concentration

Mn^{2+} has been shown to be a metallic cofactor for a number of arginases although variable effects have been reported. Enzyme incubated for 30 min in the principle assay system seemed unaffected by various Mn^{2+} concentrations at 25°C and pH 9.5 (Table IV).

Specificity

Agmatine sulfate, D-arginine, L-arginine, argininic acid, L-canavanine, γ -guanidinobutyric acid, and β -guanidinopropionic acid were tested as potential substrates. Only two were hydrolyzed to urea at an appreciable rate, L-arginine and L-canavanine (Table V). The ratio of arginase to canavanase was about 22 at pH 9.5.

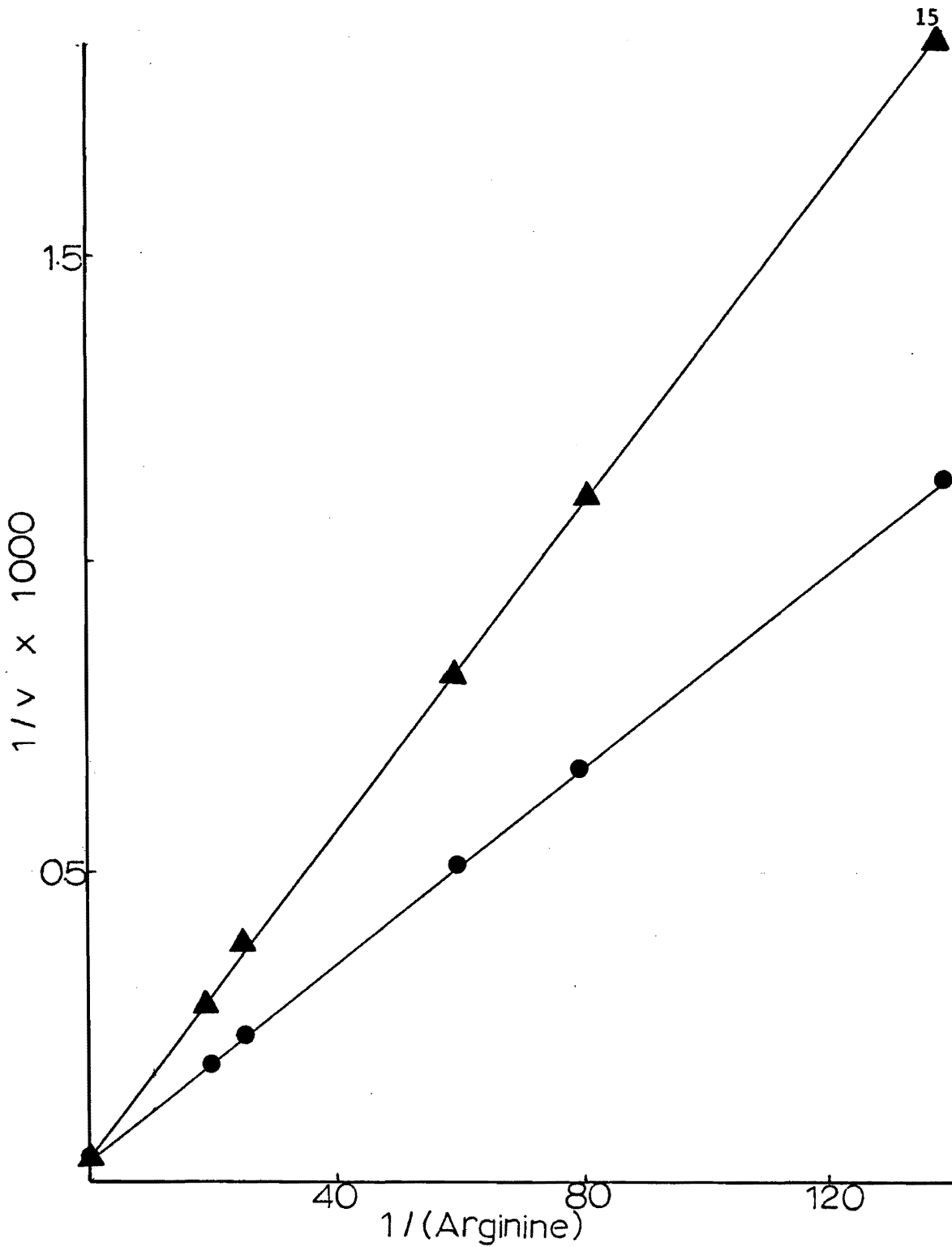


Figure 6. Kinetic behavior of *P. pacifica* arginase. A double reciprocal plot of $1/v$ versus $1/S$ for both enzyme and enzyme + inhibitor. The reaction mixture contained the concentration of arginine indicated + 0.1 ml of G-200 enzyme. ●'s represent the enzyme kinetics without ornithine; ▲'s represent enzyme activity in the presence of 0.1 ml 100 mM ornithine.

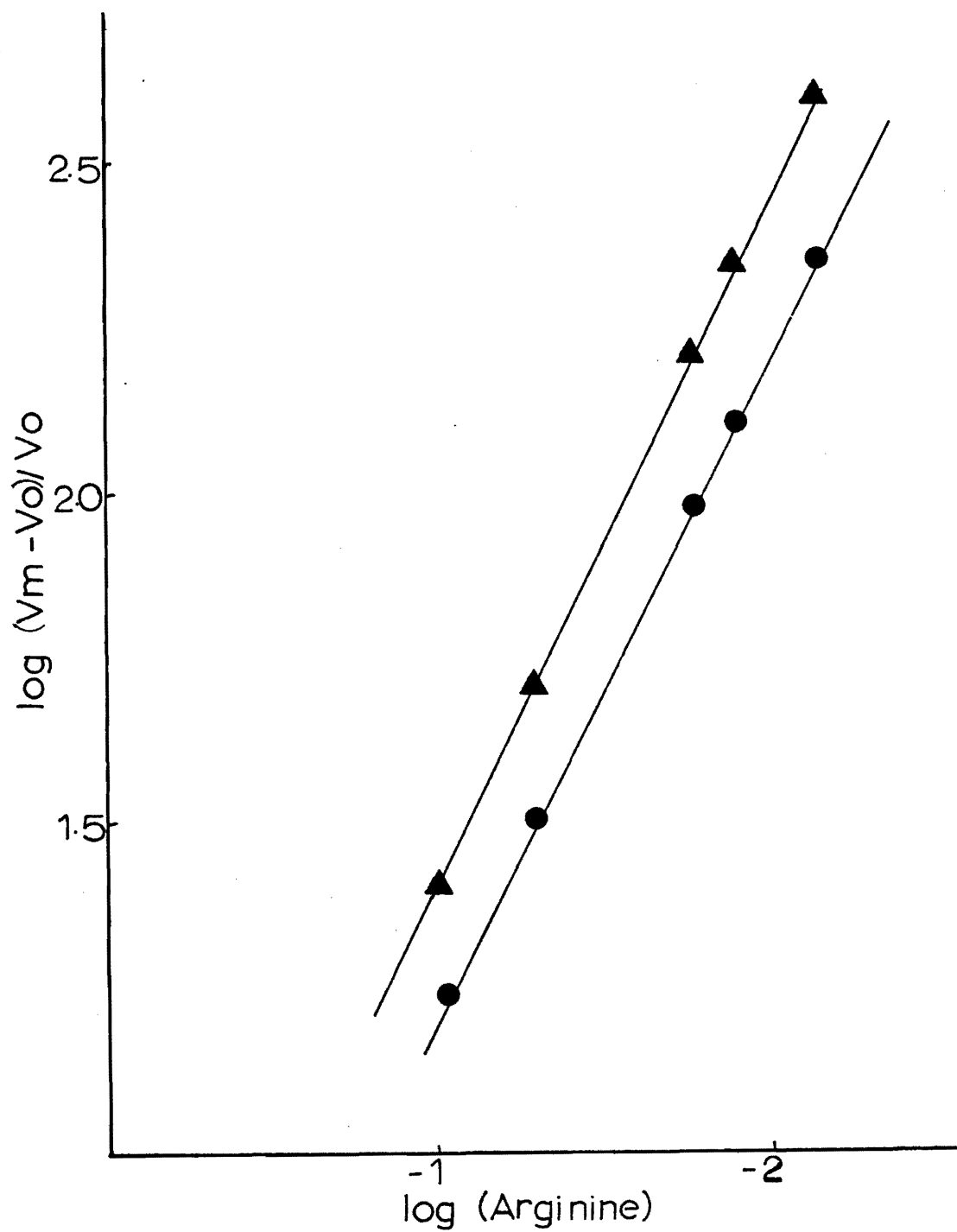


Figure 7. Hill plot of *Pista pacifica* arginase kinetics. ●'s represent the reaction mixture + enzyme; ▲'s represent the reaction mixture + enzyme + inhibitor.

TABLE IV

THE EFFECT OF Mn^{2+} CONCENTRATION ON Pista pacifica ARGINASE ACTIVITY

Mn^{2+} mM	umole urea formed/30 min
0	0.312
0.025	0.315
0.05	0.310
0.10	0.315
0.50	0.309
1.0	0.310
2.5	0.312
5.0	0.310

One-tenth ml of enzyme (1 mg/ml) from the final purification step (G-200) was incubated for 30 min in the principle assay system except that the Mn^{2+} concentration was varied as indicated.

Effect of Sulfhydryl Compounds

Cysteine, dithiothreitol, reduced glutathione, 2-mercaptoethanol were added to the assay medium and their effect noted in Table VI. All compounds and reagents tested had a stimulatory effect on polychaete arginase.

The addition of one umole of p-hydroxymercuribenzoate (PMB), a sulfhydryl inhibitor, had no effect when the enzyme and PMB were incubated for 60 min in the principle assay medium. When PMB was increased to 5 mM a slight stimulatory effect was noticed.

TABLE V
SUBSTRATE SPECIFICITY OF Pista pacifica ARGINASE

Compound	umoles urea released/hr pH 7.5	pH 9.5
Agmatine sulfate	0	0
D-arginine	0	0
L-arginine	0.072	0.620
Argininic acid	0	0
L-canavanine	0.014	0.028
γ -guanidinobutyric acid	0	0
β -guanidinopropionic acid	0	0

One-tenth ml of G-200 enzyme (1 mg/ml) was incubated for 60 min with each of the listed substrates in the principle assay system. Each of the substrates were adjusted to the indicated pH. Sodium glycinate was used as a buffer at pH 9.5 and 50 mM Tris-HCl was used at pH 7.5.

Molecular Weight

The molecular weight of Pista pacifica arginase, determined from the chromatography of the enzyme through Sephadex G-200, was estimated as 200,000 (Figure 8).

Appearance of an Active Subunit

Following heat treatment, acetone fractionation was tried as a possible purification method. One and a half sample volumes of acetone at -10°C were slowly added to the heat supernatant. The mixture was centrifuged at 13,000 x g for 5 minutes at -10°C , and the pellet resuspended in one volume 50 mM Tris-HCl containing 100 mM KCl and 50 mM MnCl_2 , pH 7.5.

TABLE VI

THE EFFECT OF SULFHYDRYL COMPOUNDS ON Pista pacifica ARGINASE

Compound	umoles urea produced/hr
None	0.685
Cysteine	1.180
Dithiothreitol	1.365
Reduced glutathione	1.190
2-mercaptoethanol	2.500

Five umoles of each sulfhydryl compound were added to the reaction medium. The reaction was started with 0.1 ml G-200 enzyme (1 mg/ml) and incubated for 60 min. Standards were prepared in the presence of the same amount of sulfhydryl compound used for colorimetric correction.

The solution was then dialyzed for 2 hours against the same buffer. The sample was then chromatographed on a Sephadex G-200 column (2x150 cm) equilibrated in the same buffer. The enzyme eluted in two peaks, one of approximately 200,000 molecular weight and an apparent dissociation product with a molecular weight about 42,000 (Figure 9).

An active subunit could also be produced when the active fractions of the G-200 enzyme in the purification scheme of polychaete arginase were pooled and lyophilized. The lyophilized material was redissolved in Tris-HCl buffer, pH 7.5, placed on a G-200 column and eluted as before. Fractions included two activity peaks, one corresponding to the large molecular weight unit and the other to 34,000. The peak fraction of the lower molecular weight was rechromatographed on the column and eluted at 15 ml/hr. There was no evidence of aggregation. Arginase activity ap-

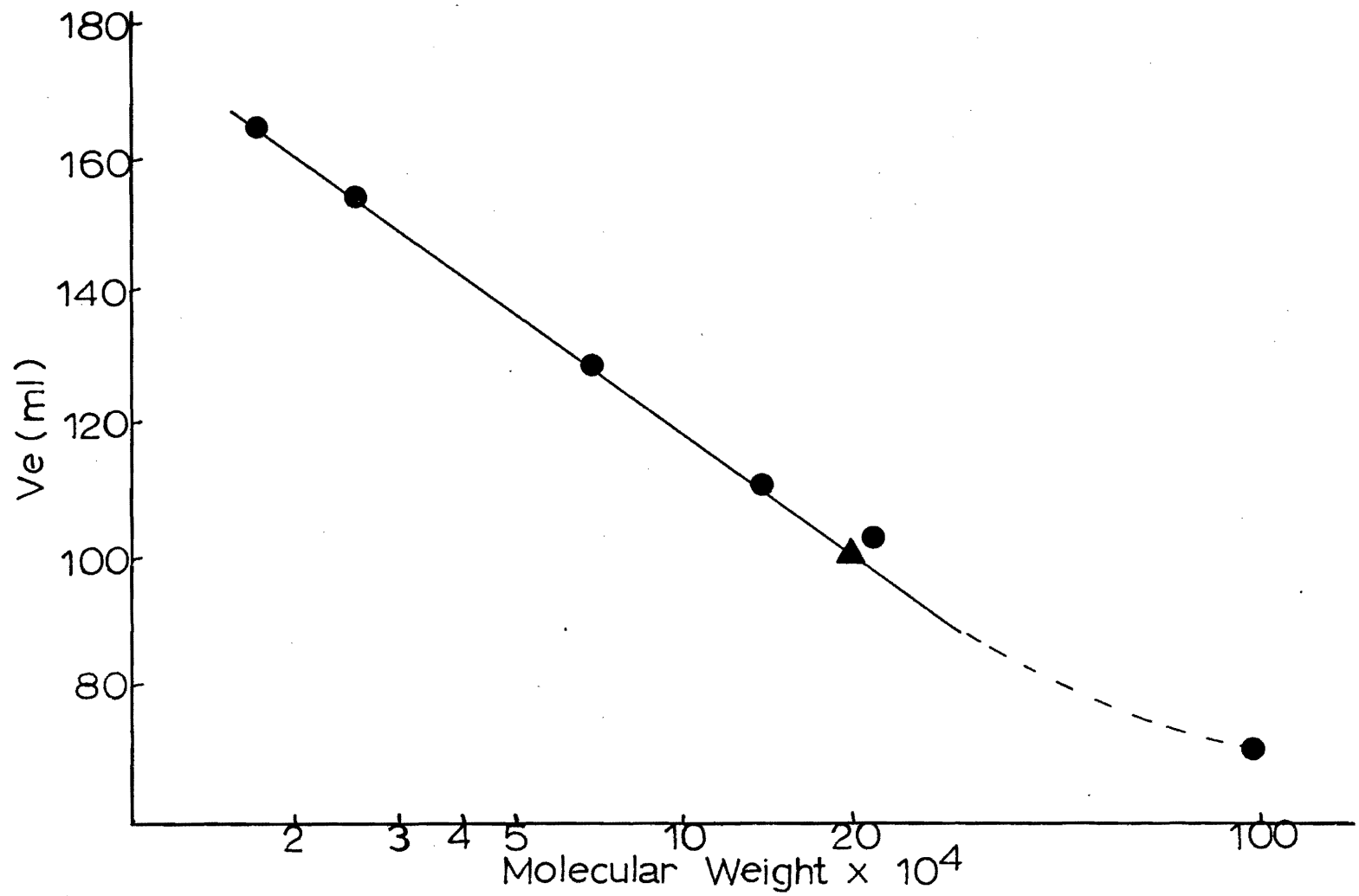


Figure 8. Estimate of molecular weight of *Pista pacifica* arginase. ●'s from left to right: sperm whale metmyoglobin, chymotrypsinogen A, bovine serum albumin, lactate dehydrogenase, catalase, and blue dextran. ▲: *Pista pacifica* arginase.

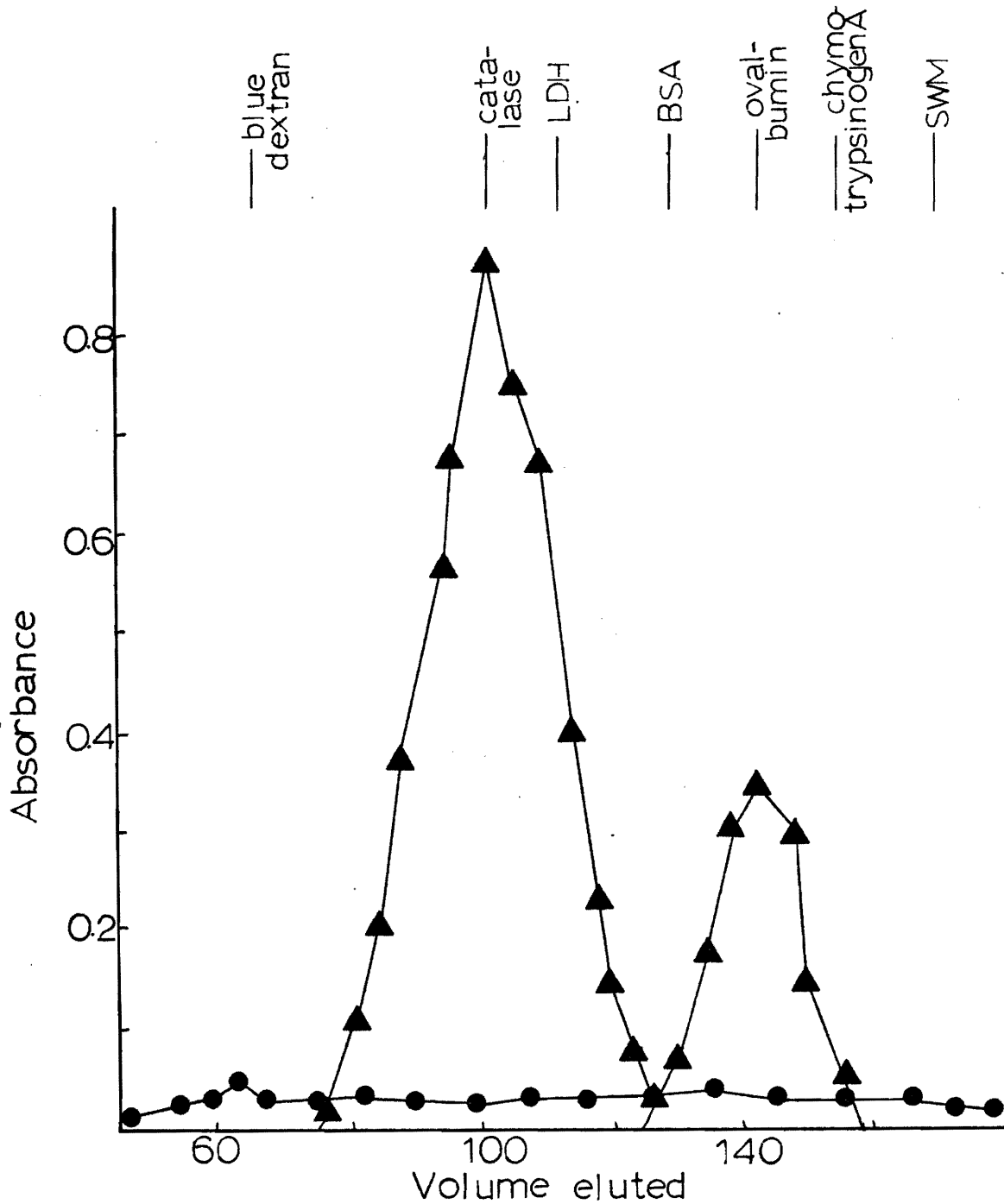


Figure 9. Breakdown of *P. pacifica* arginase after acetone fractionation. ●'s represent protein concentration measured at 280 nm. ▲'s represent arginase activity determined colorimetrically by absorbance at 540 nm. Calibrants were: blue dextran, bovine catalase, bovine serum albumin, lactate dehydrogenase, ovalbumin, chymotrypsinogen A, and sperm whale metmyoglobin.

peared in the same fractions as before: molecular weight of 34,000 (Figure 10). Active fractions were pooled and lyophilized.

Polyacrylamide Gel Electrophoresis of Subunit

Lyophilized subunit (2 mg/ml) from the dissociation of the G-200 enzyme, migrated as a single dark band during polyacrylamide gel electrophoresis at pH 9.2. Another, lighter band was observed on several occasions. Whether this band represents an impurity, closely allied subunit structure, or an artifact of electrophoresis has not yet been determined.

Michaelis Constant of Subunit

Values of 114 - 118 mM were obtained for the K_m of the subunit arginase on a double reciprocal plot of $1/V_o$ versus $1/\text{substrate concentrations}$. A Hill plot indicated an n value of 1.07 where n equals the slope of $\log(V_m - V_o)/V_o$ versus $\log S$ (Figure 11).

Effect of Mn^{2+} Concentrations on the Subunit

The subunit, incubated for 30 min in the principle assay system, appeared to be unaffected by Mn^{2+} concentrations (Table VII).

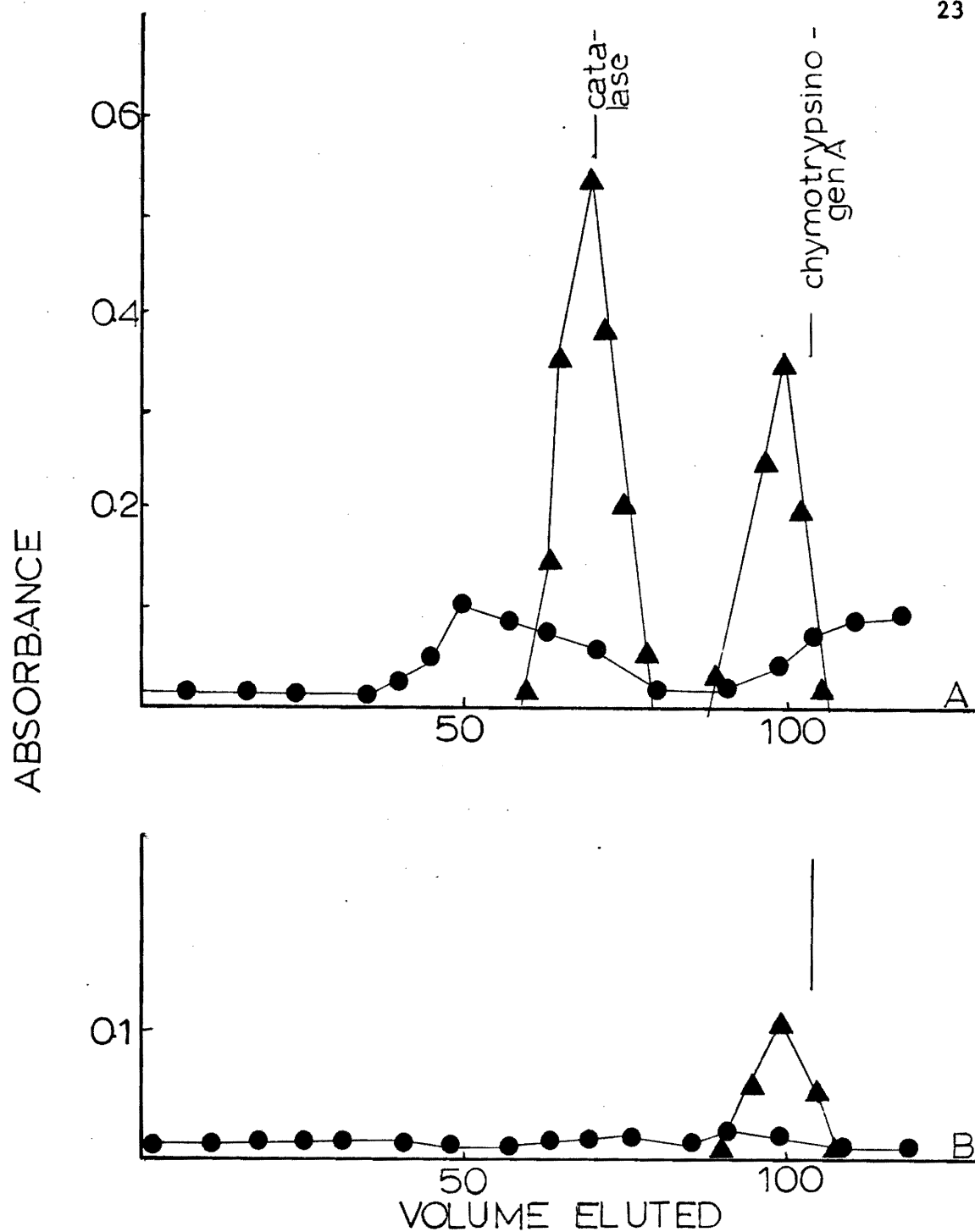


Figure 10. Appearance of subunit on Sephadex G-200. A, breakdown of enzyme after redissolution in buffer following lyophilization. B, rechromatography of subunit from A showing the absence of aggregation. ●'s represent protein concentration measured at 280 nm. ▲'s represent arginase activity measured as urea production determined colorimetrically at 540 nm.

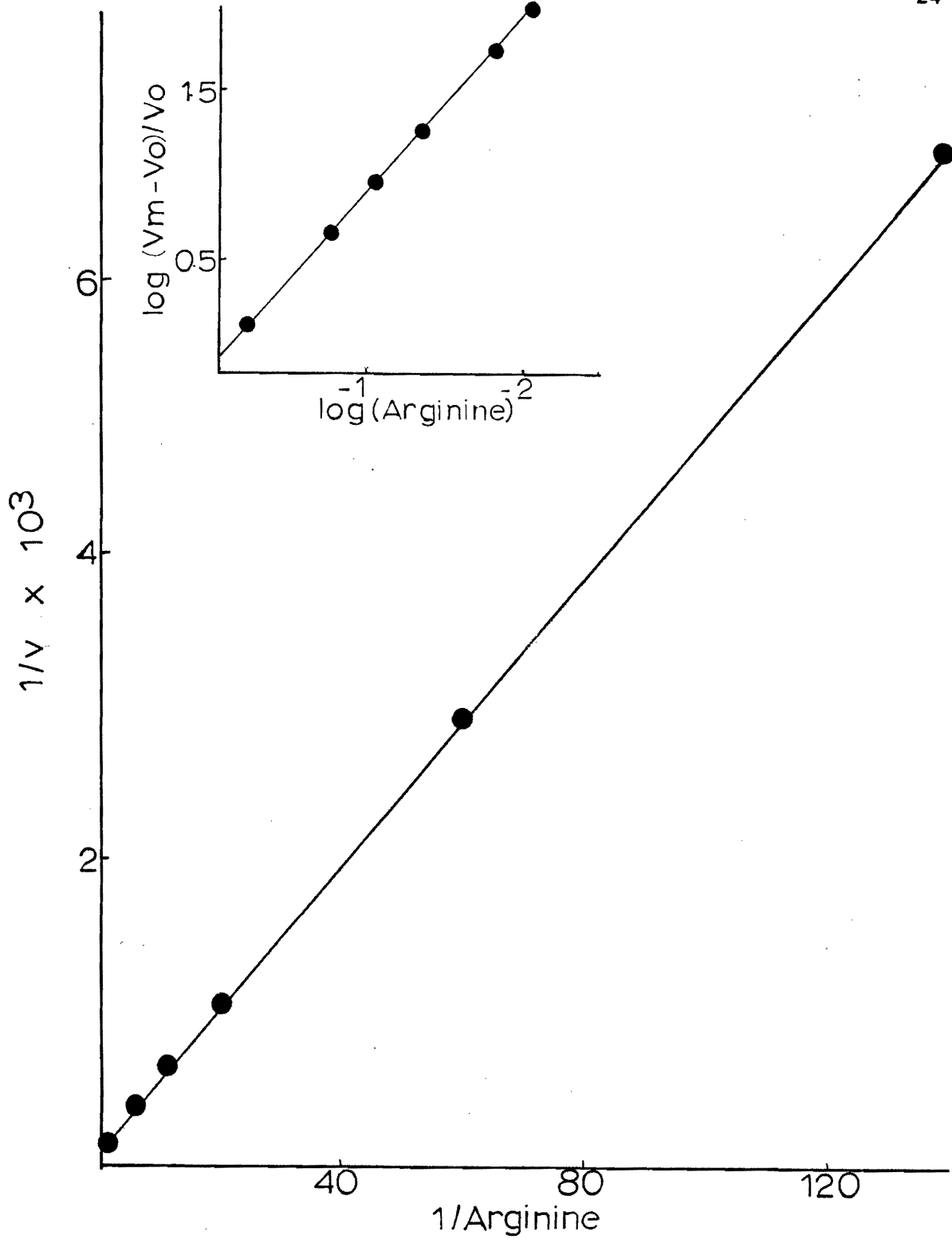


Figure 11. Lineweaver-Burke plot of affinity of subunit for substrate. Inset: Hill plot of indicated data. Enzyme source was isolated subunit (1 mg/ml) added to the assay medium.

TABLE VII

EFFECT OF Mn^{2+} CONCENTRATION ON Pista pacifica SUBUNIT ACTIVITY

Mn^{2+} mM	umole urea formed/30 min
0	0.368
0.025	0.368
0.050	0.396
0.100	0.370
0.500	0.365
1.000	0.387
2.500	0.376
5.000	0.370

One-tenth ml of subunit from dissociated G-200 enzyme was incubated in the principle assay medium with the above concentrations of manganous ion.

DISCUSSION

Despite the well established role of arginase in certain oligochaetes (Needham, 1957, 1960, 1962; Bishop & Campbell, 1965; Reddy & Campbell, 1968), few marine annelids have been investigated with regard to the presence of this enzyme, none with regard to its characteristics (Hult, 1969). Studies of arginase in oligochaetes have primarily focused on its presence, location, and role in connection with the other enzymes of the Krebs-Henseleit cycle. Needham (1960) reported that arginase activity in the gut of Lumbricus terrestris was maximal in the anterior part of the intestine declining to a minimum in the crop-gizzard region. In addition, he noted an inverse relationship between activity and the amount of chloragogen tissue. This tissue, possibly analogous to the vertebrate liver (Laverack, 1963), is characterized by a bright yellow color. In P. pacifica the occurrence of the tissue containing the yellow pigment (chloragogen tissue) is restricted to the anterior stomach and to the intestine. The anterior stomach exhibits minimal arginase activity while the intestine has relatively high activity (Table II). A cursory histological examination indicated equal amounts of chloragogen tissue in both of these anatomical regions so that in Pista there does not seem to be a correlation, either positively or inversely, between the amount of chloragogen tissue and arginase activity.

The possibility that the arginase detected in Pista pacifica tissues is of bacterial origin cannot be ruled out. Arginase has been re-

ported in Escherichia coli, Proteus vulgaris, Staph. aureus, and Bacillus stabilis (Khramov & Galaev, 1971). Unfortunately, no molecular weights or Michaelis constants are available for comparison. Fluctuations in bacterial arginase could explain the large variations observed in tissue activity (Tables II & III) though such variations could also be due to the worm's arginase responding to feeding activities, metabolic functions, or tidal patterns. Worms could only be maintained three days in sterile sea water without their tubes which may have been insufficient to notice a significant difference in activity from fresh worms.

The characteristics of arginase have undergone extensive evaluation in the past few years, in particular, their relationship to a species' mode of nitrogen excretion. A comparison of P. pacifica arginase to the enzymes of ureoteles and uricoteles summarized in Table VIII shows some similarities and some differences.

The molecular weights of arginase from uricotelic species have been found to be around 225,000 - 280,000 (Mora et al., 1965; Campbell, 1966; Reddy & Campbell, 1969a). Based on the probable subunit structure of 30,000, molecular weights in this range have been referred to as octamers (Reddy & Campbell, 1970). The value of 200,000 reported here for Pista pacifica arginase although it falls outside of this range probably represents an octamer. No heptameric arginases have been reported nor does it seem likely that they will be found as proteins containing an odd number of subunits are rare (Klotz, 1967). However, the estimation on gel filtration should be regarded as tentative until supplementary data such as ultracentrifugal studies on completely purified arginase are done. The difference in molecular weights of native Pista pacifica

TABLE VIII

CHARACTERISTICS OF ARGINASE IN RELATION TO NITROTELISM*

Nitrotelism and source	Km mM	Molecular weight x 1000	Stability during dialysis	Arginase/ canavanase	Inhibition		Reference
					----- PCMB	Arginine	
<u>Ammonoteles</u>							
crayfish body wall	2	225	+	3	+	+	Hartenstein, 1972
<u>Pista pacifica</u>	155	200	+	22	-	-	Present study
axolotl liver	16				+		Mora <u>et al.</u> , 1965
earthworm		27					Reddy & Campbell, 1968
<u>Ureoteles</u>							
rat liver	10-40	118	+	1.5	-	±	Mora <u>et al.</u> , 1966, 1967; Hirsch-Kolb & Greenberg, 1968
beef liver	2.7		+	15	+		Brown, 1966
<u>Uricoteles</u>							
chicken liver	100	276	-	7	+	-	Mora <u>et al.</u> , 1966
gull liver	2	134	-	-	+	+	Brown, 1966; Reddy & Campbell, 1970
lizard liver	100-200	276					Mora <u>et al.</u> , 1965
<u>Helix aspersa</u>	3.9	244		10		-	Campbell, 1966; Reddy & Campbell, 1970; Baret <u>et al.</u> , 1972
silkmoth	2.5	228		24	-	+	Reddy & Campbell, 1969a

* table adapted from Hartenstein (1972)

arginase (200,000) and that of Lumbricus terrestris (30,000) is notable though unexplained at this time.

Molecular weights of 120,000 (tetramers) have been reported for ureoteles (Greenberg et al., 1956; Mora et al., 1966; Hirsch-Kolb et al., 1970). Hirsch-Kolb & Greenberg (1968) have found the molecular weight of rat liver arginase to be 118,000. The enzyme is composed of four subunits each of 30,800 molecular weight. When isolated the molecular weight of P. pacifica subunit on gel filtration studies is approximately 34,000, which is in close agreement with the rat liver subunit and the monomeric arginase of the earthworm (Reddy & Campbell, 1968).

The molecular weight of the subunit following acetone treatment was approximately 42,000 (Figure 9) although the two activity peaks were not completely separated as they were with the lyophilized subunit. This could represent an association-dissociation equilibrium generated by the harsh treatment. Harsh treatment may also account for the active monomer of rabbit liver arginase. When purified at low pH's the native arginase of 118,000 molecular weight breaks down to a subunit of 36,000 (Rogers & Moore, 1963; Vielle-Breitburd & Orth, 1972).

The high K_m value obtained for polychaete arginase is in the range of the values (> 100 mM) found for chicken liver and lizard liver, both uricoteles (Mora et al., 1965, 1966). As with chicken liver, high concentrations of arginine did not inhibit the enzyme. Competitive inhibition by L-ornithine is characteristic of chicken liver, Neurospora crassa (Mora et al., 1966), silkworm arginases (Reddy & Campbell, 1969a), and Pista pacifica arginase.

Sulfhydryl compounds have had a variable effect on reported argin-

ases. Glutathione has had a stimulatory effect on arginase on several occasions (Campbell, 1966). Glutathione, dithiothreitol, 2-mercaptoethanol, and cysteine have a stimulatory effect on both bullfrog arginase and bovine arginase (Carlisky & Sadnik, 1972). The same effect was observed with Pista. On the other hand these compounds were slightly inhibitory in the case of insects (Reddy & Campbell, 1969a). The activity toward L-arginine is inhibited by p-chloromercuribenzoate in the crayfish (Hartenstein, 1971), chicken liver (Mora et al., 1966), cockroach fat body (Reddy & Campbell, 1969a), and bullfrog kidney and bovine liver (Carlisky & Sadnik, 1972). Rat liver (Mora et al., 1966) and silkworm are unaffected (Reddy & Campbell, 1969a). Pista pacifica arginase is unaffected by p-hydroxymercuribenzoate, the sodium salt of the free acid form. (The "chloro" converts to "hydroxy" when the sodium salt is prepared). Effects of sulfhydryl reagents on arginase are quite variable. This may indicate differences in enzyme structure or it may reflect experimental conditions.

The arginases present in the livers of uricotelic and ureotelic animals have a similar substrate specificity. All hydrolyze L-arginine and to some extent L-canavanine (Mora et al., 1965). Helix pomatia and Helix aspersa also hydrolyze homoarginine (Baret et al., 1972). Arginase/canavanase ratios at pH 9 - 10 for reported species have no apparent pattern. High ratios (> 10) as that obtained for Pista arginase have been reported for rat liver (Mora et al., 1966), beef liver (Brown, 1966), and silkworm (Reddy & Campbell, 1969a).

Few temperature optima have been reported. The arginase of the sockeye salmon has a temperature optimum of 45°C (Cvancara, 1971),

Helix pomatia and Helix aspersa between 60 - 65°C (Baret et al., 1972), and horse liver arginase has a temperature optimum of 45 - 50°C (Greenberg & Mohamed, 1945). The temperature optimum of Pista pacifica is approximately 60°C.

The majority of arginases appear to be activated by Mn^{2+} . In these studies P. pacifica does not exhibit this characteristic. This does not prove that Mn^{2+} is not required by the enzyme. The divalent cation may be tightly bound to the enzyme and thus be present in adequate amounts even in the absence of added Mn^{2+} . The lack of activation of the native enzyme is also observed with the subunit. Hirsch-Kolb et al., (1970) reported that four molecules of manganese are bound to one molecule of fully activated rat arginase with different binding affinities. Two are loosely bound and two molecules of manganese are tightly bound. The removal of the two loosely bound ions results in a 50% loss of activity which can be reversed by an incubation at 55°C with $MnCl_2$. When the two tightly bound ions are removed irreversible inactivation occurs. It may be that manganous ions are tightly bound to the arginase of P. pacifica. This may explain the lack of activation. On the other hand, it may be that Mn^{2+} is not required for enzyme activity or perhaps another divalent metal ion would be more effective. Experiments should be carried out in EDTA (ethylenediaminetetraacetic acid) to determine whether Mn^{2+} was required or not.

In the search to categorize arginases with respect to modes of nitrogen excretion, reports on arginases of ureo-, ammono-, and uricoteles served more to point out the disparities rather than the similarities among arginase properties. Terms such as "ammonoureoteles" and "urico-

teles with ureotelic ancestors" have been used to obscure differences and to maintain the theory of a distinction between arginases from ureotelic and uricotelic organisms. The information presented in this paper on an arginase from a terebellid worm, together with other recent data (Reddy & Campbell, 1970; Hartenstein, 1971) contradicts this theory and points out that none of the characteristics of arginase observed to date can be correlated with a particular mode of nitrotelism.

Perhaps a better way of distinguishing between arginases would be in terms of their function. A physiological function of the liver arginase is to form urea, a role which is clear from its participation in the multi-enzyme ornithine-urea cycle. Other roles for the enzyme, especially in nonhepatic tissues and in tissues where the other enzymes of the urea cycle have not been demonstrated, would include catalysis of the first step in the conversion of arginine to proline, a conversion known to occur in the udder of goats (Mephram & Linzell, 1966), the mammary gland of rats (Yip & Knox, 1972), and the fat body of insects (Reddy & Campbell, 1969b). Another functional arginase may serve to catalyze the breakdown of ingested arginine or arginine obtained from protein hydrolysis (Canedo et al., 1967; Soberón et al., 1967), as for example, via lysozymes (Tabor & Tabor, 1969). Whether P. pacifica arginase functions as an excretion arginase, a catabolic arginase, as a precursor arginase, or in a manner as yet unknown has not been empirically determined.

Although all of the ornithine cycle enzymes have not been assayed for in this animal, ornithine transcarbamylase has been detected in the intestine according to the method of Brown & Cohen (1959). Such activi-

ty is suggestive of a functional ornithine-urea cycle. However, several attempts to detect urea have been unsuccessful. A search for urease was also unsuccessful. If the arginase is not functioning to produce urea, perhaps it acts as a dietary arginase, or for the production of ornithine as a precursor of proline, or in conjunction with arginine phosphagens.

The appearance of an enzymatically active subunit is significant in comparison with the earthworm arginase and in possible terms of regulation. As previously stated, Lumbricus terrestris has a monomeric arginase of 27,000 molecular weight which is active in a functional urea cycle (Bishop & Campbell, 1965; Reddy & Campbell, 1968). Apparently a monomeric unit is sufficient for this catalysis but it raises the question of why the marine annelid developed an oligomeric arginase while retaining an active subunit, a subunit which is capable of catalyzing the reaction with a greater affinity for substrate than the intact protein. Unless, an arrangement of subunits is capable of regulating the appearance of product. Such regulation usually appears as an atypical plot (sigmoid curve) of substrate versus velocity and a Hill plot n value greater than 1. The n value of 1.034 for P. pacifica arginase would not indicate such regulation under the stated assay conditions. Possibly the enzyme has been "desensitized" (Monod et al., 1963) during purification procedures. Several research teams (Hochachka & Mostafa, 1973; Gerhart & Pardee, 1962) have noticed that the kinetics of the reaction catalyzed by a desensitized enzyme obey the Michaelis-Menten relation (hyperbolic curve) while in the presence of native enzyme the rate-concentration curve is sigmoid. Another possibility is that the

regulation requires certain small molecules for activation. Cabello (1967) has indicated that arginase modified by sodium dodecyl sulfate exhibits a sigmoidal curve as a function of arginine concentration. He also suggested that natural analogues of dodecyl sulfate, such as salts of fatty acids, may modify the properties of arginase in vivo.

However, the existence of subunits is not a necessary indication of regulation. Subunits provide stability, the conservation of DNA, and lessen the chance of a lethal mutation; many nonregulatory enzymes are composed of several subunits (Monod, 1969).

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