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PALO VERDE (*PARKINSONIA ACULEATA* L.) SEED AMINOACYLASE*

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SUMMARY

1. *N*-Acylamino acid amidohydrolase (EC 3.5.1.14), or aminoacylase, was isolated from the seeds of Palo Verde (*Parkinsonia aculeata* L.). The enzyme was localized within the cotyledons and embryo of the seed. An acetone powder preparation from the combined cotyledons and embryo furnished an active extract which was purified greater than 75-fold. Fractionation consisted of treatment with $(NH_4)_2SO_4$, cold acetone, freeze–thaw (which removed a cold-labile, inhibitory protein), and chromatography on DEAE-Sephadex A-25. The specific activity was 2650 μmoles/h per mg protein nitrogen.

2. Co^{2+} was shown to enhance activity and also to provide stability to the enzyme during the $(NH_4)_2SO_4$ fractionation.

3. *N*-Formyl-L-methionine was the best substrate and exhibited a $v_{max}/K_m = 2.9 \cdot 10^5$ at pH 7.2. The acetyl derivatives of L-methionine, L-valine, and L-leucine inhibited the enzyme at concentrations above 10 mM. The hydrolyses of *N*-acetyl-L-leucine and *N*-acetyl-L-valine exhibited non-Michaelis–Menten kinetics with an indication of two possible binding sites.

4. The molecular weight was estimated by gel filtration to be 79 500.

5. The enzyme was stable between pH 6.5 and 10.0, and up to a temperature of 50° for 10 min. The temperature optimum was 52°.

6. The enzyme catalyzed the hydrolysis of *p*-nitrophenyl acetate and was inhibited by some classical sulfhydryl reagents. Mercaptoethanol enhanced enzyme activity.

INTRODUCTION

Aminoacylase (*N*-acylamino acid amidohydrolase, EC 3.5.1.14) is a widely distributed enzyme which catalyzes the hydrolysis of a variety of N-acylated L-amino

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acids[1]. The aminoacylases from hog kidney have been particularly well studied[1-4]. At least three different aminoacylases can be distinguished in the kidney extracts by their different substrate specificities[1,5]. Renal acylase I catalyzes the hydrolysis of N-acylated L-amino acids with aliphatic side chains, and has been commonly employed in the resolution of DL-amino acids[1]. A mold aminoacylase[6] has also been employed for amino acid resolution. Acylase I and the mold enzymes are not metalloenzymes, but both activities are enhanced in the presence of Co^{2+}. Detailed knowledge of aminoacylases from higher plants is limited[7], despite the variety of plants which are known to possess aminoacylase activity[8-10]. Seeds of the Palo Verde tree (*Parkinsonia aculeata* L.), a plant indigenous to inhabited areas of the Sonoran Desert (see refs. 11-14)*, were observed to possess considerable amounts of aminoacylase activity. An extensive study of the aminoacylase from this source was undertaken with the ultimate goals of clarifying the mechanism of action and the physiological function[15], as well as to compare catalytic and stability characteristics with those of the renal aminoacylase[1-4].

MATERIALS AND METHODS

Materials

Seeds of Palo Verde (*Parkinsonia aculeata* L.) were harvested in metropolitan Phoenix, Arizona during the summers of 1966, 1967, and 1968. The seeds were removed from green, pliable pods.

Enzyme grade $(NH_4)_2SO_4$ was from Nutritional Biochemicals. Spectrograde acetone was from Matheson Coleman and Bell. DEAE-Sephadex, Sephadex G-100, and Sephadex G-200 was from Pharmacia Fine Chemicals, Inc. Protein molecular weight markers were from Mann Research Biochemicals. Hog kidney acylase was from Nutritional Biochemicals. N-Acylated L-amino acids were from General Biochemicals, Cyclo Chemical Corp., and Sigma Chemical Corp. One sample of diisopropylfluorophosphate, presumably free of sulfhydryl inhibitor, was obtained as a gift from Walter B. Gall of Merck, Sharp and Dohme Research Laboratories. All other chemicals were of reagent grade quality.

Instruments

Absorbance measurements were made with a Beckman DU spectrophotometer. When monitoring of absorbance changes with time were necessary, the Gilford model 2000 recorder was used. Fraction collection was done with a Vanguard fraction collector in conjunction with the Beckman DU spectrophotometer and a flow cell attachment. An LKB UltroRac fraction collector was also used.

All pH measurements were done with a Leeds-Northrup pH meter with expanded scale. Electrophoresis was carried out on an apparatus constructed according to the method described by DAVIS[16].

Assay procedures

Protein was assayed according to the method described by LOWRY *et al.*[17] which

* *Parkinsonia aculeata* L. is generally considered to be the Official Tree of the State of Arizona, although species of the genus Cercidium are more prevalent in the natural habitat, and are also called Palo Verde.

employs the Folin–phenol reagent and bovine serum albumin as protein standard.

The assay for aminoacylase activity consisted of equilibrating 0.9 ml of substrate (25 mM in 0.1 M phosphate buffer, pH 7.2) at 37°, then starting the reaction by the addition of 0.1 ml of the enzyme solution. At the end of the 15-min incubation period, 0.1 ml was withdrawn and added to 1.0 ml solution of ninhydrin reagent prepared according to the method of MOORE AND STEIN[18]. A marble was placed on the mouth of the tube to prevent loss of liquid and the mixture was immediately heated to 100° in a heating block for 20 min. The tube was removed from the heating block at the end of this period and immediately 5 ml of a mixture of *n*-propanol–water (1:1, by vol.) was added to it. The mixture was allowed to come to room temperature and the absorbance at 570 nm was determined. The amount of *N*-acetyl-L-amino acid hydrolyzed was calculated from a standard curve in which L-methionine was used as the reference amino acid.

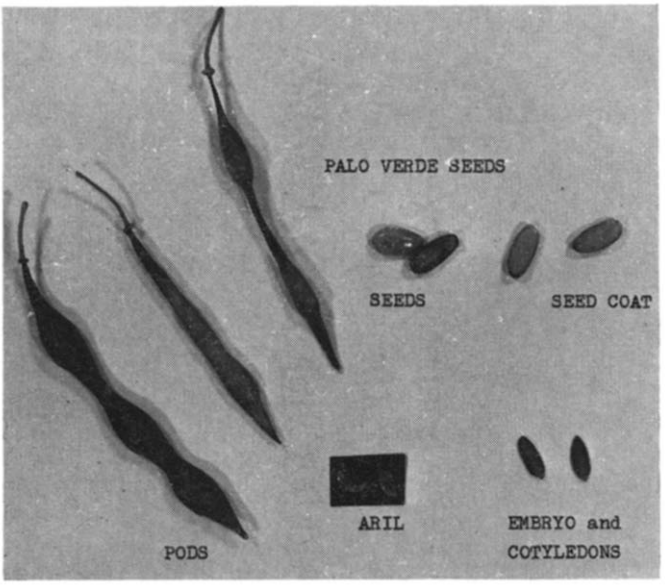


Fig. 1. Palo Verde seed pods, seeds, and seed components.

Localization of the enzyme within the seed

The seeds were soaked overnight to soften the seed coat. Twenty seeds were dissected into four major fractions (see Fig. 1) in order to determine the distribution of aminoacylase in the seed: the seed coat, the aril, the cotyledon, and the embryo. The excised fractions were dropped into chilled phosphate buffer (50 mM, pH 7.0) and then all operations were conducted at temperatures below 4°. The fractions were homogenized in a glass homogenizer and the homogenate was centrifuged at 31 000 × *g* for 15 min. The supernatant was designated as the "crude extract" and used directly in the assay for protein and aminoacylase activity.

Preparation of acetone powder of the embryo and cotyledon

The combined embryo and cotyledon was excised from seeds which had been soaked overnight. This fraction was then homogenized in an electric blendor with acetone chilled in solid CO_2. The slurry was filtered in a Buchner funnel and washed several times with chilled acetone until the powder was almost colorless. The air-dried acetone powder could be kept indefinitely refrigerated without appreciable loss of aminoacylase activity.

Extraction of aminoacylase and preliminary purification

The acetone powder was extracted with phosphate buffer (0.1 M, pH 7.20, containing 0.4 mM Co^{2+}) in an electric blendor. The extract was centrifuged at 17 000 $\times g$ for 20 min. After the residue was reextracted with no more than a total volume of buffer equivalent to 10 times the weight of the original acetone powder, the residue was discarded and the supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The fraction precipitating between 35 and 55% saturation was dissolved in a minimum amount of water and dialyzed against chilled water for 5 h. The precipitated protein was removed by centrifugation and the supernatant was subjected to acetone fractionation. Chilled acetone (−15°) was added very slowly with constant stirring and the fraction precipitating between 30 and 40% acetone (v/v) was dissolved in the minimum amount of phosphate buffer. A cold-labile protein was removed by centrifugation after freezing the solution for 48 h or longer and then thawing. The supernatant was chromatographed on DEAE-Sephadex A-25.

Chromatography on DEAE-Sephadex A-25

DEAE-Sephadex A-25 was equilibrated at room temperature in Tris–HCl buffer (0.1 M, pH 8.3). About 30 mg of protein was charged onto the 1.5 cm × 20 cm column, the flow rate was adjusted to deliver 15 ml/h, and 2-ml fractions were collected.

Polyacrylamide gel electrophoresis

Electrophoresis on polyacrylamide gel was carried out according to the method described by DAVIS[16]. Square glass tubes[19] were used to facilitate slicing of the gel. Each tube was charged with about 300 mg of protein. A current of 3 mA per tube was used. At the completion of electrophoresis the gels were removed from the tubes by rimming with a 5-cm syringe needle with a continuous jet of water and were then quick frozen on a block of solid CO_2.

The gels were sliced longitudinally into 2 or 4 segments[20], depending upon the number of assays to be carried out. One segment was stained for protein with a 1% solution of Amido Schwarz in 7% acetic acid. The remaining segments were cut into 1-mm slices[19] and dropped into 0.5 ml of the acylamino acid substrate (25 mM) in phosphate buffer (0.1 M, pH 7.20, containing 0.4 mM Co^{2+}). After incubation at 37° for 5 h, 0.1-ml aliquots were withdrawn from each tube and assayed by the ninhydrin method of MOORE AND STEIN[18]. The tubes were allowed to incubate further to 48 h and a second 0.1-ml aliquot was withdrawn from each tube and assayed as before.

Molecular weight estimation

The molecular weight of Palo Verde aminoacylase was determined chromatographically using Sephadex G-100 and protein molecular weight markers. The method

TABLE I

AMINOACYLASE DISTRIBUTION IN THE PALO VERDE SEED

Substrate was *N*-formyl-L-methionine.

Fraction	*Volume (ml)*	*Total protein (mg)*	*Total activity (μmoles/h)*	*Specific activity (μmoles/h per mg protein nitrogen)*
Seed coat	16.0	35.0	0.0	0.0
Aril	34.0	11.9	0.0	0.0
Embryo	2.0	27.4	25.1	5.7
Cotyledon	6.0	206.5	231.0	7.0

is described by ANDREWS[21]; the results were calculated and plotted according to the method of WHITAKER[22].

Kinetic constants

The kinetic constants, v_{max}, K_m, and v_{max}/K_m ("physiological efficiency"; ref. 23), were determined by use of a least-squares Fortran computer program for LINEWEAVER-BURK[24] data treatment. Initial velocities were determined in repeated experiments with varying substrate concentrations using the assay previously described. Details for the pH, temperature, and inhibitor studies are given under the appropriate figures.

RESULTS

Localization of aminoacylase in the Palo Verde seed

Pictures of the seed pods, seeds, and components are shown in Fig. 1. Table I shows the distribution of aminoacylase activity in the different excised portions of

TABLE II

PURIFICATION OF PALO VERDE AMINOACYLASE

Substrate was *N*-formyl-L-methionine.

Fractions	*Vol. (ml)*	*Total protein (mg)*	*Total activity (μmoles/h)*	*Specific activity (μmoles/h per mg protein nitrogen)*	*Activity recovery (%)*	*Purification*
I. Crude extract	71.0	1420.0	7800	34.4	100	1.0
II. $(NH_4)_2SO_4$ fraction (35–55%)	12.5	90.6	6400	441.0	82.1	12.8
III. Acetone fraction (30–40%)	8.5	38.5	6210	1001.0	79.7	29.4
IV. Removal of cold-labile protein (freezing)	8.3	30.8	6720	1365.0	86.3	39.7
V. DEAE-Sephadex A-25 Fraction (Tubes 7–9; Fig. 4)	8.0	8.6	3116	2260.0	39.9	65.7
VI. Tube 8 (peak)	2.0	2.9	1230	2650.0	15.8	77.0

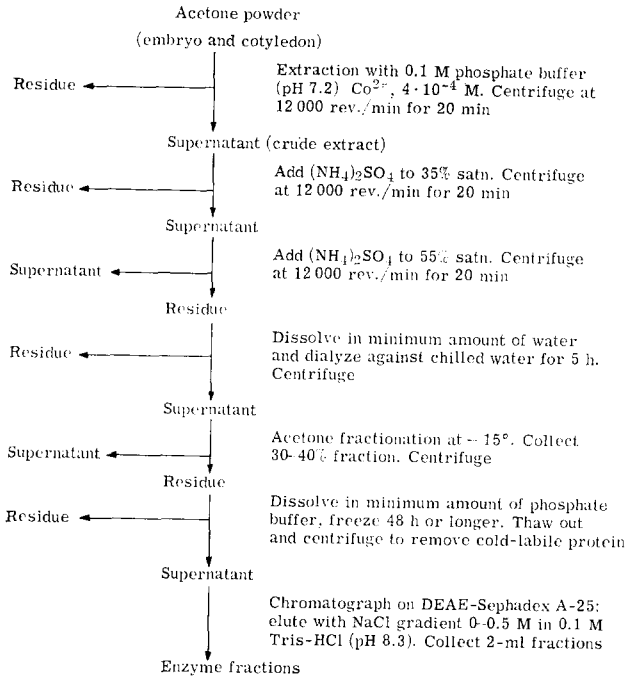


Fig. 2. Schematic diagram for the isolation of Palo Verde aminocylase.

the Palo Verde seed. All of the aminoacylase activity was found in the embryo and cotyledon fractions, with 90% in the cotyledon. The activity of aminoacylase in the embryo and the cotyledon were similar, so the extraction and purification procedure was carried through beginning with the cotyledons *plus* embryos.

Isolation of aminoacylase from the combined embryo and cotyledon

A schematic diagram of the isolation procedure is presented in Fig. 2. Table II shows a typical summary of the procedure in which the purification was 77-fold. The specific activity was higher than that sometimes observed with a higher purification factor, which probably reflects variability in batches of seeds. Under these assay conditions the rate of aminoacylase activity remained constant up to 40 min. The usual assay time employed throughout the isolation procedure was 15 min.

Effect of Co^{2+} on the activity and stability of aminoacylase

Aminoacylases from other sources are enhanced by Co^{2+} (see refs. 7, 25–28). Fig. 3 shows the stimulating effect of Co^{2+} on Palo Verde aminoacylase with optimum stimulation at 1 mM. Beyond this concentration the activity started to decrease. The enzyme used in this experiment had been purified through Stage IV of Table II. A Co^{2+} concentration of 0.1 mM, which was below the threshold for inhibition, was employed during the isolation procedure.

The aminoacylase was active in the absence of added Co^{2+} even following extensive dialysis or polyacrylamide gel electrophoresis. However, it was found that

TABLE III

ISOLATION OF PALO VERDE AMINOACYLASE. STABILIZING ACTION OF Co^{2+}

Substrate was *N*-formyl-L-methionine.

Fraction	*1 mM Co^{2+}*			*Without Co^{2+}*		
	Total activity (μmoles/h)	*Total protein (mg)*	*Specific activity (μmoles/h per mg protein nitrogen)*	*Total activity (μmoles/h)*	*Total protein (mg)*	*Specific activity (μmoles/h per mg protein nitrogen)*
Crude extract	16 950	2080	51.0	15 500	2120	54.7
$(NH_4)_2SO_4$ fractionation	10 900	149.0	450.0	4 200	152.0	172.5
Acetone fractionation	9 550	103.0	580.0	3 538	96.7	229.0
Removal of cold-labile protein	15 650	83.5	1170.0	5 790	78.5	588.0

Co^{2+} provided stability to the enzyme during isolation. It will be observed in Table III that the most pronounced effect was at the $(NH_4)_2SO_4$ fractionation stage. Almost 75% of the activity was lost in the absence of Co^{2+}, while only about 35% of the activity was lost in the presence of 1 mM Co^{2+}. The amount of protein recovered in both cases remained about the same. No appreciable effect was produced during the acetone fractionation step, nor during the removal of the cold-labile protein.

Cold-labile protein

It was observed during most of the isolation experiments that after partially purified enzyme had been stored in the freezer (−10 to −15°), a cold-labile protein

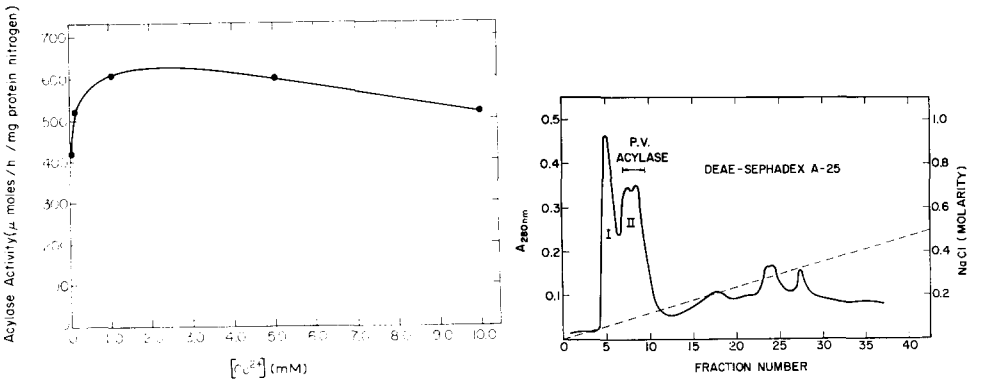


Fig. 3. Aminoacylase activity as a function of Co^{2+} concentration. 1 ml of *N*-formyl-L-methionine (37.5 mM in 0.1 M phosphate buffer, pH 7.2) containing increasing amounts of Co^{2+} was equilibrated at 37°; then 0.2 ml (297 μg protein) of Palo Verde aminoacylase solution was added. At the end of the incubation period (15 min), a 0.1-ml aliquot was withdrawn and assayed by the standard ninhydrin method[18].

Fig. 4. Separation of Palo Verde aminoacylase on DEAE-Sephadex A-25 before removal of the cold-labile protein. Column dimensions, buffer, flow rate, and other conditions of chromatography are given in the text. Peak I was inactive and corresponded to the cold-labile protein. Peak II had the aminoacylase activity.

fraction irreversibly precipitated with an accompanying increase (about 7%) in total and specific activity. The cold-labile protein was presumably bound to the aminoacylase, partially masking the catalytic activity, and was apparently not completely removed by the previous treatment with chilled acetone.

The cold-labile protein could also be removed by means of chromatography on DEAE-Sephadex A-25, an anion exchanger possessing gel filtration properties. The elution diagram of the aminoacylase preparation before the cold-labile protein was removed is shown in Fig. 4. The elution diagram after removal of the cold-labile protein did not show Peak I. Peak I in Fig. 4 did not possess any aminoacylase activity. All activity was concentrated in the region corresponding to Peak II, except for very minor amounts detected in Fraction 24.

Electrophoresis on polyacrylamide gel was carried out as described by DAVIS[16] to further characterize the Palo Verde aminoacylase. The electrophoretic pattern of Palo Verde aminoacylase is shown in Fig. 5. The cold-labile protein is represented by the dark heavy band on the cathode side near the left of the gel.

The electrophoretic patterns of fractions which have been frozen to remove the cold-labile protein showed a marked decrease in the stain intensity of the first major band. Electrophoretic patterns obtained with protein from Peak I from Fig. 4 showed an increase in the dark heavy band, which represents the cold-labile protein. Electrophoretic patterns obtained with enzyme from Peak II from Fig. 4 did not show the cold-labile protein band. Samples from Fraction 24 of Fig. 4 showed an increase in the minor bands closer to the anode; this fraction possessed very low activity. A commercial sample of hog kidney aminoacylase showed a diffused protein pattern throughout electrophoresis gels, with the aminoacylase activity located more towards the anode.

The activity profile of the Palo Verde aminoacylase after electrophoresis on

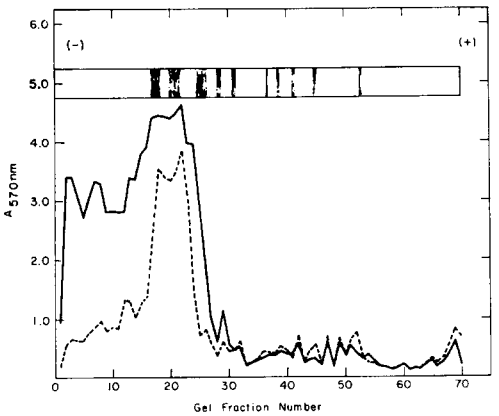


Fig. 5. Activity profile of Palo Verde aminoacylase before removal of the cold-labile protein. Electrophoresis carried out according to the method of DAVIS[16]. The gel was sliced longitudinally and stained for protein on one slice, while the other slice was cut into 1-mm segments and assayed for aminoacylase activity. The conditions of the assay are given in the text. The dotted line shows the assay results (increase $A_{570\ nm}$) after 5 h of incubation. The solid line shows the assay results after 48 h of incubation, thereby revealing which of the minor peaks were real and which were background color. Most of the aminoacylase activity was concentrated in the region of the two bands immediately below that of the cold-labile protein. The substrate was *N*-formyl-L-methionine.

polyacrylamide gel is also shown in Fig. 5. The activity was localized in a region corresponding to two adjacent bands immediately beyond the cold-labile protein. Incubation of the gel slices with *N*-formylmethionine for 5 h revealed the activity centered around gel Fraction 20. To ascertain whether minor peaks were real, the incubation was allowed to proceed to 48 h (solid line in Fig. 5); only the real aminoacylase activity peaks would increase after such an incubation. Two peaks on the cathode side of the major active aminoacylase peaks were real, and could represent degraded enzyme or very closely related aminoacylases.

The activity profile on gels when the substrate used was *N*-acetyl-L-alanine or *N*-formyl-L-methionine, and the protein stain patterns, indicated that the aminoacylase from Palo Verde might exist in at least two closely related forms. Other evidence for this observation will be presented later.

Molecular weight estimation

A molecular weight of 79 500 was obtained using Sephadex G-100, as shown in Fig. 6. This value compares well with the 76 500 obtained for hog kidney aminoacylase by BRUNS AND SCHULZE[4]. A reported[29] molecular weight of 83 000 for an aminoacylase with a high specificity for *N*-acetylhistidine, isolated from the brain of the skipjack tuna, was determined on a similar column of Sephadex G-100.

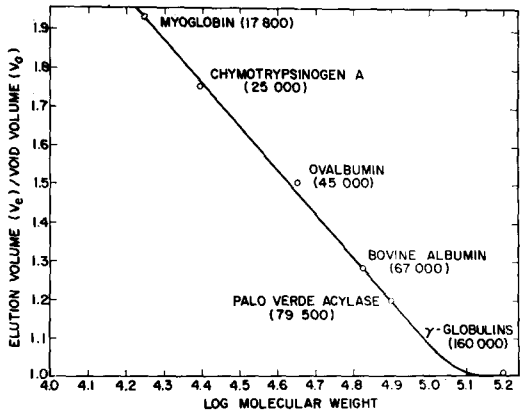


Fig. 6. The molecular weight of Palo Verde aminoacylase was determined chromatographically using Sephadex G-100 and protein molecular weight markers. The method is described by ANDREWS[21]; the results were calculated and plotted according to the method of WHITAKER[22].

Substrate specificity and reactivity

Palo Verde aminoacylase exhibited a wide range of substrate specificity as shown in Table IV. The substrate most efficiently hydrolyzed was *N*-formyl-L-methionine. Although chemical hydrolysis of formyl amino acids is generally rather facile[30], it is of interest to note that the aminoacylase hydrolysis rates for several *N*-acetyl derivatives were better than those for the corresponding *N*-formyl derivatives (Table IV). Substrates hydrolyzed by Palo Verde aminoacylase are similar to those hydrolyzed by other aminoacylases[5,6,9,10], but with some evident differences in relative reactivities, and one major exception. Table V summarizes the reactivity of

TABLE IV

PALO VERDE AMINOACYLASE SUBSTRATE REACTIVITY

Substrate	*Hydrolysis rate**	*Substrate*	*Hydrolysis rate**	*Substrate*	*Hydrolysis rate**
N-Formyl-L-methionine	1750	*N*-Acetyl-L-alanine	517	*N*-Acetyl-DL-threonine	236
N-Formyl-L-alanine	390	*N*-Acetyl-L-valine	943	*N*-Acetyl-DL-ethionine	398
N-Formyl-L-valine	127	*N*-Acetyl-L-leucine	875	*N*-Acetyl-L-norleucine	938
N-Formyl-L-leucine	738	*N*-Acetyl-L-phenylalanine	139	*N*-Acetyl-L-α-amino-butyric acid	628
N-Formyl-L-phenylalanine	100	*N*-Acetyl-L-tyrosine	73		
N-Formyl-L-tyrosine	24	*N*-Acetylglycine	276	*N*-Acetylpenicillamine	9
N-Formylglycine	92	*N*-Acetyl-L-glutamic acid	187	*N*-Acetylglucosamine	9
N-Formyl-L-aspartic acid	0	*N*-Acetyl-L-histidine	165	*N*-Acetylgalactosamine	9
N-Formyl-L-kynurenine	0	*N*-Acetyl-L-serine	282	*N*-Acetylneuraminic acid	0
N-Acetyl-L-methionine	550	*N*-Acetyl-L-cysteine	93	*N*-Acetyl-L-proline	0

* μmoles/h per mg protein nitrogen.

Palo Verde aminoacylase toward various substrates hydrolyzed by enzymes which attack amide bonds. The isolated enzyme contained no acylase II or acylase III (carboxypeptidase) activity, since it could not hydrolyze *N*-acetyl-L-aspartic acid or *N*-acetyl-L-tryptophan, respectively.[1] Palo Verde aminoacylase did not hydrolyze benzoylglycine, so the hippuricase activity reported[4] for the hog kidney enzyme was not present. The highest specific activity of 2650 μmoles *N*-formyl-L-methionine/h per mg protein nitrogen obtained for Palo Verde aminoacylase (Table II) was less than the 20 000 value for renal acylase I hydrolysis of *N*-acetyl-L-methionine[4]; however,

TABLE V

PALO VERDE AMINOACYLASE. POOR AND INACTIVE SUBSTRATES

Activity: Poor: less than 100 μmoles/h per mg protein nitrogen. Negligible: less than 10 μmoles/h per mg protein nitrogen.

Substrate	*Activity*	*Indication*
N-Acetyl-D-methionine	Poor	Requires L-enantiomer
N-Acetyl-D-alanine	Negligible	Requires L-enantiomer
N-Acetyl-L-methionineamide	Negligible	Requires free carboxyl
N-Acetyl-L-alanineamide	Negligible	Requires free carboxyl
Glycylglycine	Poor	No dipeptidase (EC 3.4.3.1)
N-Acetyl-L-tryptophan	Negligible	No acylase III (ref. 1)
N-Z-L-phenylalanine	Inactive	No carboxypeptidase (EC 3.4.2.1)
N-Acetyl-L-aspartic acid	Inactive	No acylase II (EC 3.5.1.15)
Benzoylglycine	Inactive	No hippuricase (EC 3.5.1.14)
α-*N*-Acetyl-L-ornithine	Inactive	No ornithinase (EC 3.5.1.16)
N-Formyl-L-aspartic acid	Inactive	No formylaspartate deformylase (EC 3.5.1.8)
N-Formyl-L-kynurenine	Inactive	No kynurenine formamidase (EC 3.5.1.9)
N-Formyl-L-methionyl-L-alanine	Inactive	Cannot deformylate a peptide[34–36]
Azocoll*	Inactive	No proteolytic activity

* Azocoll is an insoluble dye–collagen complex. On the action of a proteolytic enzyme, the collagen is broken down and the dye is released in solution. Rate of release is taken as a measure of enzyme activity.

it was considerably greater than the value of 91 for the purified rape seed aminoacylase hydrolysis of *N*-acetyl-L-leucine[7] and 95 for the purified mold aminoacylase hydrolysis of *N*-acetyl-L-methionine[6].

In this study of Palo Verde aminoacylase reactivity, the hydrolysis rate order for the following substrates was: *N*-acetylmethionine $\approx$ *N*-acetylalanine $>$ *N*-acetylphenylalanine. BIRNBAUM *et al.*[5] noted that renal acylase I hydrolyzed *N*-acylamino acids in the following order: *N*-acetylmethionine $\gg$ *N*-acetylalanine $>$ *N*-acetylphenylalanine. OZAKI AND WETTER[7] showed rates of hydrolysis in the following order with an aminoacylase purified from rapeseed: *N*-acetylphenylalanine and *N*-acetyltyrosine $>$ *N*-acetylmethionine $\gg$ *N*-acetylalanine. MICHI AND NONAKA[31] obtained a mold aminoacylase which showed the following hydrolysis order: *N*-acetylphenylalanine $>$ *N*-acetylmethionine $>$ *N*-acetylalanine. Although various aminoacylases may show similarities, differences can be recognized in the rates at which they hydrolyze similar substrates. An aminoacylase from *Euglena gracilis* has recently been reported[32] which selectively deformylates *N*-formyl-L-methionine, and does not catalyze the hydrolysis of *N*-acetyl-L-methionine. An enzyme from *Escherichia coli* has been prepared which will deformylate *N*-formylmethionylaminoacyl-transfer RNA and its analog, *N*-formylmethionylpuromycin, but not *N*-formylmethionine[33]. Other reports[34–36] indicate that *E. coli* extracts can deformylate *N*-formylmethionyl peptides.

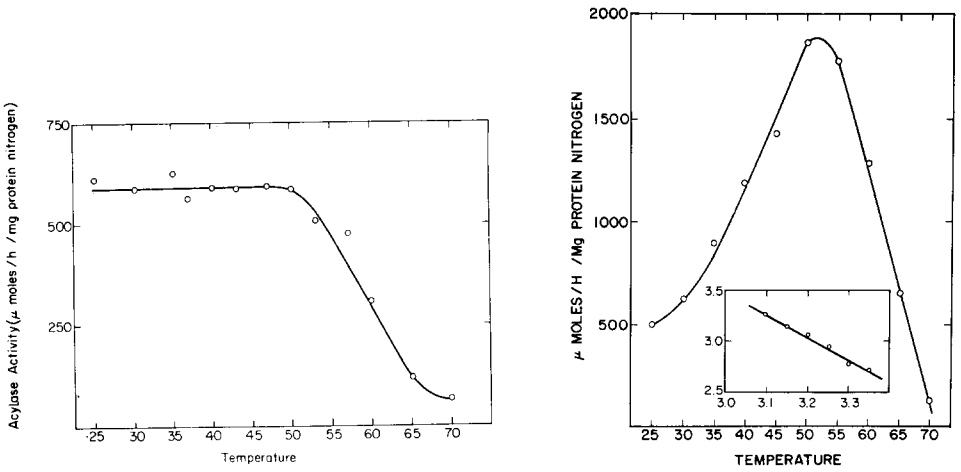


Fig. 7. Temperature stability of Palo Verde aminoacylase. The reaction tubes each contained 0.1 ml aminoacylase (192 μg protein). Each tube was heated for 10 min at a specific temperature between 25 and 70°, then cooled immediately in a 0° bath. The substrate was 3 ml of *N*-acetyl-DL-alanine (0.05 M, L-form) in 0.1 M pyrophosphate buffer (pH 9.0) containing 0.4 mM Co^{2+}. The enzyme and substrate were both brought up to reaction temperature (37°) and the reaction was started by adding the substrate to the enzyme. The rest of the assay was the same as that described under MATERIALS AND METHODS.

Fig. 8. Palo Verde aminoacylase activity as a function of temperature. The reaction tube contained 1.0 ml of *N*-formyl-L-methionine (25 mM) in 0.1 M phosphate buffer (pH 7.2) containing 0.4 mM Co^{2+} and 0.01 ml aminoacylase (70.6 μg protein). The reaction was carried out at temperatures ranging from 25 to 70°. The assay was the same as that described under MATERIALS AND METHODS. The inset shows the Arrhenius activation energy plot. The above results were replotted to obtain the apparent activation energy for the enzymatic hydrolysis of *N*-formyl-L-methionine. An apparent activation energy of 10.2 kcal/mole was obtained.

Effects of temperature on aminoacylase activity

The Palo Verde aminoacylase exhibited excellent stability. An enzyme sample stored frozen for two years retained all activity. The enzyme was shown to withstand a temperature of up to 50° for 10 min without loss in activity (Fig. 7). Chromatography of the enzyme was performed at room temperature without affecting the activity. Renal acylase I was reported[5] to be stable to 37° for 48 h and for months when stored dry at 5°. A heat stability curve reported[2] for the hog kidney aminoacylase is very similar to the one observed for the Palo Verde enzyme (Fig. 7).

The temperature optimum was 52° for the Palo Verde enzyme (Fig. 8). Assuming that saturation of the enzyme with substrate (the K_m) did not vary greatly with increasing temperature, the data of Fig. 8 could be replotted in the Arrhenius form (inset, Fig. 8). An apparent activation energy of 10.2 kcal/mole for the hydrolysis of *N*-formyl-L-methionine was determined from the slope of the plot. OZAKI AND WETTER[7] reported an activation energy of 12.6 kcal/mole for the rapeseed aminoacylase-catalyzed hydrolysis of *N*-acetyl-L-leucine. In contrast, BRUNS AND SCHULZE[4] reported 4.3 kcal/mole as the activation energy for the hydrolysis of *N*-acetyl-L-methionine by the hog kidney aminoacylase. This tends to verify the much higher specific activity possessed by the hog kidney enzyme when compared to that of the plant aminoacylases.

Effects of pH on aminoacylase activity

Palo Verde aminoacylase was found to be relatively unstable below pH 6.5 and above pH 10.0. Renal acylase I was reported[2] to exhibit complete stability between pH 6.0 and 9.0.

The determination of catalytic constants (Table VI) showed *N*-formyl-L-methionine to be the more favorable substrate at pH 7.2 on the basis of v_{max}/K_m, the "physiological efficiency" (see ref. 23). *N*-Acetyl-L-leucine showed an apparently high "physiological efficiency", but it exhibited anomalous kinetic behaviour. In particular, moderately high concentration caused substrate inhibition so that a false, and apparently high, v_{max} was obtained. The same was true for *N*-acetyl-L-methionine

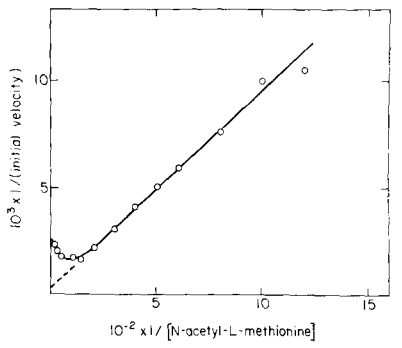
TABLE VI

PALO VERDE AMINOACYLASE

Kinetic data, pH 7.2.

Substrate	$K_m \times 10^2$ (M^{-1})	$v_{max} \times 10^{-3}$ (*μmoles/h per mg protein nitrogen*)	$v_{max}/K_m \times 10^{-3}$
N-Acetyl-L-methionine*	2.11	2.35	112.0
N-Acetyl-L-alanine	0.437	0.676	155.0
N-Acetyl-L-valine*,**	4.21	3.32	78.8
N-Acetyl-L-leucine*,**	0.285	1.27	446.0
N-Formyl-L-methionine	0.668	1.95	291.0
N-Formyl-L-alanine	2.19	0.44	20.1
N-Formyl-L-valine	14.7	0.718	4.91
N-Formyl-L-leucine	1.76	1.55	89.1

* High substrate concentrations inhibited the reaction.
** Sigmoid kinetics indicated cooperative effect.



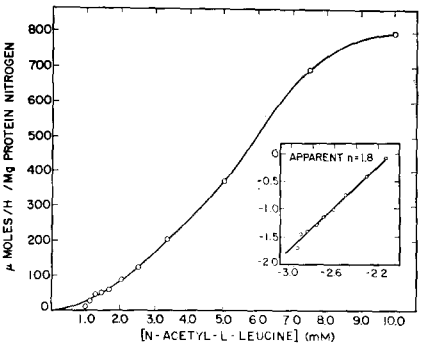


Fig. 9. Lineweaver–Burk plot for Palo Verde aminoacylase activity data. The reaction tube consisted of 1.5 ml of an appropriate concentration of *N*-acetyl-L-methionine in 0.1 M phosphate buffer (pH 7.20) containing 0.4 mM Co^{2+}. The tube was equilibrated at 37° and the reaction started by the addition of 0.02 ml of enzyme (320 μg protein). At the end of a 15-min incubation, a 0.1-ml aliquot was withdrawn and assayed. Velocity was calculated for each substrate concentration used and plotted as shown above. Kinetic parameters were usually obtained by use of a computer program.

Fig. 10. Aminoacylase activity as a function of *N*-acetyl-L-leucine concentration. The reaction tube consisted of 1.4 ml of an appropriate concentration of substrate. The reaction was started by the addition of 0.1 ml of enzyme (267 μg protein). The other conditions of the assay are mentioned under Fig. 9. Activity was plotted against *N*-acetyl-L-leucine concentration. The data obtained were replotted as indicated in the inset according to the method described by TAKETA AND POGELL[38]. In this case log $v/(v_{max} - v)$ was used in place of log $(E - S_n)/E$ for the ordinate, and log [*N*-acetyl-L-leucine] for the abscissa. The slope of the line is n and represents the apparent number of *N*-acetyl-L-leucine bound per mole of enzyme.

(Fig. 9) and for *N*-acetyl-L-valine. Another anomalous behaviour was shown in the kinetics for the hydrolysis of *N*-acetyl-L-valine and *N*-acetyl-L-leucine; a cooperative effect[37] was exhibited when these substrates were present in low concentrations. The non-Michaelis–Menten plot for *N*-acetyl-L-leucine is shown in Fig. 10. The data from Fig. 5 were replotted according to the method of TAKETA AND POGELL[38] to calculate the number of moles of substrate bound (apparent n) per mole of enzyme. As shown in the inset of Fig. 10, the apparent n for *N*-acetyl-L-leucine was 1.8. In a similar manner, the apparent n for *N*-acetyl-L-valine was calculated to be 1.3. When the actual n is 2, the apparent n cannot be greater than 2 (see ref. 39), so the actual bound substrates were probably 2 in the above two cases.

The optimum pH for Palo Verde aminoacylase varied from pH 7.0 to 9.0, depending upon the substrate employed. Single pH optima peaks were observed at 7.4 for *N*-acetyl-L-methionine and 8.0 for *N*-acetyl-L-leucine. Figs. 11–13 present the pH optimum plots for *N*-formyl-L-methionine, *N*-acetyl-L-alanine, and *N*-acetyl-L-leucine. Two buffer systems, phosphate and pyrophosphate, were used for *N*-acetyl-L-alanine (Fig. 12) to cover the pH region above and below pH 9.0. The pyrophosphate buffer (solid circles) caused a decrease in activity, but the pH optima in both buffer systems were in good agreement. A comparison of the kinetic constants at pH 7.2 and pH 9.0 for *N*-formyl-L-methionine and *N*-acetyl-L-alanine showed that v_{max}/K_m for the hydrolysis of *N*-formyl-L-methionine was the higher at the more probable physiological pH (Table VI) with both a higher v_{max} ($2.8 \cdot 10^3$ *vs.* $1.6 \cdot 10^3$) and a lower K_m ($4.8 \cdot 10^{-3}$ *vs.* $7.2 \cdot 10^{-3}$). The reverse was true for *N*-acetyl-L-alanine with a v_{max} at pH 9.0 of $1.1 \cdot 10^3$ (*vs.* $7.9 \cdot 10^2$) and K_m at pH 9.0 of $3.4 \cdot 10^{-3}$ (*vs.* $4.0 \cdot 10^{-3}$).

Fig. 11. Aminoacylase activity as a function of pH, *N*-formyl-L-methionine as substrate. The reaction tube contained 1.0 ml of *N*-formyl-L-methionine (25 mM) in 0.1 M phosphate buffer containing 0.4 mM Co^{2+}. The pH varied from 4.5 to 9.0. The tube was equilibrated at 37° and the reaction was started by the addition of 0.01 ml of enzyme (70.6 μg protein). Incubation time was 15 min; then 0.1 ml was withdrawn and assayed[18].

Fig. 12. Aminoacylase activity as a function of pH, *N*-acetyl-L-alanine as substrate. The reaction tube contained 3.0 ml of *N*-acetyl-DL-alanine (0.05 M, L-form) in buffer. Reaction was started with the addition of 0.1 ml of enzyme (192 μg protein). All other conditions were the same as that described under Fig. 11. Open circles indicate 0.1 M phosphate buffer, while the closed circles indicate 0.1 M pyrophosphate buffer.

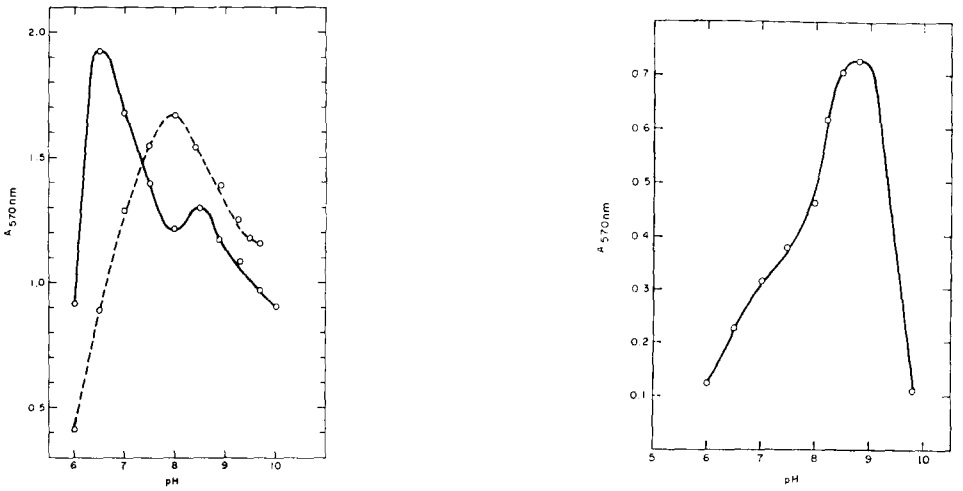


Fig. 13. Aminoacylase activity as a function of pH, *N*-acetyl-L-leucine as substrate. The reaction tube contained 0.475 ml of *N*-acetyl-L-leucine (25 mM) in 0.1 M phosphate buffer containing 0.4 mM Co^{2+}. The reaction was started with the addition of 25 μl of enzyme. The broken line represents the aminoacylase not subjected to DEAE-Sephadex separation, while the solid line represents the acylase in Fraction 7 (see Fig. 4). Fraction 7 had a protein concentration of 1.625 mg/ml. The unfractionated enzyme had a protein concentration of 0.872 mg/ml. The rest of the experiment was the same as that described under Fig. 11.

Fig. 14. Aminoacylase activity as a function of pH, *N*-acetylglycine as substrate. The reaction tube contained 0.475 ml of *N*-acetylglycine (25 mM) in 0.1 M pyrophosphate buffer containing 0.4 mM Co^{2+}. The reaction was started with the addition of 25 μl of enzyme (21.8 μg protein). The rest of the experiment was the same as that described under Fig. 11.

Unexpected results were obtained with *N*-acetyl-L-leucine (Fig. 13). When aminoacylase purified only through the acetone fractionation was used, the optimum was obtained at pH 8.0. When the enzyme used was Fraction 7 from the DEAE-Sephadex A-25 column, a pH optimum was observed at pH 6.5 with an additional increase in activity observed at pH 8.5. These dual pH optima, and previously cited evidence from gel electrophoresis experiments, indicated partial resolution in Fraction 7 of two aminoacylases. The predominant enzyme species after fractionation on DEAE-Sephadex A-25 possessed the pH optimum at 6.5. The pH optimum at 8.0 obtained with the unfractionated enzyme must have represented the overlap of two peaks. Further evidence for this phenomenon was apparent with the unfractionated enzyme and *N*-acetylglycine (Fig. 14). At least two enzymes with very similar properties must be present in the Palo Verde aminoacylase preparations. No evidence was obtained to indicate that physical characteristics reported in this paper would be altered with a more homogeneous enzyme preparation, however.

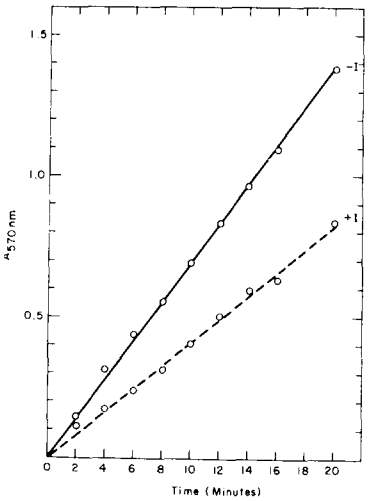


Fig. 15. Inhibition of Palo Verde aminoacylase by *p*-hydroxymercuribenzoate. The reaction tube contained 0.05 ml enzyme (43.75 μg protein) and 0.05 ml *p*-hydroxymercuribenzoate (1 mM), all in 0.1 M phosphate buffer (pH 7.2). The mixture was pre-incubated at 37° for 5 min. The reaction was started by the addition of 0.9 ml *N*-formyl-L-methionine (25 mM) in phosphate buffer. Then 0.1-ml aliquots were withdrawn at 2-min intervals and assayed by the ninhydrin method as previously described. The control (solid line)had buffer, instead of inhibitor (broken line).

Effects of inhibitors on aminoacylase activity

A series of commonly used hydrolase inhibitors was studied with Palo Verde aminoacylase. Preincubation of the enzyme with *p*-hydroxymercuribenzoate (0.5 mM) for 5 min at 37° gave 41% inhibition (Fig. 15), although only slight inhibition was obtained when the inhibitor and substrate were added together. Iodoacetamide showed no inhibition under the same conditions, while *N*-ethylmaleimide and diisopropylfluorophosphate showed negligible amounts of inhibition. The substrate concentration (2.5 mM) was about 4 times K_m, sufficiently high to prevent a reversible inhibition. When the substrate concentration was brought down to 4.5 mM (Fig. 16),

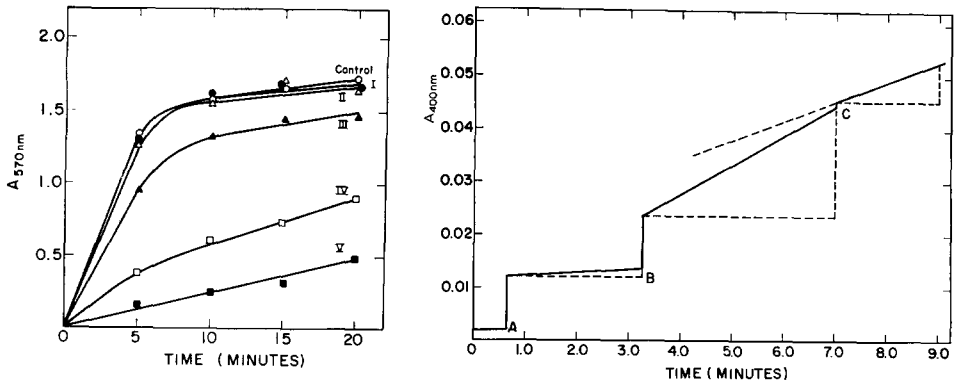


Fig. 16. Effect of inhibitors on Palo Verde aminoacylase. The inhibition experiments were carried out as described under Fig. 15, except substrate solution was 5 mM *N*-formyl-L-methionine. Inhibition was calculated from the initial velocity determined at 5 min.

Inhibitor (0.5 mM)	*Inhibition (%)*
I. Iodoacetamide	0
II. *N*-Ethylmaleimide	6
III. Diisopropylfluorophosphate	26
IV. Iodosobenzoate	70
V. *p*-Hydroxymercuribenzoate	91

Fig. 17. Aminoacylase hydrolysis of *p*-nitrophenyl acetate and competition with *N*-formyl-L-methionine. The reaction was carried out in microcuvettes with a 0.5-ml capacity and the hydrolysis was monitored at 400 nm with a Beckman DU spectrophotometer attached to a Gilford recorder. The cuvette contained 0.3 ml phosphate buffer (0.1 M, pH 7.2) at zero time. At point A, 25 μl of *p*-nitrophenyl acetate (1 M in phosphate buffer) was added. At point B, 25 μl aminoacylase (21.8 μg protein) was added. At point C, 25 μl *N*-formyl-L-methionine (25 mM) was added.

iodoacetamide still did not inhibit at all and *N*-ethylmaleimide showed only negligible inhibition. However, significant inhibition was shown by *o*-iodosobenzoate (70%) and *p*-hydroxymercuribenzoate (91%). Increased concentration of iodoacetamide to 5 mM still produced no inhibition. GOULD AND LIENER[40] reported that several commercial samples of diisopropylfluorophosphate contained an impurity which combined irreversibly with the –SH group of cysteine, GSH, and the enzyme ficin. The partial inhibition exhibited by diisopropylfluorophosphate in Fig. 16 was reinvestigated using a sample known to be free of sulfhydryl inhibitors. The diisopropylfluorophosphate which was free of –SH inhibitors had no effect on aminoacylase activity.

These inhibitor studies indicate that serine does not participate in the aminoacylase reaction and suggest that Palo Verde aminoacylase is a sulfhydryl enzyme. A 42% enhancement of activity over the appropriate control (328 μmoles substrate hydrolyzed per h per mg protein nitrogen *vs.* 232) was observed when 0.1 ml of enzyme (107.5 μg protein) was preincubated at 37° for 5 min with 0.1 ml of mercaptoethanol (1 mM), and the reaction was started by the addition of 0.7 ml of substrate (*N*-formylmethionine, 25 mM, pH 7.20). This further implicates an intact thiol group in the catalytic function of the enzyme, despite the lack of inhibition by *N*-ethylmaleimide or iodoacetamide.

Reaction with p-nitrophenyl acetate

The enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate involves the rapid formation of the acyl–enzyme intermediate, accompanied by an equally rapid release of the *p*-nitrophenolate ion[41-42]. This initial burst is followed by the steady state production of the *p*-nitrophenolate ion which can be monitored at 400 nm. Fig. 17 illustrates the hydrolysis of *p*-nitrophenyl acetate by Palo Verde aminoacylase. The increase in $A_{400\ nm}$ prior to the addition of the enzyme was the result of the spontaneous hydrolysis of the *p*-nitrophenyl acetate, but a marked rate increase resulted after addition of the enzyme. The competition for the enzyme between *N*-formylmethionine and *p*-nitrophenyl acetate is also presented in Fig. 17. The decrease in the slope on addition of *N*-formylmethionine at point C was much greater than could be expected as a result of dilution. These results suggest that the aminoacylase-catalyzed hydrolysis of *p*-nitrophenyl acetate is similar to that shown by other sulfhydryl enzymes[41].

DISCUSSION

BRUNS AND SCHULZE[4] observed that hippuric acid and thiophene-2-carboxylglycine would inhibit renal acylase I activity at relatively high concentrations. They attributed this to the formation of an inactive bimolecular complex between *ES* and *S*. They calculated the first dissociation constant for the hippuric acid *ES* to be $2.7 \cdot 10^{-3}$ moles/l and the second constant for ES_2 to be $3.7 \cdot 10^{-2}$ moles/l. They did not report any inhibition with high concentrations of *N*-acetyl-L-methionine, but hippuric acid competitively inhibited the hydrolysis of *N*-acetyl-L-methionine. The 4-fold increase in activation energy (18.0 kcal/mole *vs.* 4.3 kcal/mole), yet a K_m value only 20% greater than that for *N*-acetyl-L-methionine, indicates either an alternate site or mechanism for the hydrolysis of hippuric acid by renal acylase I.

The Palo Verde aminoacylase data for *N*-acetyl-L-methionine substrate inhibition and for *N*-acetyl-L-leucine cooperativity could be reconciled by assuming the former ES_2 complex is inactive and occurs only at high substrate concentration, while the latter ES_2 complex exhibits enhanced activity. However, *N*-acetyl-L-leucine also showed substrate inhibition at high concentrations. This does not explain why some substrates, such as *N*-formyl-L-methionine, show neither effect.

Some evidence was presented for the existence of more than one aminoacylase in the Palo Verde preparations. No gross difference in substrate reactivity was apparent between the forms, and they may just be a result of slight degradation. Variation in pH optimum for different substrates may just reflect flexibility in the catalytic mechanism. MICHI AND NONAKA[31] also observed a variation in pH optimum for several substrates used with a mold aminoacylase.

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