Brain Aminoacyl Arylamidase. Further Purification of the Soluble Bovine Enzyme and Studies on Substrate Specificity and Possible Active-Site Residues*

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ABSTRACT: An improved method for purification of a soluble bovine brain arylamidase is presented. A 600-fold purified preparation is obtained in approximately 40% yield. The enzyme is a stereospecific aminopeptidase, acting only on model substrates which have a free amino group α to the amide bond, and have the L configuration. Brain arylamidase does not appear to exhibit a dipeptidase, tripeptidase, or amidase activity. Similarly no esterase activity was demonstrable under the experimental conditions employed. The brain enzyme appears to be capable of releasing a ninhydrin-positive material from a mixture of partially digested brain proteins; however it may also be inhibited by certain polypeptides as demonstrated with the dialyzed supernatant fraction of brain homogenate and the oxidized B chain of insulin. From the energies of activation for several model substrates of arylamidase, it appears that the mechanism of hydrolysis must be the same for each substrate, and may involve a binding and/or acylation, followed by rate-limiting deacylation as deduced from the results with a poorly hydrolyzable model substrate, AlaAla-β-naphthylamide. From the pH dependency of pK_m and V_{max} for substrates and pK_i for inhibitors, the imidazole of histidine and the SH of cysteine are implicated as participating in the active site of the enzyme. Strong, reversible inhibition of arylamidase activity by Hg^{2+} and *N*-ethylmaleimide further indicates the essentiality of a free SH group for enzyme action. The irreversible inactivation by α-*N*-tosyl-L-phenylalanine chloromethyl ketone is believed to be due to modification of an essential residue other than SH, presumably the imidazole of histidine. The brain enzyme is competitively inhibited by puromycin, a characteristic reported for most other aminoacyl arylamidases.

Over the past 10 years the presence of hydrolytic enzymes that act preferentially on aminoacyl-β-naphthylamides has been demonstrated in a variety of mammalian tissues (Smith *et al.*, 1965; Behal *et al.*, 1965; Hanson *et al.*, 1967) as well as in other species (Nagatsu *et al.*, 1968; Behal and Folds, 1967; Tjeder, 1966). Arylamidase activity has also been demonstrated in the central and peripheral nervous systems (Adams and Glenner, 1962), extracts of bovine pituitary gland (Ellis and Perry, 1966; Ellis and Nuenke, 1967), and in extracts of bovine (Brecher and Barefoot, 1967) and rat brains (Marks *et al.*, 1968). Marks (1968; Marks and Lajtha, 1970) has recently reviewed the properties of arylamidases A, B, and N, the various dipeptidyl arylamidases, as well as one enzyme strongly resembling cathepsin C (McDonald *et al.*, 1966, 1969).

The function of all arylamidases is not well understood. If, as has been postulated by various workers (Tappel, 1968; Marks *et al.*, 1968), arylamidases function at some stage of protein catabolism, their presence in brain tissue, which is known to have a high arylamidase content and to exhibit high turnover of proteins (Marks, 1968), is of particular interest, and warrants a more detailed study of these enzymes.

A partial purification and characterization of the soluble, bovine brain arylamidase have been reported from this laboratory previously (Brecher and Suszkiw, 1969a).

In this paper we report on a modified purification procedure which yields an enzyme preparation of higher specific activity and in considerably improved yield. Results of studies of the substrate specificity of the highly purified bovine brain arylamidase, the nature of substrate–enzyme interactions, and kinetic investigations are also reported. Preliminary reports have appeared elsewhere (Brecher and Suszkiw, 1969b; Suszkiw and Brecher, 1969).

Experimental Section

Materials

Ammonium sulfate (enzyme grade) L-Ala-, L-Arg-, L-Leu-, L-Phe-, L-Lys-, and DL-Ala-BNA, Gly-Gly-Gly, Gly-Gly, Gly-NH_2, L forms of Ala-Gly, Lys-Lys, Leu-Gly, Lys-Gly-Gly, Ala-Gly, Gly, Leu-Gly-Gly, Phe-Gly-Gly, His-Gly-Gly, and Leu-NH_2, oxidized B chain of insulin, albumin, casein, and L-amino acids were obtained from Mann Research Laboratories, New York, N. Y. L-Ala-L-Ala-BNA,[1] L-Ala-

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[1] Abbreviations used are: ANA, α-naphthylamine; AU, activity units; Bz-L-Arg-NA, benzoyl-L-arginine β-naphthylamide; Bz-L-Arg-PA, benzoyl-L-arginine *p*-nitroanilide; BNA, β-naphthylamine; BuOH, butyl alcohol; Cbz, carbobenzoxy; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); K-P_i, potassium phosphate buffer; PMB, *p*-mercuribenzoic acid; PNA, *p*-nitroaniline; TLCK, α-*N*-tosyl-L-lysine chloromethyl ketone;

Ala, and *N*-Cbz-L-amino acids were obtained from Cyclo Chemical Corp., Los Angeles, Calif. L-Lys-PNA, L-Leu-PNA. dithiothreitol, TPCK, TLCK, β-naphthol, and β-naphthyl propionate were obtained from Sigma Chemical Company, St. Louis, Mo. All organic compounds other than those listed were from Eastman Organic Chemicals, Distillation Products, Rochester, N. Y. Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories, Richmond, Calif. DEAE-Sephadex A-50 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. All other reagents were obtained from Fisher Scientific Co., Silver Spring, Md.

Methods

Determination of Protein. Protein was measured by the method of Lowry *et al.* (1951). Protein concentrations were expressed in milligrams per milliliter from a standard curve obtained with bovine albumin, Cohn fraction V.

Routine Enzyme Assay. Arylamidase activity was measured chemically by the method of Goldbarg and Rutenburg (1958) as previously described (Brecher and Suszkiw, 1969a). Fluorometric assays were generally employed for rapid screening of eluates from the fraction collector. In the fluorometric assay of activity the increase in fluorescence due to the released BNA in the course of reaction was measured on the G. K. Turner Fluorometer, Model 111, fitted with a constant temperature door and a cuvet holder. A general utility Primary Filter, color specification 7-60, was used for the incident light, and a general utility Secondary Filter, color specification 2-A, was used for the fluorescent light. Enzyme activity is expressed in units of activity where one activity unit represents that amount of enzyme which will hydrolyze 1 μmole of AlaBNA/min at 37°, at pH 7.5.

Purification. All purification steps were performed in the cold, at 0–5°. All buffers used, unless otherwise indicated, were potassium phosphate buffers, and contained mercaptoethanol at the final concentrations of 50 mM. In addition, dithiothreitol was added to the enzyme preparation to activate it maximally just before the final dilution of the enzyme was made for the assay of activity.

A. Bovine brain fractionation. Fresh brains, or brains once frozen and thawed, were homogenized for 30 sec in a Waring blender at a ratio 1 : 4 (w/v) in 0.32 M sucrose solution containing 0.1 mM EDTA, adjusted to pH 7.4 with 0.1 N NaOH. The homogenate was centrifuged at 7000*g* for 20 min in a refrigerated Servall centrifuge. The cell debris was discarded and the supernatant fraction was further centrifuged at 44,000*g* for 90 min in the refrigerated Spinco preparative ultracentrifuge. The 44,000*g* supernatant fraction so obtained served as the starting material for the purification of the brain arylamidase.

B. Ammonium sulfate fractionation I. Solid $(NH_4)_2SO_4$ was added to the supernatant fraction from the preceding step, to give 40% saturation at pH 6.5. After 10 hr, the preparation was centrifuged in a Servall centrifuge at 17,300*g* for 20 min at 0–4°. The sediment was discarded and the supernatant fraction was brought to 70% saturation with solid $(NH_4)_2SO_4$, at pH 6.5. The mixture was allowed to stand overnight. The suspension was subsequently centrifuged as described above, and the sediment containing activity was resuspended in 0.1 M buffer (pH 6.5) to give one-tenth of the starting volume. The solution was then dialyzed exhaustively against the same buffer.

C. Ammonium sulfate fractionation II. The dialyzed fraction from step B was brought to 40% saturation with solid $(NH_4)_2SO_4$ at pH 6.5. After 5 hr the suspension was centrifuged as described before and the supernatant fraction was brought to 60% saturation with solid $(NH_4)_2SO_4$ at pH 6.5. After standing overnight, the suspension was centrifuged for 30 min at 17,3000*g*. The sediment which contained activity was resuspended in 0.1 M buffer (pH 6.5) and was dialyzed exhaustively against several changes of this buffer.

D. Hydroxylapatite column chromatography. A column of dimensions 1.8 × 26 cm was prepared with hydroxylapatite and was equilibrated overnight with 0.01 M buffer (pH 6.5). The enzyme preparation from step C, containing approximately 2.0 g of protein in a volume of 50 ml, was then applied. After washing the charged column with 200 ml of the equilibrating buffer, gradient elution under the applied pressure of 10 psi of N_2 was commenced, at the rate of flow of 1 ml/min. A linear gradient was formed between 0.05 and 0.5 M phosphate buffer (pH 6.5). The effluate fractions containing arylamidase activity were pooled and were concentrated by precipitation of the protein with ammonium sulfate at 75% saturation at pH 6.5. The sediment was resuspended and dialyzed exhaustively against 0.01 M buffer (pH 7.5).

E. DEAE-Sephadex column chromatography I. A 2.5 × 25 cm column was prepared with DEAE-Sephadex suspended in 0.05 M buffer (pH 7.5). Before application of the protein sample, the column was equilibrated overnight with 0.05 M buffer (pH 7.5). Approximately 300 mg of protein in a volume not exceeding 50 ml was applied and the charged column was washed with 200 ml of the equilibrating buffer. Subsequently, the gradient elution was started at the flow rate of 1 ml/min. Gradient vessel A contained 1 l. of the equilibrating buffer and gradient vessel B contained 1 l. of 0.50 M buffer (pH 7.5). The effluate fractions containing activity were pooled and were concentrated by ammonium sulfate precipitation as described previously. The concentrate was dialyzed exhaustively against the equilibrating buffer.

F. DEAE-Sephadex column chromatography II. A column of dimensions 1.5 × 15 cm was used. The preparation from step E was applied to the column. The applied sample normally contained no more than 30 mg of protein in approximately 10 ml of buffer. After washing the charged column with 100 ml of 0.05 M buffer, elution was begun with an NaCl gradient. An approximately linear gradient was formed by mixing equal volumes of solutions A and B, where solution A was the equilibrating buffer (pH 7.5, 500 ml). The effluate fractions containing activity were pooled and were either stored in the presence of mercaptoethanol at a final concentration of 50 mM, or were concentrated by packing solid sucrose around a dialysis sack containing enzyme, and dialyzed against 0.05 M buffer (pH 7.5).

Structural Requirements for Substrates for Arylamidase. A series of substrate analogs were synthesized. *n*-Propionyl-BNA was prepared by reacting 0.01 mole of β-naphthylamine in 20 ml of ethyl acetate with 0.10 mole of propionyl chloride, which was added dropwise over a period of 1 hr at room

TLC-SA, thin-layer chromatography–silicic acid; TPCK, α-*N*-tosyl-L-phenylalanine chloromethyl ketone.

temperature. After an additional 2 hr the reaction mixture was filtered with suction. The product was washed successively with two volumes of ethyl acetate and three volumes of distilled water. A flaky precipitate so obtained was dissolved in hot ethanol, decolorized with charcoal, filtered, and allowed to recrystallize from 50% ethanol (v/v) in the cold. The recrystallization was repeated twice, and the product was dried *in vacuo* over sodium sulfate (mp 121–122). *Anal.* Calcd for $C_{18}H_{13}ON$: C, 78.2; H, 6.6; N, 7.0. Found: C, 77.6; H, 6.7; N,7.1.

β-Ala-BNA was synthesized by the mixed-anhydride method according to Glenner *et al.* (1965). To 0.01 mole of β-*N*-Cbz-Ala dissolved in 10 ml of ethyl acetate, 0.01 mole of dry triethylamine and 0.01 mole of ethyl carbonate were added at −5°, to form the mixed anhydride. After 15 min coupling with 0.015 mole of BNA in ethyl acetate was initiated; the temperature of the reaction mixture was brought to 25°. After 14 hr the product was washed sequentially with saturated NaCl solution, 1 N HCl, distilled water, and 1 N sodium bicarbonate. The Cbz-β-Ala-BNA derivative, after being dried over sodium sulfate, *in vacuo*, was dissolved in HBr-saturated glacial acetic acid for removal of the protecting carbobenzoxy group (Greenstein and Winitz, 1961). The final product was precipitated from the glacial acetic acid solution by treating it with anhydrous petroleum ether (bp 30–60°) overnight, filtered, and washed several times with anhydrous diethyl ether (mp 232°). *Anal.* Calcd for $C_{13}H_{15}N_2O_1Br \cdot 3H_2O$: C, 44.7; H, 5.7; N, 8.2. Found: C, 45.0; H, 5.0; N, 8.2.

The α-Ala-ANA was synthesized in an analogous fashion (mp 253–254°). *Anal.* Calcd for $C_{13}H_{15}N_2OBr$: C, 52.9; H, 5.1; N, 9.5. Found: C, 53.3; H, 5.2; N, 9.5.

α-Alanyl-β-naphthyl ester was synthesized in the following way. A solution of 0.01 mole of *N*-Cbz-Ala and 0.01 mole of triethylamine in 10 ml of chloroform was cooled to 0° and 0.01 mole of ethyl chlorocarbonate was added. After 8 min the solution was treated with 0.01 mole of β-naphthol in 5 ml of chloroform and was heated to boiling for 1–2 min. The chloroform was evaporated and the residue was recrystallized from ethanol. After removing ethanol the product was treated with HBr–glacial acetic acid and was precipitated from the solution with dry ether. The crystalline product was washed several times with anhydrous ether and was stored in the refrigerator (mp 195°). *Anal.* Calcd for $C_{13}H_{14}NO_2Br \cdot H_2O$: C, 49.6; H, 5.1; N, 4.4. Found: C, 49.8; H, 5.2; N, 4.4. To test whether the above compounds served as substrates, solutions of them were made at 9.54×10^{-4} M concentrations in 0.1 M potassium phosphate buffer (pH 7.5); 1.0 ml of each, plus 0.9 ml of buffer and 0.1 ml of highly active enzyme preparation, were assayed for various lengths of time, from 15 min to 4 hr (with the exception of the alanyl ester which hydrolyzed rapidly under the conditions of the experiment). BNA was measured both chemically and fluorometrically. β-Naphthol was measured both fluorometrically and by coupling with diazo blue B and reading the color at 540 mμ (Roth, 1965).

Determination of Energies of Activation for Selected Substrates. Energies of activation were estimated graphically from the Arrhenius plots of log activity *vs.* $1/T$. Activity was determined with each substrate tested at four temperatures, usually at 21, 28, 32, and 37°. The standard chemical assay procedure was used for amino acid or dipeptidyl derivatives of BNA. For the PNA derivatives of lysine and leucine the extent of reaction was recorded at 410 mμ (Bundy, 1962).

Mode of Action of Arylamidase on Ala-Ala-BNA. Ala-Ala-BNA was made 9.14×10^{-4} M in 0.1 M potassium phosphate buffer (pH 7.5). To 1.0 ml of substrate and 0.8 ml of buffer, 0.2 ml of enzyme solution containing 0.28 μg of protein was added. The mixture was incubated for various time intervals ranging from 15 to 60 min. Reaction products at 0, 15, 30, and 60 min were identified chromatographically.

Test of Di- and Tripeptides, and Amino Acid Amides as Possible Substrates of Arylamidase. Various dipeptides and tripeptides, prepared in 0.1 M potassium phosphate buffer (pH 7.5) at concentrations ranging from 0.5 to 5 mM, were incubated in the presence of the enzyme for 1, 4, and 8 hr. The progress of the reaction was followed by the ninhydrin method (Moore and Stein, 1948). The amides were prepared in a similar way. Assay was done by recording change in optical density on a Beckman DU spectrophotometer (Mitz and Torres, 1960).

Polypeptides and Proteins as Potential Substrates of Arylamidase. Oxidized B chain of insulin, oxidized B chain of insulin after trypsinolysis and chymotrypsinolysis, heat-denatured brain homogenate before and after treatment with trypsin, casein, and bovine albumin were made up in 0.1 M potassium phosphate buffer (pH 7.5) at concentrations of 1 mg/ml. To test for activity, 1 ml of the preparation and 0.1 ml of enzyme solution containing 0.14 μg of protein plus 0.01 ml of 0.1 M dithiothreitol were incubated for 4 and 8 hr at 37°. At these times aliquots of the reaction mixtures were assayed for tyrosine increase (Udenfriend and Cooper, 1952) and increase in ninhydrin-positive material (Moore and Stein, 1948). Appropriate controls and reagent blanks were run together with the test solutions in all the experiments described above, and were used for correction of the test values.

Inhibitory Effect of the Concentrated 44,000g Supernatant Fraction of Bovine Brain and of Oxidized B Chain of Insulin and Its Fragments on Arylamidase Activity. The dialyzed, lyophilized supernatant fraction of bovine brain homogenate was resuspended in 0.01 M potassium phosphate buffer (pH 7.5). The mixture was then heated for 10 min at 100° to destroy residual arylamidase activity and to inactivate other enzymes. Aliquots of the preparation were preincubated with 0.1 ml of enzyme solution containing 0.14 μg of protein at 0–4° in a final volume of 1 ml. After 10 min 1 ml of solution of Ala-BNA was added and the reaction was allowed to proceed for 15 min at 37°.

The effect of the oxidized B chain of insulin and its chymotryptic digest on arylamidase activity was studied in the similar fashion.

Effect of pH on Kinetic Parameters of Arylamidase-Catalyzed Reactions. pK *vs.* pH curves were obtained for L-Ala-BNA. The buffers used were 0.1 M potassium phosphate for the pH range 5–8 and 0.1 M Tris-HCl buffer for the pH range 8–9. Citrate buffer was also used at low pH's. The final concentrations of substrates in the reaction mixtures were 1.25×10^{-4}, 0.5×10^{-4}, 0.25×10^{-4}, and 0.05×10^{-4} M. BNA liberated from Ala-BNA was assayed fluorometrically at 15, 30, 45, and 60 sec after initiation of the reaction. K_m was extrapolated from the initial velocities obtained for each substrate concentration (Lineweaver and Burk, 1934), and corresponding pK_m values were plotted *vs.* pH. ΔH_i for groups implicated in enzymic catalysis were estimated from K_m

TABLE I: Routine Purification of Arylamidase from the Soluble Fraction of Bovine Brain.[a]

Preparation	Substrate	Sp Act.	Total Act.	% Recov	Purificn
44,000*g* supernatant	Ala-BNA	0.925	4710	100	1
	Arg-BNA	0.661	3360	100	1
	Leu-BNA	0.510	2600	100	1
	Phe-BNA	0.293	1495	100	1
First $(NH_4)_2SO_4$, 40–70%, pH 6.5	Ala-BNA	1.99	4230	90	2.15
	Arg-BNA	1.63	3500	104	2.46
	Leu-BNA	0.85	1825	70	1.66
	phe-BNA	0.56	1205	81	1.93
Second $(NH_4)_2SO_4$, 40–60%, pH 6.5	Ala-BNA	3.68	3800	80.5	3.99
	Arg-BNA	3.04	3120	93	4.60
	Leu-BNA	1.47	1840	70.6	2.90
	Phe-BNA	1.10	1005	83.5	3.76
Hydroxylapatite chromatography potassium phosphate gradient, pH 6.5 (0.05–0.5 M)	Ala-BNA	17.65	3305	70.2	19.1
	Arg-BNA	16.20	2890	96	17.6
	Leu-BNA	7.77	1672	64.2	11
	Phe-BNA	5.75	910	61	14.1
DEAE-Sephadex chromatography. 1. Potassium phosphate (0.075–0.5 M), pH 7.5 gradient	Ala-BNA	85.0	3400	72.2	183.5
	Arg-BNA	76.5	3006	91.2	167
	Leu-BNA	37.4	1490	57.2	106
	Phe-BNA	31.3	1235	82.6	153.5
DEAE-Sephadex chromatography. 2. NaCl gradient in 0.075 M K-P_i buffer, pH 7.5 (0–0.5 M)	Ala-BNA	557	2030	43	600
	Arg-BNA	440	1600	47.6	685
	Leu-BNA	212	770	29.8	435
	Phe-BNA	156	576	38.6	533

[a] All substrates were made 9.54×10^{-4} M in potassium phosphate buffer (pH 7.5). Prior to assay, 0.9 ml of enzyme preparation was treated with 0.1 ml of 0.1 M dithiothreitol solution and then diluted with buffer as needed. The diluted enzyme preparation (0.1 ml) was incubated with 1.0 ml of substrate solution and 0.9 ml of buffer for 10 min and BNA release was determined as described in the text. Specific activity: μmoles of BNA released/min per mg of protein; unit activity: 1.0 μmole of BNA released/min.

values at 28°, obtained as described above. ΔH_i was then obtained from

$$\Delta H_i = \frac{4.56 \times \Delta pK_e \times T_1T_2}{T_2 - T_1} \text{cal/mole}$$

where ΔH_i is the heat of ionization and ΔpK_e is the difference in the pK ionization of the active groups of the enzyme at temperatures T_2 and T_1.

Study of Reversibility of Enzyme Inhibition by Means of Sephadex G-25 Gel Filtration. Sephadex gel filtration was used to study the reversibility of the formation of enzyme–inhibitor complexes. A column 1 cm in diameter and 50 cm in length was used. Prior to use the column was equilibrated with 0.1 M K-P_i buffer (pH 6.5) containing mercaptoethanol at a final concentration of 50 mM. The rate of elution of the column was 0.5 ml/min, and 2-ml fractions were collected.

Results

Enzyme Purification. The results of purification are shown in Table I. The final step yields an enzyme preparation with a specific activity approximately 600 times higher than that of the starting material, and with a recovery of the order of 40%. The 600-fold-purified enzyme preparation was used in all the experiments reported herein. When a preparation approaching homogeneity is desired the above fraction can be further purified by preparative disc electrophoresis, with 17–40% recovery.

Analytical Disc Electrophoresis. Results of analytical disc electrophoresis are shown in Figure 1. The 600-fold purified enzyme preparation is not represented, but differed only slightly from the preparative disc fraction in that the former had two to three additional faint protein bands. Tubes 2 and 4 show the zymograms of the second ammonium sulfate fraction and of the 600-fold purified fraction. On the former, one major band is observed on the zymogram as well as a faint one. It is not certain whether the faint band is an artifact of separation or not since it was not consistently found. However, since the zymogram is derived from the preparation which utilized once frozen and thawed brain, it is possible that the appearance of this band is due to partial solubilization of a particulate or lysosomal enzyme. On the other hand, more than one peak of activity, tested with the four substrates

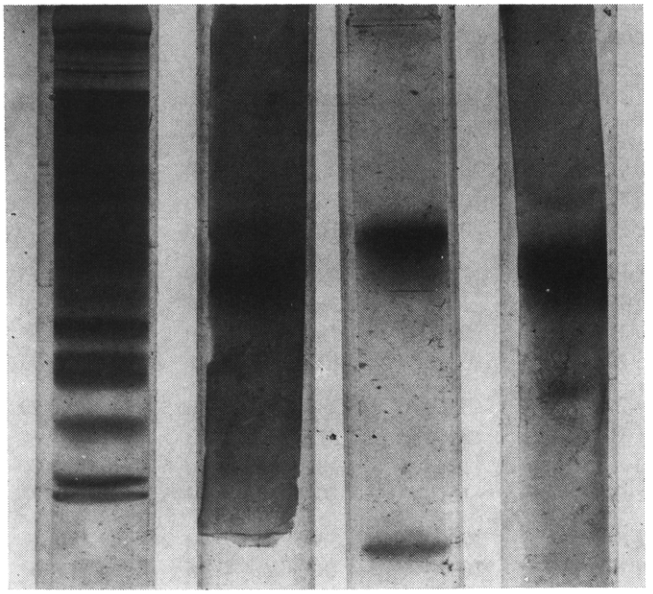


FIGURE 1: Electrophoretic protein patterns and zymograms of the starting and the highly purified arylamidase preparations. Protein electrophoregrams were obtained on polyacrylamide gels according to the method of Davis (1964). A 1:1 solution of coomasie blue in water was used to stain the protein, according to directions by the Canalco Co. Zymograms were obtained by electrophoresing 25–100 μg of protein on the polyacrylamide gels, at 0–5°. The enzyme bands were located according to the procedure of Marks *et al.* (1968) by incubating the gels in a solution of Ala-BNA, in the presence of 25 mmoles of dithiothreitol, for 30–60 min. After washing the gel with 0.1 M acetate buffer (pH 4.5) the liberated BNA in the enzyme band was detected by coupling with 0.14% solution of fast garnet GBC in 0.1 M acetate buffer (pH 4.5). Kinetic investigations were carried out with arylamidase of 557 AU, exhibiting only one activity band by the zymogram technique. (1) 44,000*g* supernatant fraction, (2) zymogram second ammonium sulfate fraction, (3) preparative disc electrophoresis fraction, and (4) zymogram 600-fold purified enzyme.

as indicated in Table I, was never encountered at any stage of the ammonium sulfate fractionation or during chromatographic separations.

Marks *et al.* (1968) were able to demonstrate three peaks of arylamidase activity in rat brain. In this laboratory, bovine brain arylamidase has never been observed in more than one peak during chromatographic purification. The question of the existence of isoarylamidases in bovine brain must at best remain unresolved at this time. Only one band of arylamidase activity was obtained with the 600-fold-purified fraction as exemplified by tube 4.

Studies of Structural Requirements for Substrates for Arylamidase Activity. Among the following compounds tested as possible substrates for arylamidase were α-alanyl-β-naphthyl ester, β-naphthyl propionate, β-naphthyl acetate, α-alanyl-ANA, propionyl-BNA, β-alanyl-BNA, and α-alanyl-BNA. Only α-alanyl-BNA was hydrolyzed, indicating that (a) a free α-amino group is necessary for activity, (b) arylamidase does not exhibit esterase properties, and (c) α-naphthylamides cannot serve as substrates, presumably for steric reasons.

Configurational Requirements for Substrates for Arylamidase Activity. Results of Table II show conclusively that arylamidase is specific to L-amino acid β-naphthylamides only.

TABLE II: Study of Configurational Requirements on the Substrate for Arylamidase Activity.[a]

Substrate	Concn in Incubn Mix. (M)	Unit Act. (μmoles of BNA/min)	% Act.
L-Ala-BNA	4.57×10^{-4}	0.796	100
DL-Ala-BNA	4.57×10^{-4}	0.860	106
L-Ala-BNA	2.29×10^{-4}	0.760	100
DL-Ala-BNA	2.29×10^{-4}	0.481	63.5
L-Ala-BNA	1.15×10^{-4}	0.515	67.8
L-Ala-BNA	0.457×10^{-4}	0.241	100
DL-Ala-BNA	0.457×10^{-4}	0.135	56.3

[a] L-Ala-BNA and DL-Ala-BNA were prepared in 0.1 M potassium phosphate buffer (pH 7.5). Enzyme preparation (0.1 ml) containing 0.14 μg of protein, 0.9 ml of buffer, and 1.0 ml of substrate was incubated for 15 min at 37°.

At saturating conditions of the L forms of the substrate an identical degree of hydrolysis occurs with either L-Ala-BNA or DL-Ala-BNA. When the concentrations of the substrates are decreased, approximately 40% decrease in the extent of hydrolysis is seen. This decrease in activity observed with 2.29×10^{-4} M DL-Ala-BNA corresponds to the decrease in activity from control when L-Ala-BNA is used at the concentration corresponding to the L form in the DL-Ala-BNA preparation, *i.e.*, 1.15×10^{-4} M. It can be furthermore concluded on the basis of results in Table II that the D forms do not compete with the L forms of substrate.

Comparison of ΔE_a and Relative Activities for Selected Substrates. Table III shows energies of activation for several substrates and the relative activities exhibited on these substrates by arylamidase. It is also observed that the ΔE_a's are relatively constant as compared to relative activities. This suggests that in the case of arylamidase-mediated hydrolysis of aminoacyl-β-naphthylamides and of aminoacyl-*p*-nitroanilides the mechanism of interactions between the substrate and the enzyme leading to the activated ES complex

TABLE III: Comparison of Energies of Activation and Relative Activities for Some Substrates of Arylamidase.[a]

Substrate	ΔE Activation (Cal/mole deg)	Rel Act.
Ala-BNA	33.4	100
Arg-BNA	23.4	71.5
Lys-BNA	10.9	65.5
Leu-BNA	23.2	55.0
Ala-Ala-BNA	24.6	37.1
Phe-BNA	20.2	31.7
Lys-pNA	21.6	14.6
Leu-pNA	21.2	9.0

[a] The experimental conditions are described in the text.

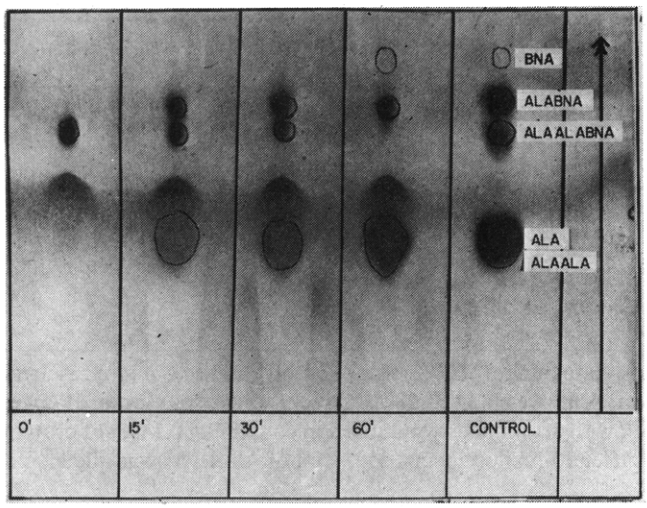


FIGURE 2: Mode of action of arylamidase on Ala-Ala-BNA I. The reaction mixture contained 1 ml of 9.14×10^{-4} M Ala-Ala-BNA in 0.1 M potassium phosphate buffer, 0.9 ml of buffer, and 0.1 ml of enzyme solution containing 0.14 μg of protein. After 0, 15, 30, and 60 min, the test solutions were lyophilized and concentrated tenfold; 10 μl of each sample was applied to the TLC-SA 20 $\times$ 30 sheets and chromatographed in a *n*-BuOH–HOAc–H_2O (60:20:20, v/v) solvent system. The sheets were then developed with 0.2% ninhydrin solution. BNA was visualized under ultraviolet light.

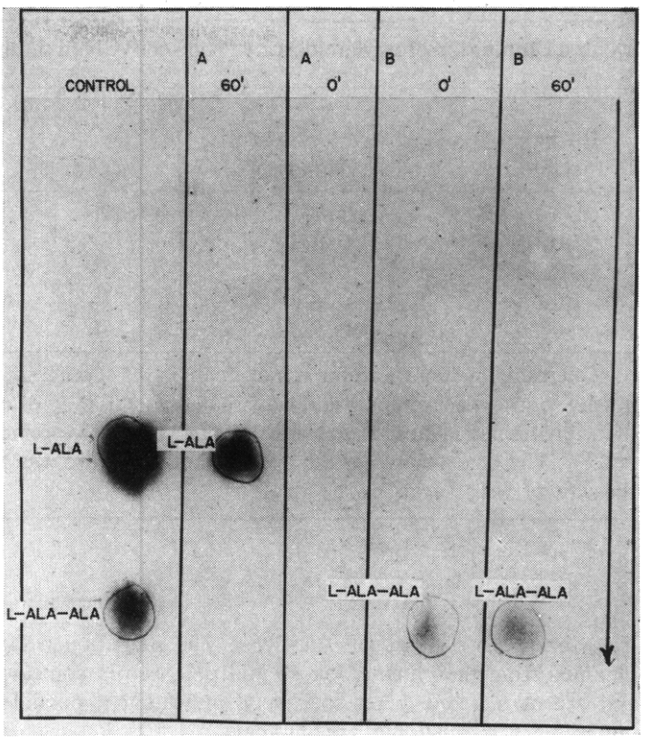


FIGURE 3: Mode of action of arylamidase on Ala-Ala-BNA II. The reaction conditions were as given in Figure 2. Contol: separation of L-Ala from L-Ala-Ala: (A) 5-μl aliquots of the reaction mixture was applied at 0 and 60 min; and (B) 5-μl aliquots of a mixture of L-Ala-Ala with arylamidase was applied at 0 and 60 min. Ascending chromatography on Whatman No. 1 paper with *n*-BuOH–HOAc–H_2O (v/v) was performed. The spots were visualized with 0.2% ninhydrin solution.

must be similar if not identical. The fact that activities do not correlate with ΔE_a values would indicate that the rate-limiting steps in the arylamidase-catalyzed reaction may be those beyond the activated ES complex and may involve decomposition of the activated ES complex, rather than its formation. One exception to the above considerations seems to be Lys-BNA for which the ΔE_a is considerably lower than for the other compounds.

Mode of Action of Arylamidase on Ala-Ala-BNA. From the results in Figures 2 and 3, it is seen that Ala-Ala-BNA is hydrolyzed sequentially from the amino end of the compound. At the start of the reaction, only Ala-Ala-BNA is present (Figure 2). At 15 and 30 min both Ala-Ala-BNA and Ala-BNA are observed but no BNA is detectable. It is also evident (Figure 3) that at the start of reaction neither Ala nor Ala-Ala is present initially. At 60 min, however, only Ala is observed. Since the enzyme does not act on Ala-Ala (see Figure 3 and the following section) it is concluded that the hydrolysis of Ala-Ala-BNA takes place sequentially from the free amino end and that Ala-Ala-BNA must be considerably depleted before the hydrolysis of the Ala-BNA can occur.

Test for Dipeptidase, Tripeptidase, and Amidase Activity. Peptidase activity on Ala-Gly, Gly-Gly, Lys-Lys, Leu-Gly, Ala-Ala, Lys-Gly-Gly, Ala-Gly-Gly, Gly-Gly-Gly, Leu-Gly-Gly, Phe-Gly-Gly, His-Gly-Gly, Leu-NH_2, Gly-NH_2, and Gly-Gly-NH_2 as potential substrates was not detected. None of the peptides tested showed any inhibitory effect on arylamidase activity.

Polypeptides and Proteins as Potential Substrates of Arylamidase. No increase in tyrosine or ninhydrin-positive material was noted even after 8-hr incubation of albumin, casein, brain homogenate, oxidized B chain of insulin, or tryptic or chymotryptic digests of the oxidized B chain of insulin with an amount of enzyme that hydrolyzed the control Ala-BNA substrate 100% within 1 min was observed. Accordingly, arylamidase shows no proteinase activity and no endopeptidase activity under the experimental conditions employed. However, after treatment of brain homogenate with trypsin to produce a partially digested mixture of proteins and peptides of various sizes, an increase in a ninhydrin-positive material is observed after incubation with arylamidase. It thus appears that the brain enzyme may act on the products of limited proteolysis, presumably oligopeptides. The nature of the oligopeptidase activity has not yet been characterized.

Inhibitory Effects of Oxidized B Chain of Insulin, Peptide Fragments of B Chain of Insulin, and of 44,000g Supernatant Fraction of Brain Homogenate. Arylamidase was inhibited by the oxidized B chain of insulin as well as by its chymotryptic digest, indicating that either a nonapeptide, hexapeptide, or tetrapeptide obtained upon chymotrypsinolysis (Sanger and Tuppy, 1951) is equally effective against arylamidase activity (Table IV). Inhibition of arylamidase by insulin reflects the ability of arylamidase to interact with endogenous peptides since the dialyzed lyophilized heat-denatured 44,000*g* supernatant fraction from bovine brain homogenates will also diminish activity markedly.

Effect of pH on Kinetic Parameters of Arylamidase. Figure 4 presents the results of pH dependence of pK_m and log

TABLE IV: Inhibition of Arylamidase by Oxidized B Chain of Insulin and Its Fragments after Chymotrypsinolysis.[a]

B Chain of Insulin (mg)	Act. Units of Arylamidase	% Act.	Chymotryptic Digest of B Chain (mg)	Act. Units of Arylamidase	% Act.
0	1.003	100	0	1.003	100
0.05	0.333	32.3	0.05	0.343	33.3
0.10	0.202	19.6	0.10	0.186	18.0
0.15	0.146	14.1	0.15	0.186	18.0
0.20	0.130	10.9	0.20	0.146	14.2

[a] Oxidized B chain of insulin was made up in 0.1 M potassium phosphate buffer (pH 7.5) at a concentration of 1 mg/ml. A 1-ml portion of the stock preparation was treated with 100 μg of chymotrypsin overnight at 28°. Aliquots of the solution of B chain of insulin treated and untreated with chymotrypsin were preincubated with 0.1 ml of enzyme solution containing 0.14 μg of protein at 0–4°. The final volume of the preincubation mixture was 1 ml. After 10-min preincubation, 1 ml of Ala-BNA was added and the activity was assayed for 15 min at 37°.

V_{max} for the hydrolysis of Ala-BNA. The pK_{E_1} and pK_{E_2} obtained from the plots of log V_0 and pK_m *vs.* pH coincide and are at 6.1 and 7.7, respectively, indicating a possible involvement of histidine and cysteine residues in enzyme action. However, involvement of an α- or an ϵ-NH_2 group which ionizes in the same pH region (Barnard and Stein, 1958) cannot be ruled out. The ΔH_i values were also obtained with Ala-BNA as substrate and were calculated to be −7123 cal/mole for ΔH_{E_1} and −11,824 cal/mol for ΔH_{E_2}. The heats of ionization at the lower pH correspond to literature values cited for histidine (6900–7500 cal/mole) (Barnard and Stein, 1958; Dixon and Webb, 1964). However it is more difficult to make a definite assignment for the heat of ionization of pK_{E_2}. Reliable heats of ionization for cysteinyl SH in proteins are not available.

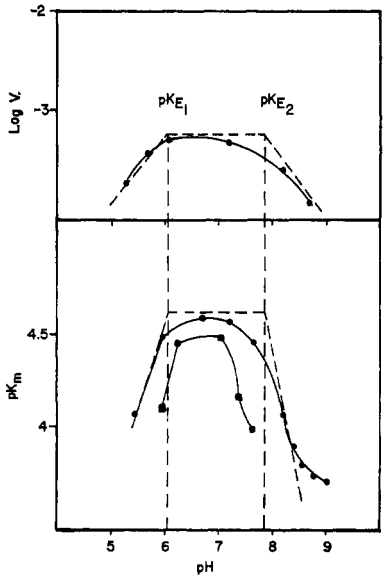


FIGURE 4: Effect of pH on kinetic parameters of arylamidase-catalyzed reactions. Ala-BNA was used as substrate. Initial velocities at different substrate concentrations were recorded fluorometrically for 10–60-sec intervals. K_m values were determined from $1/v$ *vs.* 1/(S) plots. Buffers employed were: 0.1 M potassium phosphate for pH 6.0–8.0, 0.1 M citrate for pH 5.5–6.5, and 0.1 M Tris-HCl for pH 8.0–9.0. Corrections for fluorescence quenching at high and low pH values are incorporated into the results. (●—●) Experimental at 37°, (■—■) experimental at 28°, and (- - - - - - -) theoretical.

Inhibition of Arylamidase Activity by Hg^{2+}, N-Ethylmaleimide, TPCK, and Puromycin. Table V presents the apparent K_i constant for the respective modifiers of arylamidase activity. It is observed that only TPCK and TLCK inhibit the enzyme irreversibly.

Brain arylamidase is inhibited by puromycin in a competitive fashion, in confirmation of results of Ellis and Perry (1964) and Behal *et al.* (1965). However, the aminonucleoside alone exhibits no inhibitory activity. Figure 5 shows the results of inhibition of arylamidase by Hg^{2+} and TPCK and the corresponding loss of total sulfhydryl content of the reaction mixture. Since a residual amount of the activator dithiothreitol was unavoidable in the reaction mixture, this experiment does not yield a direct stoichiometry of the reaction, and indeed the correlation between the loss of total SH and the protein SH is an oversimplification. Nevertheless,

TABLE V: Inhibition Constants for Various Modifiers of Arylamidase Activity.[a]

Inhibitor	$K_{i,app}$ (M)	Inhibition
Hg^{2+}	3×10^{-6}	Reversible
NEM[b]	4×10^{-5}	Reversible
TPCK	6×10^{-5}	Irreversible
TLCK	3×10^{-6}	Irreversible
Puromycin	5×10^{-6}	Reversible, competitive

[a] A. S. Brecher and J. B. Suszkiw, unpublished results; K_i obtained by the method of Dixon (1953). [b] NEM = *N*-ethylmaleimide.

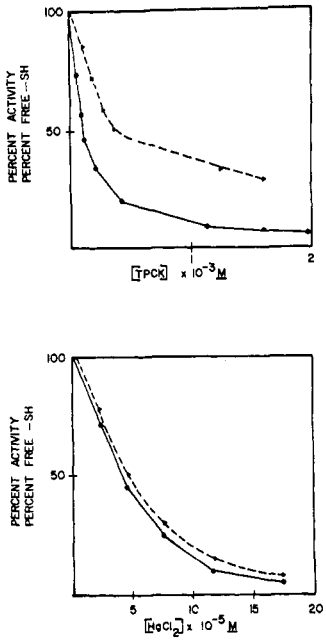


FIGURE 5: Effect of Hg^{2+} and TPCK on arylamidase activity, and on the free sulfhydryl content. To 0.1 ml of enzyme solution (0.87 AU) in 0.1 M potassium phosphate buffer (pH 7.5) aliquots of respective inhibitor solution (Hg^{2+} or TPCK) were added and the enzyme–inhibitor mixture at the final volume of 1 ml was preincubated for 10 min at 0–4°. The starting total free sulfhydryl content of the mixture was 0.39 μmole. The residual activity of the enzyme was determined by assaying against 9.54×10^{-4} M solution of Ala-BNA in potassium phosphate buffer (pH 7.5). The decrease in the sulfhydryl content was determined by DTNB method (Ellman, 1959). (●—●) Activity and (▲- - - - -▲) sulfhydryl.

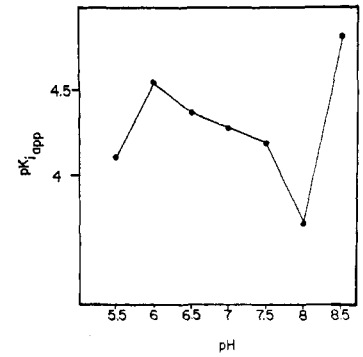


FIGURE 6: Effect of pH on pK_i for inhibition of arylamidase by TPCK. pK_i's at various pH's were determined by the method of Dixon (1953). Substrate solutions were used at two concentrations: $S_1 = 0.914 \times 10^{-4}$ M and $S_2 = 4.57 \times 10^{-4}$ M. The buffers used were pH 5.5–8.0, 0.1 M potassium phosphate and pH 7.5–8.5, 0.1 M Tris-HCl.

the qualitative picture suggests that whereas loss of enzyme activity and SH correlate well, when Hg^{2+} is used, loss of enzyme activity due to TPCK is greater than the loss of SH, indicating that TPCK may be, in addition, reacting with another group on the enzyme. Further support for this obtains from the pK_i–pH profile for TPCK inhibition of arylamidase (Figure 6) with two major inflection points at pH ~6 and ~8.

Reversibility of Arylamidase Inhibition as Studied by Gel Filtration. Table VI shows the results of gel filtration studies. The inhibition of the enzyme by Hg^{2+} is reversible. Neither Hg^{2+} nor puromycin affords any protection against the irreversible inactivation by either TPCK or TLCK. Since equimolar concentrations of modifiers (with the exception of puromycin) were employed, one can eliminate an assumption that TPCK inhibits the enzyme irreversibly by displacing Hg^{2+}, since the affinity for the latter is about 20-fold greater than for TPCK (K_i for Hg^{2+} inhibition of arylamidase = 3×10^{-6} and K_i for TPCK = 6×10^{-5}).

Discussion

In general, aminopeptidase activity has been suggested for arylamidases, and their involvement at some state of intracellular protein degradative processes has been implicated. In order to better understand the function of brain arylamidase, the nature of substrate–enzyme interaction, particularly with regard to energies of activation, mode of action, and substrate inhibition, as well as the specific requirements for compounds to act as model substrates, have been studied.

From the studies of substrate analogs, it has been found that a free amino group attached to the α carbon in the substrate is essential for arylamidase activity thereby establishing it unequivocally as an aminopeptidase. The enzyme is also absolutely stereospecific to the L configuration of the aminoacyl moiety of the model substrate as it neither hydrolyzes DL-Ala-BNA more than 50% nor is inhibited by the D form in the racemic mixture.

The energies of activation for several model substrates have been observed to be essentially independent of a substrate, thus appearing more of a property of the brain arylamidase rather than its substrates. This, in turn, implicates similar if not identical mechanisms of hydrolysis, and furthermore would indicate that the steps leading to the formation of an activated enzyme–substrate complex prior to the bond-breaking step are not only similar but also kinetically cannot be rate limiting, as evidenced by the lack of correlation between ΔE_a and relative activities. This bears directly on and corroborates our interpretation of the mode of action of brain arylamidase on Ala-Ala-BNA. The hydrolysis of Ala-Ala-BNA is sequential from the amino end confirming the absolute requirement for a free amino group α to the bond hydrolyzed. The most important conclusion that can be derived from this experiment is that the bond breaking between Ala-Ala in the Ala-Ala-BNA is followed by the release of Ala-BNA indicating once again that BNA itself plays no role in binding of the substrate, although it certainly plays a role in recognition of the substrate by enzymes. If this were the case, one would expect complete hydrolysis to either BNA + Ala-Ala or BNA + 2Ala soon after the start of reaction. Such was not observed. In view of the above discussion, one may speculate that the enzyme is most likely aminoacylated and that the liberation of the amino acid must be the rate-limiting step since otherwise it is difficult to envision the preference shown by the enzyme for Ala-Ala-BNA over that for Ala-BNA considering that a certain time is required for equilibration of the two in the immediate vicinity of the active site in order for the differences in affinity, or simple mass effect, to take place. Such an interpretation is

TABLE VI: Sephadex G-25 Gel Filtration Studies of Arylamidase Inhibition.[a]

Starting Total Act. Units	Modifier of Activity: Puromycin, 2.5 μM — Order of Addn	Puromycin, 2.5 μM — % Act.	$HgCl_2$, 10 μM — Order of Addn	$HgCl_2$, 10 μM — % Act.	TPCK, 10 μM — Order of Addn	TPCK, 10 μM — % Act.	TLCK, 10 μM — Order of Addn	TLCK, 10 μM — % Act.	Overall % Act. Prior to Gel Filtration	Total Act. Units of Effluate	% Recov of Act.
4.60									100	4.9	100
4.60	+	1.64							1.64	4.78	97.5
4.60			+	3.91					3.91	3.67	75.0
4.60					+	0			0	0	0
4.60			1	1.6	2	0			0	0	0
4.60							+	0	0	0	0
4.60			1	1.6			2	0	0	0	0
4.60	1	1.64	2	0	3	0			0	0	0
4.60	1	1.64	2	0			3	0	0	0	0

[a] Aliquots (1 ml) of enzyme solution in 0.1 M potassium phosphate buffer (pH 6.5) were treated with puromycin, $HgCl_2$, TPCK, and TLCK in various orders of addition for 30-min time intervals. A 0.5-ml sample of modified enzyme was subsequently applied to the 0.5 × 50 cm Sephadex G-25 column and was eluted with 0.1 M potassium phosphate buffer (pH 6.5) containing 50 mM mercaptoethanol. The aliquots of enzyme solution at the various stages of treatment and the column effluates were assayed for activity with Ala-BNA at 37° at pH 7.5.

not in disagreement with conclusions derived from the ΔE_a data. The mode of action of arylamidase on Ala-Ala-BNA can be thus described as

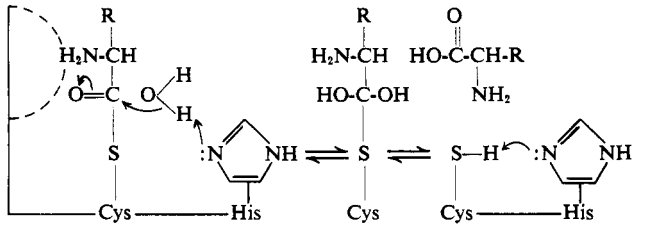
$$\begin{array}{l} E + \text{Ala-Ala-BNA} \rightleftharpoons \\ \qquad\qquad \text{E-Ala-Ala-BNA} \longrightarrow \text{E-Ala} \longrightarrow E + \text{Ala} \\ \qquad\qquad\qquad\qquad\qquad\quad \searrow \\ \qquad\qquad\qquad\qquad\qquad\quad \text{Ala-BNA} \end{array}$$

$$\begin{array}{l} E + \text{Ala-BNA} \rightleftharpoons \text{E-Ala-BNA} \longrightarrow \text{E-Ala} \longrightarrow E + \text{Ala} \\ \qquad\qquad\qquad\qquad\qquad\quad \searrow \\ \qquad\qquad\qquad\qquad\qquad\quad \text{BNA} \end{array}$$

By analogy a similar mechanism is thought to take place in the hydrolysis of other substrates of this enzyme.

Arylamidase did not hydrolyze di- and tripeptides, confirming investigations of Marks *et al.* (1968) and Hopsu *et al.* (1966), and contrasting with some results of the latter authors on basic dipeptides such as Lys-Lys. Arylamidase did not hydrolyze the oxidized B chain of insulin or endogenous polypeptides in the brain homogenate and its 44,000*g* supernatant. However, a considerable activity was detected by the ninhydrin method when the brain enzyme was incubated with a partial tryptic digest of the brain homogenate. This would indicate that the arylamidase possesses a capacity to act on certain-length polypeptide chains that may result from the action of intracellular proteases. Insulin and its chymotryptic peptides are capable of inhibiting arylamidase. Analogous productive binding of specific oligopeptides may be easily envisioned. Interestingly, a similar inhibitory capacity is exhibited by the concentrated, dialyzed 44,000*g* supernatant fraction from brain indicating that the interaction between the arylamidase and insulin is not a fortuitous phenomenon and has its counterpart in an endogenous biological system. On the basis of the pH dependence studies, imidazole, sulfhydryl, or α-amino groups were suggested as the most likely groups involved in the catalytic action of the soluble bovine brain enzyme. Inactivation of the enzyme by *N*-ethylmaleimide, PMB (Brecher and Suszkiw, 1969a), and Hg^{2+} support the involvement of SH groups in activity. Inactivation of the enzyme by photooxidation (J. B. Suszkiw and A. S. Brecher, unpublished results), TPCK, and TLCK further implicate the imidazole group. TPCK and TLCK are blocking reagents which have been shown to react specifically with one of the histidines in the active sites of chymotrypsin (Schoellmann and Shaw, 1963) and trypsin (Shaw *et al.*, 1965), respectively. These compounds, however, were also shown to inactivate papain by irreversibly reacting with the active SH group of this enzyme (Whitaker and Perez-Villasenor, 1968; Bender and Brubacher, 1966). Since loss of arylamidase activity and loss in SH content do not correlate directly with increasing TPCK concentrations, it is most likely that TPCK is capable of reacting both with the proposed necessary thiol and with the presumed necessary imidazole groups. Hence, mercuriarylamidase, which can be reactivated, may also react irreversibly with TPCK or TLCK at the imidazole site even though the Hg^{2+} may block reactivity at the thiol site. This suggestion is based in part on observation of Whitaker and Perez-Villasenor (1968) and Bender and Brubacher (1966) of the lack of inhibition of mercuripapain by TPCK. The results presented indicate that indeed mercury does not protect the enzyme against inactivation by TPCK. In conclusion, cysteine and histidine appear to participate in the catalytic action of the brain arylamidase. In this respect the brain arylamidase seems to correspond to the rat liver arylamidase for which cysteine and histidine have also been implicated in activity (Makinen and Hopsu-Havu, 1967). It is suggested that the mechanism of action of the bovine brain arylamidase could be similar to that of chymotrypsin and trypsin with the SH group of cysteine replacing the serine hydroxyl group. If such is indeed the case then the catalysis by arylamidase would entail acylation of the SH of cysteine to form an

SCHEME I: Postulated Mechanism for Arylamidase



aminoacyl-enzyme intermediate, and subsequent deacylation with catalytic participation of the histidine as shown in Scheme I.

A competitive inhibition by puromycin is noted. This appears to be a fairly specific and distinguishable characteristic of arylamidases in general and was first reported by Ellis and Perry (1964) for the pituitary aminoacyl arylamidase. It is also of interest that the aminonucleoside moiety of puromycin itself does not inhibit the enzyme indicating that an intact peptide link between *p*-methoxyphenylalanine and the 3-aminoribose moieties, together with the free amino group on the aminoacyl moiety, is essential for interaction between puromycin and arylamidase. It appears that this antibiotic behaves as a true competitive inhibitor of brain arylamidase rather than a poor substrate, in agreement with the findings of Ellis and Perry (1966) for the pituitary enzyme.

The soluble bovine brain arylamidase reported herein appears to be distinct from the anterior pituitary enzyme (Ellis and Perry, 1966) and the rat brain enzyme (Marks *et al.*, 1968) since the latter two enzymes are inactivated by treatment with or dialysis *vs.* EDTA, and the inhibition is overcome by such heavy metals as Co^{2+} and Zn^{2+}. The purified enzyme, reported herein, is not inactivated by exhaustive dialysis *vs.* 0.1 mM EDTA, nor by incubation with higher concentrations of EDTA (J. B. Suszkiw and A. S. Brecher, unpublished results). In contrast, it is inhibited by comparable levels of Co^{2+} and Zn^{2+} (Brecher and Suszkiw, 1969a,b).

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