Dipeptidyl Arylamidase II of the Pituitary

PROPERTIES OF LYSYLALANYL-β-NAPHTHYLAMIDE HYDROLYSIS: INHIBITION BY CATIONS, DISTRIBUTION IN TISSUES, AND SUBCELLULAR LOCALIZATION

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SUMMARY

An enzyme is described that was observed in extracts of rat and bovine pituitary glands. This enzyme, named dipeptidyl arylamidase II, catalyzes the cleavage of the dipeptide moiety from Lys-Ala-β-naphthylamide at pH 5.5, but not from monoamino acid arylamides. Dipeptidyl arylamidase II exhibits no metal, halide, or sulfhydryl dependences, and its specificity is restricted to substrates having an unsubstituted NH₂-terminus.

Lower but significant rates of hydrolysis were also exhibited at pH 5.5 on the β-naphthylamides of Arg-Ala and Leu-Ala, with only traces of activity on the arylamides of Ala-Ala, Gly-Pro, and Ser-Met. No activity was detected on the P-naphthylamides of benzylodicarbonyl-Lys-Ala, Lys-Lys, Arg-Arg, Gly-Arg, Gly-Phe, Ser-Tyr, or His-Ser.

Cations were exceptionally inhibitory to the hydrolysis of Lys-Ala-β-naphthylamide by dipeptidyl arylamidase II, and the sensitivity of the enzyme increased with the size of the cation: Li⁺ < Na⁺ < K⁺ < Tris < puromycin aminonucleoside < puromycin. A 50 mM concentration of Tris, pH 5.5, in the assay system gave a 96% inhibition of arylamidase II activity, while puromycin at 1 mM gave a 76% inhibition.

A Kᵣ of 1.1 × 10⁻⁵ M was determined for the hydrolysis of Lys-Ala-β-naphthylamide at pH 5.5 at 37°. The inhibition by cations was competitive for all cations tested, with Kᵣ values at pH 5.5 and 37° of 1.8 × 10⁻³ M for Na⁺, 3.2 × 10⁻⁴ M for Tris, 1.9 × 10⁻⁵ M for puromycin, and 3.0 × 10⁻⁵ M for puromycin aminonucleoside.

Dipeptidyl arylamidase II activity was detected in tissues other than pituitary; thyroid, spleen, and kidney were relatively rich sources, whereas liver, pancreas, and serum were poor. The hydrolysis of Lys-Ala-β-naphthylamide at pH 5.5 by all tissues tested showed the same sensitivity to Tris ions.

As indicated by studies with sucrose homogenates of fresh rat pituitary glands, and with subcellular fractions prepared by differential and density gradient centrifugation, dipeptidyl arylamidase II was located, along with acid phosphatase, in pituitary lysosomes. However, unlike acid phosphatase, arylamidase II appeared to be located almost exclusively in the lysosomes, and exhibited a functional latency of about 91 to 95%.

Ser-Tyr arylamidase (dipeptidyl arylamidase I) was also found to be lysosomal, whereas Arg-Arg arylamidase (dipeptidyl arylamidase III) was cytoplasmic. The aminopeptidase and dipeptidase activities measured on Lys- and Arg-β-naphthylamide were predominantly (90 to 95%) cytoplasmic.

In an earlier report (1), two enzymes that are capable of catalyzing the hydrolysis of dipeptides from their β-naphthylamide derivatives were identified in pituitary extracts. These two enzymes, designated dipeptidyl arylamidase I and II, hydrolyze Ser-Tyr-β-naphthylamide and Lys-Ala-β-naphthylamide, respectively. In that report, which dealt primarily with the properties of dipeptidyl arylamidase I, it was briefly indicated that dipeptidyl arylamidase II can be separated from dipeptidyl arylamidase I by ammonium sulfate fractionation; in addition, the former catalyzes the hydrolysis of Lys-Ala-β-naphthylamide without a chloride requirement, whereas dipeptidyl arylamidase I is a sulfhydryl enzyme which exhibits an absolute halide requirement for the hydrolysis of Ser-Tyr from Ser-Tyr-β-naphthylamide as well as for the cleavage of Ser-Tyr from a decapeptide corresponding to the NH₂-terminus of adrenocorticotropic hormone.

It was also reported previously (2) that an enzyme derived from extracts of bovine anterior pituitary catalyzes the cleavage of NH₂-terminal dipeptides from tripeptides such as Ser-Met-Glu, Ala-Ala-Ala, and Met-Met-Ala. Dipeptides and tetrapeptides through hexapeptides are not hydrolyzed. However, as will be shown in a forthcoming report, this activity can now be attributed to dipeptidyl arylamidase II, the subject of the present paper.

More recently, an enzyme was detected in pituitary extracts that is capable of catalyzing a dipeptide cleavage from Arg-Arg-

β-naphthylamide at pH 9.0. This enzyme has been purified extensively and reported under the name of dipeptidyl arylamidase III (3). Dipeptidyl arylamidases I, II, and III all behave as dipeptidyl aminopeptidases in that they only hydrolyze substrates with an unsubstituted NH₂ terminus.

The object of the present report is to describe more adequately the properties of dipeptidyl arylamidase II and thereby distinguish this enzyme from other peptidases which have already been identified in the pituitary gland. A sensitive, and apparently specific, fluorescence assay for dipeptidyl arylamidase II is described. This assay was used to investigate the cation sensitivity and subcellular localization of arylamidase II, and to ascertain its distribution among various tissues of the rat.

**EXPERIMENTAL PROCEDURE**

**Extraction and Fractionation of Pituitary Tissue—Aqueous extracts of bovine anterior pituitary glands were prepared from 20% (w/v) homogenates that were adjusted to pH 5.5 prior to centrifugation at 10,000 x g for 30 min at 5°. The clear red supernatant, designated the “pH 5.5 extract,” was either adjusted to pH 7.0 and stored at -20° or used directly for fractionation with (NH₄)₂SO₄. Most of the dipeptidyl arylamidase II activity was precipitated from the pH 5.5 extract by the addition of (NH₄)₂SO₄ to a final concentration of 1.7 M, or 43% of saturation. This constituted a significant step, since dipeptidyl arylamidase I remained in solution. The protein that precipitated at 1.7 M (NH₄)₂SO₄ was dissolved in water, adjusted to pH 7.0 with a dilute solution of NaOH, and dialyzed repeatedly against cold, distilled water maintained close to pH 7.0 with Na₃PO₄ at about 10⁻⁵ M.

**Measurement of Hydrolysis Rates on β-Napthylamide Substrates**—The direct fluorescence assay procedure, which was described in greater detail in an earlier report (1), utilizes the fluorescence properties of β-napthylamine (5). A standard reaction mixture contained 0.5 μmol of Lys-Ala-β-naphthylamide and Arg-Arg-β-naphthylamide at pH 9.0. Assays for mono- and dipeptidyl arylamidases, such as pituitary dipeptidase and aminopeptidases (6), were performed on Lys-Ala-β-naphthylamide with the use of 2-mercaptoethylamine hydrochloride, or succinic acid-sodium hydroxide buffer, pH 5.5. The buffer-substrate mixture, contained in round Pyrex cuvettes (12 x 75 mm), was first incubated at 37° for 3 min. The reaction was initiated by the addition of 0.1 ml of enzyme solution, and gave a final volume of 4.0 ml. The reaction mixture was maintained at 37° in the water-jacketed cuvette holder of a Turner fluorometer. The cuvette was irradiated with light at 335 nm, and the fluorescence intensity was continuously monitored and simultaneously recorded on a strip chart recorder. The reaction rate was indicated by the rate of increase in the intensity of the fluorescence at 410 nm. The fluorometer was calibrated with a standard solution of β-napthylamine, and the necessary corrections were made for fluorescence quenching due to reaction components and acid pH.

**Dipeptidyl arylamidase I** was assayed at pH 4.0 on Ser-Tyr-β-napthylamide with the use of 2-mercaptoethanol hydrochloride to satisfy the sulfhydryl and chloride requirements of the enzyme (1). Dipeptidyl arylamidase III (3) was assayed on Arg-Arg-β-napthylamide at pH 9.0. Assays for mono- and dipeptidyl arylamidases, such as pituitary dipeptidase and aminopeptidase (6), were performed on Lys-β-napthylamide and Arg-β-napthylamide.

**Acid Phosphatase Assays—p-Nitrophenylphosphate (7)**, a Sigma preparation, was the substrate used for all acid phosphatase assays. The reaction mixture contained 5 mm p-nitrophenylphosphate in 50 mm acetate-0.3 mm EDTA buffer, pH 5.0. The reaction was maintained at 37°, and 1.0-ml aliquots were transferred to 4.0 ml of 50 mm NaOH (to stop reactions and develop color) at time intervals of 5, 10, 20, and 40 min. The absorbance was measured at 400 nm, and the readings were determined quantitatively by reference to a standard curve prepared with p-nitrophenol. The total acid phosphatase present in sucrose homogenates and density gradient fractions was measured by incorporating Triton X-100 into the reaction mixture at a final concentration of 1% (w/v).

**Characterization of Dipeptidyl Arylamidase II Substrate**—L-Lys-β-Ala-β-napthylamide-2HCl was prepared by Fox Chemical Company by means of a condensation of N°,N°-benzyloxy carbonyl-L-lysine and L-alanine-β-napthylamide in 0.25 M sucrose-1.0 mm EDTA, pH 7.0. A 5% (w/v) suspension of glands in sucrose was homogenized according to de Duve et al. (4) in a Potter-Elvehjem homogenizer of the B type supplied by Arthur H. Thomas Company, which consists of a Teflon pestle inside a smooth walled glass tube having a chamber clearance of 0.005 to 0.007 inch. The Teflon pestle was power-driven at 900 rpm, and the pituitary glands were dispersed with three passes of the pestle to the bottom of the tube, while the tube was immersed in ice water.

A refrigerated International centrifuge, model PR-2, equipped with a swinging bucket rotor, was used for centrifugation at 500 x g. A refrigerated Lourdes Beta-Fuge, model A-2, equipped with a 9RA rotor, was used for centrifugation up to 40,000 x g. For centrifugation above this speed a Spinco model L ultracentrifuge equipped with a type 40 rotor was used except for density gradient centrifugation, when a SW 25-1 rotor was used.
the presence of \(N,N'\)-dicyclohexylcarbodiimide. The blocking groups were removed from the product by means of catalytic hydrogenation. An HCl-hydrolyzed sample of Lys-Ala-β-naphthylamide, glycine, and alanine were removed from the product by means of catalytic hydrogenation. An HCl-hydrolyzed sample of Lys-Ala-β-naphthylamide was analyzed on a Beckman Spinco amino acid analyzer and found to contain the theoretical, equimolar amounts of lysine and alanine. Other amino acids were absent. A fluorescence analysis of the hydrolysate indicated an equimolar quantity of β-naphthylamine, whereas no free β-naphthylamine was detectable prior to acid hydrolysis. Lys-Ala-β-naphthylamide was also examined by paper chromatography (Fig. 3). The preparation did not contain detectable amounts of lysine, alanine, β-naphthylamine, Lys-Ala, or Ala-β-naphthylamide.

We have examined the ultraviolet absorption spectra of many aminoacyl- and dipeptidyl-β-naphthylamides, and the absorption spectrum shown for Lys-Ala β-naphthylamide (Fig. 1), with a maximum at 243 \(\mu\) (ε, 39,000) and a shoulder at 252 \(\mu\), is typical of all arylamides tested. The maximum for free β-naphthylamine is shown to occur at 233 \(\mu\) (ε, 51,000). The molar absorbance difference at 250 \(\mu\), between β-naphthylamine and its aminoacyl derivatives, points to the feasibility of a direct arylamide assay at this wavelength. The absorption spectra for peptide- and aminoacyl-β-naphthylamides are also indicators of contamination by free β-naphthylamine or blocked (benzylocarbonylated) precursor. The latter exhibits a much lower extinction coefficient at 243 \(\mu\).

Other aminoacyl and dipeptidyl arylamides mentioned in this report were also obtained from commercial sources, except for Gly-Arg-β-naphthylamide, which was a gift from Dr. Robert E. Flapinger of the Sinai Hospital of Baltimore, Inc. All the substrates were isomers.

RESULTS

Hydrolysis of Lys-Ala-β-Naphthylamide by Aqueous Extracts—Dovine anterior pituitary extracts were found to catalyze the hydrolysis of Lys-Ala-β-naphthylamide to Lys-Ala dipeptide and β-naphthylamine. As illustrated in Fig. 2, pHi 5.5 was the optimum for the hydrolysis of Lys-Ala-β-naphthylamide, whereas Ala-β-naphthylamide was hydrolyzed best between pH 7.0 and 7.5. Since aqueous extracts of anterior pituitary tissue exhibited the same pH optimum for the hydrolysis of both Ala-β-naphthylamide and Lys-β-naphthylamide, and since both activities showed a sulfhydryl activation, the hydrolysis of these substrates was attributed to the aminopolypeptidase that Ellis and Perry (6) have described in pituitary extracts. As shown in Fig. 2, the pH curve for the hydrolysis of Lys-Ala-β-naphthylamide was asymmetric as a result of a limb extending beyond pH 7.0. This asymmetry may be a consequence of a contribution of the aminopolypeptidase at near neutral pH, since it is known that this enzyme hydrolyzes peptides and aminoacyl arylamides sequentially from the NH₂ terminus (6). Any contribution from the latter mode of attack was minimal since initial and linear rates of β-naphthylamine formation were used in order to select for the direct cleavage of β-naphthylamine from Lys-Ala-β-naphthylamide by dipeptidyl arylamidase II. Moreover, at pH 5.5, which is the optimum for arylamide activity, the aminopolymeridase has essentially no effect on the rate of Lys-Ala-β-naphthylamide hydrolysis.

Hydrolysis of Lys-Ala-β-Naphthylamide by Protein Fraction Precipitated between 0 and 1.7 \(\text{M}(\text{NH}_4)_2\text{SO}_4\)—A major portion of the Lys-Ala-arylamidase activity could be precipitated between 0 and 1.7 \(\text{M}(\text{NH}_4)_2\text{SO}_4\) with dipeptidyl arylamidase I being left in solution (1). The specific activity of dipeptidyl arylamidase II in the 1.7 \(\text{M}(\text{NH}_4)_2\text{SO}_4\) fraction was increased about 6-fold over the pH 5.5 extract. As illustrated in Fig. 3, the 1.7 \(\text{M}(\text{NH}_4)_2\text{SO}_4\) fraction hydrolyzed Lys-Ala-β-naphthylamide best at pH 5.5. As indicated, the rate of hydrolysis was reduced about 70% by an increase in the dimethylglutarate buffer concentration from 10 to 50 mM. However, as shown in Fig. 3, the pH optimum remained at about 5.5. As will be shown in a later section, the rate of Lys-Ala-β-naphthylamide hydrolysis measured in the presence of various buffers incorporated at 10 mM was approximately half the rate obtained when no buffers were added. The presence of aminopolypeptidase in the 1.7 \(\text{M}(\text{NH}_4)_2\text{SO}_4\) fraction was indicated by the hydrolysis of Ala-β-naphthylamide at about pH 7.3. This activity was not sensitive to buffer concentration, but, as seen in

![Fig. 1. Ultraviolet absorption spectra for 10⁻⁴ M aqueous solutions of Lys-Ala-β-naphthylamide and β-naphthylamine.](image)

![Fig. 2. The effect of pH on the rate of hydrolysis of Lys-Ala-β-naphthylamide (Lys-Ala-pNA) and Ala-β-naphthylamide (Ala-pNA) by a pH 5.5 aqueous extract of ovine anterior pituitary. The reaction mixtures contained 0.1 ml of an appropriate dilution of the extract and 0.8 μmole of substrate in 10 ml 3,3-dimethylglutaric acid-sodium hydroxide buffer, in a total volume of 4.0 ml.](image)
Fig. 3. The effect of pH on the rate of hydrolysis of Lys-Ala-\(\beta\)-naphthylamide (Lys-Ala-\(\beta\)NA) and Ala-\(\beta\)-naphthylamide (Ala-\(\beta\)NA) by a 0 to 1.7 m \((\text{NH}_3)\text{SO}_4\) fraction of the pH 5.5 aqueous pituitary extract. Buffer concentration and sulfhydryl (2-mercaptoethanol) effects are illustrated. Reaction mixtures contained the \((\text{NH}_3)\text{SO}_4\) fraction (4 to 100 \(\mu\)g) and 0.8 \(\mu\)mole of substrate in 10 mm 3,3-dimethylglutaric acid-sodium hydroxide buffer; volume, 4.0 ml. As indicated in the figure, one pH curve for Lys-Ala-\(\beta\)-naphthylamide was established with 50 mm dimethylglutarate (DMG) buffer. 2-Mercaptoethanol (MCE) was incorporated at 10 mm when Ala-\(\beta\)-naphthylamide served as the substrate.

Fig. 3, the characteristic sulfhydryl activation was exhibited. By comparison, the hydrolysis of Lys-Ala-\(\beta\)-naphthylamide was somewhat inhibited (about 10\%) by the incorporation of 2-mercaptoethanol at 10 mm.

The products of the hydrolysis of Lys-Ala-\(\beta\)-naphthylamide by the dipeptidyl arylamidase II contained in the 1.7 m \((\text{NH}_3)\text{SO}_4\) fraction were identified by means of a time course analysis of a pH 5.0 reaction mixture. As illustrated in Fig. 4, Lys-Ala and \(\beta\)-naphthylamine were the only products revealed by paper chromatography. Free lysine, alanine, and Ala-\(\beta\)-naphthylamide were not detected in the reaction mixture.

Inhibitory Effect of Cations on Hydrolysis of Lys-Ala-\(\beta\)-Naphthylamide by Dipeptidyl Arylamidase II—As indicated in Fig. 5, the greatest rate of Lys-Ala-\(\beta\)-naphthylamide hydrolysis was obtained when buffers were excluded from the reaction mixture. As the concentration of buffer in the reaction was increased, the hydrolysis of Lys-Ala-\(\beta\)-naphthylamide was strongly inhibited. At first it appeared as though the inhibition was attributable to an ionic strength effect; however, as shown in Fig. 5, the degree of inhibition not only increased with the concentration of the buffer, but also with the size of the basic cation. As illustrated in Fig. 6, the inhibition of Lys-Ala-\(\beta\)-naphthylamide hydrolysis was directly proportional to the size of the added cation. Large cations such as Tris and puromycin proved to be good inhibitors at concentrations less than 10 mm. The relative inhibitory effect was as follows: puromycin++ > puromycin amiononucleoside++ > Tris+ > K+ > Na+ > Li+.

When they were incorporated into the assays at concentrations of 1.0 mm, puromycin and its amiononucleoside inhibited the hydrolysis of Lys-Ala-\(\beta\)-naphthylamide by 75 and 66\%, respectively. Since 0.05 m Tris-succinate, pH 5.5, gave about 95\% inhibition of arylamidase II, this effect was used as a possible identifying characteristic when dipeptidyl arylamidase II activity was measured in extracts of other tissues. The inhibitory effect of the buffers tested did not appear to depend... 

Fig. 4. Time course study of Lys-Ala-\(\beta\)-naphthylamide (Lys-Ala-\(\beta\)NA) hydrolysis at pH 5.0. The reaction mixture contained 8.0 \(\mu\)moles of Lys-Ala-\(\beta\)-naphthylamide and 3 mg of the 0 to 1.7 m \((\text{NH}_3)\text{SO}_4\) fraction, in a total volume of 5.0 ml, adjusted to pH 5.0 with HCl. Origins on the chromatogram numbered from 1 to 6 represent samples of the reaction mixture taken after 0, 15, 30, 45, 60, and 90 min of incubation at 37°. Origins 8 and 9 represent substrate and enzyme controls, respectively, which were incubated 90 min at 37°. Each sample was combined with an equal volume of 50\% formic acid in order to halt enzyme activity prior to spotting 100\-\mu l quantities on Whatman No. 1 paper. The chromatogram underwent a 16-hour descending development with 1-butanol-formic acid-water (70:15:15). The products were detected with 0.2% ninhydrin in acetone, and their \(R_S\) values were compared with standards identified on the chromatogram from top to bottom (Origin 7) as lysine, Lys-Ala, Lys-Ala-\(\beta\)-naphthylamide (Lys-Ala-\(\beta\)NA), Ala-\(\beta\)-naphthylamide (Ala-\(\beta\)NA), and free \(\beta\)-naphthylamine. Free \(\beta\)-naphthylamine was outlined after detection with short-wave ultraviolet light (254 m). \(R_S\)'s, standards; Ant. Pit. Extr., anterior pituitary extraction; U.V., ultraviolet.
ranging from 4.5 to 90 mM. Each buffer was tested in a reaction mixture containing 4.0 µg of the 0 to 1.7 M (NH₄)₂SO₄ fraction and 0.8 µ mole of Lys-Ala-β-naphthylamide, in a total volume of 4.0 ml, pH 5.7. The five curves in the figure represent: 0, lithium succinate; ▽, sodium succinate; ◯, potassium succinate; □, Tris succinate; and ⬤, puromycin aminonucleoside succinate. ◯, reaction adjusted to pH 5.7 without buffer.

Upon the type of carboxylic acid used to prepare the buffer. Similar results were obtained with succinic acid, acetic acid, citric acid, and 3,3-dimethylglutaric acid. Only minor differences in reaction rates were observed; for example, when the different carboxylic acids were adjusted to pH 5.5 with NaOH, these differences became negligible when NaCl was added to give a common Na⁺ concentration to all the buffers. Buffers prepared as the sodium salts of these acids inhibited the hydrolysis of Lys-Ala-β-naphthylamide to about 50% when they were incorporated into a reaction mixture at 10 to 15 mM.

The inhibitory effect of the cations tested appeared to be related to their size only. Although structural requirements were not evident, it was found that streptomycin, a larger molecule than puromycin, was only one-sixth as inhibitory. On the other hand, amicetin, another Streptomyces antibiotic, with a molecular weight 30% greater than puromycin, was, consistent with its larger size, more inhibitory than puromycin. Unlike puromycin and amicetin, the cationic centers of streptomycin reside in guanidine groups. A comparison of detergents revealed that a cationic substance such as Hyamine 10 X, incorporated into the assay system at 0.02%, exhibits an 89% inhibition of Lys-Ala-β-naphthylamide hydrolysis. Similar concentrations of Triton X-100 (nonionic) and Triton X-200 (anionic) showed no inhibition.

Kinetics of Lys-Ala-β-Naphthylamide Hydrolysis—When the direct fluorescence assay technique described under "Experimental Procedure" was used to assay aqueous extracts of pituitary tissue, as well as the 0 to 1.7 M (NH₄)₂SO₄ fraction, a rate response was obtained for the hydrolysis of Lys-Ala-β-naphthylamide at pH 5.5, which was linear from the origin. The linear response was found to apply for amounts of enzyme giving rates of β-naphthylamide formation up to, at least, 1 µmole per min. All assays were therefore based on amounts of enzyme that gave rates not exceeding this value. The rate response to substrate concentration is shown in Fig. 7. A substrate concentration of 0.2 mM was found to provide zero order reaction rates, and the Fig. 4 inset illustrates the proximity of the velocity measured with the standard assay (0.2 mM substrate) to the theoretical maximum velocity when buffer salts were excluded from the reaction mixture. A Kᵣ of 1.1 X 10⁻⁴ M was determined (Fig. 7 inset) for the hydrolysis of Lys-Ala-β-naphthylamide by the 1.7 M (NH₄)₂SO₄ fraction at pH 5.5 and 37° in the absence of added buffer or other electrolytes. The presence of a buffer in the reaction mixture caused a pronounced suppression of the rate response to substrate concentration. When NaCl was added to an unbuffered reaction mixture adjusted to pH 5.5 with a trace of NaOH, an inhibition resulted which was similar to that which was obtained when carboxylate buffers were added. As illustrated in Fig. 8, Lineweaver-Burk plots showed the inhibition by NaCl to be competitive, thereby giving rise to markedly elevated Kᵣ values and a Kᵣ of 1.8 X 10⁻⁴ M. As indicated in Fig. 9, the very pronounced inhibition obtained with large cations such as puromycin and its aminonucleoside was also found to be competitive, with Kᵣ values calculated to be 1.9 X 10⁻³ M and 3.0 X 10⁻³ M, respectively.

Stability of Dipeptidyl Arylamidase II—Neutral extracts derived from 20% aqueous homogenates of bovine anterior pituitary glands could be stored at 4° for up to 1 month without detectable losses of dipeptidyl arylamidase II activity. The pH 5.5 aqueous extracts that were dialyzed and freeze dried were stable for months when stored at -20°. Similar dried preparations are presently being utilized as an enriched source of enzyme for further purification studies.

Neutral, aqueous extracts were incubated at elevated temperatures in order to ascertain the heat stability of the Lys-Ala-arylamidase activity. Relatively low rates of inactivation were observed at temperatures up to 50°. However, as shown in Fig. 10, appreciable rates of inactivation were observed when the temperature was raised above 50°. No hydrolysis of Lys-Ala-β-naphthylamide could be demonstrated with pituitary extracts subjected to 65° for 10 min. During the heat treatment studies, it was observed that the dipeptidyl arylamidase III, aminopeptidase, and dipeptidase in the aqueous extracts could be rapidly inactivated with heat at neutral pH. This effect was indicated by a 90% drop in the rate of hydrolysis of Arg-Arg-β-naphthylamide, Lys-β-naphthylamide, and Arg-β-naphthylamide, respectively, by a neutral, aqueous extract that was exposed to 55° for 10 min. As indicated in Fig. 10, only about
5% of the dipeptidyl arylamidase II activity was lost under these conditions.

Dipeptidyl arylamidase II in aqueous pituitary extracts was stable to dialysis in water at 5° and neutral pH. The stability as well as the activity of the enzyme showed no dependence upon metal or sulfhydryl requirements. The rate of Lys-Ala-β-naphthylamide hydrolysis by arylamidase II was essentially unaffected by incubation of the enzyme in 1 mM EDTA or 1 mM p-chloromercuriphenylsulfonate. The activity was slightly depressed by the added Na+. By comparison, the activity of pituitary extracts toward Ala-β-naphthylamide showed 90 and 98% inhibition when extracts were incubated in 1 mM EDTA and 1 mM p-chloromercuriphenylsulfonate, respectively. This metal and sulfhydryl requirement is characteristic of the pituitary aminopolypeptidase (6).

Specificity of Dipeptidyl Arylamidase II toward a Variety of Arylamide Substrates—The 1.7 M (NH₄)₂SO₄ fraction was used to determine the relative rates of hydrolysis, at pH 5.5, of selected dipeptide β-naphthylamides. As indicated in Table I, Lys-Ala-β-naphthylamide was the substrate hydrolyzed most rapidly, whereas the NH₂-blocked derivative of this substrate was not hydrolyzed. The rate of Lys-Ala-NH₂ hydrolysis, determined by the method of Seligson and Seligson (8), showed a zero order rate that was about 25% of the rate of Lys-Ala-β-naphthylamide hydrolysis. Neither Ser-Tyr-β-naphthylamide nor Arg-Arg-β-naphthylamide, which serve as substrates for dipeptidyl arylamidases I and III, respectively, was hydrolyzed by this pituitary fraction. Gly-Phe-β-naphthylamide, a fluorogenic substrate for cathepsin C (9), was not hydrolyzed by the pituitary fraction. Gly-Phe-β-naphthylamide, a fluorogenic substrate for cathepsin C (9), was not hydrolyzed by the pituitary fraction.

Distribution of Dipeptidyl Arylamidase II among Various Rat Tissues—An attempt was made to estimate the content of dipeptidyl arylamidase II in aqueous extracts of certain rat tissues. Table II gives the rates at which these tissue extracts hydrolyzed Lys-Ala-β-naphthylamide at pH 5.5. A buffer was incorporated into the assay system to bring the extracts to pH 5.5. However, if the reaction mixtures were adjusted to pH 5.5 without the use of buffer, the resultant rates would be about double those given in Table II. As was indicated in Fig. 5, Tris, adjusted to pH 5.5 with HCl and incorporated into the reaction mixture at 50 mM, inhibited pituitary arylamidase II activity 95%. Tris was found to have a similar inhibitory effect on the
Table I

Rates of hydrolysis of various dipeptidyl-β-naphthylamides by pituitary fraction obtained by (NH₄)₂SO₄ fractionation

Assay conditions were as follows: 5 μg of the 1.7 M (NH₄)₂SO₄ fraction and 0.8 μmole of dipeptidyl-β-naphthylamide in 9 mM sodium succinate buffer, pH 5.5; volume, 4.0 ml.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activitya</th>
</tr>
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<tbody>
<tr>
<td>Lys-Ala-β-naphthylamide</td>
<td>125</td>
</tr>
<tr>
<td>Nα,N'-benzyldi carbonyl-Lys-Ala-β-naphthylamide</td>
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</tr>
<tr>
<td>Arg-Ala-β-naphthylamide</td>
<td>37</td>
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<tr>
<td>Leu-Ala-β-naphthylamide</td>
<td>14</td>
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<tr>
<td>Ala-α-naphthylamide</td>
<td>6</td>
</tr>
<tr>
<td>Gly-Pro-β-naphthylamide</td>
<td>5</td>
</tr>
<tr>
<td>Ser-Met-β-naphthylamide</td>
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</tr>
<tr>
<td>Arg-Arg-β-naphthylamide</td>
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<tr>
<td>Gly-Phe-β-naphthylamide</td>
<td>0</td>
</tr>
<tr>
<td>Ser-Tyr-β-naphthylamide</td>
<td>0</td>
</tr>
<tr>
<td>His-Ser-β-naphthylamide</td>
<td>0</td>
</tr>
</tbody>
</table>

a A maximum of 100 μg of pituitary fraction was used in an attempt to detect low rates of hydrolysis.

Table II

Rates of hydrolysis of Lys-Ala-β-naphthylamide by variety of rat tissue extracts

Assay conditions were as follows: 0.1 ml of an appropriate dilution of a 5% aqueous extract in 0.2 mM Lys-Ala-β-naphthylamide; 3.5 mM citric acid-9.0 mM Na₂HPO₄, pH 5.5; volume, 4.0 ml.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ratea</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid</td>
<td>112 ± 10.0</td>
<td>100</td>
</tr>
<tr>
<td>Spleen</td>
<td>90 ± 9.7</td>
<td>80</td>
</tr>
<tr>
<td>Kidney</td>
<td>77 ± 3.0</td>
<td>69</td>
</tr>
<tr>
<td>Pituitary</td>
<td>68 ± 1.7</td>
<td>61</td>
</tr>
<tr>
<td>Thymus</td>
<td>53 ± 7.2</td>
<td>47</td>
</tr>
<tr>
<td>Adrenal</td>
<td>33 ± 1.0</td>
<td>29</td>
</tr>
<tr>
<td>Liver</td>
<td>22 ± 2.5</td>
<td>20</td>
</tr>
<tr>
<td>Testis</td>
<td>21 ± 0.5</td>
<td>19</td>
</tr>
<tr>
<td>Pancreas</td>
<td>14 ± 1.1</td>
<td>12</td>
</tr>
<tr>
<td>Brain (whole)</td>
<td>14 ± 0.7</td>
<td>12</td>
</tr>
<tr>
<td>Serum</td>
<td>4 ± 0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

a In millimicromoles of β-naphthylamine formed min⁻¹ ml⁻¹ of 5% aqueous extract or ml⁻¹ of serum. Rates are reported as the mean ± the standard error based on three individual rats.

hydrolysis of Lys-Ala-β-naphthylamide by all the tissues examined. The degree of inhibition was between 88 and 95% when compared with hydrolysis rates exhibited by similar tissue extracts adjusted to pH 5.5 and assayed without added buffer. Moreover, at pH 5.5, the tissue extracts apparently catalysed a dipeptide cleavage from Lys-Ala-β-naphthylamide, as indicated by rates of hydrolysis which were linear from the origin. It therefore appeared as though the same enzyme may be responsible for the hydrolysis of Lys-Ala-β-naphthylamide by all the tissues examined. Of the tissues studied, thyroid was the richest source of arylamidase II. Pituitary tissue was a relatively rich source of the enzyme in that it exceeded such tissues as thymus, adrenal, liver and brain, and blood serum.

Subcellular Localization of Dipeptidyl Arylamidase II—Whole homogenates of fresh rat pituitary glands, prepared in 0.25 M sucrose by the method described under "Experimental Procedure," were centrifuged at 500 × g for 15 min in order to rid the homogenates of nuclei, erythrocytes, and unbroken cells. An opaque, milk-white supernatant overlay a residue that was coated with a bright red layer of erythrocytes. The whiteness of the supernatant was attributed to the presence of secretory granules in the low speed supernatant (12).

The total amount of dipeptidyl arylamidase II and acid phosphatase in the whole homogenate was estimated by treating aliquots of the homogenate with Triton X-100. The non-ionic detergent in 0.25 M sucrose was incorporated into aliquots of the homogenate at concentrations of 0.1 and 1% (v/v) in order to insure the release of lysosomal enzymes (13). Since the levels of arylamidase II and acid phosphatase detected in the Triton-treated 500 × g supernatant (Table III) were the same as in the Triton-treated whole homogenate, it therefore appeared that essentially all the arylamidase II and acid phosphatase in the whole homogenate was recovered in the 500 × g supernatant. As indicated in Table III, when the 500 × g supernatant was not treated with Triton, about 95% of the arylamidase II, and 73% of the acid phosphatase activities contained in the 500 × g supernatant could be sedimented after 15 min at 30,000 × g. No amount of these activities could be sedimented at 30,000 × g if the homogenate was first treated with Triton.

With regard to other arylamidases, about 96% of the activity on Ser-Tyr-β-naphthylamide (dipeptidyl arylamidase III), as previously reported (3), could not be sedimented under these conditions.

Table III

Activity and sedimentation of dipeptidyl arylamidase II and acid phosphatase contained in 500 × g supernatant

<table>
<thead>
<tr>
<th>Treatment of 500 × g supernatant</th>
<th>Arylamidase II Concentration</th>
<th>Acid phosphatase Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute Concentration</td>
<td>Relative</td>
</tr>
<tr>
<td></td>
<td>units/ml</td>
<td>%</td>
</tr>
<tr>
<td>Triton-treatedb and assayedb</td>
<td>230</td>
<td>100</td>
</tr>
<tr>
<td>Triton-treated and centrifugedc</td>
<td>241</td>
<td>105</td>
</tr>
<tr>
<td>Centrifuged in the absence of Triton⁴</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

a Millimicromoles of substrate hydrolyzed min⁻¹ ml⁻¹ of 500 × g supernatant.

b A solution of Triton X-100 in 0.25 M sucrose-1 mM EDTA, pH 7.0, was added to the 500 × g supernatant to give a final concentration of 1% (v/v) Triton. The enzyme activities were estimated without centrifugation and considered to represent the total amount (100%) of enzyme activity.

c The supernatant was assayed after Triton X-100 was incorporated into the 500 × g supernatant at 1% (v/v), and the preparation was centrifuged at 30,000 × g for 15 min.

⁴ In the absence of Triton, 96% of the arylamidase II and 73% of the acid phosphatase were sedimented from the 500 × g supernatant at 1% (v/v) Triton.
The activity of the 500 × g supernatant on such aminoacyl arylamides as Lys-β-naphthylamide and Arg-β-naphthylamide was sedimented to the extent of only 8 and 6%, respectively. These two arylamides serve as substrates for the aminopeptidase and dipeptidase of the pituitary (6). The bulk of the monoaminoacyl arylamidase activities and all the dipeptidyl arylamidase III activity remained in solution after 1 hour of centrifugation at 100,000 × g. By way of comparison, it was recently reported by Mahadevan and Tappel (14) that the leucyl arylamidase activity of liver and kidney is primarily lysosomal.

Significant percentages of the total arylamidase II activity contained in the 500 × g supernatant could be sedimented after 15 min at relatively low centrifugal forces, e.g. 69% at 5,000 × g, 87% at 10,000 × g, and 95% at 20,000 × g. These sedimentation characteristics resemble those reported by de Duve et al. (4) for rat liver lysosomes, which were effectively sedimented at 17,000 × g for 15 min. The functional latency of dipeptidyl arylamidase II contained in the subcellular fractions was also typical of a lysosomal enzyme. The latency of the enzyme was determined by assaying the subcellular fractions on Lys-Ala-β-naphthylamide in a reaction mixture that contained sufficient sucrose to maintain the lysosomes intact. The amount of latent activity was then determined by incorporating Triton X-100 into an identical reaction mixture in order to rupture all the lysosomes. It appeared that essentially all the dipeptidyl arylamidase II of the rat pituitary was contained within lysosomes. This was indicated by a 91 to 95% latency of the arylamidase II contained in the unfractonated 500 × g supernant derived from the whole homogenate. On the basis of the sedimentation properties of dipeptidyl arylamidase II, coupled with its high latency of activity, and its solubilization along with acid phosphatase by Triton X-100, arylamidase II is, in all probability, located in the pituitary lysosomes.

Lysosomes have been shown in pituitary sections by cytochemistry and electron microscopy, and it has been demonstrated by Smith and Farquhar (15) that lysosomes of the anterior pituitary gland assimilate and degrade hormone-bearing secretory granules. The hydrolytic activity of the lysosomes appeared to vary with the secretory activity of the gland (15).

An aliquot of the 500 × g supernatant was centrifuged at 30,000 × g, and the resulting pellet was fixed and sectioned according to Smith and Farquhar (15) for examination by electron microscopy. The 500 × g supernatant was thereby shown to contain a plentiful yield of secretory granules, lysosomes, and mitochondria.

A comparable 5000 to 30,000 × g pellet was resuspended to one quarter its original volume with 0.25 M sucrose, and 0.8 ml of this suspension was layered over a 30.0-ml sucrose gradient with limits of 27 and 57% (w/w) sucrose. Equilibrium-density centrifugation was conducted in the SW 25-1 rotor of a Spinco model L ultracentrifuge at 70,000 × g for 3 hours. The gradient was then fractionated into 1.0 ml-fractions with the use of the density gradient fractionator, model D, manufactured by Instrumentation Specialties Company. The sucrose concentration in each fraction, which was determined from its index of refraction (n?), was used to plot the sucrose gradient shown in Fig. 11. The absorbance profile is a tracing of the direct scan at 254 mp acquired with the density gradient fractionator. The A254 scan shown in Fig. 11 is an approximation of the banding that is visible.

Pellets were prepared from each gradient fraction in order to examine the morphology of the constituent particles by electron microscopy. The A254 peak located at fraction 15 was seen to contain the major concentration of mitochondria. A "shoulder" of acid phosphatase activity was also located in this region of the gradient; however, dipeptidyl arylamidase II activity revealed no tendency to concentrate in this region. The A254 peak located at fraction 22 was seen to contain a heavy concentration of secretory granules, with the large (600 to 900 nm) mammotrophic secretory granules under the A254 shoulder located in fraction 26. Moving from fraction 20 to 26 it can be seen that the acid phosphatase activity did not decrease as rapidly as did the dipeptidyl arylamidase II activity, and unlike the latter activity, a shoulder of acid phosphatase activity was located in fraction 26. These
observations are in good agreement with the demonstration by Smith and Farquhar of acid phosphatase within the membranes of mammotrophic secretory granules of the lactating rat. (See Fig. 24 in Reference 15.) By comparison, dipeptidyl arylamidase II exhibited a single, near symmetrical peak of activity located between the mitochondria and granule peaks. The morphology of fractions 14 to 26, as seen by electron microscopy, revealed that the lysosome was the major subcellular structure which persisted throughout the arylamidase region. Pituitary lysosomes prepared in this manner were examined for acid phosphatase by the lead salt Barks-Anderson (16) modification of the Gomori procedure. By means of electron microscopy, the lysosomes were observed to have retained a high concentration of acid phosphatase. As expected from the studies of Smith and Farquhar (16), many of the pituitary lysosomes from these post-lactating rats were found to contain ingested secretory granules.

From these studies it appears that dipeptidyl arylamidases I and II of the pituitary are in all probability lysosomal enzymes, and, unlike acid phosphatase, almost exclusively so. Dipeptidyl arylamidase III (Arg-Arg-arylamidase) was completely cytoplasmic, and the monoaminoacyl arylamidases were predominately cytoplasmic, which is in agreement with our previous observations with both frozen and fresh bovine pituitary glands (3, 6).

**DISCUSSION**

Dipeptidyl arylamidase II of the pituitary gland appears to be a heretofore undescribed peptidease that exhibits no metal, halide, or sulfhydryl requirements for the cleavage of Lys-Ala from Lys-Ala-β-naphthylamide at a pH optimum of 5.5. The enzyme is competitively inhibited by a variety of cations. The degree of inhibition is directly proportional to the size of the cation, with an apparent linear relationship to the square root of the cationic weight of the cation. Large cations such as Tris and puromycin are extremely inhibitory. In view of this sensitivity, it is obviously essential to control and define buffer composition precisely. Maximal activity of the peptidease is displayed in the total absence of cationic solutes, including the small cations from Group I of the periodic table.

The inhibitory effect of Tris upon other enzyme systems (17) is probably the result of the tendency of Tris to form complexes with metal demonstrated by O'Sullivan and Perrin (18) and Allen, Baker, and Giller (19). In most instances (17) it was necessary to incorporate Tris into the reaction mixtures at relatively high concentrations in order to demonstrate a significant inhibition. Pavlic (20) recently reported that the inhibition of cholinesterase by Tris is competitive (Kc; 14 mm at pH 7.4). However, as in other instances of enzyme inhibition by Tris, it was not known whether the protonated or nonprotonated species of Tris was responsible.

Since dipeptidyl arylamidase II has no metal requirement, the inhibitory effect of Tris cannot be attributed to its ability to form complexes with metals. Moreover, since reaction rates were always measured at pH 5.5, the protonated species of Tris, as well as puromycin and puromycin aminonucleoside, would necessarily be the inhibitory species. Cations as small as Na+ inhibited dipeptidyl arylamidase II; the inhibition was always competitive and increased with the size of the cation. Other enzyme systems inhibited by Tris have not been reported to exhibit these characteristics.

As shown in this report, cations, at concentrations lower than usually used to buffer enzyme assay systems, have a very pronounced inhibitory effect upon the hydrolysis of Lys-Ala-β-naphthylamide by dipeptidyl arylamidase II. In view of the competitive nature of this inhibition, it appears as though the cations compete with Lys-Ala-β-naphthylamide for the active site on the enzyme. The substrate, which is itself a large cation at pH 5.5, is least able to compete with other large cations such as Tris and puromycin for the active site. The affinity of the enzyme for cationic groups is also indicated by the preferential hydrolysis of the basic substrates: Lys-Ala-β-naphthylamide and Arg-Ala-β-naphthylamide.

It is now evident from the findings described herein, as well as in previous reports from this laboratory, that at least three distinct dipeptidyl arylamidases exist within the pituitary gland. These arylamidases, which have been separated and extensively purified, can be selectively detected and assayed in tissues as a result of their distinct substrate specificities. Thus, dipeptidyl arylamidase I can be specifically assayed on Ser-Tyr-β-naphthylamide at pH 4 (1), dipeptidyl arylamidase II on Lys-Ala-β-naphthylamide at pH 5.5, and dipeptidyl arylamidase III on Arg-Ala-β-naphthylamide at pH 9.0 (3). All three of these enzymes behave as dipeptidyl aminopeptidases, since only substrates with unsubstituted NH2 termini are susceptible to hydrolysis.

With respect to their action on simple peptide substrates, the following observations are pertinent to the definition of the specificity of the dipeptidyl arylamidases. Arylamidase I has been shown to cleave Ser-Tyr from the decapeptide corresponding to the NH2 terminus of adrenocorticotropin hormone (1). In more recent work2 it has been found that highly purified arylamidase I of both the pituitary and liver also cleaves the NH2-terminal dipeptide from tetra-alanine and Gly-Phe-NH2, as well as His-Ser-β-naphthylamide, Ala-Ala-β-naphthylamide, Gly-Phe-β-naphthylamide, and Ser Met-β-naphthylamide. Arylamidase I, therefore, resembles cathepsin C of bovine spleen and may prove to be the same or closely related enzyme, particularly since both enzymes have been shown to have a halide requirement (9, 21). Arylamidase II, on the other hand, appears to possess a more limited specificity than arylamidase I both on dipeptidyl β-naphthylamidases and peptides. Of the arylamidase substrates, preferential hydrolysis occurs on Lys-Ala-β-naphthylamide and, to a much lesser extent, on Arg-Ala- and Leu-Ala-β-naphthylamidases. As reported previously (2), tripeptides such as Ser-Met-Glu, Ala-Ala-Ala, and Met-Met-Ala are cleaved by a pituitary enzyme that releases the NH2-terminal dipeptide; dipeptides and tetrapeptides on their amides were not hydrolyzed. As will be shown in a forthcoming report,1 this activity can now be attributed to dipeptidyl arylamidase II. Thus, while arylamidase I can hydrolyze peptides ranging in size from dipeptide amides to at least decapeptides, the action of arylamidase II on these substrates appears to be restricted to tripeptides, dipeptidyl arylamides, and, as will be shown in a forthcoming report,2 dipeptide esters such as Lys-Ala-OCH3. Pituitary arylamidase III differs from arylamidase I and II in having an optimum on arylamides and peptides at pH 9.0, and in being localized in the cell sap rather than in lysosomes. NH2-terminal dipeptides are cleaved from peptides having a minimum of four residues, i.e., Ala-Ala-Ala-Ala, Lys-Lys-Lys-Lys, Val-Leu-Ser-Glu-Gly; dipeptides and tripeptides are not hydrolyzed.

1 J. K. McDonald and S. Ellis, in preparation.

2 J. K. McDonald and S. Ellis, in preparation.
The existence of lysosomes within secretory cells of the anterior pituitary gland and their role in the intracellular digestion of hormone-bearing secretory granules have been convincingly shown by Smith and Farquhar (15) as a result of their studies of the pituitary with the electron microscope. With regard to peptidase content, the results of earlier subcellular studies by La Bella and Brown (22) strongly suggest that pituitary lysosomes contain proteinase I (23), a peptidase optimally active at pH 4 with a pepsin-like specificity. From the observations described in the present study, it is evident that pituitary lysosomes also contain two additional peptidases with acidic pH optima, namely, dipeptidyl arylamidases I and II. The localization, coupled with the high latency, of the two peptidases suggests that they can serve as useful markers for the detection of lysosomes during subcellular fractionation. In view of the distribution of arylamidase II in a wide range of rat tissues, the usefulness of this enzyme as a lysosomal marker in tissues other than the pituitary gland is apparent. For example, in data as yet unpublished, both dipeptidyl arylamidases I and II of the liver are localized exclusively in lysosomes.

While the function of the dipeptidyl arylamidases cannot be defined precisely at present, some tentative inferences may be drawn from their localization in lysosomes and their mode of action on peptide substrates. According to the interpretations of Smith and Farquhar (15), when the stimulus for the release of accumulated hormone-bearing secretory granules from the pituitary has been withdrawn, the surplus granules in the cytoplasm of the pituitary cell are phagocytized and digested by the lysosomes. In view of the presence of proteinase I within the lysosomes, along with the known substrate specificities of proteinase I, the hydrolysis of the protein hormone of the secretory granule must yield, principally, inactive oligopeptides. Such a hypothesis of hormone inactivation is made quite tenable as a result of the findings of Ellis (23) that 75 and 100% of the biological activity of growth hormone and prolactin, respectively, are lost as a result of the cleavage of only seven peptide bonds in each of these hormones by highly purified pituitary proteinase I. Furthermore, based on the known ability of dipeptidyl arylamidase I to cleave the terminal Ser-Tyr from the NH₂-terminal decapeptide of adrenocorticotropic hormone (1), it is expected that the arylamidase I contained within the pituitary lysosome would catalyze a rapid inactivation of the biological activity of adrenocorticotropic hormone as a consequence of a terminal Ser-Tyr cleavage from the hormone. Presumably, the peptide products resulting from the action of these lysosomal enzymes could then be degraded to amino acids by dipeptidyl arylamidase III (3) and the other peptidases (6) of the pituitary cell sap. By means of a hydrolytic sequence of this kind, the amino acids bound in excess protein hormone could be restored to the metabolic pool of amino acid for reutilization within the pituitary cell.

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Dipeptidyl Arylamidase II of the Pituitary: PROPERTIES OF LYSYLALANYL-β-NAPHTHYLAMIDE HYDROLYSIS: INHIBITION BY CATIONS, DISTRIBUTION IN TISSUES, AND SUBCELLULAR LOCALIZATION

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