An Aminopeptidase Occurring in Pig Kidney. I. An Improved Method of Preparation. Physical and Enzymic Properties*

Ernst Dieter Wachsmuth, Irene Fritze, and Gerhard Pfleiderer

ABSTRACT: An improved method for the liberation and purification of the particle-bound aminopeptidase of pig kidney is presented. Large quantities of enzyme may be prepared by separating the so-called "microsomal" particles by pH aggregation rather than by ultracentrifugation. The enzyme is distinguished from other aminopeptidases by its relative stability to heat and extremes of pH. It is denatured by alcohols, especially by long-chain alcohols, and by other organic solvents. The purest preparations were homogeneous and had molecular weights of 280,000. The most useful pH for the hydrolysis of peptides appears to be between 7 and 7.3, where the enzyme has maximum affinity for its substrate. The true maximum rate of hydrolysis is at pH 9, but here the affinity of the enzyme for its substrate is at its lowest. The turnover number is 10,450 moles of leucine *p*-nitroanilide/mole of aminopeptidase at 37°.

The majority of the aminopeptidase activity of pig kidney homogenates is present in the so-called "microsomal" fraction which sedimented at 80,000*g* in 90 min. This activity is destroyed by acetone treatment (Pfleiderer *et al.*, 1964). It is possible to separate and purify this activity from the microsomal fraction by treatment with toluene and trypsin but the method is only suitable for the preparation of small quantities of enzyme (Pfleiderer and Celliers, 1963). This paper describes a method for the preparation of large quantities of pure aminopeptidase. By precipitating the microsomal fraction at pH 5 the ultracentrifugation step is avoided. Some of the properties of the enzyme as a protein are described.

Experimental Section

Reagents. DEAE-Sephadex A-50 coarse, Sephadex G-50, G-100, and G-200 were from Pharmacia, Uppsala, Sweden. The *p*-nitroanilides of the L-amino acids and trypsin were placed at our disposal by C. F. Boehringer und Söhne, Mannheim. We should like to thank Dr. H. Determann of the Institut für Organische Chemie, Frankfurt, for the synthesis of the amino acid amides. Leucine-β-naphthylamide and fast black K salt were obtained from Serva, Heidelberg.

Methods. Optical measurements were carried out with an Eppendorf photometer (Netheler und Hinz, Hamburg) fitted with a thermostatically controlled cuvet holder.

Unless otherwise stated, the rates of hydrolysis of 2 ml of 1.66×10^{-3} M solutions of the amino acid *p*-nitroanilides (Tuppy *et al.*, 1962) were calculated from the absorption changes at 405 mμ measured in 0.06 M phosphate buffer, pH 7.0, at 37°, using molar extinction coefficient $E_{405m\mu}$ 9620 for *p*-nitrophenol. The hydrolysis of the amides was also determined in 0.06 M phosphate buffer, pH 7.0. The enzymic reaction was stopped with trichloroacetic acid and the amount of ammonia liberated was determined with Nessler's reagent after distillation in a microstill (Markham, 1942) at pH 7.0 (Pfleiderer *et al.*, 1964; Wachsmuth and Fritze 1965). Under these conditions the enzyme was some 25% more active than when tested in 0.01 M Tris buffer and 0.1 M $MgCl_2$, pH 7.2. Both Tris buffer and ammonium sulfate inhibit competitively (K_I/K_M for $NH_4Cl = 1 \times 10^2$).

Protein Determination. In the initial stages of purification protein concentrations were determined by the micro-Kjeldahl method. As soon as the protein was soluble in alkali, the method of Waddel was applied (Waddel, 1956).

Immunoelectrophoretic Analysis. Rabbits were injected three times at intervals of 1 week with aminopeptidase from pig kidney (specific activity 13.0 μmoles/mg of protein). Each injection consisted of 8 mg of enzyme dispersed in 8 ml of a mixture of equal parts of fat (Falba) and oil (Bayol). The subcutaneous injections were administered in at least two different positions on the back of the animal and 8 days after the last injection the immunoserum was removed by a heart puncture. Immunoelectrophoresis was carried out in agar gel in 0.06 M Veronal buffer, pH 8.6 (3 hr at 250 v, 30 m A). Antibody was diffused against antigen for 24 hr. In order to decrease the diffusion of naphthylamine, the coloration with leucine β-naphthylamide and fast black K salt was allowed to proceed in partially dried gel (Ishikawa and Klingmüller, 1963) for *ca.* 15 min at room temperature.

* From the Institut für Biochemie im Institut für Organische Chemie der Johann Wolfgang Goethe-Universität, Frankfurt/M, Germany. *Received June 3, 1965; revised September 23, 1965.*

TABLE I: Isolation of Pure Aminopeptidase from Pig Kidney.

Isolation Step	Total Activity (units, μmoles/min)	Spec Activity against Leucine *p*-Nitroanilide	Purification Factor
2900 g kidney homogenized, extracted, and centrifuged. Supernatant (suspension)	26,200	0.177	1
Sediment from the pH precipitation resuspended in 0.1 M Tris, pH 7.3	22,000	0.33	1.9
After toluene and trypsin treatment. Fraction precipitating between 20 and 80% saturation of ammonium sulfate	14,200	3.3	19
Reprecipitation with ammonium sulfate between 60 and 80% saturation	14,000	20.7 3 bands in IEA[a]	120
Eluate from DEAE-Sephadex at 0.2 M NaCl	8,100	31 2 bands in IEA[a]	175
Repeated reprecipitation with ammonium sulfate	5,000	37[b] 1 band in IEA[a]	210

[a] IEA = immunoelectrophoretic analysis. [b] The highest turnover number calculated from a molecular weight of 280,000 and with leucine *p*-nitroanilide as substrate was 10,450 moles/mole of enzyme.

Preparation of Pig Kidney Aminopeptidase

Pig kidney (2900 g, either fresh from the slaughterhouse or stored at −25° and then thawed at room temperature) was minced and homogenized in 0.1 M Tris buffer, pH 7.3 (9 l.), and stirred at 2° for 30 min. The supernatant after centrifuging at 3000*g* for 15 min was adjusted to pH 5.0 with acetic acid, and the sediment was centrifuged at 3000*g* for 20 min. The sediment was taken up in 0.1 M Tris buffer, pH 7.3, and diluted to approximately 6 l. Two liters of toluene was added and the suspension was homogenized for 30 min at a temperature of 38 to 40° and then slowly stirred overnight at 2°. The suspension was centrifuged at 12,000*g* for 1 hr. Four layers were visible. The topmost layer was toluene, the second layer was solid and white to light yellow in color. Beneath this was an aqueous layer and on the bottom of the tube a very small quantity of sediment. Using a filter pump, the toluene and the aqueous layers were easily removed, whereupon the second, solid layer could easily be scraped from the tube with a spatula. This second layer contained the enzyme and was suspended in 0.01 M Tris buffer, pH 7.3 (end volume 900 ml). Solid trypsin (20 mg/100 g of kidney) was added, and the suspension was incubated at 37° for 1 hr and cooled to 4°. Ammonium sulfate was added to give an end concentration equal to 20% saturation and the suspension was then centrifuged at 12,000*g*. The aqueous supernatant, containing 50–65% of the total enzymic activity, was brought to approximately 80% saturation with solid ammonium sulfate and centrifuged. The sediment was taken up in 30 ml of 0.06 M phosphate buffer, pH 7.3, and a fraction which precipitated between 60 and 80% saturation with ammonium sulfate was further purified. The enzyme solution (30 ml) was desalted on a Sephadex G-50 column (3 × 70 cm) in 0.02 M phosphate buffer, pH 7.3, and then directly separated at room temperature on a column DEAE-Sephadex A-50 coarse (1.5 × 30–40 cm, equilibrated with 0.02 M phosphate buffer, pH 7.3). The activity was eluted with a gradient of NaCl which increased from 0 to 0.3 M. The aminopeptidase activity was eluted at approximately 0.15–0.2 M NaCl. The enzyme was repeatedly precipitated between 65 and 80% saturation of ammonium sulfate in the cold until no further increase in the specific activity was achieved (Table I).

Precipitation of the enzyme at pH 5 yielded an enzyme of approximately the same purity as that previously obtained after two very laborious ultracentrifugation stages (Pfleiderer and Celliers, 1963). Only 6–13% of activity against leucine *p*-nitroanilide remained in the supernatant after the pH 5 precipitation, and this activity was rapidly destroyed if acidity of the solution was increased. As both the particle-bound aminopeptidase and that which had been dissociated from the particles were stable even at pH 3.5, it was inferred that an acid-unstable aminopeptidase activity was also present in the crude homogenate. It is therefore possible to separate a soluble from a particle-bound enzyme either by ultracentrifugation or by pH precipitation (alanine *p*-nitroanilide was a good substrate for the particle-bound enzyme with Ala:Leu 1.1, whereas in the crude homogenate Ala:Leu = 0.6). Further purification hardly altered the Ala:Leu ratio which remained between 1.1 and 1.4. The ratios of the rates of hydrolysis of the alanine to glycine *p*-nitroanilides were the same for the pure enzyme and for the crude homogenate (Ala:Gly 4.5). The velocity of proline amide

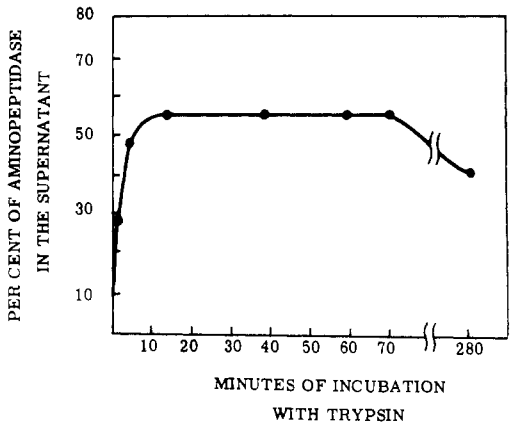

FIGURE 1: The liberation of soluble aminopeptidase from pig kidney particles by trypsin. A sample of the toluene-treated kidney particle suspension (see Results) was incubated with solid trypsin (20 mg/100 g of kidney) at 37°. After various times samples of the incubation mixture were cooled to 4° and centrifuged. The aminopeptidase activity of the resultant supernatant was measured and is expressed as per cent of that originally present in a suspension incubated without trypsin.

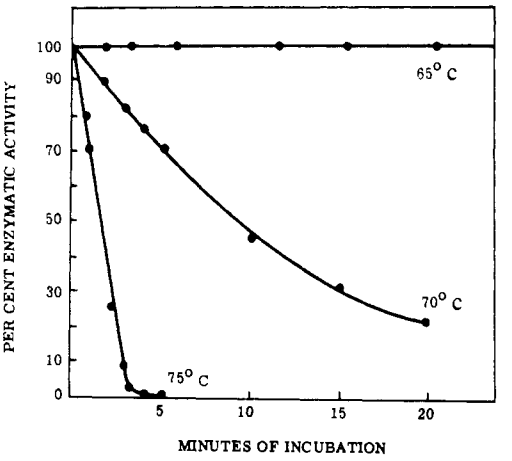

FIGURE 2: Temperature stability of aminopeptidase. The enzyme was incubated in 0.06 M phosphate buffer, pH 7.0, at the temperatures shown. Samples were removed at various times and the enzymic activity was determined against glycine and leucine *p*-nitroanilide (Methods) and is expressed as per cent of the activity of unheated controls.

hydrolysis referred to leucine amide hydrolysis did change during the purification (in the crude extract K_M was 2.5×10^{-2} M and V_{max} 53% of the leucine amide value, whereas, for the soluble preparation purified from the insoluble particle-bound enzyme fraction, K_M for proline amide was 2.5×10^{-1} M and V_{max} 1.1% of the leucine amide value). These results indicate an enzymic activity in the kidney homogenate which is more specific for proline amide hydrolysis than is the aminopeptidase. This activity could be similar to the specific prolidase described by Smith (Davis and Adams, 1955).

For the preparation of aminopeptidase it was necessary to use trypsin, and the stability of the enzyme against tryptic hydrolysis was examined by incubating samples of the toluene suspension for various times with trypsin (Figure 1). During the first 10 min the concentration of the liberated enzyme in the aqueous phase reached its maximum value and thereafter remained constant; only after several hours was it possible to detect any loss.

The aminopeptidase preparation described in this paper is apparently an enzyme bound to cell particles. The usual methods of ribosome dissolution, such as treatment in various concentrations of NaCl or $MgCl_2$, did not result in any liberation of soluble aminopeptidase, nor did they influence the yield of soluble enzyme after toluene treatment. The results of preincubation of the microsomal fraction with ribonuclease, of the removal of heavy metal ions with EDTA, or of treatment with detergents were equally negative in this respect. The enzyme–particle complex could not be dissociated between pH 5 and 11. If toluene was removed with petroleum ether (bp 60–62°), the second layer shrank to approximately one-tenth of its original size, and it was no longer possible to liberate the aminopeptidase activity with trypsin. All these results suggest that the main function of the toluene is not so much the extraction of lipids but the swelling of the aminopeptidase–particle complex so that the tryptic hydrolysis can take place under optimal conditions. Remarkably, only 5–10% of the original particle-bound aminopeptidase activity was liberated in a soluble form when the trypsin treatment was attempted in phosphate buffer. The remaining 90–95% remained bound and stable. Thus Tris, a competitive inhibitor, appears to facilitate the liberation of the aminopeptidase by trypsin.

Most previously described purification procedures for aminopeptidases use an acetone powder as a starting point. However, treatment of the homogenate with acetone, according to the method of Smith (Hill *et al.*, 1958), resulted in the loss of 91.5% of the leucine amide, 97.5% of the leucine *p*-nitroanilide, 98.3% of the alanine *p*-nitroanilide, and practically 100% of the alanine amide hydrolyzing activity (Pfleiderer *et al.*, 1964).

Properties of Aminopeptidase as a Protein

Molecular Weight. Using the method of gel filtration through Sephadex G-200 Auricchio and Bruni (1964) found the molecular weight of this aminopeptidase to be 280,000. This molecular weight was obtained by determining the ratio of elution volume of aminopeptidase to the void volume (V_0) of the column after calibrating the column with other pure proteins of known molecular weight.

Purity and Homogeneity of the Aminopeptidase. The

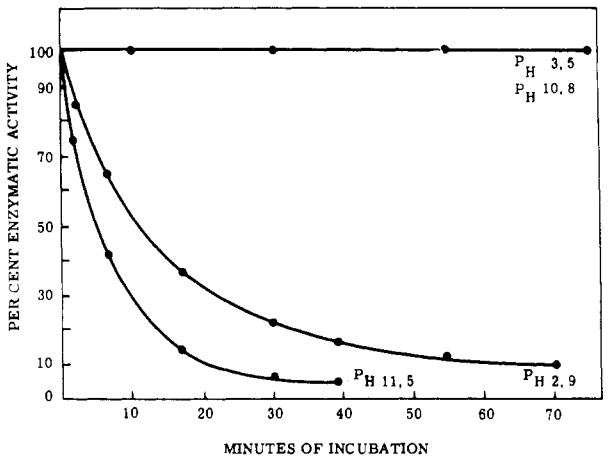

FIGURE 3: pH stability of aminopeptidase. The aminopeptidase was incubated at room temperature in 0.06 M phosphate buffers of the pH values shown. After various times the enzymic activity against leucine and glycine *p*-nitroanilides was determined (Methods) and is expressed as per cent of the activity of a control at pH 7.0.

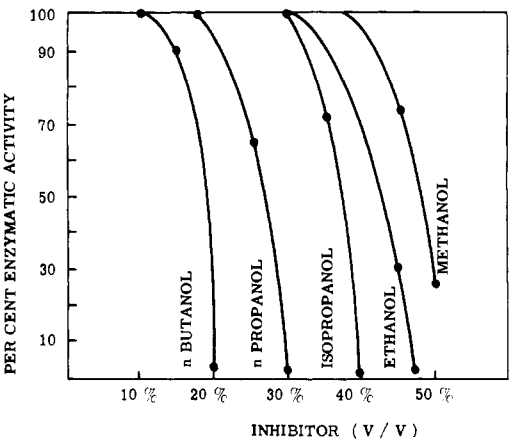

FIGURE 4: Denaturation of aminopeptidase with various alcohols. Aminopeptidase was incubated for 1 hr at room temperature in various concentrations of the alcohols in 0.06 M phosphate buffer, pH 7.0. The enzymic activity was determined (Methods) and is expressed as per cent of activity of a control incubated without added alcohol.

enzyme was rechromatographed on DEAE-Sephadex at pH 7.2 in phosphate buffer. Only one peak was eluted which contained both the ultraviolet absorption at 253 mμ and the enzymic activity. Only one peak of ultraviolet-absorption and enzymic activity was eluted from a Sephadex G-100 column (1.5 m long).

Throughout the purification, various fractions were subjected to immunoelectrophoretic analysis against a rabbit antibody serum. As is seen in Table I a preparation with a specific activity of 37 μmoles/mg is already immunochemically pure, yielding only a single band. Even when ten times as much enzyme was used for the analysis, only a single precipitation band was found. This band also contained the enzymic activity as demonstrated with specific coloration.

It has not as yet proved possible to prepare the enzyme in a crystalline form. The purest preparation of the aminopeptidase had a turnover number of 10,450 moles of leucine *p*-nitroanilide/mole of aminopeptidase when tested at 37° in 0.06 M phosphate buffer, pH 7.2. At a concentration of 1 mg of protein/ml (determined from the micro-Kjeldahl nitrogen with a factor of 6.25 times) the extinction using a cuvet, l = 1 cm, was for 280 mμ 1.63, for 266 mμ 1.228, for 225 mμ 12.50, and for 215 mμ 16.80. With the biuret reagent (Weichselbaum, 1946) in a cuvet, l = 2 cm, 1 mg of aminopeptidase protein gave $A_{546m\mu}$ 0.0506.

Temperature Stability. At 65° the enzyme is stable (Figure 2). At 70°, 50% of the enzyme activity is lost in 10 min but only 5% during the first minute. The activities against leucine *p*-nitroanilide and glycine *p*-nitroanilide decreased to the same extent, indicating that the same enzyme hydrolyzes both substrates.

pH Stability. Figure 3 shows that between pH 3.5 and pH 11 the enzyme is stable for at least 3 hr. Below pH 3 and above pH 11.5 very rapid denaturation occurs.

Influence of Denaturing Agents. Urea up to 6 M, acetone up to 35 vol %, or saturated solutions of either chloroform or toluene in water did not denature aminopeptidase during 100 hr of incubation at room temperature. Above these limiting concentrations a rapid denaturing took place which could not be reversed even after 50-hr dialysis against phosphate buffer. Guanidine treatment yielded other results in that denaturation was slow but was already evident at relatively low concentrations. For example, in 0.5 M solution after 2 hr 77%, and after 48 hr 33% of the initial activity was detected. At no point in these denaturation experiments was there any alteration in the relationship between hydrolysis rates of alanine, leucine, and glycine *p*-nitroanilide.

As can be seen from Figure 4 the length of the aliphatic chain in an homologous series of denaturing solvents is significant. As the chain length of the alcohol increased smaller and smaller concentrations were necessary to effect an irreversible denaturation of the aminopeptidase. Thus three times as much methanol as 1-butanol was required for 50% denaturation in 1 hr. Attempts to renature the inactivated enzymes of phosphate buffer 15–60 min after denaturation in ethanol, by diluting the solution with 20 times its volume of phosphate buffer, were unsuccessful. Nor did dilution with $MgCl_2$ solution and incubation in 0.05 M Tris buffer, pH 8.5, and 0.05 M $MnCl_2$ result in any renaturation. In this respect the aminopeptidase was unlike that described by Smith (Hill *et al.*, 1958). Finally it should be remarked that it was not possible to precipitate the aminopeptidase with trichloroacetic acid even in the presence of methanol, although the enzymic activity was irreversibly destroyed. The enzyme could be freeze dried without any appreciable loss of activity.

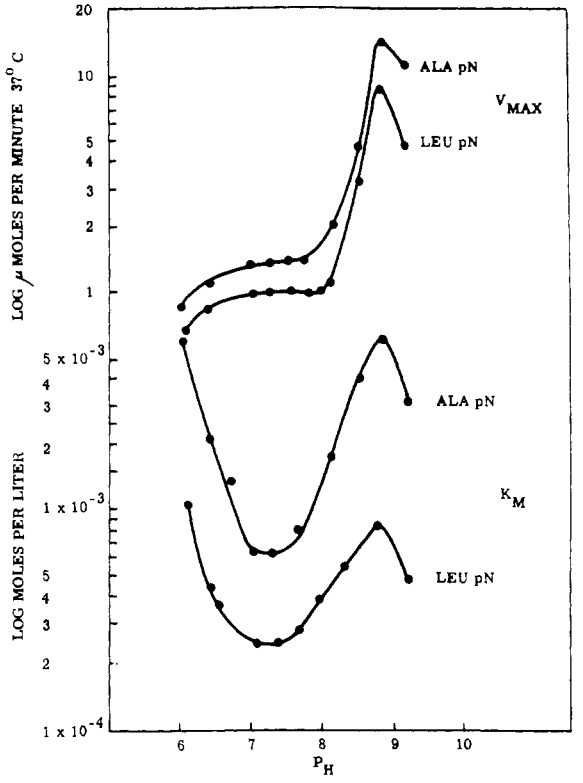

FIGURE 5: pH optimum of the aminopeptidase. The influence of pH upon the Michaelis constants (K_M) and V_{max} for the hydrolysis of leucine *p*-nitroanilide (Leu pN) and alanine *p*-nitroanilide (Ala pN) was determined from Lineweaver–Burk plots. V_{max} values are calculated for 10^{-5} μmole of enzyme. The ordinate scale is logarithmic.

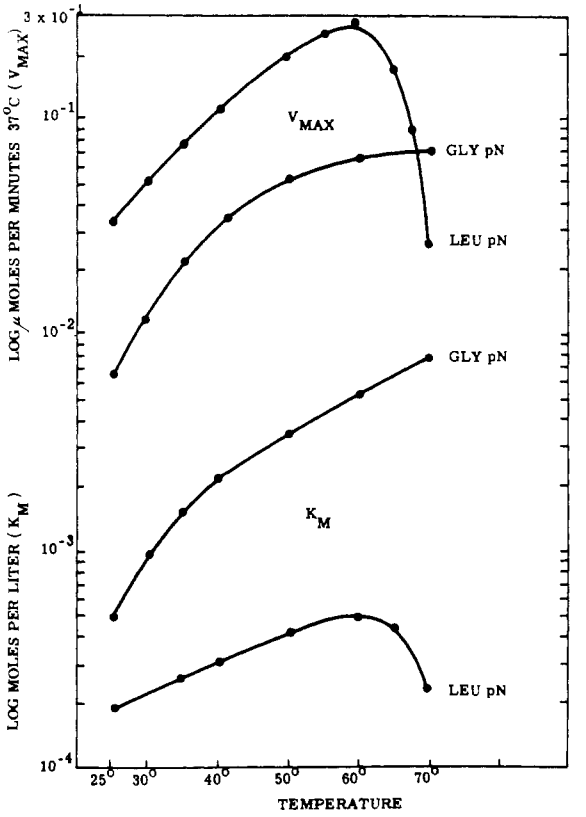

FIGURE 6: Temperature optimum for aminopeptidase. The V_{max} and Michaelis constants for the aminopeptidase hydrolysis of leucine and glycine *p*-nitroanilide were measured in a thermostatically controlled cuvet at the temperature shown. V_{max} values are calculated for 7×10^{-7} μmole of enzyme. No single measurement took longer than 1 min. The ordinate scale is logarithmic.

SH Groups. Samples of aminopeptidase were incubated in 10^{-5} M *p*-mercuriphenylsulfonate, in 10^{-2} M iodoacetate, and also in 10^{-3} M glutathione. There was no change in enzymic activity, indicating the absence of functional SH groups (Pfleiderer *et al.*, 1964).

Catalytic Properties of the Aminopeptidase

The catalytic properties of the aminopeptidase were investigated (Lineweaver and Burk, 1934) using leucine, alanine, and glycine *p*-nitroanilide as substrates.

pH Optimum. The K_M and V_{max} values for three substrates were measured in phosphate buffers of varying pH. All rates of reaction were linear with time when not more than 0.1% of the substrate was hydrolyzed. After the test was completed, the pH of the reaction mixture was remeasured with a microelectrode.

Figure 5 demonstrates that the highest rates of hydrolysis were at pH 8.8–9.0 for all three substrates. On the other hand, the enzyme has the highest affinity for the substrates between pH 7.0 and pH 7.3 and the lowest affinity at pH 8.8. The decrease of V_{max} above pH 9.0 is not due to denaturation, as it has been shown that the enzyme is stable up to pH 11. From these results it is clear why a pH optimum of 7.5 was initially reported (Pfleiderer *et al.*, 1964). The various pH optima found for the aminopeptidases described in the literature are questionable since each was tested under different degrees of saturation with substrate. At very high substrate concentrations the pH optimum is 9.0. As it is envisaged that this aminopeptidase will prove most useful for the determination of amino acid sequences of peptides, pH 7 should yield the maximum rates of hydrolysis since it would not be practicable to use the substrate concentrations required for hydrolysis at pH 9.

Optimum Temperature for Hydrolysis. K_M and V_{max} were measured for the *p*-nitroanilides of leucine and glycine. Each single measurement was carried out in less than 1 min, in order to eliminate denaturation of the enzyme. These constants were measured after pipetting 5 μl of enzyme solution into a cuvet containing the substrate solution at the desired temperature.

Figure 6 shows the expected increase of V_{max} and decrease in affinity for the aminopeptidase hydrolysis of

glycine *p*-nitroanilide up to 70°. Quite the same occurs with leucine *p*-nitroanilide up to 60°, but thereafter V_{max} decreases and the affinity increases. The only difference between the two systems is the aliphatic amino acid side chain in the leucine case. It thus appears that at about 60° some change takes place in the enzyme structure at or near the active center which affects the side chain of leucine *p*-nitroanilide and results in tighter binding of this substrate and a slower rate of hydrolysis. The importance of the side chain of the N-terminal amino acid of the substrate is clearly shown from this experiment.

Specificity of Aminopeptidase. The enzyme hydrolyzes only derivatives of α-amino acids. An attempt was made to hydrolyze asparagine, glutamine, β-alanine amide, and γ-butyramide, but even after prolonged incubation no ammonia could be detected. The amides of amino acids derived from a secondary amino group such as proline amide and sarcosine amide were hydrolyzed, but only very slowly. It was not possible to hydrolyze peptides with a D-amino acid at the amino end of the peptide. Another paper will report the detailed investigation of amide and *p*-nitroanilide amino acid derivatives (Wachsmuth *et al.*, 1966).

Discussion

According to the usual biochemical and physical criteria the exopeptidase described in this paper appears to be homogenous. It catalyses the hydrolysis only of the derivatives of the L-α-amino acids. Derivatives of secondary amines, such as proline and sarcosine, are very poor substrates. No hydrolysis of peptides occurs when the α-amino end group is substituted as in the carbobenzoxy or acetyl derivatives of peptides, showing that the enzyme contained neither endopeptidase nor carboxypeptidase activity. Since differences in the rates of hydrolysis of glycine, alanine, and leucine *p*-nitroanilides were so small, the enzyme should properly be described as an aminopeptidase and not as a leucine aminopeptidase. The leucine aminopeptidase described by Smith hydrolyzes leucine derivatives approximately 1000 times better than glycine derivatives (Smith and Hill, 1960). For the enzyme described here, there was only a factor of three between the two substrates.

The aminopeptidase is an easily soluble protein, contains no functional SH groups, is relatively stable with regard to temperature, retains its activity over a broad pH range, and does not require activation by metal ions. The enzyme was denatured by the high concentrations of alcohols which were previously used by Smith to extract leucine aminopeptidase from the same source (Hill *et al.*, 1958). The main problem in the preparation was the achievement of the soluble enzyme. Most of the aminopeptidases are only sparingly soluble (Hanson *et al.*, 1963; Patterson *et al.*, 1963; Smith and Hill, 1960). At least two aminopeptidase-active fractions were detected in kidney homogenates. The one had a high affinity for leucine and a small affinity for glycine and alanine derivatives, was soluble, only sedimented very slowly in the ultracentrifuge, and was acid unstable. The second and major fraction had approximately equal affinities for leucine, alanine, and glycine derivatives, was insoluble, sedimented with the so-called microsome fraction in the ultracentrifuge, and was relatively stable. It is only by swelling the particles with toluene so that tryptic hydrolysis can occur that we have been able to separate the enzyme from the insoluble particles. Before this treatment, the particle-bound enzyme appears very similar to the preparations described by Matheson *et al.* (1963) with respect to its stability to trypsin and alcohol denaturation. The low rate of hydrolysis of glycine derivatives compared with leucine derivatives of the dissociated Matheson aminopeptidase clearly differentiates it from the enzyme described in this paper.

None of the usual treatments for the disruption of ribosomes leads to the liberation of an easily soluble aminopeptidase. The results of further experiments will show whether the enzyme is bound to membranes of ergastoplasmatic reticulum or to cell-wall membranes. In agreement with Nachlas *et al.* (1957) we also found that the aminopeptidase activity was mainly located in the proximal tubuli contorti of the kidney, a region containing many cell-wall membranes.

References

Auricchio, F., and Bruni, C. B. (1964), *Biochem. Z. 340*, 321.

Davis, N. C., and Adams, E. (1955), *Arch. Biochem. Biophys. 57*, 301.

Hanson, H., Bohley, P., and Mannsfeldt, H. G. (1963), *Clin. Chim. Acta 8*, 555.

Hill, R. L., Spackman, D. H., Brown, D. M., and Smith, E. L. (1958), *Biochem. Prepn. 6*, 35.

Ishikawa, H., and Klingmüller, G. (1963), *Arch. Klin. Exptl. Dermatol. 217*, 340.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Markham, R. (1942), *Biochem. J. 36*, 719.

Matheson, A. T., Bjerre, S., and Hanes, C. S. (1963), *Can. J. Biochem. Physiol. 41*, 1741.

Nachlas, M. M., Crawford, D. T., and Seligman, A. M. (1957), *J. Hist. Chem. Cytochem. 5*, 264.

Patterson, E. K., Shu-Hsi H., and Kappel, A. (1963), *J. Biol. Chem. 238*, 3611.

Pfleiderer, G., and Celliers, P. G. (1963), *Biochem. Z. 339*, 186.

Pfleiderer, G., Celliers, P. G., Stanulovic, M., Wachsmuth, E. D., Determann, H., and Braunitzer, G. (1964), *Biochem. Z. 340*, 552.

Smith, E. L., and Hill, R. L. (1960), *Enzymes 4*, 37.

Tuppy, H., Wiesbauer, U., and Winterberger, F. (1962), *Z. Physiol. Chem. 329*, 278.

Wachsmuth, E. D., and Fritze, I. (1965), *Klin. Wochschr. 43*, 53.

Wachsmuth, E. D., Fritze, I., and Pfleiderer, G. (1966), *Biochemistry* 5, 175 (this issue; following paper).

Waddel, W. J. (1956), *J. Lab. Clin. Med. 48*, 311.

Weichselbaum, T. E. (1946), *Am. J. Clin. Pathol. 10*, 40.