kein **3a** mehr mit Dünnschichtchromatographie (DS.)[11] nachgewiesen werden konnte (ca. 40 Std.). Die Lösung wurde darauf im Vakuum eingedampft, mit Aktivkohle behandelt und der Rückstand aus Acetonitril/Hexan umkristallisiert. Ausbeute 2,46 g (82%); Smp. 174–205° (wurde durch 6-maliges Umkristallisieren nicht verbessert). Diese Substanz sublimiert bis 180°/0,005 Torr nicht. Bei 310° wird sie glatt zur Ausgangssubstanz **3a** pyrolysiert. Die vorgeschlagene *Dimer-Struktur* **14** wurde mit analytischen Daten belegt: IR. ($CHCl_3$): u.a. 4,42; 6,88; 11,65. UV. (CH_3CN): Endabsorption 216 nm. NMR. ($CDCl_3$): ca. 1,4–3,2/*m*, cycloaliph. H; 5,5–5,9/*m* olef. H (vier Protonen). MS: *m/e* = 290 (M^+. $C_{18}H_{22}N_2$), 145 ($M^+/2$), 92 ($M^+/2 - CH_2$ = CH-CN; Basis-Pik).

$C_{20}H_{22}N_2$ (290,4) Ber. C 82,72 H 7,64 N 9,65% Gef. C 82,84 H 7,71 N 9,44%

Pyrolyse des Photodimeren **14**. 1 g **14** wurde im Kugelrohr bei 0,02 Torr bis auf 310° erwärmt. Bei 270° begann eine ölige Flüssigkeit zu destillieren, welche spektrokopisch mit **3a** identisch ist. Ausbeute 0,73 g (73%).

LITERATURVERZEICHNIS

[1] *P. K. Friedman, D. M. Balls & D. J. Brown*, J. org. Chemistry *33*, 2211 (1968).
[2] *K. N. Houk*, Ph. D. Thesis, Harvard University 1968.
[3] *J. C. Davis Jr. & T. V. Van Auken*, J. Amer. chem. Soc. *87*, 3900 (1965).
[4] *T. J. Katz & A. Cerefice*, Tetrahedron Letters *1969*, 5970.
[5] *J. Sauer & J. Kredel*, Tetrahedron Letters *1966*, 731.
[6] *G. H. Wahl*, J. org. Chemistry *33*, 2158 (1968).
[7] *R. Criegee*, Angew. Chem., Int. Ed. *7*, 559 (1968).
[8] *E. J. Moriconi, C. F. Hummel & J. F. Kelly*, Tetrahedron Letters *1969*, 5325.
[9] *Houben-Weyl*, Bd. *5/1c*, 962 (1970); *L. A. Paquette*, J. Amer. chem. Soc. *86*, 3092 (1964); *L. A. Paquette, J. H. Barrett, R. P. Spitz & R. Pitcher, ibid. 87*, 3417 (1965); *T. Sasaki, S. Eguchi & H. Ohno, ibid. 92*, 3192 (1970); *L. A. Paquette & R. J. Haluska*, J. org. Chemistry *35*, 132 (1970); *D. I. Schuster & D. S. Sussman*, Tetrahedron Letters *1970*, 1657; *M. Sakai, A. Diaz & S. Winstein*, J. Amer. chem. Soc. *92*, 4452 (1970).
[10] *L. G. Cannell*, Tetrahedron Letters *1966*, 5967; *T. J. Katz, J. C. Carnahan, G. M. Clarke & N. Acton*, J. Amer. chem. Soc. *92*, 734 (1970).
[11] *G. D. Hunkler*, Dissertation Freiburg im Breisgau 1969.
[12] *R. Srinivasan* in «Advances in Photochemistry», Vol. 4, S. 113, Ed. *W. A. Noyes, G. S. Hammond & J. Pitts*, Interscience Publ., New York 1966.
[13] *A. G. Anastassiou & R. M. Lazarus*, Chem. Commun. *1970*, 373.
[14] *K. Witte & O. Dissinger*, J. Chromatogr. *28*, 413 (1967).

[11] Für die Dünnschicht-Chromatographie dienten *Merck*-DC-Fertigplatten F_{254} (Kieselgel). Der Substanzfleck von **3a** war sichtbar unter UV.-Licht, der Substanzfleck des Photoproduktes wurde nur nach Behandlung mit Jod-Dämpfen sichtbar.

---

47. Physical and Chemical Characterization of Pig Kidney Particulate Aminopeptidase[1]

by **Hans Wacker, Pavel Lehky, Edmond H. Fischer** and **Eric A. Stein**
Department of Biochemistry, University of Geneva (Switzerland)
and Department of Biochemistry, University of Washington (Seattle, Washington, U.S.A.)

(23. XII. 70)

Summary. Pig kidney particulate aminopeptidase (EC 3.4.1.2) was purified by a modification of the procedure of *Wachsmuth et al.* [3] to a state of homogeneity according to criteria of ultracentrifugation and polyacrylamide gel electrophoresis, and some of its physical and chemical properties were determined.

[1] A preliminary report of this work was presented at the second annual meeting of the Union of Swiss Societies for Experimental Biology at Fribourg, May 23–24, 1970 [1].

The purified enzyme (specific activity 30 ± 3 μmoles leucine-*p*-nitroanilide hydrolyzed/min/mg) has a $s_{20,w} = 9.82$ S at pH 8 and a molecular weight of about 280,000 as determined by high speed sedimentation equilibrium. Divergence between the number-, weight- and z- average molecular weights, and a sharp decrease of these values at low protein concentration, suggest that one is dealing with an associating-dissociating system.

Amino acid analysis revealed the presence of considerable quantities of carbohydrates in the enzyme. Colorimetric, gas chromatographic and enzymatic analyses demonstrated the presence of glucosamine, galactose, mannose, fucose and sialic acid residues in the ratio of 1:0.89:0.75:0.13:0.13, amounting to *ca.* 400 residues or 20% (56,000 daltons) of the molecular weight. With the exception of sialic acid, the carbohydrate content was remarkably constant from preparation to preparation.

Analyses by both atomic absorption spectrometry and the dithizone method showed that zinc – the only metal found in significant amount – was always present in the ratio of 2 atoms per molecule. It is therefore proposed that pig kidney particulate aminopeptidase is a Zn-containing glycoprotein.

Introduction. – Some years ago, *Pfleiderer et al.* [2] [3] described a particulate aminopeptidase (EC 3.4.1.2) isolated from a microsomal fraction of pig kidney. This enzyme, now commercially available under the name of 'aminopeptidase M' (*Roehm & Haas*, Darmstadt, Germany) constitutes a useful tool for the determination of amino acid sequences in proteins [4]. In further studies [5] *Wachsmuth* suggested that the enzyme might be made up of 10 subunits composed of chains of two types but of equal size (ca. 28,000 daltons). From kinetic studies using various substrates and inhibitors, three different substrate-binding sites were postulated on the enzyme [6], and tyrosyl and imidazole residues were to be involved in enzymic activity [7]. According to *Wachsmuth et al.* [4] the activity of the enzyme is not significantly affected by addition of either divalent metal ions or of EDTA, which findings suggest that this aminopeptidase is not a metalloenzyme.

Because these previous studies raised a number of intriguing questions regarding the nature and structure of this protein, the matter was reinvestigated. This publication presents some physical and chemical data suggesting that pig kidney particulate aminopeptidase is a Zn-containing glycoprotein. Although no conclusion could be drawn as to the subunit structure of the enzyme, the suggestion that it involved ten subunits of equal size could not be confirmed.

A subsequent publication (*P. Lehky et al.* [8]) will report on the role of Zn and other metal ions in the activity of the enzyme.

Methods. All reagents were of analytical grade; Sephadex products were purchased from *Pharmacia* (Uppsala, Sweden); Dowex resins from *Fluka* (Buchs, Switzerland) and Chelex-100 from *Calbiochem* (Lucerne, Switzerland). Metal-free water was obtained by double distillation of deionized water in a quartz glass still (*Westdeutsche Glasschmelze*, Geesthacht). The method of *Thiers* [9] for cleaning glassware was employed. Metal-free buffers were prepared by extraction with dithizone according to *Thiers* [9], or by passage through a column of chelating resin [10]. Disc gel electrophoreses were performed on 7.5% acrylamide gels as described by *Ornstein & Davis* [11].

Activity of the Enzyme was determined spectrophotometrically [3] [12]. Optical measurements were carried out with a *Beckman* DB spectrophotometer, fitted with a thermostatically controlled cell holder at 37°. The rate of hydrolysis of 1.66×10^{-3}M solutions of L-leucine-*p*-nitroanilide in 0.05M TES buffer at pH 7.0 was calculated from the absorption changes at 405 nm, using a molar absorbancy coefficient of 9,620 for *p*-nitroaniline. Enzyme concentration was of the order of 10^{-8} M (ca. 0.3 μg/ml).

Protein concentrations were determined spectrophotometrically by employing the absorbancy coefficient $A_{280}^{1\%} = 16.9$, based on refractometric measurements obtained from ultracentrifuge experiments, and by the method of *Lowry et al.* [13], using bovine serum albumin as standard.

The specific activity is defined as μmoles *p*-nitroaniline liberated per minute per mg of enzyme under the above conditions.

Metal analyses were performed on a *Perkin-Elmer* Model 303, atomic absorption spectrophotometer fitted with a recorder; Zn was also assayed by the dithizone method [14].

Ultracentrifugation was performed in a *Spinco* Model E ultracentrifuge equipped with temperature and electronic speed control, and focused at the two-thirds plane of the cell [15] [16]. All molecular weight distributions were based on high speed equilibrium experiments using a six channel Kel-F centerpiece designed by *Yphantis* [16]. *Raleigh* patterns were recorded on *Kodak* II-G photographic plates, and read on a modified Nikon microcomparator [17].

Points were read throughout, until the fringes could no longer be resolved at the base of the cell; computation of the data was carried out with the computer program developed by *Teller et al.* [18]. For high speed sedimentation equilibrium experiments, the enzyme was equilibrated in 0.1M Tris–HCl-0.1M NaCl buffer pH 7.8. Sedimentation velocity experiments were carried out at 18.3° in a double sector cell and at a protein concentration of 6 mg/ml in the same buffer as described above. The displacement of the Schlieren patterns was measured in the microcomparator described above. A partial specific volume of 0.74 ml/g was used in the calculations.

Amino acid analyses were performed by the procedure outlined by *Moore & Stein* [19]. The material was extensively dialyzed against several changes of twice distilled water before analysis. Aliquots (1.5 mg) were lyophilized in hydrolysis tubes; to each tube was added 3 ml of constant boiling hydrochloric acid. The tubes were flushed with nitrogen and evacuated three times in succession, then sealed under vacuum. Hydrolyses were carried out on duplicate samples at 108° ± 1° for periods of 24, 48, 72 and 96 h. After removal of HCl in a vacuum dessicator, the samples were analyzed on a *Spinco* Model 120 C automatic recording amino acid analyzer. Half-cystine was determined as cysteic acid following performic acid oxidation as described by *Moore & Stein* [19], and tryptophan was determined by the spectrophotometric method of *Bencze & Schmid* [20].

Carbohydrate Analyses: Carbohydrates were identified by thin layer chromatography on cellulose plates in three solvent systems and by gas chromatography of their trimethylsilyl derivatives [21], following hydrolysis. For this purpose, to 10–12 mg of extensively dialyzed samples in hydrolysis tubes were added 200 μg of inositol as internal standard. The mixtures were lyophilized and redissolved in 2.5 ml of water, 2.5 ml of 2N HCl was added, the tubes were evacuated several times, flushed with nitrogen, sealed, and heated at 100° for 4 and 8 h. The samples were first passed over (1 × 10 cm) columns of Dowex 1- × 8 (acetate form) to exchange the chloride ion, the effluents were evaporated in a rotatory evaporator below 35°, the residues were taken up in little water, then passed over (1 × 5 cm) columns of Dowex 50- × 8 (H^+ form) to remove cations. After evaporation of the effluents, the residues were dissolved in water and these solutions were divided equally for gas chromatography and colorimetric determinations of sugars, respectively. For control purposes duplicate samples containing 200 μg inositol and 400 μg each of fucose, mannose and galactose were carried through the same procedure.

For gas chromatography, the dried samples were dissolved in dry pyridine (400 μl); hexamethyldisilazane (200 μl) and trimethylchlorosilane (100 μl) were then added and the samples left at room temperature for 1–2 h. The solvent was evaporated and the residue extracted with petroleum ether (b.p. 40–70°). After filtration through glass wool the solution was reduced to 0.5 ml; 5–10 μl of this solution was injected into a *Perkin-Elmer* Model 900 gas chromatograph, equipped with a flame ionization detector and temperature programming. Carrier gas was N_2. For the determination of fucose an OV-1 column was used, whereas for the separation of mannose and galactose a column of carbowax 4000 was used. Semiquantitative determination of the individual components was carried out by cutting out the respective peaks from the chart paper and weighing them.

Colorimetric Methods: Total carbohydrate was determined by the orcinol-sulfuric acid method of *Winzler* [22], as modified by *François et al.* [23] but on a scaled down level. The phenol-sulfuric acid procedure [24], as modified by *McKelvy & Lee* [25] was also employed, but yielded some dif-

ferences in the colour response for mannose and galactose, in contrast to the orcinol-sulfuric acid method in which both sugars gave almost identical yields. The sum of galactose + mannose could thus be determined after correction for fucose, which was determined independently with sulfuric acid-cystein according to the procedure of *Dische & Shettles* [26]. The estimations carried out on the whole protein and on the hydrolysate were in very good agreement.

In addition to gas chromatography, galactose was also determined enzymatically with the 'Galactostat' from *Worthington* [27].

Sialic acid determinations were made after hydrolysis of the enzyme sample in 0.1N sulfuric acid at 80° for one hour [28] using thiobarbituric acid according to *Warren* [29], and, after absorption of sialic acid on an anion exchange column (Dowex 1- × 8, acetate form), following the resorcinol procedure of *Svennerholm* [28].

For hexosamine determinations, samples were hydrolyzed in 4N HCl for various intervals of time, and the hydrolysates, after evaporation and redissolution in water, were applied to columns of Dowex 50 × 8 to separate the hexosamine saccording to *Gardell* [30]. When only glucosamine could be detected, the simpler procedure of *Boas* [31] was followed. Glucosamine was determined according to *Elson & Morgan* [32], as modified by *Rondle & Morgan* [33] and *Kraan & Muir* [34].

Purification of the Enzyme was carried out essentially according to the procedure of *Wachsmuth et al.* [3], modified as follows: Pig kidney (3,000 g, fresh from the slaughter house) was homogenized in 9 l of 0.1M Tris buffer, pH 7.3 and stirred at 2° for 30 min. The suspension was centrifuged at 3,000 × *g* for 15 min, the resulting supernatant was adjusted to pH 5.0 with acetic acid, centrifuged at 1,500 × *g* for 20 min and the new supernatant solution obtained was once more centrifuged, this time at 45,000 × *g* for 30 min. The sediment from the latter centrifugation was resuspended in 400 ml of 0.01M Tris buffer and the resulting suspension was adjusted to pH 7.3. Toluene (100 ml) was added, and the mixture was vigorously stirred for 60 min at 38°. Trypsine (500 mg) was then added and the suspension was incubated at 37° for 1 h, then cooled to 4°. The mixture was centrifuged at 100,000 × *g* for 30 min yielding 4 distinct layers. The aqueous (second) layer was removed by succion and was fractionated with solid ammonium sulfate; the fraction precipitating between 70 and 80% saturation was collected and dissolved in a minimal amount of 0.02 M phosphate buffer, pH 7.3. The solution was dialyzed against the same buffer containing 0.15M NaCl and was subjected to gel filtration on a column of Sephadex G-100 pre-equilibrated with the same NaCl-phosphate solution. The pooled active fractions were concentrated by pervaporation, dialyzed against 0.02M phosphate buffer, pH 7.3, and applied to a column of DEAE-Sephadex A-50 equilibrated with the same solution. Elution was achieved by means of a linear gradient of NaCl in the same buffer. The active fractions were eluted at 0.15–0.2M NaCl. They were concentrated by pervaporation and further purified by recycling three times on a 4.5 × 40 cm column of Sephadex G-200. The active fractions were finally pooled and frozen for storage.

The whole procedure yielded approximately 200 mg of pure enzyme as judged by the finding of a single band by disc gel electrophoresis and a single symmetrical peak in the analytical ultracentrifuge (see below). The specific activity of the preparations varied from 27 to 33 units/mg.

Results and Discussion. – *Molecular weight determination:* Single symmetrical peaks were observed in sedimentation velocity experiments, as would be expected for a homodisperse solution. Fig. 1 illustrates one such ultracentrifuge run. A sedimentation coefficient $s_{20,w}$ of 9.82 S was obtained. For molecular weight determinations, high speed sedimentation equilibrium experiments were carried out on the enzyme at an initial concentration of 0.6 mg/ml (centripetal channel), 0.8 mg/ml (middle channel), and 1.2 mg/ml (centrifugal channel). Figure 2 illustrates the distribution of enzyme concentration within the first cell; the straight line obtained is a further indication of the homogeneity of the preparation. Number-average, weight-average and *z*-average molecular weights as a function of protein concentration are represented in Fig. 3. It can be seen that molecular weight averages are independent of *initial* protein concentration; values of $M_n = 251{,}564$, $M_w = 275{,}440$ and $M_z = 291{,}949$ g/mole (average of three cells) were obtained.

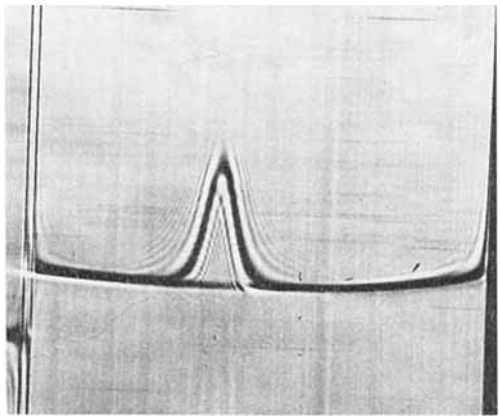

Figure 1. *Sedimentation velocity pattern of particulate aminopeptidase, 6 mg/ml in 0.1*M *Tris, 0.1*M *NaCl buffer, pH 7.8*

Temperature of the rotor maintained at 18.3°; picture taken 24 min after attainment of maximum speed (50,740 rpm)

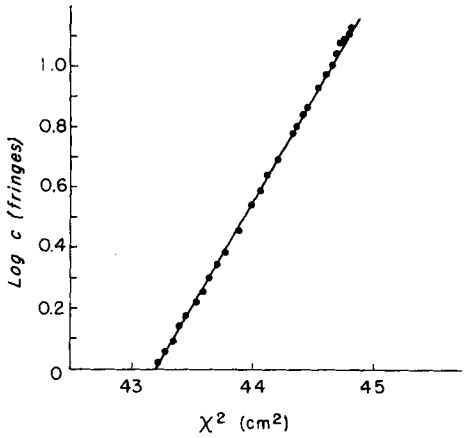

Figure 2. *Plot of logarithm of the enzyme concentration as a function of distance within the cell*

The values were obtained from the first channel in the experiment described in the legend of Fig. 3

The appreciable divergence between these three values is an indication that one might be dealing with an associating-dissociating system. Dissociation at high dilution might also be reflected by the sharp drops in number-average, weight-average and z-average molecular weights observed below 2 fringes (0.5 mg/ml)[2]. This is because in a high speed sedimentation equilibrium experiment, the concentration of protein at the meniscus of the cell approaches zero [16]. Since the rotor speed for meniscus depletion in a mixture is calculated on the basis of the molecular weight of the species present in the greatest amount [18], any protein in the mixture with a molecular weight less than that used to determine the rotor speed will be preferentially observed in the

[2] Although measurements at this low range of concentration are admittedly unreliable and, therefore, their interpretation questionable.

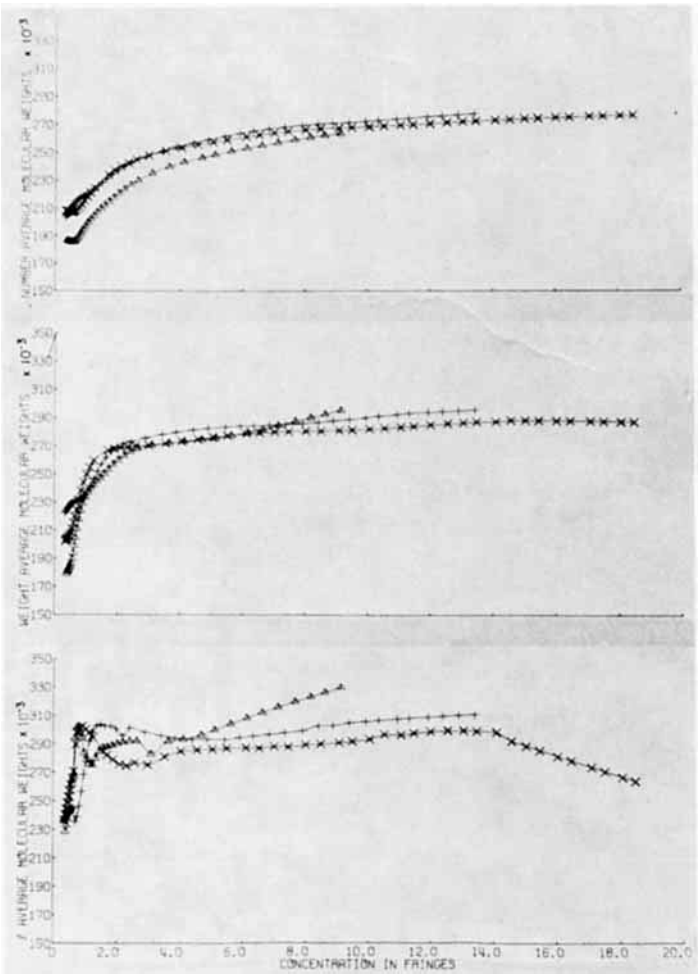

Figure 3. *Molecular weight distribution as a function of enzyme concentration*

plotted by a Cal-comp plotter directly from the data provided by the computer program of *Teller et al.* [18]. Four fringes correspond to 1 mg/ml protein; concentrations of enzyme were 0.60 mg/ml in the first (centripetal) cell (△) 0.80 mg/ml in the second (middle) cell (+) and 1.2 mg/ml in the third (centrifugal) channel (×), in 0.1M Tris, 0.1M NaCl buffer, pH 7.8

meniscus region. The number average and weight average moments reflect this partition by being lower in the meniscus region than throughout the rest of the ultracentrifuge cell; the degree to which lighter components affect the molecular weight averages at each point in the cell depends upon their concentration. Therefore, in principle, decreased molecular weights near the meniscus are a characteristic of dissociating systems whereas a homogenous, non-dissociating protein should not show such molecular weight dependence.

From the above data, it can only be concluded that the aminopeptidase appears to have a molecular weight of approximately 280,000 at high concentration and that

it might dissociate to smaller molecular weight species at low concentration. The same value of 280,000 had been previously obtained by gel filtration [3] [35].

Amino acid analyses: The values presented in Table I are the averages obtained from four times of hydrolysis; they include the usual extrapolation to zero time of hydrolysis for serine and threonine and to maximum time of hydrolysis for valine, leucine and isoleucine. Supportive analyses for half-cystine and tryptophan were carried out as indicated under Methods. The sum of the amino acid residues reported in Table I (column 3) adds up to 238,000 daltons for a total molecular weight of 294.000 if carbohydrates also present in the enzyme (see below) were included.

The amino acid composition given here is markedly different from that reported by *Wachsmuth* [5], with differences amounting to as much as 40% for certain residues; summation of the molecular weights for the amino acid residues yields a high value of 320,000 daltons, to which should be added *ca.* 15,000 daltons contributed by tryptophan and 56,000 daltons for carbohydrates that were not considered in the above mentionned analysis [5], for a total molecular weight of the order of 390,000 that is far in excess of the molecular weight (~280,000) estimated by both ultracentrifuge analysis and gel filtration. It is rather unlikely that this discrepancy could be ascribed to a basic difference in the nature of the two enzyme preparations under consideration. Other investigators [5] [7] sometimes used commercially available 'aminopeptidase M' (*Roehm & Haas*) as a starting material for their purification; however, this product is also prepared from pig kidney according to a procedure [2] [3] similar to that followed in this study. As will be discussed below, this commercial material presents enzymatic properties and a carbohydrate content almost identical to those of the purified enzyme investigated here.

Carbohydrate Analysis: Examination of the chromatograms provided by the amino acid analyzer revealed the presence of significant peaks that could not be assigned to the usual amino acids, but that occured precisely where one would expect aminosugars to emerge. Indeed, as shown in Table II, all preparations of the enzyme were found to contain rather large amounts of glucosamine, mannose and galactose, in addition to some fucose and sialic acid. Values obtained by independent analytical techniques were in close agreement; furthermore, with the exception of sialic acid, the carbohydrate content appeared to be remarkably constant from preparation to preparation. In preparation 4, neutral sugars were also determined colorimetrically on the intact enzyme without prior hydrolysis; again, values yielded by this approach for galactose plus mannose agreed closely with those obtained after hydrolysis. On the other hand, fucose gave a somewhat elevated value due to high blanks.

The carbohydrate content amounts to *ca.* 400 residues (see Table II) representing approximately 20% of the molecular weight of the enzyme or *ca.* 56,000 daltons.

Because of the discrepancies between the chemical composition reported here and that described previously [4–7] analytical tests including carbohydrate analysis were also carried out on commercial 'aminopeptidase M' (*Roehm & Haas*) on which, after further purification, some of the earlier studies were performed [5] [7]. Fig. 4 indicates that, as judged by disc gel electrophoresis, the two enzymes were very similar; the crude 'aminopeptidase M' (Fig. 4, C) shows one major band similar to that displayed by the purified enzyme investigated here (Fig. 4, A) plus, as expected, several other

Table I. *Amino Acid Analysis of Pig Kidney Particulate Aminopeptidase*

	μmoles per 0.243 mg enzyme[a])	g residues per 100 g enzyme	g residues per 280,000 g enzyme	integral number of residues per 280,000 m.w.
Lysine	0.073 ± 0.003	3.851	10,782	84
Histidine	0.036 ± 0.002	2.032	5,475	42
Ammonia[b])	0.417 ± 0.005	2.746	7,688	481
Arginine	0.059 ± 0.001	3.793	10,620	68
Aspartic Acid	0.217 ± 0.003	10.278	28,778	250
Threonine[b])	0.122 ± 0.002	5.076	13,703	141
Serine[b])	0.127 ± 0.002	4.552	12,746	146
Glutamic Acid	0.213 ± 0.003	11.816	31,685	245
Proline	0.088 ± 0.002	3.516	9,845	101
Glycine	0.080 ± 0.002	1.879	5,261	92
Alanine	0.127 ± 0.002	3.716	10,404	146
Valine[c])	0.106 ± 0.001	4.325	12,110	122
Methionine	0.043 ± 0.001	2.322	6,502	50
Isoleucine[c])	0.086 ± 0.001	4.006	11,217	99
Leucine[c])	0.178 ± 0.001	8.292	23,218	205
Tyrosine	0.074 ± 0.002	4.970	13,926	85
Phenylalanine	0.085 ± 0.003	5.147	14,412	98
$^1/_2$ Cystine[d])	0.010 ± 0.001	.425	1,190	12
Tryptophan[e])	0.072 ± 0.002	5.515	15,442	83

[a]) Averages of 24, 48, 72, and 96 h of hydrolysis on duplicate samples of the enzyme, with standard deviation.
[b]) Extrapolated to zero time of hydrolysis from least square plots.
[c]) Extrapolated to maximum time of hydrolysis in order to allow for complete release.
[d]) As cysteic acid obtained by performic acid oxidation (according to *Moore & Stein* [19]).
[e]) Obtained by the procedure of *Bencze & Schmid* [20].

Table II. *Carbohydrate Content of Four Preparations of Particulate Aminopeptidase in Residues per Molecule* (m.w. 280,000)

Preparation	Procedure	Fucose	Mannose	Galactose	Mannose + Galactose	Glucosamine	Sialic Acid
1	C	16.3			228	137	
2	C	16.3			235	134	7.1
3	C	17.1			236	130	17.6
	E			117			
4	C	19.7[a])			237[a])		
	C	16.5			236	138	13.9
	G	16.7	101.5	121	223		
	E			116			

C) Colorimetric determination
G) Analysis by gas chromatography
E) Enzymatic determination
[a]) Values determined colorimetrically on the whole enzyme without prior hydrolysis.

ones corresponding to contaminating impurities. Upon mixing the two products, the major component of each were superimposed, migrating as a single band (Fig. 4 B).

Both enzyme preparations could not be distinguished by their enzymatic properties. They had essentially identical *Michaelis* constants for leucine-*p*-nitroanilide (K_m = 0.21 mM. *vs* 0.24 mM as reported by *Pfleiderer et al.* [6] and same substrate

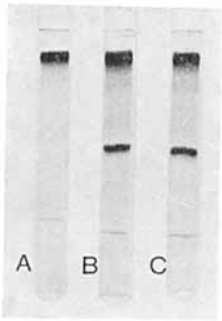

Figure 4. *Polyacrylamide gel electrophoresis of particulate aminopeptidase*

Electrophoreses were carried out on 7.5% polyacrylamide gels according to *Ornstein & Davis* [14]; gels were stained with amido black 10 B. A) aminopeptidase purified as described in this study (30 μg); B), mixture of A (15 μg) and C (50 μg); C) commercial 'aminopeptidase M' (*Roehm & Haas*) (50 μg).

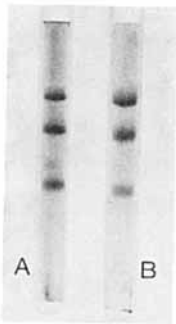

Figure 5. *Polyacrylamide gel electrophoresis of purified particulate aminopeptidase* (20 μg each) *following treatment with 0.1% sodium dodecylsulfate according to the procedure of* Shapiro et al. [36] *as modified by* Weber and Osborn [37]

A) proteins stained with coomassie brilliant blue; B) carbohydrates stained with fuchsine following periodate treatment according to *Keyser* [38]

specificities towards the *p*-nitroanilide derivative of alanine, leucine and glycine (100:76:18 *vs* 100:71:22, respectively). The specific activity of commercial 'aminopeptidase M' was 18 units/mg, *i.e.* approximately 60% that of the purified enzyme (30 units/mg).

A colorimetric determination of neutral sugars (essentially mannose + galactose[3]) performed on 'aminopeptidase M' without prior hydrolysis gave a value of 235 residues/mole (280,000 daltons) in good agreement with the value of 241 residues/mole obtained under similar conditions for the purified enzyme.

An important question, of course, is whether or not the polysaccharide is covalently linked to the enzyme, or present only as a contaminant, originating perhaps from the subcellular elements to which the enzyme was originally bound. Only preliminary experiments were carried out to answer this question.

First, the enzyme was denatured by treatment with 0.1% sodium dodecylsulfate, then subjected to polyacrylamide gel electrophoresis according to the procedure of *Shapiro et al.* [36] as modified by *Weber & Osborn* [37], bands were stained both for proteins and carbohydrates according to *Keyser* [38]. Three protein bands were consistently found (Fig. 5, A) which, when compared to marker substances of known molecular weight (Fig. 6), corresponded to material of m.w. *ca.* 60000, 100000 and

[3]) The contribution of fucose is negligible because of its low colour response in this test.

140000 respectively (on occasion, the 100000 m.w. band appeared as a doublet (as illustrated in Fig. 6). When duplicate gels were stained for carbohydrates, all three protein bands (presumably the subunits of the enzyme) gave a positive response (Fig. 5, B). In control runs, homoproteins such as bovine serum albumin that do not contain carbohydrates, were negative.

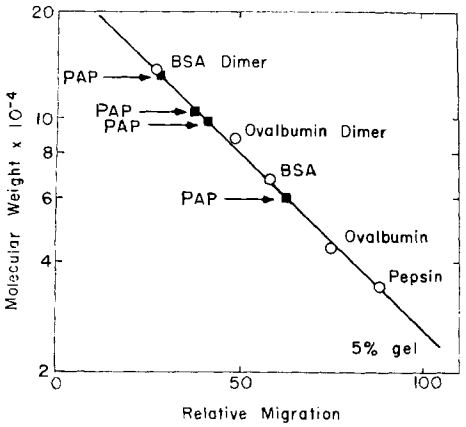

Figure 6. *Plot of relative mobilities obtained for particulate aminopeptidase* (PAP) *and protein markers as a function of molecular weight, following disc gel electrophoresis*

The enzyme (10 μg) was incubated for 30 min at 65° in 0.1% sodium dodecylsulfate prior to electrophoresis in a 5% polyacrylamide gel at pH 7.2 according to [36] and [37]. Markers were bovine serum albumin, m.w. 68000 and 136000 for monomer and dimer, respectively [39], ovalbumin, 43000 and 86000 for monomer and dimer, respectively [40], and pepsin, 35000 [41]

Second, attempts to free the enzyme of carbohydrates by enzymatic attack were uncessful. Samples (5–10 mg) of the purified enzyme were subjected to prolonged attack (at least 60 h at room temp.) by sialidase (80 μl of a solution of *V. cholerae* enzyme as obtained from *Serva*, Heidelberg), by emulsin (2 mg), and by β-galactosidase (1 mg, both from *Sigma*, St Louis, USA), respectively. Final volume was 2 ml of pH 5.0 buffer, acetate in the case of sialidase and citrate-phosphate for the two other enzymes. No loss of activity over control was observed; controls, which were incubated under identical conditions but without carbohydrases, displayed less than 15% loss of activity stressing once more the remarkable stability of the aminopeptidase. Most of the sialic acid and approximately one-half of the galactose appeared to be liberated through the action of sialidase and β-galactosidase, respectively, but in all instances, there was still some carbohydrate linked to the protein. Examination of the samples following enzymatic digestion, by disc gel electrophoresis in 0.1% sodium dodecylsulfate revealed the same three fractions as before, except that they displayed slightly higher mobilities as judged by the appearance of double bands when cochromatographed with the untreated enzyme. All three new bands gave the essentially same positive response as the original material when stained for carbohydrates. This strongly suggests that the carbohydrate moiety is an integral part of the enzyme molecule and not a contaminating polysaccharide fraction which, for some reason, had remained stuck to the enzyme during the course of purification.

Metal content of aminopeptidase: The results from metal analyses carried out on various purification steps of the enzyme are presented in Table III. Besides the metals listed (Zn, Ca, Mg, Fe, Cu, Ni), Cd, Co and Mn were also analyzed, but were always below the detection limits of atomic absorption spectrophotometry (< 0.01 ppm for

Table III. *Metal Content of Various Fractions Obtained during the Purification of Aminopeptidase*

Fraction	Specific Activity as defined p. 475	g atom metal/100,000 g protein[a]) Zn	Ca	Mg	Fe	Cu
Kidney extract	0.18	0.31	0.96	8.29	0.66	0.07
$(NH_4)_2SO_4$ precipitate (70–80% sat.)	11.0	0.84	0.06	0.01	0.39	0.22
Eluate from DEAE-Sephadex	30.2	0.86	0	0	0.08	0.04
Eluate from G-200 Sephadex	33.0	0.73	0	0	0	0

[a]) 0 indicates a value below the detection limit of the AAS *Perkin-Elmer* 303 coupled to a recording device.

these three metals, *i.e.* well below 0.05 atom per molecule of purified enzyme). The Zn-protein ratio increases approximately 2.5 fold, while the concentration of all other metals decreases rapidly. Since the Zn content is already very high in hog kidney (340 mg Zn/g protein), it is not surprising that the enrichment with respect to this metal is relatively low. In the purified enzyme Zn is the only metal present in significant quantity: 0.73 g atom per 100,000 g of protein corresponding to 2.05 atom Zn per molecule of enzyme (280,000 m.w.). The value of 2 atoms per molecule was consistently found in all preparations analyzed by atomic absorption spectrophotometry (Table IV); these data were confirmed by dithizone titration. A subsequent

Table IV. *Zn Content of Purified Aminopeptidase as Determined by Atomic Absorption Spectrophotometry*

Preparation	1	2	3	4	5
g atoms Zn per 280,000 g protein	1.99 ± 0.1	2.02 ± 0.3	2.02 ± 0.3	2.2 ± 0.2	1.98 ± 0.2
Number of determination	2	5	8	4	4

publication [8] will show that the catalytic activity of the enzyme is abolished by removal of the Zn^{2+} but fully restored upon readdition of Zn^{2+}; certain other divalent cations also reactivated the inactive apoenzyme to different levels.

Considerations on the subunit structure of the enzyme: Although only preliminary work was carried out on this problem, the data presented in this publication cast doubts on the subunit structure previously proposed [5]. Results obtained by disc gel electrophoresis following sodium dodecylsulfate treatment (see Fig. 5 and 6) gave no evidence for a dissociation of the enzyme to species of molecular weight lower than 60,000 that would be compatible with the ten-subunit postulate [5]. On the assumption that some of the bands observed could result from incomplete disaggregation of the molecule under the conditions used, or that artificial aggregation occured through the formation of disulfide linkages between subunits, the material was subjected to

drastic treatment (24 h at 65° with 0.1% sodium dodecylsulfate before and after exhaustive carboxymethylation of the protein) but with no appreciable change in the observed pattern.

Furthermore, the enzyme contains 84 lysyl and 68 arginyl residues from which 153 peptides could be theoretically generated by exhaustive tryptic digestion, assuming that the structure had no repeating sequence. From a peptide map following tryptic digestion, *Wachsmuth* [5] detected 47 peptides, a result inconsistant with a structure composed of 10 subunits made up of one or two types of chains.

Lastly, all preparations of the enzyme so far examined were found to contain 2 Zn per molecule. The metal could be either required to maintain the enzyme in the proper conformation for activity, or participate in catalysis by being involved in the active site of the enzyme, in which case up to two identical regions of the molecule (presumably two subunits) could be postulated. Too little data are available at this point to allow the formulation of a structure that would take these various observations into account; nevertheless none of the findings described herein support the hypothesis that the enzyme has an oligomeric structure that could accommodate the ten subunits consisting of the two types of chains that were previously proposed [5].

This work has been generously supported by grants 3600 and 4740 from the *Swiss National Foundation for Scientific Research*, and AM 7902 from the *National Institute of Arthritis and Metabolic Diseases*, USPHS.

The authors are indebted to Dr. *J. Deshusses* for providing access to the *Perkin-Elmer* Model 900 gas chromatograph, to Mrs. *T. Dewer* for the ultracentrifuge studies, and to Mr. *R. Olsgaard* for the amino acid analyses. The valuable technical assistance of Miss *B. Wittmann* and Miss *E. Simon* is also acknowledged. We wish to thank Drs. *J. Lisowski*, *D. P. Wolf*, and *M. Fosset* for their contribution during the early stage of this work.

BIBLIOGRAPHY

[1] *H. Wacker, P. Lehky & E. Stein*, Experientia *26*, 688 (1970).
[2] *G. Pfleiderer & P. G. Celliers*, Biochem. Z. *339*, 186 (1963).
[3] *E. D. Wachsmuth, I. Fritze & G. Pfleiderer*, Biochemistry *5*, 169 (1966).
[4] *G. Pfleiderer, P. G. Celliers, M. Stranulovich, E. D. Wachsmuth, H. Determann & G. Braunitzer*, Biochem. Z. *340*, 552 (1964).
[5] *E. D. Wachsmuth*, Biochem. Z. *346*, 467 (1967).
[6] *E. D. Wachsmuth, I. Fritze & G. Pfleiderer*, Biochemistry *5*, 175 (1966).
[7] *E. D. Wachsmuth*, Biochem. Z. *346*, 446 (1967); *U. Femfert & G. Pfleiderer*, FEBS Letters *4*, 262 (1969); *G. Pfleiderer & U. Femfert, ibid. 4*, 265 (1969).
[8] *P. Lehky, J. Lisowski, D. Wolf, H. Wacker & E. A. Stein*, in preparation.
[9] *R. E. Thiers*, 'Methods of Biochem. Analysis', *D. Glick* Ed., Vol. *5*, 273, Interscience Pub., New York 1957.
[10] *S. R. Himmelhoch, H. A. Sober, B. L. Vallee & K. Fuwa*, Biochemistry *5*, 2523 (1966).
[11] *L. Ornstein*, Ann. N.Y. Acad. Sci. *121*, 321 (1964); *B. J. Davis, ibid. 121*, 404 (1964).
[12] *H. Tuppy, M. Wiesbauer & F. Wintersberger*, Z. physiol. Chem. *329*, 278 (1962).
[13] *O. H. Lowry, N. J. Rosebrough, A. L. Farr & R. J. Randall*, J. biol. Chemistry *193*, 265 (1951).
[14] *B. G. Malmström*, 'Methods of Biochem. Analysis', *D. Glick* Ed., Vol. *3*, 327, Interscience Pub., New York 1956.
[15] *H. Svensson*, Opt. Acta *1*, 25 (1954).
[16] *D. E. Yphantis*, Biochemistry *3*, 297 (1964).
[17] *D. C. Teller*, Analyt. Biochemistry *19*, 256 (1967).
[18] *D. C. Teller, T. A. Horbett, E. G. Richards & H. K. Schachman*, Ann. N.Y. Acad. Sci. *164*, 66 (1969).

[19] *S. Moore & W. H. Stein*, 'Methods in Enzymology', *Hirs* Ed., Vol. *6*, 819, Academic Press, New York 1963.
[20] *W. L. Bencze & K. Schmid*, Analyt. Chemistry *29*, 1193 (1957).
[21] *C. C. Sweely, R. Bentley, M. Makita & W. W. Wells*, J. Amer. chem. Soc. *85*, 2497 (1963).
[22] *R. J. Winzler*, Meth. Biochem. Anal. *2*, 279 (1955).
[23] *C. François, R. D. Marshall & A. Neuberger*, Biochem. J. *83*, 335 (1962).
[24] *M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers & J. Smith*, Analyt. Chemistry *28*, 350 (1956).
[25] *J. F. McKelvy & Y. C. Lee*, Arch. Biochemistry Biophysics *132*, 99 (1969).
[26] *Z. Dische*, Methods Carbohydrate Chemistry *1*, 477 (1962).
[27] *J. M. Sempere, C. Gancedo & C. Asensio*, Analyt. Biochemistry *12*, 509 (1965).
[28] *L. Svennerholm*, Acta chem. scand. *12*, 547 (1958).
[29] *L. Warren*, J. biol. Chemistry *234*, 1971 (1959).
[30] *S. Gardell*, Acta chem. scand. *7*, 207 (1953).
[31] *N. F. Boas*, J. biol. Chemistry *204*, 553 (1953).
[32] *L. A. Elson & W. T. J. Morgan*, Biochem. J. *27*, 1824 (1933).
[33] *G. J. M. Rondle & W. T. J. Morgan*, Biochem. J. *61*, 586 (1955).
[34] *J. G. Kraan & H. Muir*, Biochem. J. *66*, 55P (1957).
[35] *F. Auricchio & C. B. Bruni*, Biochem. Z. *340*, 321 (1964).
[36] *A. L. Shapiro, E. Viñuela & J. V. Maizel*, Biochem. Biophys. Res. Commun. *28*, 815 (1967).
[37] *K. Weber & M. Osborn*, J. biol. Chemistry *244*, 4406 (1969).
[38] *J. W. Keyser*, Analyt. Biochemistry *9*, 249 (1964).
[39] *C. Tanford, K. Kawahara & S. Lapanje*, J. Amer. chem. Soc. *89*, 729 (1967).
[40] *F. J. Castellino & R. Barker*, Biochemistry *7*, 2207 (1968).
[41] *F. A. Bovey & S. S. Yanari* in *P. D. Boyer, H. Lardy & K. Myrback* (Eds.), 'The Enzymes', IV, 63, Academic Press, New York 1960.

48. ESR.-Untersuchung zur Reaktion von Hydroxylradikalen mit Glycin

von **H. Paul** und **H. Fischer**

Physikalisch-chemisches Institut der Universität Zürich, Schweiz

(12. I. 71)

Summary. Radicals $H_2N–\dot{C}H–COOH$ and $H_2N–\dot{C}H–COO^-$ which have different ESR. spectra are observed during the reaction of hydroxyl radicals with glycine in aqueous solution. Rapid and reversible exchange between the different dissociated radicals is induced by addition of phosphoric acid. The pH dependence of the ESR. spectra yields the pK value and rate constants for proton transfer reactions between the radicals and phosphoric acid.

ESR.-Spektren kurzlebiger Radikale, die bei der Reaktion von Hydroxylradikalen mit Glycin in wässrigen Lösungen entstehen, sind bereits von mehreren Autoren angegeben worden [1]–[7]. Die Spektren hängen stark vom pH-Wert der Lösungen ab. Für pH > 8 lassen sie sich mit einiger Sicherheit dem Radikal $H_2N–\dot{C}H–COO^-$ zuordnen, dessen NH_2-Protonen inäquivalent erscheinen [3] [4] [6] [7]. Im Bereich $3 <$ pH < 4 wurde ein Spektrum beobachtet, das nach *Smith* und Mitarb. [5] dem Radikal $H_2N–\dot{C}H–COOH$ zugehört und äquivalente NH_2-Protonen aufweist. Andere Autoren [1] [4] haben andere Interpretationen des in diesem pH-Bereich beobachteten