0013-7227/78/1035-1794$02.00/0
Endocrinology

Vol. 103, No. 5
Printed in U.S.A.

Identification of a Tripeptidyl Aminopeptidase in the Anterior Pituitary Gland: Effect on the Chemical and Biological Properties of Rat and Bovine Growth Hormones*

T. W. DOEBBER,† A. R. DIVOR, AND S. ELLIS

Biomedical Research Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035

ABSTRACT. The NH_2-terminal heterogeneity which is generated in bovine GH during its extraction from mildly acidified pituitary homogenates is attributable to a newly identified peptidase. The β-naphthylamide of Phe-Pro-Ala, modeled after the NH_2-terminal tripeptide sequence of the phenylalanyl monomer of bovine growth hormone, was cleaved by the peptidase into the tripeptide and β-naphthylamine and served as a substrate for assay of the enzyme. However, the β-naphthylamide of Ala-Phe-Pro, modeled after the NH_2-terminal tripeptide sequence of the alanyl monomer, was not cleaved. In harmony with this specificity, the peptidase cleaved 11 tripeptides sequentially from the NH_2-terminus of the phenylalanyl monomer of bovine GH but none from the alanyl monomer. Six of the tripeptides nearest the NH_2-terminus were unequivocally identified and their sequences were consistent with the NH_2-terminal octadecapeptide sequence of the phenylalanyl monomer of bovine GH. Five additional peptides were by composition consistent with their being tripeptides derived from residues 19–33. Because of the apparent specificity for the hydrolytic release of tripeptides and inability to cleave substituted tripeptidyl derivatives, the enzyme is considered to be a tripeptidyl aminopeptidase. In its hydrolysis of phenylalanyl monomers of rat growth hormone, a similar number of tripeptides was released, associated with which there was a 70% loss of biological activity but no reduction in immunological activity. The enzyme could be solubilized by extraction with 1% Triton X-100 at pH 3.0, precipitated between 2 and 3 M $(NH_4)_2SO_4$, and further purified by gel filtration on G-75 in M/10 acetic acid. The enzyme has a mol wt of 57,000 and is optimally active at pH 4. It can be differentiated from cathepsin D by its insensitivity to inhibition by pepstatin. (*Endocrinology* **103:** 1794, 1978)

BOVINE and ovine GH contain equimolar amounts of NH_2-terminal alanine and phenylalanine (1), when the hormone is extracted from pituitary glands at basic or acidic extremes of pH such as those used by Li (2) and by Raben and Westermeyer (3), respectively. If the hormone is isolated from extracts prepared at neutral pH, variable amounts of methionine as well as glutamic acid and serine replace the phenylalanine NH_2-terminus to an extent which depends on the pH, temperature, and duration of the extraction process (4). The new NH_2-termini appear to arise from the proteolytic cleavage of the three amino acid residues, Phe, Pro, and Ala, which precede the Met residue located in position 4 of the phenylalanyl chain of bovine GH (bGH) (4). It has not been established whether the three residues were cleaved singly and sequentially, or as a dipeptide and an amino acid, or simply as an intact tripeptide. The present investigation has revealed that the residues in question as well as up to 33 additional residues are released as tripeptides sequentially from the NH_2-terminus of the phenylalanyl monomer of bGH and from rat GH (rGH). These findings establish for the first time the existence in the pituitary of a new type of peptidase, tentatively designated as a tripeptidyl aminopeptidase (TAP), as well as its effect on the biological and immunological activity of GH.

Received February 8, 1978.

Address reprint requests to: S. Ellis, Biomedical Research Division, Ames Research Center, NASA, Moffett Field, CA 94035.

* This work was conducted during the tenure of an NRC-NASA Resident Research Associateship.

† Current address: Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, MO.

Materials and Methods

Isolation of Ala-bGH, Phe-bGH, Ala,Phe-bGH, Ala,Met-bGH, and rGH

The alanyl and phenylalanyl chains of bGH were isolated by preparative isoelectric focusing in Ampholine pH gradients as described previously (5). Ala,Phe-bGH and Ala,Met-bGH were prepared by the methods of Li (2) and Ellis (4), respectively, except that in the latter case the salted out hormone was further purified by filtration through Sephadex G-100 in 0.1 M Tris-HCl-0.1 M NaCl, pH 9.0. The rGH was isolated as reported previously (6).

Polyacrylamide gel electrophoresis of bGH and rGH digests

The conversion of the Phe-bGH to Met-bGH was monitored by polyacrylamide gel electrophoresis at pH 8.0, under nondissociating conditions (7). At this pH the Phe, Ala, and Met-bGH components could be resolved by electrophoresis in polyacrylamide gel for 40–48 h due to differences in the pK of the NH_2-terminal (8). Briefly, the electrophoresis was conducted in cylindrical resolving gels, 10 cm in length, which were prepared from 10% acrylamide and cross-linked with 0.27% methylene-bisacrylamide (in 86 mM Tris-46 mM acetic acid-2.7 mM NA_2EDTA buffer, pH 7.95). The polymerization was catalyzed with *N,N,N′,N′*,-tetramethylene ethylenediamine and ammonium persulfate at final concentrations of 0.1% and 0.056%, respectively.

The bGH digests were applied in a volume of about 100 μl which contained the following: 30 μg GH, 0.02% bromophenol blue, Tris-acetate-EDTA buffer at 1:10 dilution of the resolving and electrode buffers noted above, and 10% sucrose. Electrophoresis was conducted at 2 mA/gel until the dye entered the gel and afterwards at 4 mA/gel for 40–48 h. The electrode buffer was circulated between the anode (lower) and cathode (upper) compartments of the apparatus (Hoeffer Scientific Co., San Francisco, CA) to maintain a constant pH of 7.95; and the gels were maintained at 13 C with running tap water. The gels were stained by the method of Reisner *et al.* (9), which consists of soaking the gel in 0.04% Coomassie Brilliant Blue G-250 dissolved in 3.5% perchloric acid. Protein bands were thereby visualized directly without destaining.

Gel electrophoresis in sodium dodecyl sulfate (SDS) (0.1%) and mercaptoethanol (0.1%) was performed according to the method of Maizel (10) in 13% acrylamide gel. Ala-bGH or Phe-bGH (50 μg) were incubated with the purified peptidase fraction at a weight ratio of 12.5:1 in 0.18 ml 30 mM acetate buffer, pH 4.0, containing 0.017% Triton X-100 to facilitate solution of the bGH chains. After incubation for appropriate periods of time at 37 C, each digest was prepared for electrophoresis by adding 84 μl of a solution containing 4% SDS, 4% mercaptoethanol, 20% sucrose, 0.04% bromphenol blue, and 43 μl 59 mM Tris-32 mM phosphate buffer, pH 6.8. The diluted digests were then heated for 1 min in a boiling water bath and 100 μl containing the equivalent of 16 μg of the original GH were submitted to electrophoresis in 13% acrylamide gels with 0.1% SDS. The dye in the applied sample entered the gel after application of 1 mA for 2 h after which 2 mA were applied for an additional 3 h. The gels were fixed overnight in 12.5% trichloroacetic acid-25% isopropanol, then stained with 0.1% Coomassie Brilliant Blue R in 10% acetic acid-25% isopropanol and destained overnight by washing with 10% acetic acid-10% isopropanol. Ovalbumin, chymotrypsinogen A, ribonuclease A, and insulin served as molecular weight standards.

rGH (248 μg) was incubated with 19–58 μg of the Sephadex G-75 peptidase fraction in 1.45 ml 50 mM acetate buffer pH 4.0. Pepstatin at a final concentration of 1 μM was incorporated in the digest to inhibit small amounts of cathepsin D which contaminated the tripeptide peptidase. After incubation for 21 h at 37 C the digests were prepared for SDS electrophoresis as described above. Aliquots of the digests were neutralized with 0.5 M Na_2CO_3 and diluted with saline for measurement of growth-promoting potency in immature hypophysectomized rats (11) and for RIA (12).

Enzyme assays

The peptidase activity which cleaves the NH_2-terminal tripeptide from Phe-bGH was assayed at pH 4.0 by the means of the fluorogenic β-naphthylamide (βNA) derivative of the tripeptide Phe-Pro-Ala as a substrate. In some instances, Ala-Ala-Phe-βNA, which is hydrolyzed at a substantially lower K_m, was also employed to monitor enzyme specific activity. The rate of release of fluorescent β-naphthylamine was monitored fluorimetrically at 37 C by continuous recordings at the excitation and emission conditions previously described (13). Since the fluorescence of β-naphthylamine rapidly diminishes below pH 5, all rates of fluorescence formation were corrected by means of a calibration curve relating βNA fluorescence to pH. L-Phe-L-Pro-L-Ala-βNA·H_2O was purchased from Bachem Inc.

(Marina Del Rey, CA). The derivative yielded 94% of the theoretical content of each amino acid after acid hydrolysis and showed a single ninhydrin spot by thin layer chromatography (TLC) in two solvent systems. Stock solutions of Phe-Pro-Ala-βNA were prepared in dimethylformamide because the derivative was poorly soluble in water. Routine peptidase assay was performed at a 3 mM substrate concentration in 50 mM acetate buffer at pH 4.0. Cathepsin D was assayed spectrophotometrically by the increase in absorbancy at 280 nm of the trichloroacetic acid-soluble peptides released from denatured hemoglobin (14).

Extraction of TAP from bovine anterior lobes

Freshly collected bovine pituitary glands were dissected free of the capsule and posterior lobe then rinsed several times with saline to remove adherent blood. One kilogram of anterior lobes was passed twice through an electrically powered meat grinder using a plate with 1/16-inch holes. The ground glands were extracted successively at pH 5.5 with water, at pH 4.0 with M/10 $(NH_4)_2SO_4$, and once again at pH 5.5 with M/4 $(NH_4)_2SO_4$. The rationale for these procedures has been discussed elsewhere (4). The TAP was solubilized by suspending the final residue from the preceding extractions in a 1% solution of Triton X-100 containing 0.25 M $(NH_4)_2SO_4$, using 6 vol/kg original ground glands. After adjusting the suspension of pH 3.0 with 2 M HCl and raising the temperature to between 20 C and 25 C, the suspension was stirred vigorously for 30 min and then centrifuged at 21,000 × *g* for 30 min at 4 C. A light yellow, somewhat turbid supernatant was obtained, the temperature of which was lowered to 5 C and inert protein precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 2 M. The precipitate was removed by centrifugation at 9500 × *g* for 30 min and from the supernatant solution the peptidase was precipitated by raising the $(NH_4)_2SO_4$ concentration to 3 M. The resulting precipitate was dialyzed salt-free and the inert precipitate which formed was separated by centrifugation. The freeze-dried supernatant solution contained about 70% of the total activity on Phe-Pro-Ala-βNA. Further purification was accomplished by gel filtration at 5 C on a Sephadex G-75 column (1.6 × 90 cm) equilibrated with M/10 acetic acid.

Identification of tripeptides released from GH

Separation of the peptides released from either rGH or bGH by the peptidase was accomplished by one-dimensional TLC on precoated cellulose plates, 20 × 20 cm (E. Merck), using *n*-butanol-acetic acid-pyridine-water (30:6:24:24). Peptides were visualized under short wave ultraviolet light (254 nm) after spraying with fluorescamine according to the procedure of Felix and Jimeniz (15). Arginine-containing peptides were detected with phenanthrenequinone spray (16). Tripeptide products were tentatively identified by comparison of their R_f values with those of the authentic tripeptides Phe-Pro-Ala, Met-Ser-Leu, Ser-Gly-Leu, Phe-Ala-Asn, and Ala-Val-Leu synthesized by Bachem, Inc. In addition, the identity of the tripeptides released from GH was established by preparative TLC on cellulose followed by elution of the separated tripeptides and, after acid hydrolysis, identification of the constituent amino acids by analytical TLC on cellulose in three different solvent systems (*n*-butanol-formic acid-water, 70:15:15; *n*-butanol-acetone-acetic acid-water, 35:35:10:20; and *t*-butanol-butanone-28% NH_3-water-methanol-acetone, 40:20:5:14:1:20) according to the methods of Haworth and Heathcote (17) and Ersser and Smith (18). The reference tripeptides (Bachem, Inc.) yielded a single spot by TLC in the *n*-butanol-acetic acid-pyridine-water system and yielded at least 95% of the theoretical amino acid composition as determined by the amino acid analyzer.

Results

Hydrolysis of tripeptidyl-βNA by pituitary extracts

The conversion of Phe-bGH to Met-bGH by the peptidase in pituitary extracts was initially monitored by gel electrophoresis. At pH 8.0 Met-bGH migrates with a mobility greater than that of the Ala-bGH or Phe-bGH monomers (7) due to the lower pK_a of the methionine amino group. However, the method is not convenient for routine assay of the peptidase during purification or for kinetic studies. Phe-Pro-Ala-βNA was therefore selected as a model substitute of the NH_2-terminus of Phe-bGH. The sequence of this tripeptide derivative corresponds to the NH_2-terminus of Phe-bGH and contains the βNA analog of the —Ala-Met— bond which appears to be especially vulnerable to cleavage by anterior pituitary extracts. Successive extraction of pituitary brei at pH 5.5 with water, at pH 4.0 with 100 mM $(NH_4)_2SO_4$, and at pH 5.5 with 250 mM $(NH_4)_2SO_4$ solubilized about 30% of the total extractable β-naphthylamidase (βNAse) measured by the release of β-naphthylamine

from Phe-Pro-Ala-βNA at pH 4.0. If the residue from the successive extraction was further extracted with 1% Triton X-100 at pH 3, an additional 70% of the β-naphthylamidase was solubilized. Moreover, the specific activity of the dialyzed freeze-dried protein was 3–4 times greater than that of the detergent-free extracts (Table 1). The distinctness of the βNAse from cathepsin D is indicated by their different solubilities, cathepsin D being preferentially soluble in water, whereas solubilization of the major portion of the βNAse required treatment with Triton X-100.

Purification of Phe-Pro-Ala-βNAse by gel filtration

After concentrating the peptidase from the Triton extract by precipitation between 2 and 3 M $(NH_4)_2SO_4$ as described under *Materials and Methods,* the concentrate was fractionated on Sephadex G-75 equilibrated with M/10 acetic acid (Fig. 1). The naphthylamidase emerged at a K_{av} corresponding to a mol wt of 57,000. The yield and specific activity of the enzyme are shown in Table 2. Based on a total extractable activity of 28.5 U/kg anterior lobes the peptidase is obtained at an overall yield of 30% and a 100-fold purification. The purified enzyme cannot be freeze-dried after gel filtration since it is totally inactivated. After concentration on a PM-10 membrane to 1/10 or 1/20 vol, the enzyme could be stored in M/10 HOAc at 5 C for several months.

Although most of the cathepsin D content of the glands was solubilized into the water extract at pH 5.5 as well as the $(NH_4)_2SO_4$ extracts, some cathepsin D activity was extracted by the 1% Triton-0.25 M $(NH_4)_2SO_4$ solution at pH 3 and appeared in the 2–3 M $(NH_4)_2SO_4$ precipitate. On gel filtration most of the cathepsin D emerged simultaneously with the naphthylamidase peak and a smaller amount appeared in the exclusion volume (data not shown).

The maximal rate of Phe-Pro-Ala-βNA hydrolysis occurred between pH 3.7 and 4.0 as shown by the curve in Fig. 2, which relates hydrolysis rate to pH. The rates of hydrolysis at different concentrations of the tripeptide-βNA at pH 4.0 by the Sephadex-purified peptidase followed Michaelis-Menten kinetics. The double reciprocal plot of the data showed a K_m of 0.84 mM and a V_{max} of 84 nmol β-naphthylamine released per min per mg of enzyme protein (Fig. 3).

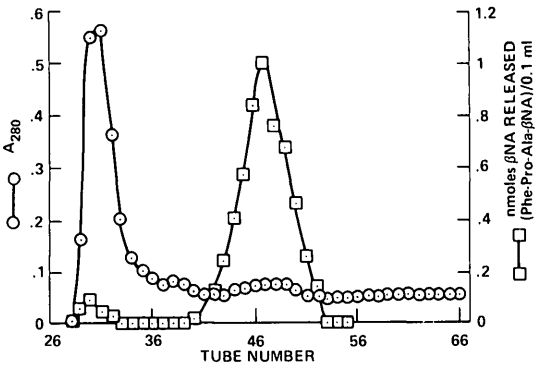

FIG. 1. Gel filtration of the 2–3 M $(NH_4)_2SO_4$ fraction on a Sephadex G-75 column (1.6 × 90 cm). The column was equilibrated at 5 C with 0.1 M acetic acid, pH 2.9. A 15-mg quantity of the 2–3 M $(NH_4)_2SO_4$ precipitate (after dialysis and freeze-drying) was applied in 0.7 ml 0.1 M acetic acid at pH 3.2. Flow rate was 8.4 ml/h and 2.0-ml fractions were collected.

TABLE 2. Purification of Phe-Pro-Ala-βNAse from a 1% Triton extract of bovine pituitary residue

Fraction	Protein yield (mg kg^{-1})	Naphthylamidase	
		mU mg^{-1}	mU kg^{-1}
1% Triton X-100, pH 3, 0.25 M $(NH_4)_2SO_4$ extract	22,800	0.89	20,300
2–3 M $(NH_4)_2SO_4$ PPT[a]	1,200	11.3	13,600
Sephadex G-75	125	62.0	8,000

[a] Precipitate.

TABLE 1. Distribution of Phe-Pro-Ala-βNAse and cathepsin D in successive extracts of bovine pituitary glands

Extract	Naphthylamidase[a]		Cathepsin D[b]	
	mU mg^{-1}	mU/kg	U mg^{-1}	U/kg
A: H_2O, pH 5.5	0.172	2,350	0.125	18,400
B: M/10 $(NH_4)_2SO_4$, pH 4.0	0.280	2,000	0.077	4,300
C: M/4 $(NH_4)_2SO_4$, pH 5.5	0.188	3,800	0.020	3,700
D: 1% Triton X-100, pH 3.0	0.890	20,300	0.003	5,600

[a] One milliunit is equal to the release of 1 nmol βNA/min at 37 C from Phe-Pro-Ala-βNA at pH 4.0.

[b] One unit is equal to an $A_{280}^{1\,cm}$ increase of 1.0 in a 1:4 dilution of the 2.5% trichloroacetic acid filtrate of hemoglobin digests incubated at pH 4.0 for 2 h at 37 C.

The naphthylamidase could be differentiated from cathepsin D by means of pepstatin. When concentrations of 1 μM or greater were present in the reaction mixture, the cathepsin D was inhibited 95% in the hemoglobin assay, where the naphthylamidase activity on Phe-Pro-Ala-Ala-βNA was essentially unaffected (Table 3). $HgCl_2$ and *p*-chloromercuriphenylsulfonate were potent inhibitors of the purified naphthylamidase. However, since the inhibition could not be reversed by an excess of mercaptoethanol, and *N*-ethylmaleimide, a sulfhydryl reagent, was not inhibitory, the peptidase cannot be considered to be a thiol-dependent enzyme.

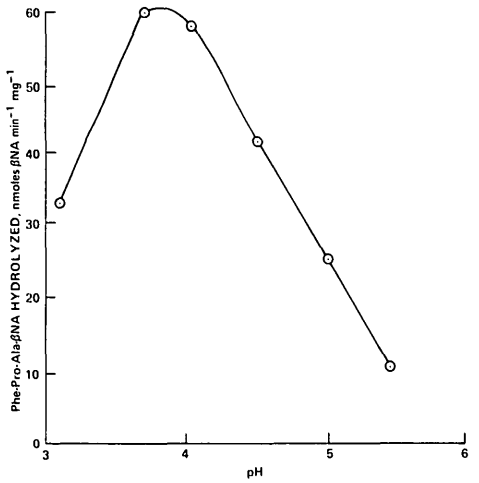

FIG. 2. Effect of pH on the hydrolysis of Phe-Pro-Ala-βNA by Sephadex G-75 purified tripeptidyl peptidase. Reaction mixtures contained 9.7 μg enzyme and 3.0 mM substrate in 50 mM acetate buffer of the indicated pH.

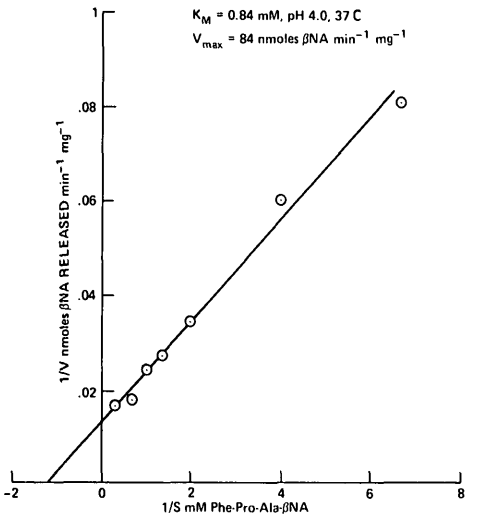

FIG. 3. Lineweaver-Burk plot of the hydrolysis of Phe-Pro-Ala-βNA by Sephadex purified tripeptidyl peptidase.

TABLE 3. Inhibition of Phe-Pro-Ala-βNA hydrolysis by Sephadex G-75-purified naphthylamidase

Reagent	Conc (mM)	% Inhibition
Pepstatin	0.001	0
Pepstatin	0.005	0
2-Mercaptoethanol	10	13
p-Chloromercuriphenylsulfonate	0.01	24
p-Chloromercuriphenylsulfonate	0.05	46
p-Chloromercuriphenylsulfonate	0.10	55
p-Chloromercuriphenylsulfonate	1.0	100
p-Chloromercuriphenylsulfonate + 2-mercaptoethanol	1.0 4.5	100
$HgCl_2$	0.01	100
EDTA	1	0
	5	0
N-Ethylmaleimide	1.0	0

Release of tripeptide from Phe-Pro-Ala-βNA by purified peptidase

In order to determine nature of the cleavage occurring during the release of β-naphthylamine, the tripeptide-βNA was incubated with the purified peptidase of the 2–3 M $(NH_4)_2SO_4$ fraction. Phe-Pro-Ala-βNA (1.5 mM) was incubated in 50 mM acetate buffer (pH 4.0) for 45 h at 37 C, at which time 39% of the substrate has been hydrolyzed as judged from the content of free β-naphthylamine. An aliquot of the digest corresponding to 30 nmol of tripeptide βNA was analyzed for amino acid content and showed the presence of 0.1 nmol proline and 0.2 nmol alanine plus an unknown ninhydrin positive peak. The unknown peptide was recovered and the equivalent of 10.4 nmol peptide were hydrolyzed with 6 M HCl. On amino acid analysis, the hydrolysate was found to contain 10.7, 10.4, and 10.6 nmol of Phe, Pro, and Ala, respectively. Thus, the peptidase cleaves the tripeptide-βNA to the free tripeptide and β-naphthylamine with the release of insignificant amounts of free amino acids and no detectable dipeptides. The presence of a benzyloxycarbonyl group at the NH_2-terminus of the tripeptide prevented cleavage of the βNA group which indicates that that enzyme is an aminopeptidase. The tripeptide derivative Ala-Phe-Pro-βNA is a model of the NH_2-terminus of the alanyl peptide chain of bGH. This derivative was hydrolyzed at only

0.5% of the rate observed with Phe-Pro-Ala-βNA and, as shown below, resembles the Ala-bGH chain in its resistance to hydrolysis by the peptidase. This finding indicates that prolyl bonds may be generally resistant to attack by the peptidase. The Sephadex-purified enzyme did not show detectable hydrolysis of dipeptide βNA of Gly-Arg, Lys-Ala, or Arg-Arg, which are relatively specific substrates for dipeptidyl aminopeptidases I, II, and III (19).

Electrophoretic detection of the conversion of Phe-bGH to Met-bGH

The progressive formation of Met-bGH accompanied by the disappearance of Phe-bGH during the course of digestion of Ala,Phe-bGH by the naphthylamidase is shown in Fig. 4. Although the pH optimum of hydrolysis of Phe-Pro-Ala-βNA was at 3.7–4.0, Phe,Ala-bGH was converted to Met,Ala-bGH maximally at somewhat higher pH. When GH was treated with the peptidase between pH 3.0 and 6.2 and the digests submitted to gel electrophoresis at pH 8 under nondissociating conditions, maximal formation of Met-bGH from Phe-bGH occurred at pH 4 and 5, with essentially no conversion taking place at pH 3 and 6.2 (Fig. 5). The Ala-bGH does not appear to be susceptible to attack by the peptidase since it remained unchanged in amount. The cleavage of Ala-bGH was blocked, presumably, by the presence of proline at the position of the third residue of the Ala-bGH peptide. This interpretation is supported by the already noted absence of significant βNA release from the Ala-Phe-Pro-βNA analog of the Ala-bGH peptide. In the Phe-bGH peptide, however, the proline residue is one removed from the susceptible bond, so that cleavage of Phe-Pro-Ala-βNA and of the Phe-bGH peptide was not impeded.

The restriction of peptidase attack to the Phe-bGH peptide was further confirmed by

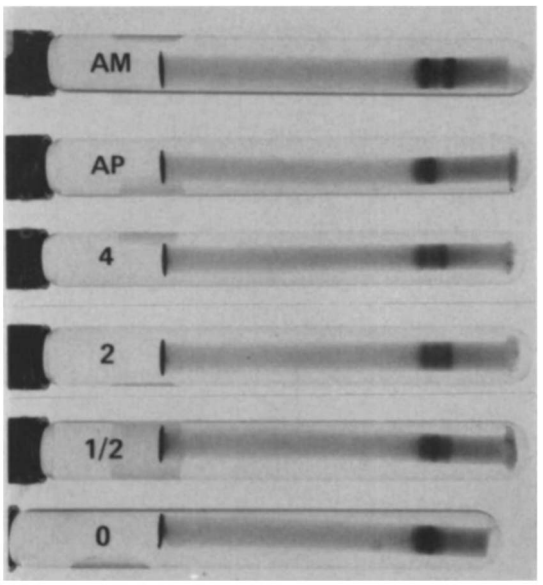

FIG. 4. Incubation of Ala,Phe-bGH with crude tripeptidyl peptidase showing the electrophoretic conversion of Phe-bGH to Met-bGH and lack of conversion of Ala-bGH. Ala,Phe-bGH (30 μg) was incubated with 60 μg extract B for the indicated time at pH 5.5 and 37 C in 0.015 M phosphate buffer. Electrophoresis was conducted for 42 h in a continuous buffer system (pH 8.0) as described in *Materials and Methods*. Relative mobilities: Met-bGH > Phe-bGH > Ala-bGH. AM: Ala,Met-bGH; AP: Ala,Phe-bGH.

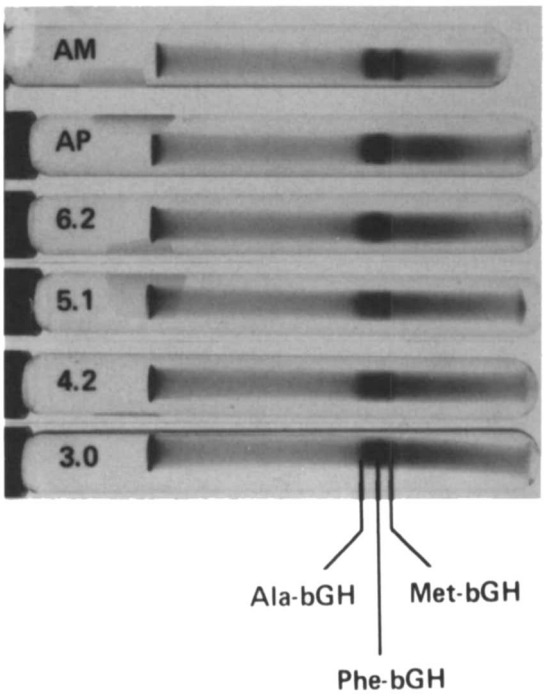

FIG. 5. Effect of pH on the electrophoretic conversion of Phe-bGH to Met-bGH by crude tripeptidyl peptidase. Ala,Phe-bGH (27 μg) was incubated with 60 μg extract B for 1.5 h at the indicated pH values in citrate-phosphate buffers. Electrophoresis conducted in a continuous buffer system (pH 8.0) as described in *Materials and Methods*.

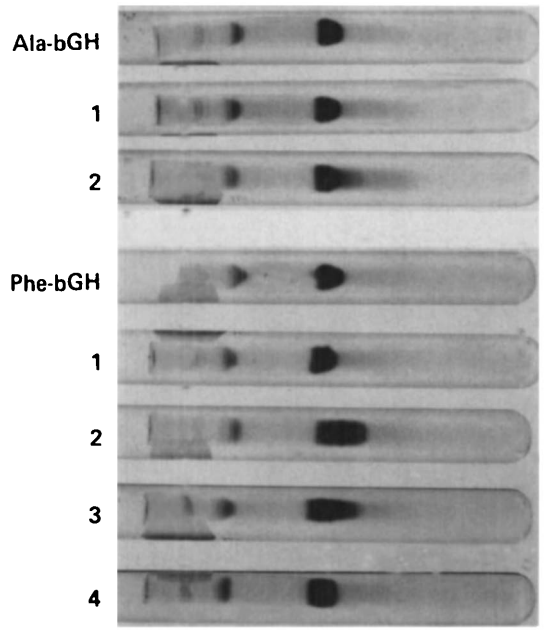

FIG. 6. Polyacrylamide disc gel electrophoresis in SDS of Ala-bGH and Phe-bGH treated with Sephadex G-75 purified tripeptidyl peptidase. The peptides were incubated with the enzyme at pH 4 and 37 C in a weight ratio of 13:1. Ala-bGH, control: with 1 μM pepstatin and 10 μM $HgCl_2$ (1) with 1 μM pepstatin (2); incubations for 6 h. Phe-bGH, control: incubated for 6 h with 1 μM pepstatin and 10 μM $HgCl_2$ (1); incubated with 1 μM pepstatin for 18 h (2); for 6 h (3); for 0.5 h (4).

incubating the individual Phe-bGH and Ala-bGH peptides with the Sephadex-purified peptidase. The bGH peptides were isolated by preparative isoelectric focusing in Ampholine gradients as described previously (5). Aliquots obtained during the course of digestion were analyzed by SDS-gel electrophoresis (Fig. 6). The Phe-bGH peptide showed a progressive formation of lower molecular weight components, the smallest of which showed a reduction in mol wt of approximately 4000 as judged from calibration curves of standard proteins. The Ala-bGH peptide, on the other hand, remained essentially unchanged in mol wt. $HgCl_2$, which was found to be a potent inhibitor of Phe-Pro-Ala-βNA hydrolysis, inhibited the degradation of the Phe-bGH peptide.

rGH, which in contrast to bGH, contains only a single type of peptide chain consisting of an NH_2-terminal Phe residue, was also degraded by the peptidase as indicated by SDS-gel electrophoresis (Fig. 7). The native rat hormone almost completely converted to a new component, the mol wt of which was reduced by 4500 in contrast to bGH, which retains the peptidase-resistant Ala-bGH peptide without modification.

Identification of hydrolysis products of bGH

The resistance of the Ala-bGH chain to cleavage by the peptidase, coupled with tripeptide release from Phe-Pro-Ala-βNA, suggested the possibility that the peptidase might release tripeptides sequentially from the NH_2-terminus of susceptible peptides. This possibility was tested by identifying the products of Ala,Phe-bGH hydrolysis by TLC. Authentic tripeptide standards corresponding to the NH_2-terminal tripeptides of the Phe-bGH peptide were used for the tentative identification of the hydrolysis products. After tentative identification by R_f, the peptide products, separated by TLC on cellulose in *n*-butanol-acetic acid-pyridine-water (30:6:24:24), were eluted and hydrolyzed with 6 M HCl for determination of amino acid composition. A time course digestion of Ala,Phe-bGH is shown in Fig. 8. Six tripeptides were unequivocally identified as originating by cleavages that occurred at the following sites (solid slashes) of the Phe-bGH peptide chain:

1 2 3 4
Phe-Pro-Ala/Met-Ser-Leu/Ser-Gly-Leu/Phe-Ala-Asn/

5 6 7 8
Ala-Val-Leu/Arg-Ala-Gln/His-Leu-His-Gln-Leu-Ala-

9 10 11
Ala-Asp-Thr-Phe-Lys-Glu-Phe-Glu-Arg-Thr-Tyr-Ile-

Pro-Glu-Gly-...

The release of the second tripeptide appears to be rate limiting, since the next four tripeptides were released in rapid succession, although this is not easily apparent from the photograph reproductions as well as from Fig. 8, due to differences in the fluorescence yield of the individual tripeptides. Sequential release of NH_2-terminal tripeptides was also obtained by the kinetic analysis of the hydrol-

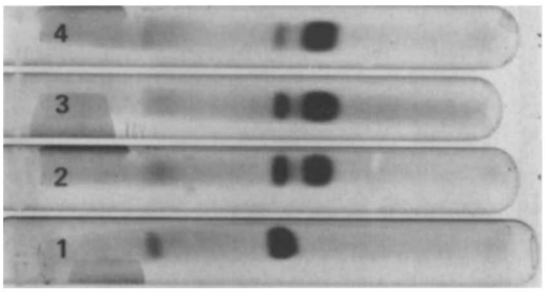

FIG. 7. Polyacrylamide disc gel electrophoresis in SDS of rGH digested by Sephadex G-75 purified tripeptidyl peptidase. The hormone (250 μg) was incubated with increasing amounts of Sephadex G-75-purified enzyme at pH 4.0 and 37 C for 21 h: control (1); with 58, 39, and 19 μg enzyme, respectively (2, 3, and 4).

ysis of other model peptides, the data on which will be presented in a separate publication. An additional five tripeptides have been tentatively identified, the composition of which corresponded to tripeptides 7 through 11. These were released by cleavages at the bonds indicated by the broken slashes. When the Ala-bGH peptide was exposed to the enzyme under the same conditions only traces of Ser-Gly-Leu and Phe-Ala-Asn could be detected but not the more distal peptides. The formation of these tripeptides may be due to the contamination of Ala-bGH with traces of bGH having NH_2-terminal serine (3). The loss of 11 tripeptides from TAP-digested bGH is consistent with the reduction of 4000 in mol wt which was observed in SDS-gel electrophoresis.

Biological activity and hydrolysis products of rGH

In order to evaluate the effect of digestion with TAP on the biological activity of GH, rGH was chosen as the substrate rather than bGH. In the case of the bovine hormone only one-half of the peptide chains would be degraded, thereby making it more difficult to evaluate the extent of inactivation of biological and immunological potencies. Digestion of native rGH with TAP was performed in the M/100 acetate buffer at pH 4.25 for 0, 1, 3, and 6 h at 25 C. The concentration of rGH and TAP were 0.5 mg and 0.02 mg/ml, respectively. The digestion mixture contained 3 μM pepstatin to inhibit traces of contaminating cathepsin D activity. The 0.5-ml aliquots were withdrawn for analysis and adjusted with 20 μl 0.5 M Na_2CO_3 to between pH 9 and 10. As shown in Fig. 9, digestion of rGH with TAP reduced its growth potency to 32% of the nonincubated control after only 1 h, after which there was no further decrease. Compared to the corresponding controls the decrease in immunological potency was negligible.

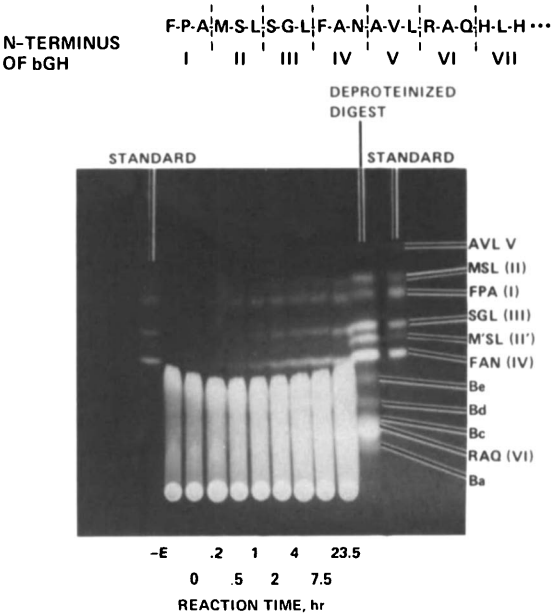

FIG. 8. Time-course analysis by TLC of the products of Phe,Ala-bGH hydrolysis by TAP, illustrating the release of NH_2-terminal tripeptides. The sequential release is more conspicuous after correction for the differences in the relative intensities of each tripeptide. The digest consisted of 8 mg bGH/ml, 5.7 μM pepstatin, and 37 mU TAP/ml (Ala-Ala-Phe-βNA substrate) in 0.1 M acetic acid-pyridine buffer at pH 4 and 25 C. For TLC, aliquots of the digests, equivalent to 2 nmol bGH, were removed and spotted without deproteinization at the indicated time intervals. The digest remaining at 23.5 h was deproteinized on Sephadex G-200 and the tripeptide fraction spotted (≅13 nmol) for identification of tripeptides which migrated within the heavily stained bands of GH. Standards consisted of equimolar quantities (2.5 nmol) of five synthetic tripeptides. TLC solvent: *n*-butanol-acetic acid-pyridine-water (30:6:24:24). Sprayed with fluorescamine and visualized with short UV light. B_a to B_e, unidentified peptides: −E, control mixture without enzyme. MS'L (II') refers to MetSO-Ser-Leu. Single letter abbreviations for amino acid residues are those employed by M. O. Dayhoff in Atlas of Protein Sequence and Structure, 1969, National Medical Research Foundation, Silver Spring, MD.

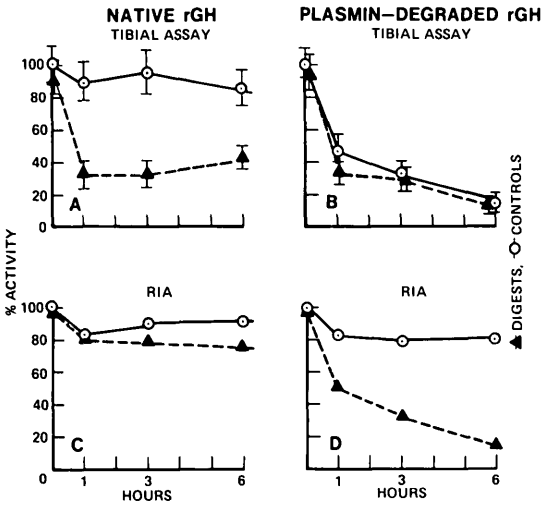

FIG. 9. Tibial and radioimmunological activities of native and plasmin-degraded rGH during digestion with tripeptidyl peptidase at pH 4 and 37 C, illustrating the loss of tibial activity (A), but retention of immunological activity (C) of native rat GH, and loss of both activities in the case of plasmin-degraded rGH (B and D). Conditions: 0.5 mg rGH and 20 μg peptidase per ml of 0.1 M acetate buffer, pH 4.25, containing 3 μM pepstatin; 0.5-ml aliquots removed at indicated intervals and brought to pH 9–10 with 20 μl 0.5 M Na_2CO_3 for bio- and immunoassay.

Since the sequences of rGH and bGH are very similar according to Wallis and Davies (20), it would be anticipated that rGH would sustain a release of NH_2-terminal tripeptides similar to that observed with bGH. A time-course analysis by TLC of the aliquots of the rGH digest with and without deproteinization is illustrated in Fig. 10, which shows a tripeptide pattern very similar to that obtained with bGH. Six major tripeptides corresponding to the 18 residues of the NH_2-terminus were unequivocally identified by their amino acid composition and R_f. In agreement with the report of Wallis and Davies, the second tripeptide from the NH_2-terminus, Met_4-Ser_5-Leu_6, and Phe_{28}-$Lys_{29}Glu_{30}$ were replaced by Met_4-Pro_5-Leu_6 and Tyr_{28}-Lys_{29}-Glu_{30}, respectively. The Met-Pro-Leu and Tyr-Lys-Glu tripeptides migrated with the same R_f as Arg-Val-Leu and Phe-Arg-Asn tripeptides, respectively (Fig. 10). The minor tripeptides have not been identified definitively but appear to be comprised of the tripeptides from His_{19}-Leu_{20}-His_{21} through Phe_{31}-Glu_{32}-Arg_{33}. Cleavage beyond the latter tripeptide would be impeded by the presence of a proline residue at the next susceptible bond, Ile_{36}-Pro_{37}. In SDS-gel electrophoresis the fastest moving portion of the digested rGH band showed a reduction in mol wt of 4500 which is in reasonable agreement with the loss of 33 residues and termination at Ile_{36}-Pro_{37}, that is, a reduction of 4000.

Plasmin-degraded rGH behaved quite dif-

F-P-A¦M-P-L¦S-G-L¦F-A-N¦A-V-L¦R-A-Q¦H-L-H...
N-TERMINAL SEQUENCE (WALLIS & DAVIES, 1976)

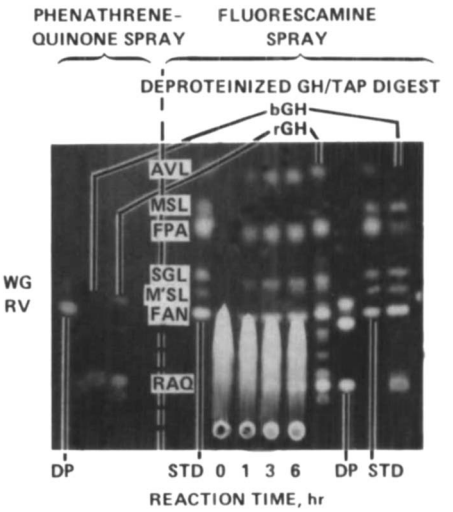

FIG. 10. Time-course analysis by TLC of the hydrolysis of rGH by TAP. Digestion mixture consisted of 10 mg rGH, 2.9 μmol pepstatin, and 100 mU enzyme (Ala-Ala-Phe-βNA substrate) per ml 100 mM acetic acid-NH_4OH buffer, pH 4, 25 C. Aliquots of the digest were sampled at 0, 1, 3, and 6 h and spotted without deproteinization. Six tripeptides, corresponding to Phe-Pro-Ala (FPA), Met-Pro-Leu (MPL) (superimposed on Ala-Val-Leu (AVL)), Ser-Gly-Leu (SGL), Phe-Ala-Asn (FAN), Ala-Val-Leu (AVL), and Arg-Ala-Gln (RAG), could be identified. The last tripeptide migrated in the region of the heavily stained protein trailing from the origin and could be revealed after deproteinization of the 6-h rGH digest on Sephadex G-100 in 30 mM $NH_4C_2H_3O_2$ buffer, pH 4.0. The resulting protein-free tripeptides were resolved as shown under the channels marked rGH which were sprayed with either fluorescamine or phenanthroquinone; a similarly treated bGH digest is shown for comparison. Note that the absence of a spot for Met-Ser-Leu in rGH is due to replacement by Met-Pro-Leu which co-migrates with Ala-Val-Leu (AVL). Std: equimolar quantities (2.5 nmol) of synthetic tripeptides corresponding to those at the NH_2-terminus of bGH; M'SL, MetSO-Ser-Leu; DP, dipeptide standards. Single letter abbreviations for amino acid residues are those employed by M. O. Dayhoff, Atlas of Protein Sequence and Structure, 1969, National Medical Research Foundation, Silver Spring, MD.

ferently from the native hormone both on digestion with TAP and control incubation at pH 4. Digestion of the hormone with TAP produced a parallel loss in biological and immunological activities to a level which was about 15% of the nonincubated control (Fig. 9). However, the plasmin-degraded hormone incubated without the enzyme also sustained a comparable loss of biological activity, which indicates that this hormone, in contrast to the native hormone, is spontaneously inactivated by incubation at pH 4.0. Despite the loss of tibial activity, the immunological activity of the plasmin-degraded hormone was essentially unchanged after incubation at pH 4 in the absence of enzyme.

Discussion

Since the original demonstration that NH_2-terminal heterogeneity in bGH is related to the mildly acidic pH used in extracting the hormone from pituitary homogenates, the identity of the responsible peptidase has remained obscure. The utilization of model tripeptidyl-β-naphthylamide substrates containing NH_2-terminal sequences of the Phe- and Ala-bGH chains facilitated identification of the peptidase by allowing for simple and rapid enzyme assay. The use of these substrates established that only the Phe-bGH was the precursor of the bGHs having NH_2-terminal Met and, to a lesser extent, Ser and Glu residues. In agreement with the resistance of Ala-Phe-Pro-βNA to cleavage by the peptidase, the Ala-bGH was not cleaved, as indicated by the absence of changes in the mobility of the Ala-bGH on gel electrophoresis at pH 8.0.

In the light of the present findings, the appearance of new NH_2-terminal residues, predominantly Met and, to a lesser extent Ser and Glu, in purified bGH, can be explained on the basis of NH_2-terminal release of tripeptidases by tripeptidyl aminopeptidase during the course of isolation. As already noted, the release of the second tripeptide Met-Ser-Leu appears to be rate-limiting for the release of succeeding tripeptides and thereby accounts for the predominance of Met-bGH over other new NH_2-termini.

TLC of Phe,Ala-bGH digested at pH 4 with the purified peptidase revealed that 11 tripeptides were cleaved from the NH_2-terminal region of Phe-bGH. The release of the tripeptides appears to be sequential provided that pepstatin, which inhibits traces of contaminating cathepsin D, is present during the digestion. In the absence of the inhibitor degradation is much more extensive. The rate of digestion appears to decrease sharply after the six tripeptides have been released, possibly because of inhibition by the tripeptide products which can be shown to act as inhibitors (unpublished data) and also to the presence of peptide bonds which are more resistant to hydrolysis, such as seems to be true for the His_{21}-Gln_{22} in Phe-bGH. Because of the sequential release of NH_2-terminal tripeptides, the enzyme has been tentatively designated as TAP.

For the purpose of assessing the effect of hydrolysis on the biological activity, rGH was selected as a substrate because it contains only one type of NH_2-terminus, namely Phe (6). The loss of 60–70% of the tibial growth-promoting activity associated with the release of 6–11 tripeptides from the NH_2-terminal region indicates that some of these residues located therein are required for full biological activity. However, the precise relationship between the loss of growth activity and the release of specific tripeptides remains to be determined. On the other hand, the retention of essentially full immunoreactivity suggests that the immunological determinants are not located in the 11 tripeptides released from the NH_2-terminus. This inference is supported by the findings with plasmin-degraded rGH from which the hexapeptide Ile-Gly-Gln-Ile-Leu-Lys has been cleaved from the large disulfide loop analogously to the plasmin cleavage obtained by Reagan *et al.* (21) with pig GH. The removal of the hexapeptide exposes an additional NH_2-terminus in the region of residue 140 and thereby offers an additional site for attack by TAP. The loss of immunoreactivity from plasmin degraded rGH may be attributed, although not as yet proven, to cleavages by TAP at this terminus in addition to those which occur at the Phe-terminus of native rGH.

The plasmin-degraded rGH lost tibial activ-

ity when incubated at pH 4.0 without TAP, either because of inherent instability or contamination with other as yet unidentified peptidases. It has not been possible, therefore, to establish the effect of TAP hydrolysis on the tibial activity of the plasmin-degraded hormone.

Acknowledgments

The authors wish to thank Dr. R. E. Grindeland for performing tibial and radioimmunoassays and Dr. P. X. Callahan for amino acid analyses. We are indebted to Professor C. H. Li for generously providing Ala,Phe-bGH and to Professor H. Umezawa for pepstatin.

References

1. Li, C. H., and L. Ash, The nitrogen terminal end groups of hypophyseal growth hormone. *J Biol Chem* **203:** 419, 1953.
2. Li, C. H., A simplified procedure for the isolation of hypophyseal growth hormone. *J. Biol Chem* **211:** 555, 1954.
3. Raben, M. S., and V. W. Westermeyer, Recovery of growth hormone in purification of corticotropin. *Proc Soc Exp Biol Med* **78:** 550, 1951.
4. Ellis, S., Studies on the serial extraction of pituitary proteins, *Endocrinology* **69:** 554, 1961.
5. Ellis, S., M. Lorenson, R. E. Grindeland, and P. X. Callahan, Separation of the phenylalanyl and alanyl chains of bovine and ovine growth hormones by electrofocusing, *In* Pecile, A., and E. E. Müller (eds.), Growth and Growth Hormone, Excerpta Medica, Amsterdam, 1972, p. 55.
6. Ellis, S., R. E. Grindeland, J. M. Nuenke, and P. X. Callahan, Isolation and properties of rat and rabbit growth hormones, *Ann NY Acad Sci* **148:** 328, 1968.
7. Lorenson, M. G., and S. Ellis, Amino terminal residue heterogeneity of bovine and ovine growth hormones as revealed by polyacrylamide gel electrophoresis, *Endocrinology* **96:** 833, 1975.
8. Wallis, M., The pK_a values of the α-amino groups of peptides derived from the N-terminus of bovine growth hormone. *Biochim Biophys Acta* **310:** 388, 1973.
9. Reisner, A. H., P. Nemes, and C. Bucholtz, The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Anal Biochem* **64:** 509, 1975.
10. Maizel, J. V., Jr., Polyacrylamide gel electrophoresis of viral proteins, *In* Maramorosch, K., and H. Koprowski (eds.), Methods in Virology, vol. 5, Academic Press, New York, 1971, p. 179.
11. Greenspan, F. S., C. H. Li, M. E. Simpson, and H. M. Evans, Bioassay of hypophyseal growth hormone: the tibia test. *Endocrinology* **45:** 455, 1949.
12. Schalch, D. S. and S. Reichlin, Plasma growth hormone concentration in the rat determined by radioimmunoassay: influence of sex, pregnancy, lactation, anaesthesia, hypophysectomy and extrasellar pituitary transplants. *Endocrinology* **79:** 275, 1966.
13. McDonald, J. K., B. B. Zeitman, T. J. Reilly, and S. Ellis, New observations on the substrate specificity of cathepsin C (dipeptidyl aminopeptidase I) including the degradation of β-corticotropin and other peptide hormones. *J Biol Chem* **244:** 2693, 1969.
14. Adams, E., and E. L. Smith, Proteolytic activity of pituitary extracts. *J Biol Chem* **191:** 651, 1951.
15. Felix, A. M., and M. H. Jimeniz, Usage of fluorescamine as a spray reagent for thin-layer chromatography. *J Chromatogr* **89:** 361, 1974.
16. Yamada, S., and H. A. Stano, Phenanthrenequinone as an analytical reagent for arginine and other monosubstituted guanidines. *Biochem Biophys Acta* **130:** 538, 1966.
17. Haworth, C., and J. G. Heathcote, An improved technique for the analysis of amino acids and related compounds on thin layers of cellulose. *J Chromatogr* **41:** 380, 1969.
18. Ersser, R. S. and I. Smith, Amino acids and related compounds, *In* Smith, I., and J. Seakins (eds.), Chromatographic and Electrophoretic Techniques, vol. 1, Yearbook Medical Publishers, Inc., Chicago, 1976, p. 75.
19. McDonald, J. K., P. X. Callahan, S. Ellis, and R. E. Smith, Polypeptide degradation by dipeptidyl aminopeptidase I (cathepsin C) and related peptidases, *In* Barrett, A. J., and J. T. Dingle (eds.), Tissue Proteinases: Enzymology and Biology, North-Holland Publishing Co., Amsterdam, 1971, p. 69.
20. Wallis, M., and R. V. Davies, Studies on the chemistry of bovine and rat growth hormones, *In* Pecile, A., and E. E. Müller (eds.), Growth Hormone and Related Peptides, Excerpta Medica, Amsterdam, 1976, p. 1.
21. Reagan, C. R., J. B. Mills, J. L. Kostyo, and A. E. Wilhelmi, Isolation and biological characterization of fragments of human growth hormone produced by digestion with plasmin. *Endocrinology* **96:** 625, 1975.