Purification and Characteristics of Porcine Growth Hormone*

Hao-Chia Chen,† Alfred E. Wilhelm,§ and Stewart C. Howard
From the Department of Biochemistry, Division of Basic Health Sciences, Emory University, Atlanta, Georgia 30322

SUMMARY

A simple procedure is described for the isolation of porcine growth hormone from the filtrate remaining after the adsorption of adrenocorticotropic hormone (ACTH) onto oxidized cellulose during the commercial manufacture of ACTH. Growth hormone is purified by ion exchange chromatography on diethylaminoethylcellulose. The purified hormone has a specific biological activity nearly twice that of the international standard of growth hormone (bovine, for bioassay), and is essentially free of other activities of the anterior pituitary. Its isoelectric point is at pH 6.3. The molecule appears to be a single polypeptide chain with phenylalanine at both the NH₂-terminal and COOH-terminal residues. The amino acid composition resembles that of bovine, canine, and human growth hormone.

In 1951, Raben and Westermeyer (1) first described a method for the isolation of porcine growth hormone from the Oxycel filtrate remaining after the adsorption of adrenocorticotropic hormone onto oxidized cellulose in the manufacture of ACTH. The product was heterogeneous, and although its partial purification was described in 1956 (2), a complete characterization of the latter product has not been reported. The preparation of porcine growth hormone from fresh-frozen glands was described by our laboratory in 1955 (3); the product, of relatively low activity, was not studied in detail (3). Ottaway (4) has also briefly reported a method for the preparation of porcine growth hormone from fresh-frozen glands, but no details of the biological activity or of the purity of the product were given. Papkoff, Li, and Liu (5) have described the isolation and purification of growth hormone from fresh-frozen porcine pituitary glands by a procedure similar to that which they have used for the preparation of bovine growth hormone (6). Their product, obtained in a yield of 0.25 g per kg of glands, wet weight, was the preparation of bovine growth hormone (6). Their product, obtained in a yield of 0.25 g per kg of glands, wet weight, was further purified by ion exchange chromatography on diethylaminoethylcellulose. Although there are small differences in detail, this highly purified porcine growth hormone resembles in most important respects the preparation from fresh porcine pituitaries described by Papkoff et al. (6).

EXPERIMENTAL PROCEDURE

Supplies of Oxycel filtrate, freshly prepared from porcine pituitary glands, were shipped refrigerated from the Wilson Laboratories, Chicago, Illinois. DEAE-cellulose was obtained either from Carl Schleicher and Schuell or from W. and R. Balston, Ltd., London, England, treated before use according to the procedure of Peterson and Sober (7), and freed of fine particles by several decantations in the course of thorough equilibration with buffer before columns were prepared. Sephadex G-100, bead type, obtained from Pharmacia, was allowed to swell in 1 M NaCl containing 0.1% EDTA for at least 24 hours, and was then washed with buffer until no chloride ion was detectable. The fine particles were removed by decantation during these washings.

Polyacrylamide gel disc electrophoresis was carried out as described by Ornstein (8). The dimensions of the cylinders of gel were 4.5 \times 45 \text{ mm} and the amount of sample applied was 0.1 mg unless otherwise indicated. Moving boundary electrophoresis was carried out in the Perkin-Elmer model 38 electrophoresis apparatus. The protein concentration was 1% and the buffers used were the same as those used by Papkoff et al. (5).

Amino-terminal analysis was carried out by the fluorodinitrobenzene method as described by Fraenkel-Conrat, Harris, and Levy (9). Carbonyl-terminal analysis was carried out by digestion of the hormone as follows with carboxypeptidase A (Sigma, lot C33B-80), treated with diisopropyl fluorophosphate to inhibit residual endopeptidase activity; 60 mg of growth hormone were dissolved in 2.6 ml of water containing 0.1 ml of 2 M NH₄HCO₃ and 0.1 ml of 0.1 N NaOH; 0.2 ml of 1% carboxypeptidase solution was added; the mixture was shaken gently at room temperature. At 10 min and again at 240 min, a 0.2-ml
sample was taken for amino acid analysis by the fluorodinitrobenzene method (9) and a 0.5-ml sample for amino acid analysis by the method of Spackman, Stein, and Moore (10) on the Beckman amino acid analyzer, model 120B. Under the same conditions, the absence of endopeptidase activity was affirmed by digestion of highly purified ovine prolactin (11) and bovine pancreatic ribonuclease purified by column chromatography on carboxymethylcellulose. After 4 hours, no amino acids were detected in the digest of ovine prolactin; with ribonuclease, 25% of the theoretical amount of valine and traces of serine were found. As described by Santoné, Wolfenstein, and Paladini (12) in a later study, the digestions of porcine growth hormone with carboxypeptidase A were repeated, with sampling at 10 min and 24 hours, in the presence and absence of 0.1% sodium lauryl sulfate.

Amino acid analyses were conducted in duplicate on 3-mg samples of the hormone hydrolyzed in 6 N HCl at 110° in sealed, evacuated tubes for 24, 50, and 77 hours. The hydrolysates were analyzed as above. The nitrogen content of carefully dried samples (4 mg) of the hormone was determined in triplicate by the micro-Kjeldahl method and tryptophan by the reaction with N-homosarcosineimide (13, 14).

All bioassays were carried out in 2 x 2 design against appropriate standards. Estimates of the potency of fractions, expressed in terms of the standard, were calculated by statistical methods for parallel line assays (15). Growth activity was determined by the gain in weight of 100 g female hypophysectomized rats, after 10 daily injections, as compared to the U.S.P. growth hormone standard (16). Prolactin activity was measured by the systemic pigeon crop-sac stimulation method as compared to the second international standard (17). Thyroid-stimulating hormone activity was determined by the 32P-labeled phosphate uptake in the chick thyroid gland, compared to the U.S.P. standard (18). Luteinizing hormone activity was measured by the ovarian ascorbic acid depletion method of Parlow (19), compared to NIH-LH-S1 and follicle-stimulating hormone activity by the ovarian weight gain method of Steelman and Pohley (20), compared to NIH-TSH-S1. ACTH activity was determined by the adrenal ascorbic acid depletion method of Sayers as modified by Munson, Berry, and Koch (21), compared to the U.S.P. standard. The estimates of prolactin, thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, and ACTH, which were tests for contamination, were therefore conducted with doses of the unknowns sufficient to detect activity of at least 1% of the respective standards.

Analytical grade chemicals were used throughout. Water for solvents and for dialysis was obtained from a glass still. The visking membranes used in dialysis were treated before use with 0.1% EDTA in distilled water.

Preparation of Porcine Growth Hormone—The entire procedure was carried out at 0-4°. The fresh filtrate was sprayed briskly with Antifoam A (Dow-Corning Corporation, Midland, Michigan). A 50-ml sample was withdrawn, neutralized, dialyzed, and lyophilized in order to determine the total nondialyzable solids and the growth hormone activity of the filtrate (Fraction X). The remainder of the filtrate was brought to 0.3 M with respect to potassium by the addition of 10 n KOH, then adjusted quickly and carefully to pH 8.5 by the addition of glacial acetic acid.

The heavy precipitate was removed by centrifuging for 1 hour in an International refrigerated centrifuge, model PR-2, at 2300 rpm. These conditions were used for all centrifugations. The precipitate was discarded, and then the clear pale yellow supernatant solution was adjusted to pH 4.0 with 4 N HCl. Solid ammonium sulfate was added slowly, with vigorous stirring, to a final concentration of 1.25 M. After 1 hour, the precipitate was collected by centrifuging, and the supernatant solution was discarded. The precipitate was taken up in half the original volume of water, and 10 N KOH was added to pH 10.0 to 10.5; a clear solution was obtained. The solution was adjusted to pH 8.5 with 4 N HCl, and solid ammonium sulfate was added slowly, with vigorous stirring, to a final concentration of 1.2 M. (A correction for the amount of ammonium sulfate in the wet pH 4.0 precipitate was calculated from the total volume of the solution at pH 8.5 and from the volumes of water, KOH, and HCl used, assuming that the volume of the precipitate, obtained by difference, was equivalent to a 1.25 M solution of ammonium sulfate.) The pH of the solution was adjusted to 7.0 with 4 N HCl, and after ½ hour, the precipitate was collected by centrifuging. The clear supernatant solution was discarded. The precipitate was dissolved in water, dialyzed, and lyophilized (Fraction A).

Fraction A was further purified by dissolving in 1% concentration in water at pH 10. The solution was adjusted quickly to pH 4.0 with 4 N HCl, and the clear solution was immediately adjusted to exactly pH 4.9 and centrifuged. The supernatant solution was decanted and adjusted to pH 7.0. Solid ammonium sulfate was added to 0.25 M. The colored, gummy precipitate was removed by centrifugation and discarded. To the clear supernatant solution, solid ammonium sulfate was added to a final concentration of 1.2 M, and after ½ hour, the precipitate was collected by centrifugation. The supernatant solution was discarded, and the precipitate was dissolved in water, dialyzed, and lyophilized (Fraction A1).

The precipitate at pH 4.9 was dissolved in half the original volume of water at pH 10. The procedure used in handling Fraction A was repeated, except that no precipitate was obtained at pH 7.0, 0.25 M ammonium sulfate. A precipitate (Fraction A2) was collected at pH 7.0, 1.2 M ammonium sulfate, and at pH 7.0, 1.6 M ammonium sulfate (Fraction A3). These fractions were taken up in water, dialyzed, and lyophilized. The second pH 4.9 precipitate and the last supernatant solution were discarded.

Further purification of the hormone was achieved by DEAE-cellulose chromatography in two systems of the enriched Frac- tions A1, A2, and A3 individually or combined. The fraction was dissolved in either 0.02 M sodium borate, pH 8.0 (System 1), or 0.01 M Tris-formate, pH 8.0 (System 2), and pumped onto a column equilibrated with the same solvent. For System 1, elution was accomplished with a linear gradient of NaCl rising from 0.033 to 0.3 M. For System 2, the Tris-formate concentration was increased stepwise. All runs were conducted at room temperature. The contents of tubes containing the main fraction were pooled, dialyzed free of salt, and lyophilized.

RESULTS AND DISCUSSION

The yield and specific activity of each fraction are summarized in Table I; the data are representative of repeated runs, which have yielded fairly uniform results. Approximately two-thirds of the initial activity of the filtrate is recovered in Fraction A, which contains only 14% of the nondialyzable solids of the filtrate. Most of the lost activity is simply entrained in the
Table I

Yield and specific activity of fractions from Oxycel filtrates

| Fraction | Yield (g/liter) | Growth hormone activity<sup>a</sup> (unit/mg) |
|----------|----------------|---------------------------------------------|
| X (dialyzed filtrate) | 19.82 | 0.087 | 1700 |
| A (first pH 7.0, 1.2 M ammonium sulfate precipitate) | 2.82 | 0.40 | 1130 |
| A<sub>1</sub> (second pH 7.0, 1.2 M ammonium sulfate precipitate) | 0.867 | 0.85 | 790 |
| A<sub>2</sub> (third pH 7.0, 1.2 M ammonium sulfate precipitate) | 0.14 | 0.64 | 90 |
| A<sub>3</sub> (pH 7.0, 1.6 M ammonium sulfate precipitate) | 0.10 | 0.97 | 97 |

<sup>a</sup> As compared to U.S.P. growth hormone standard: 1 unit = 1 mg.

Table II

Biological activities of Fraction A<sub>1</sub>

| Activity | Relative potency (units/mg) | 95% confidence limits |
|----------|-----------------------------|-----------------------|
| Growth hormone | 0.73 (0.53-1.17)<sup>a</sup> | (maximum) |
| Prolactin | 0.29 | (maximum) |
| Luteinizing hormone | 0.002 | (maximum) |
| Follicle-stimulating hormone | 0.009 | (maximum) |
| Thyroid-stimulating hormone | 0.0005 | (maximum) |
| ACTH | 0.048 (0.0076-0.30) | |

<sup>a</sup> Figures in parentheses are 95% confidence limits.

Figure 1. Elution pattern obtained by chromatography of Fraction A<sub>1</sub> on DEAE-cellulose. Column, 1.9 X 53 cm, was equilibrated with 0.02 M borate-NaOH + 0.033 M NaCl, pH 8.0. Elution with compound gradient of NaCl, 500 ml of buffer in each of four chambers: one and two contain 0.02 M borate-NaOH + 0.033 M NaCl; three and four contain 0.02 M borate-NaOH + 0.3 M NaCl, pH 8.0. Effluent, 5.3 ml per tube; flow rate, 30 ml per hour. Sample: 1.2 g of A<sub>1</sub>. Fractions I (407 mg) and II (138 mg) derived as indicated by pooling tubes, dialyzing, and lyophilizing.

Figure 2. Polyacrylamide gel electrophoresis of purified porcine growth hormone. (1 and 2) patterns of Fractions I and II (Fig. 1); 3 and 4, patterns derived by rechromatographing Fraction I on DEAE-cellulose in System 1 (Fig. 3) and Sephadex G-100 (Fig. 4), respectively. 6, pattern from the product of chromatography of Fraction I on DEAE-cellulose in System 2; 50 µg of sample were applied to each column.

The activity of Fraction A<sub>1</sub> is shown in Figure 1. The eluted material was divided, as indicated, into Fractions I and II. The disc gel electrophoretic patterns of these fractions are illustrated in Fig. 2. The major slow band characteristic of growth hormone is greatly intensified in Fraction I; this is a minor component of Fraction II, in which the minor fast band of Fraction I is intensified. The yield of Fraction I from 1.2 g of Fraction A<sub>1</sub> was 407 mg; its growth activity was 1.6 i.u. per mg; 60% of the applied activity was recovered in 34% of the weight of the starting material. Furthermore, the ACTH activity of this fraction was now only 0.00025 U.S.P. unit per mg. The major and minor components of Fraction I are not resolved either by rechromatography on DEAE-cellulose or by gel filtration on Sephadex G-100. However, resolution can be achieved by chromatography on DEAE-cellulose in 0.01 M Tris-formate buffer, pH 8.0 (System 2); the active fraction is eluted by a stepwise increase in the buffer concentration to 0.0375 M. The disc electrophoretic...
amino acid detectable after prolonged enzymatic incubation is corrected by extrapolation to zero time. The corrected value

NaOH, pH 8.0 (System 1).

pattern of one such fraction is illustrated in Fig. 2. The symmetry of the curve of elution of the hormone from Sephadex

G-100 suggests that the preparation is homogeneous in molecular size. The minor fast component is biologically active (Fraction II of Figs. 1 and 2 is as active as Fraction I). It is possible that the fast component of these fractions is a product of deamination of the native hormone, similar to those observed by Lewis (22) in preparations of bovine and human growth hormone.

Characterization of Purified Porcine Growth Hormone—The electrophoretic mobility of the hormone (Fraction I) was measured at several values of pH and constant ionic strength (0.1); the isoelectric point is pH 6.3, identical with the value reported by Papkoff et al. (5).

Amino-terminal analysis of some preparations of the purified hormone consistently yielded a single spot identifiable as phenylalanine. If the sample contained more than 9 mg of protein, two additional spots, corresponding to threonine and serine, could be seen occasionally with intensities less than 3% and 0.4% of that of the phenylalanine spot, respectively. Some preparations, however, also yielded amino-terminal alanine, in amounts up to 25%. Papkoff et al. (5) also found phenylalanine as the principal amino-terminal acid, with traces of alanine and valine.

Carboxypeptidase A mainly liberates phenylalanine from porcine growth hormone (Table III). If the samples have been previously treated with trichloracetic acid, the only other amino acid detectable after prolonged enzymatic incubation is alanine. In the presence of 0.1% sodium lauryl sulfate, alanine is liberated in nearly equal amounts with phenylalanine in 24 hours (Table III). If the molecular weight of the hormone is assumed to be 22,000, approximately 0.8 mole of each amino acid is liberated per mole of hormone. The carboxyl-terminal octapeptide released by trypsin digestion of the hormone consistently yielded a single spot identifiable as phenylalanine. If the sample contained more than 9 mg of protein, the amino-terminal sequence, -Ala-Phe-COOH, indicated by these data is corroborated by the observation of Mills (23) on the sequence of the carboxy-terminal octapeptide released by trypptic digestion of porcine growth hormone.

The amino acid composition of porcine growth hormone is presented in Table IV. The amounts of serine and half-cystine decreased with time of hydrolysis, and the values have been corrected by extrapolation to zero time. The corrected value

| Amino acid | 24 hrs | 30 hrs | 77 hrs | Value taken | Nearest integer |
|------------|--------|--------|--------|-------------|----------------|
| Aspartic acid | 15.2 | 14.9 | 15.4 | 15.2 | 15 |
| Glutamic acid | 24.4 | 24.2 | 24.7 | 24.4 | 24 |
| Lysine | 10.8 | 10.8 | 10.9 | 10.7 | 11 |
| Histidine | 2.8 | 2.8 | 2.9 | 2.8 | 3 |
| Arginine | 11.7 | 11.4 | 12.0 | 11.7 | 12 |
| Phenylalanine | 11.5 | 11.6 | 12.1 | 11.7 | 12 |
| Tyrosine | 6.5 | 6.5 | 6.8 | 6.7 | 7 |
| Half-cystine | 3.1 | 2.8 | 2.7 | 3.5 | 4 |
| Methionine | 2.8 | 2.7 | 2.9 | 2.8 | 3 |
| Serine | 13.3 | 12.3 | 12.3 | 13.5 | 14 |
| Threonine | 7.0 | 7.2 | 7.3 | 7.3 | 7 |
| Proline | 7.1 | 7.1 | 7.2 | 7.1 | 7 |
| Glycine | 8.1 | 7.9 | 8.1 | 8.0 | 8 |
| Alanine | 16.4 | 16.0 | 16.4 | 16.3 | 16 |
| Valine | 7.1 | 7.5 | 7.6 | 7.6 | 8 |
| Leucine | 22.7 | 22.9 | 23.6 | 23.8 | 24 |
| Isoleucine | 5.1 | 5.4 | 5.6 | 5.6 | 6 |
| Tryptophan | 0.99 | 1 |
| Total | | | | 182 |

* Corrected to zero time.  
* Value at 72 hours.  
* Determined by reaction with N-bromosuccinimide.
for half-cystine is still somewhat low, but on the basis of studies of partial and complete reduction of the disulfides of the same preparation of porcine growth hormone (24), it is concluded that there are 4 moles of half-cystine per mole of the hormone. Since the amounts of valine, leucine, and isoleucine increased with time of hydrolysis, the 4-hour values are taken as the best estimates. Preliminary observations of the molecular weight of the monomer of porcine growth hormone by sedimentation equilibrium in the presence of 5.5 M guanidine hydrochloride have yielded an estimate of 22,000 ± 1,500 (25). This is in accord with the most recent reports of the molecular weight of bovine (26) and of human growth hormone (27). The data of Table IV are therefore presented as moles of amino acid weight of bovine (26) and of human growth hormone (27). The estimate of the monomer weight by sedimentation equilibrium in the presence of 5.5 M guanidine hydrochloride may not be best estimates. Preliminary observations of the molecular weight of the monomer of porcine growth hormone by sedimentation equilibrium in the presence of 5.5 M guanidine hydrochloride may not be incompatible with the higher value (41,600, by sedimentation in the presence of 5.5 M guanidine hydrochloride) reported by Papkoff et al. (5), if the dimer is the form of the hormone mainly present over the range of concentrations which they used.

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