Preliminary Investigations on the Action of Pepsin on Human Pituitary Growth Hormone

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ABSTRACT A slight modification of the original procedure for the isolation of human pituitary growth hormone has been described. The purity of the preparation was further examined by chromatography on carboxymethylcellulose, zone electrophoresis on starch, and electrodialysis. The hormone was found to be stable in a pH 7.0 solution at 100°C and in 10 M urea at 25°C. Digestion with pepsin to an extent of 40 per cent did not diminish the biological activities of the hormone. Bioassays of an active core derived from such digests exhibited essentially the same specific growth-stimulating activity in the hypophysectomized rat and crop sac stimulation in the pigeon. It was concluded that the integrity of the protein is not required for the activity of human growth hormone.

Since pepsin was first isolated and characterized by Northrop (1) in 1930, it has been employed in two important areas of protein chemistry, namely, for the elucidation of amino acid sequences in polypeptide chains, and to ascertain whether the integrity of protein molecules is essential for biological activity. As an example of its application, treatment of diphtheria toxin-antitoxin with pepsin resulted in a crystalline antitoxin with only half the molecular weight of the original antitoxin (2). Furthermore, when γ-globulin was subjected to peptic hydrolysis, the immunological activity of the digestion product remained unchanged (3). Also, it was found that the COOH-terminal portion of ACTH peptides could be cleaved with pepsin without any loss of biological potency (4, 5).

Growth hormone isolated from human pituitary glands (6, 7) has been shown to be a protein consisting of a single polypeptide chain with an isoelectric point at pH 4.9 and a molecular weight (8) of 29,000. The homogeneity of the preparation was demonstrated by ultracentrifugal studies (8), terminal amino acid analyses (9, 10), and immunochemical investigations (11, 12). It was reported that the hormone retained its activity after partial
chymotryptic hydrolysis (7), as in the case of bovine pituitary growth hormone (13). The present report concerns some preliminary observations on the partial peptic hydrolysis of human pituitary growth hormone (HGH).

**PREPARATION** The hormone was isolated from fresh human pituitaries by the procedure previously described (6, 7), with the exception of the chromatographic step. Briefly, the 1.9 M(NH₄)₂SO₄ precipitate (0.8 gm from 15 gm fresh glands) was extracted twice with 100 ml of phosphate buffer of pH 5.1 containing 0.45 M (NH₄)₂SO₄. The combined extracts were applied to a column (2.5 × 25 cm) of amberlite IRC-50 (XE-97) resin which had been equilibrated with the same buffer. The hormone was absorbed under these conditions and eluted off with 100 ml of water. The water eluate was dialyzed thoroughly and lyophilized with a yield of 150 mg. Further purification of this product was achieved by isoelectric precipitation and ethanol fractionation, as described previously (6, 7).

**BIOASSAY** The growth-promoting activity of the hormone was assayed in hypophysectomized rats by the tibia test (14). Since HGH was shown to possess lactogenic activity (15), its crop sac–stimulating activity in the pigeon was also assayed. Silver King squabs, 4 weeks of age, were injected intradermally on one side of the crop sac once daily for 2 days, and autopsies were performed on the 3rd day. The sacs were dissected off, held slightly stretched against the light, and examined for signs of stimulation. A positive response can be seen with the naked eye (15 a).

**Homogeneity Studies** In addition to ultracentrifugal, terminal residue, and immunochemical studies (8–12), the preparation has been examined by chromatography on carboxymethylcellulose and by zone electrophoresis on starch. The cation exchanger, carboxymethylcellulose (61), was equilibrated with citrate-phosphate

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1 It is a pleasure to acknowledge the suggestion of Dr. H. Papkoff for this modification.
2 The author wishes to thank Dr. N. R. Moudgal for this experiment.
buffer of pH 4.0 (prepared by dissolving 6.45 gm of citric acid and 5.48 gm of anhydrous dibasic sodium phosphate in 1 liter of solution) and the column (1 X 10 cm) developed at 1°C with NaCl gradients after the hormone (3 mg) had been applied.

**TABLE I**

EFFECT OF VARIOUS TREATMENTS
ON THE BIOLOGICAL ACTIVITIES OF HUMAN PITUITARY GROWTH HORMONE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Tibia width (micra)</th>
<th>Dose (mg)</th>
<th>No. of pigeons</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>24</td>
<td>246±3§</td>
<td>2</td>
<td>9</td>
<td>- (3), 1 + (5), 2 + (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>9</td>
<td>- (2), 1 + (4), 2 + (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>6</td>
<td>1+ (1), 2 + (2), 3 + (3)</td>
</tr>
<tr>
<td>Electrodialysis†</td>
<td>5</td>
<td>240±2</td>
<td>4</td>
<td>3</td>
<td>1+ (2), 2 + (1)</td>
</tr>
<tr>
<td>Urea‡</td>
<td>6</td>
<td>260±4</td>
<td>4</td>
<td>3</td>
<td>- (1), 1 + (2)</td>
</tr>
<tr>
<td>Heat***</td>
<td>24</td>
<td>238±3</td>
<td>4</td>
<td>6</td>
<td>1+ (2), 2 + (4)</td>
</tr>
<tr>
<td>0.01 M HCl‡‡</td>
<td>5</td>
<td>242±1</td>
<td>2</td>
<td>3</td>
<td>- (1), 1 + (2)</td>
</tr>
<tr>
<td>0.10 M NaOH§§</td>
<td>9</td>
<td>213±4</td>
<td>4</td>
<td>6</td>
<td>1+ (2), 2 + (4)</td>
</tr>
</tbody>
</table>

* A total dose of 40 micrograms in 4 days was used for each assay.
† No stimulation, -; moderate stimulation, 1+; good stimulation, 2+; marked stimulation, 3+. In Tables I and II the number of pigeons is given in parentheses.
§ Mean ± standard error. The tibial width of the controls (10 animals), 157 ± 2 micra.
∥ For the conditions of electrodialysis, see text.
¶ 10 mg urea solution of pH 7.0, containing 1 mg hormone per ml, was kept at 25°C for 24 hours.
** The hormone in the amount of 5 mg was dissolved in 5 ml of water and adjusted to pH 7.0; the solution was kept in a boiling water bath for 15 minutes.
†† 25°C for 3 hours.
‡‡ 25°C for 6 hours.
It was found that all the protein was eluted from the column as a single peak at a concentration of 0.5 M NaCl (Fig. 1).

Zone electrophoresis on starch (17, 18) was carried out in a small trough (1.5 X 0.7 X 40 cm) with approximately 2 mg of hormone in a cold room at a temperature of 3°C; it was performed with a potential difference of about 200 volts across ends of the trough. The protein content in each 1 cm segment of the starch was subsequently estimated on the basis of Folin-Lowry color (19). The composition of the buffers was as follows: pH 4.0; 0.19 M acetic acid and 0.017 M NaOH; pH 11.2; 0.1 M Na₂CO₃. The electrophoretic patterns of the hormone, shown in Fig. 2, show no evidence of inhomogeneity.

<table>
<thead>
<tr>
<th>TABLE II</th>
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<tbody>
<tr>
<td>ACTION OF PEPSIN ON HUMAN PITUITARY GROWTH HORMONE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of digestion*</th>
<th>Non-protein nitrogena</th>
<th>Tibia test</th>
<th>Crop sac stimulationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>mix.</td>
<td>per cent</td>
<td>micro</td>
<td>µg</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>6</td>
<td>251±2</td>
</tr>
<tr>
<td>30</td>
<td>15.5</td>
<td>5</td>
<td>258±3</td>
</tr>
<tr>
<td>60</td>
<td>38.9</td>
<td>5</td>
<td>238±4</td>
</tr>
<tr>
<td>120</td>
<td>64.0</td>
<td>5</td>
<td>166±3</td>
</tr>
</tbody>
</table>

* Enzyme/hormone (w/w) = 1/150, 0.01 M HCl at 25°C.
† No stimulation, − ; moderate stimulation, 1+ ; good stimulation, 2+ ; marked stimulation, 3+.
§ A total dose of 40 micrograms in 4 days; mean ± standard error.

**ELECTRODIALYSIS** In order to make certain that the hormonal activity was not associated with a small polypeptide absorbed onto the protein, the preparation was submitted to electrodialysis. The hormone (49 mg) was dissolved in 17 ml of water and dialyzed against 1 liter of distilled water for 24 hours. The dialyzed solution (ca. 20 ml) was put into the middle compartment of a three-cell electrodialysis apparatus (20); the center cell was separated from the anode and from the cathode by a Visking 18/32 cellophane membrane. Both anode and cathode cells contained distilled water (ca. 20 ml). A current of 1 to 5 milliamperes was maintained by suitable variation of the applied voltage (450 to 750 volts). Electrodialysis was carried out for 5 hours in a cold room at a temperature of 3°C. Considerable precipitation occurred in the center cell and no detectable protein was found in either anode or cathode cell as examined by ultraviolet absorption at 278 mµ in a Beckman model DU spectrophotometer. The pH of the solution in the center compartment was measured in a Beckman model G pH meter and found to be 4.90, a value identical with the isoelectric point of the hormone reported earlier (7). The amount of protein recovered from the center cell was 48 mg; when assayed for growth-promoting activity by the tibia test, it was shown to have the same potency as the non-electrodialyzed material (Table I).
TABLE III
GROWTH-PROMOTING ACTIVITY OF THE CORE FROM A PARTIAL PEPTIC DIGEST OF HUMAN PITUITARY GROWTH HORMONE

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total dose</th>
<th>No. of rats</th>
<th>Tibia width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated growth hormone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µg, 8 rats</td>
<td>213±2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µg, 8 rats</td>
<td>235±2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 µg, 6 rats</td>
<td>256±2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core of peptic digest†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µg, 4 rats</td>
<td>241±2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 µg, 4 rats</td>
<td>264±3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± standard error.  † The core was obtained from a 40 per cent digest (enzyme/hormone, 1/150; 0.01 M HCl, 1 hour; 25°C).

STABILITY STUDIES. Solutions of HGH (1 mg per ml) were subjected to treatments under various conditions and assayed for growth-promoting or crop sac-stimulating activities. Unlike the bovine hormone (4), the biological activities of HGH did not diminish after being kept at 100°C for 15 minutes. In 0.1 M NaOH at 25°C for 6 hours, the growth-promoting activity of the hormone decreased considerably. There was no change in the biological potency of HGH when the hormone was...

Figure 3. Effects of HGH preparations on the crop sacs of the pigeon: A, control; B, 1 µg of ovine lactogenic hormone (2 + response); C, 4 µg of a core from peptic digests of HGH (2 + response); D, 4 µg HGH (3 + response).
kept in 0.01 M HCl of pH 2 at 25°C for 3 hours or in 10 M urea of pH 7.0 at 25°C for 24 hours. Assay data relating to these stability studies are summarized in Table I.

ACTION OF PEP SIN In a typical experiment, the hormone, in the amount of 25.0 mg, was dissolved in 2.5 ml of 0.01 M HCl and mixed with 2.5 ml of pepsin solution. The pepsin solution was prepared by dissolving 1.0 mg of crystalline pepsin (Armour) in 15 ml of 0.01 M HCl. The substrate-enzyme mixture was kept at 25°C for various periods of time; a sample was drawn at appropriate intervals for determination of the rate of hydrolysis as well as for bioassay. The degree of digestion was estimated by the amount of non-precipitable material present in 5 per cent trichloroacetic acid, determined on the basis of the Folin-Lowry colorimetric reaction (19). Results are recorded in Table II. It may be seen that no loss of biological activity is incurred when the hormone protein is hydrolyzed up to an extent of approximately 40 per cent, although further digestion with the enzyme abolishes the hormonal potency. These observations are surprising, since it has been shown that the growth-promoting activity of both bovine (21, 22) and porcine (23) growth hormones is markedly decreased by peptic hydrolysis under almost the same conditions.

An active core may be obtained from the peptic digests of HGH by dissolving the 5 per cent trichloroacetic acid precipitate of the 1 hour digest in a slightly alkaline solution and dialyzing against distilled water at 1°C, followed by lyophilization of the dialyzed solution. A multiple dose assay of the core showed its activity to be essentially the same as the untreated material (Table III). In addition, the core retained its crop sac-stimulating activity in the pigeon; a dose of 4 μg gave positive crop sac stimulation in 6 pigeons (Fig. 3).

DISCUSSION

Before the active core of HGH is isolated in highly purified form, it is worthless to investigate in detail its physical and chemical properties. Nevertheless, in preliminary studies of the core, no undigested protein has been detected by various methods including electrophoresis. It is unlikely that the biological activities of the core are attributable to any minute contamination on the part of the parent molecule. From these studies of the effect of peptic digestion together with the results that have been obtained with chymotrypsin (7) and carboxypeptidase (10), it is evident that the activity of human pituitary growth hormone does not depend upon the integrity of the protein and consequently it may be inferred that the activity resides in only a portion of the whole molecule.

It is of interest to point out that the crop sac-stimulating activity of HGH in the pigeon responded in an identical fashion to the enzymic treatment as did the growth-promoting activity of the hormone. In both stability and pepsin studies, it was demonstrated that the growth-promoting and crop sac-stimulating activities of the hormone were retained after either heat treat-
ment or partial peptic hydrolysis. Extensive peptic digestion caused a total loss of both activities. Other investigations (15) have shown that human growth hormone can exercise a luteotropic effect in hypophysectomized rats and a lactogenic effect in the rabbit and monkey. Thus, it appears that human growth hormone possesses, as intrinsic properties, all the biological effects supposed to be characteristic of animal lactogenic hormone.

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REFERENCES