N-Terminal Amino Acid Analysis of Growth Hormones from Human, Monkey, Whale, and Beef Pituitary Glands*

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The isolation of growth hormone (somatotropin) from human, monkey, and whale pituitary glands has recently been reported (1-3). Preliminary studies on the hormone proteins from these species have suggested that phenylalanine is their sole N-terminal amino acid residue (1-3), in contrast to the two N-terminal residues, alanine and phenylalanine, which have been reported for the bovine somatotropin (4, 5). In this paper are reported the results of an investigation designed to confirm the findings of earlier N-terminal studies on human, monkey (rhesus, Macaca mulatta), and whale (humpback) growth hormones, and to elucidate a partial N-terminal amino acid sequence for the somatotropins from these three species and for the bovine hormone as well.

EXPERIMENTAL

The growth hormone preparations used in this investigation were isolated from the pituitary glands of the various species by methods previously described (1, 3, 6). Oxidized whale growth hormone was obtained by treatment with performic acid according to the procedure of Hirs (7). The N-terminal amino acid residue was determined by the FDB procedure (8, 9). In 400 μl. of 5 per cent NaHCO₃, 9.0 mg. of growth hormone were dissolved. To this was added 800 μl. of 2 per cent FDB in ethanol. The reaction mixture was agitated vigorously on a mechanical shaker for 2 hours. The reaction was terminated and the mixture was immediately acidified with several drops of concentrated HCl. The DNP-protein was centrifuged and washed successively with water, ethanol, and ether. After the final washing with ether, dinitrophenol was removed by sublimation in a vacuum overnight. The DNP-protein was then dissolved in 0.5 ml. of constant boiling HCl and, after 16 hours of hydrolysis at 100°, the hydrolysate was made 1 n with respect to HCl. Extraction with ether was then performed until no yellow color appeared in the extracts. The ether extracts were then submitted to two-dimensional paper chromatography according to the procedure of Levy (9).

In order to confirm the findings obtained by the FDB procedure as well as to establish the N-terminal amino acid sequences in the hormone proteins, the paper strip modification (10) of the phenylthiocarbamyl method of Edman (11) was adopted. To a strip (2 x 8 cm.) of Whatman No. 1 filter paper 0.2 μ mole of protein in 50 to 100 μl. of 3 per cent NH₄OH was applied. The paper was dried and treated with 50 μl. of 20 per cent phenylisothiocyanate (in diisocane) and incubated for 12 hours in an atmosphere of pyridine, dioxane, and water at 40°. The strip was washed by being shaken for 15 minutes with five 15-ml. portions of a 1:1 ethanol-ether mixture. The paper strip was then placed in a desiccator which contained beakers of glacial acetic acid and 6 N HCl, and the desiccator was evacuated. After an interval of 5 or 6 hours, the amino acid phenylthiodyantoin split from the protein were washed from the paper by being shaken with 12 ml. of the ethanol-ether mixture for 2 hours. Before the next step, the paper strip was exposed to ammonia fumes to neutralize any final traces of acid which remained from the cyclization step. The ethanol-ether extract, which contained the phenylthiodyantoin, was evaporated to dryness, and the derivative was then identified by the paper chromatographic procedure of Edman and Sjögquist (12). Their procedure was followed exactly, with the "D" (xylene-formamide) and "F" (heptane-ethylene chloride-formic acid) systems. The chromatograms were examined with a fluorescent screen under ultraviolet light and then by the iodide-azide spray (13).

For the investigation of the N-terminal amino acid sequence of the bovine hormone, partial acid hydrolysis of bovine DNP-growth hormone was performed and the resulting DNP-peptides were submitted to chromatography on silicic acid-Celite columns (14). In a typical experiment 50 mg. of bovine growth hormone were allowed to react with FDB, the latter in a proportion of 6.5 per cent FDB and 2 per cent NaHCO₃ in 66 per cent ethanol; the reaction was allowed to proceed for 2 hours at 25°, with vigorous stirring. The DNP-protein was dried over P₂O₅ after being washed with water, ethanol, and ether. It was then subjected to hydrolysis in concentrated HCl for 4 days at 37° in a sealed tube. The hydrolysate was diluted until it was 3 n with respect to HCl. It was then extracted with 2 x 25-ml. and 5 x 10-ml. portions of ethyl acetate. The pooled

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‡ The abbreviations used are: FDB, fluorodinitrobenzene; and DNP, dinitrophenyl.
ethyl acetate extracts were then washed with 10 × 10-ml. portions of water, 2 drops of 6 n HCl being added to 100 ml. of the washing solution. The ethyl acetate extracts were then evaporated to dryness under a vacuum at 37°. The sample was dissolved in 2 ml. of 15AA-5A-B,4 A No. 1 column (9 × 150 mm.) was prepared as outlined by Green and Kay (14). The column was packed with a 2:1 mixture of silicic acid (Merck, lot No. 40440) and Celite 545 (Johns-Manville) and was pre-washed with 0.2 V ml.6 of ether, 1.5 V ml. of ether-acetone (1:1), 0.8 V ml. of ether, 1 V ml. of ligroin, and finally, 1.1 V ml. of developer. The sample was applied and developer was added. Fast-moving zones were collected in round-bottomed flasks; zones remaining on the column after development were cut from the extruded column of adsorbent and eluted with 25 ml. of a mixture of ethanol and ether in a proportion of 1:4. In the initial stages of fractionation, each zone was submitted to chromatography in a different solvent system. Usually the initial chromatography was performed with 8AA-4A-L and then 2AA-10A-L or 1AA-5A-B. If zones were slow-moving, a stronger developer such as 5AA-15A-L or 12AA-6A-L was used. The DNP-peptides were hydrolyzed for 16 hours under the same conditions that have been used for determinations of N-terminal residues of proteins by the FDB method. The N-terminal DNP-amino acid was then submitted to chromatography in two dimensions (9). The aqueous residue which remained after hydrolysis was submitted to chromatography on paper in the system of n-butanol-acetic acid-water, 4:1:5, to detect any remaining amino acids in the peptide.

RESULTS AND DISCUSSION

Table I presents the results of N-terminal amino acid analysis of growth hormones from various species, performed by the FDB method. It should be noted that the data obtained with the bovine hormone confirm previous findings (4), and that human, simian, and whale somatotropins were found to possess only one N-terminal residue, phenylalanine, in an amount of approximately 1 mole per mole of the hormone protein. Examination of the whole growth hormone after oxidation with performic acid again disclosed phenylalanine as the sole N-terminal residue; failure to detect cysteic acid indicated the absence of cystine from the N-terminus of the native protein. In the oxidized hormone also, the yield of phenylalanine was nearly 1 mole per mole of the hormone protein.

The sole N-terminal residue disclosed by the phenylthiocarbamyl procedure for growth hormones from whale, monkey, and human glands was again phenylalanine (Table II). This method confirmed the absence of glycine, proline, and tryptophan, which are subject to poor recovery in the form of their DNP-derivatives during the acid hydrolysis of DNP-protein. It is therefore concluded that these growth hormones possess a single phenylalanine residue at their N-terminus. These results, together with the results of the C-terminal amino acid determination presented elsewhere (16), strongly support the hypothesis that these growth hormones (whale, monkey, and human) are single-chain polypeptides. Recently, Heijkenskjold (19) formulated a similar conclusion with respect to the human somatotropin after an investigation of the N-terminus of the hormone by the phenylthiocarbamyl procedure.

Stepwise degradation of somatotropins from N-terminal sequence by phenylthiocarbamyl procedure

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample No.</th>
<th>Amino acid identified</th>
<th>Proposed N-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Step 1</td>
<td>Step 2</td>
</tr>
<tr>
<td>Bovine</td>
<td>1</td>
<td>Ala, Phe</td>
<td>Phe, Thr</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ala, Phe</td>
<td>Phe, Thr</td>
</tr>
<tr>
<td>Human</td>
<td>1</td>
<td>Phe</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Phe</td>
<td>Ser</td>
</tr>
<tr>
<td>Cetacean</td>
<td>1</td>
<td>Phe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Phe</td>
<td></td>
</tr>
<tr>
<td>Simian</td>
<td>1</td>
<td>Phe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Phe</td>
<td></td>
</tr>
</tbody>
</table>

* Corrections for destruction of DNP phenylalanine and DNP-alanine in DNP-somatotropins by acid hydrolysis are taken from earlier studies (4).
† Molecular weights of various somatotropins were assumed as follows (1–3): bovine, 46,000; cetacean, 40,000; simian, 25,000; and human, 27,000.
‡ Performic acid-oxidized sample.

These findings do not exclude the possibility of an alkyl group masking the N-terminal residue of a second peptide chain in these growth hormones. The presence of acetyl or similar moieties has been shown in tobacco mosaic virus protein (17) and in one of the melanotrops (18).

4 The abbreviations of Green and Kay (14) are used to denote the composition of the developer. 15AA-5A-B signifies 15 ml. of acetic acid and 5 ml. of acetone with sufficient benzene to make 100 ml. Similarly, 8AA-4A-L means 8 ml. of acetic acid and 4 ml. of acetone with sufficient ligroin to make 100 ml.; 2AA-10A-L represents 2 ml. of acetic acid and 10 ml. of acetone with ligroin in a volume to make 100 ml.; and 1AA-5A-B is 1 ml. of acetic acid, 5 ml. of acetone, and 94 ml. of benzene.
5 V refers to the volume of liquid required to moisten completely a column of adsorbent. With a 9 × 150-mm. column this is approximately 7 ml.
lish unequivocally the sequence Ala. Phe. Ala. ... as an N-terminal residue. It was more difficult to elucidate the sequence in the silicic acid-Celite column (14) resulted in DNP-peptides which acid hydrolysis of dinitrophenylated bovine somatotropin by the further studies were conducted on the hormones from these two species. Because of lack of material, no countered at the second step. Because of lack of material, no further studies were conducted on the hormones from these two species.

Fractionation of the DNP-peptides obtained from partial acid hydrolysis of dinitrophenylated bovine somatotropin by the silicic acid-Celite column (14) resulted in DNP-peptides which had either alanine or phenylalanine as their N-terminal residue. From these peptides, listed in Table III, it was possible to establish unequivocally the sequence Ala. Phe. Ala. ... as an N-terminal sequence in one of the polypeptide chains of the bovine hormone. It was more difficult to elucidate the sequence in the phenylalanyl chain, for although the tripeptide Phe. (Ala, Thr) was obtained the most frequently, no dipeptide containing either alanine or threonine along with the N-terminal phenylalanine appeared. However, application of the stepwise degradation procedure (10, 11) to the native bovine hormone yielded threonine in the second step, together with phenylalanine from the alanyl chain (Table II). It would therefore seem that the sequence Phe. Thr. Ala. ... is derived from the phenylalanyl chain of beef growth hormone. This and the peptide sequence Ala. Phe. Ala. ... are proposed as the N-terminal amino acid sequences of beef growth hormone.

It is of interest to note that phenylalanine is found as an N-terminal residue in all of the growth hormone studied to date. Although the bovine and ovine growth hormones have two N-terminal residues (Phe and Ala) (4, 5, 21), the other three hormones seem to consist of only a single chain at the N-terminus. This suggests the observation that cattle and sheep are closely related with respect to zoological species, and hence it is not surprising that the hormones derived from these two species should be similar. In the same way man and the monkey are closely related. It is of particular interest in the present findings to note that the whale has been found to be closer to the primates than to the ruminant ungulates.

**SUMMARY**

By means of stepwise degradation by the phenylthiocarbamyl method, together with partial acid hydrolysis in the case of bovine growth hormone, it has been possible to demonstrate the following N-terminal amino acid residues and adjacent sequences for the growth hormone from several species: beef, alanyl-phenylalanyl-alanyl ... and phenylalanyl-threonyl-alanyl ...; whale (humpback), phenylalanyl ...; monkey (rhesus, Macaca mulatta), phenylalanyl ...; and human, phenylalanyl-seryl-threonyl ... The N-terminal residues in these growth hormones have also been established by the fluorodinitrobenzene method.

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**REFERENCES**


† For a detailed discussion of these difficulties and some proposed N-terminal sequences for these two species see (20).
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