Lack of Effect of a Cyanogen Bromide Fragment of Human Growth Hormone on Some Aspects of Carbohydrate Metabolism *in Vitro* (36789)

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While the effects of the pituitary on carbohydrate metabolism have been known for many years, it is not certain whether growth hormone is the actual mediator of these effects or what the mechanisms of action might be. Recently, several groups of workers have reported finding pituitary peptides which have effects specifically on carbohydrate metabolism (1–4). Among the most extensive studies on such peptides are those by Bornstein and his co-workers. This group has reported the isolation from pituitary glands and from growth hormone of two peptides, one of which inhibits certain overall pathways of carbohydrate and lipid metabolism *in vivo* and *in vitro* as well as specific isolated enzymes involved in these pathways, and the other of which counteracts these effects (5–8). The peptides have been found in blood and urine of normal and diabetic humans (9, 10), and one of them has been shown to have potential usefulness in the treatment of certain types of diabetes (11). Most recently, the group has assigned the inhibitory activity to a peptide comprising the COOH-terminal 25 amino acids of human growth hormone and the counteracting (stimulatory) activity to the NH_2-terminal 21 amino acids (12). Bornstein *et al.* (12) have proposed that these peptides and their effects on enzymes of glycolysis and fatty acid synthesis are of importance in the control of normal carbohydrate utilization and that an imbalance in concentrations of the peptides can account for the development of some cases of diabetes (13).

If true, these findings would have far-reaching implications. They would indicate direct, intracellular effects of portions of a polypeptide hormone (as opposed to the extracellular effects of other peptide hormones). They would not only explain the origins of a metabolic disease but would offer a specific treatment.

However, Bornstein's group has been hampered by the impurity and low yields of the active factors derived from relatively nonspecific treatment of growth hormone. In an attempt to gain evidence supporting the claimed structure of the inhibitory peptide and to produce larger quantities of pure material for study, we decided to use the highly-specific cyanogen bromide reaction to cleave human growth hormone at the methionine residues and then to isolate a fragment consisting of the COOH-terminal 21 amino acids. This peptide could then be tested for inhibitory activity in some of the systems utilized by Bornstein *et al.*

Materials and Methods. Therapeutic grade human growth hormone was obtained from Auckland Public Hospital and was purified by gel filtration on a 2.5 × 105 cm column of Sephadex G100 using 0.01 *M* NH_4HCO_3 as the eluting solvent. Li, Dixon and Liu (14) and Trygstad and Foss (15) have reported good purification by this technique, and we obtained an elution profile virtually identical with that shown by the latter workers. The purified growth hormone (approx. 75 mg) was treated with approximately 110 mg cyanogen bromide (Ajax Chemicals Ltd., Sydney) by the technique of Li, Dixon and Liu (14), except that the reaction mixture was allowed to stand for 18–24 hr and the solvent and volatile reaction products were removed by lyophilization. Separation of the fragments was achieved by gel filtration on, first, a 2.5

TABLE I. The Effect of the COOH-Terminal 21-Amino Acid Peptide from Human Growth Hormone on the Glyceraldehyde 3-Phosphate Dehydrogenase Reaction.

Expt	No. of trials	Addition to cuvette[a]	pH of assay mixture	$\Delta OD_{340\,m\mu}$/min[b]
1	1	None	—	0.113
	1	Approx 0.005 μmoles peptide	—	0.114
	1	Approx 0.02 μmoles peptide	—	0.114
2	2	None	8.34	0.086
	2	0.002 μmoles peptide	8.33	0.086
	1	HCl to pH	8.30	0.083
	2	0.01 μmoles peptide	8.30	0.082
	2	HCl to pH	8.15	0.078
	1	0.015 μmoles peptide	8.15	0.075

[a] Samples of growth hormone fragment were dissolved in 0.005 *N* CH_3COOH plus a minimal volume of 0.1 *N* HCl (Expt 1) or 0.05 *N* CH_3 COOH plus a minimal volume of 1 *N* HCl (Expt 2) to assure dissolution.

[b] As the reaction is nonlinear, all $\Delta OD_{340\,m\mu}$/min are for the period extending from 60 to 120 sec after addition of enzyme.

× 105 cm column of Sephadex G25 using 0.1 *N* CH_3COOH as eluant and then a 2.5 × 105 cm column of Sephadex G50 using 0.005 *N* NH_4OH as eluant. The composition of the fragment was verified and its concentration and extent of contamination determined by hydrolysis of a sample for 20 hr at 105° in 5.7 *N* HCl and subjection of the dried residue to amino acid analysis on a Beckman Model 120C amino acid analyzer.

Samples of the purified fragment were tested for their activity in three assay systems in which Bornstein *et al.* had shown inhibition by their inhibitory fraction of growth hormone: crystalline glyceraldehyde 3-phosphate dehydrogenase (5, 6), crystalline α-glycerophosphate dehydrogenase (5), and glucose uptake by rat soleus muscle (7). Glyceraldehyde 3-phosphate dehydrogenase (from rabbit muscle, Boehringer-Mannheim Corp., Mannheim, Germany) was assayed by the method of Pihl and Lange (16); α-glycerophosphate dehydrogenase (from rabbit muscle, Sigma Chemical Co., St. Louis, MO), by the method of Shonk and Boxer (17); and glucose uptake by rat soleus muscle, by the method of Chaudry and Gould (18). Concentrations of fragment at least equivalent to those used by Bornstein *et al.* were tested in all experiments.

Results. Amino acid analysis of the purified COOH-terminal 21-amino acid fragment gave the following pattern: $asp_{1.3}$ $thr_{1.2}$ $ser_{2.0}$ $glu_{2.9}$ $gly_{2.0}$ $val_{2.1}$ $½cys_{1.5}$ $ile_{0.5}$ $leu_{1.1}$ $phe_{2.0}$ $lys_{0.9}$ $arg_{2.1}$. This compares favorably with the profile obtained by Li, Dixon and Liu (14): $asp_{1.1}$ $thr_{1.0}$ $ser_{1.8}$ $glu_{3.0}$ $gly_{2.0}$ $val_{2.4}$ $½cys_{1.3}$ $ile_{0.6}$ $leu_{1.2}$ $phe_{1.7}$ $lys_{0.9}$ $arg_{2.0}$. The analysis also revealed that there was no more than 5% contamination of the fragment by the other similar-sized cyanogen bromide fragment of human growth hormone (*i.e.*, the NH_2-terminal 14 amino acids with the COOH-terminal methionine converted to homoserine).

The results of the experiments undertaken to determine the effects of the fragment are shown in Tables I to III. In no system was a significant inhibition observed. In addition, duplicate determinations showed that samples (0.01 μmoles each) of the NH_2-terminal 14-amino acid peptide with COOH-terminal homoserine and of the central core of cyanogen bromide treated human growth hormone had no significant effects on the activities of glyceraldehyde 3-phosphate dehydrogenase or α-glycerophosphate dehydrogenase.

Discussion. We believe that our 21-amino acid fragment can be compared with the 25-amino acid peptide claimed by Bornstein *et al.*, as they are of similar size and both run to the carboxyl end of the growth hormone

TABLE II. The Effect of the COOH-Terminal 21-Amino Acid Peptide from Human Growth Hormone on the α-Glycerophosphate Dehydrogenase Reaction.

No. of trials	Addition to cuvette[a]	Mean $\Delta OD_{340\,m\mu}$/min[b]
2	None	0.081
2	0.002 μmoles peptide	0.081
2	0.01 μmoles peptide	0.080
1	None	0.076
1	0.0145 μmoles peptide	0.076

[a] All samples of growth hormone fragment were dissolved in a minimal volume of 0.01 *N* HCl and diluted with assay buffer (triethanolamine, 0.05 *M*, pH 7.6) before addition to cuvettes. The final pH of all assay mixtures was 7.58–7.60.

[b] All reactions were linear for at least 4 min, and the $\Delta OD_{340\,m\mu}$/min are the averages over the first 4 min after commencement of reaction by addition of substrate.

molecule. Furthermore, Bornstein's group has claimed that peptides with similar activity (even when administered to humans) are obtained from sheep growth hormone as well as from human growth hormone (6, 11–13), in spite of the differences in primary structure between them (14, 19). We could thus expect that the four amino acids on the amino terminus might not be an essential factor for activity of the peptide.

The fragment which we tested still had an intact internal disulfide bridge after the cyanogen bromide treatment (14), so it could have been expected to be no more denatured than that tested by Bornstein and co-workers after acid treatment of growth hormone.

As shown in Tables I–III, the purified fragment had no effect in any of the systems tested, in spite of the claims by Bornstein *et al.* that the inhibitory peptide [in amounts equivalent to 80 mg pituitary powder (5) or 25 to 200 μg of growth hormone/assay (6, 7), which would be no more than about 0.001 to 0.01 μmole growth hormone (20)] inhibited glyceraldehyde 3-phosphate dehydrogenase by almost 20% (5, 6), α-glycerophosphate dehydrogenase by about 70% (5), and glucose uptake by rat soleus muscle by about 30% (7). Small effects which we observed appeared to be due to slight alterations in the pH of the assay systems caused by the addition of the test solution.

TABLE III. The Effect of the COOH-Terminal 21-Amino Acid Peptide from Human Growth Hormone on Glucose Uptake by Rat Soleus Muscle.

Control (μmoles uptake/g/hr)	+ 0.01 μmoles peptide (μmoles uptake/g/hr)	Sig. of difference
21.3 ± 1.8[a]	20.4 ± 1.5[a]	NS

[a] The results are means ± SD from 5 experiments of 3 muscles each.

Our results indicate that unless the first four amino acids are critical for activity, the assignment of inhibitory activity to a peptide composed of the COOH-terminal 25 amino acids of human growth hormone is not warranted. Certainly the COOH-terminal 21-amino acid peptide has no such effects at the concentrations studied. In addition, some doubt has been cast onto the assignment of deinhibitory or stimulatory activity to the NH_2-terminal 21-amino acid peptide by the recent revision of the primary structure of human growth hormone in this region (21). This revision drastically affects the results on which Bornstein *et al.* (12) base their claim. The results from Bornstein's own group [Ng *et al.* (22)] showing that methionine rather than leucine is at the carboxyl end of the stimulatory peptide obtained from human urine also confuse the issue.

As it is possible that the first four amino acids are indeed essential for activity of the inhibitory peptide, we now propose to prepare the 25-amino acid peptide by addition of the appropriate amino acids to the cyanogen bromide fragment. This will then be identical to the portion of human growth hormone which is claimed to have the inhibitory activity, and its activity can be tested.

Summary. It has recently been claimed that a pituitary peptide which inhibits several phases of carbohydrate and lipid metabolism is identical with the COOH-terminal 25-amino acid portion of human growth hormone. However, the active factor has always been obtained in low yield and impure condition. We have used cyanogen bromide to prepare the COOH-terminal 21-amino acid

peptide of human growth hormone. This fails to inhibit any of three systems inhibited by the reputed active fraction: glyceraldehyde 3-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, and glucose uptake by rat soleus muscle. Unless the first four amino acids are essential, the identification of the inhibitory factor as the COOH-terminal 25-amino acid peptide of human growth hormone is not justified.

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