Metabolic Effects of Plasmin Digests of

Human Growth Hormone in the Rat and Man

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A B S T R A C T As a first step in our study of structure-function relationships among primate and non-primate growth hormones, human growth hormone (hGH) was subjected to the limited digestive activity of human plasmin. The lyophilized whole digest, containing less than 2% of unchanged hormone, had an average of 2.3 new amino-terminal groups per mole. The digest had the same potency as the native hormone (a) in causing weight gain in hypophysectomized rats; (b) in stimulating somatomedin production in hypophysectomized rats; (c) in stimulating upake of [³H]leucine into isolated diaphragm of hypophysectomized rats; (d) in accelerating transport of [¹⁴C]α-aminoisobutyric acid into isolated diaphragm of hypophysectomized rats; (e) in stimulating uptake of [3-0-methyl-¹⁴C]glucose by isolated adipose tissue of hypophysectomized rats; (f) in accelerating conversion of [¹⁴C]glucose to ¹⁴CO₂ by isolated epididymal adipose tissue of hypophysectomized rats. The digest also caused glucosuria in partially pancreatectomized rats treated with dexamethasone.

These metabolic actions of plasmin-digested hGH in the array of animal tests were confirmed by comparable effects elicited in 11 human subjects (nine pituitary-deficient children and adolescents and two nondeficient adults). A single injection of the plasmin digest caused an increase in plasma free fatty acids and a fall in plasma amino acids. Seven daily injections caused positive balances of nitrogen, phosphorous, sodium, and potassium, gain in body weight, and in two of three subjects impairment of glucose tolerance. The potency of the plasmin digest in producing these metabolic effects in man was comparable to that of native hGH.

Thus, 2–3 bonds in the hGH molecule can be cleaved by plasmin without impairing the hormone's growth-promoting, anabolic, diabetogenic, and adipokinetic actions for rat and man.

INTRODUCTION

The study of the effect of proteolytic enzymes on the activity of growth hormone is important for both fundamental and practical reasons. The identification of the minimum structural requirements for activity may improve understanding of the mechanism of action of the hormone, but it may also reveal a structure common to the growth hormones of all species, opening the way to relief of the scarcity of human growth hormone (hGH)[1] either by synthesis of the active residue or by its production from ample supplies of the growth hormone of ox, sheep, or pig. An early attempt to achieve this goal was made by Li, Papkoff, Fønss-Bech, and Condliffe (1), who showed that limited digestion of bovine growth hormone (bGH) with chymotrypsin could be carried out without significant loss of activity in the tibia test in the hypophysectomized rat. The claim was made that these digests were active in man (2), but this claim could not be substantiated (3). The problem was taken up again by Elsair, Vairel, Gerbeaux, Dartois, and Royer (4) who introduced better control of the digestion by the use of the pancreatic trypsin inhibitor of Kunitz and Northrop. The product of 30-min digestion of bGH, retaining about 50% of its original activity in the rat by the weight gain test, was given in very large daily doses to a number of pituitary dwarfs. Digests that were inhibited with acid, as in the experiments of Li et al. (1),

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were not effective, but the more carefully controlled and stabilized digests appeared to bring about some nitrogen retention and, less consistently, to cause an increase in plasma free fatty acids 4 h after injection. The whole digest was not well tolerated by the patients, and it was not further purified or characterized. Sonenberg et al. (5) in 1965 described partial digestion of bGH with trypsin; they used soy bean trypsin inhibitor to control the extent of digestion. The purification and characterization of the reaction products have been described in a series of papers (6–12). Briefly digested bGH, in which 2–3 peptide bonds were cleaved, is completely active in the rat (tibia test), and in hypopituitary human subjects at doses of 12–100 mg/day causes nitrogen retention, free fatty acid mobilization and impaired glucose tolerance. The digest has been fractionated by gel filtration in 35% acetic acid, yielding a fragment of molecular weight 16,500 and a smaller polypeptide of molecular weight 5,000 containing no cystine. The two fragments account for the weight and the amino acid composition of the original hormone. Both peptides, alone and in combination, are active in the rat but are much less potent than the undigested hormone. The smaller peptide, given in large doses to two pituitary dwarfs, induced some nitrogen retention and had a marked anti-insulin effect. This peptide is homologous in sequence with peptide 95–134 of human growth hormone (12).

Interest in plasmin as an alternative enzyme to trypsin for the partial digestion of growth hormone arises from the report of Ellis, Nuenke, and Grindeland (13) that the proteinase contaminating bovine, ovine, porcine, and human growth hormone preparations and responsible at least in part for the electrophoretic heterogeneity of the products is plasmin. The properties of the contaminating enzyme are similar to those of plasmin in respect to activation by urokinase and inhibition by pancreatic trypsin inhibitor, soy bean trypsin inhibitor, benzamidine, and aminobenzamidine. Both the contaminating proteinase and plasmin hydrolyze arginyl and lysyl bonds in synthetic peptides and thus resemble trypsin in substrate specificity. The effects of human or bovine plasmin on bovine or rat growth hormone are similar to those of the contaminating proteinase: only a limited proportion of the arginyl and lysyl bonds are cleaved, and the resulting digests are equipotent with the native hormone. New amino-terminal groups and components more acidic than the precursor molecule appear during incubation of proteinase-containing bovine growth hormone (14, 15). The more acidic components of such bovine growth hormone preparations have been isolated by Reusser (16) and by Free and Sonenberg (6) and shown to be biologically active. In our experience with incubated, proteinase-containing hGH, the more acidic components, separated

with DEAE-cellulose chromatography, are at least as active as the starting material (17).

These observations have encouraged us to undertake a systematic study of the effects of human plasmin on hGH, with the ultimate objective of defining the minimum biologically active portion of the molecule. This paper describes preparation of digests of highly purified hGH with human plasmin and assay of the resulting digests for a variety of in vitro and in vivo activities in rat and man.

METHODS

Digestion of hGH with plasmin. Human plasmin was purchased from KABI, Stockholm, Sweden. Purified hGH was prepared as described previously (18, 19). Most preparations of hGH contain multiple active components (20). The least acidic component (Type A) is believed to be native hGH, and the more acidic components are thought to be generated from the native molecule by deamidation and/or enzyme degradation. In this study preliminary observations were made on preparations in which the second least acidic component (Type B) was concentrated, but most of the work was done with preparations in which type A was the major and type B the minor component. The growth hormone potencies of homogeneous types A and B are identical.

The following method describes digestion of 100 mg hGH with plasmin, but it is applicable to 25–500 mg samples. The hormone was dissolved in 10 ml of 0.5% (wt/vol) ammonium bicarbonate, and 0.4 ml of human plasmin solution (10 mg/ml in 0.001 N HCl) was added. The mixture was incubated at 37° for 24 h and was then lyophilized. Quantitative analysis for the appearance of new amino groups during digestion was carried out by reaction of samples of the digest with trinitrobenzenesulfonic acid under conditions described previously (21). Phenylalanine was used as the standard. Disc electrophoresis of the digests was carried out on polyacrylamide gels as described by Ornstein (22).

Assays for metabolic activity in animals. A variety of in vivo and in vitro tests were used to assay the metabolic properties of the digests of hGH in animals. The weight gain test quantitatively measures the potency of the digests relative to a growth hormone standard, while the other assays provide only semi-quantitative estimates. However, these tests indicate whether a particular biological property of the hormone has been retained or lost following cleavage of the molecule with plasmin.

The ability of the digests to promote growth in hypophysectomized rats (Charles River Breeding Labs, Inc., Wilmington, Mass.) was measured by the 9-day weight gain test performed as previously described (18). The results are expressed in terms of potency (IU/mg test material) relative to the international bovine growth hormone standard.

The digests were also tested for the capacity to stimulate [³H]thymidine incorporation into the DNA of isolated costal cartilage of hypophysectomized rats (23). Test materials were injected intraperitoneally into female hypophysectomized rats (60–80 g) 48 and 24 h before sacrifice of the animals by cervical fracture. (For this and other assays described below, the rats used were purchased from the Hormone Assay Labs, Madison, Wisc.) The eighth costal cartilages were removed, trimmed, and incubated for 3 h at

TABLE I

Clinical Data on the Subjects Studied

Case	Age	Sex	Height	Weight	Bone age	Diagnosis	Duration since diagnosis	Pituitary hormone deficiencies	Hormone replacement treatment
	yr		cm	kg	yr		yr		
1	9	M	107	19.1	4	Isolated GH deficiency	4	GH	None
2	8	M	90	13.8	4	Isolated GH deficiency	3	GH	None
3	9	M	109	16.7	5	Isolated GH deficiency	4	GH	None
4	16	M	145	34.5	7	Idiopathic adenohypophyseal insufficiency	6	GH, ACTH, TSH, FSH, LH	Cortisol, thyroxine
5	16	F	162	73	13	Craniopharyngioma (resected)	4	GH, ACTH, TSH, FSH, LH, ADH	Cortisol, thyroxine, ADH
6	16	F	150	60	12	Craniopharyngioma (resected)	7	GH, ACTH, TSH, FSH, LH, ADH	Cortisol, thyroxine, ADH
7	16	M	147	36.1	12	Panhypopituitarism after head trauma	6	GH, ACTH, TSH, FSH, LH	Cortisol, thyroxine
8	16	M	149	34.8	11	Chromophobe adenoma; X-ray therapy	4	GH, ADH	ADH
9	28	M	172	77.8	Adult	Limb-girdle muscular dystrophy	10	None	None
10	51	M	178	77	Adult	Limb-girdle muscular dystrophy	31	None	None
11	9	M	109	18.5	5	Embryologic malformation prosencephalon	9	GH	None

37° in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing [³H]thymidine (2 μCi/ml) and an amino acid mixture (24). After incubation, the cartilages were boiled for 5 min and washed overnight in running water. They were trimmed of extraneous tissue, and the portion of the cartilage within 6 mm of the costochondral junction was discarded (25). The cartilages were then digested with 70% formic acid, and the radioactivity and protein concentration of the digests were determined by previously described methods (26). Results are expressed in terms of disintegrations per minute per milligram of cartilage protein.

Diabetogenic activity was assessed by the procedure of Bates and Garrison (27). Partially (80%) pancreatecto-mized female rats weighing approximately 150 g were placed in metabolism cages, and 24-h urine samples were tested for glucose with Clinitest (Ames Co., Elkhart, Ind.). Those animals free of glucosuria for 5–7 days were then injected subcutaneously twice daily for 4 days with dexamethasone (15 μg/day) and hGH or the digest in 0.9% NaCl. Controls received dexamethasone and 0.9% NaCl. 24 h urine samples were collected throughout the 4 day treatment period. Urinary glucose concentration of 0.25% or more was considered a diabetogenic response. In those rats that responded, glucosuria usually developed on the 3rd day of treatment, and urinary glucose was generally 1–2%.

The ability of the digests to stimulate the incorporation of [³H]leucine into the protein of the isolated rat diaphragm when added in vitro was tested as follows. Hy-pophysectomized female rats (60–80 g) were killed by cervical fracture, and intact hemidiaphragms were prepared. These were washed for 30 min at 37° in 30 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose (KRBG). Then the hemidiaphragms were transferred to flasks containing 10 ml KRBG, [³H]leucine (0.2 μCi/ml; 0.05 mM) and hGH or digest where appropriate. The flasks were then gassed with 95% O_2–5% CO_2, sealed and incubated at 37° with gentle shaking for 1 h. After incubation, the diaphragm muscle was dissected from the rib cage and homogenized immediately in 5 ml of 5% trichloroacetic acid (TCA). Total protein was isolated, and its radioactivity was measured by previously described

methods (28). The results are expressed as disintegrations per minute per milligram protein.

The digests were also tested for capacity to stimulate transport of the nonutilizable amino acid α-aminoisobutyric acid (AIB) into the isolated rat diaphragm when added in vitro. Intact hemidiaphragms were prepared from female hypophysectomized rats (60–80 g) and washed for 10 min in 25 ml KRBG. The tissues were then transferred to flasks containing 10 ml KRBG, [1-¹⁴C]AIB (0.1 μCi/ml; 1 mM) and the test material where appropriate. The flasks were gassed with 95% O_2–5% CO_2 and incubated at 37° with gentle shaking for 1 h. Then the diaphragm muscle was dissected from the rib cage, weighed and homogenized in 2 ml 5% TCA. Radioactivity in the TCA soluble fraction of the homogenate and in the incubation medium was measured, and the distribution ratio for [¹⁴C]AIB was then calculated as described previously (29). For these calculations, the volume of the extracellular space of the diaphragm was obtained by measuring the volume of distribution of [³H]inulin in the tissue under the conditions employed in the above experiments. The values used for the calculations were: controls = 19.5 ml/100 mg wet muscle; hGH = 16.4 ml/100 mg wet muscle; digest = 16.3 ml/100 mg wet muscle.

In vitro effects of the digests on sugar transport into the isolated rat diaphragm were detected by measuring the penetation of [3-0-methyl-¹⁴C]glucose (3-OMG) into the muscle. Intact hemidiaphragms of female hypophysecto-mized rats (60–80 g) were prepared and washed for 10 min in 25 ml Krebs-Ringer bicarbonate buffer (KRB). They were then transferred to flasks containing 10 ml KRB and test material where appropriate. The flasks were then gassed with 95% O_2–5% CO_2, sealed and incubated with gentle shaking at 37° for 30 min. Then 3-OMG was added to the medium (0.1 μCi/ml; 0.1 mM), and incubation was continued for an additional 30 min. Then the diaphragm muscle was dissected from the rib cage, weighed, and ho-mogenized in 2 ml 5% TCA. Radioactivity in the TCA soluble fraction of the homogenate and in the incubation medium was measured, and the distribution ratio for 3-OMG was calculated as previously described (29).

The in vitro capacity of the digests to stimulate the conversion of [¹⁴C]glucose to ¹⁴CO₂ by isolated epididymal
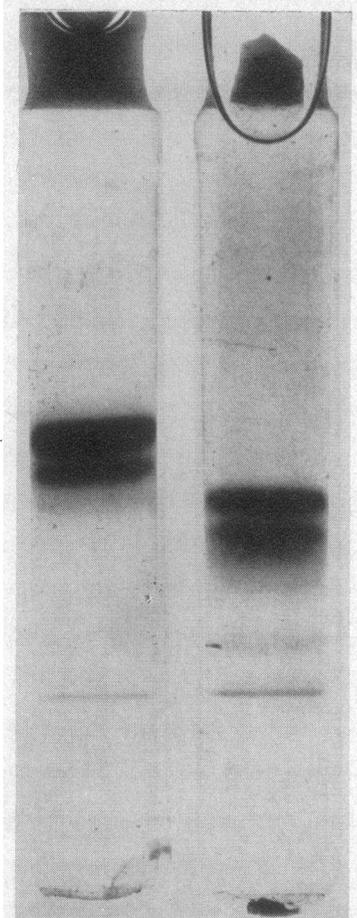
FIGURE 1 Disc gel electrophoretic patterns of undigested hGH (on the left) and the same preparation after digestion with plasmin (right). Direction of migration toward the anode at bottom. The starting material is the second major fraction obtained by DEAE-cellulose chromatography (17, 19) and contains a minor, more acidic component. Both components are more acidic after digestion with plasmin. Only a trace of the original material can be seen.

adipose tissue of male hypophysectomized rats (90–120 g) was studied using the procedure described by Goodman (30).

Tests for metabolic activity in man. Nine children and adolescents with growth hormone deficiency and two adults with limb-girdle muscular dystrophy served as test subjects. These two classes of patient were selected because of their hyperresponsiveness to exogenous hGH, compared with normal individuals (31, 32). Criteria for deficiency of growth hormone, adrenocorticotropin (ACTH), and thyroid-stimulating hormone (TSH) were as previously described (31). Clinical data are summarized in Table I. Patients deficient in ACTH or TSH received 15–20 mg of cortisol or 0.2–0.3 mg L-thyroxine daily at 7 a.m. Subjects with diabetes insipidus were treated with 2.5–5.0 U pitressin tannate in oil every 48–72 h.

Assays for growth hormone activity in man were of two types: (a) a one-day assay, measuring the effect of a single injection of test substance on plasma concentrations of free fatty acid (FFA) and α-amino nitrogen (AAN); and (b)

TABLE II

Growth Promoting Activity of Plasmin Digests of hGH in Hypophysectomized Rats

Preparation of digest	Potency of plasmin digest*	Potency of precursor hGH*
	IU/mg	IU/mg
HS 537-3	2.19 (1.39–3.52)	2.34 (1.29–4.25)
HS 538-1	1.65 (0.97–2.75)	2.17 (1.19–3.94)
HS 574	3.33 (1.90–6.52)	2.17 (1.19–3.94)
HMW600 (HS)A	1.56 (1.07–2.22)	1.97 (1.42–2.72)
HS 605	1.29 (0.39–3.15)	2.62 (1.67–4.34)
HS 621	2.99 (1.56–6.54)	2.75 (1.38–6.16)

The potency of plasmin digests of hGH, as determined by the weight gain test in hypophysectomized rats, is shown in comparison with the potency of the native precursor hGH from which each digest was prepared.
* 95% confidence limits.

a 14-day metabolic balance study measuring the effect, after a 7-day control period, of 7 days' treatment with test material on balances of N, P, Na, and K on body weight (BW), on fasting concentrations of FFA and AAN, and on the oral glucose tolerance curve (31). In both types of assay, the response of the patients to three doses of clinical grade hGH (National Pituitary Agency, lots A17 and B17, approximately 1 U/mg) were first measured. The doses were: dose A = 0.0168 U/kg BW$^{\frac{3}{4}}$; dose B = 0.053 U/kg BW$^{\frac{3}{4}}$; dose C = 0.168 U/kg BW$^{\frac{3}{4}}$. The response of the patients to 0.053 or 0.168 mg/kg BW$^{\frac{3}{4}}$ of the plasmin digest was then determined. Two preparations of digest were examined: HS 605 and HMW600 (HS)A. 1-day assays were done at 3–4 day intervals during a single admission to the metabolic research unit. 14-day assays were done at intervals of 1–2 mo.

For 3 or more days before a 1-day assay, the patient was fed a diet containing 35 kcal/kg BW and 1.5 g protein/kg BW; fat and carbohydrate furnished about 40% and 45% of calories, respectively.

On test days the patient was fasted until 2 p.m. Heparinized plasma was obtained at 8 a.m. (0 h) for analysis of FFA (33) and AAN (34). The specified dose of test sub-

TABLE III

In Vivo Effects of hGH and Plasmin Digest of hGH on [³H]thymidine Incorporation into Cartilage DNA

Material	Dose	No. of rats	Incorporation		P
			Control	Hormone treated	
	μg/day		dpm/mg protein		
hGH	5.00	6	967 ±40*	2,320 ±436	<0.02
	3.75	6	1,062 ±46	2,535 ±666	NS
	2.50	6	851 ±43	1,529 ±202	<0.01
Plasmin digest	12.50	6	1,003 ±34	2,612 ±353	<0.01
	5.00	6	1,003 ±34	1,880 ±124	<0.01

The rats received intraperitoneal injections of saline or hormone 48 and 24 h prior to sacrifice. Cartilage segments were removed and incubated for 3 h in the presence of [methyl-³H]thymidine.
* Mean ±SEM.

TABLE IV

Diabetogenic Activity of hGH and a Plasmin Digest of hGH in 80% Pancreatectomized Rats Treated with Dexamethasone

Treatment	Dose	Production of glucosuria (no. responding/no. treated)
	mg/day/4 days	
Saline		1/10
hGH	0.1	9/11
Plasmin digest	0.1	2/6
Plasmin digest	0.5	5/6

80% pancreatectomized female rats were injected subcutaneously with dexamethasone (15 µg/day) and hGH or the plasmin digest of hGH twice daily for 4 days. The urine was tested for the presence of glucose during the treatment period.

stance, dissolved in 1 ml saline, or (as a control) 1 ml saline, was then injected intramuscularly. At 3 and 6 h, fasting plasma FFA and AAN concentrations were determined again. The 14-day assay, which was preceded by 3 days' equilibration to the metabolic diet, consisted of a 7-day control period followed by a 7-day experimental periol (31). The diet has been described before (31). During days 7–14, the patient received a daily injection of test material at 11 p.m. As before, responses were expressed as $\Delta N(g)$, $\Delta P(g)$, $\Delta Na(meq)$, and $\Delta K(meq)$: [(average daily elemental balance during treatment) minus (average daily balance during control period)] divided by (kg BW$^{\frac{3}{4}}$ × 10^{-1}) ; and as ΔBW: [(BW at end of treatment period) minus (BW at end of control period)] divided by (7 × kg BW$^{\frac{3}{4}}$ × 10^{-1}). In addition, fasting plasma FFA and AAN were measured on days 5, 6, and 7 of both control and experimental periods, and the average change in these two concentrations between control and experimental periods was calculated. On the day before the equilibration period and on the day after the last injection of test substance,

TABLE V

In Vitro Effects of hGH and Plasmin Digest of hGH on [³H]leucine Incorporation into Diaphragm Protein

Material	Conc.	No. of rats	Incorporation		P
			Hormone absent	Hormone present	
	µg/ml		*dpm/mg protein*		
hGH	5.00	6	1,560±103*	2,423±175	<0.01
	2.50	6	2,578±128	3,301±286	<0.05
	2.00	6	2,022±204	2,410±345	NS
Plasmin digest	10.00	6	1,544±96	2,073±156	<0.05
	5.00	6	1,544±96	1,918±132	<0.05
	2.50	6	1,544±96	1,920±55	<0.02

Hemidiaphragms were incubated for 60 min in the presence of [³H]leucine with or without the hormone as indicated.
* Mean ±SEM.

an oral glucose tolerance test was performed according to Fajans and Conn (35).

RESULTS

Analysis of the plasmin digests of hGH with trinitrobenzenesulfonic acid revealed that 2.3 new amino groups were formed, compared with the native hormone. Therefore, plasmin cleaved an average of 2.3 peptide bonds in each hormone molecule.

On disc electrophoresis, the digestion product of hGH was found to move faster toward the anode than the native hormone, but there was no striking change in the pattern of bands (Fig. 1). A very fast diffuse band of unknown significance was sometimes seen. Type A or B hGH was not visible in the electropherogram of the plasmin digest, indicating that less than 2% of the digest consisted of uncleaved hormone.

TABLE VI

In Vitro Effects of hGH and Plasmin Digest of hGH on AIB Transport in Rat Diaphragm

Material	Conc.	No. of pairs	Distribution ratio			P
			Hormone absent	Hormone present	Mean difference ±SE	
	µg/ml					
hGH	2.00	6	0.31	0.49	0.18±0.02	<0.01
	1.00	6	0.38	0.59	0.21±0.05	<0.01
	0.75	6	0.33	0.55	0.22±0.04	<0.01
	0.50	8	0.36	0.51	0.15±0.05	<0.05
	0.25	5	0.29	0.36	0.07±0.02	<0.05
	0.10	5	0.28	0.31	0.03±0.03	NS
Plasmin digest	25.00	6	0.51	1.10	0.60±0.07	<0.01
	2.00	6	0.51	0.78	0.27±0.09	<0.05
	0.50	6	0.35	0.52	0.17±0.04	<0.01

Hemidiaphragms were incubated for 60 min in the presence of [¹⁴C]AIB with or without the hormone is indicated.

TABLE VII

In Vitro Effects of hGH and Plasmin Digest of hGH on 3-0-Methyl-Glucose Transport in Diaphragms

Material	Conc.	No. of pairs	Distribution ratio			P
			Hormone absent	Hormone present	Mean difference ±SE	
	$\mu g/ml$					
hGH	1.00	6	0.11	0.63	0.52±0.02	<0.01
	0.50	6	0.14	0.50	0.36±0.04	<0.01
	0.25	6	0.04	0.30	0.26±0.06	<0.01
	0.10	6	0.06	0.15	0.09±0.03	<0.02
Plasmin digest	2.00	6	0.16	0.47	0.32±0.03	<0.01
	0.50	6	0.01	0.35	0.31±0.07	<0.01
	0.25	6	0.01	0.33	0.31±0.05	<0.01

Hemidiaphragms were incubated for 60 min in the absence or presence of the hormone as indicated. [3-0-methyl-^{14}C]glucose was present during the last 30 min of incubation.

Six different plasmin digests of hGH were tested for the ability to stimulate growth in hypophysectomized rats in the 9-day weight gain assay. As can be seen in Table II, the digests generally retained full growth promoting potency when compared to precursor hGH.

The experiments summarized in Table III indicate that the plasmin digest of hGH also retained the ability of the native hormone to stimulate [^{3}H]thymidine incorporation into the DNA of costal cartilage of hypophysectomized rats. The dose-response relationship for the plasmin digest was not characterized in detail, but since a dose of 2.5 μg/day of native hGH usually produced only a threshold response in this test system, the data suggest that plasmin digestion probably has little or no effect on the ability of the molecule to stimulate thymidine incorporation into cartilage.

Digestion of hGH with plasmin also did not abolish its diabetogenic activity in 80% pancreatectomized-dexamethasone treated rats (Table IV). However, the finding that a dose of 0.1 mg/day of the plasmin digest was not nearly as effective as an equivalent dose of native hGH in producing glucosuria in these animals suggests that the digest may have reduced diabetogenic potency in the rat.

Compared with native hGH, the plasmin digest was fully active in stimulating incorporation of labeled leucine into protein of the isolated diaphragm of the hypophysectomized rat. The concentration of hGH producing a threshold response in this test system was 2.5 μg/ml of medium, and this concentration of the plasmin digest also produced a significant response (Table V).

The digest was also fully active in vitro in stimulating amino acid transport and sugar transport into the isolated diaphragm of the hypophysectomized rat. The in vitro stimulatory effects of hGH and the digest on transport of AIB and 3-OMG into the diaphragm are summarized in Tables VI and VII, respectively.

The experiments shown in Table VIII indicate that

TABLE VIII

In Vitro Effects of hGH and Plasmin Digest of hGH on Glucose Utilization by Isolated Adipose Tissue

Material	Conc.	No. of pairs	$^{14}CO_2$ production			P
			Hormone absent	Hormone present	Mean difference ±SE	
	$\mu g/ml$		*dpm/mg/h*			
hGH	1.00	6	47.4	102.0	54.6±9.4	<0.01
	0.50	8	39.8	93.6	53.9±6.7	<0.01
	0.25	8	39.8	68.9	29.1±9.9	<0.05
	0.10	8	39.8	52.4	12.6±5.9	NS*
Plasmin digest	0.50	6	76.5	117.3	40.9±7.5	<0.01
	0.25	8	45.1	53.0	7.9±5.3	NS*

Epididymal fat pads were incubated for 60 min in the absence or presence of the hormone.
* NS = $P > 0.05$.

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TABLE IX

Metabolic Activity of Plasmin Digest of hGH in Man (14-day Assays)

Case	Test material	Dose	ΔN	ΔP	ΔNa	ΔK	ΔBW	ΔFFA	ΔAAN
			g*	g*	meq*	meq*	kg*	μeq/liter	mg/100 ml
1	hGH	A (0.0168 U/kg BW³)	−0.02	+0.01	−1.7	+2.1	−0.1	+120	+0.2
			±0.02	±0.02	±0.6	±1.7		±60	±0.04
	"	B (0.053 U/kg BW³)	+0.41	+0.03	+4.1	+1.3	0	−90	−1.0
			±0.05‡	±0.02	±2.2	±1.0		±40	±0.09‡
	"	C (0.168 U/kg BW³)	+0.93	+0.08	+6.5	+3.4	+0.07	+210	−1.5
			±0.07‡	±0.03	±2.7‡	±1.9		±100	±0.14‡
	Plasmin digest HMW600 (HS)A	0.0532 mg/kg BW³	+0.67	+0.07	+5.9	+4.8	+0.07	+100	−1.4
			±0.07‡	±0.03	±2.9	±2.2		±30	±0.10‡
	" " " "	0.168 mg/kg BW³	+1.20	+0.10	+10.2	+6.1	+0.08	+250	−2.1
			±0.10‡	±0.04	±2.8‡	±1.4‡		±110	±0.19‡
2	hGH	A (0.0168 U/kg BW³)	0	−0.03	−2.0	1.3	0	+80	0.0
			±0.01	±0.02	±1.5	±0.5		±30	±0.02
	"	B (0.053 U/kg BW³)	+0.48	+0.03	+5.2	+1.8	+0.01	+190	−1.3
			±0.07‡	±0.01	±2.9	±0.7		±60	±0.28
	"	C (0.168 U/kg BW³)	+0.95	+0.07	+6.0	+5.1	+0.05	+140	−1.2
			±0.08‡	±0.03	±2.0‡	±1.2		±70	±0.10‡
	Plasmin digest HMW600 (HS)A	0.0532 mg/kg BW³	+0.76	+0.08	+5.3	+3.8	+0.05	+200	−0.8
			±0.07‡	±0.02	±2.6	±1.7		±40	±0.06‡
	"	0.168 mg/kg BW³	+1.4	+0.11	+12.1	+6.2	+0.08	+310	−1.9
			±0.09‡	±0.03‡	±3.4‡	±1.8‡		±140	±0.15‡
3	hGH	B (0.053 U/kg BW³)	+0.51	+0.08	+9.1	+2.0	+0.03		
			±0.08‡	±0.03	±2.0‡	±0.9‡			
	"	C (0.168 U/kg BW³)	+1.50	+0.18	+10.1	+7.4	+0.09		
			±0.09‡	±0.05‡	±3.1‡	±1.6‡			
	Plasmin digest HS 605	0.168 mg/kg BW³	+0.78	+0.11	+6.8	+4.4	+0.05		
			±0.05‡	±0.05‡	±3.4	±1.0‡			
4	hGH	A (0.0168 U/kg BW³)	−0.23	0	+2.7	−1.3	+0.01	−60	+0.2
			±0.05	±0.01	±2.0	±0.4		±30	±0.03
	"	B (0.053 U/kg BW³)	+0.86	−0.04	+5.8	+3.1	+0.03	+110	−0.7
			±0.05‡	±0.02	±3.1	±0.9		±40	±0.21
	"	C (0.168 U/kg BW³)	+1.56	+0.09	+6.5	+4.7	+0.07	+180	−1.9
			±0.11‡	±0.02‡	±1.9‡	±0.8‡		±40	±0.11‡
	Plasmin digest HMW600 (HS)A	0.0532 mg/kg BW³	+0.61	+0.04	+5.3	+3.8	+0.02	+240	−1.5
			±0.07‡	±0.02	±2.5	±0.7		±90	±0.10‡
	" " " "	0.168 mg/kg BW³	+1.40	±0.12	+9.2	+6.9	+0.09	+280	−1.7
			±0.20‡	±0.03‡	±2.9‡	±1.0‡		±100	±0.12‡
6	hGH	B (0.053 U/kg BW³)	+0.51	+0.08	+9.1	+2.0	+0.03		
			±0.06‡	±0.03	±2.4	±1.3			
	"	C (0.168 U/kg BW³)	+1.50	+0.18	+10.1	+7.4	+0.09		
			±0.14‡	±0.05‡	±2.8‡	±1.9‡			
	Plasmin digest HS 605	0.168 mg/kg BW³	+0.78	+0.11	+6.8	+4.4	+0.05		
			±0.07‡	±0.03‡	±3.0	±1.0‡			
9	hGH	B (0.053 U/kg BW³)	+0.15	+0.01	+1.5	+1.1	+0.02		
			±0.02	±0.02	±0.5	±0.5			

Each value represents [mean elemental balance, BW, FFA, or AAN concentration during experimental period] minus [corresponding mean during control period] ±SE of this difference.

* Per kg BW³ × 10⁻¹/day.

‡ $P < 0.05$ for the change in elemental balance, plasma free fatty acids (FFA) or plasma α-amino nitrogen (AAN), between experimental and control periods.

TABLE IX—*(Continued)*

"		C(0.168 U/kg BW‡)	+0.91 ±0.10‡	+0.11 ±0.05	+9.0 ±2.8‡	+3.3 ±1.7	+0.08
Plasmin digest HS 605		0.168 mg/kg BW‡	+0.78 ±0.06‡	+0.09 ±0.05	+7.4 ±2.9‡	+2.3 ±1.5	+0.08
10	hGH	B (0.053 U/kg BW‡)	+0.24 ±0.03	+0.01 ±0.02	+4.9 ±2.1	+2.5 ±1.9	+0.01
	"	C (0.168 U/kg BW‡)	+0.51 ±0.04‡	+0.05 ±0.03	+8.1 ±2.4	+6.5 ±3.1	+0.06
Plasmin digest HS 605		0.168 mg/kg BW‡	+0.40 ±0.04‡	+0.06 ±0.03	+10.5 ±3.1‡	+3.1 ±2.0	+0.03
11	hGH	B (0.053 U/kg BW‡)	+0.25 ±0.03	+0.03 ±0.02	+3.2 ±2.1	+1.6 ±1.0	0
	"	C (0.168 U/kg BW‡)	+1.3 ±0.10‡	+0.15 ±0.04‡	+6.1 ±2.5	+4.3 ±2.9	+0.08
Plasmin digest HS 605		0.168 mg/kg BW‡	+0.91 ±0.06‡	+0.08 ±0.03	+8.5 ±1.9‡	+6.7 ±2.0‡	+0.05

the digest, when added in vitro, also retained the ability to stimulate glucose utilization in isolated epididymal adipose tissue of the hypophysectomized rat. The concentration-response data suggest that, on a weight basis, the digest may be somewhat less potent that the native hormone in this test system.

To assess the metabolic activity of the plasmin digest of hGH in man, 14-day assays were performed on eight subjects, and 1-day assays were carried out on four subjects. Table IX summarizes the 14-day experiments. Native hGH caused a significant $(P < 0.05)$ retention of N at doses A, B, and C in zero, five, and eight individuals, respectively. Significant effects on P, Na, and K balance occurred with less regularity: dose A was ineffective in three trials; dose B caused significant P, Na, and K retention in only zero, one, and one of eight subjects; dose C stimulated retention of these elements in four, six, and three of eight persons, respectively. Weight gain also occurred.[2] Ratios of ΔN, ΔNa, and ΔK (36) showed about one-half the weight gain to represent protoplasm and about one-half extracellular fluid. Dose C of hGH caused a significant $(P < 0.05)$ reduction in fasting plasma AAN at days 5–7 of the 7-day course of treatment in each of three subjects but did not alter fasting FFA level significantly. Dose B lowered AAN significantly in one of three trials and dose A in none of three trials. Dose C reduced glucose tolerance in two of three patients examined for this response (Fig. 2).

The two preparations of the plasmin digest tested in these subjects reproduced all of the effects of the intact

hormone molecule. In five subjects, 0.168 mg/kg BW$^{3/4}$ of plasmin digest HS 605 produced increases in elemental balances and body weight gain intermediate in magnitude between those elicited with doses B and C of clinical grade native hGH (Table IX). Significant effects in ΔN, ΔP, ΔNa, and ΔK occurred in five, two, three, and three out of five trials, respectively. In one patient (case 11) examined in this respect, 0.168 mg/kg BW$^{3/4}$ of digest HS 605 caused a reduction in glucose tolerance similar to that produced in the same patient by dose C of hGH. Digest HMW600 (HS)A tested in three individuals was generally more potent than HS 605 (Table IX). At the 0.053 and 0.168 mg/kg BW$^{3/4}$ doses, this preparation caused retention of N, P, Na, K, and weight gain to a slightly greater extent than doses B and C of hGH, respectively. The 0.053 mg/kg BW$^{3/4}$ dose caused significant retention of N, P, Na, and K in three, zero, zero, and zero subjects. At 0.168 mg/kg BW$^{3/4}$, significant retention occurred in three, two, three, and three individuals. Both doses caused significant reduction in plasma AAN but did not alter FFA concentration. In cases 1 and 2, digest HMW600 (HS)A at the 0.168 mg/kg BW$^{3/4}$ dose reduced glucose tolerance to about the same extent as dose C of hGH (Fig. 2). In case 4, despite a brisk anabolic response to these doses of HMW600 (HS)A and hGH (Table IX), glucose tolerance remained unchanged (Fig. 2).

When the 1-day assay was employed in four subjects with GH-deficiency dose C of hGH lowered plasma AAN and increased plasma FFA significantly $(P < 0.05)$; dose B affected only FFA (Table X). Plasmin digest HMW600 (HS)A at doses 0.053 and 0.168 mg/kg BW$^{3/4}$ produced significant reduction and elevation of plasma AAN and FFA, respectively.

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[2] P value could not be estimated for ΔBW in response to hGH, since this response was calculated as the change in BW over a 7-day period divided by 7, without estimate of variance.

DISCUSSION

The above findings indicate that human plasmin cleaves only 2–3 bonds in the hGH molecule. Disc gel electrophoresis indicated that less than 2% of undigested native hormone remained in the digests. Although the digest traveled more rapidly toward the anode during electrophoresis than the native hormone, there was no evidence of the formation of more major components than were present prior to reaction of the native hormone with plasmin. This agrees with the limited cleavage predicted by the appearance of only 2.3 new amino groups at the completion of digestion. Work now in progress on the fractionation of the plasmin digest on Sephadex G-50 reveals that the action of the enzyme is mainly to set free a hexapeptide corresponding to residues 135–140 in native hGH. This small basic peptide would not necessarily appear in the gel electrophoretic pattern, but it would account for the greater anodal mobility of the residual major component and for the absence of new components in the electrophoretic pattern.

The plasmin digests of hGH retained all the metabolic actions of the native hormone in the rat: (a) to promote growth in hypophysectomized rats, (b) to stimulate thymidine incorporation into costal cartilage of hypophysectomized rats, (c) to cause glucosuria in 80% pancreatectomized-dexamethasone treated rats, (d) to stimulate protein synthesis in isolated diaphragms of hypophysectomized rats when added in vitro, (e) to stimulate amino acid transport and sugar transport into isolated diaphragms from hypophysectomized rats when added in vitro, and (f) to stimulate in vitro glucose utilization by isolated adipose tissue of hypophysectomized rats. It was of interest to examine the effects of the digests in this battery of assays that reflect the anabolic, diabetogenic, and insulin-like properties of hGH, because of the possibility that cleavage of the hormone
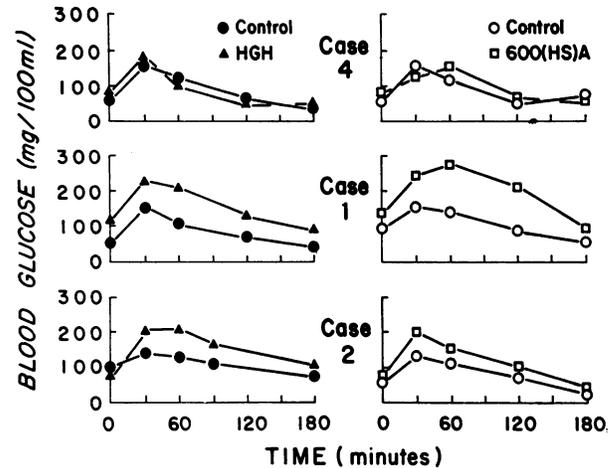

FIGURE 2 Effect of 7 days' treatment with dose C of hGH or 0.168 mg/kg BW$^{\frac{3}{4}}$ of plasmin digest HMW600 (HS)A on the oral glucose tolerance curve of three patients. The control experiment was done 10 days before the first injection of the hormonal material.

molecule with plasmin might result in the loss of one or more of these activities. In previous studies (26) of the effects of cleavage of hGH and porcine growth hormone with cyanogen bromide, we found that the large fragment (corresponding approximately to the amino terminal 2/3 of the hormone) produced by this cleavage retained the ability to stimulate protein synthesis in liver and muscle tissue and the insulin-like property of the native hormone but lost the capacity to promote growth or stimulate thymidine incorporation into rat costal cartilage. In contrast, the present study shows that cleavage of hGH with plasmin does not dissociate the various metabolic properties of the intact hormone molecule. Furthermore the digests showed little or no reduction in potency in the various animal assays. In this regard, it should be

TABLE X
Results of 1-day assays in Cases 5, 6, 7, and 8

Test material	Dose	FFA			AAN		
		0 h	3 h	6 h	0 h	3 h	6 h
		μeq/liter			mg/100 ml		
Saline	1 ml	430±50	510±70	630±80	6.5±0.5	6.3±0.4	6.3±0.2
hGH	A (0.0168 U/kg BW$^{\frac{3}{4}}$)	510±70	610±80	700±90	6.3±0.2	6.3±0.3	6.1±0.3
hGH	B (0.053 U/kg BW$^{\frac{3}{4}}$)	540±40	650±70	790±100*	6.8±0.4	5.9±0.2	5.4±0.5
hGH	C (0.168 U/kg BW$^{\frac{3}{4}}$)	560±70	750±80	920±90*	6.1±0.3	5.3±0.4	5.1±0.2*
Plasmin digest of hGH HMW600 (HS)A	0.0532 mg/kg BW$^{\frac{3}{4}}$	490±60	690±70	880±110*	6.7±0.3	6.2±0.3	5.2±0.3*
Plasmin digest of hGH HMW600 (HS)A	0.168 mg/kg BW$^{\frac{3}{4}}$	560±90	810±90	1070±130*	6.4±0.4	5.5±0.2	4.8±0.4*

Each dose was tested twice in each patient. Values represent average ±SE (n = 8).
* $P < 0.05$ for difference between this value and corresponding value at 0 h.

noted that the plasmin remaining in the digest preparations probably had no influence on the effects obtained in the animal assays. Studies now in progress indicate that a large fragment of hGH can be separated from the other components of the digest by chromatography on Sephadex G-50, and this purified fragment is fully active in the various animal assays used in the present study (37).

The experiments in Tables IX and X showed the plasmin digests also to be active in a variety of metabolic assays in human subjects, thus establishing that cleavage of the hGH molecule with plasmin does not substantially alter its properties in humans. One of the digests tested in human subjects, HS 605, was somewhat less active than the other, HMW600 (HS)A. In this connection, it is of interest that HS 605 also appeared to be less active than the precursor hGH from which it was made in the weight gain test in hypophysectomized rats (see Table II).

These experiments prove that the entire hGH sequence is not required for full anabolic potency in both the rat and man. The fully active plasmin digest becomes a useful starting material in searching for the minimum structural requirement for growth-promoting activity in man. The next step will be isolation of the active fragment in the plasmin digest and determination of its structure, followed by further fragmentation of this derivative and assay of its subunits.

ACKNOWLEDGMENTS

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