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Human Pituitary Growth Hormone:* Isolation and Properties of Two Biologically Active Fragments from Plasmin Digests

(tibia test/prolactin activity/complement fixation

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ABSTRACT Two biologically active fragments have been isolated from plasmic digests of human pituitary growth hormone. It was shown that these two fragments were derived from the cleavage of the Arg-Thr (positions 134–135) and the Lys-Gln (positions 140–141) bonds of the hormone: one has 134 amino acids and the other 51 amino acids, respectively. The two fragments were active in the rat tibia and pigeon crop-sac tests, as well as in complement fixation experiments.

It has been known for some time that human pituitary growth hormone (HGH) retained biological activity after limited digestions with chymotrypsin (1), trypsin (2), and pepsin (3). Recently, various investigators (4–7) reported that hydrolysis with plasmin did not diminish biological activity of HGH. However, no detailed chemical characterization of the cleaved products has been carried out in any of these studies. This communication reports the chemical and biological properties of two fragments isolated from plasmin digests of HGH.

MATERIALS AND METHODS

HGH was prepared from fresh human pituitary glands by a procedure described by Li *et al.* (8). Highly purified human plasminogen (20–22 casein units per mg of proenzyme) was kindly supplied by Dr. E. Shaw of Brookhaven National Laboratory, Upton, N.Y. Plasminogen was activated with streptokinase (Calbiochem) according to Robbins and Summaria (9). Human pituitary growth hormone was digested by plasmin in 0.05 M ammonium acetate buffer, pH 8.0, with an enzyme to hormone ratio of 1:100 (w/w) at 37°C for 5–30 hr. The plasmic digestion was followed by polyacrylamide gel electrophoresis and by NH_2-terminal residue analysis. The digest was submitted to purification by gel filtration on Sephadex G-75 and chromatography on DEAE-cellulose.

The disulfide bridges were reduced with 20 M excess of dithiothreitol (Calbiochem) over disulfide content in the presence of 8 M urea at pH 8.4 under nitrogen for 1 hr. Alkylation was carried out under the same conditions by addition of iodoacetamide (Calbiochem) in a 20-fold excess over reducing agent. The excess reagents were removed by gel filtration of the reaction mixture on Sephadex G-15 in 0.01 M NH_4HCO_3 pH 8.4. The plasmin fragments were separated and purified by gel filtration on Sephadex G-50 and partition chromatography on Sephadex G-25.

Digestions with carboxypeptidase A and B (Worthington) were carried out in 0.1 M $NaHCO_3$ with an enzyme to peptide ratio of 1:10 (w/w) for 15 hr at 37°C. Amino acid compositions of acid and carboxypeptidase hydrolysates were determined in an automatic amino acid analyzer (model 120, Beckman Instruments) by the procedure of Spackman *et al.* (10). For the analysis of tryptophan, the method of Liu and Chang (11) was used. NH_2-terminal residue analysis was performed by the dansyl procedure (12, 13). Disc electrophoresis in polyacrylamide gel (7%) was carried out in Tris·glycine buffer pH 8.3 (14) for 60 min at 100 V with a current of 5 mA per tube (7.5 × 0.5 cm). The gels were stained with Amido Black and destained by electrolysis in 7% acetic acid.

Growth-promoting and prolactin activities were measured by the rat tibia (15) and pigeon crop-sac assays (16), respectively. Microcomplement fixation experiments were performed by the procedure of Wasserman and Levine (17) with the guinea pig antisera to HGH.

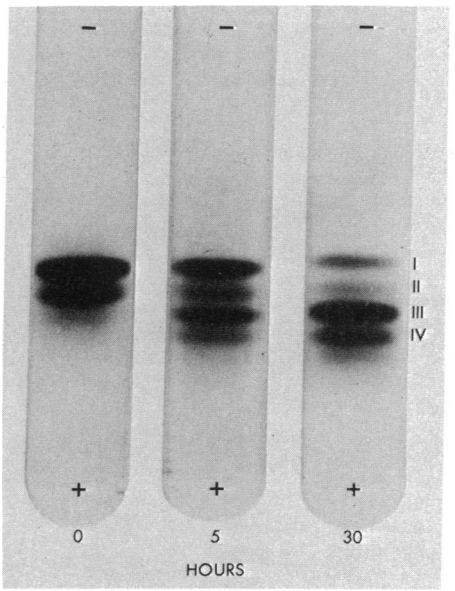

FIG. 1. Disc electrophoresis of HGH (40 μg) after 0, 5, and 30 hr of digestion with plasmin.

Abbreviations: HGH, human pituitary growth hormone; RA, reduced and alkylated.

* Paper XXXIX in the Human Pituitary Growth Hormone series.

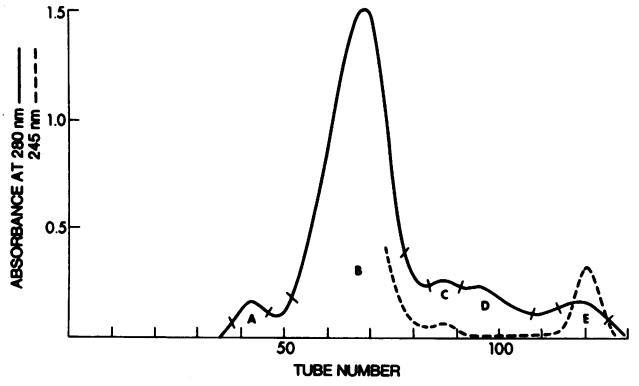

FIG. 2. Chromatography of plasmic digest of 500 mg of HGH on Sephadex G-75 (136 × 3.4 cm) in 20% acetic acid; tube volume, 10 ml; 50 ml/hr.

RESULTS

As shown in Fig. 1, HGH was resolved into two electrophoretic components (I and II). It is known that component II represents a deamidated form of the hormone (18). During the course of plasmic digestion, HGH was converted into two faster moving components (III and IV), as revealed by disc electrophoresis (Fig. 1), and two new NH_2-terminal residues, Thr and Glx, were detected in the digest. Some additional NH_2-terminal residues (Tyr, Ser, and Asx) also appeared in relatively smaller amounts as the digestion progressed, indicating further cleavages of the peptide chain. In order to restrict these less specific cleavages, the digestion was terminated after 20 hr.

The 20-hr plasmic digest of HGH (500 mg) was separated into five fractions (Fig. 2) by gel filtration on Sephadex G-75 in 20% acetic acid with the following yields: A, 25 mg; B, 360 mg; C, 25 mg; D, 35 mg; and E, 8 mg. Disc electrophoresis revealed that fraction B contained chiefly electrophoretic component III (Fig. 3), whereas fractions† A, C, D, and E were free of this component. Hence fraction B (300 mg) was further purified by chromatography on DEAE-cellulose (Fig. 4). Two fractions were obtained: B1 (45 mg) and B2 (180 mg).

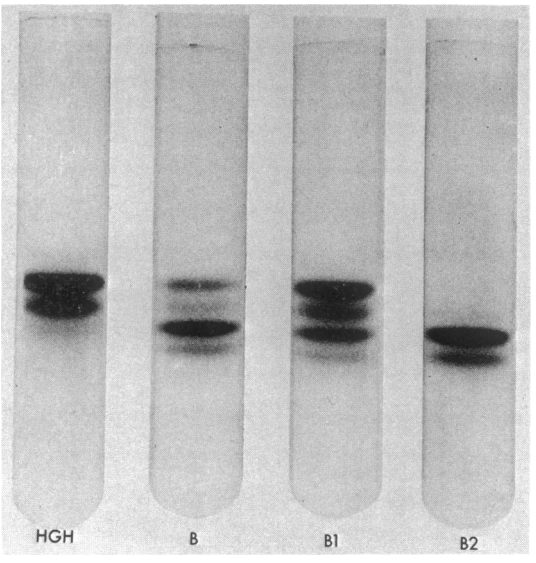

FIG. 3. Disc electrophoresis of HGH, fraction B (Fig. 2), and fractions B1 and B2 (Fig. 4); 40-μg samples.

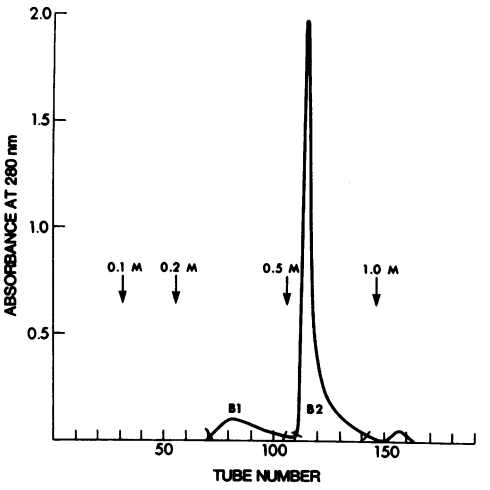

FIG. 4. Chromatography of 300 mg of material from peak B (Fig. 2) on DEAE-cellulose (20 × 2.3 cm). The column was equilibrated with 0.03 M NH_4HCO_3 buffer pH 8.0. Stepwise elution was performed with NH_4HCO_3 buffer pH 8.0, with concentrations indicated in the figure; tube volume, 8 ml; 80 ml/hr.

Disc electrophoresis (Fig. 3) showed that B1 contains mainly undigested HGH and B2 is free from the native hormone. End-group analysis of B2 revealed that Phe is the major NH_2-terminal residue with a detectable amount of Tyr and Glx. Amino acid composition of B2 is almost identical to that of HGH (Table 1). Its biological activity, as determined by the rat tibia test, was practically the same as that of native HGH (Table 2).

Fraction B2 (100 mg) was next reduced by dithiothreitol in 8 M urea and alkylated with iodoacetamide; the product (designated as RB2) was submitted to gel filtration on Sephadex G-50 in 10% acetic acid (Fig. 5). Two main fractions were obtained: RB2a (45 mg) and RB2c (22 mg).

RB2a was found to elute as a single symmetrical peak when rechromatographed on Sephadex G-75 in 20% acetic acid; it was also shown to be homogeneous by disc electrophoresis (Fig. 5), and had only phenylalanine as the NH_2-terminal residue. No amino acid could be liberated by carboxypeptidase A, but 0.7 mol of arginine per mole of protein was released in the carboxypeptidase B digest. Together with the results of the amino acid analysis (Table 1), it is evident that RB2a represented the NH_2-terminal portion, residues 1–134, of the HGH structure (Fig. 6; and ref. 19).

After rechromatography on the same Sephadex column in 10% acetic acid, RB2c (10 mg) was further purified by partition chromatography (20) in a solvent system consisting of *n*-butanol–pyridine–0.1% aqueous acetic acid (40:24:96 by volume). As shown in Fig. 5, the main peak (RB2c I, 6.5 mg) came off the partition column with an R_F value of 0.50. Disc electrophoresis of RB2c I did not show a visible band with Amido Black staining. The NH_2-terminal residue of RB2c I could not be identified as it was not accessible† for reaction with dansyl chloride; digestion with carboxypeptidase A released 1 mol of phenylalanine with a small quantity of glycine.

† The lack of NH_2-terminal residue is presumably due to the cyclization of the NH_2-terminal glutamine during the chromatography in 20% acetic acid.

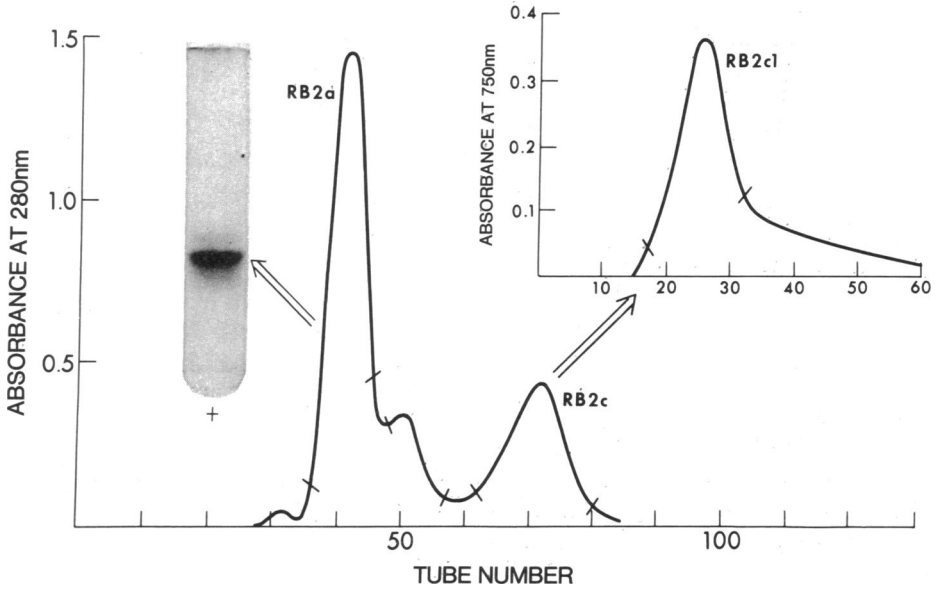

FIG. 5. Chromatography of RB2 (100 mg) on Sephadex G-50 (85 × 2.8 cm) in 10% acetic acid; tube volume, 4 ml; 30 ml/hr. Partition chromatography of RB2c on Sephadex G-25 (60 × 2.0 cm); tube volume, 3 ml; 0.2-ml aliquots were analyzed for protein content by the Folin–Lowry method. Disc electrophoretic pattern of RB2a (40 μg) is also included.

Together with the amino-acid composition (Table 1), RB2c I must be derived from residues 141–191 of HGH (Fig. 6).

The results of bioassay, summarized in Table 2, indicated that RB2a and RB2c I are biologically active, but less active when compared with the native hormone. Fig. 7 presents the complementation fixation behavior of RB2a and RB2c I; it is evident that both fragments are immunologically active.

DISCUSSION

The above data indicate that the predominant action of plasmin on HGH is the cleavage of the Arg-Thr and Lys-Gln bonds at positions 134–135 and 140–141, respectively (see Fig. 6). Thus, the main product of a limited plasmic digestion of HGH is composed of the NH_2-terminal portion, residues 1–134, and the COOH-terminal portion, residues 141–191, of

TABLE 1. *Amino-acid composition* of plasmic fragments from HGH*

	RB2a‡		RB2cI‡		B2¶		
Amino acid	Exp.	1–134§	Exp.	141–191§	Exp.	[(1–134) + (141–191)]§	HGH†
Lys	4.2	4	3.8	4	8.4	8	9
His	1.8	2	0.9	1	3.0	3	3
Arg	7.8	8	2.9	3	11.0	11	11
SCM-Cys	1.0	1	3.1	3	0	0	0
Asp	12.4	12	8.2	8	20.7	20	20
Thr‖	5.9	6	3.3	3	8.9	9	10
Ser‖	13.7	14	3.9	4	17.7	18	18
Glu	22.2	22	4.2	4	26.0	26	27
Pro	8.6	8	0.2	0	8.7	8	8
Gly	4.2	4	3.0	3	8.2	7	8
Ala	6.2	6	1.2	1	7.4	7	7
½Cys	0	0	0	0	4.5	4	4
Val	4.2	4	2.7	3	7.5	7	7
Met	2.0	2	1.1	1	3.0	3	3
Ile	5.6	6	1.0	1	6.1	7	8
Leu	20.6	21	5.3	5	25.1	26	26
Tyr	5.3	5	2.8	3	8.4	8	8
Phe	7.9	8	4.0	4	11.9	12	13
Trp	0.9	1	0	0	0.9	1	1

* Molar ratios.
† From the amino-acid sequence (see Fig. 6).
‡ See Fig. 5.
§ Residue positions in the HGH structure (Fig. 6).
¶ See Fig. 4.
‖ Corrected for destruction.

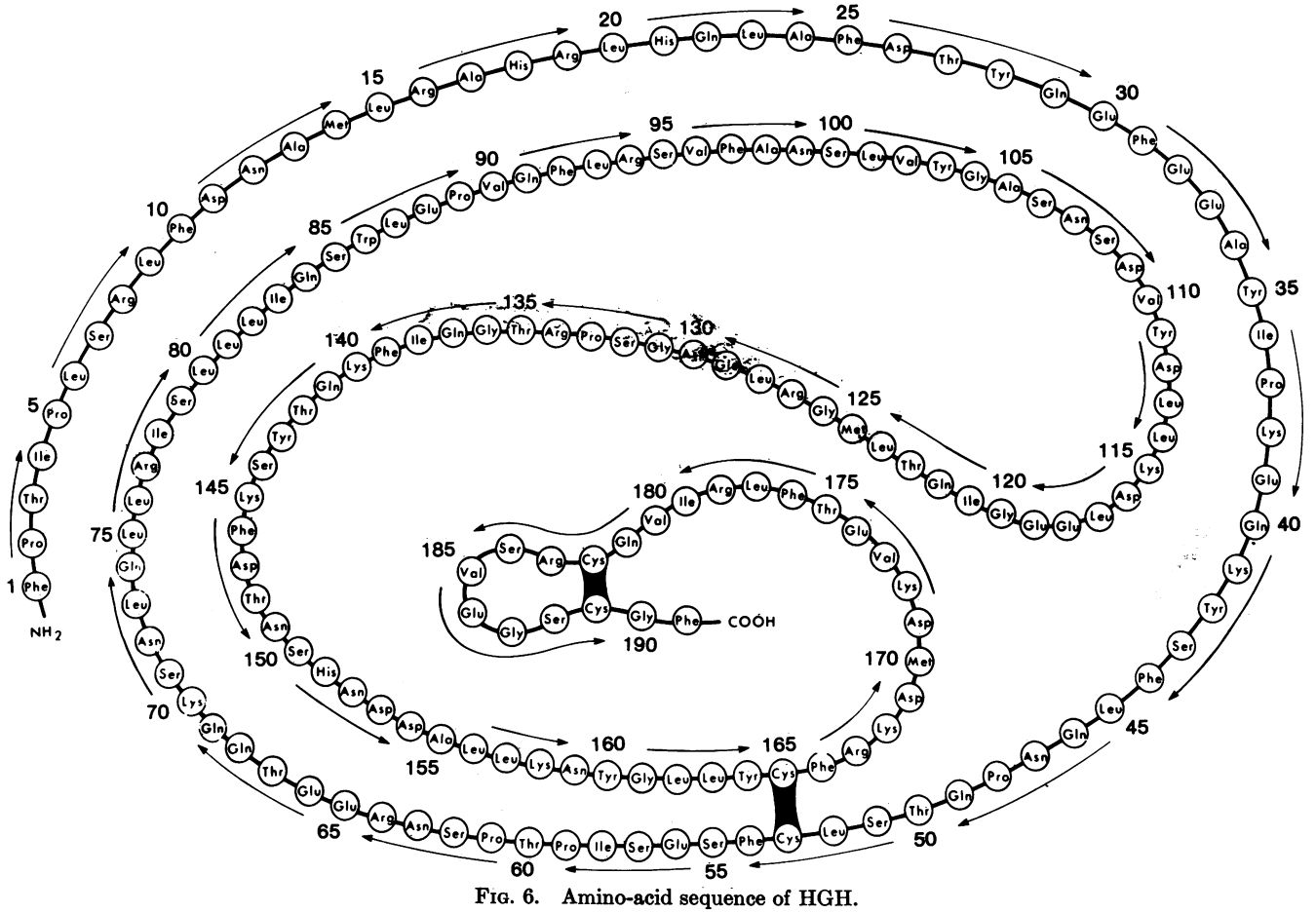

FIG. 6. Amino-acid sequence of HGH.

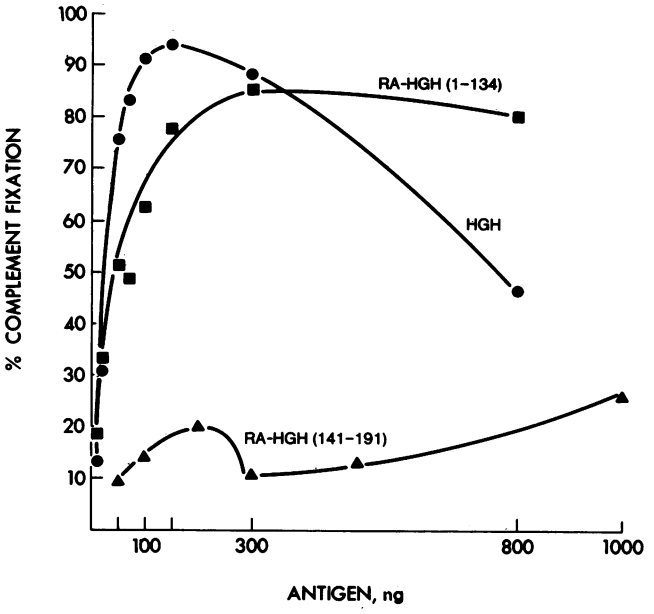

FIG. 7. Complement fixation of RB2a [RA-HGH-(1-134)] and RB2cI [RA-HGH-(141-191)] with the guinea pig antiserum to HGH.

HGH (peak B2 of Fig. 4). These are connected to each other by a disulfide linkage at sequence positions 53 and 165 (Fig. 6). The missing hexapeptide‡ (residues 135–140) was found in fraction E of Fig. 2. The removal of this small peptide from the structure of HGH results in a greater negative net charge on the molecule. This alteration was reflected in the electrophoretic change before and after plasmic hydrolysis (Figs. 1 and 3).

It is probable that the largest component of the plasmic digested HGH (B2) is similar or identical to β-HGH, a spontaneously degraded form of HGH (21); Lewis *et al.* reported that this derivative of HGH shows activity closely similar to intact HGH when measured by the tibia test. Our tibia assay data on fraction B2 (Table 2) also indicated unaltered biological potency. This apparently contradicts the observations of Yadley *et al.* (7), who reported plasmic-digested HGH to be more potent than native HGH. The question may be raised as to whether or not their plasmin-digested HGH products are chemically identical to ours.

Since it has been demonstrated that the reduction and carbamidomethylation of HGH does not influence the biological activity (22), it was of interest to determine if, when separated, the two portions of B2 originally connected by the disulfide bridges still have the full activity of intact B2. As shown in Table 2, both fragments have some response with

‡ Preliminary studies showed that whereas fraction A is undigested HGH, B and C are complex mixtures. Fraction E appeared to be homogeneous when examined by paper electrophoresis at pH 2.1. The amino-acid composition of E was found to be: $Lys_{1.0}$, $Thr_{0.9}$, $Glu_{1.1}$, $Gly_{1.1}$, $Ile_{0.8}$, $Phe_{1.0}$.

TABLE 2. *Biological potency of plasmic fragments of HGH, as measured by rat tibia and pigeon crop-sac tests*

	Rat tibia test		*Pigeon crop-sac test*	
Material*	Total dose (μg)	Tibia width (μm)	Total dose (μg)	Dry mucosal weight† (mg)
Saline	0	169 ± 11 (10)	0	10.7 ± 1.3 (4)
HGH	20	220 ± 9 (5)	2	23.7 ± 2.5 (4)
	60	263 ± 11 (5)	6	32.3 ± 3.7 (4)
B2	20	237 ± 4 (6)	5	29.4 ± 2.1 (4)
	60	273 ± 5 (5)		
RB2a	100	225 ± 9 (5)	20	26.4 ± 0.9 (4)
	300	261 ± 18 (6)	60	29.6 ± 2.0 (4)
RB2cI	100	202 ± 9 (5)	20	17.7 ± 2.2 (4)
	300	226 ± 8 (5)	60	20.8 ± 3.3 (4)

* B2, RB2a, and RB2c I, see Figs. 4 and 5. RB2a represents the fragment RA-HGH (1-134) and RB2c I fragment RA-HGH (141–191).

† Mean ± standard error of the mean; number of animals is indicated in parentheses.

either the tibia or the pigeon crop-sac assay. The biological activity of the NH_2-terminal 1–134 fragment of RA-HGH is 10–20% of that for native HGH in both tests. It is of interest to note that the COOH-terminal fragment (residues 141–191) of RA-HGH also exhibits activity in both tests, although it is less active in comparison with RA-HGH (1–134). It should be noted that both fragments are immunologically active,§ as revealed by complement fixation experiments (Fig. 7); RA-HGH-(1–134) is more potent than the COOH-terminal fragment in fixing complements.

The fact that the biological activities (Table 2) of RA-HGH-(1–134) and RA-HGH-(141–191) are less than that of B2 (Fig. 4, Table 1) or the native hormone shows that a certain interaction between the two large portions of HGH (stabilized either by the disulfide bridge in the case of B2, or by the integrity of the peptide chain in the case of the reduced and carbamidomethylated HGH) is necessary for the full biological activity of the hormone. However, experimental data herein described provide conclusive evidence that molecular integrity of HGH is not required for its biological behavior, and that peptide fragments obtained by plasmin hydrolysis of the HGH molecule are biologically active.

§ Preliminary radioimmunoassay data with the same antisera confirm the observations that both fragments are immunologically active.

Note Added in Proof. Preliminary investigations with K. Kawauchi on the circular dichroism of the biologically active fragments of HGH indicated that the NH_2-terminal fragment (RB2a) possesses approximately 40% α-helix content in solutions of pH 8.2. The COOH-terminal fragment (RB2cI) does not appear to contain α-helix but instead appears to have 20–30% β-sheet structure.

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