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Biological Properties of Plasmin Digests of *S*-Carbamidomethylated Human Growth Hormone

(human plasmin/weight gain test/cartilage metabolism/glucose oxidation)

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ABSTRACT **Reduction and carbamidomethylation of the intrachain disulfide bridges of human growth hormone did not destroy its ability to stimulate weight gain or cartilage metabolism in hypophysectomized rats. The reduced and alkylated hormone also stimulated glucose oxidation in isolated adipose tissue of hypophysectomized rats when added *in vitro*. When the *S*-carbamidomethylated hormone was incubated overnight with human plasmin, approximately 95% of the starting material was completely digested, as judged by polyacrylamide gel electrophoresis. The plasmin digest retained the ability to stimulate weight gain, cartilage metabolism, and glucose oxidation. A fraction consisting of two major electrophoretic components was isolated from the digest by chromatography on Sephadex G-50. This fraction possessed the biological properties of the whole digest.**

Plasmin digests of human growth hormone (hGH) retain the biological properties of the native hormone in the rat and man (1–4). The active component of the digests has been isolated and shown to consist of amino-acid residues 1–134 attached to residues 141–191 by the disulfide bond between residues 53 and 165 (3, 4). Reduction and *S*-carbamidomethylation of this large fragment produces two peptides, one consisting of residues 1–134 (peptide 2α) and the other comprising residues 141–191 (peptide 2β). Peptide 2α exhibits a high degree of biological activity *in vitro* but appears to be quite labile when injected subcutaneously or intraperitoneally into the rat. It must be injected intravenously in order to obtain the full expression of its biological activity in the whole animal (1, 3). Peptide 2β appears to be biologically inactive.

Since these studies suggested that the biological activity of growth hormone resides in the amino-acid sequence comprising peptide 2α, it was of interest to explore alternative and more efficient means to produce substantial amounts of this peptide for further fragmentation studies. As indicated above, the primary action of plasmin on native hGH is the cleavage of two peptide bonds resulting in the removal of the hexapeptide consisting of residues 135–140. The possibility was considered that plasmin might have the same mode of attack on reduced and *S*-carbamidomethylated hGH (RCAM-hGH) resulting in the production of peptide 2α directly. Thus in the present study, hGH was first reduced and *S*-carbamidomethylated and then digested with human plasmin. The biological properties of such digests are described below.

MATERIALS AND METHODS

Human growth hormone was dissolved in 1.4 M Tris·HCl buffer at pH 8.6 and brought to a concentration of 10 mg/ml. Reduction of its disulfide bonds was accomplished by the addition of solid dithiothreitol to give a 5-fold molar excess over protein disulfide. This solution was allowed to stand at room temperature for 30 min, and then solid iodoacetamide was added to give a 4.8-fold molar excess over dithiothreitol. The solution was then allowed to stand in the dark for 15 min, after which it was dialyzed against distilled water and lyophilized. The degree of reduction and alkylation was assessed by amino-acid analysis of the product and found to be complete.

Digestion of the *S*-carbamidomethylated hormone with human plasmin (KABI, Stockholm, Sweden) and fractionation of the digestion products by gel filtration were carried out as previously described for native hGH (2, 3).

Materials prepared as described above were assayed for biological activity in three test systems. The 9-day weight gain test (5) was used to quantitate the growth-promoting activity of each preparation relative to the International Growth Hormone Standard. Hypophysectomized female rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing approximately 100 g were used for this purpose. In addition, each preparation was tested for the ability to stimulate cartilage metabolism in female hypophysectomized rats (Hormone Assay Laboratories, Madison, Wisc.) weighing 65–86 g. The test materials were administered intraperitoneally to the animals over a 48-hr period. The costal cartilages were then removed and incubated with [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as previously described (2). Results are expressed as dpm/mg of cartilage protein. Also, the preparations were tested for the ability to stimulate *in vitro* the production of $^{14}CO_2$ from D-[^{14}C]glucose (uniformly labeled; 15 mCi/mmol; New England Nuclear Corp.) by isolated epididymal adipose tissue of male hypophysectomized rats (Hormone Assay Laboratories) weighing approximately 100 g. The details of the procedure used have been previously described (2). Results are expressed in terms of dpm of $^{14}CO_2$ produced/mg of wet tissue per hr.

RESULTS

Reduction and *S*-carbamidomethylation of hGH did not markedly alter the growth-promoting activity of the hormone. Table 1 compares the growth-promoting potency of a number of preparations of RCAM-hGH with that of several preparations of highly purified native hGH. While the relative potencies of some of the RCAM-hGH preparations were less than 1 IU/mg, the confidence limits of the assays were such that they overlapped those obtained with native hGH, suggesting no reduction in growth-promoting activity. RCAM-hGH was also quite active in stimulating the uptake of [^{3}H]-

Abbreviations: hGH, human pituitary growth hormone; RCAM-hGH, reduced and *S*-carbamidomethylated human growth hormone.

TABLE 1. *Activity of plasmin digests of RCAM-hGH in the weight gain test in hypophysectomized rats*

Preparation	Potency* IU/mg	(95% confidence limit)
hGH		
HS1744C	2.7	(1.7–4.3)
HS1863	1.6	(0.8–2.9)
HS1894	1.9	(0.9–4.0)
RCAM-hGH		
3M409B	1.6	(1.1–2.5)
5M751	1.1	(0.5–2.5)
5M772	0.6	(0.2–1.0)
5M787	0.9	(0.2–2.1)
5M791	2.7	(1.4–6.1)
Plasmin digest of RCAM-hGH		
5M730	1.9	(1.1–3.3)
5M732	0.9	(0.3–2.0)
5M737	1.2	(0.3–3.0)
5M763	1.2	(0.5–2.6)
5M792	2.1	(0.9–5.5)
Peak R2		
SS5186	1.9	(0.5–7.0)
SS5192	1.1	(0.3–2.4)
SS610	1.4	(0.6–2.9)

* Relative to the International Standard of Growth Hormone, Bovine, for Bioassay (defined as containing 1 IU/mg).

thymidine by the costal cartilage of hypophysectomized rats (Table 2). The dose of native hGH that routinely gives a threshold response in this assay is 2.5 μg/day. It can be seen that 5 μg/day of RCAM-hGH produced a significant response. Lower doses were not tested. RCAM-hGH also retained the ability to stimulate [^{14}C]glucose oxidation to $^{14}CO_2$ by isolated adipose tissue when added *in vitro*. Its potency in this system appeared to be quite high although not as great as that of native hGH (Table 3).

When various preparations of RCAM-hGH were incubated overnight at 37° with human plasmin, approximately 95% of the starting material was completely digested, as indicated by the greater mobility of the bulk of the material when subjected to disc gel electrophoresis (6). The disc gel pattern of a typical digest is compared to that of the starting material in

TABLE 2. *In vivo effects of plasmin-digested RCAM-hGH on [^{3}H]thymidine incorporation into rat costal cartilage*

Preparation	Dose (μg/day)	[^{3}H]Thymidine incorporation* (dpm/mg of cartilage protein) Control	Treated	P
hGH	2.5	851 ± 43 (6)†	1526 ± 206 (6)	<0.01
	5	1109 ± 64 (8)	2044 ± 385 (8)	<0.05
RCAM-hGH	5	928 ± 33 (8)	1598 ± 267 (8)	<0.05
	10	928 ± 33 (8)	1571 ± 185 (8)	<0.01
Plasmin digest of	5	1200 ± 68 (8)	2122 ± 358 (8)	<0.05
RCAM-hGH	10	1200 ± 68 (8)	3181 ± 730 (8)	<0.02
Peak R2	10	1044 ± 92 (6)	5988 ± 1385 (10)	<0.01
	20	1044 ± 92 (6)	8407 ± 2215 (10)	<0.01

* Rats received intraperitoneal injections of saline or test materials 48 and 24 hr prior to sacrifice. Costal cartilages were removed and incubated for 3 hr in the presence of [^{3}H]thymidine.

† Mean value ±SE; (*n*) indicates the number of rats used.

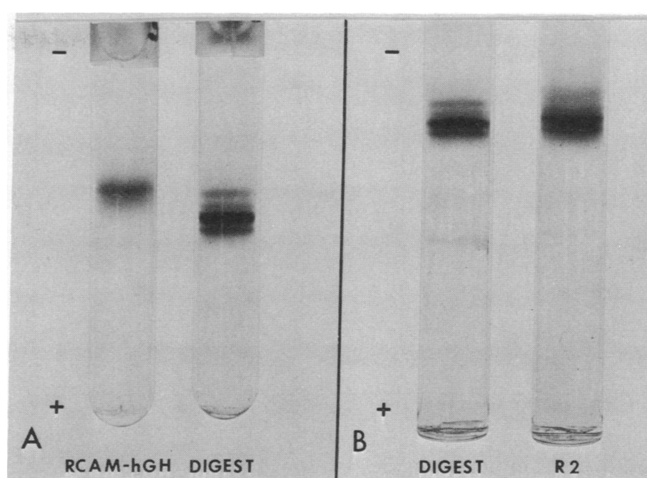

FIG. 1. (A) Disc-gel electrophoresis of precursor RCAM-hGH and a plasmin digest of RCAM-hGH (50 μg samples). The purified hGH from which the RCAM-hGH was made also had two electrophoretic components with mobilities similar to those of RCAM-hGH. (B) Disc-gel electrophoresis of precursor plasmin digest of RCAM-hGH and chromatographic fraction R2 (50 μg samples).

Fig. 1A. The plasmin digests of RCAM-hGH retained the full growth-promoting potency of native hGH, as is shown in Table 1. This activity cannot be due to the small amount of undigested RCAM-hGH present in the preparations. The results shown in Tables 2 and 3 also indicate that the digests of RCAM-hGH retained the ability to stimulate thymidine uptake into cartilage and to stimulate *in vitro* glucose oxidation by isolated adipose tissue. Again, the degree of activity is such that the effects obtained cannot be attributed to the residual amount of undigested RCAM-hGH remaining in the preparations.

In order to separate the major components of the plasmin digests of RCAM-hGH from small cleavage products, ly-

TABLE 3. *In vitro effects of plasmin-digested RCAM-hGH on glucose oxidation by isolated adipose tissue of hypophysectomized rats*

Preparation	Concentration (μg/ml)	$^{14}CO_2$ production* (dpm/mg of wet tissue per hr) Control†	Hormone	M.D. ±SE	P
hGH	0.1	39.8	52.4	12.6 ± 5.9	NS
	0.25	25.1	35.8	9.8 ± 4.1	<0.05
	0.5	44.2	89.8	45.7 ± 11	<0.01
RCAM-hGH	0.25	25.1	35.9	10.7 ± 3.3	<0.02
	0.5	44.2	66.0	21.8 ± 5.9	<0.01
Plasmin	0.5	27.7	34.8	7.1 ± 2.8	<0.05
digested	1	25.1	41.5	16.3 ± 4.1	<0.01
RCAM-hGH	2	27.7	49.1	21.5 ± 4.0	<0.01
Peak R2	0.5	16.8	27.0	10.2 ± 2.6	<0.01
	1	16.8	30.6	13.8 ± 2.2	<0.01

* Segments of epididymal adipose tissue of hypophysectomized rats were incubated for 60 min at 37° in the presence of D-[^{14}C]-glucose and test substances.

† Mean value of observations on eight rats.

M.D., Mean Difference; NS, not significant.

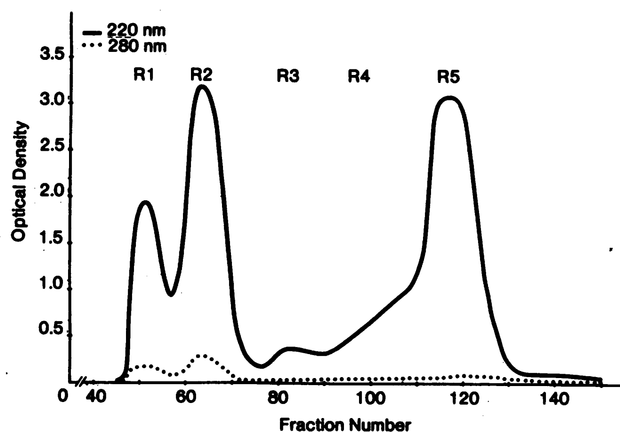

FIG. 2. Chromatographic pattern obtained by gel filtration of a plasmin digest of RCAM-hGH on a 1.6 × 150 cm column of Sephadex G-50 fine in 0.01 N HCl.

ophilized digests were dissolved in 1 M propionic acid and subjected to gel filtration on Sephadex G-50 fine in 0.01 N HCl. A typical elution pattern is shown in Fig. 2. Five absorbance peaks were identified and designated peaks R1 to R5. Peak R1 appeared to be a mixture of aggregated digestion products and undigested precursor. Peak R2 contained the major digestion products, as indicated by disc gel electrophoresis of the material (Fig. 1B). Peaks R3 to R5 contained a mixture of small peptides. Only one of these has been identified, namely, the hexapeptide consisting of residues 135–140, which eluted in peak R5. It will be recalled that this is the peptide that is cleaved from native hGH when it is digested with plasmin.

The biological properties of native hGH are retained by the material present in peak R2. The results given in Tables 1–3 indicate that peak R2 had a high degree of activity in the weight gain and cartilage metabolism tests and *in vitro* on adipose tissue glucose oxidation.

DISCUSSION

The present findings indicate that reduction and *S*-carbamidomethylation of hGH does not impair its ability to stimulate weight gain and cartilage metabolism in the hypophysectomized rat or to stimulate glucose oxidation in isolated rat adipose tissue. These results are compatible with the earlier finding of Dixon and Li (7) that RCAM-hGH retains activity in the tibia test in the hypophysectomized rat, and with the reports of Cerasi *et al.* (8) and Conners *et al.* (9) that RCAM-hGH has metabolic activity in man. Further, plasmin digests of RCAM-hGH retain the ability to stimulate weight gain, cartilage metabolism, and glucose oxidation. These biological properties are present in the major peptide components of the digests, namely, those isolated in chromatographic fraction R2.

It is of particular interest that the plasmin digests of RCAM-hGH have a high degree of activity in the weight gain and cartilage metabolism tests, in which the test substances are injected subcutaneously and intraperitoneally, respectively. As indicated earlier, peptide 2α (residues 1–134) only exhibited substantial activity in the whole animal when administered intravenously, suggesting that it is quite labile *in vivo*. If RCAM-hGH is hydrolyzed by plasmin in the same manner that hGH is, namely, that the peptide bonds between residues 134 and 135 and between residues 140 and 141 are cleaved, one might expect that the digests of RCAM-hGH would have the *in vivo* lability characteristic of peptide 2α. Since the digests of RCAM-hGH do contain hexapeptide 135–140, the action of plasmin on RCAM-hGH is in fact similar to its action on native hGH. However, digestion of RCAM-hGH may be more extensive, perhaps yielding a fragment of the hormone more stable *in vivo* than peptide 2α. Alternatively, the somewhat simpler chemical manipulations involved in the production of the digests of RCAM-hGH might account for the preservation of high *in vivo* biological activity. In any event, clarification of this issue must await the chemical characterization of the biologically active component(s) of the digests.

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1. Reagan, C. R., Kostyo, J. L., Mills, J. B. & Wilhelmi, A. E. (1973) *Fed. Proc.* **32**, 265(abstr.).
2. Mills, J. B., Reagan, C. R., Rudman, D., Kostyo, J. L., Zachariah, P. & Wilhelmi, A. E. (1973) *J. Clin. Invest.* **52**, 2941–2951.
3. Reagan, C. R., Mills, J. B., Kostyo, J. L. & Wilhelmi, A. E. (1975) *Endocrinology* **96**, 639–653.
4. Li, C. H. & Graf, L. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1197–1201.
5. Parlow, A. F., Wilhelmi, A. E. & Reichert, L. E. (1965) *Endocrinology* **77**, 1126–1134.
6. Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349.
7. Dixon, J. S. & Li, C. H. (1966) *Science* **154**, 785–786.
8. Cerasi, E., Li, C. H. & Luft, R. (1972) *J. Clin. Endocrinol. Metab.* **34**, 644–649.
9. Connors, M. H., Kaplan, S. L., Li, C. H. & Grumbach, M. M. (1973) *J. Clin. Endocrinol. Metab.* **37**, 499–504.