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A Hyperglycemic Peptide from Pituitary Growth Hormone: Preparation with Pepsin and Assay in ob/ob Mice*

(fragments of growth hormone/glucose intolerance/peptic digest/fluorescamine/diabetogenic)

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ABSTRACT **A fragment that induces glucose intolerance in hereditarily obese mice has been prepared from pituitary growth hormone (sheep and human) by controlled digestion with pepsin in 0.05 M sodium acetate buffer, pH 3.7. The digest was purified by column chromatography on Bio-Gel P-6, followed by chromatography on Bio-Rex 70 and Bio-Gel P-2; in each successive step peptide was quantitated with the fluorescamine procedure. The fragment from sheep growth hormone has these characteristics: (1) It induces glucose intolerance in fasted ob/ob female mice when injected subcutaneously in a divided dose 15 min before, and concurrently with, glucose. (2) It is a basic peptide, as judged from its elution behavior on Bio-Rex 70, H^+ form. (3) It does not crossreact with antiserum to human growth hormone in radioimmunoassay. Further purification and initial sequence determinations have been undertaken.**

Pituitary growth hormone (GH) is a growth-promoting (1) and, under some conditions, a diabetogenic (2) protein. It can be detected in the plasma of newborn infants and children (3), and in the plasma of adults (4); not until senility does the concentration of the hormone fall. Clearly, GH is not merely a juvenile hormone that is normally secreted only in childhood during a period of rapid skeletal and somatic growth. Rather, it appears that GH [molecular weight about 21,000 (5)] is a precursor protein (6), found in the plasma throughout the life of the individual, from which peptide fragments with either diabetogenic or with growth-promoting (7) activities can be formed.

To assess the validity of our proposal (6) that specific fragments with select biological activity may be formed from GH *in vivo* by enzymatic cleavage of the circulating parent hormone, we have submitted purified sheep and human GH to controlled digestion with pepsin and assayed the fractions obtained in a glucose tolerance test in genetically obese (ob/ob) female mice. Pepsin digestion under our conditions consistently yields a potent hyperglycemic peptide. Reported here are the procedures used to isolate the material and to assay for hyperglycemic activity, as well as data that identify this fragment as a basic peptide.

MATERIALS AND METHODS

Preparation of Hyperglycemic Peptide. Fragments of pituitary GH were prepared from the parent molecule by controlled digestion with pepsin (8). Each 5-mg sample of GH was dissolved in 1.0 ml of sodium acetate buffer, 0.05 M, pH 3.7. Pepsin, dissolved in the same buffer just before use, was added to give a final concentration of 1 part pepsin: 133 parts GH by weight. Samples were incubated with gentle shaking at 18°–19° for 16 or for 20 hr. The reaction was terminated by addition of sufficient ammonium hydroxide to raise the pH to 7.5–8.

After 30 min at room temperature, the mixture was made 0.5 M with respect to acetic acid by addition of 60 μl of glacial acetic acid, and the digest was chromatographed on Bio-Gel P-6 in 0.5 M acetic acid. Individual fractions to be lyophilized were identified by the fluorescamine method (9); in our procedure sheep GH and serum albumin (Armour) = 280 fluorescence units (FU)/nmol, where 1 FU = 1% of a scale 1–100 on the Turner fluorometer model 110.

Purification of Active Fraction from Bio-Gel P-6. Active fractions from 12 experiments on Bio-Gel P-6 were redissolved in 0.01 M acetic acid, pooled, and lyophilized. They were then taken up in 500 μl of 0.01 M acetic acid, the solution was transferred to a polypropylene tube, the lyophile jar was rinsed with an additional 500 μl of 0.01 M acetic acid, and the pH of the combined washes was adjusted to pH 6.8 with NaOH. This material was applied to a Bio-Rex 70 column (1.2 × 9 cm), prepared in the H^+ form, that had been washed extensively with ion-free water just before use; 3.8-ml fractions were collected. The adsorbed material was then subjected to stepwise elution with water (150 ml), 0.015 N HCl (38 ml), and 0.04 N HCl (38 ml) each step being terminated when a decrease in fluorescence indicated that no significant amount of peptide was being eluted, or an increase indicated that a new species was probably appearing. The HCl eluates were neutralized with NaOH and chromatographed separately on Bio-Gel P-2.

Assay in a Glucose Tolerance Test in C57 Bl/J6 (ob/ob) Female Mice. For assay, lyophilized fractions were taken up in 0.1 M $NaHCO_3$ that contained 0.1% bovine-serum albumin; controls received an equal volume of the injection medium.

Female mice, of the same age and paired by weight, were injected with a priming dose of dexamethasone (2 μg subcutaneously)†. The mice were then fasted 6 hr, after which

Abbreviations: GH, growth hormone; FU, fluorescence unit.

* A portion of the data has been reported in abstract form [Lostroh, A. J., Krahl, M. E. & Marshall, L. B. (1973) *55th Annual Meeting of the Endocrine Society*, Chicago, June 20–22].

† At this dose level dexamethasone, like natural glucocorticoid, accentuates the inhibition by GH of glucose uptake in muscle (10). At relatively large doses, adrenocorticotrophic hormone alone (11), like GH (12), has diabetogenic effects in rats, and both adrenocorticotrophic hormone and GH are required for maximum diabetogenicity (13).

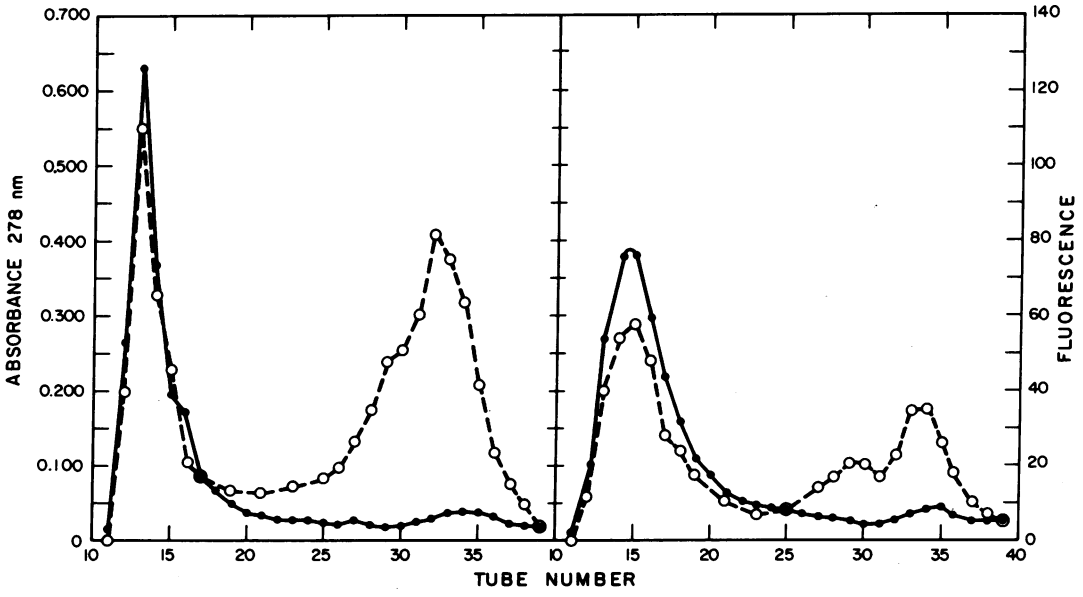

FIG. 1. Chromatography of pepsin digests of sheep GH (*left*) and of human GH (*right*) on Bio-Gel P-6 in 0.5 M acetic acid. 1.0 ml (5.0 mg dry weight) of partially digested GH was applied to the column (1.2 × 41 cm). For sheep GH, 1.30-ml fractions were collected; for human GH, 1.25-ml fractions were collected. An aliquot (10 μl) was taken from each tube for fluorescence determinations (O——O), and the absorbance of the residual solution was measured at 278 nm (●——●). Solutions in select tubes were then pooled, lyophilized, and used for assay or submitted to further fractionation. Hyperglycemic activity in the digest of sheep GH appeared exclusively in fraction II tubes 30–32); fractions I (tubes 27–29), III (tubes 33–35), and M (tubes 13–15) were inactive. Activity in the digest of human GH also appeared exclusively in fraction II (tubes 32–34).

they were given glucose (1 mg/g of body weight intraperitoneally) and bled from the tail 25 min later. Test solutions and control diluent were given subcutaneously in divided doses 15 min before, and at the same time as, the glucose injection. Glucose was estimated by the glucose oxidase method. Mice were rested for 2 weeks or longer and then used again for assay.

Materials. Genetically obese female mice of the C57Bl/6J (ob/ob) strain were obtained from Jackson Laboratories, Bar Harbor, Maine. They were used for assays from 9 weeks to 9 months of age.

The human GH used in these studies was supplied by Dr. Alfred Wilhelmi and the National Pituitary Agency, and the sheep GH by the Endocrine Study Section. The preparations were: human GH, NIH-GH-H51523D; sheep GH, NIH-GH-S10. Pepsin (3100 units/mg) and glucose oxidase reagent were purchased from Worthington Biochemical Corp. To avoid loss of peptide due to adsorption to glass surfaces, glassware was siliconized and polypropylene tubes were used wherever feasible.

The fluorescamine (4-phenylspiro[furan-2 (3H), 1'-phthalan]-3,3'-dione) was a generous gift from Dr. W. E. Scott and Dr. S. Udenfriend of the Roche Institute of Molecular Biology; it was prepared as a stock solution in acetone (50 mg/ml) and was stable for months when stored at 4°.

Materials for column chromatography purchased from Bio-Rad Laboratories included: Bio-Gel P-2 (200–400 mesh, exclusion limit 1800 daltons); Bio-Gel P-6 (100–200 mesh, exclusion limit 6000 daltons); Bio-Rex 70 (100–200 mesh, sodium form). Bio-Rex 70, a weakly acidic carboxylic cation exchanger, was prepared for use by passing it through three successive cycles of 0.5 M HCl and 0.5 M NaOH. Finally, it was converted to the H^+ form in 0.5 M HCl and poured; the column was washed extensively with ion-free water before each use.

RESULTS

Controlled digestion of sheep GH and human GH with pepsin yields the patterns shown in Fig. 1 when digests are chromatographed in 0.5 M acetic acid on Bio-Gel P-6. The first peak

TABLE 1. *Identification of fraction in 16-hr pepsin digest of sheep GH that induces glucose intolerance in ob/ob mice*

Test preparation*	Blood glucose (mg/100 ml)	*P*	Activity as hyperglycemic agent†
Control	443 ± 29 (5)‡		
SGH-P-P6-I	475 ± 19 (6)	ns	−
Control	475 ± 16 (7)		
SGH-P-P6-II	633 ± 20 (7)	<0.001	+
Control	574 ± 38 (5)		
SGH-P-P6-III	562 ± 14 (6)	ns	−
Control	428 ± 27 (6)		
SGH-P-P6-M	469 ± 16 (4)	ns	−
Control	556 ± 20 (5)		
SGH, undigested	570 ± 38 (5)	ns	−

* Test fractions are lyophilized eluates from Bio-Gel P-6 (for numbering of test preparations, see Fig. 1) that were dissolved in 0.1 M $NaHCO_3$ that contained 0.1% serum albumin; each preparation was administered subcutaneously in a total dose of 50 nmol (14 × 10^3 FU)/0.60 ml. The values in one experiment cannot be compared with those in a different experiment because of the different ages and weights of the mice.

† The glucose tolerance test, as used here, is a null-point assay that detects significant difference from the control.

‡ Mean ± standard error; number of mice in parentheses.

ns, not significant.

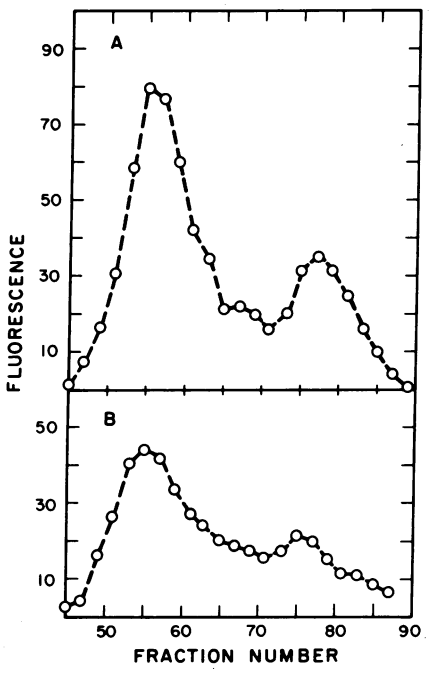

FIG. 2. Chromatography on Bio-Gel P-2 of HCl eluates of sheep GH fractions from a Bio-Rex 70 column. The 0.015 N HCl and 0.04 N HCl fractions were neutralized with NaOH and chromatographed separately on Bio-Gel P-2 (column 4 × 30 cm) in 0.01 M acetic acid. Fractions of 3.8 ml were collected, and pooled eluates were lyophilized. Hyperglycemic activity was found in the major fraction eluted with 0.015 N HCl (*A*, tubes 50–60). No activity was found in the major fraction eluted with 0.04 N HCl (*B*, tubes 49–61).

(tubes 12–18) probably is comprised of undigested GH, large fragments, and pepsin; it is inactive in our assay (Table 1). The smaller peptides (tubes 26–36) yield distinctly different patterns for sheep GH and human GH. All the hyperglycemic activity in both preparations is localized in fractions on the rising side of the final peak (Tables 1 and 2).

When the active fraction from sheep GH (tubes 31 and 32) was submitted to further purification by column chromatography on Bio-Rex 70, approximately 20% (22 × 10^3 FU) was eluted in the water fraction, 35–40% (63.5 × 10^3 FU) in the 0.015 N HCl fraction, and 30–35% (52.8 × 10^3 FU) in the 0.04 N HCl fraction. A major peptide (Fig. 2) with hyperglycemic activity was present in the 0.015 N HCl eluate. The major peptide obtained in the 0.04 N HCl eluate lacked hyperglycemic activity. The water fraction had no effect on blood sugar.

DISCUSSION

Controlled digestion of sheep GH with pepsin yielded a fragment with the following characteristics: (*1*) It induces glucose intolerance in fasted genetically obese (ob/ob) female mice when injected subcutaneously in a divided dose 15 min before, and concurrently with, glucose. (*2*) It is a basic peptide, as judged from its elution behavior on Bio-Rex 70, H^+ form. (*3*) It does not crossreact with antiserum to human GH in radioimmunoassay (14).

That pituitary GH is a precursor molecule from which active fragments can be formed *in vivo* has been considered by several investigators (6, 15–17). A mechanism for generating these materials, and a potential relationship of a hyperglycemic peptide to *diabetes mellitus*, also have been proposed (6). The experiments reported here demonstrate that a peptide with some diabetogenic properties can be generated, reproducibly, by enzymatic (pepsin) digestion. Other hyperglycemic proteins of pituitary origin also have been reported (18, 19).

Further purification of the pepsin digest and initial sequence determinations have been undertaken. Once the peptide has been identified, the mechanism of its production *in vivo* can be better understood, and its effects on other biological parameters assessed.

TABLE 2. *Identification of fraction in 20-hr pepsin digest of human GH that induces glucose intolerance in ob/ob mice*

Test preparation	Blood glucose (mg/100 ml)	*P*	Activity as hyperglycemic agent
Control	528 ± 18 (4)		
HGH-P-P6-I	520 ± 32 (4)	ns	−
Control	423 ± 16 (5)		
HGH-P-P6-II	542 ± 10 (4)	<0.001	+
Control	484 ± 14 (9)		
HGH-P-P6-M	449 ± 23 (8)	ns	−

Derivation of test fractions and conditions of assay are the same as in Table 1.

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