A comparison of the growth-promoting, lipolytic, diabetogenic and immunological properties of pituitary and recombinant-DNA-derived bovine growth hormone (somatotropin)

Ian C. HART,* Patricia M. E. CHADWICK,* Tom C. BOONE,† Keith E. LANGLEY,†
Chris RUDMAN† and Larry M. SOUZA†

*National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.,
and †Amgen, Thousand Oaks, CA 91320, U.S.A.

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The physiological mechanisms by which growth hormone (somatotropin) exerts its several metabolic activities remain poorly understood. In particular, there is disagreement as to whether the diabetogenic and lipolytic activities of the hormone are intrinsic properties of the molecule or are the result of contamination with other pituitary components. The availability of recombinant-DNA-derived bovine growth hormone (rebGH) presented an opportunity to compare the biological activities of rebGH and pituitary bGH in the absence of pituitary contaminants. Pituitary bGH and rebGH were immunologically identical in the radioimmunoassay for bGH, and good agreement was obtained for the potency of the latter measured by radioimmunoassay (1.6 units/mg) and the dwarf-mouse bioassay (1.4 units/mg). The lipolytic activity *in vitro* was examined by measuring the release of glycerol from rat epididymal fat maintained in the presence of dexamethasone (0.2 µg/ml) and the material to be tested (0.1 and 0.2 mg/ml). Although two preparations of pituitary bGH stimulated a significant ($P < 0.01$) increase in glycerol production, neither rebGH nor recombinant-DNA-derived chicken GH was lipolytic. However, when rebGH was intravenously injected into three sheep (0.15 mg/kg), the increase in plasma non-esterified fatty acids was similar to that measured with the same dose of pituitary bGH. Insulin-tolerance tests were conducted in sheep before and after treatment with rebGH and pituitary bGH (four subcutaneous injections of 0.15 mg/kg). Although the effect of rebGH was less than that of the pituitary hormone, both significantly impaired the ability of insulin to lower the concentration of plasma glucose. These data suggest that the lipolytic and diabetogenic activities of bGH are intrinsic properties of the molecule. However, the lipolytic activity may only become apparent after either modification of the molecule *in vivo* or activation of a lipolytic intermediate.

The expression of bGH with additional *N*-terminal methionine by means of recombinant-DNA techniques has provided a potentially limitless source of the hormone to stimulate milk production in domestic ruminants (Hart, 1983) and has renewed interest in the physiological mechanism(s) by which the hormone achieves this response. Close arterial infusion of bGH into the

mammary gland of goats and sheep failed to stimulate galactopoiesis (McDowell & Hart, 1983), and attention has turned to the possibility that the hormone partitions nutrients (glucose and triacylglycerols) away from tissue deposition and makes them available for milk synthesis by exerting its diabetogenic and lipolytic properties (Hart *et al.*, 1978; Hart, 1983; McDowell *et al.*, 1983). However, there has been considerable debate over the question of whether GH is intrinsically lipolytic and/or diabetogenic.

The lipolytic activity of pituitary GH has been recognized since Lee & Schaeffer (1934) dis-

Abbreviations used: GH, growth hormone (somatotropin); bGH, bovine GH; rebGH, recombinant-DNA-derived bGH; hGH, human GH; NEFA, non-esterified fatty acids; SDS, sodium dodecyl sulphate.

covered that growth-promoting extracts of the anterior pituitary depleted fat stored in rats. As purer preparations of the hormone became available, this property of GH underwent considerable investigation to determine the role of GH in fat metabolism [see Goodman & Schwartz (1974) and Rao & Ramachandran (1977) for reviews]. More recently, evidence has accumulated to suggest that the pure hormone may not be lipolytic and that this activity is stimulated by either pituitary contaminants or cleaved forms of GH (Salaman, 1972; Assa & Laron, 1977; Schleyer & Voigt, 1977; Frigeri, 1980; Frigeri *et al.*, 1982, 1983).

Raben & Westermeyer (1952) were among the first to suggest that pig GH could be prepared without diabetogenic activity, and more recently homogeneous forms of hGH and bGH have been prepared which had markedly decreased diabetogenic activity (Lewis *et al.*, 1977; Hart *et al.*, 1984). Lewis *et al.* (1977) partially purified a diabetogenic component from crude hGH ($M_r < 5000$), and furthermore were able to convert non-diabetogenic hGH into a diabetogenic form by proteolytic cleavage, thereby suggesting that the active material is a modified form of GH. This contention is supported by the fact that short sequences of hGH have been found to be diabetogenic in rats and rabbits (Lostroh & Krahle, 1976; Bornstein, 1978).

The availability of rebGH presented a unique opportunity to examine the biological properties of the hormone in the absence of contamination with other pituitary factors and to gain additional information on the heterogeneous nature of the molecule.

Materials and methods

Preparation of recombinant-DNA-derived bovine growth hormone

Bovine GH was expressed in *Escherichia coli* by using a recombinant plasmid (414 Syn, bGH 22) which contained a synthetic copy of the bGH gene under the transcriptional control of an *E. coli* tryptophan promoter (L. Souza, unpublished work).

A 40 g batch of the *E. coli* cells containing the rebGH was suspended in 200 ml of buffer (20 mM-Tris/HCl/50 mM-NaCl, pH 7.5) and was disrupted by three passages through a Gaulin homogenizer. The broken cells were centrifuged (7000 g for 15 min at 4°C), and the pellet was resuspended in 50 ml of distilled water, to which 50 ml of acetic acid was added over a period of 10 min. The mixture was stirred for 3 h and then centrifuged (60000 g for 30 min at 4°C). The rebGH in the supernatant was purified by gel chromatography on a column (5 cm × 95 cm) of Sephadex G-75 which was equilibrated with acetic acid (10%, v/v).

The fractions containing rebGH were pooled, dialysed against distilled water and redialysed against glycine/HCl (0.01 M; pH 3.6)/lactose (0.2%)/mannitol (0.2%).

The rebGH was similar to preparations of recombinant-DNA-derived hGH in that it possessed an additional methionine residue at the *N*-terminus (Goeddel *et al.*, 1979). Furthermore, it possessed leucine at position 127. The integrity of rebGH was assessed by high-pressure liquid chromatography and SDS/polyacrylamide-gel electrophoresis in the presence and absence of 2-mercaptoethanol (Hart *et al.*, 1984).

Radioimmunoassay

The rebGH was assessed in the radioimmunoassays for ruminant GH (Hart *et al.*, 1975) and prolactin (Hart, 1972) with modifications described by Tindal *et al.* (1982).

Growth-promoting activity

The growth-promoting activity of rebGH was measured with hypopituitary dwarf mice and the method described by Wallis & Dew (1973).

Lipolytic activity (in vitro)

This was examined by measuring glycerol production from rat epididymal fat which had been incubated for 4 h in the presence of the test material and dexamethasone (Hart *et al.*, 1984). The activity of rebGH was examined at two concentrations (0.1 and 0.2 mg/ml) and compared with two preparations of pituitary bGH prepared by the method of Ellis (1961). A preparation of recombinant-DNA-derived chicken GH (Boone *et al.*, 1983) was included in one of the experiments.

Intravenous injection into sheep

Four groups of three castrated male sheep (23–41 kg) were individually housed in metabolism crates and fed on a diet of concentrates and hay *ad libitum*. The sheep were starved for 22 h before the start of the experiment, during which they were allowed access to water. On the day of the experiment, three blood samples (6 ml) were taken from a jugular catheter, inserted at least 24 h previously, and the stability of the pre-injection glucose values was immediately established with a Hypocount blood glucose monitor (Hypoguard Ltd., Woodbridge, Suffolk, U.K.). Each animal was then injected, via the catheter, with 3 ml of the test material (0.15 mg/kg) followed by 2 ml of sterile saline (0.9% NaCl). The treatments were as follows: group 1, glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 2, pituitary bGH dissolved in sterile saline (0.9%, pH 11.0); group 3, rebGH dissolved in glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol

(0.2%); group 4, pituitary bGH dissolved as for group 3. The pituitary bGH had been prepared by the method of Ellis (1961) and its growth-promoting activity (1.6 units/mg) determined by the dwarf-mouse assay. Blood samples (5ml) were collected every 30min for 2h and then at 1h intervals for up to 9h after the intravenous injection.

The plasma was stored at -20°C before analysis for GH (Hart *et al.*, 1975; Tindal *et al.*, 1982), glucose, urea and NEFA (Hart *et al.*, 1978). Only selected samples were analysed for NEFA.

Diabetogenic activity

In this study the diabetogenic activity of rebGH was defined as its ability to impair insulin sensitivity in sheep as measured by the insulin-tolerance test.

Intravenous insulin-tolerance tests were carried out in three groups of four castrated male sheep which had been pre-treated (0.15 mg/kg injected subcutaneously twice daily for 2 days) with the test material: group 1, pituitary bGH (1.6 units/mg) dissolved in sterile saline (0.9%, pH 11.0); group 2, pituitary bGH dissolved in glycine/HCl (0.01M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 3, rebGH dissolved as for group 2. The sheep, which had previously been fed *ad libitum*, were starved for 17h before each insulin-tolerance test. On the morning of the test, four blood samples (3ml) were taken at approx. 10min intervals, from a jugular catheter which was inserted at least 24h previously, and the stability of the pre-insulin glucose values was established with the blood glucose monitor. Bovine insulin (0.08 unit/kg, in 2ml of sterile saline, 0.9%) was then injected via the catheter, followed by 2ml of saline. Blood samples were taken at 8min intervals for 32min after the insulin injection, and the plasma was analysed for glucose by using a Chemlab continuous-flow analyser. Each test of experimental material was preceded, 2 days earlier, by an identical control insulin-tolerance test in which the goats had been pre-treated with the vehicle for 2 days. Thus for each goat there was a comparison between the insulin-stimulated fall in blood glucose before and after treatment with the test material.

Results

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis in the absence of 2-mercaptoethanol revealed the expected heterogeneity of the pituitary bGH extracted by the salt precipitation technique of Ellis (1961) (Fig. 1). There was a major component at M_r 22000 and eight minor components ranging from M_r < 16000

Fig. 1. Approximate M_r values for (a) pituitary bGH and (b) rebGH obtained by SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in 10mM-imidazole/HCl buffer, pH 7.0, at 70mA and 0.8 V for 4h.

to 53000. The rebGH contained a similar major component at M_r 22000 and three minor components with M_r > 22000, which corresponded to similar bands in pituitary bGH. The rebGH contained no material of M_r < 22000. The high- M_r components were almost absent when the same hormone was run in the presence of 2-mercaptoethanol, thereby suggesting they were disulphide aggregates (results not shown).

High-pressure liquid chromatography

The results for the high-pressure liquid chromatography are illustrated in Fig. 2 and support the data obtained with SDS/polyacrylamide-gel electrophoresis. The rebGH contained no low- M_r components (i.e. M_r < 22000), but contained three high- M_r components (i.e. M_r > 22000), two of which corresponded to those found by SDS/polyacrylamide-gel electrophoresis. The pituitary bGH contained at least four peaks of M_r > 22000 and a number of low- M_r (i.e. < 22000) components.

Radioimmunoassay

When compared on a weight basis, serial dilutions of rebGH were parallel with the pituitary bGH standard (NIH-GH-B2; 1.5 units/mg) in the radioimmunoassay for bovine GH (Fig. 3). The potency of rebGH, derived from these data, was equivalent to 1.6 units/mg. In the radio-

Fig. 2. Approximate M_r values for rebGH and pituitary bGH obtained by high-pressure liquid chromatography. High-pressure liquid chromatography was performed with an Ultropac column (TSK 9 3000 SW; 7.5 mm $\times$ 600 mm) eluted with 0.1 M-potassium phosphate buffer, pH 7.5, at a flow rate of 0.75 ml/min. M_r values are marked on the Figure.

Fig. 3. Binding displacement characteristics in the radioimmunoassay for ruminant GH (Hart *et al.*, 1975). —, pituitary bGH (NIH-GH-B2; 1.5 units/mg); ·····, rebGH.

Fig. 4. Effect of pituitary bGH and bGH and recombinant-DNA-derived chicken GH (recGH) on the release of glycerol from rat epididymal fat maintained *in vitro*.

Rat epididymal fat was incubated for 4 h (37°C) with either buffer (control; □) or the material to be tested (0.1 or 0.2 mg/ml, ▤) in the presence of dexamethasone (0.2 μg/ml). Glycerol was analysed by the Boehringer fully enzymic method [$n = 6$ rats/treatment for (a) and 5 rats/treatment for (b)]. Results are means $\pm$ S.E.M.: ** $P < 0.01$ and *** $P < 0.001$, for comparison of treatment with control, by Student's t test for paired observations.

Table 1. Growth-promoting activity of recombinant-DNA-derived bovine GH as determined by the hypopituitary-dwarf-mouse assay

The method of Wallis & Dew (1973) was used for the dwarf-mouse assay. Index of precision (λ) was 0.34 and the 95% fiducial limits of potency were 1.13–2.01. Results are means $\pm$ s.e.m. for eight mice.

	Treatment (μ g/day)	Weight gain (g)	Potency (units/mg)
Control (saline)		0.12 $\pm$ 0.11	
Standard	5	2.05 $\pm$ 0.21	
Bovine pituitary GH (NIH-GH-B5; 1.0 unit/mg)	20	2.95 $\pm$ 0.32	
	80	3.75 $\pm$ 0.19	
Recombinant-DNA-derived bovine GH	10	2.68 $\pm$ 0.25	1.4
	40	3.48 $\pm$ 0.30	

immunoassay for bovine prolactin, rebGH caused no displacement of 125 I-prolactin when tested at concentrations as high as 12.8 μ g/ml.

Growth-promoting activity

The results of the hypopituitary-dwarf-mouse assay are given in Table 1 and show that rebGH had a biological activity of 1.4 units/mg.

Lipolytic activity (in vitro)

Fig. 4(a) shows the effect of rebGH (0.1 mg/ml) and of pituitary bGH (ICH/bGH/5; 1.2 units/mg; 0.1 mg/ml) on glycerol release from rat epididymal fat maintained *in vitro*. Fig. 4(b) illustrates a similar comparison using another preparation of pituitary bGH (ICH/bGH/12; 1.5 units/mg) and recombinant-DNA-derived chicken GH, but the concentration of rebGH was increased to 0.2 mg/ml of medium. Vaughan (1962) had previously established that glycerol is not readily metabolized by rat adipose tissue, and thus its release can be taken as a reliable indication of the lipolytic activity of the material tested. Although both of the pituitary preparations stimulated a significant increase in glycerol production, neither the bovine nor the chicken recombinant-DNA-derived GH was lipolytic *in vitro* at the doses tested.

Intravenous injection into sheep

Single intravenous injection of three preparations of bGH into sheep stimulated similar changes in circulating NEFA (Fig. 5). Pituitary bGH, dissolved in saline at pH11.0 (group 2), stimulated a significant increase in plasma NEFA after 2 h, which remained elevated for up to 7 h. Similar responses were measured with rebGH (group 3) and pituitary bGH (group 4), which were dissolved in glycine/HCl, pH 3.6, but in both cases the magnitude of the average rise in NEFA was considerably less than that measured in group 2 (group 2, +0.66 mM; group 3, +0.39 mM; group 4, +0.36 mM at 2 h after injection). The initial changes in plasma glucose were different in each

Fig. 5. Effect of intravenous pituitary bGH and recombinant-DNA-derived bGH on the percentage change in plasma NEFA in sheep

Four groups each of three sheep were intravenously injected with: group 1, glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 2, pituitary bGH (0.15 mg/kg) in sterile saline (0.9%; pH 11.0); group 3, rebGH (0.15 mg/kg) in glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 4, pituitary bGH (0.15 mg/kg) dissolved as for group 3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the pre-injection value.

group (Fig. 6). For groups 2 and 4, circulating glucose increased within 15 min of injection and then declined to reach a trough after 60–90 min. The sheep treated with rebGH (group 3) showed no initial rise in plasma glucose, but a small fall

Fig. 6. Effect of intravenous pituitary bGH and recombinant-DNA-derived bGH on the percentage change in plasma glucose in sheep

For details, see legend to Fig. 5.

was noted 30 min after treatment. Thereafter all of the group exhibited an increase in glucose such that, after 3 h, the average concentration of each group was significantly higher than the average pre-treatment value (group 2, +0.67 mM; group 3, +0.40 mM; group 4, +0.44 mM; $P < 0.001$). None of the preparations provoked significant changes in plasma urea.

Diabetogenic activity

Pre-treatment with pituitary bGH dissolved both in saline (pH 11.0) and in glycine/HCl (pH 3.6) significantly decreased the insulin sensitivity of the sheep tested (Fig. 7; groups 1 and 2). The rebGH was also found to be diabetogenic (group 3), but, at the dosage used, its ability to inhibit the insulin-stimulated fall in plasma glucose was somewhat less than that of the pituitary hormone.

Discussion

The data obtained from the radioimmunoassay for ruminant GH clearly indicated that pituitary bGH and rebGH were immunologically indistinguishable with this antiserum. Furthermore, the potency of rebGH, calculated from the radioimmunoassay (1.6 units/mg) was slightly

Fig. 7. Intravenous insulin-tolerance tests in sheep. Intravenous insulin (0.08 unit/kg) tolerance tests were conducted in sheep ($n = 4$) before and after subcutaneous injection (0.15 mg/kg, twice daily for 2 days) of: group 1, pituitary bGH in sterile saline (0.9%, pH 11.0); group 2, pituitary bGH in glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 3, rebGH dissolved as for group 2. Values are changes in plasma glucose concentrations ($\pm$ S.E.M.). Statistics refer to a comparison of mean values at each time interval for the experimental treatment (.....) and the control (—): $\dagger P < 0.1$, $* P < 0.05$, $*** P < 0.001$.

higher than that of the pituitary bGH standard (NIH-GH-B2, 1.5 units/mg), which has the highest biological activity of any bGH preparation distributed under the programmes of the National Institute of Arthritis Metabolism and Digestive Diseases (Reichert & Wilhelmi, 1973, 1978). Allowing for the precision of the dwarf-mouse assay for growth-promoting activity, good agreement was obtained between the potency of rebGH calculated from the radioimmunoassay and that derived from the bioassay (1.4 units/mg). Similar agreement was obtained for recombinant-DNA-derived hGH (Goeddel *et al.*, 1979; Olson *et al.*, 1981). Research in rats has suggested that GH may be modified, both before and after secretion from the pituitary, to a form which either retains or has

enhanced growth-promoting activity but cannot be detected by radioimmunoassay (see Vodian & Nicoll, 1979, for references). The present data indicate that the rebGH used in the present study contains none of these modified forms of GH and, in terms of immunological and growth-promoting activities, is identical with the hormone normally extracted and purified from anterior pituitaries.

The question of whether GH is intrinsically lipolytic or whether this activity is the result of the pituitary hormone being contaminated with either modified forms of GH or other pituitary hormones (e.g. thyrotropin, corticotropin, lipotropin) has occupied researchers for several years (Goodman & Schwartz, 1974; Rao & Ramachandran, 1977). Frigeri (1980) and Frigeri *et al.* (1983) found that highly purified pituitary hGH and bGH failed to stimulate glycerol production from epididymal fat or adipocytes taken from starved rats and that the lipolytic activity *in vitro* was associated with an acidic fraction which was removed during the chromatographic purification. They further demonstrated that recombinant-DNA-derived hGH was not lipolytic when incubated with rat fat *in vitro* (Frigeri *et al.*, 1982).

Our results, with two different recombinant-DNA-derived growth hormones, support those of Frigeri and co-workers. Neither of the preparations increased glycerol production from rat epididymal fat maintained *in vitro*, whereas both of the pituitary preparations were markedly successful in that respect. Although it is possible that rat adipose tissue is insensitive to the lipolytic activity of highly purified bGH, it is more likely that these data support the contention that pituitary bGH, extracted by salt precipitation (Ellis, 1961), contains either modified lipolytic forms of bGH, which may be the result of proteolytic cleavage before or during extraction, or other lipolytic pituitary factors. In this respect it is relevant that the pituitary bGH used in this study contained low- M_r components (i.e. <22000), which were absent from rebGH. Furthermore, it has been shown that commercially available preparations of hGH contain substantial quantities of β - and α -lipotropin (Kuhn *et al.*, 1983).

Intravenous injection of pituitary bGH stimulated a rise in plasma NEFA which was similar to that previously found by others (Williams *et al.*, 1963; Bassett & Wallace, 1966), although the lipolytic activity appeared to be decreased when the hormone was dissolved at the lower pH. The fact that intravenous rebGH increased circulating NEFA in a manner similar to that of the same dose of pituitary hormone suggests two alternatives. Either bGH is directly lipolytic and the molecule is converted into a lipolytic form *in vivo*, or the hormone stimulates/facilitates the activity of a

lipolytic intermediate. The former is an attractive possibility, as Pankov *et al.* (1982) have indicated that hGH may have to be proteolytically cleaved before it is able to increase plasma NEFA in rabbits, and evidence has subsequently been obtained to suggest that bGH is modified before it can inhibit the activity of acetyl-CoA carboxylase and thus fatty acid synthesis (Bornstein *et al.*, 1983).

It should be noted that our results and those of Frigeri *et al.* (1982) do not agree with data obtained by Goodman & Grichting (1983) and Goodman (1984), who demonstrated an increase in glycerol production from rat epididymal fat maintained *in vitro* with both highly purified hGH and recombinant-DNA-derived hGH. It is difficult to account for these disparate results; a possible explanation might be that the last group used fed rats in their studies, whereas the other two groups used rats which had been starved for 48 h. Ruiz *et al.* (1981) have shown that the lipolytic response of rat tissue can be influenced by prior feeding, and recombinant-DNA-derived hGH was found to stimulate lipolysis weakly in fat taken from fed rats, but the response was not dose-related (Frigeri *et al.*, 1982).

Similar controversy surrounds the diabetogenic activity of bGH (Lewis *et al.*, 1980). The changes in plasma glucose after intravenous injection of pituitary bGH and rebGH into sheep, combined with the results of the insulin-tolerance test, conclusively indicated that, although the diabetogenic activity of rebGH may be slightly decreased, the hormone can be regarded as intrinsically diabetogenic. This agrees with data in which the diabetogenic activity of recombinant-DNA-derived hGH was examined by using oral glucose-tolerance tests in humans (Rosenfeld *et al.*, 1982). The plasma glucose response to the intravenous injection of pituitary bGH was similar to that found by Bassett & Wallace (1966). It was noteworthy that the increased plasma glucose was associated with the decreased insulin tolerance. In other experiments, in which non-diabetogenic forms of bGH have been examined (Hart *et al.*, 1984), neither of these responses occurred (I. C. Hart, unpublished work), which suggests that the increased circulating glucose is the result of a decrease in the rate of its uptake by body tissues rather than the hormone stimulating glycogenolysis or gluconeogenesis.

The lack of a technique *in vitro* for examining diabetogenic activity prevented us from obtaining further information on the form of the hormone which stimulates this response. However, the conversion of the molecule into a diabetogenic peptide is a likely possibility, as short sequences of hGH are diabetogenic in rats and rabbits (Lostroh

& Krah1, 1976; Bornstein, 1978). Furthermore, Lewis *et al.* (1977, 1980) have increased the diabetogenic activity of purified hGH by proteolytic cleavage with subtilisin and have partially purified a diabetogenic component from crude hGH which has a M_r of less than 5000. The present data do not support the contention that GH can be purified to the point where it is no longer diabetogenic *in vivo*. Preparations of hGH and bGH have been isolated which were growth-promoting but exhibited no diabetogenic activity in three different test systems *in vivo* (Lewis *et al.*, 1977; Hart *et al.*, 1984). Although both preparations had the physical and chemical properties of monomeric GH, the possibility should now be considered that they may have undergone conversion into a non-diabetogenic form of the hormone either before or after extraction from the pituitary.

In conclusion, these results show that the lipolytic and diabetogenic properties of bGH are not the result of contamination with other pituitary factors, but are derived from the bGH molecule. It remains to be seen whether the activities are stimulated by bGH itself, or by proteolytically cleaved forms of the molecule, or whether the hormone activates lipolytic or diabetogenic intermediates *in vivo*.

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