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Trypsin-resistant forms of human growth hormone have diabetogenic and insulin-like activities

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Although diabetogenic and insulin-like activities are intrinsic properties of the growth hormone (GH) molecule, it has been frequently suggested that the hormone must be proteolytically processed for these activities to be expressed. If this is correct, then derivatives of GH having resistance to appropriate proteolytic attack might not have diabetogenic and/or insulin-like activity. The purpose of the present study was to prepare derivatives of human GH that are resistant to digestion by trypsin and to determine whether they possess diabetogenic or insulin-like activity. Three derivatives were prepared from purified native human GH in which lysine residues were modified with methyl acetimidate, citraconic anhydride or *S*-ethyl-thioltrifluoroacetate, and one in which arginine residues were modified with camphorquinone-10-sulfonic acid. Comparisons of peptide maps of tryptic digests of these derivatives with that of unmodified human GH indicated that all four were resistant to proteolysis by trypsin. All of these trypsin-resistant forms of human GH were found to possess significant growth-promoting, diabetogenic and insulin-like activities, although all activities were attenuated to some extent in each derivative. The relative potencies of the human GH derivatives in a radioimmunoassay for human GH were somewhat similar to their order of potency in the growth-promoting and diabetogenic assays. These results suggest that if proteolytic processing of the GH molecule is involved in the expression of one or more of its biological activities, such processing probably does not involve a trypsin-like proteinase.

Introduction

Pituitary growth hormone (GH) exhibits several distinct biological actions in mammals [1]. In addition to growth-promoting activity, GH can be diabetogenic, producing hyperinsulinemia, hyperglycemia and glucose intolerance when given in doses greater than those required to promote growth. In GH-deficient subjects, the hormone also exhibits transient insulin-like effects, stimulating glucose uptake and utilization by peripheral tissues. It has been suggested that the intact GH molecule is not itself diabetogenic, but that it must be proteolytically processed in the organism to smaller peptides for diabetogenic activity to become manifest [2]. This concept is supported by studies showing that controlled digestion of human GH with pepsin [3,4] produced a product capable of causing acute glucose intolerance in the genetically obese (ob/ob) mouse. Additionally, Lewis et al. [2] reported that digestion of human GH with subtilisin resulted in a product which produced fasting hyperglycemia and glucose intolerance within 10 h after its administration to

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dogs, whereas intact human GH was not diabetogenic in this assay. That proteolytic processing could also be important for the insulin-like property of GH has been suggested by recent findings that a synthetic peptide representing residues 31–44 of human GH exhibits significant in vitro insulin-like activity on glucose uptake by isolated rat epididymal adipose tissue [5].

If it is indeed correct that proteolytic processing of the native GH molecule by the organism is required for the expression of diabetogenic activity, and perhaps insulin-like activity, then modified forms of GH that are resistant to appropriate proteolytic attack might not exhibit these activities. In the present study, chemical derivatives of human GH were prepared in which lysine or arginine residues were modified to render the molecule more resistant to proteolysis by trypsin. The various biological activities of these derivatives were then characterized.

Methods

Native human GH that had been purified as previously described [6] and chemically and biologically characterized in our laboratory was used as the precursor for all modified forms, and as the reference standard in the various bioassays. It was also used to prepare ^{125}I-labeled human GH by the lactoperoxidase method of Thorell and Johansson [7]. Citraconic anhydride, methyl acetimidate, *S*-ethylthioltrifluoroacetate and camphorquinone-10-sulfonic acid were purchased from Pierce Chemical Company, Rockford, IL.

Three human GH derivatives were prepared involving the chemical modification of the ε-amino groups of lysine residues, as well as the free amino group of the N-terminal phenylalanine. Two preparations were produced by reaction of 20 mg and 30 mg of human GH with citraconic anhydride [8]. Following dialysis and lyophilization, 15.6 and 29.7 mg, respectively, of citraconylated GH were recovered. Samples of one of these preparations were submitted to gel filtration on a column of Sephacryl S-200 that had been calibrated previously with unmodified human GH. The column, which had been equilibrated with 0.5% (w/v) ammonium bicarbonate, was eluted with the same solution, and absorbance of the fractions was monitored at 220 nm. In duplicate experiments, citraconylated GH was found to contain approx. 75% monomeric hormone derivative, judging from the absorbance of the peaks having elution volumes equivalent to that of monomeric human GH. The extent of modification of the hormone was monitored by polyacrylamide gel electrophoresis [9], which indicated that virtually all of the starting material had been converted to a more negatively charged form. Determination of free amino groups in the derivative with 2,4,6-trinitrobenzenesulfonic acid [10] indicated that three of the ten free amino groups in the molecule had been modified.

The degree to which the citraconylated GH preparations were resistant to proteolytic attack by trypsin was determined by comparison of peptide maps of tryptic digests of the derivative with that of unmodified human GH by a modification of the method of Seavey et al. [11]. Digestion of the hormones with trypsin (TPCK trypsin, Worthington, Freehold, NJ) was carried out in 0.5% sodium dodecyl sulfate at 37°C for 4 h. The enzyme/substrate ratio was 20 : 1 (w/w). Mapping of the digests was performed on 20 × 20 cm plastic thin-layer chromatography sheets coated with cellulose (0.1 mm, Pierce Chemical Company Rockford, IL). Approx. 200 μg of digest were applied to the thin-layer sheet, and ascending chromatography was then performed in the first dimension with *n*-butyl alcohol/glacial acetic acid/pyridine/water (15 : 3 : 12 : 10). Electrophoresis was carried out in buffer containing glacial acetic acid/pyridine/acetone/water (2 : 1 : 8 : 40), pH 4.4, for 1.5 h at 25 V/cm. Peptides were detected by spraying the sheet with 0.001% fluoran/0.01% pyridine in acetone and examining it under ultraviolet light. Fig. 1 shows typical tryptic peptide maps of unmodified human GH and citraconylated GH. It can be seen that trypsin digestion of human GH results in the production of numerous small peptides, as expected. When the tryptic peptide map of citraconylated GH is compared with that of unmodified human GH, however, it is apparent that modification of the lysine residues with citraconic anhydride rendered the hormone resistant to proteolytic attack by trypsin, judging from the reduction in the number of cleavage products produced and alterations in their electrophoretic mobilities.

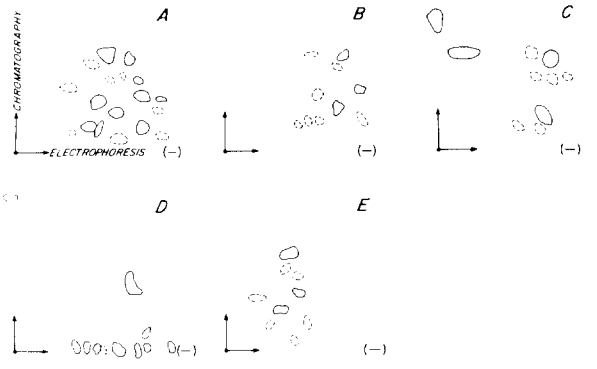

Fig. 1. Peptide maps of trypsin digests of unmodified human GH (A), citraconylated GH (B), amidinated GH (C), trifluoroacetylated GH (D) and camphorquinone-10-sulfonic acid-modified GH (E). The dotted shapes indicate weakly staining peptides. (−) indicates the cathode.

Two preparations of amidinated GH were produced by reaction of 30 and 20 mg of native human GH with methyl acetimidate [12]. Following dialysis and lyophilization, 23.5 and 18.1 mg, respectively, of amidinated GH were recovered. These preparations were subjected to gel filtration on a column of Sephacryl S-200 that had been equilibrated with 0.5% (w/v) ammonium bicarbonate and found to contain approx. 75% monomeric hormone derivative. The fractions representing monomer were pooled and lyophilized, yielding 12.0 and 12.8 mg, respectively, of monomeric amidinated GH. The extent of modification of the amidinated GH preparations was monitored by amino acid analysis [9], which revealed the emergence of a peak which was presumed to represent amidinated lysine residues. The calculated mole percentage of this peak was equivalent to the mole percent that was lost from the lysine peak and suggested four or five of the nine lysine residues had been amidinated (data not shown). As shown in Fig. 1, it is apparent that amidination of human GH rendered the hormone molecule resistant to digestion by trypsin.

One preparation of modified human GH was produced in which the terminal amino group and the ε-amino groups of lysine residues were trifluoroacetylated [13]. This derivative was prepared by reaction of 30 mg of native human GH with *S*-ethylthioltrifluoroacetate. Following dialysis and lyophilization, 19 mg of trifluoroacetylated GH were recovered. When samples of trifluoroacetylated GH were submitted to polyacrylamide gel electrophoresis, no indication of starting material was observed on the gels. Estimation of free amino groups in the derivative with 2,4,6-trinitrobenzenesulfonic acid indicated that six amino groups had been trifluoroacetylated. As is again shown in Fig. 1, trifluoroacetylated GH exhibited resistance to proteolytic attack by trypsin, judging from the reduced number of digestion products detected.

Finally, two hormone preparations were produced in which the arginine residues were modified by reaction of 20 and 30 mg of native human GH with camphorquinone-10-sulfonic acid [14]. Following dialysis and lyophilization, 16.0 and 25.8 mg, respectively, of camphorquinone-10-sulfonic acid-modified GH were recovered. When one of these preparations was subjected to gel filtration on a column of Sephacryl S-200 that had been equilibrated with 0.5% (w/v) ammonium bicarbonate, it was found to contain approx. 80% monomeric hormone derivative (data not shown). Polyacrylamide gel electrophoresis of these preparations revealed a poorly staining, more acidic band with no evidence of remaining starting material (data not shown). Again, as shown in Fig. 1, modification of arginine residues with camphorquinone-10-sulfonic acid rendered the resulting hormone derivative resistant to proteolytic attack by trypsin.

The bioassays used to assess the growth-promiting activity [15], diabetogenic activity [16] and in vitro insulin-like activity [9] of the modified GH preparations have been described in detail. All modified GH preparations were also tested for their ability to compete with ^{125}I-labeled human GH for binding to guinea pig antiserum to human GH as previously described [15].

Results

The growth-promoting activities of the modified GH preparations and the native human GH which served as precursor were assessed in the 9-day weight-gain test in hypophysectomized rats and compared to the International Standard of GH, Bovine (defined as 1.0 IU/mg). In this assay, the GH preparations were injected subcutaneously once daily for 9 days at the following daily doses

TABLE I

GROWTH-PROMOTING POTENCIES OF TRYPSIN-RESISTANT HUMAN GH PREPARATIONS

Values were estimated in the 9-day weight-gain test in hypophysectomized rats using the International Standard of Growth Hormone, Bovine (1.0 IU/mg) as the standard. Potency estimates are shown with their 95% confidence limits given in parentheses. CQSA, camphorquinone-10-sulfonic acid.

Material	Growth-promoting potency (IU/mg)
Unmodified human GH	2.4 (0.8–7.8)
Amidinated GH	1.44 (0.5–3.3)
Citraconylated GH	1.22 (0.9–1.8) [a]
Trifluorocetylated GH	0.43 (0.2–0.7)
CQSA-modified GH	0.29 (0.02–0.78)

[a] Pooled estimate for two assays.

(number of rats used per dose are given in parentheses): bovine GH standard, 20 μg (5) and 100 μg (5); unmodified human GH, 10 μg (4) and 50 μg (5); amidinated GH, 10 μg (4) and 50 μg (5); citraconylated GH, 20 μg (8) and 100 μg (7); trifluoroacetylated GH, 20 μg (5) and 100 μg (5); and camphorquinone-10-sulfonic acid-modified GH, 20 μg (4) and 100 μg (4). As shown in Table I, both amidinated GH and citraconylated GH retained substantial growth-promoting activity, having estimated potencies greater than 1 IU/mg. In contrast, the growth-promoting potency of trifluoroacetylated GH, in which lysine residues are also modified was markedly reduced. Whether the reduced growth-promoting activity of trifluoroacetylated GH is due to the introduction of trifluoroacetyl groups in the molecule or to its reduced water solubility compared to that of unmodified human GH remains to be established. It can be seen in Table I that camphorquinone-10-sulfonic acid-modified GH, in which arginines have been modified, also possessed reduced but significant growth-promoting activity.

The human GH derivatives were tested for diabetogenic activity in the genetically obese (ob/ob) mouse by their ability to increase fasting blood glucose concentration and decrease glucose tolerance when administered chronically. Groups of ob/ob mice (5–6 months of age) were injected subcutaneously with saline for 3 consecutive days, and on the fourth day a control glucose tolerance test was performed [16]. 7 days after the beginning of saline treatment, the same mice were injected with the test substance for 3 days, and on the fourth day the tolerance test was repeated. Results obtained when native GH was administered at doses of 5, 10 or 25 μg/day for 3 days are depicted in Fig. 2. It can be seen that unmodified human GH at a dose of 5 μg/day had no effect on fasting blood glucose concentration or glucose tolerance. However, a significant increase in fasting blood glucose concentration and decreased glucose tolerance were produced when the dose of hormone was increased to 10 μg/day. A very marked diabetogenic response was produced with a dose of 25 μg/day of unmodified human GH. Fig. 3 shows the results obtained when amidinated GH was tested for diabetogenic activity in this assay. A dose of 5 μg/day of amidinated GH had little effect on fasting blood glucose concentration and glucose tolerance, whereas a dose of 25 μg/day produced marked effects on both. Thus, when compared to native human GH, amidinated GH appears to retain high diabetogenic activity. When citraconylated GH was tested in this assay (see Fig. 4), a dose of 25 μg/day produced a slight effect on fasting blood glucose concentration but had no effect on glucose tolerance. When the dose

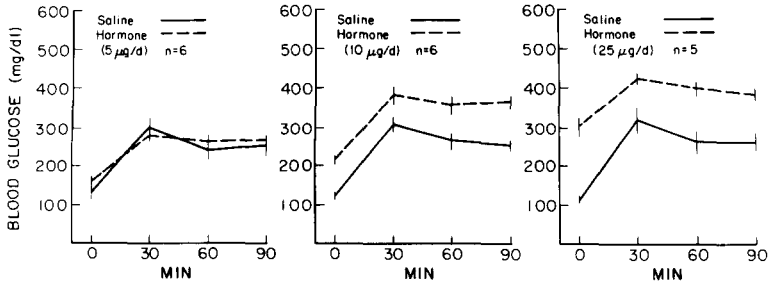

Fig. 2. Effects of treatment with three doses of unmodified human GH for 3 days on glucose tolerance of ob/ob mice. Each point represents the mean of n observations. Vertical lines through the points indicate 2 S.E. The effects of the 10 μg/day and 25 μg/day doses of hormone were significant ($P < 0.05$) by paired t test at all time points examined.

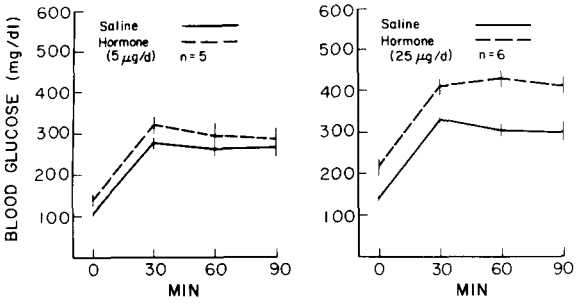

Fig. 3. Effects of treatment with two doses of amidinated GH for 3 days on glucose tolerance of ob/ob mice. Each point represents the mean of n observations. Vertical lines through the points indicate 2 S.E. The effects of the 5 μg/day dose of amidinated GH were statistically significant ($P < 0.05$) at the 0 and 30 min time points. The effects of the 25 μg/day dose were statistically significant ($P < 0.05$) at all time points examined.

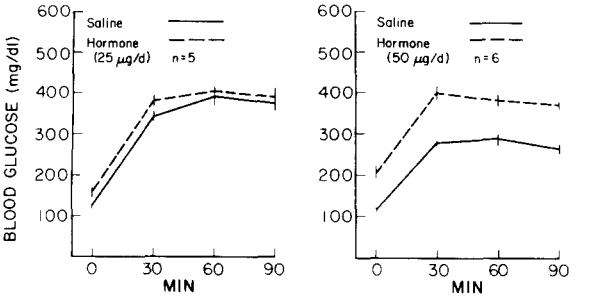

Fig. 4. Effects of treatment with two doses of citraconylated GH for 3 days on glucose tolerance of ob/ob mice. Each point represents the mean of n observations. Vertical lines through the points indicate 2 S.E. The effects of the 25 μg/day dose of citraconylated GH were only statistically significant ($P < 0.05$) at the 0 min time point. The effects of the 50 μg/day dose of hormone derivative were significant at all time points examined.

of citraconylated GH was raised to 50 μg/day a very marked increase in fasting blood glucose concentration and decrease in glucose tolerance were obtained. Thus, citraconylated GH appears to retain 10–20% the diabetogenic activity of unmodified human GH in this assay. The diabetogenic activity of trifluoracetylated GH is depicted in Fig. 5. It can be seen that no activity was detected when the derivative was tested at a dose of 50 μg/day, but a large diabetogenic response was obtained when the dose was raised to 250 μg/day. Thus, it appears to retain 5–10% the diabetogenic activity of the native hormone. Rather similar results were obtained with camphorquinone-10-sulfonic acid-modified GH (see Fig. 6). A dose of 50 μg/day of the derivative produced a slight

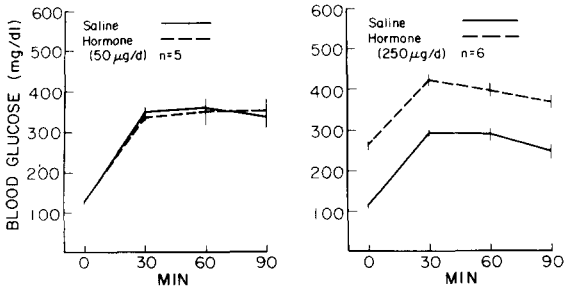

Fig. 5. Effects of treatment with two doses of trifluoroacetylated GH for 3 days on glucose tolerance of ob/ob mice. Each point represents the mean of n observations. Vertical lines through the points indicate 2 S.E. The effects of the 250 μg/day dose of trifluoroacetylated GH were statistically significant at all time points examined.

effect on fasting blood glucose but had no effect on glucose tolerance. When the dose was raised to 250 μg/day, marked effects on fasting blood glucose concentration and glucose tolerance were noted. Thus, this arginine-modified form of human GH retained approx. 5–10% of the diabetogenic activity of native human GH.

Insulin-like activity of the trypsin-resistant human GH derivatives was assessed by their in vitro ability to stimulate [^{14}C]glucose oxidation to $^{14}CO_2$ by isolated epididymal adipose tissue of hypophysectomized rats. The results obtained are summarized in Table II. Unmodified human GH pro-

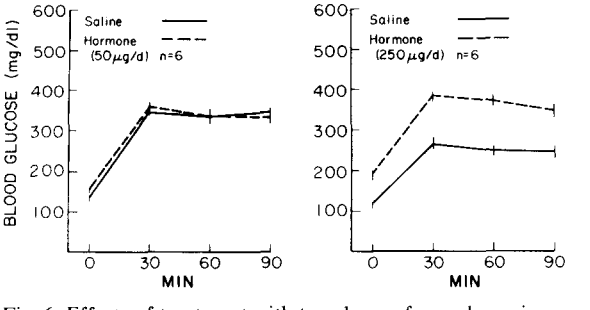

Fig. 6. Effects of treatment with two doses of camphorquinone-10-sulfonic acid-modified GH for 3 days on glucose tolerance of ob/ob mice. Each point represents the mean of n observations. Vertical lines through the points indicate 2 S.E. The effects of the 50 μg/day dose of hormone derivative were only statistically significant at the 0 min time point. The effects of the 250 μg/day dose were significant at all time points examined.

TABLE II

IN VITRO EFFECTS OF TRYPSIN-RESISTANT HUMAN GH PREPARATIONS ON GLUCOSE OXIDATION BY EPIDIDYMAL ADIPOSE TISSUE OF HYPOPHYSECTOMIZED RATS

Segments of epididymal adipose tissue were incubated for 60 min at 37°C in medium containing [^{14}C]glucose with or without the indicated concentration of test substance. $^{14}CO_2$ production values represent the means ± S.E. of n observations. CQSA, camphorquinone-10-sulfonic acid.

Material	Concn. (nM)	n	$^{14}CO_2$ production (dpm/mg per h)	
			control	hormone
Unmodified human GH	2	7	47.8 ± 5.5	66.8 ± 7.5
	20	7	47.8 ± 5.5	85.0 ± 4.9 [a]
Amidinated GH	2	7	47.8 ± 5.5	59.5 ± 6.2
	20	7	47.8 ± 5.5	79.1 ± 10.0 [a]
Unmodified human GH	2.5	16	65.5 ± 8.6	106.2 ± 15.5
	25	16	65.5 ± 8.6	156.8 ± 21.4 [a]
Citraconylated GH	25	16	65.5 ± 8.6	134.3 ± 18.2 [a]
	250	16	65.5 ± 8.6	165.5 ± 21.6 [a]
Unmodified GH	2.5	8	73.3 ± 9.9	135.4 ± 11.3 [a]
	25	8	73.3 ± 9.9	196.4 ± 22.3 [a]
Trifluoroacetylated GH	25	8	73.3 ± 9.9	108.1 ± 17.2
	250	8	73.3 ± 9.9	160.4 ± 26.6 [a]
Unmodified GH	5	8	49.0 ± 4.0	82.5 ± 9.9 [a]
	25	8	49.0 ± 4.0	105.5 ± 11.6 [a]
CQSA-modified GH	50	8	49.0 ± 4.0	77.0 ± 11.0 [a]
	250	8	49.0 ± 4.0	103.9 ± 11.7 [a]

[a] Statistical comparisons were made with Dunnett's test. A difference is considered to be significant when $P < 0.05$.

duced statistically significant stimulatory effects on glucose oxidation when employed at concentrations of 2.5–5 nM in this assay. It can be seen in Table II that amidinated GH and citraconylated GH retained substantial activity in this assay, whereas trifluoracetylated GH and camphorquinone-10-sulfonic acid-modified GH appeared to have approx. 10% the activity of native human GH.

Finally, the trypsin-resistant derivatives of human GH were tested for their ability to compete with ^{125}I-labeled human GH for binding to antibodies to human GH. The potencies of the derivatives in this assay relative to that of unmodified human GH were estimated by determining the amount of test substance required to displace 50% of antibody-bound ^{125}I-labeled human GH. All displacement curves obtained with the derivatives were parallel to that produced with unmodified human GH. Assuming a potency of 100% for unmodified human GH, the following relative potencies (± S.E.) were estimated from repeated (2–6) assays: amidinated GH, 90.5 ± 3.8; citraconylated GH, 71.5 ± 3.1; trifluroacetylated GH, 40.0; and camphorquinone-10-sulfonic acid-modified GH, 43.3 ± 3.3. Thus, the relative potencies of the derivatives in this assay are somewhat similar to their order of potency in the growth-promoting and diabetogenic assays.

Discussion

In the present study, four derivatives of GH were prepared in which lysine or arginine residues were modified chemically to render the hormone molecule more resistant to proteolysis by trypsin. All of these trypsin-resistant forms of human GH were found to possess significant growth-promoting, diabetogenic and insulin-like activities, although all activities were attenuated to some extent in each derivative. Of the four derivatives studied, amidinated GH, in which positive charges on lysine residues were retained, was the most active, having nearly full growth-promoting activ-

ity and a high degree of diabetogenic and insulin-like activities. This finding is in agreement with a recent report by de Satz and Santome [17], indicating that bovine GH, in which four or five of the lysine residues were amidinated, retained essentially full growth-promoting activity. As these authors suggested, retention of a positive charge on certain lysine residues in GH may be important for the expression of high biological activity. Citraconylated GH, in which negatively charged groups were present on certain lysine residues, retained substantial growth-promoting and insulin-like activities, but its diabetogenic activity was reduced. All activities were substantially attenuated in the trifluoroacetylated derivative being approx. 10% those of the native hormone. The arginine-modified derivative also exhibited a significant but attenuated biological activity profile. From these observations, it would seem reasonable to conclude that the alterations in biological activity profile exhibited by the various derivatives are not due to their trypsin-resistance but are most likely due to changes in configuration of the hormone molecule resulting from the chemical modification of the lysine and arginine residues. If proteolytic processing of the GH molecule is involved in the expression of one or more of its biological activities, such processing probably does not involve a trypsin-like proteinase.

Acknowledgements

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References

1 Kostyo, J.L. and Reagan, C.R. (1976) in Pharmaceutical Therapy B, Vol. 2, 591–604, Pergamon Press, Oxford
2 Lewis, U.J., Singh, R.N.P., VanderLaan, W.P. and Tutwiler, G.F. (1977) Endocrinology 101, 1587–1603
3 Lostroh, A.J. and Krahl, M.E. (1974) Proc. Natl. Acad. Sci. USA 71, 1244–1246
4 Lostroh, A.J. and Krahl, M.E. (1976) Proc. Natl. Acad. Sci. USA 73, 4706–4710
5 Yudaev, N.A., Pankov, Yu.A., Keda, Yu.M., Sazina, E.T., Osipova, T.A., Shwachkin, Yu.P. and Ryabtsev, M.N. (1983) Biochem. Biophys. Res. Commun. 110, 866–872
6 Mills, J.B., Ashworth, R.B., Wilhelmi, A.E. and Hartree, A.S. (1969) J. Clin. Endocrinol. Metab. 29, 1456–1459
7 Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta 251, 363–369
8 Atassi, M.Z. and Habeeb, A.F.S.A. (1972) Methods Enzymol. 25, 546–553
9 Mills, J.B., Kostyo, J.L., Reagan, C.R., Wagner, S.A., Moseley, M.H. and Wilhelmi, A.E. (1980) Endocrinology 107, 391–399
10 Habeeb, A.F.S.A. (1966) Anal. Biochem. 14, 328–336
11 Seavey, B.K., Singh, R.N.P., Lindsey, T.T. and Lewis, U.J. (1973) Gen. Comp. Endocrinol. 21, 358–367
12 Hunter, M.J. and Ludwig, M.L. (1962) J. Am. Chem. Soc. 84, 3491–3504
13 Goldberger, R.F. (1967) Methods Enzymol. 11, 317–322
14 Pande, C.S., Pelzig, M. and Glass, J.D. (1980) Proc. Natl. Acad. Sci. USA 77, 895–899
15 Reagan, C.R., Kostyo, J.L., Mills, J.B., Moseley, M.H. and Wilhelmi, A.E. (1978) Endocrinology 102, 1377–1386
16 Kostyo, J.L., Gennick, S.E. and Sauder, S.E. (1984) Am. J. Physiol. 246, E356–E360
17 De Satz, V.B. and Santome, J.A. (1981) Int. J. Peptide Res. 18, 492–499