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Synthetic Position 5 Analogs of Adrenocorticotropin Fragments and Their in Vitro Lipolytic Activity†

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ABSTRACT: Twenty-three analogs of the ACTH-(4–10)-heptapeptide sequence, which forms the "active core" of adrenocorticotropin (ACTH) and related hormones, have been synthesized by the solid-phase method. These analogs all contain structural modifications at or near the 5-glutamic acid residue of ACTH. The peptides were purified to electrophoretic and chromatographic homogeneity. The peptides were assayed for lipolytic activity in an isolated cell system derived from rabbit adipose tissue. In this system, it was determined that residue 5 plays a very important "spacer" role in the peptide, but that this spacer function is not very dependent on the nature of the side chain of the position 5 amino acid. It was found, however, that a number of analogs containing *basic* residues (arginine or lysine) in position 3 and/or position 5 of ACTH-(3–10) and ACTH-(4–10) fragments have 5 to 10 times the activity of the respective parent peptides. The presence of a latent anionic locus in the rabbit fat-cell receptor for ACTH is suggested by this study.

The ACTH-(4–10)-heptapeptide sequence (Met-Glu-His-Phe-Arg-Trp-Gly) has been found to be absolutely invariant in the adrenocorticotropins (ACTH), melanocyte-stimulating hormones (MSH), and lipotropins (LPH)[1] of all species sequenced to date (Riniker et al., 1972; Li, 1972; Hechter and Braun, 1972; Lee et al., 1963; Lowry and

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; LPH, lipotropin; Boc, *tert*-butoxycarbonyl.

Chadwick, 1970; Gráf et al., 1971; Cseh et al., 1972; Chrétien et al., 1972). The sequence is believed to be the "active core" of each of these hormones (Hechter and Braun, 1972; Ramachandran, 1973), and has thus been the focus of many structure–function studies. It is clear that 8-arginine and 9-tryptophan play vital and very specific roles in the function of ACTH and related hormones (Ramachandran, 1973; Bodanszky et al., 1964; Yajima et al., 1966; Chung and Li, 1967; Hofmann et al., 1970b; Tesser et al., 1973; Fujino et al., 1971b), that 6-histidine and 7-phenylalanine play important but somewhat less specific parts (Ramachandran, 1973; Fujino et al., 1971a,b; Hofmann et al., 1970a; Blake and Li, 1972), and that 4-methionine is involved in an important hydrophobic interaction with the receptor (Hofmann et al., 1964; Geiger et al., 1969; Fujino et al., 1970). Until recently, 5-glutamic acid, the only acidic residue of the sequence, has received very little attention (Schnabel and Li, 1960; Moroder and Hofmann, 1970; Li and Hemmasi, 1972). A preliminary communication (Draper et al., 1973a) has indicated that this position may play a spacer role in the peptide, and in a previous report (Draper et al., 1973b; Draper, 1974) it has been shown that 5-Arg-ACTH-(4–10) has significantly increased activity over that of ACTH-(4–10) itself, when activities are measured in the rabbit adipocyte system. We wish to report here a study involving the synthesis and lipolytic assay of 23 analogs of the ACTH-(4–10) sequence, in which attention was focused on position 5 of this sequence, and in which explanations and extensions of the previous observations were sought.

Experimental Section

Synthesis of Peptides (Merrifield, 1969). Boc-glycine-resin (1.0 g, 0.18 mmol of glycine) (Draper et al., 1973b) was placed in the reaction vessel of a Schwarz BioResearch peptide synthesizer and treated as follows: (1) three washings with 40-ml portions of methylene chloride; (2) prewash with 25 ml of 35% trifluoroacetic acid (CF_3COOH) in methylene chloride containing 5 mg/ml of dithiothreitol, followed by a 30-min treatment with 25 ml of the same CF_3COOH solution; (3) five washings with 40-ml portions of methylene chloride; (4) two washings with 25-ml portions of isopropyl alcohol; (5) five washings with 40-ml portions of methylene chloride; (6) three 2-min treatments with 25-ml portions of 5% diisopropylethylamine in methylene chloride; (7) five washings with 40-ml portions of methylene chloride; (8) addition of 0.45 mmol of Boc-L-tryptophan in 1 ml of dimethylformamide and 7 ml of methylene chloride, followed by brief shaking; (9) addition of a 5-ml solution of methylene chloride containing 0.45 mmol of dicyclohexylcarbodiimide, followed by shaking the mixture for 30 min; (10) five washings with 40-ml portions of methylene chloride; (11) two washings with 25-ml portions of isopropyl alcohol; (12) two washings with 40-ml portions of methylene chloride; (13) two washings with 25-ml portions of isopropyl alcohol; (14) five washings with 40-ml portions of methylene chloride; (15) a repeat of steps 6 through 14. In the next cycle, the same steps were repeated but with Boc-N^g-tosyl-L-arginine in the place of the Boc-L-tryptophan. Additional cycles were performed for the synthesis of each peptide as were appropriate to build the desired sequence from the C terminus toward the N terminus. Boc-L-phenylalanine, Boc-N^{im}-tosyl-L-histidine, Boc-L-glutamic acid γ-benzyl ester, Boc-L-methionine, Boc-glycine, Boc-*O*-benzyl-L-threonine, Boc-L-leucine, Boc-L-alanine, Boc-β-alanine, Boc-D-methionine, Boc-D-glutamic acid γ-benzyl ester, Boc-D-alanine, Boc-L-proline, Boc-N^ϵ-(2,6-dichlorocarbobenzoxy)-L-lysine, and Boc-*O*-benzyl-L-serine were used in approximately threefold molar excess, dissolved in 8 ml of methylene chloride. The entire synthesis was performed under a nitrogen atmosphere. Each synthesis was completed by brief drying under positive nitrogen pressure, followed by overnight drying at room temperature under high vacuum. Some of the syntheses (especially peptides II–XV) were performed using a previously reported protocol (Draper et al., 1973b) which differs from the above mainly in that an acetylation step was included following each double coupling cycle. Peptides I and XVI were synthesized several times by each of the two protocols.

All of the protected peptide–resin products were deprotected, cleaved, and purified in essentially the same manner. The protected peptide–resin (usually 0.8–1.3 g) was placed in a Kel-F container along with about 80 mg (2 equiv) of tryptophan and 1 ml of anisole. About 10 ml of anhydrous hydrogen fluoride was distilled into the flask and the mixture was stirred at 0° for 30 min. The hydrogen fluoride was removed below 0° by evaporation under high vacuum, and the residue was extracted three times with ethyl ether to remove the anisole. To the residue was added 50% CF_3COOH in methylene chloride (containing 5 mg/ml of dithiothreitol) and the mixture was filtered. The resin was thoroughly washed with this CF_3COOH solution and the total filtrate collected. The CF_3COOH and methylene chloride was evaporated off, and the residue was dissolved in 2 ml of 1 *N* acetic acid. The milky solution was placed on a 110 × 1 cm Bio-Gel P-2 column and eluted with 1 *N* acetic acid. The peptides in this study all emerged at 150–250 ml of elution volume, well ahead of the free tryptophan peak (550 ml) and other smaller impurity peaks. The large peak in the 150–250-ml range was isolated and lyophilized to a white powder. This material was dissolved in 1 ml of 0.1 *N* NH_4OAc (adjusted to pH 5 with acetic acid) and placed on a carboxylmethylcellulose (CM-cellulose) column (35 × 0.8 cm) and eluted with either a discontinuous or a continuous NH_4OAc gradient extending from 0.1 to 4.0 *N* (adjusted to pH 5 with acetic acid). The major peak in each case was pooled and lyophilized. Each product was subjected to one–three additional passes through this same CM-cellulose system with the NH_4OAc gradient until a single, symmetrical peak free of contaminants was obtained. This material was lyophilized and desalted on a 55-cm Bio-Gel P-2 column and again lyophilized from acetic acid to a fluffy white powder. An 8–17% yield was obtained in each case, based on the loading of the original Boc-glycine-resin.

Amino acid analyses were performed on acid and enzymatic hydrolyses of each of these peptides as previously described (Draper et al., 1973b). Amino acid ratios agreed well with theory (Table I), enzymatic hydrolysis gave complete digestion in every case, and less than 1% oxides of methionine were detected in the enzymatic hydrolysates of the methionine-containing peptides. High voltage electrophoresis (30 min at 3000 V, 120 mA, 10°) was carried out at pH 1.9 in every case and in most cases at pH 3.5 and 6.3 as well. All electrophoretic results showed a single spot for each purified, unhydrolyzed peptide. It was demonstrated that impurities could be detected at the 1–2% level. Some of these results are included in Figures 1a and 1b. In addition, in several cases electrophoretic analysis (Draper et al., 1973b) of a tryptic digest of the peptide was performed. Representative results along with interpretation of spots are

Table I: Amino Acid Analyses.

	3 4 5 6 7 8 9 10	Ser	Lys	Pro	Ala	Leu	Thr	Met	Glu	His	Phe[a]	Arg	Trp	Gly
I	Met - Glu-His-Phe-Arg-Trp-Gly							0.96	1.02	1.00	1.00	0.99	0.92	1.01
II	Glu-His-Phe-Arg-Trp-Gly								0.96	0.97	1.00	1.02	0.88	1.01
III	His-Phe-Arg-Trp-Gly									0.95	1.00	0.99	0.89	0.96
IV	Gly - Gly-His-Phe-Arg-Trp-Gly									1.03	1.00	1.07	0.91	3.30
V	Met - Thr-His-Phe-Arg-Trp-Gly						0.97	1.08		1.03	1.00	1.01	1.02	1.05
VI	Met - Leu-His-Phe-Arg-Trp-Gly					1.00		1.04		1.12	1.00	1.04	1.00	1.15
VII	Met - Ala-His-Phe-Arg-Trp-Gly				0.96			0.98		0.92	1.00	0.98	0.95	0.97
VIII	Met - Gly-His-Phe-Arg-Trp-Gly							0.99		1.06	1.00	1.09	0.99	1.83
IX	Met-His-Phe-Arg-Trp-Gly							0.95		0.99	1.00	0.96	0.94	0.99
X	Met-β-Ala-His-Phe-Arg-Trp-Gly				1.09[b]			1.12		1.08	1.00	0.98	0.98	1.05
XI	Met-Gly - Gly-His-Phe-Arg-Trp-Gly							0.95		0.93	1.00	1.07	0.89	2.96
XII	D-Met - Glu-His-Phe-Arg-Trp-Gly							1.02	0.99	0.97	1.00	0.96	0.89	1.00
XIII	Met-D-Glu-His-Phe-Arg-Trp-Gly							1.02	1.00	1.03	1.00	1.02	0.92	1.00
XIV	Met-D-Ala-His-Phe-Arg-Trp-Gly				1.00			0.99		1.04	1.00	1.02	0.89	1.00
XV	Met - Pro-His-Phe-Arg-Trp-Gly			0.98				0.93		1.04	1.00	1.08	0.87	1.01
XVI	Met - Arg-His-Phe-Arg-Trp-Gly							0.98		1.04	1.00	2.05	0.97	0.99
XVII	Met - Lys-His-Phe-Arg-Trp-Gly		1.07					1.09		1.06	1.00	1.07	0.97	1.02
XVIII	Arg-His-Phe-Arg-Trp-Gly									1.02	1.00	2.06	0.95	1.02
XIX	Gly - Arg-His-Phe-Arg-Trp-Gly									1.06	1.00	1.97	1.02	1.94
XX	Arg - Gly-His-Phe-Arg-Trp-Gly									0.98	1.00	2.03	0.96	1.93
XXI	Ser -Met - Glu-His-Phe-Arg-Trp-Gly	0.96						0.91	1.08	1.03	1.00	1.03	0.80	1.05
XXII	Arg-Met - Glu-His-Phe-Arg-Trp-Gly							0.94	1.03	1.06	1.00	2.00	0.99	1.00
XXIII	Arg-Met - Arg-His-Phe-Arg-Trp-Gly							0.91		1.02	1.00	2.99	0.83	1.00

[a] Defined as 1.00. [b] β-Alanine.

included in Figures 1a and 1b. The purified peptides were stored in the following way. The peptide was dissolved in 1 *N* acetic acid, aliquots were placed in small siliconized glass tubes, the acetic acid was removed by lyophilization, and the tubes were flushed with nitrogen, sealed, and stored in the cold.

Bioassays. Collagenase-dispersed rabbit adipocytes were used in these studies. Net free fatty acid production during a 45-min incubation with the peptide was used as a measure of lipolytic activity. Free fatty acids were measured as the ^{63}Ni complexes by the method of Ho (1970). Details of the cell preparation and assay are included in a previous communication (Draper et al., 1973b).

Results

The activity of each of the synthetic analogs in the lipolytic assay is shown in Table II. Activity is expressed as a comparison of the midpoints of dose–response curves rising to the same maximal level, with ACTH-(4–10) (peptide I) defined as a reference standard. This peptide was found to stimulate lipolysis in this assay system to the same maximum as ACTH itself, when present at 10^3 times the concentration (w/w) of the parent hormone (Draper et al., 1973b). The activity of some of these peptides (II, III, IV, IX, XV, XVIII, XIX, and XX) cannot be quantitatively compared in this system, either because they showed *no* stimulation of lipolysis at the highest concentrations tested, or because they showed *partial* stimulation of lipolysis, but the maximal stimulation achieved by the standard ACTH-(4–10) was never reached. We have considered the activity to be qualitatively "low or absent" in such cases.

Several peptides were synthesized in an attempt to define the role of the position 5 glutamic acid in the core sequence. Peptide II, without 4-methionine, and peptide III, lacking both 4-methionine and 5-glutamic acid, were inactive at high concentrations, indicating that both glutamic acid and methionine are somehow important to the activity of the peptide. Filling the 4 and 5 positions with glycines (peptide

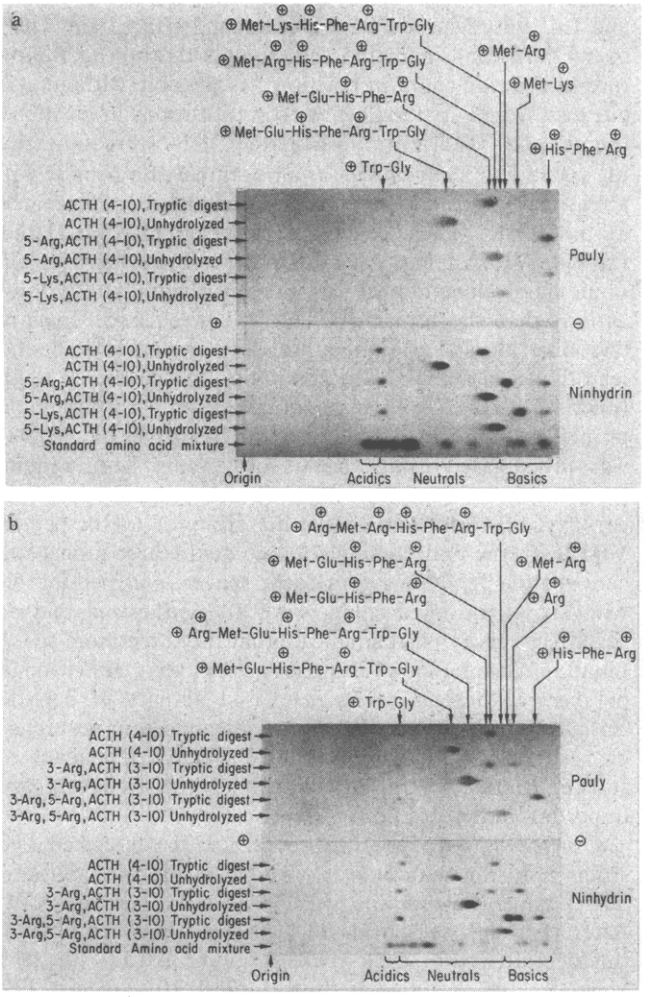

FIGURE 1: (a,b) Electropherograms of unhydrolyzed and tryptic digest of some of the synthetic peptides used in this study compared with the standard Beckman amino acid mixture. Electrophoresis was run at pH 1.9 for 30 min at 3000 V/50 cm at 10°. Upper strip was stained with Pauly spray and lower strip with ninhydrin in each case.

Table II: Analogs of the ACTH Core Sequence.

	3 4 5 6 7 8 9 10	Act., SD	No. of Trials
I	Met - Glu-His-Phe-Arg-Trp-Gly	100 (def.)	
II	Glu-His-Phe-Arg-Trp-Gly	<0.1	3
III	His-Phe-Arg-Trp-Gly	<0.05	3
IV	Gly - Gly-His-Phe-Arg-Trp-Gly	<1	3
V	Met - Thr-His-Phe-Arg-Trp-Gly	87 (±14.2)	5
VI	Met - Leu-His-Phe-Arg-Trp-Gly	76 (±21.6)	3
VII	Met - Ala-His-Phe-Arg-Trp-Gly	98 (±6.0)	4
VIII	Met - Gly-His-Phe-Arg-Trp-Gly	108 (±18.6)	7
IX	Met-His-Phe-Arg-Trp-Gly	<1	2
X	Met-β-Ala-His-Phe-Arg-Trp-Gly	31 (±8.9)	3
XI	Met-Gly - Gly-His-Phe-Arg-Trp-Gly	7 (±2.5)	4
XII	D-Met - Glu-His-Phe-Arg-Trp-Gly	37 (±11.6)	3
XIII	Met-D-Glu-His-Phe-Arg-Trp-Gly	2 (±0.7)	4
XIV	Met-D-Ala-His-Phe-Arg-Trp-Gly	18 (±15.7)	7
XV	Met - Pro-His-Phe-Arg-Trp-Gly	<0.5	2
XVI	Met - Arg-His-Phe-Arg-Trp-Gly	560 (±139)	8
XVII	Met - Lys-His-Phe-Arg-Trp-Gly	520 (±151)	5
XVIII	Arg-His-Phe-Arg-Trp-Gly	<1	4
XIX	Gly - Arg-His-Phe-Arg-Trp-Gly	<10	3
XX	Arg - Gly-His-Phe-Arg-Trp-Gly	<1	2
XXI	Ser -Met - Glu-His-Phe-Arg-Trp-Gly	125 (±20.3)	3
XXII	Arg-Met - Glu-His-Phe-Arg-Trp-Gly	570 (±160)	6
XXIII	Arg-Met - Arg-His-Phe-Arg-Trp-Gly	1370 (±227)	4

IV) was not sufficient to restore activity to the inactive ACTH-(6–10) sequence. Since previous studies (Schnabel and Li, 1960; Moroder and Hofmann, 1970; Li and Hemmasi, 1972) had indicated that some alteration of the nature of the side chain in position 5 is possible without serious loss of activity, analogs with substitutions in position 5 (peptides V–VIII) were investigated. The 5-threonine analog (peptide V) was chosen as an example of a peptide with a reasonably isosteric and polar, yet uncharged replacement at position 5. The 5-leucine (peptide VI) and 5-alanine (peptide VII) analogs gave less polar substitutions for glutamic acid. None of these peptides had significantly lower activity than the natural ACTH-(4–10) sequence. Even reduction of the side chain to a proton (peptide VIII) does not lead to loss of activity. The possibility that position 5 might function as a spacer, holding an important 4-methionine in proper position for its binding role (Draper et al., 1973a), was investigated using spacers of various sizes, ranging from 0 to 6 atoms. Peptide IX, with no atoms between the carboxyl of 4-Met and the amino group of 6-His, peptide VIII, with the natural single amino acid (three atom) spacer, peptide X, with a four-atom spacer, and peptide XI, with six atoms intervening, were all synthesized and assayed. The assay results indicate that the three-atom spacer found in nature allows for higher activity than either longer or shorter "bridges". The decreased activity of peptides XII–XIV, in which D residues are introduced in positions 4 or 5, suggests some conformational specificity, and the results on peptide XV raise the possibility that free rotation is important in this position.

Our previous study (Draper et al., 1973b) described a position 5 analog with a *basic* residue in place of glutamic acid, namely 5-arginine ACTH-(4–10) (peptide XVI). After thorough evaluation of two additional syntheses of this peptide, it was clear that 5-arginine-ACTH-(4–10) is about 5 times as active as the natural 4–10 sequence. Peptides XVII–XXIII were synthesized in an attempt to explain this effect. The 5-lysine analog (XVII) was as active as its arginine counterpart, demonstrating a lack of specificity for the nature of the basic side chain. The 4-methionine residue is still very important in these analogs as was shown by the low activity of peptide XVIII, in which 4-methionine is omitted in a 5-arginine analog, and peptide XIX, in which 4-methionine is replaced by glycine. Also, the basic function cannot be moved to position 4 as was shown by the inactivity of 4-arginine,5-glycine-ACTH-(4–10) (peptide XX). Substitution of a basic residue in position 3 of ACTH-(3–10) resulted in an analog (peptide XXII) with about 5 times the activity of ACTH-(3–10) (XXI). The enhancing effects of basic residues in positions 3 and 5 on lipolysis appear to be additive. Thus, Arg-Met-Arg-His-Phe-Arg-Trp-Gly (peptide XXIII) is over 10 times as active as the parent ACTH-(3–10)-octapeptide (peptide XXI). Analysis of these data by the Student t test shows differences in activity between peptides I and XVI, I and XVII, XXI and XXII, and XXI and XXIII all to be highly ($P < 0.001$) significant.

Discussion

Assay results on peptides I–XV strongly suggest that when considering interaction with the rabbit adipocyte receptor, the important role of the position 5 residue in ACTH-(4–10) is that of a spacer. The nature of neutral or acidic side chain replacements in position 5 makes little difference to activity. The low activity of peptides IX–XI compared to that of peptide VIII shows clearly that a single residue (or three atom) width of the spacer is crucial. The results on peptides XII–XV, although far from suggesting any conformational model of the peptide, do indicate that conformational restrictions exist. Overall, the picture emerges of the position 5 residue forming a vital bridge between an important binding function, 4-methionine, and the active "center" of the peptide chain, positions 6–9.

In light of the invariance in position 5 within all hormones of the ACTH family, it is of interest that a complete reversal of charge in the side chain of that position would lead to an analog with increased activity in *any* specific bioassay system. The results suggest that this increased activity is a *binding* phenomenon, dependent on an electro-

static interaction without specificity for the basic side chain. The finding of parallel, superimposable dose–response curves displaced from each other only on the concentration axis suggests differences in strength of binding to the receptor (Ariëns and Simonis, 1964). The results further indicate that the proposed binding role of 4-methionine persists in these new analogs (see peptides XVIII–XX); that is, the *hydrophobic* binding function of methionine is *not* effectively supplanted by this apparent introduction of an *ionic* binding function. Substitution of a basic residue in position 3 of the ACTH-(3–10) sequence (peptide XXII) results in a similar increase in activity to that seen with position 5 substitution, and these effects are additive (peptide XXIII). It thus appears that, due to the presence in the receptor of a latent electrostatic binding site, appropriate introduction of positive charge can result in analogs which bind up to 10 times as strongly as the natural ACTH-(3–10) sequence. This charge can be introduced in the side chains of positions 3 and/or 5 of the sequence, where the side chains of the naturally occurring residues are apparently not playing a crucial role. The charge *cannot,* however, be introduced in the position 4 side chain, as it then interferes with a crucial function in the natural sequence. The observation of a large increase in activity with the substitution of the basic residue in position 3 recalls the fact that β-MSH (a peptide containing a basic residue in its natural sequence in the equivalent of position 3 in ACTH) is a potent lipolytic agent in the rabbit (but not in the rat) (Tanaka et al., 1962).

The most likely explanation for these data is, then, that the rabbit adipocyte receptor for ACTH contains a latent anionic binding site accessible to the N-terminal region of the peptide hormone in its receptor-bound state. The observations on position 3 analogs, position 5 analogs, and on β-MSH itself could all be part of the same phenomenon. Our own preliminary data and above-mentioned observations by others (Draper, 1974; Tanaka et al., 1962) suggest that this phenomenon may not be the same in rat (fat cell and adrenal) ACTH receptors. The fact that the natural-sequence peptide, containing a negatively charged side chain in position 5, is not relatively *less* active than analogs with neutral side chains in this position (compare, for instance, peptides I and VIII) may be explained by the suggestion (Flynn et al., 1972) that the charge of glutamic acid is neutralized under physiological conditions.

Other possibilities must also be considered. It is certainly conceivable that, due to differences in solubility in various hydrophobic environments, *access* to the receptor is affected in some analogs. Increased resistance to degradation, which has often been responsible for apparent increases in activity of peptide hormone analogs, is unlikely in this case. There is nothing obvious in the structure of peptides XVI, XVII, XXII, or XXIII to suggest increased resistance to degradation, and our preliminary observations on the time course of both aminopeptidase M hydrolysis and in vitro hormone incubation with adipocytes give no suggestion of any unusual resistance to inactivation. The possibility that different mechanisms of action are involved in the activity of the parent sequence vs. the unusually active analogs seems remote. The shape of the dose–response curves (Draper et al., 1973b) certainly does not support the idea. Furthermore, preliminary data (Draper and Ho, 1974) show that the relative activities of peptides I, XVI, XVII, and XVIII are the same when cyclic AMP production is used as an index of activity, as when lipolysis is the assay end point. Such results suggest, at least, that similar mechanisms of action are involved for these peptides.

The significance of these results in the full ACTH molecule and in other (especially adrenal) assay systems remains to be established. Presently, these studies and those from other labs are delineating important characteristics of this crucial "core" peptide of ACTH. Such work suggests that peptide hormones may be important yet subtle tools in the investigation of the plasma membrane receptors with which they interact.

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Growth Hormone Covalently Bound to Sepharose or Glass. Analysis of Ligand Release Rates and Characterization of Soluble Radiolabeled Products†

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ABSTRACT: Purified bovine growth hormone labeled enzymatically with iodine-125 was covalently coupled to cyanogen bromide activated Sepharose 4B gel and to diazotized zirconia-clad glass beads. Under the conditions employed, an average of 0.8 and 7.3 mg of hormone were bound per ml of Sepharose and glass, respectively. When the conjugates were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4), three separate radioactive species were detected in the incubation supernatant by chromatography on Sephadex G-75. The elution volumes of two of the species were identical with those of ^{125}I-labeled growth hormone and Na^{125}I controls, while the third component eluted as a molecule of intermediate size. The rate of release of each species from the solid matrix was linear with time over 4 days and increased with temperature from 4 to 37°. Although significantly less growth hormone was released from glass (0.14%/day) than from Sepharose (0.40%/day) at 37°, active hormone in amounts sufficient to be detectable in a biological assay was nevertheless liberated from the former after as little as 4 hr of incubation. By contrast, the rate of release of $^{125}I^-$ and the intermediate-size compound from glass was significantly greater than from Sepharose, suggesting that protein bound to glass supports is more susceptible to degradation from exposure to ionizing radiation.

The coupling of proteins and other ligands to insoluble supports has provided an important tool for the investigation of molecular interactions (Cuatrecasas, 1970). Insolubilized hormones have been particularly useful in the areas of antibody purification (Wofsy and Burr, 1969; Fellows et al., 1973; Sairam et al., 1974) and hormone receptor isolation (Sica et al., 1973; Shiu and Friesen, 1974). In addition, protein hormone conjugates have been employed in experiments designed to show that receptors for these hormones are located on the surface of the target cell (Cuatrecasas, 1969; Turkington, 1970; Selinger and Civen, 1971). Several recent reports, however, suggest that the interpretation of biological studies carried out with such conjugates is complicated by the slow release of hormones and hormonally active fragments not only from agarose derivatives (Davidson et al., 1973; Fritz, 1971) but also from glass (Yong, 1973) and polyacrylamide supports (Davidson and Van Herle, 1973). When this occurs, it becomes difficult to determine whether part or all of the biological activity of the hormone conjugate may be due to free rather than immobilized hormone.

Previously, we observed that bovine growth hormone covalently coupled to Sepharose released a component with growth hormone activity into the medium after incubation with rat epididymal fat pads (Schwartz et al., 1973). The following studies were undertaken in an attempt to define experimental conditions under which protein hormones would remain covalently bound to a solid matrix for sufficient time to permit investigation of biological activity with the conjugate. The consistent observation of hormone release from both glass and agarose supports compels a reevaluation of investigations attributing biological activity to hormone conjugates unless the amount of hormone released under the conditions employed is clearly demonstrated to be less than the threshold for the biological response.

Materials and Methods

Bovine growth hormone (NIH-BGH-B17) was kindly provided by the Hormone Distribution Program, NIAMDD. Lactoperoxidase (lot No. 300011), grade B, was obtained from Calbiochem; trypsin-TPCK[1] (lot No. 2GB), 190 U/mg, was from Worthington Biochemical Cor-

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[1] Abbreviations used are: HPP, 3-(4-hydroxyphenyl)propionate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.