responding fractions were pooled and evaporated *in vacuo* to about 30 ml. A concentration of 1.0 mg/ml was determined by uv [λ_{max} 278 nm (ϵ 1400)] and quantitative amino acid analysis; $[\alpha]^{22}D$ −26.2° (*c* 0.1, 1 *N* AcOH); tlc showed a trace amount of an impurity and only a single strong spot R_f^A 0.19, R_f 0.58 (*n*-PrOH-NH_4OH, 7:3). The minor spot might be due to polymerization of the product on the tlc plate itself. This tendency for polymerization interfered with further examinations by paper chromatography and electrophoresis. Amino acid analysis gave Asp. 1.0; Glu. 1.0; cystine, 1.0; Tyr, 0.9; Pro, 1.0; Hnl, 1.0; Gly, 1.0; Phe, 1.0.

1-Deamino-8-L-homonorleucine-vasopressin (XIV) was prepared from compound XII (0.22 g, 0.17 mmol) in the same manner as described for 8-L-homonorleucine-vasopressin. Although insoluble in O_2-free water containing 0.05% AcOH, the reduced material formed a disulfide linkage on air oxidation (determined by the nitroferricyanide test) in suspension. The product, collected by filtration (0.1 g, 58%), was found to dissolve readily in concentrated NH_4OH but not in H_2O: mp 253-254° dec; homogeneous on tlc, R_f^A 0.39. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; half-cystine, 0.87; mixed disulfide, 0.24; Tyr, 0.9; Phe, 0.9; Hnl, 1.0. *Anal.* ($C_{47}H_{65}N_{11}O_{12}S_2 \cdot H_2O$) C, H, N, S.

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Lipolytic Activity of Met-Arg-His-Phe-Arg-Trp-Gly, a Synthetic Analog of the ACTH (4–10) Core Sequence

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A new analog of the ACTH-(4-10)-heptapeptide has been synthesized by the solid-phase method. The thoroughly purified and characterized peptide, Met-Arg-His-Phe-Arg-Trp-Gly, was found to have about four times the lipolytic activity in isolated rabbit fat cells of the synthetic peptide, Met-Glu-His-Phe-Arg-Trp-Gly, corresponding to the natural sequence.

The heptapeptide sequence, Met-Glu-His-Phe-Arg-Trp-Gly, common to all the adrenocorticotropic (ACTH), melanotropic (MSH), and lipotropic (LPH) hormones yet investigated,[1,2] has been the subject of a large number of structure–function studies.[3-26] Of these seven amino acids, the pentapeptide sequence, His-Phe-Arg-Trp-Gly, positions 6–10 in ACTH, or even the position 6–9 tetrapeptide is either the minimal core of activity or is an essential element of the minimal core in all biological actions ascribed to any of these hormones.[1,3,27-31] The Met-Glu dipeptide sequence, positions 4 and 5 in ACTH, is not an absolutely essential element in most biological actions, but is always found in nature associated with the essential pentapeptide, and virtually always greatly enhances the minimal biological activity of the pentapeptide.[3,30,32-38] Several studies have produced data consistent with the hypothesis that the 4-methionine is involved in a hydrophobic interaction with the receptor,[12,13,15,20,22,26] and some of our recent work has suggested that the 5-glutamic acid may play a "spacer" role with little side-chain specificity.[39] We now wish to report that an analog of the heptapeptide sequence, in which the 5-glutamic acid is replaced by arginine, has about four times the activity of the natural sequence in the stimulation of free fatty-acid release in isolated rabbit fat cells.

Both the natural ACTH-(4–10)-peptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine, and the analog of interest, methionylarginylhistidylphenylalanylarginyltryptoptophylglycine, were synthesized by the solid-phase method.[40,41] These syntheses were performed on a Beckman Model 9900 peptide synthesizer, and the conditions for the two syntheses were virtually identical. Deprotection of the amino-protected intermediates on the resin was accomplished with 20% trifluoroacetic acid (TFA) in methylene chloride to which 5 mg/ml of dithiothreitol (DTT) had been added to protect against acid-catalyzed oxidations.[42] Double coupling of each amino acid was performed. The second coupling was followed by acetylation of any remaining free amino groups with acetylimidazole.[43] The protected peptide was

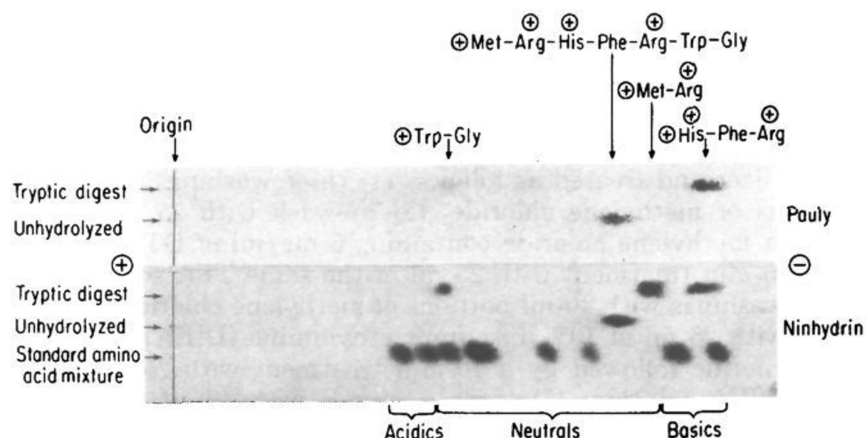

Figure 1. Electropherogram of unhydrolyzed and tryptic digest of synthetic [Arg^5]-ACTH (4–10) compared with the standard Beckman amino acid mixture. Electrophoresis was run for 50 min at 3000 V/50 cm at 10°. Upper strip was stained with Pauly spray and lower strip with ninhydrin.

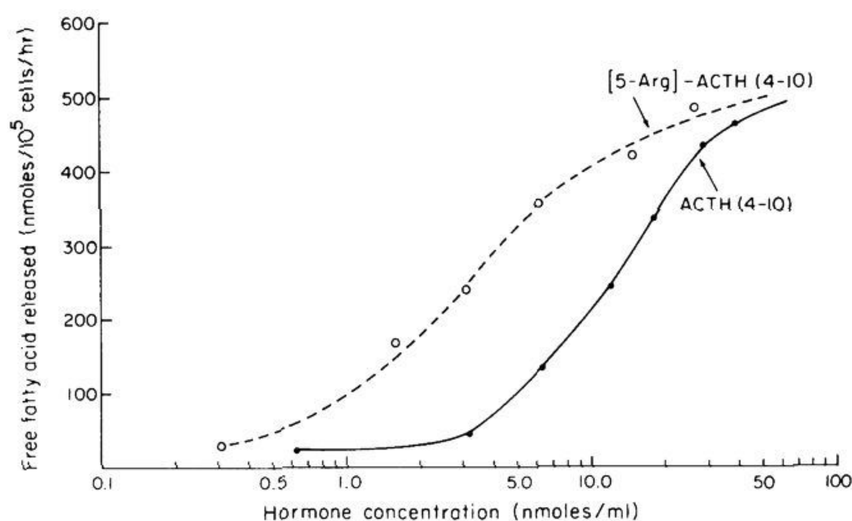

Figure 2. Pair of dose-response curves representing one complete assay in isolated rabbit adipocytes. Free fatty-acid release was calculated from the cpm of the ^{63}Ni complex. See text.

cleaved from the resin and simultaneously deprotected by treatment with anhydrous hydrogen fluoride[44,45] in the presence of anisole and free tryptophan. Purification of the crude, deprotected peptide was accomplished on a series of Biogel P-2 and carboxymethylcellulose (CMC) columns. The identity and purity of the resulting peptides were evaluated by amino acid analysis after both acid and enzymatic hydrolysis, high voltage paper electrophoresis at three different pH values, and, in the case of the 5-arginine analog, by electrophoretic analysis of the tryptic digest. Amino acid analyses agreed well with theory, electrophoreses gave single spots, and the tryptic digest was very clear in helping to verify the identity of the analog (see Figure 1).

Biological activity of the peptides was determined in an isolated rabbit fat-cell system prepared by a modification of the procedure of Rodbell.[46] Stimulation of lipolysis was the criterion of biological potency used. The release of free fatty acids into the medium was measured by forming the ^{63}Ni-fatty acid complex as described by Ho.[47] The lipolytic system was chosen for bioassay because of its convenience, precision, reproducibility, and high sensitivity to short sequences of ACTH.[34,48] The dose-response curve of the 5-arginine analog was compared with that of the natural sequence, ACTH-(4-10)-peptide. A pair of representative dose-response curves may be seen in Figure 2. Both synthetic peptides stimulated lipolysis to the same maximum value and had dose-response curves with about the same slope, but for half-maximal response only about one-fourth as much of the 5-arginine analog was required. If the activity of the ACTH (4-10) sequence is defined as 1.0, the activity of the 5-arginine analog is 3.7 (see Table I). Natural porcine-ACTH was about 10^3 times as potent on a molar basis.

An attempt was made to assess the effect of changes in pH on relative activities of ACTH itself, the ACTH (4-10) sequence, and the 5-arginine analog of the 4-10 sequence. Fat cells were washed and incubated in various Krebs-Ringer-triethanolamine buffers of pH as high as 8.0 and as low as 6.8. At the extreme pH values, marked depression of hormone-stimulated lipolysis was noted overall, along with indications that metabolic and physical deterioration of the cells was occurring. However, over the entire pH range investigated, no significant variations in the *relative* activities of ACTH, ACTH (4-10), and [Arg^5]-ACTH (4-10) were noted.

Experiments are now in progress to determine whether these observations can be extended to other biological assay systems and to longer sequences of the ACTH chain incorporating the 5-arginine substitution. In addition, we are investigating the mechanism of this increased stimulation of lipolysis by the 5-arginine analog. A number of possible explanations can be put forward at this time. Similarly shaped dose-response curves rising to the same maximum suggest that the observed lipolytic activities of the two heptapeptides are a result of differing affinity for the receptor.[49] The observations of Li and Oelofsen[50] that there is a correlation of net charge and activity in ACTH analogs with variations in the strongly basic 15-18 region suggest a possible explanation for our observations. The strength of binding of the amino terminus of the 4-10 peptide could be charge dependent in this system. A *simple* correlation of net charge and activity is not observed, however, since a number of neutral substitutions for the glutamic acid that effectively increase the net charge in this region have resulted in peptides with activity no greater than that of the natural, glutamic acid containing peptide.[39]

Table I. Relative Lipolytic Activity of [Arg^5]-ACTH-(4-10)-heptapeptide

Synthetic peptide	Rel. lipolytic potency
ACTH (4–10)	1.0 (defined)
[Arg^5]-ACTH (4–10)	3.7 (2.5–4.8)[a]

[a]Reported potency is the average of five separate assays. Range is given in parentheses.

Another possible explanation would involve the neighboring 6-histidine, an important residue for biological activity.[9,14,15] The nearby positive charge of arginine could affect the pK of the histidine and account for the enhanced activity of this analog. Hofmann, *et al.*, have virtually ruled out a vital role for the acid-base behavior of 6-histidine in steroidogenic and melanocyte-stimulating systems,[15] but the *lipolytic* system may be different. For example, Blake and Li[9] found that replacement of 6-histidine by phenylalanine in ACTH (1-19) resulted in a large drop in steroidogenic and melanocyte-stimulating activity but even a larger drop in lipolytic activity in both the rabbit and the rat. Although our experiments in which the pH of the assay medium was varied did not confirm this possibility, it is still not ruled out.

A third possible explanation envisions that there is a latent negative charge in the receptor site, near the area in which the 4 and 5 positions of ACTH lie in the receptor-bound state. This would greatly enhance the binding of the 5-arginine analog of the hormone to the receptor. A very interesting observation of Yajima, *et al.*, on synthetic monkey β-MSH intermediates should be considered.[51] In their synthetic procedure, intermediates of increasing length at the N terminus were available and were assayed for melanocyte-stimulating activity. A comparison of their

Table II. Amino Acid Analyses of Synthetic Peptides

	ACTH (4–10)			[5-Arginine]-ACTH (4–10)		
Amino acid	Theory	Acid hydrolysis	Enzymatic hydrolysis	Theory	Acid hydrolysis	Enzymatic hydrolysis
Arg	1	0.94	0.93	2	2.10	2.04
Glu	1	1.02	1.02	0	0	0
Gly	1	1.00[a]	0.96	1	1.00[a]	1.00
His	1	0.96	1.01	1	1.04	1.05
Met	1	0.95	0.98[b]	1	1.01	1.12[b]
Phe	1	0.97	1.00[a]	1	1.03	1.00[a]
Trp	1	0.86[c]	0.90	1	0.93[c]	1.03

[a]Arbitrarily defined as 1.00. All other values relative to Gly in acid hydrolysis; to Phe in enzymatic hydrolysis. [b]Traces (<0.01) of methionine oxides were noted. [c]The recovery of tryptophan by this method is 80–90%. See text.

peptide containing an N-terminal methionine (the methionine of the core heptapeptide) with the next peptide in their synthetic series, in which an additional N-terminal arginine had been added, shows that addition of the basic residue results in a large increase in biological activity. An arginine in this position is the natural residue in monkey β-MSH and is displaced by two residues from the position in our analog, but the comparison is still of interest, especially since melanocyte and rabbit fat-cell receptors for ACTH and related peptides have been noted to have some important characteristics in common.[52] Another notable study of Li, *et al.*,[53] is one in which basic residues were attached directly to the C terminus of ACTH (1-10). We would conclude from these and other data[7,11,16,34,35,53-55] that addition of the basic residues results in little increase in steroidogenic or melanocyte-stimulating activity but a large increase in lipolytic activity in the rabbit fat cell.

Many analogs of ACTH have been synthesized that display increased activity over natural sequences of the same length. These have usually been peptides in which replacements near the N terminus by various modified or D-amino acids have led to longer lifetimes in biological systems.[20,22,31,56-67] Such results have most plausibly been explained in terms of susceptibility of the peptide to attack by various degradative enzymes which may be present in the assay system *in vitro* or *in vivo*. The analog considered in this study is the first example we know of in which replacement of a single residue within a fragment of the natural sequence of ACTH by another common, naturally occurring, L-amino acid has resulted in a large increase in any type of biological activity.

Our previous study[39] characterized the 5-glutamic acid as a "spacer," with little, if any, side-chain specificity. The fifth position in ACTH peptides might thus be considered an "unimportant" residue in the sequence from a structure-function point of view. The possibility that such "unimportant" sites in peptide hormones may be candidates for profitable use as important loci for synthetic replacements that subtly influence hormonal activity is suggested by the present study.

Experimental Section

tert-**Butyloxycarbonylglycine-resin.** A batch of 16.7 g of polystyrene-1% divinylbenzene resin (Bio-Rad Biobeads S-X1, 200-400 mesh, Lot 6718), chloromethylated to the extent of 1.7 mmol of Cl/g of resin, was placed in a vessel with 150 ml of dimethylformamide, 4.0 mmol of Boc-glycine, and 4.0 mmol of $CsHCO_3$ and stirred at 50° for 48hr.[68] The degree of esterification was determined to be 0.18 mmol of Boc-glycine/g of resin by hydrolysis for 5 hr at 137° in 1:1 propionic acid-hydrochloric acid followed by amino acid analysis.

Methionyl-*O*-benzylglutamyl-N^{im}-tosylhistidylphenylalanyl-N^{g}-tosylarginyltryptophylglycine-resin. Boc-glycine-resin (1.0 g, 0.18 mmol of glycine) was placed in the reaction vessel of the synthesizer and treated as follows: (1) three washings with 25-ml portions of methylene chloride; (2) prewash with 25 ml of 20% TFA in methylene chloride containing 5 mg/ml of DTT followed by a 25-min treatment with 25 ml of the same TFA solution; (3) three washings with 40-ml portions of methylene chloride; (4) prewash with 25 ml of 10% diisopropylethylamine (DIEA) in methylene chloride followed by a 10-min treatment with 25 ml of the same DIEA solution; (5) three washings with 40-ml portions of methylene chloride; (6) addition of 0.45 mmol of Boc-tryptophan in 1 ml of dimethylformamide and 7 ml of methylene chloride, followed by a brief stirring; (7) a 5-ml solution of methylene chloride containing 0.45 mmol of dicyclohexylcarbodiimide was then added and the entire mixture stirred for 150 min; (8) three washings with 40-ml portions of methylene chloride; (9) a treatment of 10 min with 25 ml of 10% DIEA in methylene chloride; (10) three washings with 40-ml portions of methylene chloride; (11) a repeat of steps 6-8; (12) a 45-min treatment with 25 ml of 5 mg/ml of acetyl imidazole in methylene chloride; (13) three washings with 40-ml portions of methylene chloride; (14) three washings with 40-ml portions of absolute ethanol; (15) three washings with 40-ml portions of methylene chloride. In the next cycle, the same steps were repeated by with Aoc-N^{g}-tosylarginine in place of the Boc-tryptophan. In the next four cycles, the identical procedure was used but with 0.45 mmol of Boc-phenylalanine, Boc-N^{im}-tosylhistidine, Boc-glutamic acid γ-benzyl ester, and Boc-methionine (each dissolved in 8 ml of methylene chloride), respectively, as the amino acid derivative used in the coupling steps. A final deprotection was performed by adding 25 ml of 20% TFA in methylene chloride (5 mg/ml of DTT) and stirring for 30 min. Several washings with methylene chloride were followed by brief drying under positive nitrogen pressure and drying overnight under high vacuum. All steps of the synthesis were performed under a nitrogen atmosphere.

Methionylglutamylhistidylphenylalanylarginyltryptophylglycine. The above protected heptapeptide resin (1.25 g) was placed in a Kel-F container along with 80 mg (2 equiv) of tryptophan and 1 ml of anisole. Addition of 10 ml of anhydrous hydrogen fluoride by distillation was followed by 30 min of stirring of the mixture at 0°. The hydrogen fluoride was removed 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% TFA in methylene chloride (containing 5 mg/ml of DTT) and the mixture was poured onto a filter. The resin was thoroughly washed with this TFA solution and the total filtrate collected. The TFA and methylene chloride were evaporated off, and the residue was dissolved in 1 ml of 1 *N* acetic acid. The milky solution was placed on a 110 × 1 cm Biogel P-2 column and eluted with 1 *N* acetic acid. A peak containing peptide material with modified (oxidized) tryptophan emerged in the void volume (120 ml) followed by a large peak (150-210 ml) consisting of the crude desired peptide. A number of smaller peaks followed, containing peptide material of undesired composition, and then the very large tryptophan peak emerged at 550-ml effluent volume. DTT was washed off the column much later. The large peak (150-200 ml) containing material of amino acid composition corresponding to the desired peptide was pooled and lyophilized. The fluffy powder was dissolved in 1 ml of 0.1 *N* NH_4OAc (adjusted with acetic acid to pH 5) and placed on a CMC column (35 × 0.8 cm) and eluted with a discontinuous gradient of NH_4OAc extending from 0.1 to 2.0 *N* (adjusted to pH 5 with acetic acid). The major peak was pooled and lyophilized. The lyophilized material was dissolved in 1 ml of 0.1 *N* NH_4OAc (pH 7), placed on another 0.8 × 35 cm CMC column, and eluted with a 0.1-2.0 *N* gradient of NH_4OAc at pH 7. A large, single peak, eluted with 0.1 *N* buffer, was pooled and lyophilized. This material was desalted on a 55-cm Biogel P-2 column and again lyophilized from acetic acid to a fluffy white powder (12.1 mg). After hydrolysis in 6 *N* HCl containing 2% by volume mercaptoacetic acid,[69] amino acid analysis was performed (Table II). These hydrolyses conditions were found to yield an 80-90% recovery of tryptophan in our hands and allowed tryptophan to be routinely determined on the amino acid analyzer. The peptide was completely hydrolyzed by aminopeptidase M (see Table II). Little if any methionine oxide was detected. High-voltage paper electrophoresis of the peptide was carried out at three different pH values: (1) pH 3.46 pyridine

acetate, 1500 V, 60 min; (2) pH 6.32 pyridine acetate, 1500 V, 60 min; and (3) pH 9.22 sodium borate, 1500 V, 60 min. Each of the above runs gave a single homogeneous spot with the R_f expected from the net charge of the synthetic molecule. The purified peptide was weighed into small tubes in 0.2-2.0-mg portions. The tubes were flushed with nitrogen, sealed, and then stored in the cold. The peptide was found to be stable in the bioassay over a period of months when stored in this way.

Methionyl-N^g-tosylarginyl-N^{im}-tosylhistidylphenylalanyl-N^g-tosylarginyltryptophylglycine-resin. Boc-glycine-resin (650 mg, 0.12 mmol of glycine) was treated exactly as in the previous synthesis, except that Aoc-N^g-tosylarginine was used in place of the glutamic acid derivative.

Methionylarginylhistidylphenylalanylarginyltryptophylglycine. Protected peptide resin (790 mg) was deprotected and cleaved from the resin in hydrogen fluoride under identical conditions to those above. The elution pattern from the 110-cm Biogel P-2 column was very similar to that of the previous peptide. The elution pattern from the CMC column was similar to that of the previous case, except that the major peak eluted with 0.5 *N* buffer. Final desalting followed by lyophilization gave a fluffy white powder (10.9 mg) with amino acid analyses given in Table II. Electrophoreses on paper were carried out simultaneously with the 5-glutamic acid peptide, and single homogeneous spots were found with the expected mobilities in each case. Tryptic digestion of the purified peptide was carried out as follows. Incubation at 37° for 2 hr (in 0.5% NH_4HCO_3) of 0.93 mg of the peptide with 0.01 mg of trypsin (total volume 0.06 ml) was followed by spotting 20 μl of the hydrolysate, 20 μl of the unhydrolyzed peptide at the same molar concentration as the hydrolysate, and 10 μl of a 0.5 m*M* Beckman amino acid standard mixture. A 50-min electrophoresis at 3000 V, 120 mA, at 10° was carried out. Parallel strips were stained with ninhydrin and Pauly spray. A photograph of the resulting electropherograms along with interpretations is shown in Figure 1. The peptide was stored in small portions as with the 5-glutamic acid peptide and was also found to be stable under these conditions.

Bioassay. Young, 2-kg, New Zealand White male rabbits (Carver's Rabbitry, Somerville, N. J.) were used in this study. Older, larger rabbits were found to have larger, more fragile adipocytes. The animal was tranquilized with chlorpromazine 1 hr before sacrifice with an overdose of nembutal. The abdominal cavity was immediately opened and the perirenal fat pads were removed, cut in pieces, and placed in plastic vials. Krebs-Ringer-triethanolamine buffer (pH 7.4, containing 3% albumin and 1 mg/ml of glucose) was added (about 1 ml/g of tissue weight) along with 150 units of purified Worthington collagenase per gram of tissue weight. The vials were agitated in a 37° water bath for 30-40 min, which caused disaggregation of most of the original tissue into single cells. The mixture was strained through nylon mesh to remove tissue aggregates. The suspension of cells was centrifuged for 3 min at 3000g and the lower aqueous layer containing most of the blood residue removed. The cells were washed twice by resuspending them in fresh buffer. The cells were transferred to a plastic, stirred vessel and the concentration of cells was adjusted to *ca.* 15-20% by volume with buffer. An aliquot of 0.5 ml of cell suspension was transferred to cellulose nitrate tubes containing the various concentrations of hormones used. The tubes were capped and shaken gently at 37° for 45 min. Blanks, containing cells but no hormone, both incubated and nonincubated, were included. At the end of the incubation time, 1 ml of Dole's fatty acid extraction mixture[70] was added and each tube vortexed. Heptane (0.6 ml) and H_2O (0.4 ml) were then added to each tube and vortexing was followed by a 3-min spin at 3000g, causing sharp separation of the upper organic and lower aqueous phases. Ho's method[47] for determination of free fatty acids by complexation with ^{63}Ni was used as follows. A 50-μl aliquot of the organic layer was transferred to a small cellulose nitrate tube; 100 μl of chloroform-heptane (4:1) followed by 10 μl of ^{63}Ni (1-2 μCi as nicklous nitrate) in a saturated salt solution were added. Each tube was vortexed for at least 30 sec and then spun at 3000g for 3 min. A 50-μl sample of the organic layer was transferred to a scintillation vial and 18 ml of scintillation fluid (64:750:750 liquifluor-toluene-2-ethoxyethanol) was added. The vials were counted on the standard ^{14}C channel of a Packard Tri-carb liquid scintillation counter for 10 min. The moles of free fatty acid released from a standard number of cells per unit time were calculated from the net counts per minute and plotted against the molar concentration of the peptide. The same bioassay was also used with cells from rat epidydimal fat pads. Both synthetic heptapeptides were inactive at concentrations of 500 nmol/ml in the rat cell system.

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2-Aminoindans of Pharmacological Interest

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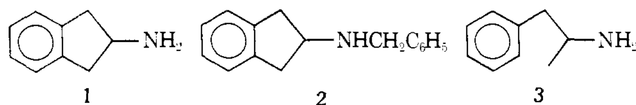
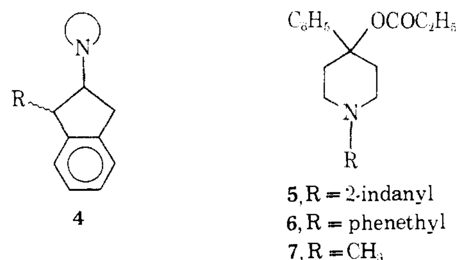
2-Aminoindans in which the amine moiety consists of various cyclic amine derivatives including morpholino, hexamethyleneimino (potential sympathomimetic agents), and 4-acyloxy-4-phenylpiperidino derivatives (potential analgetic agents) were prepared and tested for antiinflammatory, analgetic, pharmacodynamic, and neuropharmacologic activity. No marked antiinflammatory or blood pressure effects were noted. Compound **10j** exhibits strong amphetamine type activity; **10h** and **18c**, respectively, possess analgetic activity approximately equal to and one-half that of meperidine.

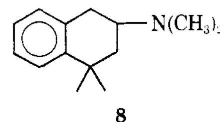
2-Aminoindans have been noted to possess interesting biological properties. For example, 2-aminoindan (**1**) exhibits significant bronchodilating[1] and analgesic properties[2] and 2-benzylaminoindan (**2**) possesses significant bronchodilating properties.[1] Both **1** and **2** may be considered cyclic analogs of amphetamine (**3**).



Because of (a) the limited information available on 1-(2′-indanyl) cyclic amine derivatives, (b) the pronounced analgesic activity of 2-aminoindan, and (c) other significant biological activity[3] of existing 2-aminoindans, a number of 1-(2′-indanyl) cyclic amines **4** were investigated. Furthermore, 1-(2′-indanyl)-4-phenyl-4-piperidyl propionate (**5**) represents a conformationally restrained relative of the corresponding analgesic **6**. The former also is a hybrid of 2-aminoindan and 1-methyl-4-phenyl-4-piperidyl propionate (**7**), both analgesics. In compounds such as **6**, the *N*-arylalkyl substituent is free to assume various conformations. In view of the variation of biological activity with changes in conformation of drug molecules, it was of interest to synthesize and to study pharmacologically compounds such as **4** and **5** in which the conformational variations have been restrained. The in-



vestigation of **4** (R = CH_3) was undertaken with the goal of producing derivatives in which rotation about the indan C-2 to nitrogen bond is restrained by steric interaction of the methyl group with the methylene hydrogens adjacent to the nitrogen. Further impetus for the synthesis of de-



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