Further Studies on the Structure-Activity Relationships in the C-Terminal Part of Luteinizing Hormone-Releasing Hormone†

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Nine new analogs of luteinizing hormone-releasing hormone (LH-RH), *i.e.*, [Des-Gly-NH_2[10],Pro-*n*-butylamide[9]]-LH-RH, [Des-Gly-NH_2[10],Pro-OMe[9]]-LH-RH, [Des-Gly-NH_2[10],Pro-OEt[9]]-LH-RH, [Des-Gly-NH_2[10],Pro-dimethylamide[9]]-LH-RH, [Des-Gly-NH_2[10],Pro-piperidineamide[9]]-LH-RH, [Pro[10]]-LH-RH, [Des-Gly-NH_2[10],Pro-isopropylamide[9]]-LH-RH, [Des-Gly-NH_2[10],Pro-isobutylamide[9]]-LH-RH, and [Des-Gly-NH_2[10],Pro-cyclohexylamide[9]]-LH-RH, were synthesized by the solution method and some biological properties of these analogs were measured. On the basis of the LH- and FSH-releasing activities and the ovulation-inducing activities of these analogs and our previously reported nonapeptide-alkylamide analogs, structure-activity relations in the C-terminal part of this releasing hormone were discussed in detail.

Since the characterization of luteinizing hormone-releasing hormone (LH-RH) of porcine hypothalamus as the decapeptide amide, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2,[2] much work on the synthesis of analogs of LH-RH has been made to determine the structure-activity relationships of this hormone.[3-9] We reported the synthesis and biological activity of a number of LH-RH analogs in which one amino acid in the sequence was replaced by an analogous amino acid and suggested that a total molecular shape of the hormone defined by all the amino acid residues in the sequence may play a vital role in the biological functions of the releasing hormone.[10] We subsequently reported the synthesis and some biological properties of six nonapeptide analogs in which the C-terminal glycine amide was replaced by alkylamines, *i.e.*, [Des-Gly-NH_2[10],Pro-methylamide[9]]-LH-RH (I), [Des-Gly-NH_2[10],Pro-ethylamide[9]]-LH-RH (II), [Des-Gly-NH_2[10],Pro-propylamide[9]]-LH-RH (III), [Des-Gly-NH_2[10],Pro-ethanolamide[9]]-LH-RH (IV), [Des-Gly-NH_2[10],Pro-pyrrolidineamide[9]]-LH-RH (V), and [Des-Gly-NH_2[10],Pro-morpholineamide[9]]-LH-RH (VI), and showed that analogs II and III exhibit a much higher *in vitro* LH-release activity and ovulation-inducing activity than those of the natural hormone.[11,12]

In order to derive more detailed information on the structure-activity relationships in the C-terminal part of this hormone, we have prepared nine new analogs of LH-RH and examined their *in vitro* hormonal activity and ovulation-inducing activity. The activities of the newly synthesized analogs were compared with those of LH-RH and our previously reported nonapeptide-alkylamide analogs. The newly synthesized analogs are as follows: [Des-Gly-NH_2[10],Pro-*n*-butylamide[9]]-LH-RH (VII), [Des-Gly-NH_2[10],Pro-OMe]-LH-RH (VIII), [Des-Gly-NH_2[10],Pro-OEt[9]]-LH-RH (IX), [Des-Gly-NH_2[10],Pro-dimethylamide[9]]-LH-RH (X), [Des-Gly-NH_2[10],Pro-piperidineamide[9]]-LH-RH (XI), [Pro[10]]-LH-RH (XII), [Des-Gly-NH_2[10],Pro-isopropylamide[9]]-LH-RH (XIII), [Des-Gly-NH_2[10],Pro-isobutylamide[9]]-LH-RH (XIV), and [Des-Gly-NH_2[10],Pro-cyclohexylamide[9]]-LH-RH (XV).

Synthesis of Peptides. For the synthesis of these analogs the key intermediates, H-Leu-Arg(NO_2)-Pro-R (R = the corresponding alkylamine, amino acid amide, or alcohol moiety), were prepared by the conventional solution method using the activated ester method. The resulting intermediates were coupled with the N-terminal hexapeptide, <Glu-His-Trp-Ser-Tyr-Gly-OH, in a manner similar to that described for our synthesis of LH-RH itself,[13] to yield the protected peptides which were then treated with hydrogen fluoride for removal of the nitro group in the molecules. The resulting crude peptides were purified by column chromatography on CMC in a way similar to the purification of synthetic LH-RH.[14] All the analogs thus obtained were chromatographically pure and gave the correct amino acid ratios and reasonable uv spectra.

Discussion

The data in Table I indicate that those compounds in each series (straight-chain monosubstituted, branched chain monosubstituted, and disubstituted) which have the greatest conformational similarity to the N-C-C unit of the replaced glycine amide are the most active. Since introduction of a bulky group having more than C_4-alkyl (monoalkylamine) or C_5-alkyl (dialkylamine) at position 10 reduces the biological potency, this portion of the molecule may make a significant contribution to the binding of the hormone to its receptor(s). The data also show that the peptide bond between positions 9 and 10 does not play an important role in the binding affinity because compound IX still exhibits a relatively high potency.

The reason for the enhanced hormonal activity of II, III, IV, and XIII is not known at the present time. It may, however, be reasonable to assume that the replacement of the glycine amide by a suitable alkylamine should result in an increased binding affinity of the hormone for its receptor(s) at the target organ, pituitary, and that the absence of the C-terminal glycine amide and introduction of alkylamines may prolong the lifetime of these compounds *in vivo* by protecting them from a specific deamidase or other hydrolytic enzymes.

Experimental Section

Melting point determinations were performed on Yamato Model MP-21 melting point apparatus and are uncorrected. Evaporations were carried out with a rotary evaporator. The purity of intermediates was checked by tlc (silica gel G plates) and that of the products by precoated tlc sheets (Woelm silica gel F 254/366). The solvent systems employed were: R_f^1, $CHCl_3$–

† For the preceding paper, see ref 11. Abbreviations used here are in accordance with the IUPAC-IUB Tentative Rules on Biochemical Nomenclature[1a] and with suggestions in ref 1b, and other abbreviations used are OSu = *N*-hydroxysuccinimide ester, HONB = *N*-hydroxy-5-norbornene-2,3-dicarboximide, DC-urea = *N,N'*-dicyclohexylurea. The amino acids (except Gly) are of the L configuration.

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Table I. Relative Activity of 10-Substituted LH-RH Analogs: <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-R

Compound	R	Ovulation-inducing activity (rat, sc),[a,b] %	In vitro LH release, %	In vitro FSH release, %
LH-RH[c]	Gly-NH_2	100	100	100
Des-Gly[10]-LH-RH	NH_2		11[d]	
I	$NHCH_3$[e]	130 (95–202)	47 (2)[i]	56 (2)
II	$NHCH_2CH_3$[f]	672 (521–867)	300 (5)	280 (5)
III	$NHCH_2CH_2CH_3$[e]	384 (328–449)	190 (3)	210 (2)
IV	$NHCH_2CH_2OH$	126 (92–174)	210 (3)	220 (2)
VII	$NHCH_2CH_2CH_2CH_3$	4 (3–5)	7.2 (2)	4.3 (3)
VIII	OCH_3	10 (8–12)	6.2 (2)	2.9 (2)
IX	OCH_2CH_3	43 (34–54)	11.5 (2)	6.4 (3)
X	$N(CH_3)_2$	9 (6–14)	15 (3)	9 (3)
V	c-$N(CH_2)_4$[e]	78 (65–94)	110 (3)	92 (2)
VI	c-$N(CH_2CH_2)_2O$[e]	22–36[g]	17 (2)	16 (3)
XI	c-$N(CH_2)_5$	0.5–2.2[g]	1.5 (3)	2.0 (2)
XII	Pro-NH_2	≪22[g]	0.18 (3)	0.14 (2)
XVI	Ala-NH_2[h]	2.2–22[g]	6.0 (3)	3.5 (2)
XIII	$NHCH(CH_3)_2$	283 (214–372)	150 (4)	100 (2)
XIV	$NHCH_2CH(CH_3)_2$	6 (4–10)	3.0 (3)	2.8 (2)
XV	NHC_6H_{11}	<2.2[g]	0.7 (3)	0.6 (3)

[a] Value = (ED_{50} of LH-RH/ED_{50} of analog) × 100; mean ED_{50} ± S.E. of synthetic LH-RH standard = 215 ± 12 ng/100 g of body weight. [b] 95% confidence limits shown in parentheses. [c] Standard. [d] Taken from Rivier, *et al.*, ref 5. [e] See Fujino, *et al.*, ref 12. [f] See Fujino, *et al.*, ref 11. [g] Preliminary results. [h] See Fujino, *et al.*, ref 10. [i] Denotes number of two-level assays involved in mean value.

Table II. Physicochemical Properties of Intermediates: Z-Leu-Arg(NO_2)-Pro-R

Compd	R	Yield, %	Mp, °C	$[\alpha]^{22}D$, deg (*c*, solvent)	$R_f{}^1$	Formula	Analyses
5	$N(CH_3)_2$	72.5	103–107	−58.4[a] (1.0, MeOH)	0.50	$C_{27}H_{42}O_7N_8$	C, H, N
6	c-$N(CH_2)_5$	65.5	156–161	−56.5[b] (1.0, MeOH)	0.51	$C_{30}H_{47}O_7N_8 \cdot 0.5H_2O$	C, H; N[c]
7	Pro-NH_2	73.3	156–160	−63.2 (0.5, EtOH)	0.22	$C_{30}H_{45}O_8N_9$	C, H, N
8	$NHCH(CH_3)_2$	85.0	114–116	−48.2 (1.0, EtOH)	0.51	$C_{28}H_{44}O_7N_8$	C, H, N
9	$NHCH_2CH(CH_3)_2$	72.0	110–111	−47.6 (0.5, EtOH)	0.53	$C_{29}H_{46}O_7N_8$	C, H, N
10	NHC_6H_{11}	89.7	104–106	−52.0 (0.5, EtOH)	0.60	$C_{31}H_{48}O_7N_8 \cdot H_2O$	C, H, N

[a] Temperature 27°. [b] Temperature 24°. [c] N: calcd, 17.49; found, 16.95.

MeOH-AcOH (9:1:0.5); $R_f{}^2$, *n*-BuOH-AcOH-EtOAc-H_2O (1:1:1:1); $R_f{}^3$, *n*-BuOH-pyridine-AcOH-H_2O (30:20:6:24). Where analyses are indicated only by symbols of the elements, analytical results were obtained for the elements within ±0.4% of the theoretical value. Amino acid analyses were run on acid hydrolysates prepared in 5.7 *N* HCl at 105° for 20 hr under N_2. The analyses were performed on a Hitachi KLA-3B amino acid analyzer.

Synthesis of the Intermediates. Z-Leu-Arg(NO_2)-Pro-OMe (1). Z-Arg(NO_2)-Pro-OMe[15] (30.0 g) was dissolved in 25% HBr-AcOH (100 ml), and the solution was kept for 30 min. The solution was diluted with dry Et_2O to give a precipitate. The precipitate was filtered, washed with Et_2O, and dried over NaOH. The free peptide hydrobromide was dissolved in dioxane (200 ml) together with Z-Leu-OSu[16] (16.8 g), and TEA (18 ml) was added. After being stirred at room temperature for 15 hr, the reaction mixture was diluted with H_2O to give an oily precipitate which was extracted with AcOEt. The AcOEt solution was washed with 0.5 *N* HCl, 5% $NaHCO_3$, and H_2O, dried over Na_2SO_4, and evaporated. The residue was precipitated from MeOH-Et_2O to afford 1: 30.0 g (80.5%); mp 105–107°; $[\alpha]^{22}D$ −65.0° (*c* 0.5, EtOH); $R_f{}^1$ 0.53. *Anal.* ($C_{26}H_{39}O_8N_7$) C, H, N.

Z-Leu-Arg(NO_2)-Pro-OH (2). Compound 1 (26.5 g) was dissolved in MeOH (100 ml), and to this was added 1 *N* NaOH (60 ml) with cooling. After being stirred at room temperature for 1.5 hr, the solution was neutralized with 1 *N* HCl (60 ml), and the bulk of MeOH was removed by evaporation. The formed precipitate was extracted with EtOAc, and the organic layer was washed with H_2O, dried (Na_2SO_4), and evaporated to give a solid which was purified by reprecipitation from EtOH-Et_2O: 21.8 g (84.3%); mp 113–117°; $[\alpha]^{22}D$ −55.5° (c 1.0, EtOH); $R_f{}^1$ 0.39. *Anal.* ($C_{25}H_{37}O_8N_7 \cdot 0.5H_2O$) C, H, N.

Z-Leu-Arg(NO_2)-Pro-OEt (3). $SOCl_2$ (0.4 ml) was added dropwise to precooled EtOH (3 ml) at −10°, and to this was added compound 2 (1.69 g). After being stirred at room temperature during 10 hr, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc (100 ml) and the solution was washed with 0.5 *N* HCl, 5% $NaHCO_3$, and H_2O. The organic layer was dried (Na_2SO_4) and evaporated. The residue was solidified by trituration with Et_2O to give pure 3: 1.06 g (59.8%); mp 89–93° dec; $[\alpha]^{29}D$ −64.1° (c 1.0, MeOH); $R_f{}^1$ 0.55. *Anal.* ($C_{27}H_{41}O_8N_7$) C, H, N.

Z-Leu-Arg(NO_2)-Pro-$NHCH_2CH_2CH_2CH_3$ (4). To a solution of compound 2 (1.12 g) and HONB (430 mg) in a mixture of dioxane (5 ml) and DMF (5 ml) was added DCC (495 mg) at 0°, and the mixture was stirred at 0°for 4 hr. The formed DC-urea was filtered off, and to this filtrate was added *n*-butylamine (146 mg). After being stirred at room temperature for 10 hr, the reaction mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ (50 ml) and the solution was washed with 0.5 *N* HCl, 5% $NaHCO_3$, and H_2O and dried (Na_2SO_4), and the $CHCl_3$ layer was evaporated to give a solid which was purified by reprecipitation from EtOH-Et_2O: 950 mg (76.8%); mp 143–145° dec; $[\alpha]^{22}D$ −55.8° (c 1.0, EtOH); $R_f{}^1$ 0.53. *Anal.* ($C_{29}H_{46}O_7N_8 \cdot 0.5H_2O$) C, H, N.

The other intermediary protected peptides were prepared by the HONB procedure from compound 2 and the corresponding alkylamine or amino acid amide as described above for compound 4. The yields and the physicochemical properties of isolated intermediates are listed in Table II.

Synthesis of the LH-RH Analogs. <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-c-N(CH_2)$_5$ (XI). Compound 6 (177 mg) was dissolved in 25% HBr-AcOH (2 ml), and the solution was stirred at room temperature for 30 min. The reaction mixture was diluted with Et_2O to give a precipitate which was collected by filtration, washed with Et_2O, and dried over NaOH. The resulting peptide hydrobromide and <Glu-His-Trp-Ser-Tyr-Gly-OH·HCl[13] (190 mg) were dissolved in DMF (2 ml) together with HONB (54 mg) and *N*-ethylmorpholine (0.08 ml), and the mixture was cooled to 0°. To this was added DCC (62 mg) and the solution was stirred at 0° for 2 hr. After being stirred for an additional 12 hr at room temperature, the reaction mixture was filtered to remove the formed DC-urea and the filtrate was evaporated. The

residue was dissolved in 10% EtOH in H_2O (2 ml) in the presence of urea (100 mg) and the solution was applied to an Amberlite XAD-2 (200–300 mesh) column (2.4 × 18 cm). The column was washed with 10% EtOH (50 ml) and the product was then eluted by a gradient elution method (10% EtOH–80% EtOH = 200 ml/240 ml). The fractions containing the product showing a single spot on tlc were combined and evaporated to a small volume and lyophilized to constant weight: yield, 110 mg.

The material (100 mg) was dissolved in anhydrous hydrogen fluoride (*ca.* 4 ml) together with anisole (0.04 ml) and 2-mercaptoethanol (0.02 ml) at −70°, and the mixture was stirred at 0° for 40 min. Volatile components were evaporated off, and the residue was dissolved in H_2O (20 ml) and the solution was passed through a column of Amberlite CG-400 (AcO^-, 8 ml). The eluate and washings (total 50 ml) were combined and lyophilized to give the crude product as an acetate form. The acetate was dissolved in H_2O (10 ml), and the solution was applied to a CMC column (1 × 30 cm) which was eluted with pH 6.8 NH_4OAc buffer (gradient, 0.005 *M*/0.2 *M* = 190 ml/190 ml). The desired peptide XI was located in fractions 150–180 (one tube = 4 ml). The fractions were combined and lyophilized to give a white fluffy powder which was then dried over P_2O_5 *in vacuo* at 50° for 6 hr: 69 mg; $[\alpha]^{24}D$ −58.2° (*c* 0.5, 5% AcOH). Amino acid ratios in acid hydrolysate gave His 0.95, Arg 1.00, Ser 0.84, Glu 0.95, Pro 1.05, Gly 1.00, Leu 1.00, Tyr 0.84 (average recovery, 89%); R_f^2 0.39; R_f^3 0.59; single spot with Pauly, Ehrlich, Sakaguchi, and Cl–tolidine reagents. The product was also found to be homogeneous by electrophoresis (500 V, 60 min) on paper at pH 1.8 (formic acid–AcOH buffer), exactly the same mobility as LH-RH itself.

Other LH-RH analogs were prepared by exactly the same procedure from the N-terminal hexapeptide and the corresponding intermediate as described above for the analog XI.

Analog VII: $[\alpha]^{29}D$ −56.0° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.98, Arg 1.05, Ser 0.81, Glu 1.00, Pro 0.98, Gly 1.02, Leu 1.02, Tyr 1.02 (average recovery, 85%); R_f^2 0.48; R_f^3 0.66.

Analog VIII: $[\alpha]^{23}D$ −45.0° (*c* 0.5, 5% AcOH); amino acid analysis gave His 1.00, Arg 1.08, Ser 0.92, Glu 1.08, Pro 1.16, Gly 1.10, Leu 1.11, Tyr 0.98 (average recovery, 90.8%); R_f^2 0.50; R_f^3 0.59.

Analog IX: $[\alpha]^{28}D$ −56.8° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.98, Arg 1.00, Ser 0.96, Glu 0.94, Pro 1.00, Gly 1.00, Leu 1.00, Tyr 1.00 (average recovery, 89%); R_f^2 0.51; R_f^3 0.60.

Analog X: $[\alpha]^{25}D$ −56.2° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.91, Arg 1.00, Ser 1.00, Glu 1.03, Pro 1.00, Gly 1.02, Leu 1.00, Tyr 1.00 (average recovery, 89%); R_f^2 0.25; R_f^3 0.53.

Analog XII: $[\alpha]^{24}D$ −75.0° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.91, Arg 1.00, Ser 0.92, Glu 0.92, Pro 2.00, Gly 1.00, Leu 1.00, Tyr 0.92 (average recovery, 78%); R_f^2 0.40; R_f^3 0.50.

Analog XIII: $[\alpha]^{29}D$ −55.4° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.97, Arg 1.00, Ser 0.78, Glu 1.00, Pro 0.97, Gly 1.03, Leu 1.00, Tyr 0.97 (average recovery, 75%); R_f^2 0.41; R_f^3 0.65.

Analog XIV: $[\alpha]^{22}D$ −55.2° (*c* 0.5, 5% AcOH); amino acid analysis gave His 1.00, Arg 1.00, Ser 0.96, Glu 1.00, Pro 1.00, Gly 1.00, Leu 1.00, Tyr 1.00 (average recovery, 87%); R_f^2 0.55; R_f^3 0.63.

Analog XV: $[\alpha]^{22}D$ −51.0° (*c* 0.5, 5% AcOH); amino acid analysis gave His 0.92, Arg 1.04, Ser 0.92, Glu 0.96, Pro 1.04, Gly 1.00, Leu 1.00, Tyr 0.92 (average recovery, 87%); R_f^2 0.55; R_f^3 0.64.

The uv spectra of all the peptides were identical with that of the synthetic LH-RH[13,14] indicating that the Trp residue in the above analogs was intact, and the mobilities of all the peptides on paper electrophoresis (Whatman No. 1, pH 1.9 formic acid-AcOH buffer, at 500 V, 60 min) were identical with that of the synthetic LH-RH. The observation on electrophoresis merely indicates that the C terminal of each analog is blocked.

Biological Assays. *In vitro* LH- and FSH-releasing activities were measured by modification[17] of the method of Mittler and Meites[18] using hemisected anterior pituitaries from 250–300-g mature male Sprague–Dawley rats (Holtzman Co., Madison, Wis.). The equivalent of five anterior pituitaries (ten halves) was randomly assigned to a flask containing 4 ml of a complete tissue culture medium composed of Earle's salts, vitamins, and 0.5% lactalbumin hydrolyzate. After a 30-min preincubation at 37° in an atmosphere of 95% O_2 + 5% CO_2, the media were changed and the incubation was continued for a 6-hr experimental period. FSH activity of the media was determined by the HCG augmentation method of Steelman and Pohley[19] and by radioimmunoassay.[20] Purified ovine FSH (Abbott Lot 3154-098-A; 0.225 × NIH-FSH-S1) and NIAMD-Rat-FSH-RP-1 were used as standards for these assays, respectively. LH was measured by the radioimmunoassay method of Niswender, *et al.*,[21] using NIH-LH-S16 as standard and a purified ovine LH (Abbott-Lot 2418-245-2; 1.2 × NIH-LH-S1) for iodination. The statistical methods of Bliss[22] were used to compute relative potencies, 95% confidence limits, and departures from parallelism. If separate determinations of the relative potency of the analogs differed by more than 50%, at least one additional two-level assay was performed.

Ovulation-inducing activity was determined by the methods described by Yamazaki and Nakayama[23] using 4-day cycling female Sprague–Dawley rats maintained under the illuminating condition from 7:30 to 21:30. LH-RH standard or the analogs were subcutaneously administered at 14:30 on the day of diestrus, and the ovulated ova in the ampulla of the oviduct were inspected under a differential interference microscope on the morning of the next day. The ED_{50}'s and 95% confidence limits were calculated by the dose–response linear line regressed between the probits of frequencies in ovulating rats and the logarithmic doses according to the method of Litchfield and Wilcoxon.[24]

The hormonal activities of the analogs synthesized in the present paper and the analogs previously reported are compared with those of a synthetic LH-RH standard[25] which has been exhaustively compared with the pure natural porcine hormone[26] and the relative hormonal activities of the analogs are summarized in Table I.

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Synthesis and Structure–Activity Relationship of Some Thiol Adducts of Ethacrynic Acid†,‡

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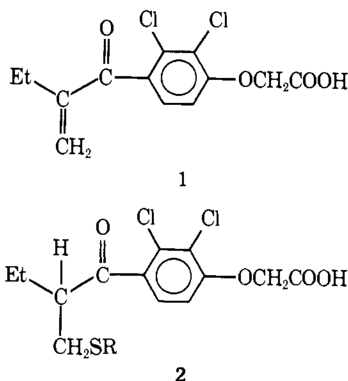
β-Mercaptoacylphenoxyacetic acid derivatives of ethacrynic acid were synthesized. Those derivatives that possess diuretic activity were shown to release ethacrynic acid and the accompanying thiol under appropriate *in vitro* conditions. This finding reemphasizes the importance of an intact α,β-unsaturated system in ethacrynic acid for diuretic activity. The mechanism of the reaction leading to the liberation of ethacrynic acid and thiol is postulated to be an intramolecular base-catalyzed retro-Michael-type reaction.

Most of the evidence accumulated to date supports the idea that the pharmacological activity of ethacrynic acid (1) is dependent on the α,β-unsaturated ketone moiety which allows it to react, *via* a Michael-type reaction, with various nucleophiles (*i.e.*, sulfhydryl-containing substances) *in vitro* as well as in *in vivo*.[2-5] It has been suggested that a reaction with protein-bound sulfhydryl groups (PBSH) in renal tissue accounts for the diuretic response induced by 1.[6]



Although the β-mercaptoacylphenoxyacetic acid derivatives 2 (also referred to as thiol adducts) which result from the *in vitro* reaction of various thiols with 1 no longer possess an intact α,β-unsaturated ketone system, some of them are effective diuretics.§,= This apparent discrepancy is the subject of this paper.

There are at least three possible explanations for the fact that only certain β-mercaptoacylphenoxyacetic acid derivatives possess diuretic activity. First, ethacrynic acid (1) may be the biologically active form and the β-mercaptoacylphenoxyacetic acid derivatives 2 that induce a diuretic response do so by liberating 1 and the corresponding thiol under appropriate *in vivo* conditions. 1 generated from such a reaction would then be free to react with a nucleophilic receptor in renal tissue (such as PBSH). The quantity of 1 generated from the thiol adducts 2 would determine the magnitude of the diuretic response. Beyer, *et al.*,[7] have reported that 1 undergoes rapid, extensive, and reversible conjugation *in vivo*, but no mention was made as to the importance of the reversible conjugation insofar as diuretic activity is concerned.

The second possibility is that 1 may not be the species responsible for inducing the diuretic response. An *in vivo* reaction ("metabolic activation") with a nucleophile such as cysteine or glutathione could lead to the formation of a β-mercaptoacylphenoxyacetic acid derivative 2 which is the active form of 1. It would be essential that the endogenous thiol-containing substance meet certain structural requirements to form an active diuretic agent. This second possibility appears to be supported by at least three findings. (a) 1 reacts with cysteine *in vitro* with a $T_{1/2}$ of 0.8 min.[2a] If metabolic activation of 1 is a prerequisite for diuretic activity then the rapid reaction of cysteine with 1 may explain the rapid onset of the diuretic response when 1 is administered intravenously. (b) 1 and its cysteine adduct 2 [R = $-CH_2CH(NH_2)COOH$] are equipotent as diuretic agents,§,= and (c) a radioactive substance which appears to be indistinguishable from the cysteine adduct of 1 is formed *in vivo* and excreted in the urine of dogs after administration of [2-^{14}C]ethacrynic acid.[7]

A third possibility is that the PBSH groups or other nucleophilic receptors present in renal tissue are able directly to displace (SN2 reaction) the thiol present in those β-mercaptoacylphenoxyacetic acid derivatives that are diuretic.

The objective of this paper is to describe the results of a study which focused attention on whether or not the diuretic effect of certain β-mercaptoacylphenoxyacetic acid derivatives could be correlated with their ability to liberate 1 and the accompanying thiol under appropriate conditions.

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‡ Portions of this work were presented at the 57th Annual FASEB Meeting, Atlantic City, N. J., 1973. See ref 1.
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