Stereoisomeric Lactoyl-β-methylcholine Iodides. Interaction with Cholinesterase and Acetylcholinesterase

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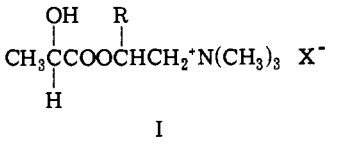
The preparation and absolute configuration of the stereoisomeric forms of lactoyl-β-methylcholine iodide together with the two racemic forms of the molecule are reported. None of the stereoisomers were substrates for the enzyme acetylcholinesterase, whereas two stereoisomers (L-lactoyl-L-β-methylcholine and D-lactoyl-L-β-methylcholine iodide) were poor substrates for cholinesterase. The results were analyzed in the light of previous studies of the chiral requirements of these two enzymes and an attempt was made to define the rate-limiting or prohibited step in the enzyme-catalyzed reaction with these compounds.

The isolation of choline esters other than acetylcholine from animal tissues, *e.g.*, propionylcholine, acrylylcholine,[1,2] has led to the suggestion that these and other as yet unidentified choline esters may contribute to the acetylcholine-like activity of nervous and nonnervous tissue.

Lactoylcholine (I, R = H) has been proposed as a potential candidate in this respect[3] and studies of the muscarinic and nicotinic agonist activity[3,4] and substrate activity toward acetylcholinesterase[5] and cholinesterase[6] have been extensively studied.



I

(±)-Lactoylcholine iodide is a weak muscarinic agonist (0.004–0.001 of the activity of acetylcholine), the activity being predominantly nicotinic in origin (approximately equal to acetylcholine). The pharmacological activity is resident predominantly in the D enantiomer (enantiomeric ratio of 10:1 at the muscarinic receptor and approximately 5:1 at the nicotinic receptor),[3] these differences in activity arising mainly from differences in the affinity of the agonist for the receptor rather than to differences in their intrinsic efficacies.[4]

In studies of the interaction of lactoylcholine and its enantiomers with acetylcholinesterase and cholinesterase, a reversal of the above-mentioned stereoselectivity is shown, the L configuration enantiomer being the better substrate and being hydrolyzed at a rate faster than acetylcholine when compared at their respective optimum substrate concentrations.[5,6]

Although lactoylcholine shows measurable activity when interacting with each of the above-mentioned acetylcholine binding sites, lactoyl-β-methylcholine iodide (I, R = Me) is reported to be hydrolyzed at a measurable rate by cholinesterase only and to produce an agonist response at the muscarinic receptor only, although the response is weak.[3] The compound employed in these experiments, however, is a racemate with sharp melting point (mp 122.8–124.4°)[3] and thus probably represents only one of the two racemic modifications possible for this compound.

Because of the high substrate activity of (±)-lactoylcholine toward acetylcholinesterase and cholinesterase and the known selectivity of these enzymes when interacting with acetyl-β-methylcholine,[7] coupled with the stereospecificity of L-(+)-acetyl-β-methylcholine iodide toward acetylcholinesterase,[8] it was considered that preparation of the four stereoisomers of lactoyl-β-methylcholine iodide would be useful in studies of the chiral requirements of these two enzymes. The present paper thus reports the preparation of the four stereoisomeric forms of lactoyl-β-methylcholine iodide, the absolute configuration of each isomer, and the testing of these compounds as substrates for the enzymes acetylcholinesterase and cholinesterase. In addition, the racemic form previously employed by Sastry, *et al.*,[3] has been identified.

The lactic and glyceric acid esters of choline have previously been prepared by reacting the hydroxy acid in the presence of a large excess of the appropriate amino alcohol hydrochloride salt (to reduce lactone formation) and removing the water as a benzene–water azeotrope. Application of such a technique to the present work, however, would require fairly large quantities of optically pure reagents and would be wasteful of such reagents. Thus, it was considered that a more appropriate synthetic route to obtaining the four stereoisomeric forms of lactoyl-β-methylcholine iodide would be to esterify each of the enantiomers of 1-dimethylaminopropan-2-ol with the enantiomeric acid chlorides of a suitably protected lactic acid derivative followed by selective removal of the protective group and quaternization.

Initial use of *O*-benzyllactic acid led to esters resistant to hydrogenolysis and thus we report here the use of an acetyl protective group which was removed, after quaternization of the *O*-acetyllactoyl-β-methylnorcholine, by controlled acid-catalyzed hydrolysis.

(±)-1-Dimethylaminopropan-2-ol was resolved into its enantiomers using D- and L-tartaric acid.[9] The absolute configuration of each enantiomer has been previously established.[9-11]

(±)-*O*-Acetyllactic acid was prepared by a literature method[12] and resolved using (+)- and (−)-α-methylbenzylamine as resolving agent.[13] The absolute configuration of (−)-*O*-acetyllactic acid has previously been established as of the L configuration by preparation from L-lactic acid.[14]

Esterification of the enantiomeric amino alcohols with the enantiomers of *O*-acetyllactoyl chloride† yielded the four stereoisomeric *O*-acetyllactoyl-β-methylnorcholines which, after quaternization with methyl iodide and recrystallization, were subjected to a controlled acid-catalyzed hydrolysis (0.05 *N* hydriodic acid at 40° for 30 min). Evaporation of the reaction mixture to dryness under reduced pressure followed by fractional crystallization of the residue from pentan-2-one allowed the isolation of the stereoisomeric lactoyl-β-methylcholine iodides.

Attempts to isolate the two racemic forms of lactoyl-β-methylcholine iodide by fractional crystallization of a product prepared according to the method reported by Sastry, *et al.*,[3] using racemic reactants yielded only the previously reported racemate, mp 125–126° (lit.[3] mp 122.8–124.4°), as the least soluble fraction. Examination of

†Cocolas, *et al.*,[15] recently prepared (*R*)-(−)-1-dimethylaminobutan-2-ol from (*R*)-2-acetoxybutyryl chloride and obtained a product of equal optical purity to that obtained by resolution. Thus, racemization of *O*-acetyllactoyl chloride is unlikely to occur.

Table I. Kinetic Parameters for the Hydrolysis of Choline and β-Methylcholine Iodide Esters

Substrate	AchE		ChE	
	K_m (app), $M \times 10^4 \pm$ S.E.	$V_{max} \times 10^6$ [a]	K_m (app), $M \times 10^4 \pm$ S.E.	$V_{max} \times 10^6$ [a]
Acetylcholine perchlorate	6.1 ± 0.25	1.40 ± 0.03	4.7 ± 0.5	0.27 ± 0.02
(±)-Acetyl-β-methylcholine iodide	14.9 ± 1.1	0.24 ± 0.01	Inactive	
(±)-Lactoylcholine iodide	18.7 ± 0.7	1.2 ± 0.02	5.0 ± 0.25	0.57 ± 0.01
(±)-Lactoyl-β-methylcholine iodide, mp 125–126°	Inactive		2.4 ± 0.5	0.03 ± 0.01
(±)-Lactoyl-β-methylcholine iodide, mp 113–116°	Inactive			
L-Lactoyl-L-β-methylcholine iodide	Inactive		12.8 ± 4.0	0.06 ± 0.002
L-Lactoyl-D-β-methylcholine iodide	Inactive		Inactive	
D-Lactoyl-L-β-methylcholine iodide	Inactive		27.5 ± 3.0	0.07 ± 0.03
D-Lactoyl-D-β-methylcholine iodide	Inactive		Inactive	

[a] Experimental values of V_{max}(app) have been normalized by dividing by the enzyme concentration in μM units/ml. Units are thus M min^{-1} μM unit^{-1}.

the mother liquors yielded fractions, all of which were contaminated with this product.

Using (±)-*O*-acetyllactoyl chloride and (±)-1-dimethylaminopropan-2-ol, followed by quaternization and fractional crystallization of the product ester, the two racemic forms of *O*-acetyllactoyl-β-methylcholine iodide were isolated, mp 125–127 and 141–142°. Controlled hydrolysis of these racemates, followed by fractional crystallization, allowed the isolation of the two racemic forms of lactoyl-β-methylcholine iodide (mp 113–116 and 125–128°) and by mixture melting point studies it was possible to determine that the racemate prepared by Sastry was a mixture of equal parts of D-lactoyl-L-β-methylcholine iodide and L-lactoyl-D-β-methylcholine iodide (Chart I).

Chart I. Mixture Melting Points Determined with Stereoisomers Showing Constitution of Racemic Forms of Lactoyl-β-methylcholine Iodide

L-*O*-AcLact-D-β-MeChI → L-Lact-D-β-MeChI
mp 150–152°, $[\alpha]^{22}_D$ −25.3° mp 137–140°, $[\alpha]^{22}_D$ −24.8°
mmp 139–143° mmp 122–128°
D-*O*-AcLact-L-β-MeChI → D-Lact-L-β-MeChI
mp 149–151°, $[\alpha]^{22}_D$ +23.5° mp 135–137°, $[\alpha]^{22}_D$ +23°

(±)-*O*-AcLact-β-MeChI → (±)-Lact-β-MeChI
mp 141–142° mp 126–129°

(±)-*O*-AcLact-β-MeChI → (±)-Lact-β-MeChI
mp 125–127° mp 113–116°

L-*O*-AcLact-L-β-MeChI → L-Lact-L-β-MeChI
mp 152–155°, $[\alpha]^{22}_D$ −14° mp 120–122°, $[\alpha]^{22}_D$ +19.5°
mmp 127–130° mmp 108–112°
D-*O*-AcLact-D-β-MeChI → D-Lact-D-β-MeChI
mp 154–157°, $[\alpha]^{22}_D$ +19° mp 118–121°, $[\alpha]^{22}_D$ −24.6°

Results and Discussion

Within the present work, the four stereoisomeric forms of lactoyl-β-methylcholine iodide and the two racemic modifications of this molecule have been prepared and tested as substrates of acetylcholinesterase (bovine erythrocytes) and cholinesterase (horse serum) and the results presented in Table I.

Turning initially to the results obtained using acetylcholinesterase, it has been previously shown that this enzyme is stereospecific toward L-acetyl-β-methylcholine iodide[8] and stereoselective toward L-lactoylcholine iodide.[5] It is therefore not surprising that the racemic form of lactoyl-β-methylcholine having mp 125–126°, previously tested by Sastry,[5] and shown here to be the racemic modification formed from L-lactoyl-D-β-methylcholine and D-lactoyl-L-β-methylcholine iodides is inactive as a substrate for the enzyme. It is, however, surprising that L-lactoyl-L-β-methylcholine iodide, that stereoisomer having at each asymmetric center the reported optimal configuration for substrate activity, similarly fails to show any substrate activity.

Acetylcholinesterase catalyzed hydrolysis is usually depicted as proceeding *via* a three-step reaction sequence

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} E\text{-}Ac \xrightarrow{k_3} E + P$$

and a potential substrate may fail to show any enzyme-catalyzed hydrolysis due to an extremely slow rate constant at any of these stages. Thus, carbamoylcholine and other carbamate esters fail to act as substrates for the enzyme due to an extremely slow deacylation rate constant. However, since the relative rate of hydrolysis of L-lactoylcholine at its optimum substrate concentration[5] is slightly greater then that of acetylcholine, it follows that the L-lactoyl enzyme is not formed when L-lactoyl-L-β-methylcholine iodide is tested as a substrate and that the prohibited step is either formation of the Michaelis complex or formation of the acyl enzyme.

Recent studies employing electric eel acetylcholinesterase[16] have shown that propionyl-β-methylcholine iodide is a substrate for the enzyme with V_{max} and K_m(app) values only slightly different to those of acetyl-β-methylcholine. In addition, the racemate of L-lactoyl-D-β-methylcholine and D-lactoyl-L-β-methylcholine has been shown to have some weak affinity for the enzyme active site.[5] Thus, it would not be unreasonable to assume that L-lactoyl-L-β-methylcholine iodide has some affinity for the enzyme and the prohibited step therefore is formation of the acyl enzyme intermediate, the step thought to be rate limiting in the hydrolysis of acetyl-β-methylcholine iodide.

Turning now to the results obtained using cholinesterase isolated from horse serum, the compounds L-lactoyl-L-β-methylcholine and D-lactoyl-L-β-methylcholine iodide were hydrolyzed at a slow rate by the enzyme. It can be seen therefore that the configuration of the acyl moiety has little significant effect upon the various stages in the reaction sequence, in agreement with previous studies using the enantiomers of lactoylcholine iodide.[6] It is interesting to note that, although cholinesterase does not hydrolyze acetyl-β-methylcholine, increasing the length of the acyl group leads to stereospecific enzyme-catalyzed hydrolysis, *e.g.*, L-butyryl-β-methylcholine is hydrolyzed

by the enzyme.[17] The results obtained using the stereoisomeric lactoyl-β-methylcholines further confirm the stereospecificity of cholinesterase toward the L configuration of β-methylcholine esters and would suggest that the inability of cholinesterase to hydrolyze acetyl-β-methylcholine is the results of a poor affinity of the compound for the enzyme active site.

Experimental Section‡

Resolution of 1-Dimethylaminopropan-2-ol. (±)-1-Dimethylaminopropan-2-ol was resolved by the method reported by Beckett, *et al.*,[9] using D-(+)- and L-(−)-tartaric acid. Using D-(+)-tartaric acid, the salt, after 24 recrystallizations, had mp 106–108°, $[\alpha]^{22}D$ −10.1° (c 2.18, H_2O) [lit.[9] −10.84° (c 4.9, H_2O)] and gave, on liberation of the amino alcohol, D-(−)-1-dimethylaminopropan-2-ol, bp 119–124° (lit.[18] bp 124–126°), $[\alpha]^{22}D$ −23.7° (c 1.11, CH_3OH) (overall yield 7%).

Using L-(−)-tartaric acid as resolving agent, the salt, after 22 recrystallizations, had mp 108–110°, $[\alpha]^{22}D$ +9.5° (c 1.89, H_2O), and gave on basification L-(+)-1-dimethylaminopropan-2-ol, bp 118–122° [lit.[9] bp 125°), $[\alpha]^{22}D$ +22.85° (c 1.02, CH_3OH) [lit.[9] $[\alpha]^{22}D$ +23.22° (c 0.9, EtOH)].

(±)-*O*-Acetyllactic acid was prepared by a literature method[12] and had bp 130–131° (12 mm) [lit.[12] 127° (11 mm)], mp 35–37° (lit.[12] mp 57–60, 39–40°), ν_{max} (Nujol) 1750 (br, -COOH), 1200 cm^{-1} (acetoxy).

Resolution of (±)-*O*-Acetyllactic Acid. (±)-*O*-Acetyllactic acid was resolved by a literature method[13] using (+)- and (−)-α-methylbenzylamine as resolving agents and methylene chloride–petroleum ether as recrystallizing solvent. Using (+)-α-methylbenzylamine the salt (three recrystallizations), mp 141–145° (lit.[13] 147–149°), had $[\alpha]^{22}D$ −12.9° (c 0.8, ethanol) [lit.[13] $[\alpha]^{20}D$ −13.0° (c 6.0, ethanol)] and the liberated L-(−)-*O*-acetyllactic acid, bp 78–80° (0.15 mm) [lit.[13] bp 80–82° (0.1 mm)], had $[\alpha]^{22}D$ −42.9° (c 0.42, benzene) [lit.[13] $[\alpha]^{20}D$ −44° (c 2, benzene)] (overall yield 20%).

Using (−)-α-methylbenzylamine, the salt (three recrystallizations), mp 135–138° (lit.[13] 147–149°), had $[\alpha]^{22}D$ +13.1° (c 0.53, ethanol) [lit.[13] $[\alpha]^{22}D$ +13.3° (c 3, ethanol)] and the liberated D-(+)-*O*-acetyllactic acid, bp 78–82° (0.2 mm) [lit.[13] bp 80–82° (0.1 mm)], had $[\alpha]^{22}D$ +44° (c 0.6, benzene) [lit.[13] $[\alpha]^{20}D$ +44.7° (c 2, benzene)].

Preparation of Stereoisomeric *O*-Acetyllactoyl-β-methylnorcholines and Methiodide Salts. L-(+)-1-Dimethylaminopropan-2-ol (0.9 g) was dissolved in benzene (7 ml) and D-*O*-acetyllactoyl chloride (1.2 g, prepared from the acid by reaction with thionyl chloride) in benzene (10 ml) added slowly with stirring. The stoppered reaction vessel was stirred at room temperature overnight, water was added, and the solution was basified (NaOH solution) and extracted with chloroform (5 × 20 ml). The bulked extracts were dried (Na_2SO_4), filtered, and concentrated under reduced pressure and the residue was fractionally distilled, the fraction with bp 70–71° (4.5 mm) being collected: ν_{max} (liquid film) 1740 (C=O), 1220 cm^{-1} (acetyl); $[\alpha]^{22}D$ +37.1° (c 1.06, CH_3OH); methiodide (acetone) mp 149–151°; $[\alpha]^{22}D$ +23.5° (c 0.63, CH_3OH). *Anal.* ($C_{11}H_{20}O_4NI$) H; C: calcd, 36.76; found, 37.28.

The following compounds were similarly prepared. **L-*O*-Acetyllactoyl-L-β-methylnorcholine:** bp 76–78° (4 mm); $[\alpha]^{22}D$ −24.7° (c 0.67, CH_3OH); methiodide (acetone) mp 152–155°; $[\alpha]^{22}D$ −14.0° (c 0.52, CH_3OH). *Anal.* ($C_{11}H_{22}O_4NI$) C, H. **D-*O*-Acetyllactoyl-D-β-methylnorcholine:** bp 68–69° (4 mm); $[\alpha]^{22}D$ +19° (c 0.86, CH_3OH); methiodide (acetone) mp 154–156°; $[\alpha]^{22}D$ +11.85° (c 0.58, CH_3OH). *Anal.* ($C_{11}H_{22}O_4NI$) C, H. **L-*O*-Acetyllactoyl-D-β-methylnorcholine:** bp 74–76° (5 mm); $[\alpha]^{22}D$ −46° (c 0.81, CH_3OH); methiodide (acetone) mp 150–152°; $[\alpha]^{22}D$ −25.3° (c 0.72, CH_3OH). *Anal.* ($C_{11}H_{22}O_4NI$) C, H.

Preparation of Stereoisomeric Lactoyl-β-methylcholines. L-Lactoyl-D-β-methylcholine Iodide. L-*O*-Acetyllactoyl-D-β-methylcholine iodide (400 mg) was dissolved in dilute hydriodic acid (8 ml, 0.05 *N*) and the solution maintained at 40° for 30 min. The excess HI and liberated acetic acid were removed at reduced pressure without heat and the solid residue crystallized from acetone. The product was fractionally recrystallized from pentan-2-one, and fractions were initially identified by melting point, fractions having mp 170–175° being assumed to be D-β-methylcholine iodide (lit.[9] mp 175–176°). The product had mp 137–140°, $[\alpha]^{22}D$ −24.8° (c 0.41, CH_3OH). *Anal.* ($C_9H_{20}O_3NI$) H; C: calcd, 34.1; found, 34.6.

The following compounds were similarly prepared. **D-Lactoyl-L-β-methylcholine iodide:** mp 135–137°; $[\alpha]^{22}D$ +23.08° (c 0.45, CH_3OH). *Anal.* ($C_9H_{20}O_3NI$) C, H. **L-Lactoyl-L-β-methylcholine iodide:** mp 120–122°; $[\alpha]^{22}D$ +19.5° (c 0.36, CH_3OH). *Anal.* ($C_9H_{20}O_3NI$) C, H. **D-Lactoyl-D-β-methylcholine iodide:** mp 118–121°; $[\alpha]^{22}D$ −24.6° (c 0.35, CH_3OH). *Anal.* ($C_9H_{20}O_3NI$) C, H.

Preparation of (±)-Lactoyl-β-methylcholine Iodide (mp 125–126°). This compound was prepared according to the method of Sastry.[3] The product was fractionally recrystallized from ethanol and gave a fraction with mp 125–126° (lit.[3] mp 122.8–124.4°): nmr (D_2O) two doublets centered at δ 1.90 and 1.97 (J = 6 Hz, 6 H, β-CH_3 and acyl CH_3), 3.76 [s, 9 H, $^+N(CH_3)_3$], 4.0–4.6 (m, 3 H, α-CH_2, β-methine proton), 5.02 (q, 1 H, lactate methine proton). Examination of the mother liquors gave fractions with mp 95–103°.

Preparation of Racemic *O*-Acetyllactoyl-β-methylcholine Iodide. This compound was prepared as reported for D-*O*-acetyllactoyl-L-β-methylcholine iodide but using racemic reagents. Fractional crystallization of product from ethanol gave a fraction, mp 141–142°, as the least soluble fraction: nmr (D_2O) two doublets centered at δ 1.79 and 1.94 (J = 6 Hz, 6 H, β-CH_3 and acyl CH_3), 2.6 (s, 3 H, CH_3CO), 3.62 [s, 9 H, $^+N(CH_3)_3$], 5.60 (q, 1 H, lactate methine). *Anal.* ($C_{11}H_{22}O_4NI$) C, H.

Examination of the mother liquors and recrystallization from an EtOH–ether mixture gave a further fraction, mp 125–127°. *Anal.* ($C_{11}H_{22}O_4NI$) C, H.

Selective Hydrolysis of Racemates of *O*-Acetyllactoyl-β-methylcholine Iodide. Using the method reported for the preparation of L-lactoyl-D-β-methylcholine iodide, the racemate (mp 141–142°) gave (±)-lactoyl-β-methylcholine iodide, mp 126–129° [*Anal.* ($C_9H_{20}O_3NI$) C, H], and the racemate (mp 125–127°) gave (±)-lactoyl-β-methylcholine iodide, mp 113–116° [*Anal.* ($C_9H_{20}O_3NI$) H; C: calcd, 34.1; found, 34.7].

Enzyme Kinetics. Bovine erythrocyte acetylcholinesterase (Sigma Chemical Co., Type I) and horse serum pseudocholinesterase (Sigma Chemical Co., Type IV) were used. All kinetic measurements were carried out by the pH-stat method in a medium previously made 0.04 *M* in $MgCl_2$ and 0.05 *M* in NaCl, at a temperature of 25 ± 0.1° and at pH 7.4 as previously described.[19] The kinetic parameters V_{max} and K_m(app) were obtained from plots of S *vs.* S/V and using a least-squares regression analysis to obtain the best line fitting the experimental data points.

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‡Melting points were obtained on a Thomas-Hoover Uni-melt apparatus and are uncorrected. Infrared spectra were recorded on a Beckmann IR-8 spectrophotometer. Microanalyses were by Microanalysis Laboratories, Ltd., Toronto, and, where indicated by symbols of elements only, results were within ±0.4% of theoretical values.

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Luteinizing Hormone-Releasing Hormone and Analogs. Synthesis and Biological Activity

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A fragment synthesis of LH-RH is described which lends itself to large-scale preparation. Fragment 1–3 is coupled with fragment 4–6 followed by reaction with the tetrapeptide 7–10 to yield the unprotected decapeptide. The preparation of analogs follows the same synthetic pattern. The biological activity of the analogs is compared with that of synthetic LH-RH.

The isolation and structure elucidation of the porcine hypothalamic luteinizing hormone-releasing hormone (LH-RH) by Schally and collaborators[1] and its apparent diagnostic and therapeutic value[2] have caused a flurry of synthetic activity.[3-17] The choice of our synthetic approach to LH-RH was influenced by two considerations: the synthesis should be simple enough to lend itself to large-scale preparation, and extensive separation procedures should be avoided after the final coupling step. The fragment synthesis selected permitted considerable scale up. Since most of the partial sequences were crystalline, time-consuming purifications of the intermediates could be minimized. Only one separation step was required to obtain the LH-RH in the desired state of purity.[18] The synthesis of the LH-RH analogs was quite similar to that of LH-RH.

Chemistry. The synthesis of LH-RH, Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is represented in Scheme I. The partial sequence 1–3 (A) was coupled with the partial sequence 4–6 (B) and the hexapeptide (C) was coupled with fragment 7–10 (D) to yield directly the unprotected decapeptide.

Fragment A was prepared by coupling of Pyr-His-$NHNH_2$[19] with H-Trp-OMe[20] by the azide method. The resulting tripeptide methyl ester was hydrazinolyzed to give A. B was obtained by hydrolysis of Z-Tyr(Bzl)-Gly-OMe[21] to the acid and conversion of the acid by the mixed anhydride method with *tert*-butyl carbazate to Z-Tyr(Bzl)-Gly-NHNHBoc, which was hydrogenolyzed to H-Tyr-Gly-NHNHBoc. This intermediate was condensed with Z-Ser-ODnp[22] to give Z-Ser-Tyr-Gly-NHNHBoc which was hydrogenolytically transformed into B. For the partial sequence D, H-Pro-Gly-NH_2 was coupled with Boc-Arg(NO_2)-OH[23] to Boc-Arg(NO_2)-Pro-Gly-NH_2. After removal of the Boc group and coupling with Z-Leu-OTcp,[24] the tetrapeptide was hydrogenolyzed with concomitant removal of Z and NO_2 to yield D.

The condensation of A and B to yield C, followed by reaction with D and chromatographical purification of the product gave LH-RH (AY-24,031).

LH-RH Analogs. The procedures employed were essentially the same as those used in the LH-RH synthesis. The methods for preparing the 7–10 tetrapeptide amides were governed by the individual changes in amino acid composition and are described in detail in the Experimental Section. Table I provides a summary of the analogs prepared and analytical and biological data.

Endocrinology. Induction of Ovulation in Hamsters. The method used was similar to that described in the literature by Arimura, *et al.*[25]

The estrus cycle of Syrian hamsters (*ca.* 100 g) was checked by inspecting vaginal discharge which occurred every 4 days in most of the animals. Experiments were started after the animals had shown two regular 4-day cycles. At 1:00 p.m. on the day of proestrus, 13 mg of phenobarbital/100 g of body weight was injected ip to block

Scheme I