loss in sensitivity was observed as well as the appearance of interfering peaks in the chromatogram. At this time, a new column was installed and primed.

The isosorbide dinitrate human plasma levels following a 5-mg dose of chewable isosorbide dinitrate were highest at 15 min after administration in all four subjects (Table III). No isosorbide dinitrate levels were detectable at 120 min or more after administration.

The overall recovery of ^{14}C-isosorbide dinitrate in the 2.2-90-ng/ml concentration range is shown in Table IV. The recovery of 75–80% was independent of concentration.

Previous attempts to quantitate isosorbide 2-mononitrate and isosorbide 5-mononitrate in the same concentration range as isosorbide dinitrate were unsuccessful. Certain plasma components that appeared in the benzene extract from time to time interfered with the determination of the mononitrates of isosorbide dinitrate.

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Potency of Synthetic Luteinizing Hormone Releasing Hormone Preparations in Rat Anterior Pituitary Cell Cultures

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Abstract □ Selected synthetic luteinizing hormone releasing hormone preparations were assayed, and their potencies were determined relative to one sample utilizing primary cultures of enzymatically dispersed rat anterior pituitary cells. Preliminary cell culture experiments indicated that luteinizing hormone releasing hormone had to be in constant contact with cells for continued luteinizing hormone secretion. Luteinizing hormone levels in media reached a maximum concentration after 4 hr of continuous luteinizing hormone releasing hormone exposure. Cell culture bioassay was selected over the bioassay employing chronically ovariectomized steroid-blocked rats due to greater sensitivity and economy. The assay of each luteinizing hormone releasing hormone preparation was replicated four to seven times. Preparations from several companies were less potent ($p < 0.05$) than the reference product. Contaminants were disclosed by TLC in preparations with potencies lower than the reference product.

Keyphrases □ Luteinizing hormone releasing hormone—various synthetic preparations analyzed by TLC and radioimmunoassay, potencies determined, rat anterior pituitary cells □ TLC—analysis, luteinizing hormone releasing hormone, various synthetic preparations □ Radioimmunoassay—analysis, luteinizing hormone releasing hormone, various synthetic preparations □ Hormones—luteinizing hormone releasing hormone, various synthetic preparations analyzed by TLC and radioimmunoassay, potencies determined, rat anterior pituitary cells

Although luteinizing hormone releasing hormone has been synthesized in many laboratories, no potency comparisons of various synthetic preparations have been published. However, it was reported that luteinizing hormone releasing hormone, synthesized by several laboratories (1–3), exhibited biological activity equivalent to an isolated natural porcine luteinizing hormone releasing hormone preparation[1]. These results were obtained utilizing either short-term incubations of minced pituitary tissue or ovariectomized estrogen–progesterone-blocked rats. This report presents potency comparisons of various synthetic luteinizing hormone releasing hormone preparations assayed with cultures of rat anterior pituitary cells.

EXPERIMENTAL

Various synthetic luteinizing hormone releasing hormone preparations (Samples 1–6[2–6]) were obtained commercially. These preparations were compared to Sample 7[7] luteinizing hormone releasing hormone.

The reference product (Sample 7) was prepared by fragment condensation and purified by partition chromatography[8] with 1-butanol[9]–acetic acid–water (4:1:5, upper phase). The major fraction from the partition column was homogeneous in the following TLC systems on silica gel 60 F-254 glass plates[10]: System 1, chloroform–methanol–water (45:45:10); System 2, chloroform–methanol–water–acetic acid (60:45:10:1); System 3, chloroform–methanol–32% acetic acid (60:45:20); System 4, 1-butanol–acetone–water–acetic acid–5% ammonium hydroxide (45:15:20:10:10); System 5, 2-propanol-1 M acetic acid (2:1); System 6, 1-butanol–acetic acid–water (upper phase) (4:1:5); System 7, pyridine–ethyl acetate–acetic acid–water (5:5:1:3); and System 8, 1-butanol–pyridine–acetic acid–water (30:20:6:24).

The reference product corresponded to 89% peptide content (1.4 acetate salt, 3.5 hydrates) with a molecular weight of 1329.6 and $[\alpha]_D^{25} - 55.8°$ (c, 1 in 1% CH_3CO_2H)[11].

Molecular weights and peptide contents (Table I) of comparative luteinizing hormone releasing hormone preparations were obtained from package inserts or the company of origin. Peptide content varied from 80 to 88%. The purity of these preparations was examined using TLC System 2. Preliminary experiments indicated that System 2 was superior

[1] AVS 77-35, 215-269.

[2] Sample 1, lot 208052, and Sample 2, lot 221040, Spectrum Medical Industries, Torrance, Calif.
[3] Sample 3, lot A0402, Beckman Instruments, Palo Alto, Calif.
[4] Sample 4, lot 4562, Bachem, Marina Del Rey, Calif.
[5] Sample 5, lot 19-192AL, Abbott Laboratories, North Chicago, Ill.
[6] Sample 6, lot SY-1, Sankyo Co., Tokyo, 140 Japan.
[7] Sample 7, lot 10746 X71A, Parke, Davis & Co., Ann Arbor, Mich.
[8] Sephadex G-25 coarse, Pharmacia Fine Chemicals, Piscataway, N.J.
[9] All analytical reagents, Mallinckrodt, St. Louis, Mo.
[10] EM Laboratories, Elmsford, N.Y.
[11] Model 141, Perkin-Elmer Corp., Norwalk, Conn.

Table I—*In Vitro* Potency of Commercial Synthetic Luteinizing Hormone Releasing Hormone Preparations[a]

Preparation	Slope ± 95% *CL*[b]	Relative Potency	95% Fiducial Limits	λ[c]
Samples 1 and 2, mol. wt. = 1338, 88% peptide	37.1 ± 6.0	0.35[d]	0.31–0.39	0.95
	40.5 ± 5.7	0.29[d]	0.23–0.37	0.52
	27.5 ± 3.9	0.73[e]	0.57–0.95	0.92
	51.0 ± 12.3	0.50[d]	0.41–0.62	0.64
	72.6 ± 25.1	0.75[e]	0.55–0.94	0.85
	47.9 ± 10.5	0.44[d]	0.36–0.54	0.72
	38.7 ± 9.3	0.36[d]	0.27–0.48	0.67
	Average ± *SEM*[f]	0.489 ± 0.070		
Sample 3, 80% peptide	38.4 ± 2.5	0.45[d]	0.42–0.49	0.73
	46.6 ± 13.8	0.66[d]	0.53–0.82	0.89
	43.7 ± 11.2	0.72[d]	0.61–0.85	0.82
	53.3 ± 31.9	0.44[d]	0.35–0.55	0.99
	42.5 ± 6.9	0.60[d]	0.52–0.70	0.87
	54.0 ± 8.0	0.56[d]	0.47–0.67	0.89
	Average ± *SEM*	0.572 ± 0.046		
Sample 4, mol. wt. = 1338, 88% peptide	44.9 ± 9.7	0.54[d]	0.42–0.71	0.55
	31.0 ± 6.2	0.60[d]	0.46–0.77	0.87
	45.7 ± 13.4	0.73[d]	0.59–0.90	0.95
	24.8 ± 13.4	0.67[e]	0.46–0.92	0.91
	Average ± *SEM*	0.635 ± 0.040		
Sample 5, mol. wt. = 1375, 86% peptide	48.8 ± 8.5	0.88	0.72–1.08	0.90
	39.0 ± 4.6	0.79[e]	0.65–0.97	0.95
	33.0 ± 11.4	0.70[d]	0.55–0.89	0.93
	41.5 ± 9.4	0.83[e]	0.71–0.98	0.95
	44.5 ± 7.4	0.71[e]	0.59–0.87	0.97
	Average ± *SEM*	0.782 ± 0.035		
Sample 6, mol. wt. = 1338, 88% peptide	34.0 ± 6.0	0.86	0.69–1.07	0.99
	39.4 ± 13.2	0.89	0.72–1.10	0.99
	45.6 ± 6.1	0.95	0.83–1.09	0.99
	53.4 ± 10.9	1.04	0.86–1.26	0.99
	Average ± *SEM*	0.935 ± 0.039		

[a] Potency determined relative to Sample 7. [b] Confidence limits. [c] Relative bioassay precision. [d] $p < 0.01$. [e] $p < 0.05$. [f] Standard error of the mean.

to the other systems in elucidation of contaminants in luteinizing hormone releasing hormone preparations. Preparations were dissolved in 95% ethanol, and 30 μg of each sample was applied to thin-layer plates followed by one-dimensional chromatography and exposure to iodine vapor for visualization.

Anterior pituitary cells were isolated from diestrous female rats[12] (200–250 g) and incubated as described previously (4), except that Hanks balanced salt solution[13] (calcium and magnesium free) was substituted for *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid buffer and enzymatic cell dispersion was carried out in a humidified carbon dioxide incubator[14]. The sensitivity of the primary cell culture system was compared to the classical luteinizing hormone releasing hormone bioassay (5) employing ovariectomized estrogen–progesterone-blocked rats as described previously (6, 7).

Fresh luteinizing hormone releasing hormone solutions were prepared for each assay from the lyophilized powder stored over a desiccant. Samples, 1–2 mg, were weighed[15] on the day of assay, and solutions were prepared at three or four treatment levels to extend over the effective dose–response range of luteinizing hormone releasing hormone in the cell culture (0.06–0.60 ng/ml). Included in each assay was Sample 7; all curves within an assay were compared to the curve for the standard.

Luteinizing hormone concentrations of media removed from cell cultures were evaluated by double antibody radioimmunoassay (8) employing reagents for rat luteinizing hormone[16]. Anti-rat luteinizing hormone serum-2 (final dilution 1:250,000) and ^{125}I-rat luteinizing hormone-I-3[17] were added per test tube. A computer program (9) was employed for analysis of radioimmunoassay data[18].

Comparison of 10 luteinizing hormone assays, selected at random, indicated that 31.59 ± 1.98% (mean ± *SEM*) of labeled hormone was found in the absence of unlabeled hormone, the midrange of the luteinizing hormone dose–response curve was 1.31 ± 0.05 ng/ml, and the minimum detectable concentration was 77.51 ± 10.87 pg/ml. Within assay variability at the 80 and 20% binding points on the luteinizing hormone curve was 13.93 ± 2.54 and 7.00 ± 1.29%, respectively. Within each radioimmunoassay, analyses of two rat serum pools containing either high (71.69 ± 2.91 ng/ml) or low (6.87 ± 0.20 ng/ml) luteinizing hormone levels were employed for determining between assay variability. The coefficients of variation between assays for these two serum pools were 8.87 and 9.37%, respectively.

Potency estimates were determined within each cell culture experiment relative to Sample 7 (potency = 1.0). Each luteinizing hormone releasing hormone dose–response curve was first tested for linearity (*t* test) and then for parallelism (*F* test) to the Sample 7 curve prior to estimation of potency. Relative precision (λ) for symmetrical experimental designs was determined according to Finney (10).

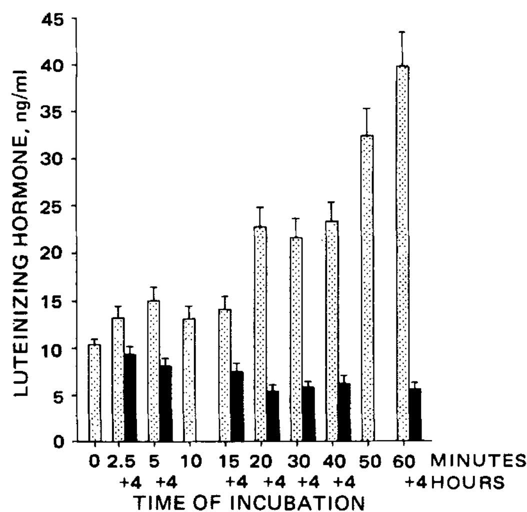

Figure 1—*Luteinizing hormone concentrations in the presence (dotted bars) and following removal (black bars) of luteinizing hormone releasing hormone. The 4-hr samples following luteinizing hormone releasing hormone for 10 and 50 min were lost. Values represent the mean* ± SEM *of triplicate observations.*

RESULTS AND DISCUSSION

Some preliminary studies were necessary to validate and standardize the *in vitro* system. Cell culture experiments were performed to determine if luteinizing hormone releasing hormone (0.6 ng/ml) had to be in

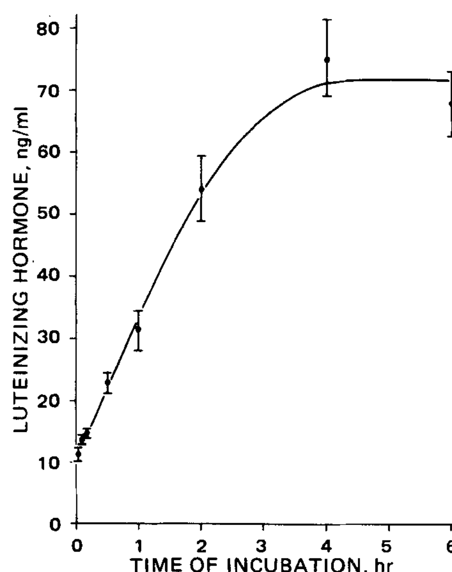

Figure 2—*Time course of luteinizing hormone releasing hormone-induced luteinizing hormone release. Values represent the mean* ± SEM *of triplicate observations.*

[12] Holtzman Laboratory Animals, Madison, Wis.
[13] Grand Island Biological Co., Grand Island, N.Y.
[14] Model 329, Forma Scientific Co., Marietta, Ohio.
[15] H 10 T, Mettler Instrument Co., Hightstown, N.J.
[16] Reagents supplied by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolic and Digestive Diseases, Bethesda, Md.
[17] Counted in model 588 gamma counter, Micromedic Systems, Niles, Ill.
[18] IBM 370/158.

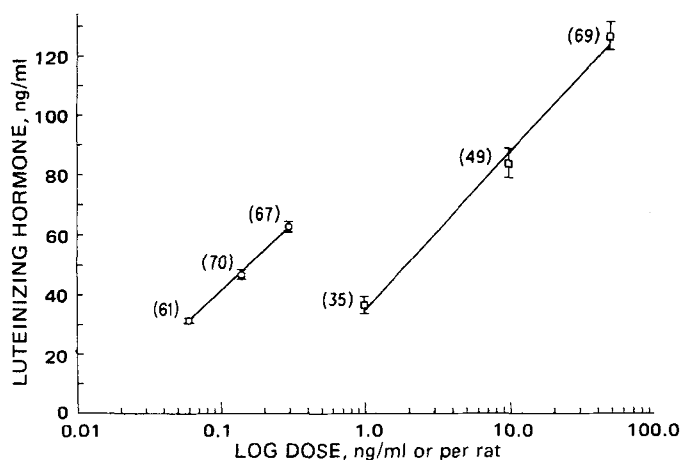

Figure 3—*Sample 7 dose–response curves* in vitro *(tissue culture, O) and* in vivo *(chronically ovariectomized steroid-blocked rats, □). Figures in parentheses indicate the number of observations at each point. Vertical bars denote the standard error of the mean.*

constant contact with cells for continued luteinizing hormone secretion and at what time maximum luteinizing hormone concentrations were found in the medium. Media were removed at selected times and assayed for luteinizing hormone (Fig. 1). Cells were washed and fresh medium, without luteinizing hormone releasing hormone, was added; incubations were continued for an additional 4 hr. In the presence of luteinizing hormone releasing hormone, the increase in luteinizing hormone concentration in the medium was linearly related to time during the 60-min observation. Following removal of luteinizing hormone releasing hormone, there was a dramatic fall in the luteinizing hormone concentration observed at the end of 4 hr of additional incubation. Data indicate that luteinizing hormone secretion depends upon constant cell contact with luteinizing hormone releasing hormone. Maximum luteinizing hormone levels in the medium were observed after 4 hr of incubation with luteinizing hormone releasing hormone (Fig. 2); therefore, all subsequent experiments involved 4-hr incubation periods.

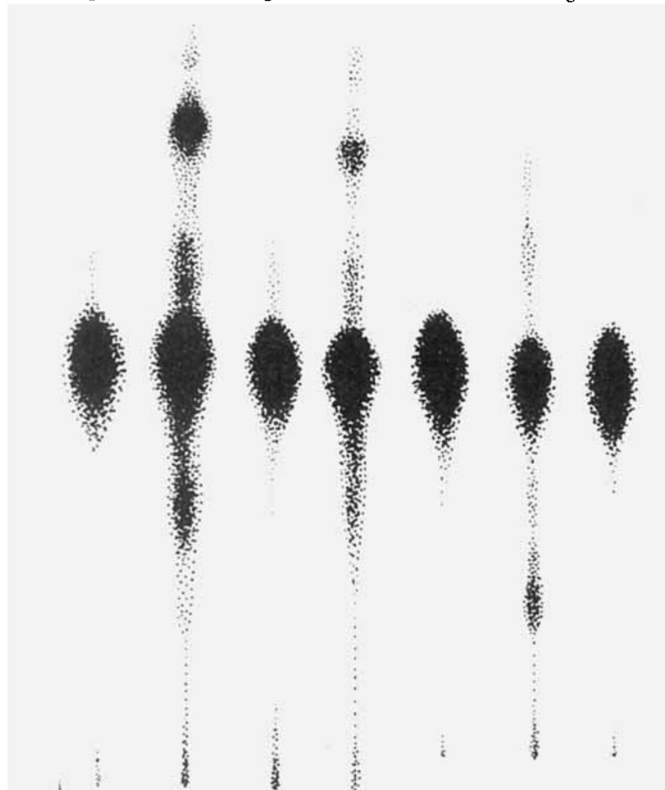

Figure 4—*Thin-layer chromatograms of luteinizing preparations. In Solvent System 2, the R_f of luteinizing hormone releasing hormone was 0.25 visualized by iodine vapor. From left to right, the preparations (30 μg each) were: Samples 7, 2, 5, 4, 7, 3, and 6.*

Various assays may be employed to determine the potency of luteinizing hormone releasing hormone preparations. Dose–response curves were compared (Fig. 3) for bioassays employing chronically ovariectomized, steroid-blocked rats (7–9) and primary pituitary cell culture. The slope of the dose–response curve obtained *in vivo* was 53.84 ± 5.15 (n = 5) over the dose range of 1–50 ng/rat. The average slope of Sample 7 dose–response curves in cell cultures was 44.32 ± 3.20 (n = 14). Similar luteinizing hormone responses of 30.90 ± 2.21 ng/ml in cell cultures and 39.64 ± 4.73 ng/ml *in vivo* were obtained in response to 0.06 ng/ml and 1.0 ng/rat, respectively. Primary cell cultures were selected for evaluation of synthetic luteinizing hormone releasing hormone preparations due to greater assay sensitivity and economy.

Of the five preparations compared to Sample 7, all but Sample 6[6] were less potent ($p < 0.05$) (Table I). The average potency of the preparations varied from 0.49 to 0.94. Differences in molecular weights were minor (8%) and could not account for the potency differences. Thin-layer chromatograms of 30-μg samples of the preparations (Fig. 4) showed the presence of materials that do not migrate with luteinizing hormone releasing hormone. These contaminants may account for the lower potencies of Samples 1–4. Identification of the impurities was not germane to the current investigation. However, the contaminants may create difficulties in radioimmunoassays (11), where the purity of the labeled antigen is critical.

The data indicate that a sensitive and precise assay for luteinizing hormone releasing hormone was obtained by utilizing primary cultures of enzymatically dispersed rat anterior pituitary cells. This assay procedure demonstrated differences in biological potency of synthetic luteinizing hormone releasing hormone preparations that might account for certain reported biological and immunological differences.

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