Sustained-Release Hormonal Preparations XV: Release of Progesterone from Cholesterol Pellets *In Vivo*

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Abstract □ Progesterone–sterol pellets were made that provided a zero-order release of progesterone for 80 days. 4-^{14}C-Progesterone was used to measure the release *in vitro* and *in vivo*. The dissolution rate *in vitro* (distilled water as the desorbing medium) for progesterone–cholesterol (59:41 w/w) and progesterone–β-sitosterol (47:53 w/w) pellets was 72 μg/100 mm^2/24 hr. The average *in vivo* absorption from subcutaneously implanted pellets in rabbits was 2 ± 0.1 μg/ml of plasma/cm^2 of surface area. Of this amount, 20–25% was progesterone; the remainder was progesterone metabolites and conjugates. Zero-order release (plasma levels) was obtained for approximately 80 days or until about 70% of the available progesterone was exhausted. During this time, the level of excreted radioactivity in urine continuously decreased, indicating that monitoring only this parameter would lead to erroneous conclusions. A long-term effect and increased effectiveness were obtained with a 5–20-mg progesterone equivalent dose, using gel prepared from 2% methylcellulose as the suspending medium.

Keyphrases □ Progesterone—in sustained-release cholesterol implant pellets, *in vivo* and *in vitro* release, biological effectiveness, rabbits and rats □ Cholesterol—implant pellets containing progesterone, *in vivo* and *in vitro* release and biological effectiveness, rabbits and rats □ Dosage forms—sustained-release progesterone–cholesterol implant pellets, *in vivo* and *in vitro* release and biological effectiveness of progesterone, rabbits and rats □ Sustained-release implant pellets—progesterone–cholesterol, *in vivo* and *in vitro* release and biological effectiveness of progesterone, rabbits and rats □ Hormones—progesterone in sustained-release cholesterol implant pellets, *in vivo* and *in vitro* release, biological effectiveness, rabbits and rats

Over the past several years, the biological effectiveness of drugs as a function of dosage form has been evaluated. In most studies, subcutaneously implanted sustained-release poly(dimethylsiloxane) capsules were used as the drug carrier (1). Animal studies demonstrated a significant increase in the potency of steroid hormones given in a sustained-release form; when released continuously from silicone rubber implants, considerably less hormone was needed to produce a given biological effect than when the same compound was given by injection or orally (2). The lower dose resulted in lower plasma levels and less accumulation of the steroid and of its metabolites in various organs (3).

Steroid–lipid implants as drug carriers have also been used (4–8). Mixing of steroids and lipids in arbitrary proportions resulted in steroid release in sufficient amounts to provide a pharmacological effect, but "the reproducibility of pellets of the same composition but made at different times varied . . . ." (4).

The present experiments were designed to test the release and biological effectiveness of steroid–sterol pellets combined in the region of eutectic mixtures.

EXPERIMENTAL

Pellet Preparation—Weighed amounts of progesterone and the respective sterol were dissolved in minimal amounts of chloroform. The solvent was removed by evaporation in a rotary evaporator, and the resulting crystalline mass was dried at 37°. The dry powder was transferred into a hand press preheated to about 100°. The temperature of the brass block was then slowly raised until the mass began to soften (115–120°). The soft mass was extruded through a perforated disk; the emerging rods (3 and 1 mm thick), which cooled and hardened rapidly, were cut into 1–3-mm long, cylindrical sections and marumerized to spherical pellets.

Marumerizer—The marumerizer was made from a Plexiglas block containing a circular chamber 6.4 cm in diameter. The wall was inlaid with aluminum oxide-coated abrasive[1]. For operation, the chamber was loaded with about 2–5 g of material; a current of air led in through tangential air inlets imparted a rapid rotation to the pellets. The average time for a 3-g load was 15 min with an air flow of about 200 liters/hr.

To minimize the loss of material, the outlets of the marumerizer were connected to a small Plexiglas cyclone separator. Five different pellet sizes between 177 and 1705 μm were obtained by screening marumerized material through a set of analytical sieves.

Preparation of Radioactive Progesterone—A known amount of 4-^{14}C-progesterone was added to cold progesterone dissolved in chloroform. The solvent was removed by distillation in a rotary evaporator, and the crystalline material was dried *in vacuo*. Specific activity of the resulting material was determined by weighing two or three samples on an analytical microbalance. Weighed samples were dissolved in toluene, and the radioactivity was determined.

Determination of Radioactivity—Toluene-based scintillation medium containing 1% of a fluor was used; plasma samples (1 ml) were solubilized with 1 ml of a tissue solubilizer[2] by digesting at 45–50° for about 1.5 hr. When a clear solution was obtained, 10 ml of a medium[3] was added. Prior to counting, the samples were kept at 0° for approximately 24 hr to eliminate photoluminescence. Urine and feces samples (*vide infra*) were decolorized with 30% aqueous hydrogen peroxide solution (0.5 ml) by incubating at 37° for 48–64 hr. A solubilizing solution (10 ml) prepared from two parts of toluene and one part of a surfactant[4] and containing 1% of a fluor was added. Counting efficiency (liquid spectrophotometer[5]) was determined by using external (and where appropriate, internal) standards. The efficiency in toluene scintillation medium was 80–85%; the efficiency in aqueous scintillation medium was 20–45%.

Studies *In Vitro*—A known weight (and number) of pellets was gently shaken[6] in 100 ml of distilled water at room temperature. To determine the amount of progesterone in the desorbing medium, 2-ml aliquots were taken periodically. Water was evaporated (IR lamps), and the radioactivity present was counted upon addition of toluene scintillation medium. Fresh desorbing medium was used every 24–72 hr. Other conditions were as stated previously (4).

Metabolic Studies in Rabbits—With the use of a local anesthetic[7], ethylene oxide-sterilized pellets (suspended in a sterile medium prepared from 2% aqueous gel of methylcellulose; 0.75 ml of the medium to suspend 100 mg of pellets) were implanted in the left hindleg of rabbits[8] by means of a thin walled No. 9 trocar. One-milliliter graduated pipets were used to deliver the desired amount. The wound was closed by clips[9].

Two rabbits were implanted with 23 and 27 tablets (compressed in a small press, hardness >5 kg) having a surface area of 456 and 535 mm^2, respectively. Two rabbits were implanted with 67 and 70 pellets with an average diameter of 1705 μm (surface area of 612 and 639 mm^2, respectively), and two rabbits were implanted with 78 and 94 pellets with an average diameter of 1205 μm (surface area of 356 and 429 mm^2, respectively). The average hardness of the pellets was <1 kg.

The animals were kept in metabolic cages, and water and food were given *ad libitum*. Peripheral blood (by ear puncture), urine, and feces were collected as stated. For blood collection, the ear and the collection

[1] Norton 120-J, Behr-Manning Division, Troy, N.Y.
[2] NCS solubilizer, Amersham/Searle, Arlington Heights, Ill.
[3] PCS, Amersham/Searle.
[4] Triton X, Rohm and Haas Co., Philadelphia, Pa.
[5] Model 720, Nuclear Chicago.
[6] Burrel wrist-action shaker, Burrel Corp., Pittsburgh, Pa.
[7] Xylocaine.
[8] Rockland, Inc., Gilbertsville, Pa.
[9] Justerite wound clips, 11 mm, Clay Adams, Parsippany, N.J.

Table I—Dissolution of Progesterone from Lipid Pellets into Water (Based on Unit Weight)

Lipid Used (% w/w)	Micrograms of Progesterone/24 hr/100-mg Pellets ± *SE*				
	1705[a]	1205	750	375	177
Cholesterol (41%)	560 ± 22	905 ± 33	1320 ± 96	3620 ± 289	5015 ± 409
β-Sitosterol (53%)	640 ± 25	1115 ± 72	1210 ± 93	4090 ± 435	3960 ± 278
	Based on Unit Area, μg of Progesterone/24 hr/100-mm² Area				
Cholesterol (41%)	252	224	239	245	Not calculated
β-Sitosterol (53%)	288	276	219	278	Not calculated

[a] Average pellet size, micrometers (obtained by sieving).

Table II—Observed Plasma Progesterone[a] Levels in Rabbits following Subcutaneous Implantation of Progesterone–Cholesterol Pellets

Animal	Progesterone, μg/100 ml of Plasma											
	1[b]	3	8	18	25	32	39	57	64	71	81	89
1	NS[c]	98	97	75	99	94	84	121	NS	94	56	76
2	226	NS	83	86	109	102	118	116	102	102	103	74
3	211	NS	97	134	121	120	117	132	127	131	NS	68
4	180	NS	98	125	112	112	101	129	128	127	NS	73
5	165	NS	80	135	110	121	109	(Died)				
6	226	NS	79	110	114	91	NS	93	88			

[a] Based on the amount of detected radioactivity. [b] Days after implantation. [c] NS = no sample.

vessel were siliconized to minimize hemolysis. Citrate solution, 1 ml/10 ml of blood, was added, and plasma was separated by centrifugation. At autopsy, body weights were recorded and blood was collected by heart puncture. Liver and kidneys were excised, weighed, and frozen. The implantation site was examined for capsule formation, and the pellets were collected.

Sample Preparation—*Urine*—Weekly collections were combined, and the volume was reduced about fourfold by evaporation in a rotary evaporator under reduced pressure. The resulting volume was recorded, and a 30-ml aliquot was diluted with 70 ml of absolute ethanol. The solution was kept for 48 hr at −20° to precipitate the proteins and was then centrifuged, and the clear supernate was separated. Radioactivity was determined in 2-ml aliquots.

Feces—Feces collected separately two or three times a week were combined and weighed. A 30-g sample was ground in a blender[10] with 100 ml of 70% aqueous methanol, and the resulting slurry was centrifuged. An aliquot of the clear supernate diluted with an equal volume of absolute methanol was kept at −20° for 48 hr. Radioactivity was determined in 1-ml aliquots.

Implantation Site—Surrounding tissue, including the pellets, was excised; the tissue was exhaustively extracted at room temperature with acetone–dichloromethane (1:3), the extracts were combined, and the solvent was removed by evaporation. Then the residue was redissolved in methanol, the volume was brought to 10 ml, and the residual radioactivity was determined by counting 1-ml aliquots in a toluene scintillation medium.

Calculations—Data were usually expressed in micrograms of progesterone per milliliter of plasma per surface area of the implant in square centimeters. *In vivo* data were analyzed with a two-factor repeated-measures design analysis of variance (9). Factors found to be significant were further analyzed with Duncan's new multiple range tests (10). All data met the assumptions of variance homogeneity and independence of means and variance (11, 12).

Two separate analyses were performed because data for some individuals were not collected for the entire experimental period. Data for all six animals were available for the first 5 weeks, and these data were utilized in one analysis. Data for four animals, the two given hard tablets and the two given large (1705-μm diameter) pellets, were available for Weeks 6 and 8–10. These data were analyzed independently.

The amount of radioactivity excreted in urine was submitted to regression analysis to determine if there was a significant change in the amount of progesterone excreted as a function of time. Data were expressed as $\log_{10}$ micrograms of progesterone equivalent per day per 100-mm² surface area of implanted pellets. Only the data from four animals, the two given hard tablets and the two given large pellets, were available for the entire period, and only these data were analyzed.

Biological Experiments—Pellets having an average particle size of 750 μm suspended in 2% aqueous methylcellulose gel were used in all experiments. Subcutaneous insertion was used in both tests.

Antiovulatory Tests—Following subcutaneous insertion, ovulation was induced in rabbits (different periods of therapy) by intravenous injection of 0.3 ml of 1% copper acetate–sodium chloride solution (13). Laparotomy was performed 18–24 hr after the injection, and the ovaries were examined for ovulation points.

Maintenance of Pregnancy—Rats[11] were bilaterally ovariectomized (ether anesthesia) on Day 5 following insemination, and progesterone pellets were inserted in the neck region. At autopsy, on Day 18 of the test, the number of viable (and resorbing) fetuses was counted and recorded. On the day of the ovariectomy and 1 week later, some animals received (in addition to progesterone) 1 μg of estradiol benzoate dissolved in 0.2 ml of sesame oil. A small group of ovariectomized animals with no treatment was included for comparison.

RESULTS

The present studies confirmed previous observations (4) that the amount of progesterone found in the desorbing medium is dependent upon the frequency of sample collection and the total pellet surface area exposed to the desorbing medium. As noted (4), dissolution rates were high during the first few days of the test, most likely due to the liberation of small particles loosely attached to the surface of the pellets; after a few days, the values became more uniform and variations between different samples of the same pellet size from day to day were small. In addition to the sampling frequency, the *in vitro* release rate was also influenced by the volume and temperature of the desorbing medium and the frequency (or speed) of agitation.

Table I shows average dissolution of progesterone from cholesterol (41% w/w) and β-sitosterol (53% w/w) pellets of various sizes based on 4- and 6-hr sampling (observation period of 6–28 days) calculated as micrograms of progesterone released per 24-hr interval from 100-mg pellets. The relative dissolution rate of progesterone per unit weight increased with the decrease in pellet size. This finding is in accord with the observation that the dissolution rate is dependent upon the surface area of the pellets; smaller pellets have a large surface area per unit weight. When calculated on the basis of unit area (100 mm²), good correlation between the various sizes was obtained.

Progesterone absorption (as total radioactivity) from pellets of three different sizes, monitored by measuring plasma levels, is shown in Table

[10] Warington Products, New Hartford, Conn.

[11] Holtzman Co., Madison, Wis.

Table III—Material Balance of Progesterone Used in Implantation Studies

Animal	Progesterone Available, mg	Progesterone Equivalent[a] Found, mg			
		Urine, %	Feces, %[b]	Implant Site, %	Total Recovered, %
1	43.04	24.05 (55.4)	0.94 (2.2)	13.46 (31.0)	88.6
2	63.09	28.22 (44.7)	0.97 (1.5)	27.00 (42.8)	89.0
3	92.09	72.97 (78.7)	5.30 (5.7)	3.60 (3.9)	88.3
4	93.82	55.68 (59.3)	5.05 (5.4)	17.39 (18.5)	93.3
5	37.39	19.14 (51.2)	3.39 (9.1)	—[c]	—[c]
6	45.43	38.15 (84.0)	3.92 (8.6)	2.97 (6.5)	99.1

[a] Based on the amount of detected radioactive material. [b] Collected 54 days of the experiment. [c] Not autopsied.

II. The release of radioactive material was high the 1st day after implantation; there was then a decrease in circulating levels, which remained stable until about Day 80. On Day 90, the amount of radioactivity in peripheral blood was found to be declining. Statistical evaluation (calculated in terms of unit surface area) revealed no differences in values between the three types of pellets used. The high levels noted immediately after implantation were significantly higher ($F = 6.79$, $df = 41$, $p < 0.005$) as compared to Weeks 1–4. This difference resulted because the plasma level for Week 0 was significantly greater ($\bar{x} \pm SE = 3.8 \pm 0.6$ μg/ml/cm^2) than that of the other 4 weeks, which did not differ from each other (overall $\bar{x} \pm SE = 2.1 \pm 0.2$ μg/ml/cm^2).

For Weeks 6 and 8–10, there was no significant difference between individual animals and there was no significant difference due to the time factor. The mean overall plasma level of 2.0 ± 0.1 μg/ml/cm^2 for this period did not differ significantly from that for Weeks 1–4 ($t = 0.75$, $df = 38$, $p > 0.40$). These data indicate that high plasma progesterone levels are reached shortly after implantation. Within less than 1 week, the plasma progesterone level decreased to a stable level, which was maintained for at least 10 weeks.

Of the total radioactivity in plasma, about 40% was ether extractable. The remaining 60% was in the aqueous phase, presumably associated with polar progesterone metabolites and conjugates. Partial purification of the ether extract by TLC in silica separated 40–60% of material with the same mobility as progesterone. This result indicates that the 100-mm^2 surface area of pellets provided 0.3–0.5 μg of progesterone/ml of plasma; the balance was presumably free and conjugated progesterone metabolites.

Elimination of radioactive progesterone metabolites was significantly lower in feces than in urine (Table III). The average values were below 5% for Animals 1–4 and less than 10% for Animals 5 and 6. Since excretion of progesterone metabolites in feces represented usually less than 5% of the total metabolic products (occasional higher values being due most likely to contamination from urine), the metabolic cages were modified to minimize this possibility; feces were not collected during the last 2 weeks of the experiment.

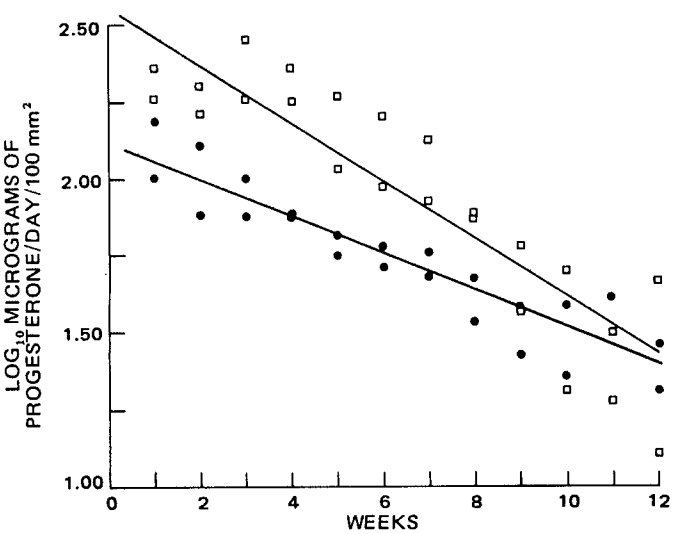

Figure 1—*Excretion of radioactivity in rabbits implanted with progesterone–cholesterol acetate pellets. Key: ●, Animals 1 and 2 (hard tablet); and □, Animals 3 and 4 (large pellet).*

The patterns of excreted radioactivity, expressed as $\log_{10}$ micrograms of progesterone per day per 100-mm^2 surface area implanted pellets, are shown in Fig. 1. There was a significant difference between the slopes of the two regression lines shown ($t = 4.06$, $df = 44$, $p < 0.001$); the amount of progesterone excreted by the animals implanted with the large pellets was initially at a higher level and declined more rapidly than in those animals implanted with the hard tablets. For both treatments there was no significant deviation from linearity; the r^2 values (the proportion of the variation in progesterone excreted explained by a linear relationship with time) were 0.81 for Animals 1 and 2 and 0.86 for Animals 3 and 4.

There was a significant increase in body weight during the 10-week experiment. The average initial body weight was 1430 g (1110–1680 g), and the final body weight was 2100 (1860–2445 g); this difference represents an average increase of 47%. Because the animals implanted with the large pellets had a higher average proportional weight increase than did those implanted with the hard tablets (60 *versus* 50%), the data were submitted to the following transformation to adjust for any differential effects of weight gain. For each individual for each week, the amount of progesterone excreted ($\log_{10}$ micrograms progesterone equivalent per day per 100-mm^2 surface area of implanted pellets) was multiplied by the estimated weight of that individual for that week and divided by that individual's initial weight.

Only initial and final body weights were recorded, so estimation of an individual's weight for each week was made by linear interpolation. For the data so treated, the regression equations are: for the hard tablet, $y = 2.10 - 0.005x$; and for the large pellets, $y = 2.59 - 0.029x$. The difference between the slopes is not significant ($t = 1.46$, $df = 44$, $p > 0.10$), which suggests that the difference in the slopes of the excretion rates in Fig. 1 is probably a function of the difference in weight gain.

Pellets were routinely recovered from the deposits under the skin; some migrated into the proximal muscle. In a few animals, the implant site was covered with a thin membrane, forming a pouch. No macroscopic manifestation of an inflammatory reaction at the implantation site was observed in any animal. The amount of radioactivity left in the remaining pellets varied from 4 to 27%. The overall recovery of ^{14}C-radioactive material in urine (and feces) and from the subcutaneous tissue at implant site varied from 70 to 99%; the average value was about 85%.

The results of antiovulation tests in rabbits are summarized in Table IV. The weights of pellets used in the experiment were 25, 75, and 225 mg, representing a dose of progesterone from 13.5 to 121.5 mg. Ovulation was inhibited for at least 45 days (duration of experiment) at the highest dose used. Based on the *in vivo* release data, it can be assumed that this dose released about 145 μg of progesterone/day.

Gravid rats deprived of progesterone by ovariectomy lost the ability to maintain pregnancy, and fetuses were rapidly resorbed; none was viable after 2 weeks (Table V). A single dose of 25 mg of progesterone pellets (13.5 mg progesterone equivalent) provided an incomplete fetal support;

Table IV—Duration of Antiovulatory Activity with Progesterone–Cholesterol Pellets

Progesterone Dose, mg	Rabbits Used	Average Ovulation ± *SE*	
		15 Days	45 Days
0	6	4.5 ± 0.4	6.5 ± 0.7
13.5	4	2.5 ± 0.4	4.0 ± 1.0
40.5[a]	5	0.5 ± 0.1	2.0 ± 0.3
121.5	5	0.0	0.0

[a] Estimated daily release, 450 μg.

Table V—Pregnancy Maintenance in Ovariectomized Rats with Progesterone–Cholesterol Pellets

Progesterone Dose, mg	Number of Rats			Mean Number (Range)	
	Total	with Fetuses	with Resorbed Fetuses	Fetuses	Resorbed Fetuses
4	6	0	0	0	0
13.5	6	0	3	0	7.2 (0–14)
13.5[a]	5	4	4	3.8 (3–13)	4.2 (0–6)
27.0[b]	4	2	4	4.5 (4–5)	4.8 (2–9)
27.0[a]	5	5	4	11.6 (10–14)	1.2 (0–2)
54	8	3	5	3.8 (0–8)	6.9 (3–12)
54[a]	3	3	0	12.0 (11–13)	0

[a] Indicates groups injected twice with 1 μg of estradiol benzoate. [b] Estimated daily release, 300 μg.

only resorption sites were found at autopsy. Addition of 1 μg of estradiol at weekly intervals was sufficient to increase significantly fetal salvage. In animals given 50-mg pellets (27 mg progesterone equivalent), there was an average of 11.6 live fetuses (a value usually seen in our colony) and only a few resorbing sites. Based on the same assumption as already given, the dose used was about 32 μg of progesterone/day.

DISCUSSION

Properties that influence absorption rates of drug implants, such as area, dissociation constant, solubility, and the diffusion coefficient, have been recorded (14). Most investigators considered absorption of materials released from pure crystalline implants of spherical shape. Mathematical relationships have been derived to predict the rates, but such relationships are not valid for drug systems combining two substances of unequal dissolutions.

Progesterone dissolution from steroid–sterol pellets was more rapid than sterol dissolution. When a "diffusion" constant was calculated using the slope of the regression line of cumulative release rate ($b^2\pi/16/3600$), similar values were obtained for progesterone regardless of pellet diameter. The values varied from 1.90×10^{-11} to 2.22×10^{-11} cm^2/sec for 750-, 1205-, and 1705-μm diameter pellets independent of period. The calculated value for 375-μm diameter pellets was 1.05×10^{-11} cm^2/sec, while the value for a "powder" (particle size <177-μm diameter) was 3.48×10^{-12} cm^2/sec. Corresponding values for β-sitosterol pellets (1205 and 1705 μm) were 1.87×10^{-11} and 1.99×10^{-11}, respectively.

Measurement of radioactivity in peripheral plasma showed a steady dissolution of progesterone, indicative of zero-order release rates in the presence of continuously declining excretion of radioactive material in urine and feces. The overall decline in the amount of progesterone excreted with time was most likely associated with the increase in body weight of the individuals. The difference in the origins in the regression equations (Fig. 1) suggests that animals implanted with the large pellets had a higher rate of progesterone excretion than did animals implanted with the hard tablets. However, this possible difference was not a function of any difference in circulating levels of progesterone in plasma, because these levels did not differ significantly as a function of treatment difference. Also, circulating plasma progesterone levels did not change as a function of time, as did the levels of excreted progesterone, which further indicates that plasma and excretory levels may vary independently.

Weight gain could have been the result of progesterone therapy; overeating and weight gain induced by progesterone have been observed in several species (15). Since there was no control group in these experiments and food consumption was not measured, additional experiments would be needed to confirm this conclusion. However, it is well documented that many steroid hormones, including progesterone, are deposited selectively in lipid tissues. In the present study, the concentration of radioactivity in intestinal fat (five observations) was 0.4 μg progesterone equivalent/g of fat. This observation supports the data showing that, in part, released progesterone was stored in the continuously formed lipid tissue.

These observations lead to the conclusion that, in studying absorption dynamics from implanted material, measurement of plasma levels provides more reliable information than measurements of excretion rates.

Progesterone was released from the pellets in a sufficient amount to exert biological effects. The effectiveness of sustained-release progesterone in maintaining pregnancy in ovariectomized rats was potentiated by injecting small amounts of estradiol as reported by others (16). Based on previously published data, it is possible to estimate that progesterone in a lipid delivery system is about 20 times more active than a daily injection of progesterone dissolved in oil. This value is in good agreement with previous findings (2).

REFERENCES

(1) F. A. Kincl and H. W. Rudel, *Acta Endocrinol.*, **66**, Suppl. 151 (1971).

(2) C. C. Chang and F. A. Kincl, *Fertil. Steril.*, **21**, 134 (1970).

(3) F. A. Kincl, I. Angee, C. C. Chang, and H. W. Rudel, *Acta Endocrinol.*, **64**, 508 (1970).

(4) F. A. Kincl, K. Sundaram, C. C. Chang, and H. W. Rudel, *ibid.*, **64**, 253 (1970).

(5) M. B. Shimkin and J. White, *Endocrinology*, **29**, 1020 (1941).

(6) C. D. Kochakian, *Am. J. Physiol.*, **145**, 549 (1945).

(7) A. Lipschutz and R. Iglesias, *Nature (London)*, **190**, 174 (1961).

(8) A. F. Pi, A. Oriol, L. H. Lasso, M. Maqueo, R. I. Dorfman, and F. A. Kincl, *Acta Endocrinol.*, **48**, 602 (1965).

(9) J. L. Bruning and B. L. Kintz, "Computational Handbook of Statistics," Scott, Foresman & Co., Glenview, Ill., 1968.

(10) J. C. R. Li, "Statistical Inference," vol. 1, Edwards Brothers, Ann Arbor, Mich., 1964.

(11) C. I. Bliss, "Statistics in Biology," vol. 1, McGraw-Hill, New York, N.Y., 1967.

(12) R. R. Sokal and F. J. Rohlf, "Biometry," Freeman, San Francisco, Calif., 1969.

(13) F. A. Kincl, *Endokrinologie*, **44**, 67 (1963).

(14) B. E. Ballard, *Ph.D. thesis, University of California, San Francisco, Calif., 1961.*

(15) S. Roberts, W. S. Kenney, and D. G. Mook, *Horm. Behav.*, **3**, 267 (1972).

(16) W. R. Lyons, *Proc. Soc. Exp. Biol. Med.*, **54**, 65 (1943).

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