The Biological Activity of Estrogen-Free Norethindrone. (30174)

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The synthesis of 19-norsteroids, which excludes the use of ring A aromatic compounds as starting materials, leads to estrogen-free norethindrone (17α-ethynyl-17β-hydroxyestr-4-en-3-one)(1). The biological activity of this material is the subject of this report.

Materials and methods. Estrogen-free norethindrone was synthesized in the Syntex Chemical Laboratories. Estrogenic activity was determined by the method of Rubin *et al*(2) using the end-point of uterine weight of the immature mouse and by the vaginal smear method of Allen-Doisy(3). Test compounds were administered in sesame oil solution either by subcutaneous injection or by gavage. Antiestrogenic activity was determined either by subcutaneous injection or by gavage by procedures previously described (4,5). These methods measure the ability of a compound to inhibit the growth-promoting activity of estrone on the uterus of the immature mouse. Progestational activity was evaluated by a modification of the Clauberg method(6), inhibition of ovulation by the method of Kincl and Dorfman(7), and parabiosis rat assays by the method of Shipley (8).

Inhibition of fertility was measured in adult mated rats. Treatment, by gavage, was started in the proestrous stage of the cycle and was continued once daily for 7 days. The animals were caged for the first 2 days of the test with fertile males and mating was confirmed by the presence of sperm in vaginal smears. At autopsy, performed 48 hours after the last treatment, the uterine horns were examined for the number of implantation sites. The test material was suspended in an aqueous vehicle (CMC). The suspending medium consisted of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxy-methylcellulose (0.5%), and benzyl alcohol (0.9%).

To measure the inhibition of spermatogenesis in the immature male rat the test material, suspended in CMC, was administered by subcutaneous injection once daily for 21 days. At autopsy, at the age of 43 days, weights of the testes, ventral prostate, seminal vesicles and levator ani muscle were determined and spermatogenesis evaluated histologically as previously described(9).

Norethindrone was administered once daily in CMC to pregnant rats beginning on day 17 of pregnancy and the anogenital distance of male and female offspring determined on day 1 of life and also on day 22.

Some of these studies were performed at The Endocrine Laboratories, Madison, Wis., under the supervision of Dr. Elva G. Shipley.

Results. The uterotrophic activity of estrogen-free norethindrone, as determined by gavage and by subcutaneous injection, is illustrated in Table I. When administered by gavage norethindrone produced a statistically significant increase in uterine growth at doses as low as 20 μg, but with increasing

TABLE I. Uterotrophic Activity of Norethindrone in the Immature Mouse.

Route	Material administered	Total dose, μg	No. of mice	Mean uterine ratio ± S.E.
Gavage	0	.0	24	.89 ±.054
	Estrone	.8	12	1.30 ±.057
		1.6	11	2.06 ±.163
		3.2	12	2.43 ±.143
		6.4	12	3.02 ±.200
	Norethindrone	5	20	.99 ±.049
		20	20	.48 ±.089
		80	19	1.51 ±.071
		320	19	2.14 ±.132
		1000	18	2.51 ±.213
Subcut inj	0	.0	9	1.01 ±.072
	Estrone	.05	9	1.78 ±.197
		.1	9	2.83 ±.320
		.2	9	4.56 ±.420
		.4	8	6.28 ±.305
	Norethindrone	5	9	1.69 ±.100
		20	9	2.36 ±.120
		80	9	2.34 ±.170
		320	9	3.09 ±.133
		1000	9	4.23 ±.243

TABLE II. Vaginal Cell Cornification in Ovariectomized Rat Following Subcutaneous Administration of Norethindrone.

Material administered	Total dose, μg	Total No. of rats	% Positive
Estrone	.22	20	0
	.55	20	30
	.88	20	70
Norethindrone	4	20	0
	20	20	5
	100	20	10
	500	30	25

dose only a shallow curve was obtained. On the basis of these data, the estrogenic potency of norethindrone determined graphically was 0.3% that of estrone by gavage and by subcutaneous injection. A similar degree of activity was seen in the Allen-Doisy test (Table II) when the compound was injected subcutaneously and was found to be 1/1000 as active as the standard estrone.

Norethindrone, free of estrogens, produced a statistically significant inhibition ($P<0.01$) at a total dose of 9 μg by gavage and 3 μg when injected subcutaneously while a sample of this steroid which contained some estrogenic material was found one-third to one-fifth as active (Table III).

The progestational response to orally administered norethindrone and norethindrone containing estrogenic material in the Clauberg assay were of the same order and not significantly different (Table IV). The two forms of norethindrone were studied in an inhibition of ovulation test in the adult estrus rabbit following oral administration. The biological activities of these materials were not significantly different. A dose of 0.3 mg produced an incomplete inhibition of ovulation with an average of 2.4 and 2.6 ovulation points, respectively, and a complete suppression of ovulation was achieved with a dose of 2.7 and 2.5 mg, respectively.

The antifertility activity of estrogen-free norethindrone was compared to mestranol when both compounds were given by gavage. Efficacy was judged by the number of rats which became pregnant, and the average number of implantation points within each group. A dose of 1.2 mg of norethindrone produced a moderate decrease in fertility and a 4-fold increase in dosage was needed for complete suppression. Two-hundredths mg of mestranol (3-methoxy ethynyl estradiol-17β) was equivalent in inhibitory activity to 1.2 mg of norethindrone and 0.04 mg of the estrogen produced complete suppression.

TABLE IV. Response to Orally Administered Norethindrone in the Clauberg Test.

Material	Total dose, mg	No. of rabbits	Mean response (range)
Norethindrone, estrogen-free	.1	6	.5 (.0–1.0)
	.3	8	2.0 (1.0–3.0)
	.9	8	2.3 (1.0–3.5)
	2.7	8	3.7 (3.0–4.0)
Norethindrone with estrogen*	.7	9	1.3 (.5–2.0)
	1.3	14	2.9 (1.5–4.0)
	2.5	3	3.5 (3.0–4.0)
	5.0	5	3.8 (3.5–4.0)

* Kincl(11).

Inhibition of gonadotropin synthesis and/or release measured by the parabiotic rat assay technique is given in Tables V-VII. The test material was given either by gavage or subcutaneously for 10 days. The response to oral administration, using intact female-

TABLE III. Anti-Estrogenic Activity of Norethindrone.

Route	Material administered	Total No. of mice	Dosage range studied, μg	Minimum dose to produce inhibition	Maximum inhibition, %
Gavage	Norethindrone, estrogen-free	102	1– 750	9	60
	Norethindrone* with estrogen	238	2–1000	32	40
Subcut inj	Norethindrone, estrogen-free	88	1– 243	3	44
	Norethindrone with estrogen†	180	1–4000	16	56

* Dorfman *et al.*(5)

† Dorfman *et al.*(4)

ovariectomized female parabiont is given in Table V and mestranol was used as the standard. A significant decrease in ovarian weight (expressed as tissue ratio, *i.e.*, tissue weight in mg/body weight in g) occurred at a dose of 0.5 μg of mestranol; a dose of 1 μg produced about 50% reduction in weight. A dose of 400 μg norethindrone was needed to produce a comparable response. By subcutaneous injection (Table VI) a total dose of 80 μg was needed for a significant response. From these data the oral potency of norethindrone in the female-female parabiotic pair may be estimated to be about 0.2% that of mestranol when given by gavage and about 0.03% of estradiol-17β by subcutaneous injection. When the gonadotropin inhibiting activity was measured in the castrated male-intact female parabiotic pair, a total dose of 300 μg of subcutaneously ad-

TABLE V. Activity of Norethindrone Assayed by Gavage in Castrated Female - Intact Female Parabiotic Pair.

Treatment	Total dose, μg	Total No. of rats	Intact female ovarian ratio ± S.E.	Castrated female uterine ratio ± S.E.
0	0	28	1.71 ± .011	.55 ± .004
Mestranol	.25	8	1.32 ± .224	.56 ± .006
	.5	24	1.46 ± .021	.55 ± .004
	1	29	.88 ± .010	.56 ± .001
	2	8	.72 ± .026	.69 ± .005
	5	15	.46 ± .007	1.05 ± .010
	10	10	.38 ± .001	1.48 ± .104
Norethindrone	5	8	2.26 ± .009	.71 ± .006
	10	7	1.76 ± .195	.74 ± .007
	20	8	1.91 ± .283	.66 ± .010
	50	13	1.40 ± .170	.38 ± .001
	100	12	1.67 ± .122	.56 ± .003
	200	8	1.59 ± .200	.52 ± .001
	400	7	.96 ± .249	.71 ± .001

TABLE VI. Activity of Norethindrone Assayed by Subcutaneous Injection in Castrated Female - Intact Female Parabiotic Pair.

Treatment	Total dose, μg	Total No. of rats	Intact female ovarian ratio ± S.E.	Castrated female uterine ratio ± S.E.
0	0	8	2.44 ± .179	.60 ± .007
Estradiol-17β	.05	8	1.29 ± .289	.41 ± .007
	.1	8	.87 ± .129	.55 ± .008
	.2	8	.43 ± .038	.60 ± .001
Norethindrone	20	8	2.09 ± .285	.57 ± .004
	40	8	1.09 ± .126	.56 ± .004
	80	8	1.56 ± .221	.43 ± .006
	160	8	1.08 ± .209	.36 ± .001

TABLE VII. Activity of Norethindrone Assayed by Subcutaneous Injection in Castrated Male - Intact Female Parabiotic Pair.

				Castrated male, organ ratio ± S.E.		
Treatment	Total dose, mg	Total No. of rats	Intact female ovarian ratio ± S.E.	Ventral prostate	Seminal vesicles	Levator ani
0	0	10	1.90 ± .155	.18 ± .009	.13 ± .007	.25 ± .008
Testosterone	.5	9	.93 ± .192	.57 ± .077	.39 ± .087	.42 ± .032
	1.5	9	.38 ± .028	1.20 ± .072	1.22 ± .151	.70 ± .036
Norethindrone	.03	5	2.23 ± .176	.12 ± .001	.10 ± .001	.24 ± .002
	.1	10	1.81 ± .158	.16 ± .009	.12 ± .006	.25 ± .016
	.3	10	.93 ± .152	.19 ± .009	.12 ± .023	.25 ± .020

TABLE VIII. Inhibition of Spermatogenesis by Subcutaneously Administered Norethindrone in 21-Day-Old Male Rats.

Treatment	Daily dose, μg	No. of rats used	Body wt, g ± S.E.	Organ weights, mg ± S.E.				Spermatogenetic index (range)
				Testes	Ventral prostate	Seminal vesicles	Levator ani	
0	0	12	166 ± 3	1850 ± 35	64.4 ± 4.1	51.1 ± 4.2	67.4 ± 2.7	.0 (0–0)
19 Nortestosterone	50	8	151 ± 7	1560 ± 34	52.1 ± 4.8	53.1 ± 5.9	62.0 ± 6.5	.0 (0–0)
	150	8	163 ± 5	1290 ± 28	66.4 ± 6.4	39.5 ± 3.4	76.8 ± 4.3	.0 (0–0)
	400	5	159 ± 3	974 ± 15	38.5 ± 5.4	24.6 ± 3.3	82.4 ± 11	1.6 (1–2)
Norethindrone	50	8	163 ± 3	1540 ± 81	60.8 ± 6.4	46.4 ± 4.1	73.5 ± 5.3	.5 (0–1)
	150	8	154 ± 5	380 ± 69	17.8 ± 1.1	16.5 ± 1.2	54.7 ± 2.9	1.9 (1–2)
	400	5	156 ± 8	340 ± 15	44.9 ± 9.6	30.2 ± 1.5	81.8 ± 9.3	3.0 (3–3)

ministered norethindrone was needed to produce inhibition of the same order as that produced by 500 μg of testosterone. The 300 μg dose did not stimulate the ventral prostate or seminal vesicles, indicating lack of an androgenic response (Table VII).

In the inhibition of spermatogenesis test, 19-nortestosterone was used as a standard (Table VIII). A daily dose of 150 μg of 19-nortestosterone significantly inhibited the testes and levator ani weights, but not the weights of ventral prostate or seminal vesicles and histologically the seminiferous epithelium was normal. At a dose of 400 μg per day, the weights of all 4 parameters studied were decreased and a mean spermatogenetic index of 0.6 was produced. Norethindrone was more active. At a dose of 50 μg per day, the testes were slightly atrophied with only modest damage to the seminiferous epithelium and a mean value of 0.5. A dose of 150 μg per day produced an intense atrophy of testes and accessory sex tissue and a mean spermatogenetic index of 1.9. Maximum inhibition of spermatogenesis resulting in a mean value of 3.0 and atrophy of the testes was seen in animals treated with a daily dose of 400 μg. This dose also produced a modest increase in levator ani weights but the weights of the ventral prostrate and seminal vesicles were well below those of the control groups.

Orally administered norethindrone at a dosage up to 1 mg per day for the last 5 days of pregnancy in rats produced no significant differences in the anogenital distances in either males or females at day 1 or day 22.

Discussion. In assays where ring A phenolic steroid exhibits a low degree of activity such as assays for progestational activity or inhibition of ovulation, no significant difference was noted between estrogen-free and estrogen-containing norethindrone. In assays where estrogens in microgram quantity are markedly active, as parabiotic rat, inhibition of spermatogenesis or antifertility activity, all previously published results have to be interpreted with caution.

Pure norethindrone appears to be only weakly estrogenic, possessing less than 0.5% the activity of estrone, or 0.05% the activity

of mestranol(12) when given by gavage and the compound is a potent antiestrogen. Besides its well recognized progestational effect, norethindrone appears to decrease the output of the luteinizing hormone (rabbit anti-ovulation test) but is less effective in blocking the release of FSH (parabiotic rat assay). In a daily dose of up to 400 μg in the immature male rat and a dose of 1000 μg in the pregnant female rat no androgenic effects were noted. These findings are in agreement with our previously published results(10).

Summary. Norethindrone (17α-ethynyl-17β-hydroxyestr-4-en-3-one) (estrogen-free) is 0.3% as active as estrone by gavage and by subcutaneous injection by a mouse uterotrophic assay. This steroid, free of estrogens, is about 3 to 5 times more active than previously studied material containing an estrogen contaminant. Both materials exhibit the same progestational activity when administered orally in the Clauberg assay and when studied in an oral rabbit anti-ovulation assay. Norethindrone (estrogen-free) has been studied in castrated female-intact female and castrated male-intract female parabiotic pairs in a rat spermatogenesis inhibition test, and in the pregnant rat.

1. Bowers, A., Villotti, R., Edwards, J. A., Denot, E., Halpern, O., J. Am. Chem. Soc., 1962, v84, 3204.
2. Rubin, B. L., Dorfman, A. S., Black, L., Dorfman, R. I., Endocrinology, 1951, v49, 429.
3. Emmens, C. W., in Methods in Hormone Research, Vol. II, p59, ed., R. I. Dorfman, Academic Press, New York, 1962.
4. Dorfman, R. I., Kincl, F. A., Ringold, H. J., Endocrinology, 1961, v68, 17.
5. ———, ibid., 1961, v68, 43.
6. Miyake, T., in Methods in Hormone Research, Vol. II, p135, ed., R. I. Dorfman, Academic Press, New York, 1962.
7. Kincl, F. A., Dorfman, R. I., Acta Endocrinol., 1963, v62, Suppl. 73.
8. Shipley, E. G., in Methods in Hormone Research, Vol. II, p179, ed., R. I. Dorfman, Academic Press, New York, 1962.
9. Kincl, F. A., Maqueo, M., Dorfman, R. I., Acta Endocrinol., 1965, in press.
10. Kincl, F. A., Dorfman, R. I., ibid., 1962, v41, 274.
11. Kincl, F. A., Endokrinologie, 1961, v40, 17.
12. Dorfman, R. I., unpublished.

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Toxicity of Ant Venom, Further Studies of the Venom from *Pogonomyrmex barbatus.** (30175)

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McCook(1), in his excellent monograph, The Natural History of The Agricultural Ant of Texas, *Pogonomyrmex barbatus,* presents observation of the aspects of the sting of this myrmicinae which parallel closely the observations of this author(2).

These earlier studies of venom prepared from homogenates of the entire abdominal segments of the desert ant, *Pogonomyrmex barbatus, barbatus,* indicated the presence of a highly toxic cholinergic material. Blum and Ambrose(3) described a water soluble cholinergic and histamine-like venom from the army ant, *Eciton burchelli.* Venom from the fire ant, *Solenopsis soevissima,* was described by Caro *et al*(4), who also mentioned one human fatality resulting from this agent. Cavill(5) in a recent study of the venom of the Australian bull ant, *Myrmecia gulosa,* found a water soluble proteinaceous material having ultraviolet absorption peaks at 204 and 277 to 282 mμ.

The purposes of the present study were to expand upon collection procedures previously described(2) and relate the amount of venom extracted by these new techniques with the

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