Displacement of 17β-Estradiol from Uterine Receptor Sites by an Estrogen Antagonist (33018)

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CN-55, 945-27[1], a potent nonsteroidal estrogen antagonist (1), has previously been shown to block the uptake of 17β-estradiol by the uterus (2). One of the interesting biologic activities of this compound is its ability to inhibit uterine contractility both *in vitro* (1,3) and *in vivo* in unanesthetized animals (4). Since CN-55, 945-27 can rapidly suppress myometrial activity in animals which are actively secreting estrogen or under *in vitro* conditions in uteri previously exposed to estrogen, it occurred to us that this agent might be acting by displacing bound estradiol from the uterus as well as by preventing its binding. Therefore, experiments were carried out in the present study to test this hypothesis both *in vitro* and *in vivo*.

Materials and Methods. Adult female Holtzman rats (200 ± 5 gm) were used in this study. The animals were housed in an air-conditioned room (22°C) in which the lights were automatically controlled to provide a 12-hour photoperiod (lights on 7 a.m.–7 p.m.). Rockland rat pellets and tap water were supplied *ad libitum*.

In Vitro *Study*. Intact animals were injected subcutaneously with approximately 0.5 ml of peanut oil containing 17β-estradiol-4-^{14}C (1 μg/kg; 31.8 mC/mmole; Nuclear of Chicago). Six hours were allowed to elapse following injection of radioactive estradiol to allow maximal uptake by the uterus (5). At this time, the rats were sacrificed with diethyl ether and the uteri were dissected free of surrounding tissue. Following removal of the uterus, each horn (duplicate determinations) was slit longitudinally, divided into thirds, and placed in a 10-ml vessel containing 5 ml of incubation medium to which CN-55, 945-27 (20 μg/ml) nonradioactive 17β-estradiol (0.08 μg/ml) or vehicles for these compounds had been added. Vehicle for CN-55,945-27 was 0.5 ml of H_2O while that for estradiol was 0.5 ml of ethanol. The incubation medium was composed of Krebs–Ringer–phosphate solution (6):potassium dihydrogen phosphate–disodium hydrogen phosphate, pH 7.4 (7) (13:3/v:v), glucose (0.2%), albumin (5%) and the following cofactors (0.1 μM) : NAD, NADP, NADH, NADPH, and ATP. The tissues were then incubated at 37°C for 3 hours under 95% O_2–5% CO_2 in a Dubnoff metabolic shaker (60 oscillations /min). A 1-ml aliquot was removed from each vessel at 1, 2, and 3 hours after the incubation was initiated and analyzed for estradiol (^{14}C content) as previously described (8). Uterine estradiol was determined in a similar manner at the end of the 3-hour incubation period.

In Vivo *Study*. Rats, which had been ovariectomized under ether anesthesia 21 days previously, were injected subcutaneously with approximately 0.1 ml of peanut oil containing 17β-estradiol-6,7-^{3}H (20 μg/kg; 445 mC/mmole; Nuclear of Chicago). One group of animals was sacrificed and their uteri removed as described above 6 hours following administration of radioactive estradiol. The remaining animals were divided equally into two additional groups, one of which received CN-55,945-27 (5 mg/kg) by gavage in an aqueous suspending medium (1) whereas the other group was dosed with 0.5 ml of the vehicle alone. Five animals from each group were killed and the uteri removed at 8, 10, 14, and 22 hours following injection of 17β-estradiol-6,7-^{3}H. The uteri obtained from all of the animals were dried and the estradiol (radioactivity) content was determined by the oxygen flask combustion method as previously described (8) with the exception that

[1] CN-55,945-27, (1-[2-(*p*-[α-(p-methoxyphenyl-β-nitrostyryl] phenoxy) ethyl] pyrrolidine, monocitrate). Structural formula given in Ref. (1).

TABLE I. *In Vitro* Displacement of Uterine 17β-Estradiol-4-^{14}C by CN-55,945-27 or 17β-Estradiol.

Treatment	No. of determinations	dpm/ml of medium[a]			Completion of 3-hour incubation	
		1 hour	2 hours	3 hours	Uterine dry wt. (mg)[a]	dpm/mg of uterine dry wt.[a]
Control	6	10.64 ± 1.72	14.09 ± 1.11	18.39 ± 0.93	34.73 ± 2.79	3.27 ± 0.09
CN-55,945-27 (20 μg/ml)	7	11.16 ± 0.51	21.37 ± 2.07[b]	33.19 ± 3.20[b]	31.77 ± 1.94	1.61 ± 0.13[b]
17β-Estradiol (0.08 μg/ml)	7	11.56 ± 0.99	21.70 ± 2.36[b]	31.38 ± 3.43[b]	31.34 ± 1.74	1.77 ± 0.16[b]

[a] Mean ± SEM.
[b] $p < 0.01$, Rx vs control.

absolute ethanol was used in place of phenethylamine:methanol as the trapping medium for trititum. This study was carried out using doses of CN-55,945-27 and estradiol in a ratio of 250:1 because we had previously observed a good degree of estrogen antagonism with this proportion of these agents (1).

Detection of estradiol. As indicated above, measurement of radioactivity in the uterus following subcutaneous injection of labeled 17β-estradiol was considered as evidence for the presence of estradiol per se. Similarly, when uteri obtained from animals previously treated with radioactive 17β-estradiol were incubated *in vitro*, it was assumed that the levels of isotope in the tissue and medium were indicative of estradiol which was bound and released, respectively. Although such assumptions would not be valid for all species, Jensen and his coworkers (5,9–11) have shown rather conclusively by a variety of chemical methods that only estradiol is bound by the uterus of the rat subsequent to *in vivo* administration of radioactive 17β-estradiol. Moreover, these investigators have also demonstrated that neither the immature nor the actively growing rat uterus has the ability to metabolize estradiol. Therefore, in the present study estradiol binding was assessed by following the level of radioactivity in the uterus of rats previously treated with tritium- or ^{14}C-labeled 17β-estradiol.

It is possible that the estrogen antagonist studied in this investigation may evoke the metabolism of estradiol by the rat uterus. Hence, the radioactivity detected in the *in vitro* study within the incubation medium might not be estradiol per se but rather could be associated with a metabolite of this steroid. If this were the case, such radioactivity would still be indicative of displaced 17β-estradiol since only the latter steroid would be bound in the uterus following *in vivo* administration of radioactive estradiol. However, it should be emphasized that the data concerning the level of isotope in the incubation medium must be considered together with the estradiol concentration in the uterus.

Results. The effect of incubating uteri of animals previously treated with 17β-estradiol-4-^{14}C in a medium containing CN-55,945-27 or unlabeled 17β-estradiol is shown in Table I. It is evident that uteri placed in an incubation medium which contained neither of these agents released some ^{14}C-radioactivity during the course of the 3-hour incubation period. However, those uteri incubated with CN-55,945-27 or nonradioactive estradiol showed a significant ($p < 0.01$) increase in the rate of release of ^{14}C-radioactivity derived from previously bound radioactive estradiol at the end of 2 and 3 hours. Moreover, there was no significant difference between the effect of CN-55,945-27 and cold estradiol on a temporal basis which suggests that these agents exert their effect on estradiol binding in a similar manner. Although the control uterine horns and those incubated with the above compounds had dry weights which were not significantly different, the uteri incubated with

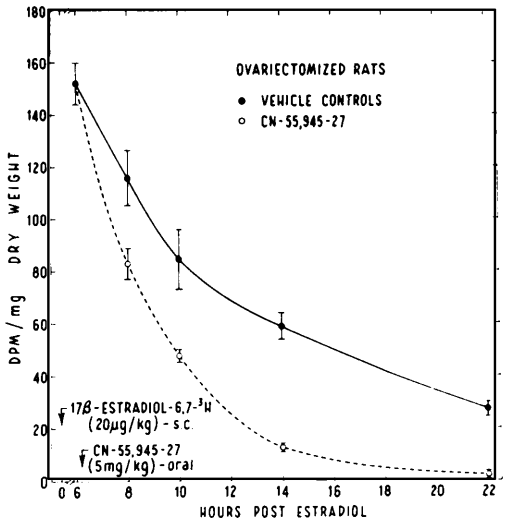

FIG. 1. Displacement of uterine 17β-estradiol-6, 7-^{3}H by CN-55,945-27 *in vivo*. Estradiol was injected s.c. in peanut oil whereas CN-55,945-27 was given orally by gavage in an aqueous vehicle. Each point on graph represents mean response of five animals; brackets indicate SEM.

either CN-55,945-27 or unlabeled 17β-estradiol contained significantly ($p<0.01$) less estradiol than those incubated in the control medium. Uterine estradiol concentration was the same in tissues obtained from medium containing CN-55,945-27 or unlabeled estradiol. Therefore, it would appear that both CN-55,945-27 and nonradioactive estradiol can displace estradiol from the uterus since a decrease in uterine estradiol concentration occurred concomitantly with a progressive increase in ^{14}C-radioactivity in the medium during the incubation period.

The results obtained when CN-55,945-27 was given by gavage to ovariectomized rats previously injected with 17β-estradiol-6,-7-^{3}H are illustrated in Fig. 1. Six hours following injection of labeled estradiol the uterus contained a high level of steroid which decreased progressively in the control animals treated with vehicle alone. However, within 2 hours after oral administration of CN-55,945-27 the uterus showed a significant ($p<0.01$) increase in the rate of uterine estradiol release over that observed in the control animals. This difference in uterine estradiol concentration between the control and CN-55,945-27 treated animals was observed to increase progressively during the next 6 hours. Within 16 hours practically all of the estradiol had been displaced from the uterus in rats receiving CN-55,-945-27 whereas the controls still exhibited an appreciable level of this steroid. Thus, it is apparent that CN-55,945-27 can enhance the rate of release of estradiol bound in the uterus when adminsitered orally to ovariectomized rats. These *in vivo* observations corroborate those of the foregoing *in vitro* study.

Discussion. The results of the present investigation show that CN-55,945-27 can displace previously bound 17β-estradiol from the uterus. *In vitro,* this response was found to be similar to that exerted by nonradioactive estradiol (Table I). That is, the uterus, when incubated for 3 hours with either of these compounds, showed a decrease in estradiol concentration which was accompanied by an increase in the level of radioactivity in the medium. In addition to these findings, CN-55,945-27 was observed to increase the rate of release of 17β-estradiol from the uterus *in vivo* (Fig. 1). Thus, it appears that CN-55,945-27 can displace uterine estradiol under physiologic conditions as well as *in vitro*.

Previous reports have demonstrated that 17β-estradiol is selectively bound by several target tissues including the hypothalamus (13–16), adenohypophysis (14, 16, 22), uterus (5, 12, 13, 18, 19), and vagina (18–20). All of these tissues apparently contain a limited number of binding sites since it is possible to saturate them with relatively small doses of 17β-estradiol (13, 16). Although the specific nature of this uptake and binding phenomenon has not been elucidated, it appears that a protein component of the cells is responsible for this action in the uterus (21). In addition, it has been shown that the protein receptor molecule for 17β-estradiol is stereospecific since 17β-estradiol is bound to a much greater degree than is 17α-estradiol (12, 16). However, it should be noted that while several other steroids including androgens

(13,14,21), corticoids (12,14,21) and progestins (12,13,14), do not influence 17β-estradiol binding, certain synthetic compounds such as norethynodrel (13), TACE (23), Clomiphene (17), and CN-55,945-27 (2) do have an appreciable influence on this phenomenon. Thus, the receptor molecule is apparently stereospecific for 17β-estradiol as opposed to other *endogenous* hormones, but still receptive to certain synthetic agents. All of the compounds known to influence the binding of 17β-estradiol have some level of estrogenicity, albeit low in some cases. Therefore, we might expect that a direct relationship exists between the estrogenic potency of a compound and its binding ability. This possibility is substantiated by studies carried out quantitatively with various doses of 17α- and 17β-estradiol in which these compounds were compared in terms of their ability both to increase uterine dry weight and to block the uptake of 17β-estradiol-^{3}H by the uterus (12). The data from this study indicated that the more estrogenic isomer had a greater affinity for the uterine receptor sites. Conversely, however, it should be noted that the direct relationship between estrogenicity and the ability to block the uptake of 17β-estradiol does not hold true for the estrogen antagonist employed in this study. That is, CN-55, 945-27 has been shown to exhibit a constant degree of mild estrogenicity on the basis of uterine weight over a wide range of doses (5–1000 μg/kg) but to progressively inhibit the uptake of 17β-estradiol-4-^{14}C over this same dosage range (2). Thus, although a compound does not require a high degree of estrogenicity to compete with 17β-estradiol for its receptor molecule, it apparently does need to possess some level of estrogenicity. When the magnitude of estrogenicity is low, as with CN-55,945-27, then a large quantity of the antagonist as versus the amount of 17β-estradiol (250:1, Table I and Fig. 1) is required. These observations support the concept that an affinity for the 17β-estradiol receptor molecule is required for estrogens to act. Various actions of estrogen (e.g., imbibition of water by the uterus, myometrial activity, behavioral estrus) may be related to the affinity of a molecule for estrogen receptor sites on both a qualitative and quantitative basis. Whether or not the magnitude of estrogenicity exhibited is dependent on the degree of binding and/or the intrinsic properties of a given molecule, which may in turn dictate the magnitude of its affinity for the recptor site, are questions for further investigation.

It is obvious from the foregoing discussion that CN-55,945-27 may exert two distinct inhibitory effects on estradiol binding in the uterus of the rat. Firstly, this agent has been shown to block the incorporation (uptake) of radioactive 17β-estradiol by the uterus of the immature ovariectomized rat when given orally prior to subcutaneous injection of labeled estradiol (2). Secondly, as shown in the present study, CN-55,945-27 can cause the displacement (release) of previously bound estradiol from the uterus of the rat. Hence, these observations may, at least in part, account for the inhibitory action of this agent on contractility of the estrogen-dominated uterus (1,3,4) and fallopian tube (24). The temporal aspects of estradiol binding and fallopian tube contractility will be discussed in a subsequent communication.

Summary. A potent nonsteroidal estrogen antagonist (CN-55,945-27) was found to displace previously bound radioactive 17β-estradiol from the uterus when added to an incubation medium containing uterine horns obtained from intact rats or when administered by gavage to ovariectomized rats. *In vitro,* this phenomenon was indicated by a significant decrease in the uterine estradiol-4-^{14}C with a concomitant and progressive increase in the level of radioactivity within the medium during a 3-hour incubation period. Similar results were obtained *in vitro* when nonradioactive 17β-estradiol was incubated with uteri obtained from rats previously treated with 17β-estradiol-4-^{14}C *in vivo*. When CN-55,945-27 was given orally to rats injected subcutaneously 6 hours previously with 17β-estradiol-6,7-^{3}H, it markedly increased the rate of release of estradiol from the uterus over

that observed in animals receiving oral vehicle. This effect, as shown by a decrease in uterine estradiol, was significant within 2 hours, increased markedly during the next 6 hours, and continued to nearly complete depletion of uterine estradiol within 16 hours after administration of CN-55,945-27. Uteri obtained from vehicle-treated control rats still contained an appreciable amount of estradiol 16 hours post treatment. These observations indicate that CN-55,945-27 can displace uterine estradiol under physiologic conditions as well as *in vitro*. Moreover, this action may partially account for the ability of this agent to suppress myometrial activity both *in vitro*, and *in vivo* in unanesthetized animals.

Addendum Recently, we have found that CN-55,945-27 will significantly ($p < 0.01$) displace previously bound 17β-estradiol-6,7-^{3}H (0.8 μg/kg; 42.2 C/m*M*) from uteri of immature ovariectomized rats within 1 hr under *in vitro* conditions as described above.

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