Action of Progesterone and Synthetic Progestogens on Estrogen-Induced Ovarian Growth. (31908)

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(Introduced by A. C. Bratton, Jr.)
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Estrogens can markedly influence ovarian growth as well as its functional activities, *viz.*, ovulation and steroidogenesis. These effects of estrogen are exerted in two ways: *indirectly* by altering pituitary gonadotrophin secretion (1,2), and *directly* by acting upon the cells of the ovary(3,4). The latter action results in either stimulating growth mechanisms *per se* and/or increasing ovarian responsiveness to gonadotrophins(5). Thus, it is clear that estrogen, which is produced by the ovary, is also fundamental to the function of this organ. Moreover, any agent capable of antagonizing the action of estrogen might be expected to exert part of its influence at the level of the ovary.

Several types of compounds have been shown to antagonize one or more actions of various estrogens. Notable among these are both natural and synthetic steroids including progestogens, androgens, corticoids, and other estrogens(6). Since progesterone is known to synergize with estrogen in some cases and to inhibit the action of estrogen in others(7), it was of interest to study this steroid and "20α-ol"* in terms of their effect on estrogen-induced growth at the level of the ovary. In addition, we investigated under similar conditions the action of 5 synthetic progestogens which are structurally related to 19-nortestosterone or 17α-hydroxyprogesterone. The results of this study suggest that the synthetic progestogens behave rather specifically, and that their biological activity is not necessarily associated with their structural similarity to either of the latter steroids.

Materials and methods. Holtzman female rats (22 days old) were received from Hormone Assay Laboratories (Chicago, Ill.) where they had been hypophysectomized 6-24 hours earlier. The animals were housed in an air-conditioned room (22°C) in which the lights were automatically controlled to provide a 12-hr photoperiod (lights on 7 A.M.-7 P.M.). Rockland rat pellets and tap water containing 5% glucose were supplied *ad libitum*.

Growth studies. Our initial experiments were carried out according to the following treatment regimen. Animals, 26 days of age (60 ± 2 g), were randomized and injected subcutaneously once daily with 0.1 ml peanut oil containing diethylstilbestrol (DES, 1 mg/rat/day) or the peanut oil vehicle alone for 14 days. DES was administered similarly to a third group of animals which in addition received a given steroid (1 mg/rat/day) in peanut oil subcutaneously during the last 7 days of the treatment period. The animals were weighed and then sacrificed with carbon dioxide 24 hours following the last injection. At this time, their ovaries were rapidly removed and freed from the fallopian tubes and surrounding tissues. They were then weighed, and frozen by dropping them into a collecting vial immersed in an acetone-dry ice mixture. Subsequently, the ovaries were analyzed for their nucleic acid (RNA, DNA) content as previously described(8).

Uptake of diethylstilbestrol. A second type of experiment was conducted to determine the effect of norethindrone on uptake of ^{14}C-DES by the ovary. The animals employed in this experiment were identical with respect to age and endocrine state to those utilized in the above experiments. However, the length of the treatment regimen was different in that the DES (1 mg/rat/day; 1.8 μc/mg) was injected subcutaneously in peanut oil once daily for only 4 days. Likewise, norethindrone (1 mg/rat/day) was also administered for 4 days in peanut oil. Twenty-four hours following the last injection the animals were sacrificed and their ovaries removed as described above. The organs were weighed fresh and then dried by placing them in a vacuum oven (45°C) for 48 hr. Subse-

* 20α-hydroxy-pregn-4-en-3-one.

quently, they were allowed to cool for 1 hour at room temperature and then weighed on a Mettler (Model M-5) microbalance. Diethylstilbestrol uptake (ovarian ^{14}C content) was determined by the oxygen flask combustion method as described below.

Combustion analysis. The method used in this study was similar to that originally described by Schoniger(9) in which the sample is placed in a cellophane bag and combusted in an oxygen atmosphere. The $^{14}CO_2$ is collected in a trapping medium and counted directly by liquid scintillation. Various modifications of this method as well as its advantages have been discussed elsewhere(10-13). In this study, each pair of dried ovaries was placed in a cellophane ladle prepared from dialysis tubing (size 20, Food Casing Division, Union Carbide). The ladle, to which a paper wick with a black end was attached for ignition purposes, was in turn positioned in a nichrome basket attached to the glass head of a 1 liter round heavy-walled combustion flask having a trap at the bottom. Eight combustion flasks were mounted upright in a metal rotation rack with the trap of each flask immersed in an acetone-dry ice bath. A solution (11.9 ml) of phenethylamine: methanol (1:1/v:v) was pipetted into the trap of each flask and allowed to cool for 3 minutes. Each combustion flask was then flushed with 100% O_2 for 1 minute prior to placing the combustion head, to which the nichrome basket containing the sample was attached, on the flask. Vacuum grease was used to seal the joint and the combustion head was then secured with a metal clamp. An infra-red lamp was used to ignite the sample by focusing the light on the black spot of the paper wick attached to the ladle. Following combustion of each sample, the metal rack containing the flasks was rotated continuously for 5 minutes by an air motor. Next, the rotation was stopped, the flasks turned in the rack 90°, and rotation continued for another 5 minutes. Subsequently, a 10 ml aliquot was pipetted from the combustion flask into a liquid scintillation counting vial to which 10 ml toluene liquid scintillation solution [5 g PPO (2,5-diphenyloxazole) and 100 mg POPOP [(2,2-p-phenylenebis)(5-phenyloxazole)] in 1 liter of toluene] was added. The vials were then shaken for 30 seconds and counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003). Internal standard (0.1 ml benzoic acid-^{14}C) (1.7×10^5 DPM) was added to each vial after the initial counting and the vials recounted under the same conditions to correct for quenching. The counting efficiently and final data were processed by a computer. A study on ovarian tissue to which a known amount of ^{14}C-diethylstilbestrol was added showed a recovery of 100%. Radiochemical purity of the ^{14}C-diethylstilbestrol utilized in the study was confirmed prior to carrying out any of the experiments by means of thin-layer chromatography and liquid scintillation counting.

Results. The data concerning the effect of DES on ovarian growth when administered with and without various progestogens are shown in Table I. DES treatment alone (Group II) caused a marked increase in ovarian weight and nucleic acid (total RNA, total DNA) content. In addition, the ratio of RNA to DNA in the ovaries was also significantly ($P<0.01$) increased in rats receiving DES (Group II) as compared to those injected with peanut oil vehicle (Group I).

The effect of various steroids administered concomitantly with DES during the last 7 days of treatment is also shown in Table I. Norethindrone (Group VI), norethindrone acetate (Group VII) and medroxyprogesterone acetate (Group IX) were found to inhibit the stimulatory action of DES on ovarian growth in the immature hypophysectomized rat. That is, the ovaries obtained from animals which received one of these 3 steroids weighed significantly ($P<0.01$) less and contained less RNA and DNA than those of the DES-treated animals (Group II). However, the ratio of RNA/DNA in ovaries obtained from animals treated with norethindrone, norethindrone actate, or medroxyprogesterone acetate was not significantly different from that observed in the DES-treated controls. Conversely, when norethynodrel was superimposed on DES treatment there was a significant *increase* in the weight, total RNA, and

TABLE I. Action of Progestogens on Diethylstilbestrol (DES)-Induced Ovarian Growth in Immature Hypophysectomized Rats.

Group	Treatment	No. of animals	Ovarian wet wt (mg)*	Total nucleic acids (μg/ovary)* RNA	DNA	RNA/DNA
I	Peanut oil controls	116	6.31 ± .11	28 ± 1	195 ± 4	.14 ± .003
II	DES + peanut oil	52	24.39 ± .55†	124 ± 3†	578 ± 10†	.21 ± .004†
III	DES + progesterone	8	25.59 ± 2.33	131 ± 9	617 ± 34	.21 ± .006
IV	DES + 20α-hydroxy-pregn-4-en-3-one	8	24.45 ± 1.76	121 ± 10	569 ± 37	.22 ± .006
	Related to 19-nortestosterone:					
V	DES + norethynodrel	8	29.17 ± 1.19†	136 ± 5†	649 ± 27†	.21 ± .004
VI	DES + norethindrone	10	15.85 ± .70†	71 ± 4†	339 ± 14†	.21 ± .005
VII	DES + norethindrone acetate	8	13.45 ± 1.10†	60 ± 5†	283 ± 23†	.21 ± .006
	Related to 17α-hydroxyprogesterone:					
VIII	DES + 17α-hydroxyprogesterone caproate	8	23.32 ± .88	126 ± 5	579 ± 20	.22 ± .003
IX	DES + medroxyprogesterone acetate	12	14.61 ± 1.01†	66 ± 5†	320 ± 19†	.21 ± .005

* Mean ± SEM.

† $P < .01$, Rx *vs* control.

total DNA content of the ovaries (Group V). The ovarian RNA to DNA ratio was not significantly different from that observed in DES-treated rats. Progesterone (Group III), 20α-hydroxy-pregn-4-en-3-one (Group IV), and 17α-hydroxyprogesterone caproate (Group VIII) had no significant effect on DES-induced growth in terms of the weight or nucleic acid content of the ovary.

Table II shows the data obtained when norethindrone was administered concomitantly with ^{14}C-DES to immature hypophysectomized rats. This steroid caused a significant ($P<0.01$) decrease in ovarian dry weight and the total content of ^{14}C radioactivity. However, norethindrone treatment did not significantly ($P>0.05$) alter ^{14}C-uptake (DES) per unit weight of ovarian tissue.

Discussion. It is generally agreed that neither progesterone nor various synthetic progestogens affect ovarian responsiveness to gonadotrophins(14-17). However, since estrogen has been shown in this study and others(3,4) to exert direct stimulatory action on ovarian growth, the possibility existed that progestogens might influence this response by antagonizing or synergizing with estrogen at the level of the ovary. Our present findings, although not entirely conclusive from a physiologic point of view since a synthetic estrogen was employed, lend support to this hypothesis.

Diethylstilbestrol (DES) was found to stimulate ovarian growth in terms of its weight and nucleic acid content (Table I). The marked increase in total RNA, which is intimately associated with protein synthesis, can partially be explained on the basis of hyperplasia since total DNA (a measure of cell number) also increased significantly. Increased synthesis by the existing cells apparently also contributed to the increase in total RNA as indicated by a significantly ($P<0.01$) higher RNA/DNA ratio.

Three of the progestogens studied, *viz.*, norethindrone (Group VI), norethindrone acetate (Group VII), and medroxyprogesterone acetate (Group IX), antagonized the stimulatory effect of DES on ovarian growth (Table I). In each case, DES-induced hyperplasia (total DNA) was significantly ($P<0.01$) decreased. This action resulted

TABLE II. Effect of Norethindrone on Uptake of ^{14}C-Diethylstilbestrol (DES) by the Ovary in Immature Hypophysectomized Rats.

Treatment	No. of animals	Ovarian dry wt (mg)*	DPM/single ovary*	DPM/100 mg dry wt*
^{14}C-DES + peanut oil	10	3.97 ± .15	29.2 ± 3.8	1478 ± 201
^{14}C-DES + norethindrone	10	2.76 ± .22†	15.6 ± 2.1†	1250 ± 273

* Mean ± SEM.

† $P < .01$.

in a marked drop in total ovarian RNA content. However, it is important to note that the RNA level in the cells (RNA/DNA ratio) of ovaries obtained from rats treated with the above steroids was not significantly ($P>0.05$) different from that observed in ovaries of DES-treated rats. Such an inhibitory effect of norethindrone acetate on DES-induced ovarian growth in the immature hypophysectomized rat was not observed by Smith and Bradbury(17). It is possible that the gonadotrophin (Pergonal®) which they administered to their animals masked the antagonistic action of this progestogen.

The specificity of action of the progestogens studied is further emphasized by the results obtained with norethynodrel (Group V) and 17α-hydroxyprogesterone caproate (Group VIII, Table I). Norethynodrel significantly increased the degree of ovarian growth observed with DES by virtue of its stimulatory effect on cell division (total DNA). Although this action resulted in an increased ovarian RNA content, the ratio of RNA to DNA remained the same. These observations may reflect the greater estrogenicity of norethynodrel as compared to the other progestogens studied. On the other hand, 17α-hydroxyprogesterone caproate, like progesterone (Group III) and "20α-ol" (Group IV, Table I), did not influence DES-induced ovarian growth either in terms of stimulating or inhibiting cell division or RNA synthesis. In general, the changes observed in ovarian nucleic acid (RNA, DNA) content were paralleled by similar changes in ovarian weight. Moreover, it is obvious from the data shown in Table I that although some progestogens antagonize DES-induced ovarian growth, others may have no effect or evoke a totally opposite (stimulatory) response. No consistent effect was exhibited by those progestogens related to either 19-nortestosterone or 17α-hydroxyprogesterone. Thus, it appears that their action under these conditions is rather specific and cannot be correlated with their structural similarity to either of the latter steroids.

Several non-steroidal estrogen antagonists are known to block the uptake of estrogen by various target organs including the pituitary, uterus and vagina(18-21). The data presented in Table II indicate that norethindrone does not inhibit ^{14}C-DES uptake per unit weight of ovarian tissue. However, since the ovaries of the norethindrone-treated animals weighed less, they contained a significantly ($P<0.01$) lower total content of ^{14}C radioactivity (DES) even though the uptake of DES was the same in both groups on a concentration basis. Thus, these data, coupled with those in Table I, suggest that norethindrone acts to inhibit DES-induced hyperplasia rather than by preventing binding of this estrogen by the ovary. Whether or not norethindrone acetate and medroxyprogesterone acetate exert their inhibitory effect on estrogen-induced ovarian growth in a similar manner is as yet open to question.

Summary. Five synthetic progestogens along with progesterone and 20α-hydroxypregn-4-en-3-one were compared for their ability to influence diethylstilbestrol (DES)-induced ovarian growth in the immature hypophysectomized rat. Ovaries of DES-treated rats showed a marked increase in ovarian weight, total RNA, total DNA and the RNA/DNA ratio. Norethindrone, norethindrone acetate, and medroxyprogesterone acetate inhibited DES-induced ovarian growth by preventing cell division as indicated by significant decreases in total DNA and RNA with no change in the ratio of RNA to DNA. Conversely, norethynodrel enhanced ovarian hyperplasia in DES-treated rats since total DNA and RNA were increased and the RNA/DNA ratio remained unchanged. Progesterone, 20α-hydroxy-pregn-4-en-3-one, or 17α hydroxyprogesterone caproate did not alter DES-induced ovarian growth. Norethindrone was observed to have no effect on the uptake of ^{14}C-DES by the ovary and therefore appears to antagonize the action of DES on ovarian growth rather than by blocking the incorporation of this estrogen by the ovary.

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Cytotoxicity of Dichlorodiphenylacetic Acid (DDA) Upon Cultured KB and HeLa Cells, and its Reversal by Mevalonic Acid. (31909)

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DDA [2,2-bis (p-chlorophenyl) acetic acid], constantly present in the tissues of most humans and other mammals, is the principal urinary metabolite of DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane](1). Structurally, it falls into the pattern of a large series of substituted arylacetic acids which have been shown to inhibit the incorporation of acetate and mevalonate into cholesterol(2). A typical example is diphenylylethylacetic acid, which has been extensively studied in this regard(3,4). DDA, like diphenylylethylacetic acid, has been shown to inhibit the acetylation of choline(5) and sulfanilamide(6) by interfering with the formation of acetyl-coenzyme A. The present studies are concerned with the cytotoxicity of DDA, and its reversal by mevalonic acid, in cultured KB and HeLa cells.

Materials and methods. Stock cultures of KB and HeLa cells were grown as monolayers in Roux and Brockway bottles in Mixture 199(7) as modified by Salk *et al*(8), supplemented by 5% calf serum. For rapid growth the medium was supplemented with 10% serum, and for maintenance the amount of calf serum was reduced to 3%. A cell suspension was obtained from a 5 day culture by trypsinizing (0.05-0.1% trypsin) the monolayer from the glass surface and dispersing the cells with gentle, repeated pipetting. Cell populations were determined by counting in Neubauer hemocytometer chambers.

Cytotoxic effects produced by the test compounds were graded according to the rating scale described by Toplin(9) in which the cytotoxic end-point (CE) is that concentration of test substance causing significant cellular degeneration, and the lethal end-point (L.E.), that concentration causing complete disintegration.

The DDA used in this work was prepared

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