A Micro-Bioassay Method for Estrogen.* (25994)

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Among responses of the uterus of rat and mouse to estrogen, 2 of the most specific are hypertrophy of the Golgi apparatus and depletion of basal phospholipid in uterine epithelium. These effects have been described (1) in rats which had received estradiol benzoate subcutaneously and earlier observations of lipid depletion have been summarized by Alden(2). The amount of hormone needed to produce these changes has been reduced drastically(3) by administering it by intrauterine injection, a method employed successfully by Hooker and Forbes(4) for progestin assay in mice. Positive estrogenic responses have been produced by this method with as little as 0.00002 μg of estradiol benzoate dissolved in 2 μl of sesame oil when injected into a 10 mm segment of castrate rat uterus.

Materials and methods. A 2 μl volume of test solution was injected by means of a microsyringe into the lumen of a 10 mm segment of one horn of the uterus of 5-months-old Long-Evans rats which had been ovariectomized 14 days. After 48 hr the animals were killed with chloroform, the injected segment removed and divided into 2 pieces and 2 similar tissue samples taken from the contralateral uninjected horn of the uterus. One piece of tissue from each horn was processed by the direct silver method for the Golgi apparatus(5) and the other was fixed overnight in dichromate-sublimate(6), all of the tissue being imbedded in paraffin the following day and cut into 5 μ sections. The Golgi apparatus was available for study immediately after clearing the sections. Phospholipids were visualized by the technic of controlled chromation(6,7,8) for which purpose tissue fixed in dichromate-sublimate and imbedded in paraffin was stained with Sudan black B in 70% alcohol and mounted in Apathy's solution. Glycogen was studied in the dichromate-sublimate fixed tissue by the periodic acid-Schiff technic, with diastase digested controls, followed by nuclear staining with Harris' hematoxylin.

After preliminary experiments to determine the optimum volume of injection fluid and optimum time between injection and autopsy, the effect of graded doses of estrogen was investigated by subjecting castrate rats, in groups of 3, to 2.0, 0.2, 0.02, 0.002, 0.0002 and 0.00002 μg of estradiol benzoate dissolved in 2 μl of sesame oil. For comparison, pairs of rats were given subcutaneous doses of 0.5, 0.4, 0.2 and 0.1 μg of the same hormone with 48 hr intervening before autopsy. Pilot experiments were also conducted with Swiss albino mice.

Results. The changes produced in the Golgi apparatus and basal phospholipid of the uterine epithelium by 0.00002 μg of estradiol benzoate are shown in Fig. 1. With the particular dosage employed, the uninjected horn is essentially unchanged from the castrate condition. The Golgi apparatus of this horn consists of narrow tubules, darkened by silver, occupying a shallow space between nucleus and uterine lumen. In the injected horn the Golgi apparatus has responded by marked hypertrophy. The phospholipid of the uninjected horn is characterized by numerous droplets, stained by Sudan black B, in the basal cytoplasm with subsidiary amounts between nucleus and lumen. In the injected horn the basal phospholipid has been depleted although some droplets remain with this dosage. Between the nucleus and lumen phospholipid of a different significance is present, some of it associated with Golgi apparatus and mitochondria.

Hypertrophy of the Golgi apparatus and depletion of basal phospholipid have proved the most useful criteria of estrogen activity in our experiments since they apply to the mouse as well as the rat and are not subject to distortion by the mechanics of injection or of tissue fixation. In the rat a useful third

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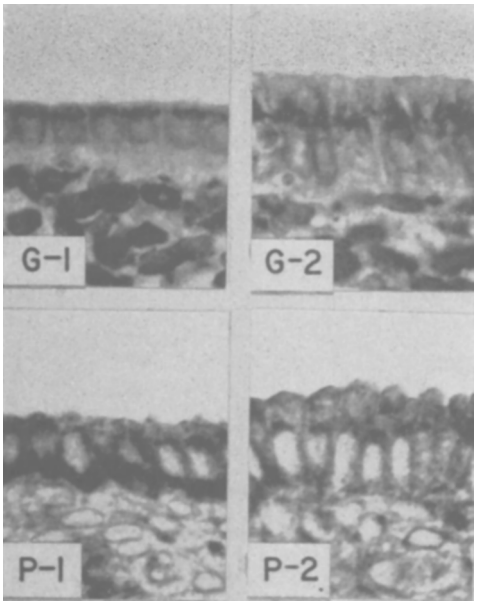

FIG. 1. Photomicrographs (×800) of uterus of castrate rat 48 hr after inj. of 0.00002 μg of estradiol benzoate into 10 mm segment of one horn. G-1 shows Golgi apparatus of epithelium of uninj. horn in the form of narrow black tubules above the nucleus. G-2, inj. segment, has hypertrophied Golgi apparatus. P-1 shows phospholipids of uninj. horn with dense aggregations of black globules basal to the nucleus. P-2, inj. segment, has been depleted of much of the basal phospholipid.

criterion is the appearance of cytochemically demonstrable glycogen(9). Glycogen is found in the longitudinal uterine musculature with a dose of 0.00002 μg of estradiol benzoate. The circular muscle may respond slightly at this dose, especially in the region of the mesometrium, but the epithelium does not respond below a dose of 0.002 μg. The mouse does not give a useful glycogen response but offers instead as a third criterion the mobilization of alkaline phosphatase in uterine epithelium and circular muscle(10). This response also is differential, the epithelium being more sensitive.

Comparison of the uninjected uterine horn with that which received the hormone directly shows that it takes a dose of 0.2 μg before the uninjected horn will present the response evoked in the injected segment by 0.00002 μg. Intrauterine injection is only slightly more effective on the uninjected horn than is subcutaneous administration.

Discussion. Determination of the estrogenic activity of test substances by intrauterine injection allows experiments to be conducted on much smaller amounts of material than has hitherto been possible. This method has additional advantages in by-passing many of the problems of absorption, transportation and distribution inherent in subcutaneous and intraperitoneal administration. It also decreases the confusion which may be introduced by systemic side-effects since the material tested is more nearly confined to the target tissue.

Quantitative measurement of estrogenic potency can be made by testing successively greater dilutions until one is found which produces incipient Golgi hypertrophy, basal lipid depletion and cytochemically demonstrable glycogen in the longitudinal muscle of the rat or alkaline phosphatase in the epithelium of the mouse. The augmentation of these responses which accompanies higher doses may be used for quantitative determinations when methods of measurement have been developed.

Our criteria of estrogenic response have been selected because they involve a minimum of technical skill and produce results which can be evaluated without prolonged training. Many additional cytochemical effects of estrogen can be used and advantage may be taken of increase in epithelial cell height and presence of edema if proper allowance is made for the mechanical interference provided by injection and ligation. Smaller test samples can be handled by employing shorter segments of uterus. This would further increase the sensitivity of the method which is already 10,000 times as sensitive as subcutaneous administration when 10 mm of uterus are used. It is also possible that use of other solvents might enhance the response as it does with intravaginal application(11).

Local application of estrogen to other target tissues has been used in previous assay methods with opening of the vagina in the guinea pig(12) or the appearance of a cornified smear in at least half of the test rats(13) as the criterion of estrogenic activity. The guinea pig test was positive with a minimum dose of 0.0004 μg of estradiol dipropionate

and the cornification test in rats required slightly more than 0.0005 μg of estradiol benzoate, in both cases dissolved in oil. It is possible that the sensitivity of these tests would approach that of intrauterine injection more closely if smaller areas of target tissue could be used or more delicate cytological criteria employed.

The effectiveness of small amounts of hormone when applied directly to the target organ emphasizes their physiological potential. The effect of estrogen is apparent not only in the epithelium but as far peripherally as the longitudinal muscle. Its concentration there must be exceedingly small since our minimum effective dose, if distributed evenly throughout the tissue of the segment into which it was injected, would provide only 4 molecules of steroid for each cubic micron of tissue.

The small amount of test material required has enabled us to carry out successful preliminary experiments with small pieces of tissue excised from organs of estrogenic interest. Although the rat is the more convenient animal for this purpose, it is possible to make simultaneous observations on progestational activity with the criterion of Hooker and Forbes(4) by using the mouse.

Summary. Very small amounts of test substances can be assayed for estrogenic activity by injecting them into the lumen of the castrate uterus of rat or mouse and observing hypertrophy of the Golgi apparatus and depletion of basal phospholipid of the epithelium. Additional criteria are appearance of glycogen in the uterine muscle of the rat and mobilization of alkaline phosphatase in the mouse.

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Uptake of Free Fatty Acids by Skeletal Muscle During Stimulation.* (25995)

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Recent work from several laboratories has suggested that free fatty acids (FFA) of the plasma might be utilized by skeletal muscles (1,2,3,4). However, direct proof of uptake has been hard to obtain, chiefly because of technical difficulties in obtaining blood from the muscles and in measuring blood flow through the muscle. In the present experiments uptake of FFA by the stimulated skeletal muscle was demonstrated in the dog by simultaneous determinations of blood flow and of arterio-venous FFA differences.

Methods. Adult mongrel dogs of both sexes were studied in postabsorptive state under sodium pentobarbital anesthesia (30 mg/kg i.v.). Using the method of Issekutz *et al.* (5), blood flow was determined in the profunda femoris veins which drain predominantly muscular areas. The femoral vein was prepared on the right side and tied 2-3 cm below the junction of profunda femoris veins. A "T" cannula was inserted into the femoral

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