al skin to estrogen following maturation.

The biochemical nature of both the edematous reaction of the skin and the development of the endometrium in response to a series of injections of estrogen is still quite obscure. It has been found that edema of the sexual skin of the perineum involves an increase in both plasma protein(5,6) and hyaluronic acid (7). The available evidence seems to support the hypothesis suggested by Zuckerman(8) that hyaluronic acid affects the water content of the sexual skin by causing retention of serum protein in the extracellular space. Biochemical changes involved in the development of refractoriness to estrogen has not been determined.

Summary. The marked edematous effect of large doses of estrogen on the skin of monkeys decreases progressively on repeated treatments. The response finally attained is characterized by a general absence of edema though some growth and wrinkling of the skin may occur. A concomitant effect is a lengthening of the interval between discontinuance of an estrogen treatment and subsequent withdrawal bleeding, and in certain animals such bleeding may cease to occur. These conditions in the skin and uterus once established seem to persist indefinitely.

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Effects of Clomiphene Citrate on the Mouse Uterus.* (31052)

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Clomiphene, 1-p (β-diethylaminoethoxy) phenyl, 1-2-diphenyl-2-chloroethylene, is an analogue of the nonsteroidal estrogen, chlorotrianisene (TACE). Clomiphene has been shown in rats to have gonadotrophin inhibiting, antiovulatory, and antifecundity effects (1,2), but in the human, it has been shown to be capable of inducing ovulation in anovulatory women(3,4). Recently Roy, Greenblatt and Mahesh(5,6), have shown that clomiphene in rats has both estrogenic and antiestrogenic effects on the uterus. The uterotrophic action was observed in hypophysectomized immature female rats without any evidence for ovarian stimulation(5). The uterotrophic action of exogenous estrogen was inhibited by clomiphene(6).

Similarly, another nonsteroidal compound, ethamoxytriphetol (MER-25), has been shown to have uterotrophic effects, stimulating an increase in alkaline phosphatase enzymatic activity as well as producing uterine weight gain over a similar time course(7).

The present study was attempted to demonstrate that the uterotrophic action of clomiphene is typically estrogenic; that is, stimulating an estrogen sensitive enzyme system as well as stimulating uterine weight gain. The antiestrogenic effects of clomiphene were detected and studied.

Materials and methods. Bilateral ovariectomy was performed on immature female Swiss mice weighing 8-10 g, and they were kept on a normal diet *ad libitum* for 12 days before treatment. The mice were killed by cervical dislocation and examined at 0, 6, 12, 24, 36, 48, 72 and 96 hours after a single subcutaneous injection of either 0.1 μg estradiol dipropionate or 0.2 mg clomiphene citrate. Other groups of mice were pretreated for 3 days with 0.1 μg estradiol, 0.1 mg

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TABLE I. Response of Gonadectomized Mouse Uterus to Single Injection (0.1 μg) Estradiol Dipropionate.

Time after injection	No. of mice	Body wt	Uterine wt	Alkaline phosphatase activity
(hr)		(g)	(mg)	(μg PHT/ mg/hr)
0	15	19.7	6.71 ± .56*	1.80 ± .28*
6	15	18.8	6.87 ± .61	1.83 ± .26
12	15	19.4	8.43 ± .81†	2.57 ± .40
24	14	17.4	8.91 ± .76	3.00 ± .48†
36	14	18.1	15.96 ± 2.72	4.55 ± .86
48	15	17.4	18.47 ± 2.40	5.03 ± 1.23
72	14	18.3	14.22 ± 1.97	3.08 ± .40
96	14	17.6	13.71 ± 1.47	3.12 ± .82

* Standard errors.
† Initial significant ($P < .05$) increases.

chlorotrianisene (TACE), or 0.2 mg of clomiphene and on the fourth day were injected at a different site with 0.1 μg estradiol dipropionate in addition to the clomiphene or estrogens and were examined 0-96 hours as before. The estrogens and clomiphene were dissolved and given in 0.1 ml sesame oil. Immediately the mice were killed, the uteri were dissected free from all surrounding tissue and were quickly weighed. They were then homogenized in double glass distilled water at 5°C, the volume being adjusted to give concentrations of 5-10 mg of tissue per ml of homogenate. The alkaline phosphatase enzymatic activity of the tissue homogenate was determined immediately by the method of Huggins and Talalay as modified by Clitheroe and Leathem(7). Optical densities of the samples were read at 540 mμ on a Zeiss spectrophotometer. Alkaline phosphatase enzymatic activity was expressed as μg phenophthalein liberated per mg of tissue homogenate per hour (μg PHT/mg/hr).

Results. Twelve hours following a single subcutaneous injection of 0.1 μg estradiol dipropionate there was a significant gain in uterine weight ($P<0.05$). At 48 hours the maximum increase was detected (Table I). A slight increase (42%) in alkaline phosphatase enzymatic activity was noted at 12 hours, reaching a significant level by 24 hours and a maximum level at 48 hours. The increase and decline of enzyme activity followed closely the changes in uterine weight.

Administration of 0.2 mg clomiphene alone induced a significant gain in uterine weight within 12 hours ($P<0.01$) and a maximum 5-fold peak occurred at 48 hours. The activity of alkaline phosphatase showed no change for 24 hours. By the end of 36 hours there was a significant 3-fold increase which peaked at 48 hours corresponding to the maximum weight gain (Table II).

Pretreatment for 3 days with 0.1 μg estradiol dipropionate did not prevent the response of the uterus to a further dose 0.1 μg estradiol. There was a significant gain in uterine weight ($P<0.05$) by 24 hours and the maximum increase was detected at 48 hours. There was also a significant increase ($P<0.05$) in alkaline phosphatase activity by 36 hours (Table III). Similar treatment of a group of mice with the estrogen dose increased to 0.5 μg indicated that this level of stimulation is not a maximum level. (Table III—footnote.)

Pretreatment for 3 days with 0.1 mg TACE did not prevent the further stimulation of the uterus by 0.1 μg estradiol. A significant ($P<0.05$) increase in uterine weight was found at 24 hours. The increase in weight continued up to 72 hours. The alkaline phosphatase enzyme activity showed a significant increase to estradiol at 12 and 24 hours ($P<0.05$) although the initial control level of alkaline phosphatase activity was 32 μg PHT/mg/hr. This level of enzyme activity was significantly higher ($P<0.001$) than the control levels for estradiol or clomiphene pretreatments (Table IV).

After pretreatment for 3 days with 0.2 mg

TABLE II. Response of Gonadectomized Mouse Uterus to Single Injection (0.2 mg) Clomiphene Citrate.

Time after injection	No. of mice	Body wt	Uterine wt	Alkaline phosphatase activity
(hr)		(g)	(mg)	(μg PHT/ mg/hr)
0	10	17.1	6.30 ± .70*	2.28 ± .78*
6	9	18.3	8.80 ± .79	2.51 ± .92
12	9	15.6	9.71 ± .84†	2.30 ± .87
24	9	18.9	14.87 ± 2.74	2.60 ± .63
36	8	16.4	19.18 ± 3.42	6.25 ± 1.09†
48	9	17.6	33.28 ± 4.01	8.25 ± 1.29
72	9	16.8	32.24 ± 2.79	7.08 ± 1.0
96	9	18.4	30.16 ± 2.10	5.63 ± 1.13

* Standard errors.
† Initial significant ($P < .01$) increases.

clomiphene the uterotrophic effect of a stimulating dose of estradiol was blocked, both for weight gain and stimulation of alkaline phosphatase enzymatic activity (Table V).

Discussion. The single injection of estradiol dipropionate produced both an increased uterine weight and alkaline phosphatase enzymatic activity which followed similar time-response curves. The effect of a single dose of clomiphene produced changes in uterine weight and alkaline phosphatase similar to those of estradiol. This uterotrophic action

TABLE III. Influence of Three Days of Pretreatment with 0.1 μg Estradiol Dipropionate on Response of Gonadectomized Mouse Uterus to a Further Injection of 0.1 μg Estradiol.

Time after last injection	No. of mice	Body wt	Uterine wt	Alkaline phosphatase activity
(hr)		(g)	(mg)	(μg PHT/ mg/hr)
0	8	19.6	19.81 ± 1.64*	5.17 ± .42*
6	8	18.6	22.56 ± 1.38	5.50 ± .97
12	9	23.5	24.06 ± 1.80	5.67 ± .62
24	9	21.5	28.02 ± 3.49†	6.44 ± .69
36	8	21.8	31.23 ± 2.33	6.73 ± .61†
48	9	20.5	31.28 ± 2.50‡	6.06 ± .26‡
72	8	21.2	29.91 ± 1.74	6.10 ± .50
96	8	20.8	24.25 ± 1.96	4.70 ± .21

* Standard errors.
† Initial significant (P <.05) increases.
‡ Similar treatment of a group of mice with 0.5 μg estradiol dipropinate gave levels at this time interval of uterine weight 74.1 ± 8.9 mg and alkaline phosphatase 13.1 ± 2.6 μg PHT/mg/hr.

TABLE IV. Influence of 3 Days of Pretreatment with 0.1 mg Chlorotrianisene (TACE) on Response of Gonadectomized Mouse Uterus to a Further Injection of 0.1 μg Estradiol Dipropionate and 0.1 mg Chlorotrianisene.

Time after last injection	No. of mice	Body wt	Uterine wt	Alkaline phosphatase activity
(hr)		(g)	(mg)	(μg PHT/ mg/hr)
0	11	18.3	29.30 ± 1.95*	32.72 ± 2.66*
6	10	18.9	33.65 ± 2.25	29.82 ± 1.19
12	11	18.6	31.66 ± 2.56	42.18 ± 3.19†
24	10	18.2	36.39 ± 2.44†	40.62 ± 2.60
36	11	18.9	33.20 ± 2.35	36.18 ± 3.56
48	10	17.9	36.87 ± 3.38	36.40 ± 3.42
72	8	19.3	43.4 ± 2.93	32.16 ± 6.56
96	8	20.5	43.3 ± 2.50	24.50 ± 2.89

* Standard errors.
† Initial significant (P <.05) increases.

TABLE V. Influence of 3 Days of Pretreatment with 0.2 mg Clomiphene Citrate on Response of Gonadectomized Mouse Uterus to Further Injections of 0.1 μg Estradiol Dipropionate and 0.2 mg Clomiphene.

Time after last injection	No. of mice	Body wt	Uterine wt	Alkaline phosphatase activity
(hr)		(g)	(mg)	(μg PHT/ mg/hr)
0	7	18.7	28.73 ± 1.23*	6.24 ± .78*
6	7	17.7	26.84 ± 1.56	6.41 ± .63
12	8	17.7	26.64 ± 1.47	6.23 ± .92
24	7	17.3	28.80 ± 2.23	6.84 ± .84
36	8	19.5	26.84 ± 2.41	6.56 ± 1.19
48	8	19.3	30.12 ± 3.13	7.15 ± .87
72	8	19.9	29.57 ± 1.86	6.56 ± .69
96	8	20.3	30.02 ± 2.49	6.59 ± 1.02

* Standard errors.

adds support to the suggestion that clomiphene has retained some estrogenic activity from its parent compound chlorotrianisene and that clomiphene can act upon estrogen receptor or binding sites.

It is well known that consecutive doses of estrogen lead to summation of effects. An additional estradiol treatment after 3 days pretreatment with either estradiol or chlorotrianisene produces a further significant rise in uterine weight and alkaline phosphatase enzyme activity. Consequently, it would be reasonable to suppose that after pretreatment with clomiphene exogenous estrogen would have a further stimulating action. However, this does not occur, and since the uterine activity is not at maximal level, it may be concluded that the estrogen has been blocked. This antiestrogenic effect of the clomiphene producing a blockage of the exogenous estrogen is not contradictory. Roy, Greenblatt and Mahesh(6) have shown in the rat that clomiphene had an antiestrogenic effect as shown by an antiuterotrophic action at least 12 times as great as its estrogenic action.

It is feasible to suggest, as did Roy and his co-workers, that clomiphene could act by occupying the estrogen sites in the uterus; exhibiting some inherent estrogenic activity, but blocking the more potent estrogenic steroid.

Summary. The nonsteroidal clomiphene was shown to have inherent estrogenic stimulating potency on alkaline phosphatase and uterine

weight gain in mice. It was also shown to inhibit the increases in enzyme activities and uterine weight stimulated with estradiol dipropionate.

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Stimulation by Insulin of Protein Synthesis in Isolated Fat Cells.* (31053)

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This communication presents evidence that insulin, in physiological concentration, stimulates synthesis of protein from amino acids by individual fat cells of rat epididymal adipose tissue.

Methods. The isolated fat cell preparation is obtained by a modification of Rodbell's technique(1). Male Sprague-Dawley rats, weighing 110-210 g, were utilized. Eight animals were used in each experiment. The rats, in a fed state, were sacrificed by decapitation. Distal segments of epididymal adipose tissue were quickly excised, weighed, and added to 8-10 ml of Krebs-Ringer-bicarbonate solution containing 1 mg/ml glucose, 4% bovine albumin, and 20 mg of collagenase.† The albumin was previously purified by filtering through #42 Whatman paper followed by dialysis, utilizing Krebs-Ringer solution, for 18-24 hours. Before incubation, the pH of the medium was adjusted precisely to 7.4. Following incubation for one hour at 37°C in a Dubnoff metabolic shaker, the adipose tissue preparation was filtered through surgical gauze. The filtrate was suspended in 8-10 ml of Krebs-Ringer-bicarbonate‡ solution and centrifuged for 1-2 minutes at 600×. The infranate, containing blood vessels and connective tissue, was then aspirated. Resuspension of the preparation, centrifugation, and aspiration were repeated twice. The fat cell preparation was finally suspended in 6-8 ml of Krebs-Ringer-bicarbonate solution.‡ An amino acid mixture§ and Krebs-Ringer-bicarbonate,‡ with or without insulin, were added in 0.05 ml volumes each to plastic vials ("Analocup," Aloe Co.). The final insulin¶ concentration was 800 micro-units/ml. With constant agitation, 0.5 ml of the fat cell preparation and approximately 0.1 microcurie of radioactive Amino Acid-C^{14} (U.L.) Mixture‖ were added to each plastic vial. A 0.1 ml aliquot was taken after 5 minutes had elapsed, and the mixture was then incubated at 37°C in a Dubnoff metabolic shaker for 2 hours, when a second 0.1 ml aliquot was taken. In 2 experiments, aliquots were also taken following one hour of incubation. Disposable plastic vials, test tubes, and pipettes were utilized throughout these procedures.

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† Worthington Biochemical Corp., Freehold, N. J.

‡ Containing 1 mg/ml glucose and 4% bovine albumin.

§ Slightly modified from Borsook, J. Biol. Chem., 1957,, v229, 1059.

¶ From Eli Lilly & Co., through the kindness of Dr. Otto K. Behrens.

‖ New England Nuclear Corp., Lot No. 127-72-50a.