Stimulation of *in Vitro* ^{3}H-Uridine Uptake and RNA Synthesis in Mouse Blastocysts by 17β-Estradiol (37585)

NANCY I. F. LAU, B. K. DAVIS, AND M. C. CHANG
(Introduced by C. Terner)
Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Blastocyst implantation is known to be estrogen dependent in mice and rats (1–5). In ovariectomized females treated with progesterone, implantation will not occur until estrogen is given (6–9), and blastocyst development can be arrested for several days by delaying the administration of estrogen. Autoradiographic studies indicated that estrogen administration to rats induced nucleic acid and protein synthesis in both the uterus and blastocyst (10–12). Injection of estrone also increased subsequent *in vitro* RNA synthesis by rat blastocysts (13). Amino acid uptake and protein synthesis was stimulated by estradiol in mouse blastocysts cultured *in vitro* (14).

These findings may be interpreted as evidence that estrogen acts directly to activate specific blastocyst genes, which code for proteins necessary for the occurrence of implantation. In this event it should be possible to demonstrate an increase in blastocyst RNA synthesis by estrogen *in vitro*. In the present study, nucleotide uptake and RNA synthesis by mouse blastocysts under *in vitro* conditions have been investigated in the presence of estradiol.

Materials and Methods. Animals. Charles River CD-1 female mice (6–8 wk old) were injected (ip) with 5 IU PMS, followed 48 hr later by an injection of 2.5 IU HCG. Treated females were caged with mature males and the day on which a vaginal plug was found has been designated as Day 1 of pregnancy. The pregnant females were ovariectomized by a dorsolateral approach on Day 4 between 08.00–10.00 hr under Nembutal anesthesia. Progesterone (1 mg in 0.05 ml corn oil) was injected (sc) daily from Days 5 to 9 for the maintenance of blastocysts.

In vitro incubation. On Day 9, between 14.00–16.00 hr, blastocysts were flushed from excised uteri with medium 199 (Difco) and washed once to remove endometrial tissues and blood cells. Recovered blastocysts were incubated (5–10 at a time) in 100 μl medium 199 supplemented with 10 μCi/ml ^{3}H-uridine (27.3 mCi/mmole, New England Nuclear) and with 10^{-10}–10^{-6} M 17β-estradiol in treatment groups. Control blastocysts were similarly treated without estrogen. Estradiol dissolved in a small volume of ethanol and diluted with saline to 10^{-5} M served as a stock solution. Blastocysts in these media were placed in a 35-mm watchglass inside a water-vapor saturated petri dish and incubated under an atmosphere of 5% CO_2 in air at 37° for various periods. After incubation, the blastocysts were washed three times with 300 μl ice-cold medium 199. Blastocysts tended to adhere to glassware and this was minimized by coating the glassware and transfer micropipettes with Dricote (Fisher), or by chilling the incubation medium.

Blastocyst volume before and after incubation was measured with the aid of an oculomicrometer under a dissecting microscope (×25). The volume of the blastocysts was calculated according to the formula for a prolate spheroid: $\frac{4}{3}\pi ab^2$, where a represents the major radius, and b is the minor radius of the blastocyst.

^{3}H-Uridine uptake. Blastocysts were incubated at 37° in medium 199 with ^{3}H-uridine for various times (0–120 min). In the zero time series, they were immediately transferred to ice-cold medium. At indicated in-

tervals, a single blastocyst in 5 μl medium, was removed from the incubation medium, washed three times, and then transferred by micropipette onto a Millipore filter. An equal amount of medium from the final wash bath was deposited onto another Millipore filter to furnish background radioactivity. The Millipore filter was dried and counted with a toluene base phosphor in a Nuclear Chicago Mark II liquid scintillation counter.

The effects of estradiol on nucleic acid uptake and RNA synthesis were determined in another series of experiments in which blastocysts had been incubated for 2 hr in ^{3}H-uridine containing medium 199.

RNA extraction. Following incubation in the presence of ^{3}H-uridine, washed blastocysts were transferred to 500 μl of 0.1 *M* acetate buffer (pH 5.0) and stored at —28° until RNA extraction. To the thawed acetate buffer containing these blastocysts, 50 μl purified RNA (10 mg/ml) and 600 μl water-saturated phenol (redistilled) were added. The whole mixture was shaken vigorously for 20 min at 4° and centrifuged (1200*g*) for 20 min to separate phenol and aqueous phases. The aqueous phase was aspirated and the phenol phase was then re-extracted with 250 μl acetate buffer. Both aqueous phases were pooled. Extracted RNA was precipitated with 2 vol of absolute alcohol at —28° overnight. The precipitate was collected by centrifugation, washed twice with 70% alcohol and dissolved in 200 μl 0.5 *N* KOH. The total RNA solution was deposited onto a Millipore filter, dried and assayed for radioactivity.

Results. ^{3}H-Uridine uptake. Figure 1 shows the kinetics of ^{3}H-uridine uptake by blastocysts recovered from mice 9 days after mating. A linear relationship expressing this uptake is given by the regression formula $\hat{Y} = 9.3 + 25.2\ X$ ($r = 0.997$), where $\hat{Y}$ is the ^{3}H-uridine uptake (cpm/μm^3 $\times$ 10^5) and X is the time of incubation (min).

Figure 2 depicts estradiol stimulated ^{3}H-uridine uptake by mouse blastocysts. ^{3}H-Uridine uptake was about 135% of the control value in the presence of 10^{-9} and 10^{-8} *M* estradiol. In contrast, 97% of the control level was obtained with 10^{-6} *M* estradiol treated blastocysts. Statistical analysis of these data by a sequential range test showed that no significant difference ($p > 0.05$) occurred in ^{3}H-uridine uptake between control and 10^{-6} *M* estradiol treated blastocysts, or among 10^{-10}, 10^{-9} and 10^{-8} *M* estradiol treatment. A significant difference ($p < 0.05$)

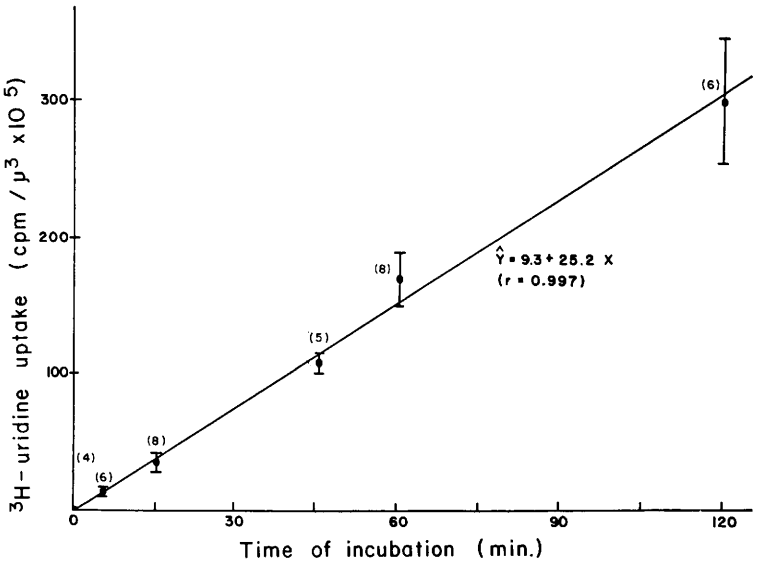

FIG. 1. Kinetics of ^{3}H-uridine uptake by Day 9 mouse blastocysts incubated in medium 199. The number of blastocysts studied are indicated in parentheses.

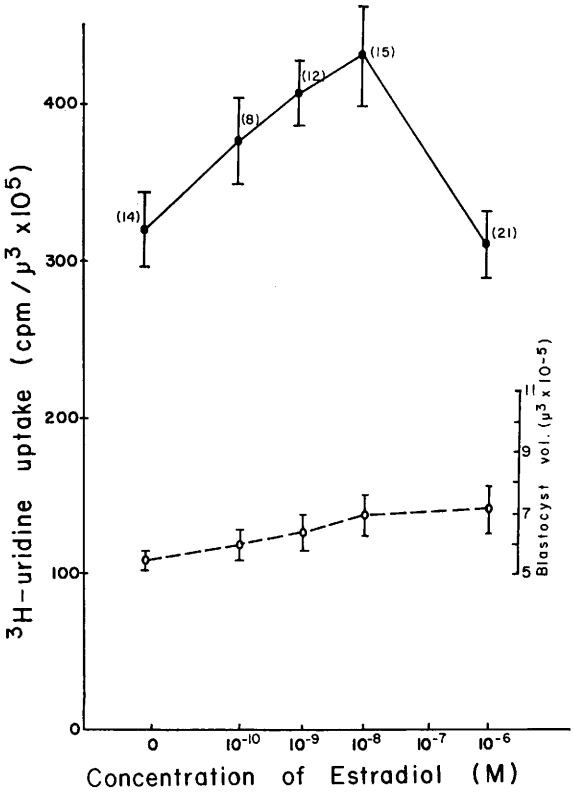

FIG. 2. Effect of 17β-estradiol on *in vitro* ^{3}H-uridine uptake in mouse blastocysts incubated at 37° for 2 hr under an atmosphere of 5% CO_2 in air. Numbers in parentheses indicate the number of blastocysts. Blastocyst volumes at the completion of incubation are given by the lower line. The mean preincubation volume of these blastocysts was 9.1 × 10^5 μm^3.

existed between the tritium concentrations of the control and 10^{-6} *M* estradiol versus the 10^{-10} to 10^{-8} *M* estradiol treated groups of blastocysts.

Blastocyst volume following incubation is also shown in Fig. 2. A significant decrease in volume accompanied incubation. This decrease, however, diminished in the presence of estradiol. Before incubation the average blastocyst volume was estimated to be 9.1 × 10^5 μm^3. Interestingly, addition of 5 m*M* cAMP to the culture medium also prevented this change in blastocyst volume.

Incorporation of ^{3}H-uridine into RNA. Table I shows that ^{3}H-uridine was incorporated into RNA in mouse blastocysts after culture *in vitro* for 2 hr. Incorporation increased to 195% of the control value in the presence of 10^{-8} *M* estradiol. At 10^{-9} *M* estradiol ^{3}H-uridine incorporation into RNA was 167% of the control value. An analysis of variance revealed that estradiol significantly enhanced RNA radioactivity.

Discussion. The results of the present investigation demonstrate that RNA precursor uptake and RNA synthesis can be rapidly stimulated at physiological levels of estradiol in mouse blastocysts under *in vitro* condi-

TABLE I. Effect of 17β-Estradiol on *in Vitro* Incorporation of ^{3}H-Uridine into RNA by Mouse Blastocysts Cultured for 2 hr.

Treatment	No. of blastocysts	RNA (cpm/blastocyst) mean ± SE
Control	106	33.6 ± 3.7
17β-Estradiol, 10^{-8} *M*	82	65.5 ± 3.6[a]
10^{-9} *M*	41	56.2 ± 2.0[a]

[a] Statistically significant at $p < 0.05$.

tions. About 2–3% of the ^{3}H-uridine taken up by these blastocysts during a 2-hr incubation period was recovered as RNA. Since the increase in RNA synthesis exceeded the elevation in blastocyst tritium concentration in the presence of estradiol, it seems reasonable to conclude that the steroid induced an increase in the amount of RNA synthesized. Characterization of this rapidly labeled RNA and of the proteins made following estrogen treatment is obviously of interest to understanding the action of this hormone and the mechanism of blastocyst implantation.

Optimal estradiol concentrations of 10^{-6} M and also of 10^{-10} M for amino acid uptake and protein synthesis have been reported (14). By comparison 10^{-8} to 10^{-9} M of 17β-estradiol were optimal in the present study. The complex concentration dependence reported in the former study may be accounted for by the presence of albumin in the culture medium, as estradiol binds to serum proteins (15); consequently, some of the added estrogen would be inaccessible to the blastocysts.

The present finding that a change in blastocyst volume during incubation and the partial inhibition of this change by estradiol presumably reflect different degrees of water efflux (16). It has been suggested that estrogen effects on the blastocysts may involve a change in membrane permeability (12). When these volume changes are taken into account, it is obvious that the stimulatory effect of estradiol on ^{3}H-uridine uptake by the whole blastocyst exceeds that shown in Fig. 2, which is based on tritium concentration in the blastocysts.

Although it was suggested that the uterus may exert an influence on the blastocysts during delayed implantation (17), present observations establish estradiol directly promotes new blastocyst RNA synthesis. It would be interesting to know if the elevated uptake of uridine and amino acids (14) following estradiol treatment is directly dependent on this new RNA synthesis. Since RNA synthesis has been stimulated by estradiol it seems reasonable to infer that the hormone exerts at least part of its action on the mouse blastocyst through gene induction. How these genes are rendered inducible by estrogen shortly before implantation appears fundamental to understanding the molecular basis of blastocyst development.

Summary. It was found that physiological levels of estradiol (10^{-8}–10^{-16} M) enhanced ^{3}H-uridine uptake by mouse blastocysts cultured in a protein-free medium and that 10^{-8} and 10^{-9} M 17β-estradiol increased the amount of RNA synthesized during the 2-hr incubation period. The volume of the blastocysts decreased during incubation, the reduction in size was partly prevented by 17β-estradiol.

Financial support was received from NIH Grant GM 14370, USAID Contract CSD/2837 and the Ford Foundation. We are indebted to D. M. Hunt for drafting the figures.

1. Nutting, E. F., and Meyer, R. K., *in* "Delayed Implantation" (A. C. Enders, ed.). Univ. of Chicago Press, Chicago (1963).
2. Davis, B. K., J. Endocrinol. **27**, 99 (1963).
3. Psychoyos, A., Arch. Anat. Microsc. Morphol. Exp. **54**, 85 (1965).
4. Psychoyos, A., Ciba Found. Study Groups **23**, 4 (1966).
5. Krehbiel, R. H., Anat. Rec. **81**, 381 (1941).
6. McLaren, A., J. Endocrinol. **42**, 453 (1968).
7. McLaren, A., Nature (London) **221**, 739 (1969).
8. Yoshinaga, K., and Adams, C. E., J. Reprod. Fert. **12**, 593 (1966).
9. Humphrey, K. W., Steroids **10**, 591 (1967).
10. Prasad, M. R. N., Dass, C. M. S., and Mohla, S., J. Reprod. Fert. **16**, 97 (1968).
11. Mohla, S., Dass, C. M. S., and Prasad, M. R. N., J. Reprod. Fert. **27**, 295 (1971).
12. Mohla, S., and Prasad, M. R. N., J. Endocrinol. **49**, 87 (1971).
13. Jacobson, M. A., Sanyal, M. K., and Meyer, R. K., Endocrinology **86**, 982 (1970).
14. Smith, D. M., and Smith, A. E. S., Biol. Reprod. **4**, 66 (1971)
15. Westphal, U., *in* "Mechanism of Action of Steroid Hormones" (C. A. Ville and L. L. Engel, eds.). Pergamon, New York (1961).
16. Bowman, P., and McLaren, A., J. Embryol. Exp. Morph. **24**, 203 (1970).
17. Psychoyos, A., *in* "Advances in the Biosciences" (G. Raspé, ed.). Pergamon Press, New York (1969).

Received June 1, 1973. P.S.E.B.M., 1973, Vol. 144.