was centrifuged (13,800*g*, 30 minutes) and the supernatant stored as a lyophilized powder. The extract was reconstituted with 50 m*M* tris-HCl, *p*H 7.4, containing 150 m*M* NaCl and 0.5 m*M* EDTA and applied to a Sephadex G-100 column equilibrated in the reconstitution buffer. The keratinocyte growth-promoting activity chromatographed with a K_a of approximately 0.3. Preparative isoelectric focusing (Bio-Rad Labs) between *p*H 10 and *p*H 3 was also performed with the brain extract. The extract was dialyzed (5000 molecular weight cutoff) against 100 μ*M* tris-HCl, *p*H 7.0, for 18 hours prior to isoelectric focusing. The keratinocyte growth-promoting activity migrated to an isoelectric point between *p*H 4 and *p*H 5.

4. J. G. Rheinwald and H. Green, *Cell* **6**, 331 (1975).
5. ———, *Nature (London)* **265**, 421 (1977); T-T. Sun and H. Green, *Cell* **9**, 511 (1976).
6. B. A. Gilchrest, R. E. Nemore, T. Maciag, *Cell Biol. Int. Rep.* **4**, 1009 (1980).
7. E. Engvall and E. Ruoslahti, *Int. J. Cancer* **20**, 1 (1977).
8. W. L. McKeehan, W. G. Hamilton, R. G. Ham, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2023 (1976).
9. J. Bottenstein, I. Hayashi, S. Hutchings, H. Masui, J. Mather, D. B. McClure, S. Ohasa, A. Rizzino, G. H. Sato, G. Senero, R. Wolfe, R. Wu, *Methods Enzymol.* **58**, 94 (1979).
10. P. Honegger, D. Lenoir, P. Favrod, *Nature (London)* **282**, 305 (1979); I. Hayashi, J. Larner, G. Sato, *In Vitro* **14**, 23 (1978).
11. E. Rinderknecht and R. E. Humbel, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2365 (1976).
12. K. A. Thomas, M. C. Riley, S. K. Lemmon, N. C. Baglan, R. A. Bradshaw, *J. Biol. Chem.* **255**, 5517 (1980).
13. H. N. Antoniades, C. D. Scher, C. D. Stiles, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1809 (1979); R. Ross, J. Glomset, B. Kariya, L. Harker, *ibid.* **71**, 1207 (1974).

14. We thank R. M. Goldwyn for the provision of tissue. These studies were supported by grants AG 0599 and HO 07374 from the National Institutes of Health.

12 January 1981

Estrogen Receptors in the Nidatory Sites of the Rat Endometrium

Abstract. *In ovariectomized rats treated with progesterone, implantation was induced by a minute dose of 17β-estradiol. Twenty-four hours later, the concentrations of estradiol receptor in nuclear and cytosol fractions prepared from the endometrium surrounding the blastocyst and the inter-implantation areas remained very low. This indicates that estrogen was not secreted by the blastocyst. The higher receptor content in cytosol from inter-implantation sites may reflect modifications accompanying the decidual reaction since our results show that there is no translocation of the receptor to the nuclei. The choice of the dye used to reveal the implantation sites is critical, since Trypan blue but not Evans blue binds steroids and thereby interferes with receptor measurements.*

For ovoimplantation to be initiated, egg development and preparation of the endometrium must be closely synchronized. In the rat, the timing of endometrial preparation depends on a precise sequence of hormonal reactions. Thus only after the endometrium has been primed with progesterone for at least 48 hours can it be induced by estradiol to become receptive to the blastocyst 18 hours later (*1*). Although progesterone priming may be essential in all species, estradiol intervention does not appear to be a common process (*2–4*). It has been hypothesized that, at the time of implantation, the blastocyst itself influences endometrial function locally by releasing estradiol (*5–6*). Synthesis of estradiol by the blastocyst has been described in some species, and it has been suggested in other species that the egg accumulates estradiol in the ovary and releases it later in the uterus (*6–7*).

In the rat there is no direct evidence that estrogens are synthesized or released by the blastocyst. Furthermore, in rats that are ovariectomized before the evening of day 4 of pregnancy and then treated daily with progesterone, ovoimplantation is delayed until a minute amount of estradiol is administered intravenously or locally (*1*). If, indeed, the rat embryo released estradiol locally, this should induce translocation of the estradiol receptors from the cytoplasm to the nucleus in the adjacent endometrial cells.

In the study described here, pregnant rats were ovariectomized under a dissecting microscope (*8*) on day 3 of pregnancy (appearance of vaginal sperm was designated day 1 of pregnancy) and were then treated daily with 4 mg of progesterone; thus any circulating estrogens of maternal origin were removed. Implantation was induced on day 8 postcoitum by a single injection of estradiol (0.25 μg per rat) and the animals were killed 24 hours later. The implantation sites were revealed by injecting intravenously 1 ml of 0.5 percent Evans blue via the femoral vein 20 to 30 minutes before the rats were killed (*9*). The uteri were removed and the blue (implantation sites) and nonblue (inter-implantation) areas of the endometrium were collected and assayed for estradiol receptor. Previous studies in our laboratory had shown that the concentration of estrogen receptor in the myometrium is not modified during implantation (*10*). Control animals, in which the oviducts containing fertilized eggs were removed during ovariectomy, were treated in a similar way.

The concentrations of estradiol receptor in cytosols prepared from uteri of the control animals and from the inter-implantation (nonblue) areas of the uteri from the pregnant animals were similar (Table 1), whereas estradiol receptor in cytosols from implantation (blue) areas was significantly decreased ($P < .001$, *t*-test). This decrease amounts to 53 percent when the receptor content is expressed per microgram of DNA and 63 percent when expressed per milligram of protein. However, in both the implanta-

Table 1. Estradiol receptors in the implantation (blue area) and inter-implantation (nonblue area) sites. Wistar rats were prepared as described in the text; the uteri were removed, slit longitudinally, and placed at 0°C. All other operations were carried out at 0°C unless otherwise specified. The blue and nonblue regions of the endometrium were collected by gentle scraping and homogenized in buffer (250 m*M* sucrose, 3 m*M* $MgCl_2$, 10 m*M* tris-HCl, *p*H 7.4). Cytosolic and nuclear fractions were prepared by centrifugation (700*g* for 10 minutes). The cytosol receptor was assessed by the exchange technique as described by Katzenellenbogen *et al.* (*18*), slightly modified (*19*), and protein determinations were carried out according to Lowry (*20*). The nuclei were washed in a 0.5 percent Triton-X 100 solution in buffer, and washed twice in buffer, then assayed by the [^{3}H]estradiol exchange method as described by Clark *et al.* (*13*). At the end of the incubation period, the nuclei after being washed twice were subjected to acid hydrolysis; one half of the hydrolysate was counted for radioactivity, the other was used for DNA determination (*21*).

Fraction	Uteri from control rats		Uteri from rats injected with Evans blue			
			Inter-implantation sites		Implantation sites	
	Per microgram of DNA (fmole)	Per milligram of protein (10^{-13} mole)	Per microgram of DNA (fmole)	Per milligram of protein (10^{-13} mole)	Per microgram of DNA (fmole)	Per milligram of protein (10^{-13} mole)
Cytosol	7.31 ± 0.52	4.45 ± 0.31	7.25 ± 0.61	4.11 ± 0.70	3.40 ± 0.43	1.50 ± 0.38
Nuclear	0.060 ± 0.035		0.056 ± 0.099		0.032 ± 0.011	

0036-8075/81/0327-1454$00.50/0

tion sites and the inter-implantation area there were very few receptor sites in the nuclear fraction (Table 1), suggesting the absence of effective estrogen concentrations in both regions.

Several authors have reported that the uptake of [^{3}H]estradiol (which cannot be considered a measurement of receptor concentration), decreases in the nidatory uterine segments compared to the internidatory segments (*11, 12*). It has been postulated from this observation that estrogen receptors in the implantation sites are not able to bind the [^{3}H]estradiol because they are already saturated by estrogen diffusing from the blastocyst. Our results do not support this hypothesis because, according to previous data (*13, 14*), estrogen production by the blastocyst should have induced translocation of the estradiol receptor to the nuclear fraction in the adjacent endometrial cells, and this did not occur. However, in agreement with the studies of [^{3}H]estradiol uptake (*11, 12*), we did observe a decrease of the cytoplasmic estradiol receptor concentration in the endometrium surrounding the blastocyst; this decrease was evident when the data were expressed per unit of DNA or of protein. Whether this decrease reflects a true decline of the receptor remains to be determined. In our opinion, conclusions should be drawn with caution because total DNA or protein content, or even wet weight, all of which are used to express receptor concentrations, change dramatically during the early stage of decidualization.

Recently, Logeat *et al.* (*15*) observed a dramatic increase in the concentration of both estradiol and progesterone nuclear receptors at implantation sites in uteri of rats on day 6 of pregnancy. In an attempt to understand the reasons for this discrepancy with our own data, we studied the interaction between various steroids and the dyes used to reveal the implantation sites. The choice of the dye used for visualization of these sites appears to be important (Table 2). Unlike the Evans blue that we used, the Trypan blue used by Logeat *et al.* (*15*) binds various steroids in a nonspecific but saturable way, and thus interferes with the receptor measurement when assayed by the unlabeled competitor technique. Evans blue and Trypan blue have similar chemical formulas; however, the different positions occupied by the $NaSO_3$ groups confer on these molecules strikingly different chemical properties (*16*) that may explain the differences between our data that those of Logeat *et al.* (*15*).

Probably because of its steric conformation, Trypan blue crosses the capillaries and diffuses in the intercellular space more easily than Evans blue. For these reasons it may be a poor marker of the implantation sites. In fact, the inter-implantation sites are also stained after injection of Trypan blue, although considerably less than the implanted sites. This may also explain why Logeat *et al.* (*15*) obtained some unexpected results, such as a tenfold difference in the estradiol binding capacity between the pseudopregnant sterile horns of control animals not injected with Trypan blue, and the inter-implantation areas (contaminated with blue) of the animals that were injected with blue. The binding of steroids to Trypan blue may also explain the large amount of estrogen receptor found by Logeat *et al.* (*15*) in the nuclei of the implantation sites (estimated by these authors to be around 35,000 molecules per nucleus). In normal cycling rats, even at proestrus when estradiol secretion is at a maximum, only 3000 receptor sites are translocated into the nucleus (*13*).

Our data indicate that the rat blastocyst at day 5 of pregnancy does not release estrogens, at least not in amounts sufficient to induce a functionally important local change in the intracellular distribution of estrogen receptor in the adjacent endometrium. However, our results do not rule out such a mechanism in other species. It is also possible that the rat trophoblast secretes estrogens at a later stage of development (*17*).

We do not know whether the decrease in cytosolic receptor concentrations that we observed in the endometrium adjacent to the embryo was an artifact of the experiment or reflected a real decrease in receptor level. If a real decrease occurred, its mechanism and the participation of the blastocyst in this phenomenon remain to be investigated.

Table 2. Binding of steroids to Trypan blue and Evans blue. Solutions containing increasing concentrations of Trypan blue or Evans blue were incubated at 20°C for 20 minutes in two sets of tubes (A and B) with the steroid concentrations usually used to measure steroid receptors by the following exchange technique (addition of unlabeled competitor): (i) (A) 10 n*M* [^{3}H]estradiol and (B) 10 n*M* [^{3}H]estradiol plus unlabeled 1 μ*M* estradiol; (ii) (A) 10 n*M* [^{3}H]R 5020 and (B) [^{3}H]R 5020 plus unlabeled 1 μ*M* progesterone; (iii) (A) 20 n*M* [^{3}H]progesterone and (B) 20 n*M* [^{3}H]progesterone plus unlabeled 2 μ*M* progesterone. The final volume was 0.3 ml. At the end of the incubation period, 0.5 ml of a dextran-coated charcoal solution was added. The incubation was continued for 7 minutes at 0°C, then the charcoal was removed by centrifugation (700*g* for 5 minutes). The charcoal treatment was repeated on the supernatant by the addition of 50 μl of a tenfold concentrated charcoal suspension. The supernatant was counted, the radioactivity in B was subtracted from that in A, and the results were expressed as disintegrations per minute.

Dye concentration	Incubation with					
	Estradiol		R 5020		Progesterone	
	Trypan blue	Evans blue	Trypan blue	Evans blue	Trypan blue	Evans blue
0	42	22	148	0	44	16
1/100,000	30	0	58	0	372	40
1/50,000	166	24	0	0	490	40
1/10,000	1,702	42	1,218	0	7,028	406
1/5,000	4,346	122	5,208	0	13,804	138
1/1,000	13,500	216	14,964	0	30,338	528

Our results do not rule out such a mechanism in other species. It is also possible that the rat trophoblast secretes estrogens at a later stage of development (*17*).

We do not know whether the decrease in cytosolic receptor concentrations that we observed in the endometrium adjacent to the embryo was an artifact of the experiment or reflected a real decrease in receptor level. If a real decrease occurred, its mechanism and the participation of the blastocyst in this phenomenon remain to be investigated.

DOMINIQUE MARTEL
ALEXANDRE PSYCHOYOS
E.R. 203 du CNRS, Hôpital de Bicêtre, 94270 Bicêtre, France

References and Notes

1. A. Psychoyos, *Vitam. Horm. (N.Y.)* **31**, 201 (1973).
2. M. W. Orsini and R. K. Meyer, *Anat. Rec.* **134**, 619 (1959).
3. B. M. Bindon, *Aust. J. Biol. Sci.* **24**, 149 (1971).
4. J. K. Kwun and C. W. Emmens, *ibid.* **27**, 275 (1974).
5. J. S. Perry, R. B. Heap, E. C. Amoroso, *Nature (London)* **254**, 45 (1973).
6. Z. Dickmann, S. K. Dey, J. S. Gupta, *Vitam. Horm. (N.Y.)* **34**, 215 (1976).
7. R. M. Barland, G. F. Erickson, T. Ducibella, *J. Reprod. Fertil.* **49**, 219 (1977).
8. A. Psychoyos and J. J. Alloiteau, *C. R. Soc. Biol.* **156**, 46 (1962).
9. A. Psychoyos, *C. R. Acad. Sci.* **251**, 3073 (1960).
10. D. Martel and A. Psychoyos, *J. Endocrinol.* **76**, 145 (1978).
11. P. Sartor, *Acta Endocrinol.* **84**, 804 (1977).
12. W. F. Ward, A. G. Frost, M. Ward Orsini, *Biol. Reprod.* **18**, 598 (1978).
13. J. H. Clark, J. Anderson, E. J. Peck, Jr., *Science* **176**, 528 (1972).
14. J. Anderson, J. H. Clark, E. J. Peck, Jr., *Biochem. J.* **126**, 561 (1972).
15. F. Logeat, P. Sartor, M. T. Vu Hai, E. Milgrom, *Science* **207**, 1083 (1980).
16. F. Hartwell, *Org. Synth.* **16**, 12 (1936); L. Lewers, *Ind. Eng. Chem.* **17**, 1289 (1925).
17. S. R. Glasser and S. A. McCormack, *Adv. Biosci.* **25**, 165 (1979).
18. J. A. Katzenellenbogen, J. H. Johnson, K. E. Carlson, *Biochemistry* **12**, 4092 (1973).
19. We used an additional charcoal step which considerably decreased the background that is elevated after the first charcoal treatment because of the high steroid concentrations used for the exchange.
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
21. K. Burton, *Biochem. J.* **62**, 315 (1956).
22. This work was supported by the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, and the Ford Foundation. We thank Marie-Noelle Monier for excellent technical collaboration.

1 July 1980; revised 24 October 1980