into the brain ventricular system rapidly penetrates into the brain parenchyma with uptake being highest in the hypothalamus. This indicates that melatonin in CSF has access to brain areas important for neuroendocrine control.

The reason that melatonin concentration in CSF is higher than that in plasma is not known. Melatonin could be secreted directly into the third ventricle (*18*); it could be actively transported into the CSF from the blood; or, it could be the result of retrograde flow in the superior sagittal sinus (*19*). Secretion directly into the third ventricle, although the most attractive hypothesis, is not supported by anatomical evidence. The bovine pineal gland has a relatively dense glial layer between the pineal parenchyma and the lumen of the third ventricle (*20*), and it does not exhibit the intimate contact with the suprapineal recess such as occurs in some lagomorphs and rodents (*18*).

There is a degree of uncertainty about the initial source of plasma and CSF melatonin in mammals, because retinae, Harderian glands, and pineal glands are all capable of melatonin synthesis (*21*). However, in chickens, removal of the pineal gland reduces melatonin to nondetectable levels in plasma, confirming that the pineal gland is the primary source of melatonin in this species (*8*). Although it seems likely that most of the melatonin found in the plasma of rats, sheep, humans, and calves, as well as the melatonin in calf and human CSF, originates from the pineal gland, critical experimental support is still lacking.

LAURENCE HEDLUND
MICHAEL M. LISCHKO
Department of Dairy Husbandry, University of Missouri, Columbia 65201
MARK D. ROLLAG
GORDON D. NISWENDER
Department of Physiology and Biophysics, Colorado State University, Fort Collins 80523

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13. Illumination by 40-watt fluorescent bulbs (GE F40 WW) located 3 m above the floor. Light intensity during the day was about 431 lux at the level of the calf's head, while at night the room was dimly lighted (much less than 22 lux) by two shaded fluorescent bulbs located about 10 m from the calf pens.
14. Cannula guides were 15-gauge syringe needles, 2 cm in length and fitted with a flange on the needle hub to facilitate anchoring to the skull. The cannula guides were placed on the frontal bone with the aid of a stereotaxic apparatus; we used coordinates of 0.9 to 1.2 cm lateral from the skull midline and 0.8 to 1.5 cm anterior from the ear bar. The cannula guides were secured to the skull with screws and dental acrylic cement. When not in use, the cannula guides were capped. The calves were prepared with these implants about 7 months before use in the present study.
15. Plasma and CSF samples, packed in Dry Ice, were shipped by air from Columbia to Fort Collins where all assays were performed.
16. From analysis of variance (probability of the F value), individual sample time means compared by least significant differences with a $P = .05$ (Student's t-test).
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Does "Blastocyst Estrogen" Initiate Implantation?

Abstract. *Fertilized eggs were incubated for 2 hours in a medium containing estradiol-17β and then transferred into the uteri of day 5 pseudopregnant rats. These eggs, but not estrogen-free control eggs, induced a local increase in capillary permeability. We suggest that the blastocyst factor which induces the local increase in capillary permeability during early pregnancy is estrogen synthesized by the blastocyst.*

In previous publications we introduced a new concept which states that the preimplantation embryo (PIE) has the capacity to synthesize steroid hormones, and that these PIE steroids are essential for preimplantation embryogenesis and for implantation of the blastocyst (*1–3*). In support of this concept, it has been shown that the PIE contains steroid hormones (*2, 4*) and can synthesize them (*2, 3, 5*). However, the definitive support for the concept will have to come from evidence demonstrating the function of PIE steroids. Accordingly, the purpose of the present study was to determine whether "blastocyst estrogen" plays a role in the initiation of implantation in the rat.

In the rat, and in a few other species which have been studied, the earliest macroscopically demonstrable reaction of the uterus to the presence of a blastocyst is a local increase in capillary permeability. This reaction can be demonstrated experimentally by injecting intravenously a macromolecular dye, such as Chicago Blue, and inspecting the uteri 15 minutes later: in the area where each blastocyst is located, a discrete blue band is seen across the uterus. Apparently, the dye can leave the circulation only in loci where an increase in capillary permeability has occurred. The increase in permeability is a necessary preliminary for the decidual reaction (*6*), and the decidual reaction, in turn, is a prerequisite for the implantation process. It seems obvious that the blastocyst provides the stimulus for the induction of increased capillary permeability, since the reaction occurs only in the immediate vicinity of the blastocyst. Nevertheless, we have not encountered in the literature any suggestion concerning the nature of the blastocyst's stimulus.

In castrated rats and rabbits (*7*) and in immature rats (*8*), systemic injection of estrogen induces an increase in capillary permeability throughout the length of the uterus. We have shown that the rabbit blastocyst contains estrogen and suggested that this estrogen is synthesized by the blastocyst (*2*). Taken together, these findings led us to propose that the stimulus for the local increase in capillary permeability in the intact pregnant rat is estrogen secreted by the blastocyst, and that this stimulus is effective only in a uterus which has been properly primed with systemic progesterone and estrogen. The design of our experiments was based on this hypothesis.

The animals used were adult, virgin female rats of the Holtzman strain weighing 180 to 220 g. They were housed in temperature- and humidity-controlled quarters with lights on from 0600 to 2000 hours. To induce pregnancy, females

were caged with fertile males on the day of proestrus, and were examined the next morning (day 1 of pregnancy) for the presence of vaginal plug or spermatozoa in the vagina. To induce pseudopregnancy, females were cervically stimulated with a vibrating rod on the day of estrus (day 1 of pseudopregnancy) according to the method described by DeFeo (*9*).

To provide background controls, we injected 1 ml of a 1 percent solution of Chicago Blue into the tail vein of female rats on day 5 of pregnancy between 2200 and 2300 hours. Fifteen minutes later, the rats were killed, and the uteri were excised and inspected for blue bands. The number of blue bands per rat corresponded to the expected number of blastocysts, that is, 8 to 12.

According to our hypothesis, a minute quantity of estrogen released from the blastocyst causes the local increase in capillary permeability which, under the above experimental condition, is manifested as a blue band. To test this hypothesis, day 5 pseudopregnant rats were anesthetized with ether between 1000 and 1600 hours, and their uteri were exteriorized through a midventral incision. Each uterus was punctured with a fine needle so as to create a passageway to the uterine lumen. Through the passageway a fine pipette was inserted, and a minute quantity of estrogen dissolved in water was instilled into the uterine lumen. Each uterus received such an instillation at three separate loci. As in the background controls, between 2200 and 2300 hours the rats were injected with Chicago Blue and later killed and checked for blue bands. The procedure did induce blue band formation. However, when we instilled into uteri water not containing estrogen, and when we inserted the pipette without instilling anything, blue bands also formed. These results could have been predicted, because trauma induces increased capillary permeability. However, we were hoping that the trauma would be sufficiently mild not to induce increased permeability, because we employed the method used for egg transfer (*10*), which is gentle enough not to induce deciduomata.

Our next objective was to find a way by which to deliver estrogen to local areas in the uterine lumen without physically traumatizing these same areas. This problem was resolved in the following manner.

Fertilized eggs at the 2- to 4-cell stage were recovered from pregnant rats on day 3 between 1200 and 1400 hours. The eggs were incubated for 2 hours in a balanced salt solution containing 2 μg of estradiol-17β (*11*) per milliliter [this concentration is innocuous for mouse PIE's grown in vitro from the 4-cell stage to blastocysts (*12*)], and were then transferred into the uteri of day 5 pseudopregnant recipients. Depending on the number available, between three and six eggs were transferred into each uterus. For reasons which will be clarified below, the uteri were punctured and the transfer pipette inserted in the ovarian quarter of the uterus. On the same day, between 2200 and 2300 hours, the recipients were injected with Chicago Blue; 15 minutes later they were killed and the uteri checked for blue band formation. Normally, during day 5 of pregnancy, blastocysts are spaced throughout the length of the uterus; therefore, we were hoping that the transferred day 3 eggs would also be spaced, and once they settled on a particular spot, they would lose there the estrogen which they picked up during the incubation in vitro. Then, if our hypothesis was correct, the estrogen released from an egg would result in the induction of a blue band. The presence of one or more blue bands in the ovarian quarter of the uterus would be interpreted as having been induced by the physical trauma of the puncture needle and the transfer pipette, but blue bands in the other three quarters of the uterus would be regarded as resulting from a stimulus by the transferred eggs. Control transfers were done in the same way as experimental transfers, except that the eggs were incubated in estrogen-free balanced salt solution. Nine recipients for experimental transfers and ten recipients for control transfers were used. In the controls, blue bands were present only in the ovarian quarter of the uterus, whereas in the experimental transfers, blue bands were present in all four quarters. Thus, the estrogen-carrying eggs, but not the estrogen-free eggs, induced an increase in capillary permeability.

These results lend support to our hypothesis that blastocyst estrogen induces the local increase in capillary permeability, if we assume that the rat blastocyst, like the rabbit blastocyst, contains estrogen (*2*) and has the capacity to release it. There is no evidence that estrogen has a direct effect on capillary permeability, but estrogen is known to cause the release of vasoactive substances. Thus it has been shown that estrogen treatment of adult, ovariectomized rats causes release of uterine histamine (*13, 14*), uterine prostaglandin (*15*), and uterine 5-hydroxytryptamine (*14*). We therefore suggest that, singly or in combination, these substances or other vasoactive substances are released as a result of stimulation with blastocyst estrogen.

A less plausible interpretation of our results is that the estrogen stimulated the release of a vasoactive substance from the egg itself. It could also be postulated that during pregnancy, blastocyst estrogen triggers the release of a vasoactive substance from the blastocyst. Whether blastocysts can synthesize and release vasoactive substances remains to be determined; however, it has been shown that rabbit blastocysts contain prostaglandins (*16*).

It is thus our contention that blastocyst estrogen plays a key role in initiating the local increase in capillary permeability which is a necessary preliminary for implantation. Future studies will determine whether the target for the blastocyst estrogen is the uterus, the blastocyst, or both. We have discussed previously (*3*) additional lines of evidence which we interpret as supportive for the hypothesis that blastocyst estrogen plays a key role in the process of implantation.

ZEEV DICKMANN
JAYASREE SEN GUPTA
SUDHANSU K. DEY
Department of Gynecology and Obstetrics and *Ralph L. Smith Human Development Research Center, University of Kansas Medical Center, Kansas City 06103*

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