protoplasm of the mammalian, plant, and algal cells are far greater than those in lyophilized samples or in isolated phospholipids. In these three cells, tempone experiences much greater freedom of molecular motion in cellular hydrophobic zones than in cellular aqueous zones. These data suggest that, in cells where internal membrane structure abounds, the aqueous regions have such a high microviscosity that diffusion along the plane of the membrane or within the membrane structure may be faster and more efficient than through the three-dimensional aqueous protoplasm. Our observations indicate that the lateral translational diffusion in membranes reported by Frye and Edidin (*12*), Träuble and Sackmann (*13*), Devaux and McConnell (*14*), and Scandella *et al.* (*15*) may be extremely important to vital cellular logistic processes.

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References and Notes

1. W. Wilson and L. Heilbrun, *Q. J. Microsc. Sci.* **101**, 95 (1960).
2. F. Ashton, *Biol. Bull. (Woods Hole)* **113**, 319 (1957).
3. F. Crick and A. Hughes, *Exp. Cell Res.* **1**, 37 (1950).
4. J. Tanner and E. Stejskal, *J. Chem. Phys.* **49**, 1768 (1968).
5. In addition to tempone we have used 2,2,6,6-tetramethylpiperidinol-*N*-oxyl, the hydroxyl derivative of tempone; 3-carbamyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl, a small water-soluble nitroxide having a molecular size close to that of tempone; and the carboxylic acid derivative of the latter nitroxide. All yield rotational correlation times that are very similar. Wagner and Hsu reported [T. E. Wagner and C. J. Hsu, *Anal. Biochem.* **36**, 1 (1970)] that $NaBH_4$ causes the ketone of tempone to form a covalent linkage with amine groups. They thought that the reaction is mediated through a Schiff base. Preparations of cells reported on here were washed twice in medium containing no tempone. These cells contained no detectable spin, which illustrates that the spin species was fully diffusible and not covalently attached.
6. The line amplitude of an *Escherichia coli* sample at low field increased linearly with cell concentration and extrapolated through zero, demonstrating that the signal observed comes totally from the cells and not from the extracellular medium.
7. G. Pake, *Paramagnetic Resonance* (Benjamin, New York, 1962), pp. 79–89.
8. The toxicity of nickel to cells is variable; for example, exposure of *E. coli* to 0.5*M* $NiCl_2$ for 1 hour kills less than 25 percent of the cells, whereas exposure of *Pseudomonas* sp. BAL-31 to 0.1*M* $NiCl_2$ for the same time kills about half the cells.
9. Values were calculated from $\tau_c = K(W_{-1} - W_1)$, simplified to use line height (h) ratios, where $W_{-1} - W_1 = W_1[(h_1/h_{-1})^{1/2} - 1]$. This equation, or modifications of it, has been treated elsewhere both theoretically [D. Kivelson, *J. Chem. Phys.* **33**, 1094 (1960)] and in application to membranes [A. Keith, G. Bulfield, W. Snipes, *Biophys. J.* **10**, 618 (1970)].
10. W. Snipes and A. Keith, *Res. Develop.* **21**, 22 (1970).
11. These samples also had varying percentages of a highly immobilized signal trapped in an unknown manner.
12. L. Frye and M. Edidin, *J. Cell Sci.* **7**, 319 (1970).
13. H. Träuble and E. Sackmann, *J. Am. Chem. Soc.* **94**, 4499 (1972).
14. P. Devaux and H. McConnell, *ibid.*, p. 4475.
15. C. Scandella, P. Devaux, H. McConnell, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2056 (1972).
16. N. A. Lange, Ed., *Handbook of Chemistry* (McGraw-Hill, New York, 1967), p. 1681.
17. Supported by AEC contracts AT(11-1)-2223 and AT(11-1)-2311. A.D.K. is also thankful for NIH grant 1-K4-GM70593-01. We thank E. Pollard, P. Scheie, and many other members of the biophysics department, Pennsylvania State University, for their interest, useful suggestions, and helpful criticism.

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Cell Culture of Mammalian Endometrium and Synthesis of Blastokinin in vitro

Abstract. *Endometrial cells obtained from mature female estrous rabbits can be grown in cell culture with the aid of insulin. These cells, after several days in culture, are capable of synthesizing blastokinin by induction with progesterone for 48 hours.*

Recent evidence on the action of steroids on target cells of chick oviduct (*1*), mammary explants (*2*), and ovarian follicular cells in vitro (*3*), indicates that the interaction of hormones is required for cell proliferation and subsequent cell differentiation. Previous workers in attempts at in vitro cultivation of mammalian endometrium failed to take this into consideration (*4*), which probably accounts for their inability to maintain long-term viable tissue cultures that respond to steroid hormones. It had been observed, however, that a cell line from human endometrial adenocarcinoma has recently been established without exogenous addition of steroids or other hormones (*5*). On the other hand, survival of short-term human endometrial tissue in organ culture increased when insulin was added to the culture prior to stimulation by progesterone (*6*). Studies on chick oviduct epithelium also indicated that estrogen is necessary for cell division and that progesterone added to cells previously stimulated by estrogen blocked cell proliferation.

These results and those from mammary explant cultures (*2*) suggested to us the possibility that tissue cultures of rabbit endometrium could be established if insulin alone or in combination with estrogen was added to the medium prior to administration of progesterone. In vitro cultures of rabbit endometrium would serve as an excellent model system for testing the interaction of various hormones, since it has been shown in vivo that rabbit endometrium secretes "blastokinin" in the progestational state from days 3 to 9 of pregnancy (*7*). Blastokinin has been found to be an inducer of blastocyst growth in vitro (*7*), stimulates mitotic activity in cells of diapausing blastocysts (*8*), and stimulates RNA and protein synthesis in rabbit blastocysts (*9*). Blastokinin is synthesized in uteri of ovariectomized rabbits by exogenous addition of progesterone (*10*). Therefore, we have developed a cell culture system to investigate the control and regulation of hormones on the production of blastokinin. Monolayers (mixed culture) of uterine endometrium have been established in our laboratory both as primary and secondary tissue cultures which proliferate under the influence of insulin, and primary cultures secrete blastokinin after stimulation with progesterone.

Primary tissue cultures of rabbit endometrium were established from uterine tissues obtained from mature estrous New Zealand white female rabbits with no previous history of matings. Whole uteri were removed surgically from rabbits, washed several times in 0.9 percent sterile saline, and exposed to ultraviolet light in a sterile transfer hood for 10 to 15 minutes to ensure sterile organs. With the ultraviolet off, uteri were cut open and the uterine epithelium was carefully removed with forceps and curved surgical scissors. Endometrium was finely minced, with curved scissors, in sterile saline (0.9 percent) and placed in sterile, 40-ml capacity, screw-cap centrifuge tubes containing four to five volumes of 0.25 percent sterile trypsin. Tissues were agitated gently by hand for 20 to 30 minutes at room temperature and centrifuged. The trypsin solution was decanted and cells were suspended in four to five volumes of medium 199 containing Hanks basal salts and 10 percent fetal calf serum (Colorado Serum Co.).

Cells were placed down (2×10^3 to 5×10^3 cells per milliliter) in 60-mm sterile Falcon plastic petri dishes containing 3 to 5 ml of medium 199 with Hanks base containing 10 percent fetal calf serum, antibiotic-antimycotic mixture (Grand Island Biological Co.), and insulin (bovine pancreas, Calbiochem),

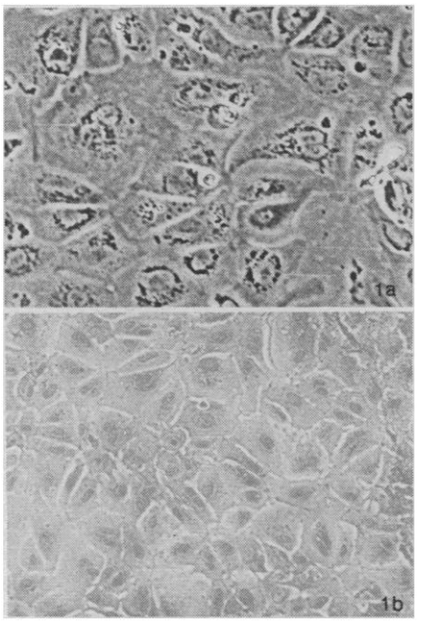

Fig. 1. Photomicrographs of rabbit uterine endometrial cells in growth in tissue culture (× 290). (a) A phase contrast micrograph of primary cells grown for 5 days in medium 199 with insulin. (b) A photomicrograph of the same culture fixed in Bouin's fluid after 8 days in culture and stained with Harris hematoxylin and aqueous eosin.

40 μg/ml. Cells were allowed to set for 5 days, medium was removed, and fresh medium containing insulin was added for three more days. After the second wash, medium containing both insulin (40 μg/ml) and estradiol 17β (0.1 μg/ml) or insulin (40 μg/ml) and progesterone (10 μg/ml) (Calbiochem) was added to the cells. The media containing steroids were allowed to remain in contact with monolayers of endometrium for 48 hours. After this treatment secondary cell cultures were established making a 1 to 3 split of cells in medium 199 containing only insulin. Cells were dislodged from each plate with a rubber policeman.

A representative phase contrast photograph of a 5-day primary culture of endometrial cells is shown in Fig. 1a, and a hematoxylin and eosin stained preparation of the same culture after 8 days is shown in Fig. 1b. The culture reached maximum density after three more days in fresh medium with insulin. Cells are not always typically epithelioid in appearance. Some appear vacuolated, others have long protoplasmic extensions assuming a more fibroblastic nature, some appear binucleate, and several exhibit pleomorphic nuclei.

Medium (washes) from primary cultures after various stages of hormonal treatment were analyzed for blastokinin. The medium was centrifuged at 10,000*g* to remove cells and cellular debris. High-molecular-weight components of serum in the medium were removed by an Amicon XM-50 membrane filter under nitrogen pressure. This particular membrane excluded molecules greater than 50,000 daltons. Blastokinin has a molecular weight of about 15,000 (*11*) and if present would appear in the filtrate. Vacuum dialysis was next used to concentrate the filtrate to a final volume of approximately 0.1 ml. This entire filtrate was subjected to polyacrylamide gel electrophoresis with the use of a slab gel (Ortec). The results are shown in Fig. 2. Gel 1 is an insulin control and gel 2 is a standard in vivo uterine flushing taken from a 5-day pregnant rabbit. The position of blastokinin in the gels is shown by the arrows. It can be seen (gel 3) that endometrial cells cultured for 5 days produced very little blastokinin, if any. The same cells after 3 days more in culture (gel 4) had a definite blastokinin band. These cells subsequently treated with estrogen for 48 hours produced lesser blastokinin (gel 5), indicating an inhibitory effect by this steroid. Gel 6 is a preparation from cells treated for 48 hours with progesterone. A preparation treated as that in gel 6 was subjected to immunoelectrophoresis in polyacrylamide gel (*12*). Goat antiserum against 5-day pregnant rabbit uterine flushings was used in these experiments after absorption with rabbit serum and rabbit uterine flushings which lacked blastokinin. The region of the gel occupied by the band which we designate as blastokinin produced a faint but discernible arc when allowed to diffuse against the antiserum. No additional immunoelectrophoretic reactions were observed. Therefore, our designation of the blastokinin band is confirmed. The distinct blastokinin band indicates that progesterone can induce its production in vitro. At present it is unclear which cell type in our cultures produces blastokinin; however, data from in vivo experiments (*13*) suggest that the source of blastokinin is epithelial.

It is also interesting that media from the cell cultures (second wash) prior to treatment with gonadal steroids contained some blastokinin (although much less than the progesterone-treated cells). This is a consistent result in our experiments and may reflect a dilution of estrogen already present in the cells at the initial time of culture. In the absence of estrogen, cells that reach confluency at about day 8 or 9 may become secretory once they stop proliferating in culture.

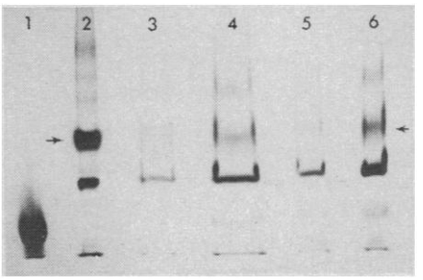

Fig. 2. Polyacrylamide slab gel electrophoresis of filtered media (washes) removed from cell cultures after various treatment with hormones. Arrows point to the position of blastokinin present in the various gels. See text for details.

Initial attempts to culture endometrial cells from pregnant (progestational) rabbits failed. In these attempts we used all of the above procedures, including 1- to 2-mm explants of endometrium in the same medium mentioned above containing no steroids or insulin. It is now apparent that cells from progestational uteri do not proliferate without the aid of insulin and estrogen. These results agree with the findings on chick oviduct cells, where it has been shown that progesterone blocks cells in the cell cycle (*1*). Other attempts at cell culture with progestational endometrium with estrogen-supplemented medium without the aid of insulin allowed a low level of cell growth and failure to establish complete monolayers.

Secondary cell cultures have been established through four serial transfers of uterine endometrium from estrous rabbits, grown only in insulin-containing medium. Prolonged treatment with progesterone, over 48 hours, prevents cells from entering back into the cell cycle when insulin-containing medium is added again to these cells. These results suggest that progressive differentiation is mediated by progesterone and that if cells are stalled for long periods in the cell cycle they are no longer capable of further proliferation or recruitment. In a typical experiment, with six petri plates of monolayers of primary cultures treated with progesterone for 48 hours, only two plates were found to produce viable secondary cultures of rabbit endometrium when placed in medium containing only insulin.

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References and Notes

1. S. H. Socher and B. W. O'Malley, *Dev. Biol.* **30**, 411 (1973).
2. R. W. Turkington, *Developmental Aspects of the Cell Cycle*, I. L. Cameron, G. M. Padilla, A. M. Zimmerman, Eds. (Academic Press, New York, 1971), p. 315; I. S. Owens, B. K. Vonderhaar, Y. J. Topper, *J. Biol. Chem.* **248**, 472 (1972).
3. H. S. Grob, *Biol. Reprod.* **5**, 207 (1971).
4. K. L. Barker and J. M. Anderson, *Endocrinology* **83**, 585 (1968); R. H. Maurer and R. G. Chalkley, *J. Mol. Biol.* **27**, 431 (1967); G. Shyamala and J. Gorski, *J. Cell Biol.* **35**, 125A (1967); E. J. Sitko, thesis, University of Colorado (1969); T. W. Glenister, in *Methods in Mammalian Embryology*, J. C. Daniel, Jr., Ed. (W. H. Freeman, San Francisco, 1971), p. 320;
5. D. Williams and J. Gorski, *Biochemistry* **12**, 297 (1973).
6. H. Kuramoto, S. Tamura, Y. Notake, *Am. J. Obstet. Gynecol.* **114**, 1012 (1973).
7. E. I. Kohorn and R. Tchao, *J. Endocrinol.* **45**, 401 (1969).
8. R. S. Krishnan and J. C. Daniel, Jr., *Science* **158**, 490 (1967).
9. J. C. Daniel, Jr., and R. S. Krishnan, *J. Exp. Zool.* **172**, 267 (1969).
10. A. A. El-Banna and J. C. Daniel, Jr., *Fertil. Steril.* **23**, 101 (1972).
11. M. A. Urzua, R. Stambaugh, G. Flickinger, L. Mastroianni, *ibid.* **21**, 860 (1970).
12. F. A. Murray, R. W. McGaughey, M. J. Yarus, *ibid.* **23**, 69 (1972).
13. M. H. Johnson, B. D. Cowan, J. C. Daniel, Jr., *ibid.*, p. 93.
14. C. Kirchner, *ibid.*, p. 131.

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The Casparian Strip as a Barrier to the Movement of Lanthanum in Corn Roots

Abstract. *The effectiveness of the Casparian strip as a barrier to apoplastic movement of solutes from cortex to stele of corn roots was investigated by using lanthanum in combination with electron microscopy. Lanthanum deposits were found only in cell walls and on the outside of the plasma membrane of epidermal, cortical, and endodermal cells up to the Casparian strip. Lanthanum was completely absent from the stele, indicating that the Casparian strip provides an effective barrier to apoplastic movement of solutes. Inhibitory effects of trivalent lanthanum ions on the absorption of potassium ions are discussed in relation to the nature of the lanthanum ion binding site on membranes.*

The endodermis of roots separates the cortex from the vascular tissue (stele). Endodermal cells have a chemically distinct band of hydrophobic wall material known as the Casparian strip which is presumed to act as a barrier to the diffusion of solutes through cell walls from cortex to stele. Electron microscopic studies of the uptake of uranyl ions by barley roots (*1*) and of the distribution of lead-ethylenediaminetetraacetic acid in wheat and carrot roots (*2*) provide evidence for this contention.

The cation La^{3+} does not penetrate cell membranes and can be visualized with the electron microscope. Because of these features, La^{3+} has been used to define extracellular channels in animals (*3*) and the cell wall continuum (apoplast) in plants (*4*). We report here the use of La^{3+} to examine the apoplast of corn roots with particular reference to the function of the Casparian strip.

Primary roots of 4-day-old corn seedlings were incubated in a solution containing La^{3+} (*p*H 5.7) and prepared for electron microscopy (*5*). Fixation at *p*H 7.3 or above resulted in localized deposition of La in cell walls and along the outer leaflet of the plasma membrane of epidermal (not shown) and cortical cells (Fig. 1, A and B). As expected, La^{3+} did not cross the plasma membrane into cells (Fig. 1, A–C).

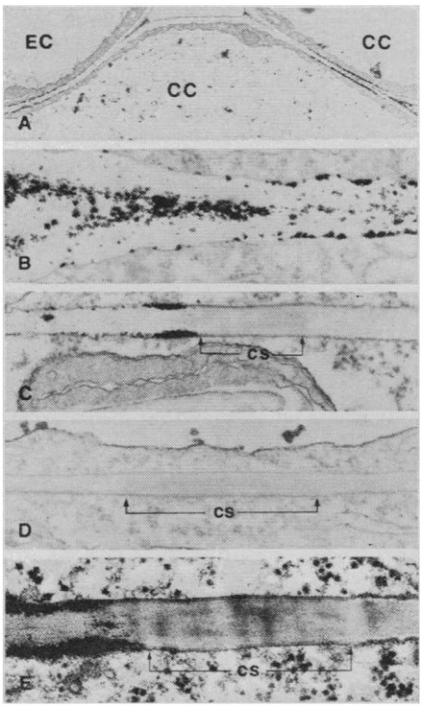

Fig. 1. (A) Lanthanum deposition between two cortical cells (*CC*) and between a cortical cell and an endodermal cell (*EC*) (unstained, × 41,000). (B) High magnification of lanthanum deposition between two cortical cells (unstained, × 27,500). (C) A portion of a radial wall between two endodermal cells showing lanthanum deposition up to the Casparian strip (*CS*). The Casparian strip region is the dark area of the wall designated by the arrows (unstained, × 33,500). (D) Untreated control of the Casparian strip (between arrows) (unstained, × 27,500). (E) Stained preparation showing lanthanum deposition up to the Casparian strip (between arrows) (× 69,000).

Lanthanum was also found deposited along the cortical side of endodermal cells up to the Casparian strip (Fig. 1, C and E). The stelar side of the Casparian strip and the cells in the stele were completely free of La deposits, as were those of untreated control tissue (Fig. 1, C and D).

High magnification of the cell wall region between two endodermal cells (Fig. 1, C and E) showed that La was deposited in the cell wall and along the plasma membrane up to the Casparian strip (dark area of cell wall). Plasma membrane associated with the Casparian strip had a tripartite structure and formed a smooth, tight junction with the wall material (Fig. 1E) (*6*).

Our results indicate that the Casparian strip of corn roots provide a barrier to the diffusion of La^{3+} (and presumably other solutes) in the apoplast from the cortex to the stele. Solutes entering vascular tissue of roots possessing an intact endodermis must do so by first being absorbed into epidermal, cortical, or endodermal cells and then moving through the cytoplasmic continuum (symplast) to the stele.

The association of lanthanum with the outside of the plasma membrane (Fig. 1, A and B) (*3*, *4*) suggests that La^{3+} binds to some site on the membrane. It has been proposed that La^{3+} binds to Ca^{2+} sites on membranes because of correlations between Ca^{2+} exchange induced by La^{3+} and several physiological effects (*7*). Further, in atomic absorption spectroscopy La^{3+} is routinely used to displace Ca^{2+} from complexes with phosphate (*8*). Hence, La^{3+} is believed to bind to, and (or) displace Ca^{2+} from, membrane sites normally occupied by Ca^{2+}.

In corn roots, Ca^{2+} (0.5 m*M* or less) has been shown to be an inhibitor of