the increase in a^{i}_{Ca} of the resting muscle may be due to the release of Ca^{2+} from the sarcoplasmic reticulum or to some other mechanism not related to a sodium-calcium exchange at the sarcolemma.

This study shows that the a^{i}_{Ca} of ventricular muscle cells is 38 n*M* (mean value). This value is equivalent to a sarcoplasmic-free Ca^{2+} concentration of 119 n*M* if one assumes that the activity coefficient of Ca^{2+} in the sarcoplasm is the same as that of extracellular solution (0.32). This assumption is justified provided that the sarcoplasmic ionic strength is not too different from that of the extracellular solution. The value of 119 n*M* is similar to a sarcoplasmic-free Ca^{2+} concentration (*2*) of sheep cardiac Purkinje fibers. The value is also consistent with the range of the sarcoplasmic Ca^{2+} concentrations expected from actin-myosin interaction and the threshold for tension development (*3*). The measurement of a^{i}_{Ca} enables one to calculate that the Ca^{2+} equilibrium potential across the cell membrane is +128 mV. The results we obtained by changing $[Na]_o$ are consistent with those observed by other investigators (*4*). They show that Ca^{2+} movements across sarcolemma depend on both the external and the internal Na^+ concentration. Glitsch *et al.* (*4*) observed that their results are compatible with the idea of an exchange ratio of two sodium ions for each calcium ion and that the energy required for calcium extrusion by the Na-Ca exchange system seems to be provided by the Na gradient across the sarcolemma rather than by metabolic energy. If two sodium ions exchange for each calcium ion (electroneutrality), at equilibrium state the following relation is given: $(a^{i}_{Na})^2/(a^{o}_{Na})^2 = (a^{i}_{Ca})/(a^{o}_{Ca})$. If the ratios are calculated with the a^{i}_{Ca} measured in this study and the a^{i}_{Na} measured by Lee and Fozzard (*5*), $(a^{i}_{Na})^2/(a^{o}_{Na})^2$ is at least an order of magnitude greater than $(a^{i}_{Ca})/(a^{o}_{Ca})$. Whatever the exchange ratio of Na^+ and Ca^{2+} is, this study shows that the sarcoplasmic Ca^{2+} activity was increased by a reduction in the Na^+ activity gradient across the sarcolemma. This is consistent with the close correlation between contractility and intracellular Na^+ activity of sheep cardiac Purkinje fibers in which the Na^+ activity gradient was reduced by an increase in the intracellular Na^+ activity (*6*).

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Estrogen and the Growth of Breast Cancer: New Evidence Suggests Indirect Action

Abstract. *The growth of the MCF-7 human breast cancer cell line is unresponsive to the presence of estrogen in culture media. Paradoxically, in nude mice, growth of these cells and formation of solid tumors are dependent on estrogen. Tumors fail to develop in ovariectomized mice, but do develop in intact mice and in ovariectomized mice given estrogen. Primary cultures derived from MCF-7 tumors revert to unresponsiveness to estrogen. However, when these cultures are again transplanted into nude mice, estrogen is required for tumor formation. The continuous culture, the solid tumor, and the primary cultures therefrom have similar estrogen-binding capacities and affinities. These results indicate that mammary carcinoma cell growth in vivo is subject to inhibition that can be overcome by estrogen.*

Regression of human mammary carcinoma after ovariectomy has been linked to the presence of an estrogen receptor in the tumor (*1*). Indeed, tumors lacking the estrogen receptor seldom regress after ovariectomy. The presence of an estrogen receptor, however, does not always guarantee that ovariectomy will induce regression; about half of the tumors with estrogen receptors do not regress after ovariectomy (*2*).

Estrogen is believed to act on mammary cells by binding to the receptor (*3*). The influence of the estrogen-receptor complex on the growth of mammary epithelium is, however, not well understood. We therefore studied the effects of 17β-estradiol and insulin on the growth of the MCF-7 human mammary carcinoma cell line (*4*) in vitro and in vivo. Despite the presence of estrogen receptor at comparable levels in vitro and in vivo, MCF-7 cells grew without estrogen in vitro, but could not grow in vivo unless estrogen was present. In contrast, insulin, which also acts by binding to its receptor, enhanced the growth of MCF-7 cells both in vitro and in vivo. These findings have been interpreted to indicate that the effect of estrogen and its receptor on mammary cell growth in vivo must involve additional intermediary factors.

Soule and McGrath (*5*) reported that 4 weeks after MCF-7 cells were inoculated into 30 Swiss nude mice given supplementary estrogen, 70 percent developed tumors. The karyotypes of the tumor cell cultures derived from the nude mice were human and contained chromosomal markers characteristic of the MCF-7 cells in the continuous culture. In the present study, within 7 days after 2×10^6 actively growing (log phase) MCF-7 cells were inoculated into the mammary fat pad of each of 142 intact, athymic nude mice, BALB/c strain, 100 percent developed tumors. Tumors did not develop in ovariectomized mice or in mice made diabetic with streptozotocin (*6*). This failure could not be overcome even when the inoculum was increased to 20×10^6 cells for each mouse and when a latency period of 90 days was allowed. Tumors were obtained with 100 percent frequency and within 7 days in ovariectomized mice given a single injection of 0.1 mg of estradiol valerate at the time of cell inoculation. Similarly, tumors were obtained when each of the ovariectomized nude mice was given 10 μg of 17β-estradiol in the form of a pellet placed subcutaneously in the interscapular region. Tumors were also obtained with 100 percent frequency in nude mice made diabetic and given injections of insulin (0.2 I.U.) daily for 2 weeks.

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Table 1. Effect of insulin and 17β-estradiol on growth of MCF-7 cells in culture. Two cultures were tested for growth response: (i) passage 246 to 251 of the continuous culture and (ii) passage 3 of cell culture 1, derived from the enzymatic dissociation (*7*) of MCF-7 tumors grown in nude mice. Cells were regularly passed into flasks once every 2 weeks. Every other day, minimum essential medium–Hanks balanced salt solution (20 ml), supplemented with 2 m*M* L-glutamine, 10 μg of bovine insulin per milliliter, 100 units of penicillin, and 100 μg of streptomycin per milliliter, and 10 percent fetal calf serum were added to each flask. For hormone treatments, the cells (5×10^6 per flask) were grown for 3 days in the above medium before they were transferred to a hormone- and serum-free medium (control) supplemented with either insulin (10 μg/ml) or 17β-estradiol (10^{-11} to 10^{-8}*M*). Media were changed every other day for six additional days. Cell counts were determined after this period by a hemocytometer. Data shown are the means of 8 to 15 determinations ± standard error of the mean (S.E.M.).

Culture	Percent of cell number (100 percent = 18.5×10^6 cells)		
	Control	Control + insulin	Control + 17β-estradiol
Continuous	100 ± 16	212 ± 39	106 ± 21
Cell culture 1	100 ± 16	191 ± 33	96 ± 16

Table 1 shows a comparison of sensitivity of cell growth to 17β-estradiol and to insulin between the continuous culture and the primary culture. Cell culture 1 was derived from the enzymatic dissociation (*7*) of MCF-7 tumors (T_1) developed by an estrogen-dependent process in nude mice. The data demonstrate two important points. (i) The growth stimulation by insulin in vitro is similar to that in the nude mouse system. Even under serum-free culture conditions, insulin treatment doubled the cell number, an indication that there is direct interaction between insulin and the tumor cell. (ii) In contrast to the in vivo situation, estradiol at 10^{-11} to 10^{-8}*M* had no effect on the rate of cell growth in vitro. That cell growth was not sensitive to estrogen was demonstrable in the absence or presence of 2 to 10 percent fetal calf serum or in serum stripped with dextran-coated charcoal (data not shown).

The inability to demonstrate a growth effect on MCF-7 cells by estrogen in vitro has also been reported by other investigators (*5, 8*). It is not likely that this lack of growth response is due to the presence of residual estrogen in the medium or in the cell, because the induction of progesterone receptor (*9*) and plasminogen activator (*10*) by estrogen can be demonstrated under conditions in which there is no effect on the growth rate.

Curiously, growth and solid tumor (T_2) formation in nude mice induced by cell culture 1 were again estrogen-dependent. Dissociated and cultured cells (cell culture 2) from T_2 tumors responded in vitro to insulin with increased growth, but did not respond to 17β-estradiol. Similarly, T_3 tumors were induced in nude mice by cell culture 2 in an estrogen-dependent process.

The in vitro–in vivo environments under which sensitivity of cell growth to estrogen is altered could not be linked to changes in the estrogen receptor. As shown in Table 2, specific binding in the cytosol of the continuous culture was similar to that in the cytosol of T_1, T_2, and T_3 cells, whether freshly dissociated or maintained in short-term culture. The binding affinity was also unchanged ($K_d \approx 5 \times 10^{-10}$*M*). Because of the unusual cytoplasmic and nuclear estrogen receptor distribution in the MCF-7 cells (*11*), receptor levels were also determined in intact cells (Table 2) by the method described in (*12*). The similarity in estrogen-binding affinity and capacity under these different conditions indicates that estrogen responsiveness in vivo and the lack of it in vitro cannot be attributed to changes in the estrogen receptor.

Estrogen-dependent growth of breast cancer cannot be reproduced under classical culture conditions. The back-and-forth in vivo–in vitro extrapolation with MCF-7 cells described in this report demonstrates unequivocally that the cells have retained their hormone response system. Because MCF-7 cells do not grow without estrogen in vivo, but can grow without estrogen in cultures, these cells must be subject to inhibitory control in vivo. Estrogen may be influencing cell growth through an indirect pathway, presumably by depressing the inhibitory effect.

The effect of estrogen in vivo described above is obtained with estrone, estradiol, and estriol, but not with progesterone, testosterone, or hydrocortisone (*13*). This selective estrogen effect, therefore, may well involve a specific estrogen-receptor complex that controls the induction or stimulation of gene product synthesis by the target cell. The gene product may be a differentiation product, such as the progesterone receptor, or a product that could function as a growth de-inhibitor. Should the synthesis of such a gene product occur constitutively in certain tumors, this would explain why some malignant cells retain their estrogen receptor, but can also be endocrine resistant. Confirmation of such hypothesis would first require that the inhibitor be defined and identified.

Table 2. Levels of 17β-estradiol receptor in MCF-7 tumor cells and cytosol. Receptor levels shown below were determined as described by Shafie and Brooks (*12*). The designation T_1 represents freshly dissociated cells of MCF-7 tumors derived from the implantation of the continuous culture into nude mice; cell culture 1 represents T_1 cultures at day 9 of the third passage (approximately 40 days in culture). Similarly, T_2 represents freshly dissociated tumor cells derived from cell culture 1, and T_3 represents tumor cells derived from cell culture 2. The binding affinity ($\approx 5 \times 10^{-10}$*M*) and capacity were estimated from a graphical analysis of the binding data according to the Scatchard equation (*14*). Data shown are the means of five to six determinations ± S.E.M.

Culture	Receptor levels	
	Cytosol (fmole/mg protein)	Intact cells (fmole/mg DNA)
Continuous culture	120 ± 23	1603 ± 338
T_1	84 ± 14	1401 ± 218
Cell culture 1	115 ± 16	1806 ± 313
T_2	88 ± 22	1299 ± 289
Cell culture 2	122 ± 26	1497 ± 114
T_3	90 ± 18	1507 ± 163
Cell culture 3	114 ± 18	1550 ± 201

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