# Effect of Estradiol on Human Breast Cancer Cells in Culture

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## ABSTRACT

Conditions are described for growing and maintaining the estradiol sensitivity of the human breast cancer cell line ZR-75-1 both in monolayer and suspension cultures. Either new-born calf or fetal calf serum can be used in the culture medium, but an effect of estradiol on growth of the cells was only observed reproducibly if the serum was first treated with dextran-charcoal. Sulfatase treatment of the sera prior to dextran-charcoal treatment did not decrease cell growth in the absence of added estradiol, indicating that estrogen sulfates are unlikely to contribute to cell growth in dextran-charcoal-treated sera.

In monolayer cultures, estradiol increased both the growth rate and final saturation density of the cells for each individual plating density tested in a dose-dependent manner with maximal stimulation occurring between $10^{-10}$ and $10^{-8}$ M estradiol. Estradiol also markedly increased the ability of the cells to grow both in suspension and semisolid Methocel cultures. In suspension, the cells grew as tight balls which clustered together to give small organoid-like structures reaching diameters of 4 mm and composed of an outer shell of living cells containing a central cavity of necrotic cells.

In the absence of estradiol in both monolayer and suspension, the cells went through a limited and constant number of divisions and then stopped, such that the final cell number was determined by the initial plating density. In the presence of estradiol, this block was removed such that in monolayer cultures the final cell number was independent of plating density. A major loss of estradiol response was found if the cells were grown for 7 to 14 days in the absence of estradiol. This loss of response appeared to be due to a loss of ability to grow rather than to selective cell death within the population.

## INTRODUCTION

Breast cancer cell lines that will respond to sex steroids in culture are limited, and for cells of human origin only 4 choices are available, MCF-7, T-47-D, CAMA-1, and ZR-75-1. Most attention has been directed at the MCF-7 cells particularly with regard to hormone effects on the estrogen (5, 10, 12, 18) and progesterone receptor machinery (7, 10). The latter topic has also been discussed with T-47-D (9, 11, 13), CAMA-1 (28), and ZR-75-1 (1) cells. These data attest to the estrogen sensitivity of all these cell lines but are of dubious relevance to the question of estrogen effects on proliferation. Several publications indicate that hormone effects on progesterone receptor are not necessarily related to effects on cell number or thymidine incorporation (1, 7, 11, 28). Proliferation of T-47-D cells is not influenced by estrogens (11) while the CAMA-1 cells which were originally sensitive became unresponsive with pas-

sage (28). The effect of estrogen on MCF-7 cell number has been controversial, but experiments using [$^3$H]thymidine have yielded more consistent data (16, 17). As Lippman and Aitken (16, 17) point out, estrogen effects on [$^3$H]thymidine incorporation into MCF-7 cells are complex, and we therefore thought that a human mammary tumor cell line that reproducibly responded to estrogen by changes in cell number was required. The published data indicated that ZR-75-1 cells might be such a line.

The ZR-75-1 cells respond to estrogens (8) even in serum-free conditions (2) and contain receptor proteins for several classes of steroid (8, 16), the progesterone receptor concentration being estrogen sensitive (1) as is the secretion of certain proteins (25). However, many features of the behavior of ZR-75-1 cells in culture have not been described. The original major publication described the morphology, karyology, and growth kinetics of monolayer cultures in 5% serum with and without $10^{-8}$ M estradiol (8). A subsequent study reported the requirements and estradiol sensitivity of the cells in serum-free medium (2). The present paper describes in greater detail the dose response effects of estradiol, FCS,[3] and new-born calf serum on ZR-75-1 cells in monolayer culture. We further describe the growth behavior of these cells in suspension culture and the culture conditions under which we obtain reproducible estrogen effects on cell number.

## MATERIALS AND METHODS

**Cell Culture.** The ZR-75-1 cells were kindly provided by Dr. M. Lippman (National Cancer Institute, Bethesda, Md.) such that they were originally of about the same passage generation as previous experiments (2, 8). Stock ZR-75-1 cells were grown routinely as monolayer cultures in DMEM supplemented with 5% FCS (Gibco-Biocult, Glasgow, Scotland) and $10^{-8}$ M estradiol in a humidified atmosphere of 10% carbon dioxide in air at 37°. Cells were subcultured at weekly intervals.

**Growth of Cells as Monolayer Cultures.** Cells from stock plates were suspended by treatment in 0.06% trypsin buffered with 0.02% EDTA (pH 7.3) and counted on a hemocytometer. Cells were added to the overall required volume of DMEM containing 10% DC-CS but no estradiol and, unless otherwise specified, were seeded at a density of between 0.1 and 0.2 × $10^5$ cells/ml. Cells were plated into 50-mm plastic tissue culture dishes (Nunc, Denmark) in 5-ml aliquots for 24 hr, and then the medium was changed to the appropriate amount and type of serum with or without estradiol. To investigate the effects of estradiol deprivation, the cells were kept in medium lacking estradiol for the appropriate number of days and then the medium was changed to DMEM containing estradiol. Plating efficiency of the cells was determined by counting the cells in monolayer after 24 hr for 3 dishes by the method described below. The entire culture medium on the cells was changed routinely in all dishes every 3 to 4 days.

**Treatment of Serum.** DC treatment of sera was carried out by incubation with 0.5% charcoal (Sigma, Poole, United Kingdom) plus

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[3] The abbreviations used are: FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; DC-CS, dextran-charcoal-stripped newborn calf serum; DC, dextran-charcoal; DC-FCS, dextran-charcoal-stripped fetal calf serum.

0.05% dextran T-70 (Pharmacia, Uppsala, Sweden) for 30 min at 55°. Treatment of serum with sulfatase was by incubating 2 units sulfatase (Sigma) per ml DC-treated serum for 2 hr at 37°. The serum was then restripped with DC in the usual way.

**Measurement of Estradiol and Testosterone in Serum.** The immunoassays were kindly performed by Dr. I. Rincon at the Chelsea Hospital for Women, London, England.

**Growth of Cells as Suspension Cultures.** Cells were grown in the same medium as for monolayers in 50-mm plastic bacteriological dishes (Sterilin, Teddington, England) to which less than 2% of the cells could attach. As the medium could not be completely renewed periodically, 1 ml of fresh extra medium was added to each dish every 3 to 4 days. When grown in Methocel, the cells were grown in the same way but the medium contained 1% methyl cellulose.

**Cell Counts.** Cells in monolayer cultures were washed in situ with phosphate-buffered saline and then lysed in 2 ml 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma) containing 1.5 mM $MgCl_2$ plus 4 drops of Zaponin (Coulter Electronics, Ltd., Harpenden, England) for 5 min. The nuclei released were counted in Isoton (Coulter Electronics, Ltd.) in triplicate on a Model ZB1 Coulter Counter.

Cells in suspension were harvested in phosphate-buffered saline, spun down on a bench centrifuge, and washed twice. The cells were lysed and counted as for monolayer cultures, except that lysis took 1 hr.

Viability of cells was checked by washing the cells in situ 3 times with 0.9% NaCl solution and adding 5 ml of trypan blue (0.1%) in 0.9% NaCl solution.

**Histology of Cells in Suspension Cultures.** Cells were harvested and washed in phosphate-buffered saline, allowing the cells to settle under gravity without centrifugation. Cells were fixed in Bouin's fixative for 2.5 hr, transferred to 70% ethanol, and then embedded in wax prior to sectioning and staining with hematoxylin and eosin.

**Time Lapse Photography.** Two 50-mm dishes of cells were set up as for monolayer cultures, and after 24 hr the medium was changed so that one dish contained $10^{-8}$ M estradiol but the other contained no estradiol. Time lapse photography was carried out using an Olympus camera programmed to take one frame of film every 6 min for 13 days.

## RESULTS

ZR-75-1 cells grown in untreated FCS show variable growth responses to estradiol dependent on the batch of FCS used. One batch of FCS gave little if any growth response (Chart 1A), but other batches have shown small growth responses (data not shown). However, a pronounced and reproducible effect is seen if DC-FCS is used (Chart 1B). DC treatment of the FCS results in the removal of a growth factor(s) the activity of which can be replaced by addition of estradiol. Estimation of estradiol in the serum (used in Chart 1A) before and after DC treatment indicated a reduction from 84.0 to 0.6 pmol/liter while testosterone levels fell from 0.99 to 0.09 pmol/liter. Thus, DC treatment removed greater than 90% of unconjugated steroids measured. Complementary experiments in which the FCS was equilibrated with various $^3$H-steroids prior to DC treatment indicated that the latter removed greater than 90% of the added steroid. We therefore conclude that the DC effect on cell growth can be explained by the removal of estrogens and/or growth factor(s) whose absence can be overcome by readdition of estradiol. The limited proliferation still occurring in DC-FCS without added estradiol was further investigated. The residual estradiol would provide a final concentration of about $10^{-14}$ M in the incubation medium (5% DC-FCS) used for the experiments shown in Chart 1B. Serum contains large amounts of estrogen sulfates so we tested the possibility that they might

be the growth factor in FCS. FCS was hydrolyzed with sulfatase prior to DC treatment and then tested in the culture system. Sulfatase treatment had no effect on cell number either in the absence or presence of estradiol. Furthermore, equilibration of FCS with a steroid sulfate ([$^3$H]dehydroepiandrosterone sulfate) prior to DC treatment indicated that greater than 98% of the sulfate had been removed.

FCS can be replaced by newborn calf serum without detriment to the estradiol response, provided that the serum is DC treated. The growth response of ZR-75-1 cells to estradiol in 10% DC-CS is identical to that shown for 5% DC-FCS in Chart 1B. Optimal growth in the presence of estradiol was obtained with 10% DC-CS (Chart 2). In the absence of estradiol, high concentrations of either serum were inhibitory. This inhibitory effect in the absence but not in the presence of estradiol
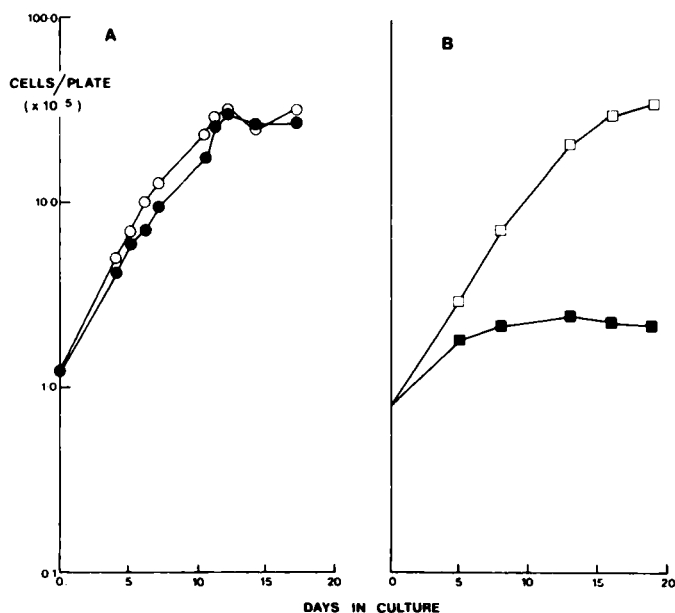


Chart 1. Growth of ZR-75-1 cells in monolayer culture in 5% FCS (A) or 5% DC-stripped FCS (B) in the presence (O, □) and absence (●, ■) of $10^{-8}$ M estradiol. Symbols, mean ± SE. No error bars are shown if the variation was too low for visual display.
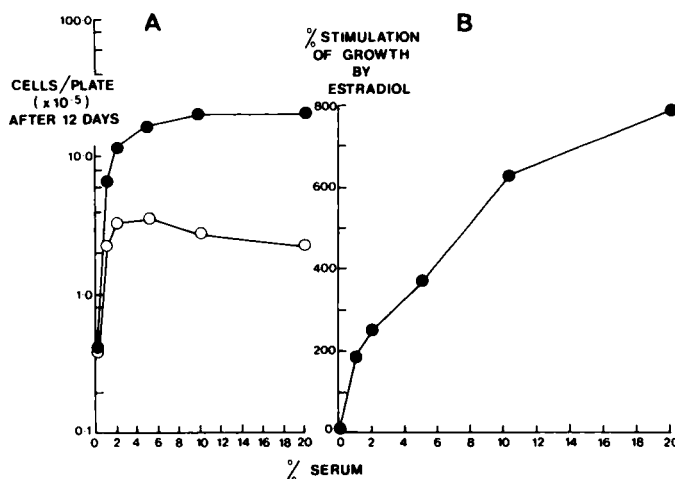


Chart 2. Effects of serum concentration on the growth of ZR-75-1 cells for 12 days in monolayer culture. A, growth in 10% DC-CS in the presence (●) and absence (O) of $10^{-8}$ M estradiol; B, percentage of stimulation by estradiol of the growth of the cells in DC-CS.

resulted in a more pronounced stimulation by estradiol at high serum concentration (Chart 2). We decided that for routine experiments either 5% DC-FCS or 10% DC-CS would be used. For reasons of economy, DC-CS was preferred to DC-FCS. The plating efficiency in either 5% DC-FCS or 10% DC-CS was always about 100% and was unaffected by presence or absence of estradiol. Experience with different batches of either serum indicated that it is important to check cell growth in each batch after DC treatment. Sometimes a second DC treatment is required. However, when effectively stripped, the DC serum gives reproducible results which are independent of batches or type of serum. A total of 32 growth curves have been done over a period of 10 months, giving identical data to that in Chart 1B on every occasion irrespective of batch of serum.

Estradiol also markedly affected the ability of ZR-75-1 cells to grow in suspension culture (Chart 3). Unlike monolayer cultures, the cells do not distribute themselves uniformly in suspension but rather grow as tight balls of cells which themselves tend to cluster together. These clusters can attain diameters of 4 mm. The tight balls are made up of viable cells while the clusters have an outer layer of viable cells enclosing a cavity containing necrotic cells (Fig. 1).

Estradiol effects on growth in 1% methyl cellulose were similar to those described for suspension culture. Growth curves were determined and were identical to those shown in Chart 3. The only difference was that the balls of cells were smaller (maximal size, 0.1 mm) and did not cluster together.

Chart 4 shows the correlation between initial plating density and final saturation density both in monolayer and suspension cultures in the absence (Chart 4A) and presence (Chart 4B) of estradiol. Final saturation density was determined in each case from the plateau of a full growth curve. A highly significant linear relationship was observed between these 2 parameters in the absence of estradiol. Statistical analysis including the data on Chart 4A for both monolayer and suspension cultures yielded a correlation coefficient of 0.98, and the slope of the

line (determined by least squares analysis) indicated that, regardless of plating density, the cells went through a mean 1.5 doublings and then stopped. Chart 4B shows that, in the presence of estradiol, saturation density was independent of plating density for cells in monolayer (correlation coefficient, 0.53) but not in suspension. The lower cell density seen with suspension as compared with monolayer culture in the presence of estradiol may be an artifact caused by either the limited diffusion of essential nutrients into the center of the clusters or medium deprivation or build-up of toxic materials because medium could not be completely changed as for monolayers.

For routine purposes, an initial plating density of $0.5 \times 10^5$ cells/5-cm dish was chosen, and Chart 5 shows the effects of different concentrations of estradiol on cell growth in monolayer at this plating density. Optimal growth required $10^{-10}$ M
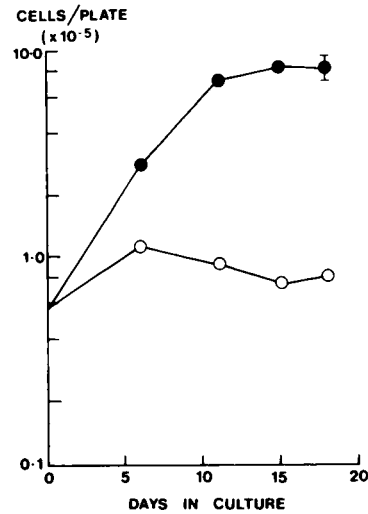


Chart 3. Growth of ZR-75-1 cells in suspension culture in 10% DC-CS in the presence (●) and absence (O) of $10^{-8}$ M estradiol. *Bars*, S.E. No error bars are shown if the variation was too low for visual display.
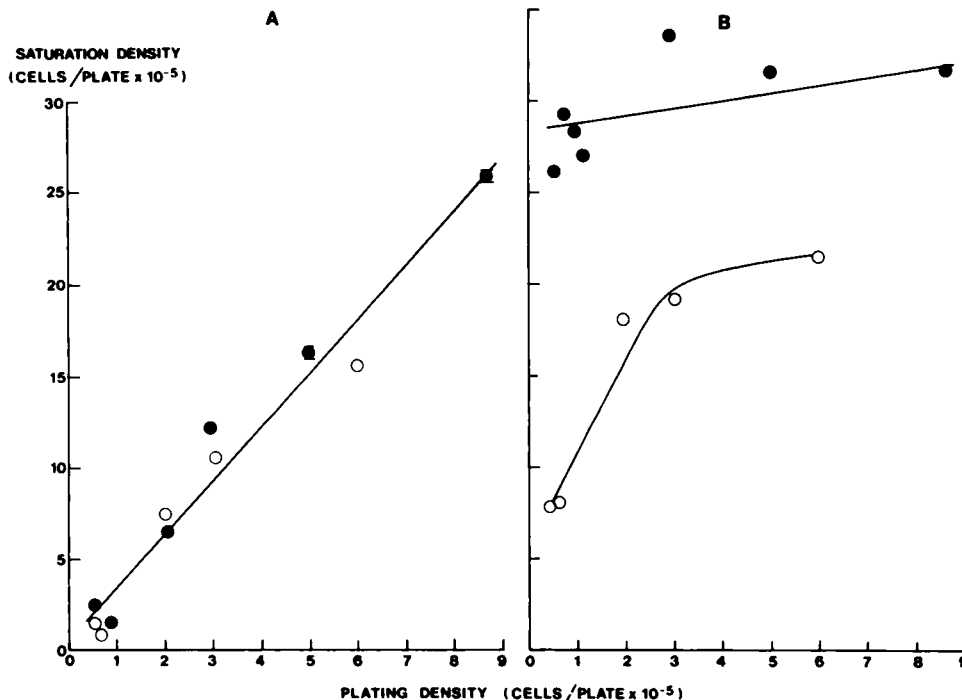


Chart 4. Effect of plating density of ZR-75-1 cells grown in 10% DC-CS on saturation density in 5-cm tissue culture dishes in the absence (A) and presence (B) of $10^{-8}$ M estradiol for both monolayer (●) and suspension (O) cultures. *Bars*, S.E.
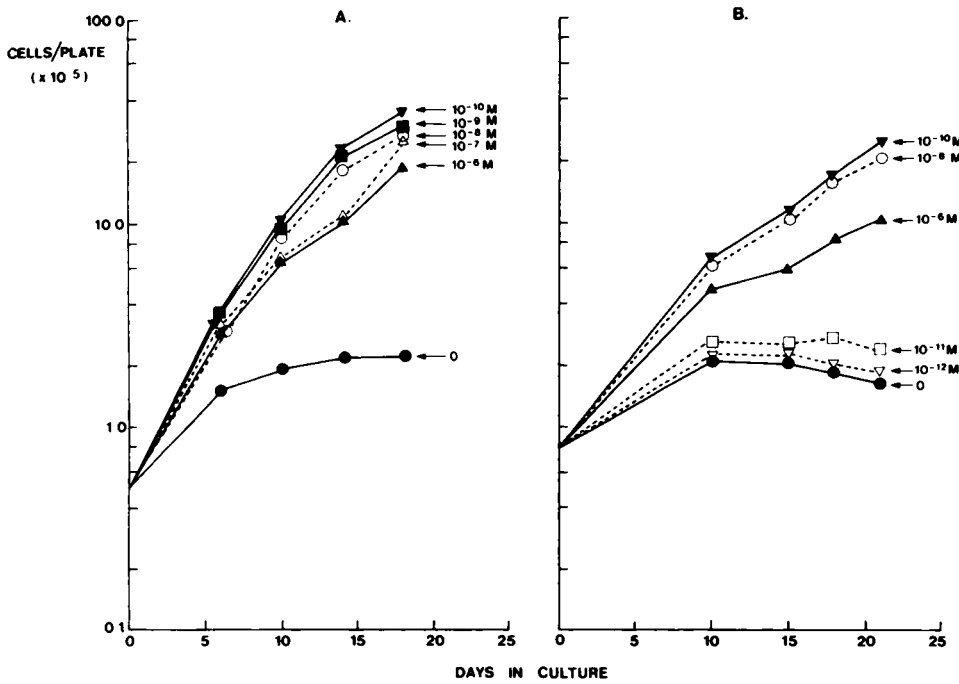
Chart 5. Effect of different concentrations of estradiol on the growth of ZR-75-1 cells in 10% DC-CS in monolayer cultures. A and B, 2 experiments carried out at different times.

estradiol, and a dose-dependent inhibition of growth was noted at concentrations in excess of $10^{-8}$ M. Similar responses were seen in DC-FCS (data not shown).

Chart 6 shows the effects of short-term estradiol deprivation on subsequent growth of ZR-75-1 cells in monolayer in the presence of $10^{-8}$ M estradiol. A major loss of estradiol response was found between 7 and 14 days after withdrawal of estradiol.

Time lapse photography indicated very clearly that in the absence of estradiol ZR-75-1 cells gradually stop dividing until by 7 days they divide no longer. However, between 7 and 13 days, the cells were clearly still alive, in particular because they could still move. Cell death was very rare. In addition, trypan blue staining of the cells at intervals up to 17 days indicated at least 99% viability of the cells.

## DISCUSSION

For reasons outlined in the introduction, we felt that most of the currently available human mammary tumor cell lines had major disadvantages if one wished to study estrogen effects on cell growth. From our own experience with MCF-7 cells (data not shown) and with ZR-75-1 cells reported here, we feel that the latter cells are more useful for studying proliferative effects of estrogens. We report here details of the culture conditions required to obtain reproducible estradiol effects on cell number that supplement those provided in the earlier publications (2, 8). Our studies on the androgen sensitivity of S115 mouse mammary tumor cells indicated that reponsiveness was lost if the cells were cultured for 1 to 2 weeks in the absence of androgen (15, 26, 27). Analogous data have been reported for the estrogen sensitivity of CAMA-1 cells (28), and the experiments reported here indicate a similar loss of response for the ZR-75-1 cells. The cause of this lost sensitivity is not known but is unlikely to be due to inactivation of the purine biosynthesis pathway (17) since thymidine will not prevent loss of sensitivity (data not shown).

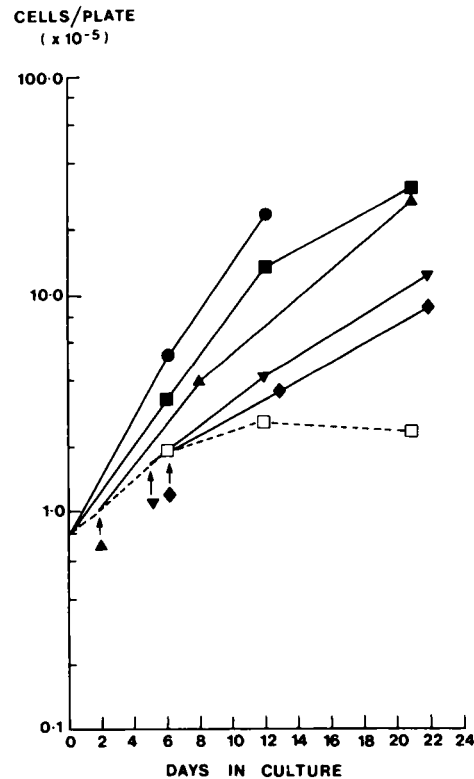Although it is customary to refer to "growth and proliferation

CELLS/PLATE
( x 10⁻⁵ )



Chart 6. Effect of estradiol deprivation for various lengths of time on the subsequent growth in the presence of $10^{-8}$ M estradiol of ZR-75-1 cells in DC-CS in monolayer cultures. After plating out the cells in the absence of estradiol, estradiol was added immediately to dishes for one growth curve (●). The rest of the cells were left to plate, as usual, for 24 hr in the absence of estradiol. The cells were then grown as follows: □, no estradiol; ■, plus estradiol; deprived of estradiol for 2 (▲), 5 ;(▼), or 6 (◆) days and then plus estradiol.

effects of estradiol," an alternative explanation is that the hormone is necessary to maintain cell viability such that culture in the absence of estradiol results in cell death. Under the conditions used here, most of the cells do remain viable as

judged by their continued attachment to the substrate, negative trypan blue staining, and characteristic life movements seen on time lapse photography. Thus, culture in the absence of estradiol does not result in an equilibrium between growth and cell death but rather in the cells remaining alive, unable to divide. This indicates that ZR-75-1 cells do appear to be far more dependent on estradiol for continued growth (and not just viability) in culture than other breast cancer cell lines described to date.

Several of our data are worth comment in relation to the question of how steroids affect the proliferation of mammary tumor cells. It is still uncertain as to whether estrogens are mitogenic on their own or whether they exert their proliferative effects indirectly (19, 20). Indirect effects via other organs (19) are not relevant to this test system and will not be discussed further whereas indirect effects causing changes in sensitivity to other agents could be important (14, 20, 26). Part of the argument about direct or indirect mitogenic effects of estrogens centers on the ability of cells to grow in serum without additional hormones. This can be interpreted either by saying that estradiol is changing a growth rate determined by other agents in the culture system or that DC-treated serum still contains sufficient estrogen to maintain a basal growth rate. Such estrogens could be derived by their long-term retention within the cell (22) or from sulfate conjugates in the serum (24). Our data with sulfatase-treated sera do not support the latter suggestion.

Our studies with androgen-responsive S115 mouse mammary tumor cells in culture have led us to suggest that steroids alter proliferation by changing cell structure such that the cells are more sensitive to other agents (26, 27). In particular, they enable cells to grow to higher saturation densities and to grow in suspension culture. The data presented here indicate that the same is true for ZR-75-1 cells. Indeed, the present results indicate that in the absence of estradiol cell division is very limited. As changes in growth rate and saturation density of cultured cells can occur independently of each other (4, 21), experiments on steroid-modulated growth of cultured cells should take cognizance of the fact. With S115 cells, the changes in growth rate are accompanied by dramatic changes in cell morphology and adhesion to the substratum (6, 27). Our preliminary impression with ZR-75 cells is that morphological changes do occur but that they are less well defined than in the mouse cells. The same is true for MCF-7 cells (3, 23). This topic is being investigated further.

Finally, our observation that ZR-75-1 cells grown in the absence of estradiol stop after a mean 1.5 doublings but continue past this point in the presence of hormone could provide a clue as to the site(s) of action of estradiol within the cell cycle. Estradiol enables the cells to overcome a block and cell kinetic analysis might help to define the locus of that block.

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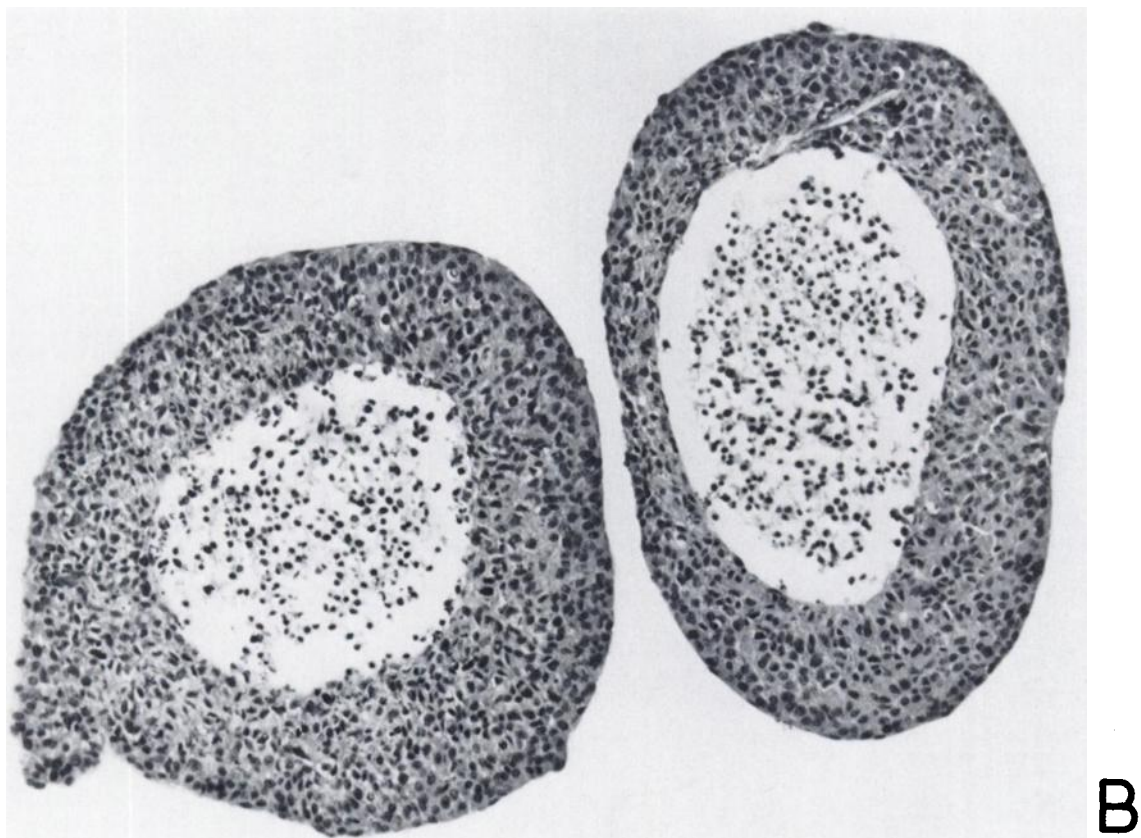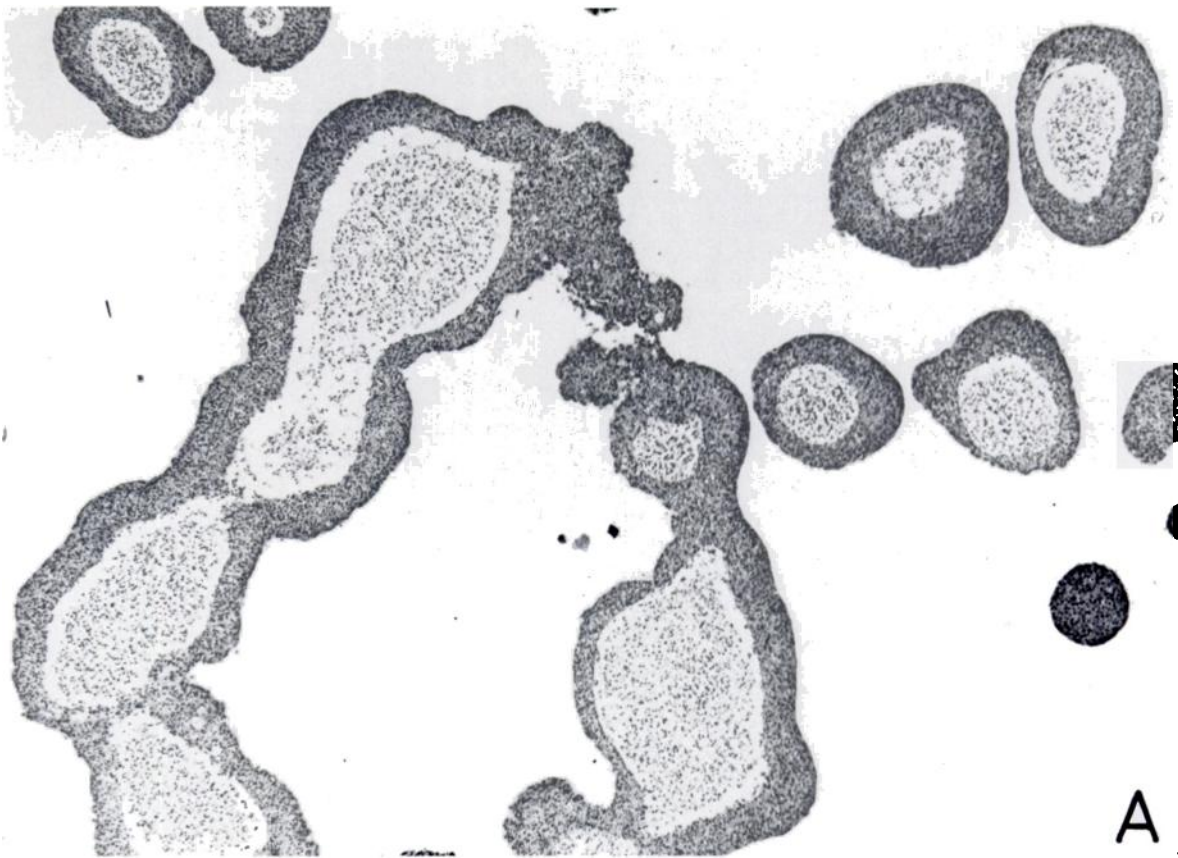
Fig. 1. Growth of ZR-75-1 cells in suspension culture. Cells grow as balls of cells which tend to cluster. The small tight balls are made up only of viable cells (*A, lower right side*) while the larger balls and clusters contain an inner cavity of necrotic cells (*A* and *B*). *A*, H & E, × 10; *B*, H & E, × 40.