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## Effects of Natural Estrogens on L Strain Fibroblasts in Tissue Culture.* (27494)

ROBERT J. KUCHLER AND ROBERT C. GRAUER
*Department of Microbiology, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.*

Several hormonally active steroids inhibit growth of tumor cells(1), microorganisms(2) and tissue cultured cells(3). The most frequently observed physiological effects on microorganisms are involved with suppression of cell growth, or cell function such as ion, sugar or amino acid accumulation(4). Holden and Adams have described the inhibitory reaction by corticoids on L strain fibroblasts (5), and estrogens have been shown to exert an inhibitory action on HeLa cells comparable to that induced by corticoids(6). The L strain system, because of its accessibility to quantification in a serum free medium, was chosen as a model animal cell to study *in vitro* to clarify the nature of the primary interaction between mammalian cells and estrogens.

*Materials and methods.* A replicate series of L strain (929) fibroblast cultures was set up in 150-ml milk dilution bottles; a series consisted of approximately 100 bottles. Each culture was established by inoculating a 10-ml portion from a large suspension containing about $3.0 \times 10^5$ cells/ml in medium 199 supplemented with 0.5% bacto-peptone (199P) (7). After 24 hours at 37°C, the populations of cells attached to the glass reach the early exponential phase of the growth cycle; at this time the used medium was decanted, and 10 ml of fresh 199P medium containing the test steroid was placed on the cell sheets.

Estrone, estradiol-17$\beta$ and estriol were dissolved in propylene glycol at a concentration of 1 mg/ml. These stock concentrates were sterilized by autoclaving. Chromatography in a 2 phase system showed that the steroids were not altered(8). A 1:100 dilution of the stock in medium 199P gives an estrogen concentration of 10 $\mu$g/ml; this is $3.5 \times 10^{-5}$ M for each of the estrogens. The limiting solubility for estrone and estradiol in this medium is about 20 $\mu$g/ml; estriol is more readily soluble. Dialyzed horse serum was added to medium 199P in some experiments which increased the solubility of these steroids, and at a 20% serum concentration, the solubility of estrone and estradiol was doubled. Experiments were controlled by setting up a series of cultures which were bathed in 199P containing 1% propylene glycol.

At the time the medium containing the test steroids was added to a culture series, 9 bottles were set aside, and harvested as the zero time controls. At subsequent 24-hour intervals, through a period of 6 days, 9 bottles from the control set and 9 bottles from the test set were harvested. In harvesting, the medium was decanted from the bottles and centrifuged to pack the cells that became detached from the glass at the higher estrogen concentrations. The bottles were allowed to drain onto a paper towel for a few minutes. Three milliliters of cold 5% TCA was added to each to bathe the cell sheet. The cells were then scraped from the glass into the cold TCA and this precipitated suspension was combined with its respective pack of detached cells. The contents of 3 bottles from each set were combined in a 15-ml conical centrifuge

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tube; this provided 3 zero time samples, and 3 samples for each 24-hour test point. The precipitated cells in the TCA were preserved at −20°C until completion of the experiment, at which time the samples were analyzed for DNA by the method previously described(9) and for protein by the Folin reaction(11).

In another experiment, a series of 52 bottle cultures was set up. Estrone or estradiol at $3.5 \times 10^{-5}$ M in 199P media was added to one-half of the bottles after 48 hours; fresh media was placed into the remaining 26 bottles which served as controls. After 24 hours, 3 bottles from each set were harvested by scraping the cells into the bathing medium. Ten-ml aliquots were centrifuged in vaccine tubes to determine the packed cell volumes (9). Five ml of the media was removed for glucose determinations(10). The cells were resuspended and the diameters of 100 cells from each set were measured with an ocular micrometer(10). Twenty-four hours later, the remaining 46 bottles were harvested. The packed cell volumes and cell diameters were again measured using 3 bottles from each set. The 2 suspensions from the remaining 40 bottles were packed into large conical centrifuge tubes. These packed cells were washed once with Hanks' balanced salt solution. After decanting the washing fluid, the cells were extracted with 5% cold TCA to separate the intracellular amino acids(7). The cold TCA extract was analyzed quantitatively for the constituent free intracellular amino acids by the methods of Moore, Spackman and Stein (12). Fresh media 199P and used media 199P were analyzed to determine the amount of each amino acid consumed. All samples were extracted with 10 volumes of ether, and properly acidified before being placed onto 150 cm cation exchange columns.

*Experimental results.* Fibroblast cultures were established, so that a uniform sheet of about $3.5 \times 10^6$ cells in the early logarithmic growth phase covered one of the inner surfaces of each milk dilution bottle after 24 hours. At this time 199P medium containing the test steroid was placed on the cultures. Fig. 1 shows the effect of the 3 common estrogens on DNA and protein synthesis in these populations during the growth cycle. When

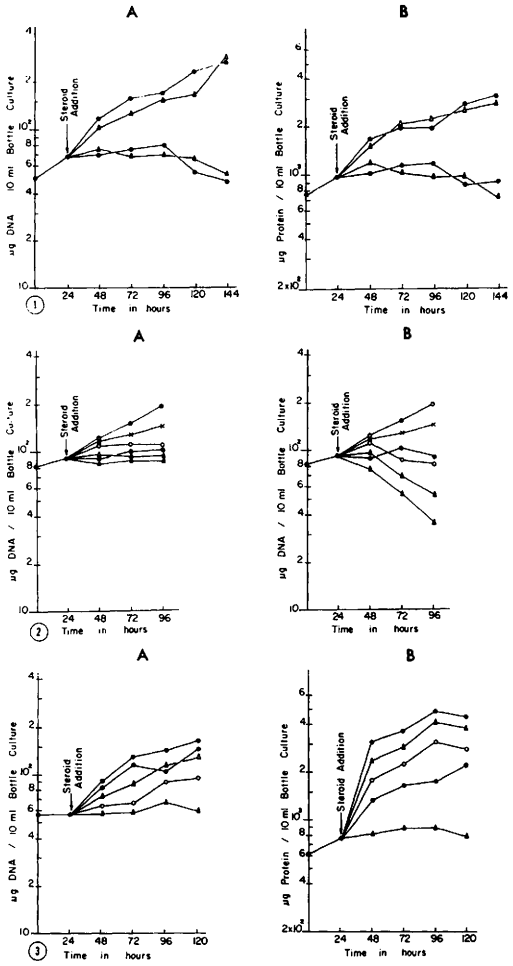


FIG. 1. Effect of estrogens at $3.5 \times 10^{-5}$M in tissue culture. Medium 199P on synthesis of DNA and protein in L strain fibroblast populations.
(⊙), control (△), estriol
(▲), estradiol (●), estrone

FIG. 2. Effect of varying estradiol concentration in medium 199P on synthesis of DNA in L strain fibroblast populations.
(⊙), $10^{-7}$M (X), $9 \times 10^{-6}$M
(○), $1.9 \times 10^{-5}$M (●), $2.7 \times 10^{-5}$M
(△), $3.7 \times 10^{-5}$M (▲), $5.0 \times 10^{-6}$M

FIG. 3. Reversal of estradiol inhibitory reaction by horse serum.
(⊙), control, no estradiol (△), 20% horse serum
(○), 10% horse serum (●), 5% horse serum
(▲), estradiol control

the steroid additions were made, each culture contained approximately 66 μg of DNA and 950 μg of total cell protein. Estrone and estradiol exerted an inhibitory effect on these cell syntheses to about the same extent. This occurs almost immediately upon contact since only small additional increments in DNA and

TABLE I. Estrogen Effects on Population Volume, Individual Cell Size and Glucose Uptake in L Strain Cultures.

| | Control cultures in 199P | | | Estrone | | Estradiol | | Estriol | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 3.5 × $10^{-5}$M in 199P | | | | | |
| | 0 time | 24 hr | 48 hr | 24 hr | 48 hr | 24 hr | 48 hr | 24 hr | 48 hr |
| Packed cell vol/ 10 ml bottle culture, μl | 12 | 27 | 34 | 14 | 16 | 14 | 15 | 23 | 31 |
| Avg cell diameter, μ | 16.2 | 16.8 | 15.2 | 16.2 | 15.5 | 16.0 | 15.6 | 15.8 | 15.5 |
| Residual glucose, mg/ml of media | 1.10 ±.08 | .86 ±.06 | .70 ±.07 | — | — | 1.06 ±.09 | .91 ±.08 | — | — |

protein synthesis were noted thereafter. Estriol does not affect these syntheses at a comparable concentration. Cell populations in the presence of this estrogen as well as control cultures produce about 220 μg of DNA and 2400 μg of protein in the course of the experiment.

Fig. 2A shows that the rate of DNA synthesis varies inversely with the external estradiol concentration in medium 199P. There was no inhibition at $10^{-7}$ M and complete cessation occurred at about 3.5 × $10^{-5}$ M. In the completely inhibitory range, about 3.5 × $10^{-5}$ M, another aspect was obesrved in these populations, depicted in Fig. 2B. The first visible evidence of estrogen inhibition was noted a few hours after initial estradiol-cell contact; the cells began to round up and eventually fell off the glass, indicating that their capacity to form stable bonds with the glass surface was impaired. The number of cells in a population that became detached from the glass was a function of time and estradiol concentration; a greater number of cells fell off the glass at the higher estradiol concentrations in any given time period. These aspects of cell detachment from glass were reflected by the fact that the slopes of the lines at the higher estradiol concentrations dropped off more steeply, when only cells remaining attached to the glass surface were analyzed. The cells that became detached from the glass were still viable, since transfer to a fresh medium devoid of steroid permitted them to grow and synthesize DNA and protein at rates similar to those before exposure. Further, the ratio of DNA to protein in cells floating in the medium was similar to fibroblasts in control populations growing in the logarithmic phase.

In an attempt to clarify the nature of the primary interaction between estrogens and fibroblasts, other aspects of population development were studied. Data presented in Table I show that fibroblasts did not imbibe water after estrogen exposure, as might be expected if the initial reaction broke down cellular permeability. The diameters of the rounded fibroblasts were measured with an ocular micrometer. The results indicate that their volumes were similar to randomly distributed normal cell populations in the logarithmic phase of the growth cycle. The chart also shows that the packed cell volume from a fibroblast population did not appear to increase after the interaction with estrone or estradiol, while a control population increased from 12 μl to 27 μl in 24 hours, and to 34 μl in 48 hours.

Table I shows that only small amounts of glucose were taken up by fibroblasts after exposure to estradiol. This was reflected in the complete and abrupt cessation of macromolecular syntheses, and other endergonic reactions.

Table II depicts the complete data on uptake of amino acids by L cells, and the nature of the pool of free amino acids in control populations and those exposed to estradiol for 48 hours. L cells take up mostly essential amino acids from 199P medium; the results were similar to other results on amino acid consumption by L cells from protein free media (13). In 48 hours about 50% of the leucine, 41% of the valine and about 25% of each of the other essential amino acids were consumed

TABLE II. Effect of Estradiol on Extracellular and Intracellular Amino Acid Concentrations in L Strain Cultures.

| | 199 peptone medium | | | Free amino acid pool | |
|---|---|---|---|---|---|
| Amino acid | 0 time | Control 48 hr | $3.5 \times 10^{-5}$M E2 48 hr | Control 48 hr | $3.5 \times 10^{-5}$M E2 48 hr |
| | mM | | | | |
| Aspartic acid | .59 | .56 | .62 | .53 | .64 |
| Threonine | .77 | .52 | .72 | 1.51 | 1.71 |
| Serine | 1.01 | .78 | 1.05 | 3.09 | 2.98 |
| Glutamic acid | .97 | 1.06 | .94 | 2.96 | 3.81 |
| Proline | .39 | .34 | .38 | 1.20 | 1.69 |
| Glycine | 1.15 | 1.16 | 1.24 | 8.51 | 9.78 |
| Alanine | .96 | .84 | .79 | 2.55 | 2.59 |
| Cystine | .34 | Trace | Trace | Trace | Trace |
| Valine | .77 | .45 | .71 | .51 | .66 |
| Methionine | .51 | .26 | .48 | .38 | .32 |
| Isoleucine | .47 | .36 | .49 | .28 | .41 |
| Leucine | .91 | .43 | .86 | .53 | .93 |
| Tyrosine | .45 | .37 | .40 | .35 | .53 |
| Phenylalanine | .61 | .53 | .56 | .53 | .71 |
| Lysine | .62 | .43 | .59 | .32 | .48 |
| Ammonia | 1.52 | 1.25 | 1.50 | 1.08 | 2.27 |
| Histidine | .18 | — | — | .11 | .20 |
| Arginine | .96 | .78 | .94 | .48 | .82 |

from 199P medium. However, in medium containing $3.5 \times 10^{-5}$ M estradiol, amino acid consumption was impaired; leucine uptake dropped to 7%, valine to 7% and each of the other essential amino acids to about 5%. A previous study showed that L cells preferentially concentrated glycine, alanine, glutamic acid and serine when grown in 199P medium(7). Again, if cell permeability were disturbed, one might expect that amino acid entry or exit might be altered. It was interesting to note that these amino acids were found free in the pool at the same concentrations as in cells growing actively in the logarithmic phase even in cells that have rounded up and fallen off the glass.

Many attempts have been made to reverse this inhibitory reaction, so that something might be learned of the specific locale of the steroid action. Other steroids were added as competitors(14); glycolytic intermediates, coenzymes, vit. E and K were all tested at various concentrations(15). Only by addition of whole or dialyzed serum to the tissue culture medium could a reversal be effected. The extent of the reversal could be related to the serum concentration as is shown in Fig. 3. When 20% dialyzed serum was added DNA was synthesized at almost the same rate as in control cultures bathed in 199P medium. The protein increases were somewhat misleading, since cells grown in 199P without serum never showed as much protein as cells grown in the presence of serum. The excesses occur despite washing, and it has been presumed by us that the serum is adsorbed to the surface of the cells(16).

*Discussion.* Numerous steroids inhibit or suppress the growth of cells in tissue culture. Bimes *et al.* have reported that estradiol at 20 $\mu$g/ml ($7 \times 10^{-5}$M) in a tissue culture medium inhibits growth of HeLa cells after a 3-hour contact period(6). Inhibition of L cell growth by hydrocortisone at 125 $\mu$g/ml(3), and progesterone at 10 $\mu$g/ml(17) have also been observed. Estradiol benzoate stopped the multiplication of chick heart fibroblasts at 0.03 $\mu$g/ml in primary cell cultures(18). In all of these previous studies, serum was a component of the medium. In the study reported here estrone and estradiol completely inhibited synthesis of DNA and protein in L strain fibroblast populations at $3.5 \times 10^{-5}$ M in a tissue culture medium devoid of serum. Partial inhibition was noted below this level and the extent of suppression could be directly related to the concentration in the medium down to $10^{-5}$ M (0.026 $\mu$g/ml). With addition of 5% dialyzed serum to medium 199P, approximately twice as much estradiol was required to effect a complete inhibition, and degree of protection provided by serum could

be related to quantity in the medium. Hence, serum afforded protection against the deleterious effects of estrogens on L cells by directly competing with the steroid.

The estrogens displayed similarities to other steroids in inducing morphological changes in cells grown in tissue culture. Observations made in this study have shown that rounding up and detachment of the cells from a glass surface occurs after about 6 hours of contact. Further, detachment was shown to be a quantitative reaction varying with time and external estrogen concentration. Rounding up and detachment of cells from glass was also noted in tissue cultures exposed to corticoids(19). Electron micrographs of cells exposed to hydrocortisone have shown a hypervacuolization of the cytoplasm(20), and we have observed the same reaction in L cells exposed to estradiol(21).

These parallelisms indicate that the steroid inhibitory reaction is exerted through a common mechanism. There is evidence that this involves the steroids reacting near the cell surface. Other work in this laboratory dealing with application of physico-chemical concepts to biological interpretation of interaction between steroids and surfaces has led to the development of equations based on the Langmuir adsorption isotherm(22). Berliner and Dougherty noted with radioautographic and histological methods that cortisol localizes on or just inside the cell membrane in tissue culture(19). Human red cells rapidly and reversibly bind stilbesterol at superficial sites on the surface(23). Our study would support the contention that the estrogens are bound at the cell surface and block substrate access to the interior of the L cell for the following reasons. [1] The inhibition elicited by the 3 estrogens was related to their solubilities. This suggests that a larger number of molecules of the less soluble steroids could be complexed at the lipoprotein membrane in accordance with the "polarity rule" at equilibrium(24). Hence, in separate cultures containing equimolar concentrations of each estrogen in an aqueous medium, more molecules of estrone or estradiol than estriol would bind in the lipoprotein phase of the cell membrane(25). This was confirmed in a study in which diethylstilbesterol and progesterone were found to be the most potent inhibitors of L cell growth, and conjugated steroids were the least effective(21). [2] There was an abrupt arrest of the increase in cell volume immediately after the cell-steroid contact, and the free amino acids were retained in intracellular pools at fixed levels for 48 hours. This denotes that there was no drastic alteration in the cell's permeability which would permit leakage of small molecules, or the free passive diffusion of substances which would cause the cells to swell. The population of cells became statically suspended after the interaction. [3] The cells survived for long periods after exposure at high estrogen levels. The fact that L cells grew at optimal rates almost immediately after being transferred to an estrogen free medium indicated that cell permeability was not impaired after the cell-steroid dissociation. This would favor the speculation that dilution merely reversed the binding equilibrium. [4] The transport of glucose and amino acids was increasingly suppressed coincident with elevated estradiol levels in the medium, indicating that as more steroid molecules are adsorbed at the membrane, there is less opportunity for essential metabolites to be transported across that cell membrane. Support for this proposal was found in a microbial system in which steroids inhibited growth of *Neurospora crassa*. Deoxycorticosterone (DOC) was shown to interfere with sugar transport, and not to some subsequent event(s) in sugar utilization since DOC inhibited cellular respiration with sucrose as a substrate. At the same time the steroid had no effect on the endogenous respiration(4). [5] The inhibition elicited by the estrogens could be reversed by addition of serum to the medium. An hypothesis can be submitted for the ability of serum proteins to reverse the inhibitory reaction. The proteins probably compete with the lipoproteins of the cell membrane for the "free" available steroid molecules in the medium. Since serum proteins have a high binding capacity for steroids, there would be a reduction in the number of these molecules capable of interacting with the sites that are used for transport of metabolites into the cells. Serum albumin

could be the protein restraining a large fraction of the steroid molecules, since it possesses a large binding capacity (85%) relative to the other serum proteins(26).

*Summary.* The effect of the 3 natural estrogens on L strain fibroblasts was studied. Estrone and 17$\beta$-estradiol at a concentration of $3.5 \times 10^{-5}$ M in medium 199 supplemented with 0.5% bacto-peptone completely inhibit the synthesis of deoxyribonucleic acid and protein in L strain fibroblasts. Estriol is not an inhibitor of L cell growth at the same concentration; in fact, DNA and protein synthesis occur at rates similar to control cultures. The extent of suppression of fibroblast growth can be related to the external concentration of estrone or estradiol below the level of $3.5 \times 10^{-5}$ M, and a direct relationship exists between degree of inhibition and external concentration to $10^{-7}$ M; below this level there is no inhibition. Morphological observation has shown that the initial reaction involves a reduced cellular adhesiveness, which is followed by a rounding up of the cells in the population; these cells eventually fall off the glass surface. Swelling of the fibroblasts has not been observed. The reaction is reversible since cells that have fallen off the glass will spread and grow when placed into fresh medium devoid of these steroids. The suppression of macromolecular synthesis appears to be associated with the inability of glucose and amino acids to be transported into fibroblasts. The free amino acids that have accumulated into intracellular pools are retained. The magnitude of individual free amino acids in pools is comparable to control culture levels. Introduction of dialyzed horse serum into the medium results in resistance to the inhibitory action, and extent of the reversal can be related to the serum concentration.

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