droxyphenylbutyric acid and N(3-hydroxybenzyl)-N-methylhydrazine. Their inhibitory action is approximately 40 times higher than, *e.g.*, that of L-α-methyldopa.

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Received July 23, 1962. P.S.E.B.M., 1962, v111.

Estradiol-Cellular Interaction in Tissue Culture.* (27926)

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

Estrogens are inhibitors of mammalian cell growth *in vitro*. This activity is believed to be associated with the fact that these steroids physically block the uptake of essential metabolites(1). Substances as unlike as amino acids, monosaccharides, and inorganic phosphate are hindered in their normal passage into cells by free and sulfated estrogens. Included are inactive mediations, active transports and one apparently unmediated migration(2). LeFevre and Marshall have shown that glucose transport is inhibited when stilbesterol is fixed to superficial sites on the human red cell surface (3). In a previous report we presented evidence in a tissue culture system indicating that substrate utilization and cell growth were inhibited when estrogens were bound at the cell membrane(1). In this report we have attempted to clarify some aspects of the cell-estradiol interaction since this compound is not metabolized by L strain fibroblasts.

Materials and methods. Large numbers of stationary bottle cultures of L strain mouse fibroblasts were set up in a replicate series in 150-ml milk dilution bottles in medium 199 supplemented with 0.5% bacto-peptone (199P). After 48 hours the cells on the bottles were in the exponential phase of the growth cycle. At this time the medium was replaced with fresh 199P containing estrone-16-C^{14}† or estradiol-16-C^{14}† at tracer levels which did not inhibit cell growth. These radioactive steroids were solubilized in propylene glycol, sterilized by autoclaving and added to the tissue culture medium at a final concentration of 1 μg/ml. The cultures were harvested at 24 hour intervals for the next 72 hours. When harvested, the media were decanted and the fibroblasts attached to the glass were washed with BSS,

* This work was supported in part by grants from U. S. Public Health Service and from the Health Research and Services Foundation, United Fund of Allegheny County, Pa.

† 1 mg/2.7 μc, Charles E. Frosst and Co., Montreal, Canada.

then lysed with distilled water. Fresh media, the used media, the washing solution and the cell lysate were extracted repeatedly with benzene. Samples from the solvent and the aqueous phases were pipetted onto planchets in order to locate the radioactivity in the course of the experiment. Counting of the isotope was carried out in a windowless flow-gas counter.

To identify the labeled compounds extracted with benzene, the solvent was evaporated and the residue dissolved in a minimal amount of absolute alcohol. Known radioactive estrogens were treated in the same manner. Each sample was spotted onto washed paper, and chromatographed by the descending method of Migeon *et al*(4). These paper chromatograms were scanned on a paper chromatogram scanner which automatically moves the paper in front of a windowless flow-gas counter; the particles are counted on a rate meter which continually records the disintegrations. R_F values of the knowns were compared with those of the samples in the various extracts.

To study the early cell-estrogen interaction, cell suspensions were used. Exponentially growing cells were harvested and pooled. The cells were sedimented by centrifugation; the used medium was decanted and fresh 199P medium was added to make a suspension which contained approximately 10^7 cells/ml. Ten milliliters of this suspension were inoculated into 10 separate 50-ml Erlenmeyer flasks. These flasks were placed on a rotary shaker at 110 rpm in an incubator at 37°C. A constant quantity of tracer steroid was added to each flask and they were covered with aluminum foil. The contents of individual flasks were transferred at various time intervals to centrifuge tubes. The cells were sedimented and washed with Hanks' BSS. Benzene extracts were prepared from cell lysates and pipetted onto planchets for counting.

The following experiment was designed to determine the relationship between quantity of estradiol present in medium 199P and quantity that associates with the fibroblasts. Cell suspensions as in the previous experiment were distributed into Erlenmeyer flasks and placed on the shaker at 37°C. Radioactive estradiol was added to the flasks at varying concentrations and the contents of the flasks were harvested after 30 minutes. The cells were separated from the medium and extracted with benzene as before to recover the radioactivity for counting.

Since serum proteins are known to bind estrogens, this experiment was set up to determine how the quantity of estradiol associated with the cells varies as the serum concentration is increased in 199P medium. Estradiol-16-C^{14} was added to 5 flasks of cells suspended in 10 ml of 199P medium which contained horse serum concentrations ranging from 0 to 40%. These cell suspensions were harvested after 30 minutes and analyzed as in the previous experiments.

To test definitively whether the competition occurred between serum proteins and cells for free steroid molecules, or between serum proteins and free steroid for cell surface sites, the following experiment was designed. Six stationary bottle cultures were set up and radioactive estradiol was added to the 199P medium bathing the cells during the exponential growth phase. Two milliliters of a 5% albumin solution in 199P were added to 2 cultures. A dialysis bag containing 2 ml of the 5% albumin solution was introduced into 2 other cultures. The third pair served as controls. After 18 hours the dialysis bags were removed from the cultures. The contents inside the bag as well as the various culture mediums, and a distilled water cell lysate were each extracted 3 times with benzene. Aliquots from the extracts were placed onto planchets which were dried and counted for their radioactivity.

Results. Estrone and estradiol completely inhibit L cell growth at 3.5×10^{-5} in medium 199P, and extent of inhibition is directly related to concentration. The experiments reported here were designed to clarify the nature of the interaction between L cells and estrogens in order to specify the mechanism of growth inhibition. Isotopically labeled estrogens were employed. The study included estrogen levels in culture media which inhibit cell growth and concentrations which

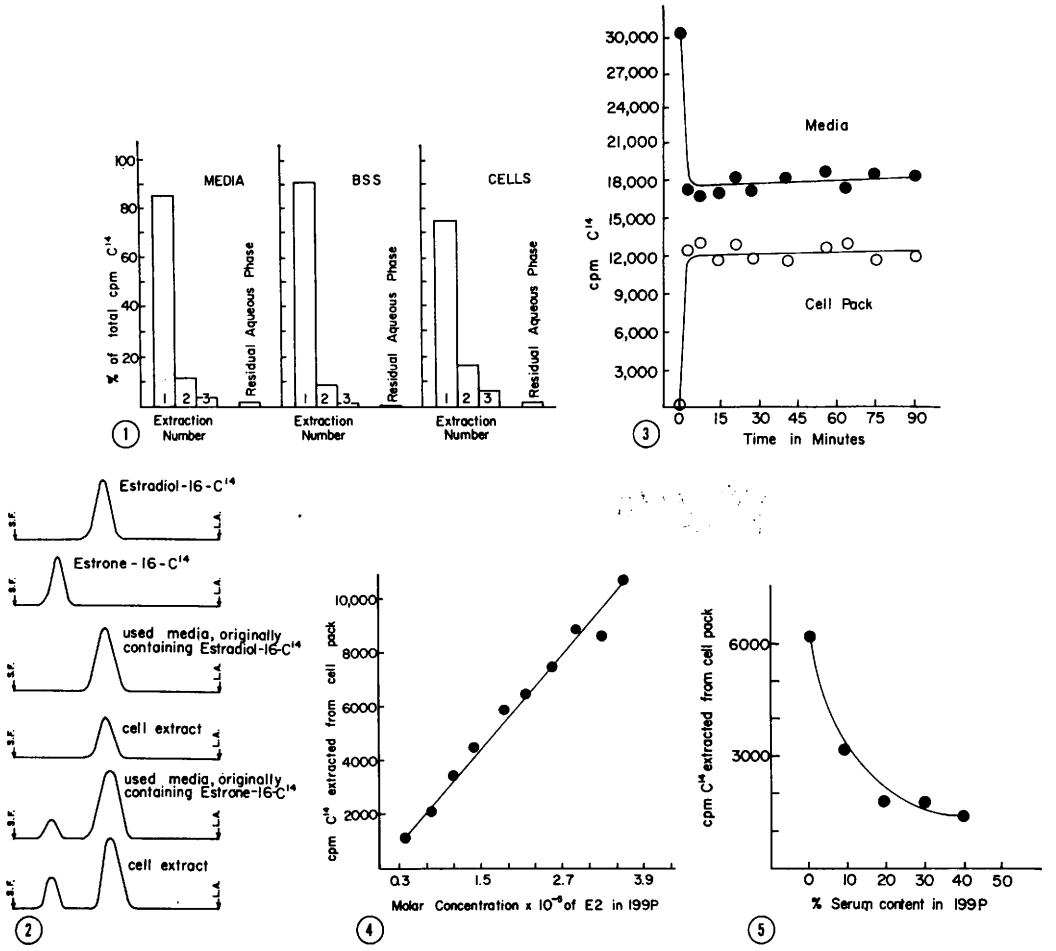

FIG. 1. Percentage of total radioactivity extracted from used medium, cell washing fluid and cell lysate after L cell cultures were grown for 72 hr in presence of estradiol-16-C^{14}.

FIG. 2. Paper chromatograms of radioactive material extracted from used media and cells after exposure to either estrone-16-C^{14} or estradiol-16-C^{14} for 72 hr.

FIG. 3. Interaction of estradiol-16-C^{14} with L strain cells as a function of time.

FIG. 4. Quantity of estradiol-16-C^{14} adsorbed to fibroblasts as a function of estradiol concentration in medium 199P.

FIG. 5. Effect of increasing serum content in 199P medium on quantity of estradiol-16-C^{14} adsorbed to L cells.

are not inhibitory. In the first experiment L cells were grown in a medium containing 10^{-7} M estradiol-16-C^{14} for 72 hours. At harvest the cells were separated from the medium and washed once with BSS. Fig. 1 shows percent of total radioactivity found in each of 3 benzene extractions from used medium, cell washing fluid and cell lysate. Eighty-five percent of the total radioactivity in the used tissue culture medium could be removed with one benzene extraction, and 99% could be recovered in the solvent phase after 3 separations. The chart also shows that 75% of the total isotope in the cell lysate could be obtained with one benzene extraction and 99% could be removed with 3 separations. Ninety percent of the radioactivity in the cell washing solution could be obtained with one extraction. Exhaustive extraction with benzene eventually reduced the last 1% of isotope in the various aqueous phases to background radioactivity. Approximately 90% of the tracer originally present in the fresh medium could be accounted for in these various benzene extracts. The results were similar at 24 and 48 hours and

estrone-16-C^{14} could be substituted for estradiol with essentially the same results. Labeled water soluble compounds were not detected. Other studies have indicated that this is the case with corticoids(5) and progesterone(6) in tissue cultures.

Fig. 2 illustrates a series of paper chromatograms of the radioactive material extracted by benzene from used media and cells when either estrone-16-C^{14} or estradiol-16-C^{14} was present in the original medium. The chart shows only the 72 hour result, since cultures grown for 24 and 48 hours were similar qualitatively. Estradiol was not changed during the period of contact with the fibroblasts. Paradoxically, estrone was largely converted to estradiol in the cultures. Somehow, the reduction of estrone is catalyzed only in the presence of cells since used medium alone could not effect the reaction. After 72 hours, only 10% of the estrone remained as the original compound.

Since estradiol was not altered by L cells, this compound could be used to study the interaction between mammalian cells and steroid molecules during the early minutes of their association and for extended time periods. Fig. 3 shows that the interaction is abrupt, a maximum value is reached in 3 minutes and is not significantly changed even after hours of contact. The rate of interaction was similar at the various exogenous concentrations studied, but the plateau level was dependent on the external estradiol content. This result led us to investigate the quantity of estradiol that would associate with fibroblasts at widely different concentrations of estradiol in the medium. The range included concentrations which inhibited L cell growth. Fig. 4 shows that the quantity of estradiol associated with fibroblasts fluctuates by a constant ratio when the medium concentration varied between 3×10^{-6} and 3.7×10^{-5} M. Approximately 45% of the steroid was bound to 10^7 cells in 1 ml of medium at any estradiol concentration throughout the range. Washing the cells one time with 1 ml of balanced salt solution reduced the amount of radioactive estradiol associated with the cells by 50%. The reduction was independent of the quantity bound by equivalent cell masses, and subsequent washes continued to reduce the amount by one-half each time. It was also possible to reduce the estradiol bound to cells by 50% by diluting a cell suspension containing estradiol-16-C^{14} with an equivalent volume of 199P medium devoid of steroid.

It has been observed that a washing operation permitted the growth of L cells after growth was inhibited for 48 hours(1). Many attempts were made to reverse the inhibitory reaction by other means than elution or dilution. We have found that by addition of serum this inhibition could be reversed; we suggested that serum was binding estrogen and effectively reducing the free steroid capable of binding to fibroblasts. Fig. 5 shows that this is the situation since the constant ratio noted above can be altered by addition of serum. The radioactivity associated with the cells is decreased with addition of serum. The quantity bound to the cells is a function of serum concentration; at 40% serum in medium 199P, only one-fifth as much estradiol is associated with the fibroblast mass after 30 minutes of contact. Serum apparently protects the fibroblasts because it binds some of the estradiol, and reduces the amount free in the medium that is capable of combining with the cells. There may also be some competition between serum proteins and steroids for direct binding to surface sites.

To clarify the nature of the competitive reaction, stationary bottle cultures were set up with inhibitory concentrations of isotopically labeled estradiol in medium 199P. Serum albumin was added to some of the cultures, some served as controls while dialysis bags containing albumin were placed into others. The inhibitory reaction noted in the control cultures was not apparent in cultures containing albumin either in the medium or within the dialysis bag. The cell cultures grew as if the inhibitor were not present. Table I shows that 25% of the labeled estradiol migrated into the dialysis bag, while only 3% of the isotope was associated with the cells in the culture. In contrast, 10% of the estradiol became associated with

TABLE I. Effect of Albumin on Estradiol Absorption.

Culture No.	Phases extracted	CPM (avg/ culture)
1 & 2 (No serum)	1. 199P medium	25,084
	2. Cell lysate	8,276
		33,360
3 & 4 (Albumin added)	1. 199P + albumin	30,247
	2. Cell lysate	2,592
		32,829
5 & 6 (Dialysis bags containing albumin added)	1. 199P medium outside dialysis bag	21,148
	2. Cell lysate	790
	3. Contents inside dialysis bag	8,729
		30,667

The quantity of radioactivity extracted from cell masses grown in presence of estradiol-16-C^{14} in medium 199P and from those cultures containing bovine albumin added either directly to 199P medium or contained within a dialysis bag.

the cells when albumin was included directly in the medium. In the absence of albumin 30% of the radioactivity was found associated with the cells, and in these cultures growth was completely inhibited; rounding up and detachment of the fibroblasts was also noted. These results indicate that one form of competition does occur between serum proteins and the cell surface for free steroid molecules.

Discussion. We have used the observation that estradiol is not metabolized by L cells to study the characteristics of the estrogen-fibroblast interaction. The system could be quantitatively analyzed by separating the cells from the medium and measuring the radioactivity after solvent extraction. The results indicate that L cells rapidly adsorb the steroid molecules from the medium; a packed mass of these cells will contain 40 times more estradiol than an equivalent amount of bathing media in less than 3 minutes. Further accumulation of the isotope does not occur even after hours of contact. Approximately 10,000,000 cells (30 μl packed cells) can adsorb 45% of the isotope from 1 ml of bathing media. The interaction is extremely labile since 50% of the steroid can be removed from the cells by diluting with an equal quantity of media or by washing the cells one time with an equal volume of Hanks' balanced salt solution. This evidence supports the hypothesis that the steroids are bound at the membrane and little, if any, permeates the cell. Other studies using molds and bacteria suggest that steroids are largely excluded from cell water(7,8). Abundant supportive evidence is available for the fixation of stilbesterol and estrone on the red cell surface(3,9), estradiol disulfate on the Ehrlich carcinoma cell membrane(10), and deoxycorticosterone on or within the membrane of a soil amoeba(11).

Previously we showed that the transport of metabolites was increasingly suppressed in L strain populations coincident with the elevated estradiol level in the medium(1). We concluded that estrogens blocked the movement of glucose and amino acids into as well as out of cells. The results obtained here have shown that there is a direct relationship between amount of radioactive estradiol in the medium and quantity associated with the cells. This holds for low exogenous levels which are not transport inhibiting and persists through exogenous levels which completely inhibit substrate penetration and cell growth. The results indicate that the steroid molecules block the passage of polar molecules, perhaps by blocking a pore used for transport or by altering the potential at surface sites necessary for attachment of essential molecules before transport. When the transport system is reopened by simply eluting the steroid from the cell membrane, L cells resume their synthetic activities almost immediately. The fact that a single agent can influence several transport systems would indicate that a common aspect of membrane structure is modified by surface binding of certain steroids(2).

The inhibition elicited by the estrogens could be reversed by addition of serum to the medium(1). The serum proteins hinder adsorption of estradiol to the cell membrane, since the amount of radioactivity bound to cells is decreased as serum concentration is increased. The serum can be added to the cultures with the steroid, or after the cell-steroid interaction with similar results. The

rounding-up reaction noted in monolayer cultures can be reversed by addition of serum, and cells that have already become detached will reattach and spread out over the glass upon addition of serum.

Albumin is a serum protein which has a high affinity for estrogen binding as observed in equilibrium dialysis(12). We found that albumin could reverse the growth inhibitory reaction as did whole serum when it was either placed directly into the bathing medium or placed inside a dialysis bag which was then placed into inhibited cultures. When radioactive estradiol was put into cultures containing albumin in dialysis bags, it was noted that much of the isotope moved into the dialysis bag. The results imply that albumin competes with the surface lipoproteins for the binding of steroid molecules. However, it does not ediminate the possibility that serum could replace some estradiol bound at the surface by direct competition.

The bond at the site of interaction at the fibroblast surface is of low energy because reversal can be effected by simple dilution. The fibroblasts appear to compete with serum protein for free steroid to approximately the same extent. One might speculate that the bond which associates steroids with the cell membrane is similar to those associating steroids to serum proteins. These are hydrogen bonds and van der Waal's forces (13). However, the bond that forms is sufficiently stable, so that polar substrates are not able to be transported into L cells.

Since inhibition is related to the amount of steroid bound to the cell, it is possible to calculate the extent of surface that must be covered to obtain complete inhibition of cell growth. The average diameter of an L cell in exponential growth is 16 μ's. The surface area of an L cell would, therefore, be approximately 810 μ^2. Estradiol is a planar molecule with a surface area of about 140 $A^{\circ 2}$. If the steroid binds from the under or α-side as proposed for the binding to albumin (13), then one can estimate that 5×10^8 molecules could coat the entire surface of the cell. Experimentally, we have found that 10^9 molecules combine per cell at a completely inhibitory concentration. The calculation infers that the cell is at least entirely coated when substrate uptake is inhibited.

Estrone is reduced by L cells to estradiol. It is difficult to reason that this conversion occurs extracellularly. It does not appear that enzyme is leaking from cells or that cells are being broken since used media cannot effect this transformation. It must be reasoned that this conversion is non-specific since there would be an equilibrium existing for enzymatic conversion and some estradiol should be oxidized; however, this is not the case. It is our belief that the reduction of estrone is non-specifically catalyzed at the site of interaction on the cell membrane. Estradiol was the main reaction product of estrone with rabbit and human red cells(14).

Summary. L strain fibroblasts were grown in medium 199P containing estrone-16-C^{14} and estradiol-16-C^{14} at a concentration ranging from 10^{-7} to 10^{-5} M. These cells did not convert either steroid into water soluble conjugates in 72 hours. The estradiol in the medium and that associated with the cells was unchanged, but a large portion of the estrone was converted to estradiol. In experiments using cell suspensions, some of the early events in the interaction between estradiol and fibroblasts were studied. The primary reaction appeared to involve a rapid adsorption of the compound to the cell surface. There is evidence indicating that very little, if any, of the steroid penetrated the cell. The amount of estrogen that adsorbs to the cell surface could be directly related to the estradiol content in the medium. When estradiol content in the medium was 3.5×10^{-5} M, there was inhibition of cell growth. This inhibition has been previously shown to be associated with the blockage of substrate transport. Approximately 10^9 molecules per cell were required to effect complete inhibition of cell growth. Adsorption of estradiol could be impaired by addition of horse serum to the medium, and under such conditions a greater amount of estradiol was required to attain the extent of inhibition observed in a serum free medium. The competition by serum proteins occurred between cell surface sites and proteins in the medium for free steroid rather than between serum

protein and steroid for surface sites.

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Received September 4, 1962. P.S.E.B.M., 1962, v111.

Coxsackie A9 Myocarditis in Adult Mice.* (27927)

A. MARTIN LERNER† AND JAMES A. SHAKA (Introduced by Maxwell Finland)
Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, and Mallory Institute of Pathology, Boston City Hospital, and Department of Medicine, Harvard Medical School, Boston, Mass.

In a previous report on the replicating capacity of Coxsackie A9 virus, Strain 13, in mice varying in age from less than 24 hours to 8 months, it was found that virus production occurred at all ages, but the relative yields of virus from myocardium and skeletal muscle varied markedly(1). In striated muscle the highest titers of virus were found in suckling mice, whereas in the myocardium the highest titers were in the oldest mice. A diffuse myositis was observed in suckling mice, moderate lesions in 20- and 40-day-old mice, and only focal areas of infiltration were seen in striated muscle of the 8-month old animals. No lesions were noted in the hearts of the sucklings, or of 20- or 40-day old mice, but frank myocarditis was seen in 8-month old animals.

Since multiplication of the Coxsackie A group of viruses with concomitant pathology in cardiac muscle of mice had not been reported previously and the number of adult mice included in the initial study was small, further experiments were done to determine the incidence of these myocardial infections and concomitant lesions. Further data on strain specificity were also obtained.

Materials and methods. Swiss mice from the Hauschka and Marand Institute of Cancer Research‡ or Webster§ strains were used. Three strains of Coxsackie A9 virus were employed, *viz.,* Strain 13, used as second rhesus kidney (MKTC) passage, NIH-1, and Wiederhold. The sources and passage histories of the first 2 strains have been described(1); the Wiederhold strain was obtained from Dr. Sidney Kibrick; it was isolated originally by Dr. Frederick C. Robbins(2) in suspended cell culture of mature human kidney inoculated with feces of a patient diagnosed as having nonparalytic poliomyelitis and was then passaged in the same tissue and in human embryonic skin muscle cultures and was received after many additional passages in

* Aided by a grant from Nat. Inst. Health.

† Fellow of The Medical Foundation, Inc. Present address: Dept. of Biol., Mass. Inst. of Technol., Cambridge, Mass.

‡ Supplied by Charles River Breeding Laboratories, Inc., Boston.

§ Obtained from New Animal Farm, Harvard Med. School, Boston.