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FURTHER STUDIES ON THE REGULATION OF CULTURED RABBIT ENDOMETRIAL CELLS BY DIETHYLSTILBESTROL AND PROGESTERONE

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SUMMARY

The addition of diethylstilbestrol to cultured rabbit endometrial cells increased the number of DNA-replicating cells; progesterone had an opposite effect. However, both hormones induced a significant increase in the incorporation of labeled uridine and aminoacids into TCA precipitates. These hormonal effects were rapidly reversed upon removal of the hormones from the culture medium and were also inhibited by actinomycin D and cycloheximide. Hydrocortisone did not change the effect produced by diethylstilbestrol on DNA replication but inhibited the progesterone action. Dibutyril 3′,5′-cyclic AMP did not alter the effect of the same hormones on DNA replication. However it was surprisingly found to inhibit the hormonal effects on the incorporation of labeled uridine and amino acids. Neither sex hormone altered the specific activity of alkaline, acid phosphatase, or lactic dehydrogenase, or the uptake of 3-*O*-methyl-D-glucose or α-aminoisobutyric acid.

It is suggested on the basis of our findings, (a) that progesterone induces these endometrial epithelial cells to differentiate *in vitro* into non-dividing, secretory cells and (b) that there is no need for hyperemic and/or cell membrane transport changes for the expression of these estrogenic effects.

INTRODUCTION

A technique to culture rabbit endometrial cells in chemically defined medium, for a limited period of time, has been described. The growth of the primary explanted cells was found to be increased by the addition of diethylstilbestrol and decreased by the presence of progesterone in the culture medium. These hormones were determined to have concentration-dependent and antagonistic effects upon the DNA replication of the cultured cells as determined by radioautography after a ^{3}H-Tdr pulse [1].

One of the earliest and most dramatic estrogenic effects described in rat uterus is an inflammatory-like reaction [2]. Some authors [2, 3] have proposed that this phenomenon, with the subsequent increased availability of nutrients plays a primary role in the estrogenic effect on uterus. The liberation of endogenous histamine [3], increase in cyclic 3′,5′-AMP [4] and lysosomal migration and labilization [5] have been linked to the above mentioned hyperemia and the mechanism of action of estrogens in uterus. In addition, increased cell membrane-mediated transport of amino acids [6] and glucose [7] has also been shown in uterus after administration of estrogens to ovariectomized animals.

The development of a endometrial culture system, which is growth-responsive to ovarian hormones, therefore provides a unique opportunity for the investigation of possible causal relationships between the above mentioned early estrogenic phenomena, cell membrane transport changes and the regulation of growth by sex steroids on endometrium. The object of the present investigation was to determine the effects elicited in cultured rabbit endometrial cells by diethylstilbestrol or progesterone on; (a) the incorporation of labeled uridine and amino acids in TCA precipitates as a general estimate of RNA and protein synthesis; (b) the uptake of 3-*O*-methyl-D glucose and α-aminoisobutyric acid; (c) the specific activity of acid and alkaline phosphatases and lactic dehydrogenase; and (d) the effect of hydrocortisone, DBCAMP, actinomycin D and cycloheximide on the hormonal effects.

MATERIALS AND METHODS

General techniques. Six- to eight-month-old virgin female New Zealand rabbits were used in the experiments. The techniques to isolate and culture cells, as well as the autoradiography procedures have been described previously [1]. Protein was determined in all the experiments by the method of Lowry *et al.* [8].

Chemicals were purchased from Sigma or Calbiochem, tissue culture plastic containers and pipettes from Falcon Plastics.

Radiochemicals. Thymidine [G-^{3}H]-(specific radioactivity 9·82 Ci/mmol) [6,7-^{3}H (N)] estradiol-17β (specific radioactivity 46·6 Ci/mmol), [5,6-^{3}H] uridine (specific radioactivity 40·4 Ci/mmol), l-amino acid mixture [G-^{3}H] (specific radioactivity 1 mCi/ml), 3-*O*-methyl-D-glucose (methyl-^{14}C) (specific radioactivity 40 mCi/mmol) and α-aminoisobutyric acid [1-^{14}C] (specific radioactivity 8 mCi/mmol) were obtained from

New England Nuclear, Boston, Mass. and diethylstrilbestrol [G-^{3}H] (specific radioactivity 46·6 Ci/mmol) was purchased from Amersham & Searle Corp., Arlington Heights, Ill.

Endogenous estrogens in cultures. Animals were injected in the ear vein with 250 μCi of labeled estradiol or DES solubilized in 1 ml of absolute ethanol. Two hours later the animals were killed by a lethal i.v. dose of Diabutal. The endometrial cells were then isolated and cultured as mentioned above in dishes of 10 cm. dia. The culture medium was poured off at indicated times, the cells were rinsed 5 times with cold saline and then scraped with a rubber policeman in 1 ml of cold 0·1 M Tris buffer pH 7·4 and sonicated for 10 s using a Biosonik III sonicator, equipped with a microprobe, at a setting of 20. A 0·5 ml aliquot of the homogenate was then placed in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Beckman Scintillation Counter with a 60% efficiency. Proteins were determined in the rest of the homogenate.

Uridine and amino acid incorporation. Incorporation of [^{3}H]-labeled amino acids or [^{3}H]-uridine into total soluble protein or RNA was monitored by the retention of radioactivity on Millipore filters after several cold trichloroacetic washes [9, 10]. The actual counts measured were always above 500 c.p.m.

3-O-*Methyl*-D-*glucose and* α-*aminoisobutyric acid uptake.* Cells were plated in 5 cm. dia. dishes. At indicated times of culture 2 μCi per dish of ^{14}C-α-aminoisobutyric acid or ^{14}C-3-*O*-methyl-D-glucose diluted in 3 ml fresh culture medium were added to the cells. The final concentrations of the amino acid and the glucose analogue were 8 mmol and 0·5 mmol respectively. Unlabeled compounds were used to dilute the isotopes. The culture medium used was a modification of Ham's F-12 described in a previous publication [1], but devoid of glucose for the 3-*O*-methyl-D-glucose experiments or without amino acids for the study of α-aminoisobutyric acid uptake. The standard technique for both determinations was as follows: Cells were incubated at 25°C for 20 min with 3-*O*-methyl-D-glucose and for 2 h with α-aminoisobutyric acid, the reaction was then stopped at indicated times within 20 s by pouring off the culture medium and rinsing the cultures five times with 3 ml cold saline. One ml 0·5 M NaOH was then added to each dish and they were kept at 4°C for 18 h. A 500 μl aliquot of this extract was placed in 10 ml of Aquasol, neutralized with 1 N HCl and counted in a scintillation counter with an efficiency of 75%. Proteins were determined in the rest of the extract by the method mentioned above.

Enzyme determinations. The cells were harvested at different times by scraping and centrifugation. After washing with cold saline once, the cells were resuspended in 1·2 ml of 0·1 M Tris buffer, pH 7·4 and sonicated for 10 s at setting 10 with a Biosonik III furnished with a microprobe. The homogenates were then centrifuged at 10,000 *g* for 20 min and the supernatant fractions were used to determine the enzyme activities.

Alkaline and acid phosphatases were measured using kits 104-LS and 104-AS respectively, obtained from Sigma, Saint Louis, Mo., Lactic dehydrogenase was determined using kit LDH-P Stat-Pack obtained from Calbiochem, La Jolla.

RESULTS

Determination of "carry-over" endogenous hormones. The cultured cells used in these experiments were isolated from rabbits in estrus. Therefore, they could have carried over estrogens, which might complicate the interpretation of the results. A simple experiment was designed to investigate this problem. Labeled estrogens were injected i.v. in rabbits and after a period of time the rabbits were killed, the endometrium was isolated and the cells were cultured.

Figure 1, A and B, shows that the cellular radioactivity, originated in the labeled estradiol or DES injected in the animals, could be detected in freshly isolated cells. However, it was found to decrease with time in culture, reaching minimal levels at the third or fourth day of culture.

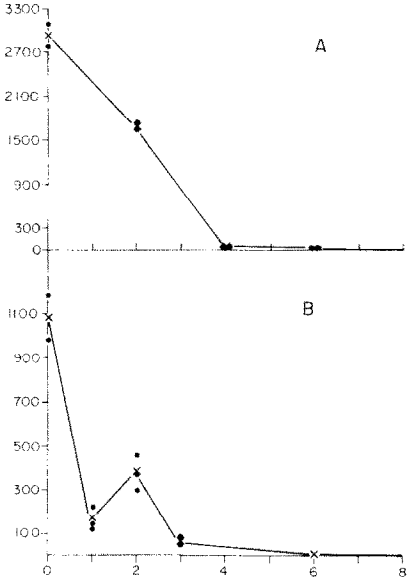

Fig. 1. Abscissa, Days in culture; ordinate, c.p.m. per Petri dish. Determination of remaining endogenous estrogens in cultured cells. (A) Diethylstilbestrol; (B) Estradiol-17β. Rabbits were injected i.v. with 250 μCi of labeled diethylstilbestrol or estradiol-17β and after 2 h the rabbits were killed and the endometrial cells isolated, and evenly plated. An aliquot of the freshly isolated cells was used for a zero point. This aliquot, containing the same cell number plated in one Petri dish, was centrifuged at 500 *g* for 10 min at 4°C, the pellet was then resuspended in 10 ml cold saline and the procedure repeated five times. The final pellet was sonicated and radioactivity determined as described in Materials and Methods. The radioactivity of the cultured cells was determined after various times in culture. ● = c.p.m. per Petri dish. × = average c.p.m. per Petri dish.

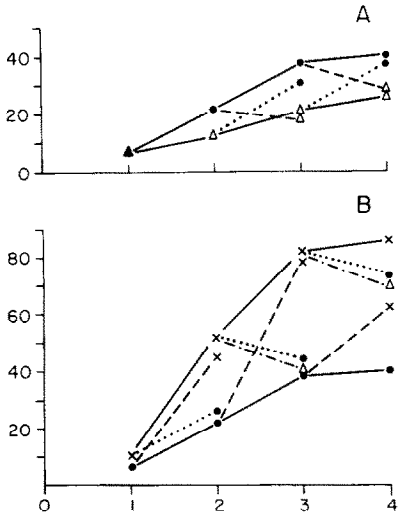

Fig. 2. Abscissa, days in culture; ordinate, Percentage of (^{3}H-Tdr)-labeled epithelial cells per total number of cells. Sensitivity to—and reversibility of—hormonal influences at different times of culture. (A) Progesterone effect and reversibility by hormone removal. (B) Diethylstilbestrol effect and reversibility by its removal. Endometrial cells were plated at 0 time in medium without additions (●) or containing 10^{-7} M diethylstilbestrol (×) or 10^{-7} M progesterone (▲). In some cultures the hormones were removed by rinsing them gently 5 times with 5 ml of culture medium minus hormones at 37°C and then culturing the cells in the same medium. When progesterone was used to reverse the effect of diethylstilbestrol, the cells were rinsed as described above and medium plus 10^{-7} M progesterone was added to the cultures. The addition of hormones to control cultures was made after rinsing the cells as above and then medium plus hormone was added. Changes from one culture medium to another are indicated by broken lines and the medium to which they are changed is indicated by the symbol at the end of the line. Incubation with ^{3}H-Tdr followed by radioautography and scoring was performed as described previously [1]. Total Tdr concentration was 10^{-4} M and 4 μCi of ^{3}H-Tdr was added to each Petri dish (dia., 2 cm.) in a 2 ml vol. Each point is the average of 5 Petri dish scorings.

Cell responsiveness and reversibility of hormonal effects. Figure 2, A and B, presents evidence that the cultured endometrial cells respond to the DES-stimulating effect and the P-inhibitory effect on DNA replication during the first 4 days in culture, and effects can be seen as early as 24 h. The same effects were easily reversible after removal of the hormones and addition of medium devoid of steroid hormones.

Time study of DES effect and Actinomycin inhibition. A more detailed study of DES effects is illustrated in Fig. 3. The addition of the synthetic estrogen induced an increase in the percentage of (^{3}HTdr.)-labeled cells after a lag period of 12 h. The effect could be seen after 18 h and is fully expressed after 24 h. Simultaneous addition of Actinomycin D inhibited the estrogenic action.

Hydrocortisone and DBCAMP effects. Figure 4 shows that the addition of hydrocortisone did not have any effect on the cell labeling by itself or on the stimulation by DES. However, it was found to decrease the P-inhibitory effect. DBCAMP was not found to influence the same parameters.

Hormonal effects on general RNA synthesis. DES or P were found to increase the incorporation of labeled uridine in TCA insoluble precipitates. A preliminary time course study (Gerschenson, L. E., unpublished results) showed that this increase could not be observed after 2 h of incubation with either hormone, but it was detectable and maximal after 6 h.

Figure 5 shows the result of two experiments after a 6 h incubation with DES. Actinomycin D and cycloheximide inhibited these effects while DBCAMP obliterated the hormonal effects, depressing slightly uridine incorporation.

Hormonal effects on general protein synthesis. In the experiments illustrated in Fig. 6 it was found that addition of DES or P for 24 h, induced an increase in the incorporation of labeled amino acids in TCA precipitates. Preliminary experiments (Gerschenson, L. E. unpublished results) showed no detectable increase after 2 or 6 h. However, it was possible to see an increase after 18 h, which became maximal after 24 h of incubation. Simultaneous addition of actinomycin D or cycloheximide inhibited the hormonal effects. DBCAMP had the paradoxical effect of increasing the values of the control group, therefore the differences between this group and the hormone-treated ones became statistically insignificant.

Hormonal effects on glucose and amino acid transport. Figure 7, A and B, illustrates two experiments showing that the addition of DES or P did not

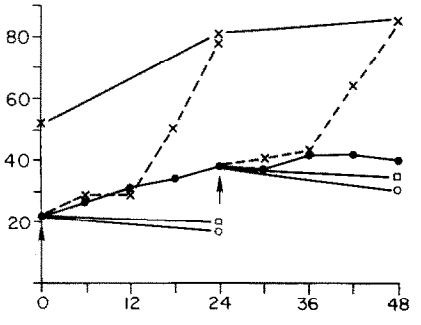

Fig. 3. Abscissa, Hours in culture; ordinate, Percentage of [^{3}H-Tdr]-labeled epithelial cells per total number of cells. Endometrial cells were obtained from a rabbit as described before [1] and then placed in Petri dishes in culture medium minus hormones (●) or with 10^{-7} diethylstilbestrol (×). Therefore at 0 time the cells were already preincubated with (×) or without (●) diethylstilbestrol for 3 days. After 3 or 4 days in culture the medium was removed and medium plus diethylstilbestrol was added to some control cultures. 2×10^{-7} actinomycin D was added to some control (○) or diethylstilbestrol-treated (□) cultures for 24 h. The arrows indicate the addition of hormone and/or actinomycin D to cultures. Incubation with ^{3}H-Tdr followed by radioautography and scoring was performed as described in the legend of Fig. (2) and the isotope was added to the cultures in 25 μl vol., 6 h before each determination. Each point is the average of five Petri dish scorings.

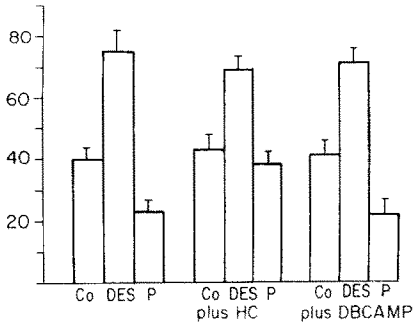

Fig. 4. Abscissa, CO = Control, DES = 10^{-7} M diethylstilbestrol, P = 10^{-7} M progesterone, HC = 5×10^{-7} M hydrocortisone, DBCAMP = 10^{-4} M dibutyril 3',5'-cyclic AMP; ordinate, Percentage of (^{3}H-Tdr)-labeled epithelial cells per total number of cells. Influence of hydrocortisone and dibutyril 3',5'-cyclic AMP on the sex hormones-induced changes in DNA synthesis. Cells were cultured for 3 days without sex hormones. They were then incubated for 24 hours with medium containing ^{3}H-Tdr and other additions indicated above. Radioautography was employed to determine the percentage of labeled cells. Each bar represents the average of 5 Petri dish scorings ±S.E.M.

change the uptake of labeled 3-*O*-methyl-D-glucose or α-aminoisobutyric acid by cultured endometrial cells.

DES and P effects on enzyme activities. The addition of the two sex hormones used in this paper was determined to have no effect at different time intervals (5 min to 48 h) upon the specific activities of acid and alkaline phosphatase, and lactic dehydrogenase. The initial drop in phosphatase activities was observed in all experimental conditions, and was probably due to the medium change. The specific activity of all three enzymes was found to be similar in cultured cells and freshly isolated cells. It should be noted that this last preparation is contaminated with red blood cells. The enzymes were also measured in the culture medium and the levels were found to be very low and not to change with hormonal additions (Table 1).

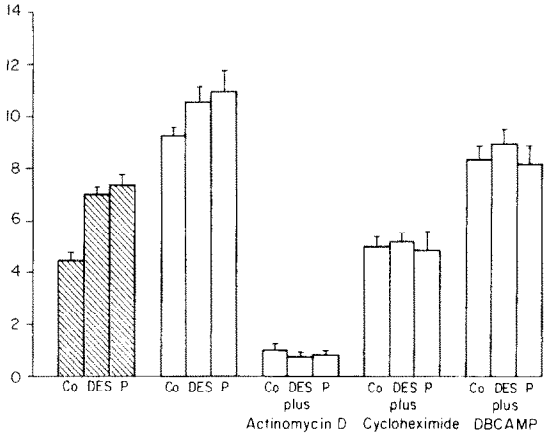

Fig. 5. Abscissa: See legend of Fig. 4; ordinate, c.p.m. per μg. protein. Hormonal effects on RNA synthesis. After 3 days of culture without hormones, hormones were added to the cultures as indicated for 6 h. Two experiments (striped and empty bars) are depicted to illustrate that there are quantitative differences in hormonal responses between different experiments. In the first bar grouping (striped), no other additions was made during the 6 h period. In the last bar groupings (empty) 2×10^{-7} M Actinomycin D or 10^{-4} M Cycloheximide 10^{-4} M dibutyril 3',5'-cyclic AMP were also present during the 6 h incubation with hormones. After this period of time the cells were pulsed for 1 h with 2 μCi of ^{3}H-uridine, added in 25 μl vol. to each Petri dish (diameter 5 cm) containing 5 ml medium and incorporation into TCA-insoluble precipitates was determined as referred to in Material and Methods.

DISCUSSION

The present and previous results [1] show that the DNA replication of cultured endometrial cells can be regulated by the addition to the culture medium of DES or P. It was observed, in the previous paper, that cells cultured without addition of hormones showed a considerable incorporation of ^{3}H-Tdr. This was thought to be due to the fact that the cells, isolated from normal adult rabbits, might have carried over endogenous steroid hormones, which could complicate the results as well as decrease the expression of the hormonal effects, by increasing the "background." The experiments described above demonstrate that after 3–4 days in culture only a very small fraction (1–5%) of estrogenic hormones is retained by the cultured cells. Russell and Thomas [11] have published similar experiments using organ cultures of rabbit uterus. However, their results show as much as 60% of the radioactivity still present in the tissue after three days of culture. The difference between their results and ours may be due to the use of different experimental systems, organs vs cell culture or different thoroughness (number and volume of washes) in rinsing the cultures during the medium changes.

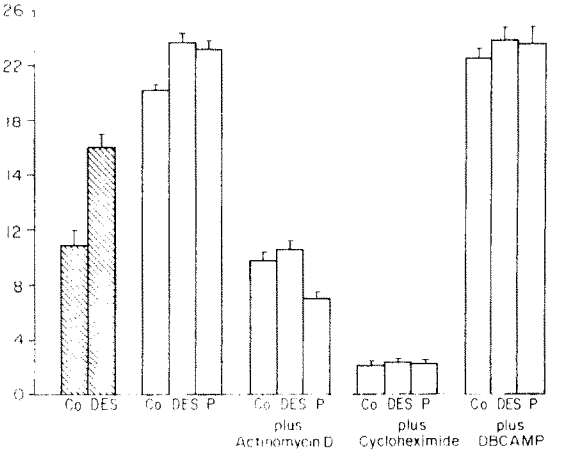

Fig. 6. Determination of hormonal effects on labeled amino acids incorporation in TCA precipitates. See legend of Fig. 5 for general experimental design, abcissa and ordinate. The cultures were incubated with hormones for 24 h and pulsed for 1 h with 2 μCi of a mixture of amino acids universally labeled with ^{14}C in 25 μl vol., added to each Petri dish (dia. 5 cm.) containing 5 ml of culture medium.

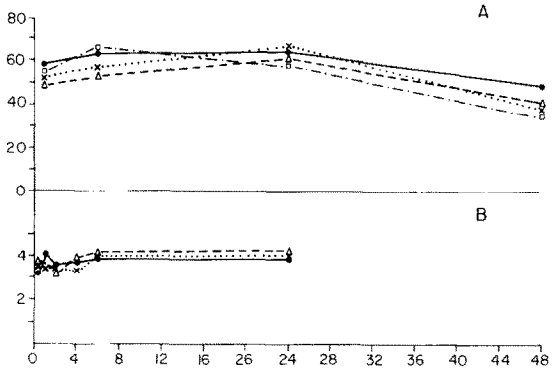

Fig. 7. Abscissa, hours after addition of hormones; ordinate, c.p.m. per μg protein, Uptake of ^{14}C-α-aminoisobutyric acid or ^{14}C-3-*O*-methyl-D-glucose. The cells were isolated and cultured as described in the legend of Fig. 3 with control medium for 3 days. Then the cells were incubated with 10^{-7} M diethylstilbestrol (×) or 10^{-7} M progesterone (▲) or with both hormones at the same concentration (□) or with medium minus hormones (●). At the indicated times after hormonal addition the uptake of the isotopes was determined after incubating the cells with them and radioactivity was measured using techniques described in Materials and Methods. (A) Uptake of α-aminoisobutyric acid, (B) uptake of 3-*O*-methyl-glucose. Each point is the average of six individual determinations in 5 cm. dia. Petri dishes.

DES was found to induce a significant increase in the percentage of (^{3}H-Tdr)-labeled cells. This effect could be detected as early as 18 h after hormonal addition and it was rapidly reversed after DES removal. The need for RNA synthesis in the expression of this phenomenon was demonstrated by its Actinomycin D inhibition shown in Fig. 3. The P effect in decreasing the percentage of (^{3}H-Tdr)-labeled cells was also detectable after 24 h of this ovarian steroid addition and it was found to be reversible in the same period of time, after hormonal removal.

The results described above show that DES rapidly increased proliferation in cultured endometrial cells. This increase could be due either to the entrance of a "dormant" cell population, such as stem cells, into the cell cycle or a shortening of the cycle duration. It is generally accepted that increased uterine epithelial cell division results from a shortening of the cell cycle, mostly at the expense of G_1 and S, and possibly at the expense of G_0 (non-dividing) phase [12]. On the other hand it has been suggested that progesterone may suppress cell division, in uterine or chick oviduct epithelium, by inducing cells to go to a G_0 stage [13, 14, 1]. With these suggestions in mind it was thought that DES removal or P addition might cause a decrease in the number of DNA-replicating cells by separate mechanisms which could operate at different velocities, but removal of DES or its antagonism by P addition inhibited within the same extent and time the DES-induced increase in number of labeled cells. Hsueh *et al.* [15] proposed that progestogens may block estrogen action by reducing the amount of available estrogen receptors. Current experiments performed in our laboratory on hormonal regulation of the cell cycle and hormonal receptors using our experimental system may help to further the understanding of these phenomena.

The increase in incorporation of labeled uridine and amino acids into TCA precipitates, induced by DES and P, suggests increased RNA and protein synthesis. These changes may very well not be evidence for RNA or protein synthesis but express changes in pool sizes. However, qualitative and quantitative sex hormone-induced changes have been described in uterus during *in vivo* experiments [16] and experimental work using chick oviduct has also provided strong evidence for sex steroid induction of mRNA and specific protein synthesis [17, 18]. The inhibition by cycloheximide of the hormone-mediated increase in uridine incorporation, is similar to the one described by Gorski *et al.* [19] using animals and suggests that the hormonal effects in general RNA synthesis may be mediated through synthesis of one or more proteins. It is noteworthy that P was also found to bring about significant increases in RNA and protein synthesis since the same hormone was found in a previous paper [1] and here to inhibit DNA replication. In view of the known progestogenic effects on rabbit uterus [20] and chick oviduct [18] where this hormone induces the secretion of specific proteins, blastokynin and avidin respectively, it is proposed that P causes the cultured rabbit endometrial cells to differentiate into non-dividing, secretory cells. This process may be also related to the decidualization phenomenon [21]. Furthermore, preliminary experiments performed in our laboratory (Gerschenson, L. E., in preparation) have demonstrated that in the culture medium of P-treated cells, a still unidentified protein appears and this phenomenon is inhibited by protein synthesis inhibitors.

Ultrastructural studies on the hormonal effects on nuclear structures of cultured rabbit endometrial cells suggest transcriptional effects [22] and support the interpretations described above of sex steroid effects on RNA and protein synthesis. The same authors demonstrated an increase in the amount of rough endoplasmic reticulum in P-treated cultured cells, which also adds weight to our contention that these cells may be involved secretory activity, regulated by progestins.

The administration of estradiol to ovariectomized animals is known to rapidly induce inflammatory changes in uterus [2, 7]. These hormonal manifestations have been considered to be related to growth as causative factors [2, 23] and to result, among other things, from 3′,5′-cyclic AMP increases which may have lysosomal labilization effects. The anti-estrogenic effects of glucocorticoids were then related to their lysosomal stabilizing or anti-inflammatory effects [23].

The present study demonstrates that neither the addition of DBCAMP or hydrocortisone had any effect on the DES-mediated stimulation of DNA rep-

Table 1. Hormonal effects on enzyme activities

Time	Alkaline phosphatase (Units) Co	DES	P	Acid phosphatase (Units) Co	DES	P	Lactic dehydrogenase (mUnits × 10^{-2}) Co	DES	P
Freshly extracted cells	502			10·7			35		
5 m	450 ± 15	425 ± 20	432 ± 30	9·5 ± 0·5	8·9 ± 0·4	9·0 ± 0·5	30 ± 1·4	32 ± 1·0	34 ± 1·1
15 m	400 ± 12	395 ± 18	400 ± 15	7·6 ± 0·7	7·2 ± 0·3	8·0 ± 0·3	36 ± 0·8	29 ± 0·9	36 ± 0·8
30 m	350 ± 14	340 ± 17	325 ± 20	6·0 ± 0·3	6·1 ± 0·6	6·2 ± 0·2	29 ± 0·5	32 ± 0·9	29 ± 0·8
1 h	420 ± 16	400 ± 20	380 ± 18	7·8 ± 0·3	7·4 ± 0·7	7·0 ± 0·3	32 ± 1·2	32 ± 0·7	32 ± 1·0
2 h	445 ± 17	460 ± 21	450 ± 18	10·1 ± 0·4	9·6 ± 0·6	10·0 ± 0·3	32 ± 0·9	30 ± 1·2	33 ± 1·2
4 h	450 ± 20	430 ± 18	460 ± 17	9·6 ± 0·6	9·6 ± 0·2	9·8 ± 0·4	30 ± 0·7	36 ± 0·8	30 ± 1·0
8 h	435 ± 22	450 ± 18	470 ± 21	10·4 ± 0·5	9·7 ± 0·4	9·7 ± 0·3	32 ± 1·5	31 ± 1·0	31 ± 1·2
12 h	460 ± 30	460 ± 21	442 ± 22	9·5 ± 0·3	9·6 ± 0·4	9·7 ± 0·4	29 ± 1·2	29 ± 0·5	32 ± 0·9
24 h	430 ± 22	435 ± 20	430 ± 20	10·0 ± 0·6	9·4 ± 0·3	9·5 ± 0·2	30 ± 0·9	32 ± 0·8	29 ± 0·9
48 h	442 ± 19	450 ± 12	432 ± 22	10·6 ± 0·6	9·8 ± 0·3	9·7 ± 0·5	29 ± 1·0	32 ± 1·0	30 ± 1·2

The cells were isolated and cultured as described in the legend of Fig. 3 with control medium for three days. Then the cells were incubated with 10^{-7} M diethylstilbestrol (DES) or 10^{-7} M progesterone (P) or with medium minus hormones (Co). At the indicated times after hormonal additions, the enzyme activities were determined as described in Material and Methods. Freshly isolated cells were isolated as described previously [1], centrifuged at 500 *g* for 10 min, resuspended in cold saline and the same procedure used for the cultured cells was then followed. The results are expressed as Mean ± Standard Error of triplicate determinations in five different Petri dishes (5 cm. dia). Phosphatase Unit is the amount of enzyme contained in 1 mg protein which liberates 1 mmole of p-nitrophenol/hour at 37°C. Lactic dehydrogenase Unit is the amount of enzyme contained in 1 mg protein which converts 1 μmol of substrate/min at 25°C.

lication of cultured endometrial cells. The cyclic nucleotide however, inhibited unexpectedly both the hormone-induced increases in labeled uridine and amino acid incorporation. It should be pointed out that sex steroids have been shown to exert their effects on chick oviduct [24] or prostate [25] without changing the adenyl cyclase activity and that Kaye *et al.* [26] were unable to mimic estrogen effects by injecting DBCAMP into immature rats.

The levels of acid phosphatase, a lysosomal enzyme, were also found to be unchanged by hormonal additions to our cultures. The lack of inflammatory reaction in our *in vitro* system is also supported by the findings of Berliner and Gerschenson [22] demonstrating a lack of ultrastructural quantitative or qualitative changes in primary lysosomes in steroid hormone-treated cultured endometrial cells. At present the reasons for these differences between the *in vitro* vs *in vivo* responses are not known. The following possibilities should be considered: (a) a need for tissue-level organization; (b) the hyperemic effects may occur mainly in the endometrial stroma or myometrial cells which are not present in our experimental system [1]; (c) the lack "in culture" of vascular structures, which may be the real indirect or direct target for the inflammatory effects; and (d) the inflammatory estrogenic effects may not be at all a primary cause for the hormonal manifestations. We favour this last hypothesis and believe that the inflammatory reaction is secondary and has possibly been acquired during evolution as an advantageous change to amplify estrogenic effects, by increasing the availability of nutrients.

It is interesting to note the hydrocortisone inhibition of the progesterone effect in decreasing the percentage of labeled cells, since it has been shown in other systems that progesterone is a competitive inhibitor for glucocorticoids [27].

Alkaline phosphatase and lactic dehydrogenase specific activities have been shown to increase in uterus of ovariectomized animals injected with estrogens [28, 29] and the transport of amino acids and carbohydrates was also found to increase under the same experimental conditions [6, 7]. The present findings demonstrate that such changes could not be detected in cultured endometrial cells, which otherwise were responsive to ovarian hormones. This lack of response may also be, as mentioned above, the result of the loss of absence in culture, of a specific cell population or may indicate that the estrogenic stimulation of enzyme activities and cell membrane transport observed in the animal is indirect and is not due to an action of the hormone directly on the endometrial cells. Further experiments are required to answer these important questions.

The presence of estrogenic and progestogenic effects apparently dissociated from metabolic and cell membrane transport changes, in epithelial endometrial cells cultured in chemically defined medium should prove useful in further studies on the hormonal control of growth, and the mechanism of steroid hormone actions and interactions.

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