Journal of Steroid Biochemistry, 1976. Vol. 7, pp. 153–158. Pergamon Press. Printed in Great Britain

SEX STEROID INDUCED MORPHOLOGICAL CHANGES IN PRIMARY UTERINE CELL CULTURES

J. A. BERLINER and L. E. GERSCHENSON

Laboratory of Nuclear Medicine and Radiation Biology, and Department of Pathology, UCLA School of Medicine, 900 Veteran Avenue, Los Angeles, California 90272, U.S.A.

(*Received* 8 *July* 1975)

SUMMARY

The ultrastructure of primary cultures of endometrial cells after treatment with diethystilbestrol, progesterone, and hydrocortisone was examined using the method of electronmicroscopic stereology. Cells treated with diethylstilbestrol had the appearance of a rapidly dividing culture having large euchromatic nuclei and prominent nucleoli with a cytoplasm containing many free ribosomes. Progesterone appeared to convert the cells to a more secretory type with large amounts of RER and smaller nucleoli. Mitochondrial enlargement was induced by sex hormones but not by hydrocortisone.

INTRODUCTION

Sex steroids have been shown *in vivo* to induce numerous morphological changes in uterine epithelium[1–3]. However, due to the multiple homeostatic mechanisms operating in the body, it is difficult to determine which cell types are directly affected and which hormones are directly responsible for the observed changes. We have thus developed a primary uterine cell culture system that has been shown to be sensitive to the actions of diethylstilbestrol and progesterone which induced changes in the percentage of ^{3}H-TdR labelled cells, as determined by autoradiography, and the uptake of ^{3}H uridine and ^{3}H aminoacids into TCA- precipitates[4, 5]. In this paper we have examined the ultrastructural changes induced by these hormones using the technique of electron microscopic stereology. We have found that the cell ultrastructure reflects the changes in the growth rates and macromolecular synthesis mentioned above. Overall organelle changes are generally similar to those reported in rabbit uterus *in vivo*[3] with the exception of changes in the Golgi apparatus. Fragmentary reports of mitochondrial alterations in response to estrogen and progesterone have been made in the literature[1–3] indicating that such alterations may be part of the mechanism of hormone action. In the present detailed study we found that in fact both hormones produced mitochondrial enlargement with a concomitant decrease in mitochondrial number.

MATERIALS AND METHODS

Endometrial cells were obtained from rabbit uterus and cultured for 4 days in defined medium as described previously[4]. For this four day period some cultures were supplemented with 10^{-7} M diethylstilbestrol (DES), 10^{-7}M progesterone (P), a combination of the two (D + P), or 10^{-7} M hydrocortisone (HC).

For electron microscopy cells were fixed *in situ* in 0·135 M sodium phosphate buffer containing 2% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in Luft's epon on the dishes with capsules placed over epithelial colonies before polymerization. The epon covered dishes and capsules were polymerized at 60°C for 1 day, after which the plastic was stripped from the dishes and then polymerized an additional 2 days. This stripping at 1 day allows for easy removal of epon and is an essential step to obtain clearly visible preparations. Blocks were sectioned, and grids stained for 30 min with alcoholic uranyl acetate and 5 min with lead citrate.

For morphometric analysis the following sampling procedure was employed. For each treatment 4 separate isolates were examined and eight separate blocks (2 from each isolate) were sectioned. For low magnification analysis, to determine organelle volume density, surface density, number and volume, 3 micrographs were made at a magnification of 3000 × using the corner sampling method[6] and enlarged 3 times. Thus for each treatment a total of approx 7000 u^2 of cellular area was analyzed for all the data in Tables 1 and 2 except cristae area of mitochondria. A 1 cm. grid was employed in the morphometric analyses. The data in Table 1 is expressed as Vv, volume density of the component or Sv, surface density of the component[6]. Volume density is the percent of a given area occupied by a component, for example the Vv of the Golgi in Table 1 represents the average % of the cytoplasm containing Golgi. The surface density represents the % of the total visible surface covered by the component.

To determine overall cell size/nucleus phase contrast micrographs were made of 16 randomly selected fields for each treatment. Micrographs were taken at

Table 1. Effect of sex hormones on general cell ultrastructure. The fraction of the total cell occupied by nucleus (Nucleus/cell), and the fraction of the nucleus occupied by the nucleolus (nucleolus/nucleus) were determined by morphometric methods. Also the fraction of the cytoplasm containing Golgi (Golgi/cytoplasm), primary lysosomes (1° lysosomes/cytoplasm) and secondary lysosomes (2° lysosomes/cytoplasm) were examined. Finally the fraction of the visible surface area occupied by rough endoplasmic reticulum (RER/cytoplasm) was measured. The data are given as the mean (± standard error) of 24 micrographs

		Co	DES	P	DES & P
Nucleus/cell	Vv	0·26 ± 0·03	0·29 ± 0·03	0·27 ± 0·03	0·38 ± 0·63*
Nucleolus/nucleus	Vv	0·09 ± 0·02	0·13*** ± 0·01	0·06*** ± 0·01	0·15 ± 0·02
RER/cytoplasm	Sv	0·11 ± 0·02	0·16 ± 0·03	0·24 ± 0·04*	0·20*** ± 0·02
Golgi/cytoplasm	Vv	0·016 ± 0·003	0·011 ± 0·002	0·013 ± 0·002	—
1° lysosomes/cytoplasm	Vv	0·008 ± 0·002	0·007 ± 0·001	0·006 ± 0·001	—
2° lysosomes/cytoplasm	Vv	0·051 ± 0·001	0·04 ± 0·008	0·07*** ± 0·001	—

* P 0·005 vs control
** P 0·01 vs control
*** P 0·025 vs control

a magnification of 50 times, enlarged to 200 times and analyzed for cell size/nucleus using A 0·5 cm. grid.

RESULTS

The general appearance of uterine epithelial cells in culture is similar to that seen *in vivo*[3]. We reported previously some initial observations on their appearance and the more extensive studies reported here reveal further similarities. The cells in culture form colonies with junctional complexes between cells being composed of desmosomes and areas which appear to be tight and gap junctions (Plate 1a). There are extensive "adherens zones" with microfilaments connecting to the terminal web regions. The cells themselves contain prominent Golgi complexes, often several per cell and considerable rough endoplasmic reticulum (RER). Mitochondria are of a similar size and form to those seen *in vivo*.

The ultrastructural appearance of cells treated with DES suggests a rapidly growing population which is engaged in little secretory activity, while cells treated with P and DES + P appear to be engaged in the production of proteins for secretion and not rapidly dividing (Fig. 1, 2 and Table 1). In DES-treated cultures this is indicated by the presence of large highly euchromatic nuclei with large prominent nucleoli and small amounts of RER but many free ribosomes. Nuclei were more heterochromatic and nucleoli small in P treated cultures; large nuclear indentations were more prominent (Fig. 2). Nuclei of DES + P treated cells were intermediate in morphology to those with DES or P alone (Fig. 2, 2). The amount of RER was increased by about 2-fold with P and DES ± P and only about 0·4 fold with DES treatment. The volume density of primary lysosomes and Golgi were unaltered by these hormones as was their position in the cell; however, there was a slightly significant increase in the Vv of secondary lysosomes with P. The overall cell area/nucleus was approximately the same for control and DES cultures as determined by phase microscopy (Co = 1·16 ± 0·11; DES = 1·06 ± 0·08), but with P and especially DES + P, cells were increased in size (P = 1·25 ± 0·15; DES + P = 1·49 ± 0·11).

Mitochondria were increased in volume 1·6-fold by DES, P and DES + P and decreased in number by the same amount (Table 2). More branched mitochondria were seen in these preparations as compared to controls (Control = 0·05%. Hormone-treated 2·3%.) As would be expected with an increase in volume and a decrease in number, the surface to volume ratio of an average mitochondrion was decreased by the sex steroids. The effects of DES and P were not additive. The cristae Vv in enlarged mitochondria were not altered from that seen in mitochondria of control cultures indicating that enlarge-

Table 2. Effect of sex hormones and hydrocortisone on mitochondrial morphology. The fraction of the cytoplasm containing mitochondria, (Mit/Cyt) and the fraction of the mitochondria containing cristae (Cristae/Mit) were determined by morphometry. Also the surface to volume ratio of an average mitochondrion, the average volume of a mitochondrion and the number/100 μ^3 of cytoplasm were determined by morphometric measures. The data are given as the mean (± standard error) of 24 micrographs

		Co	P	DES	DES & P	HC
Mit/cyt	Vv	0·06 ± 0·004	0·06 ± 0·005	0·06 ± 0·007	0·05 ± 0·001	0·06 ± 0·002
Surface/volume	Sv/Vv	9·99 ± 0·77	6·91* ± 0·34	6·74* ± 0·59	6·79* ± 0·37	8·89 ± 0·73
No./100 μ ^{3}Cyt	N/100 μ^3	23·00 ± 2·33	14·17** ± 1·51	15·43** ± 1·71	15·61** ± 1·63	20·10 ± 1·02
Volume	V	0·30 ± 0·04	0·49** ± 0·04	0·51** ± 0·08	0·47** ± 0·05	0·31 ± 0·03
Cristae/mit	Vv	0·38 ± 0·03	0·38 ± 0·04	0·38 ± 0·02	0·40 ± 0·009	—

* P < 0·001 vs. control
** P < 0·005 vs. control

ment is not a result of swelling. Pictures of control and treated mitochondria are shown in Fig. 3. Hydrocortisone produced no change in mitochondrial shape or size (Table 2).

DISCUSSION

The ultrastructural appearance of primary uterine cell cultures after treatment with steroids generally resembles that seen *in vivo* in pre- and post-ovulatory states in the rabbit uterus[3]. The morphology of our DES-treated cells corresponds approximately with the morphology of endometrial epithelium of rabbits in estrus, DES + P to 3 days post ovulatory, and P alone to 6 days post ovulatory. In control and DES treated cells the nuclei were very euchromatic and contained large multiple nucleoli; nuclei from P

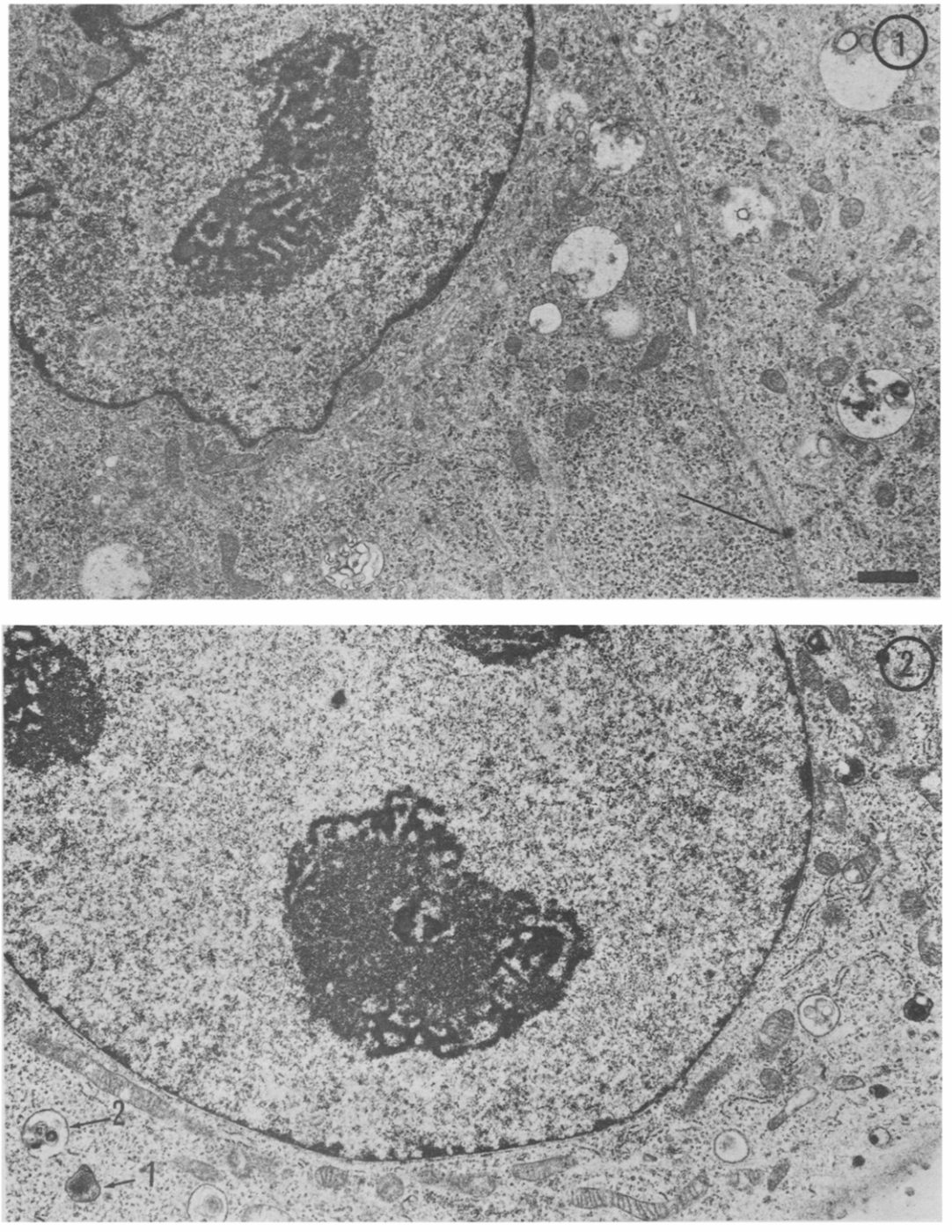

Fig. 1. Micrographs of control (1) and diethylstilbestrol treated (2) cultures to show general cell appearance. The arrow in (1) points to a desmosome; just above this area some microfilaments can be barely seen leaving the junction area and further up an area of very close junctions can be seen. In (2) a primary lysosome is shown at arrow 2. Mag = 9000 × Mark represents 1 μm.

treated cells were more heterochromatic and contained smaller nucleoli; in DES + P cells intermediate states were found for chromatin and nucleoli. The amounts of rough endoplasmic reticulum was found to increase primarily in the presence of P, while there appeared to be a larger number of individual ribosomes in control and DES treated cultures as seen *in vivo*. Biochemical studies indicated an increase in general RNA synthesis with both hormones[5] and the morphological data described above suggest that

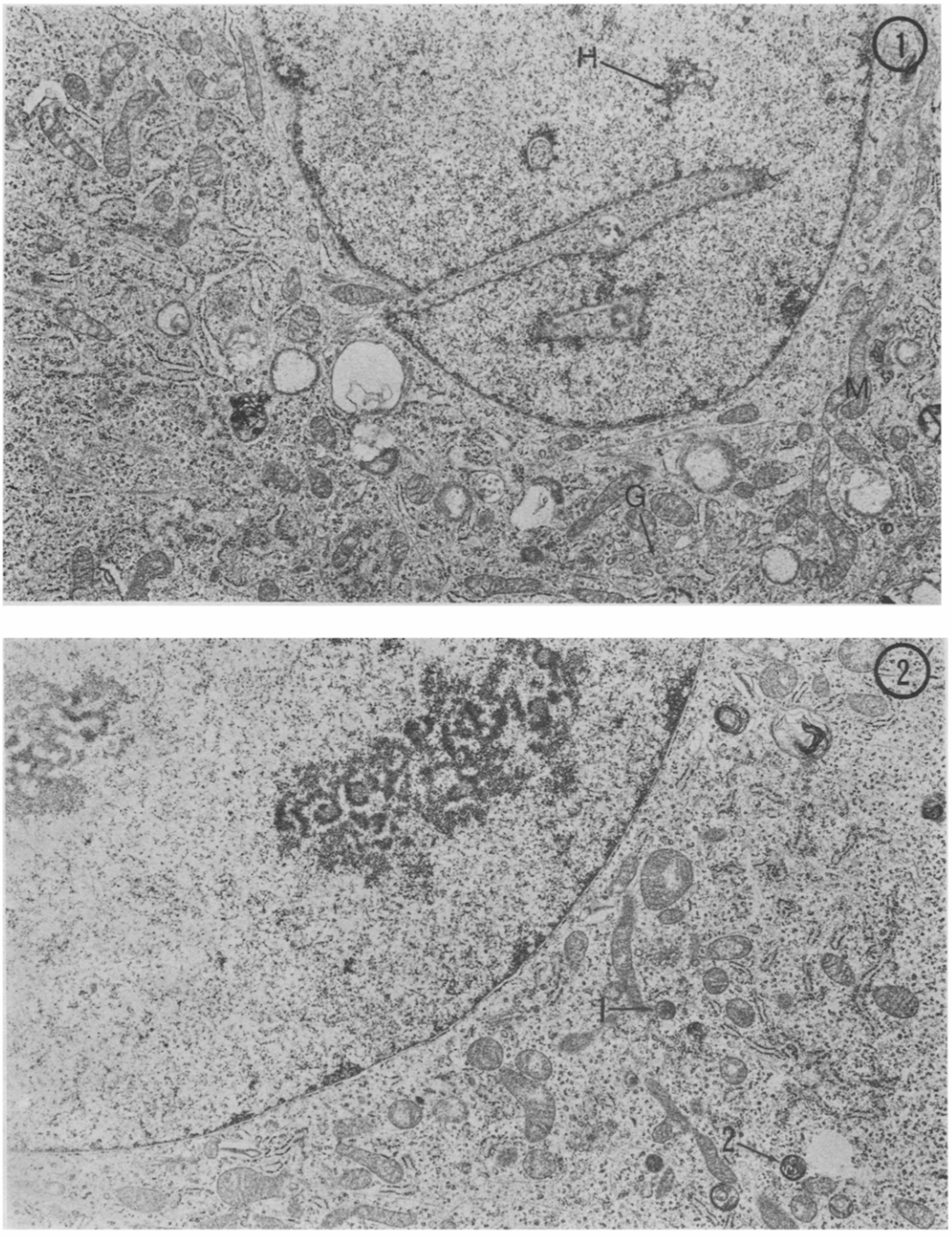

Fig. 2. Micrographs of P (1) and DES + P (2) treated cells. (1) Progesterone treated cells showed increased amounts of RER and mitochondrial enlargement. Many nuclear invaginations were observed as shown here and more condensed chromatin (H) was present. G = Golgi. Nucleoli were small and similar to those seen with DES + P but half the size (not shown). (2) Cells treated with DES + P had nucleoli intermediate in size to those with either hormone separately. Mitochondria were enlarged but not more than with each hormone alone. As in all cells, many primary lysosomes (arrow 1) and secondary lysosomes (arrow 2) were present. Same Mag. as Fig. 1.

DES and P may effect in a different way nucleolar and nucleoplasmic RNA synthesis. In previous studies we showed that there was an overall increase in protein in cultures treated with DES+P greater than that observed with DES alone though the DNA synthetic rate was higher with D[4]. Our morphological

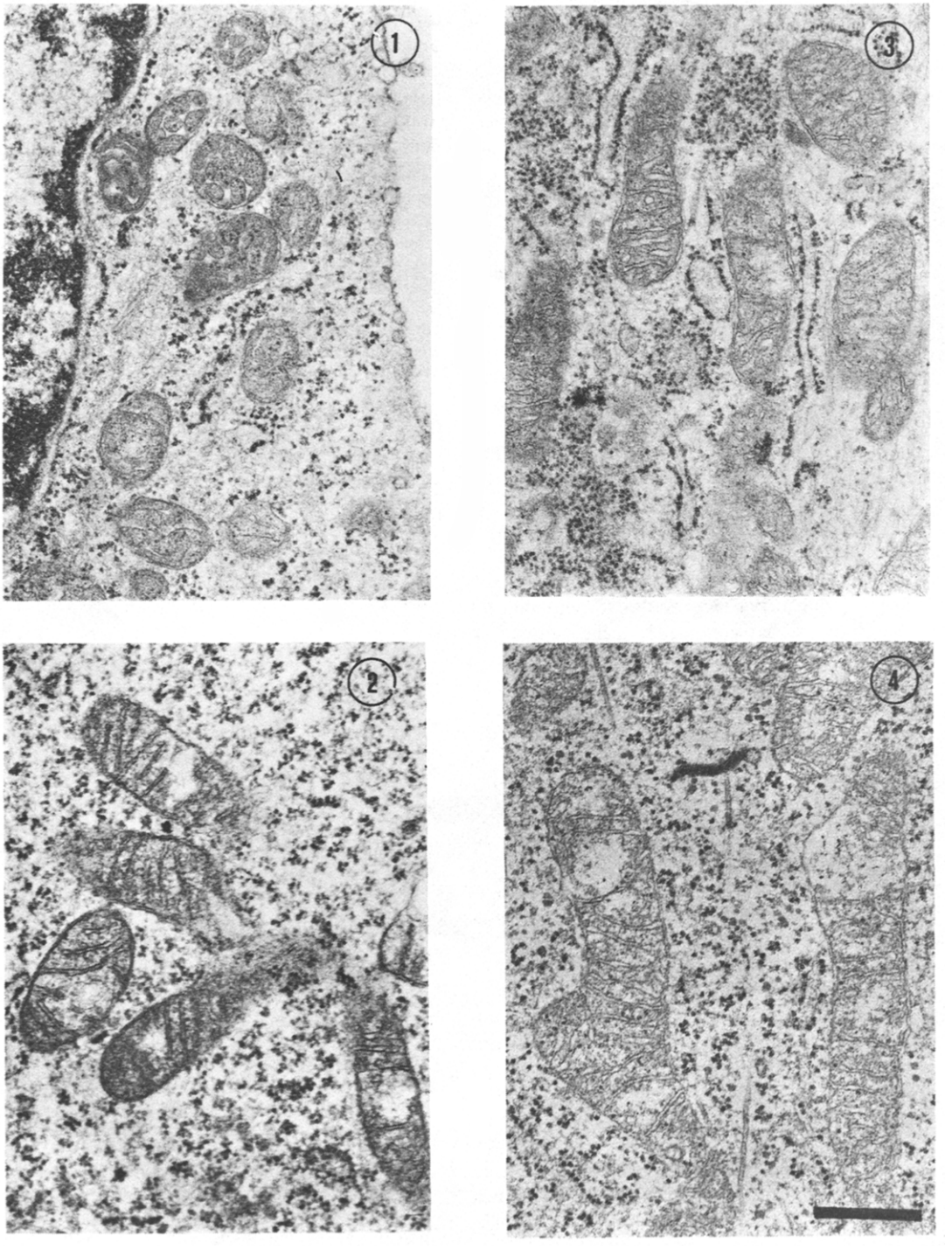

Fig. 3. Higher magnification micrograph of the mitochondria from control and hormone treated cultures. (1) Control. (2) Diethylstilbestrol. (3) Progesterone. (4) Diethylstilbestrol + progesterone. The mitochondria for these pictures were selected at random and are not meant to represent exactly the dimensional differences shown in Table 2. The control mitochondria were generally more dense than mitochondria from treated cells which could perhaps represent a different respiratory state. Many mitochondria showed some indications of small amounts of swelling; some of these are seen here. Mag = 30,000. Mark represents 1/2 μm.

observations suggest that the cells treated with DES+P are hypertrophic compared to DES alone which may explain our earlier result.

There was no qualitative or quantitative alterations in primary lysosomes with DES or P treatment though there was a slight increase in the Vv of secondary lysosomes with progesterone. It is noteworthy to point out that no biochemical difference was found in a lysosomal enzyme activity measured in these cells[5]. The general lack of lysosomal differences we obtained might not have been expected from some previous observations[8] though other authors also have observed no change in lysosomes in rabbit uterine endothelium with sex steroids[3]. The lack of difference in these cultured cells might however be explained by the fact that the number of primary lysosomes per cell is reduced as compared to uterine endothelial cells *in vivo* (a rough estimate suggests a 60% reduction per unit area of cytoplasm) and more secondary lysosomes are present. The major difference in the morphology of cultured cells compared to cells *in vivo* in the lack of changes in the Golgi apparatus with progesterone. A possible cause for this observation could be the absence of vitamin A in our culture medium which has been found necessary for the maintenance of mucus secretion in several glandular epithelia *in vitro*[9, 10].

The fact that we found general morphological and biochemical similarity between cultured cells and cells *in vivo*, suggests that endometrial cells cultured in defined medium can be used as a reliable system to study the specific direct actions of these hormones. The data from stereology closely confirm previously obtained experimental information and suggests the usefulness of this method as an indicator of overall cell alterations.

The addition of DES and P also caused mitochondrial enlargement with a concomitant decrease in mitochondrial number. The increased number of branched forms in hormone treated cells suggest the formation of enlarged mitochondria by fusion; a similar conclusion for the method of formation of enlarged mitochondria in cortisone treated liver was drawn by Kimberg and Loeb[11]. These mitochondrial changes induced by sex steroids in uterus resemble those induced by corticosteroids in liver cells in culture[12] or in *in vivo* experiments using animals[13] and possibly those produced by testosterone in prostate epithelium[14]. All of these observations indicate the possibility that the induction of mitochondrial fusion is a consistent effect of steroids. That this is not a non-specific effect of steroids is shown here by the lack of HC induced effects in uterine cells, and the lack of P induced changes in liver cell mitochondria (Berliner, J. A. unpublished observation).

What are the implications of mitochondrial fusion for cellular metabolism and how might any changes in metabolism contribute to the overall function of these hormones? The answer to these questions is still unclear. Some studies have been made on mitochondrial function in "fused" mitochondria using livers from cortisone treated[15] and riboflavin deficient rats[16]. These studies show several defects in enlarged mitochondria, chief among them being a decreased efficiency in oxidative phosphorylation expressed as a decrease in P:O ratios. However, the universality of defective mitochondrial function in enlarged mitochondria is far from certain due to the possibility of greater liability of mitochondria isolated from treated animals. Defective function would be difficult to reconcile with the well-known anabolic effects of steroids hormones in uterus[17]. Further experimental data will be required for clarification of this problem.

Note added in proof. The effects on mitochondria of DES and P at a concentration of 10^{-9} M were evaluated and found to be similar to the effects at 10^{-7} M. The surface/volume ratio for DES was $7{\cdot}22 \pm 0{\cdot}35$ and for P was $7{\cdot}00 \pm 0{\cdot}41$. This data should be compared with the one presented in Table 2.

Acknowledgements—We thank J. Yang, S. Beydler and L. Gumz for excellent assistance. This work was supported by Grant CA-12136 from the NIH, by Contract E(04-1)Gen-12 between ERDA and the University of California, and by grants from UCLA Fund and the California Institute for Cancer Research.

REFERENCES

1. Wynn R. M. and Harris J. A.: *Fertil. Steril.* **18** (1967) 632–648.
2. Wynn R. M. and Woolley R. S.: *Fertil. Steril.* **18** (1967) 721–738.
3. Davies J. and Hoffman L. H.: *Am. J. Anat.* **142** (1975) 335–366.
4. Gerschenson L. E., Berliner J. and Yang J.: *Cancer Res.* **34** (1974) 2873–2880.
5. Gerschenson L. E. and Berliner J. A.: *J. steroid Biochem.* **7** (1976) 159–165.
6. Weibel E. R. and Bolender R. P.: *Principles and Techniques of Electron Microscopy* (Edited by M. A. Hayat) Vol. 3 pp. 237–296. Van Nostrand Reinhold, New York (1973).
7. Loud A. V.: *J. cell Biol.* **37** (1968) 27–46.
8. Szego C. M.: *The Sex Steroids* (Edited by K. W. McKerns) pp. 1–52. Appleton-Century-Crofts, New York (1971).
9. Lasnitzki I.: National Cancer Institute Monograph **12** (1963) 381–393.
10. Fell H. B. and Rinaldini C.: *Cells and Tissues in Culture* (Edited by E. N. Willmer) Vol. I pp. 659–699. Academic Press, New York (1965).
11. Kimberg D. V. and Loeb J. H.: *J. cell Biol.* **55** (1972) 635–643.
12. Berliner J. A.: *J. cell Biol.* **64** (1975) 711–716.
13. Wiener J., Loud A. V., Kimberg D. V. and Spiro D.: *J. Cell Biol.* **37** (1968) 47–61.
14. Groth D. P. and Brandes D.: *J. Ultrastruct. Res.* **4** (1960) 166–181.
15. Kimberg D. V., Loud A. V. and Weiner J.: *J. cell. Biol.* **37** (1968) 63–78.
16. Tandler B. and Hoppel C. L.: *Meth. Achiev. Exp. Path.* (Edited by E. Bajusiz and G. Jasmin) Vol. 6, pp. 25–47. Karger, Basel (1972).
17. McKerns K. W.: *The Sex Steroids* Appleton-Century-Crofts, New York 1971.