

EFFECTS OF 5-BROMODEOXYURIDINE ON THE ACTH-DEPENDENT
MITOCHONDRIAL BIOGENESIS IN CORTICAL
CELLS OF FETAL RAT ADRENALS IN TISSUE CULTURE

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Mitochondrial division and development of the inner membranes of mitochondria in adrenocortical cells can be inhibited in tissue culture by many inhibitors, all of which seem to have a more or less specific locus in the mitochondrial protein synthesis system (4-6). As yet, it is not certain whether inhibited proteins are necessarily coded by mitochondrial DNA.

In fetal rat adrenal glands in tissue culture two phases of active DNA synthesis occur in cortical cells. First, most of the undifferentiated cortical cells actively synthesize DNA during cultivation. This nuclear DNA synthesis is very strongly suppressed by ACTH stimulation during the first 24 h. Upon further stimulation, an increase in mitochondrial DNA synthesis occurs and the cells differentiate from the undifferentiated zona glomerulosa type to a differentiated zona fasciculata type of cortical cells (3).

5-bromodeoxyuridine (BrdU) has been shown to displace thymidine moieties in both nuclear (12) and mitochondrial (2) DNA during the replicative cycle. We decided to study the effects of BrdU addition on both phases of DNA synthesis of cortical cells and thus, possibly, gain more insight into the roles of these DNAs during the ACTH-induced differentiation of cortical cells.

MATERIALS AND METHODS

Tissue Culture

Primary cultures of fetal rat adrenals were used (3). Rats of the Sprague-Dawley strain served as the source of the experimental material. The medium was changed every fifth day and consisted of 50% Melnick solution (Hanks' balanced salt solution + 0.5% lactalbumin hy-

drolysate) and 25% Eagle's minimum essential medium (both from Pharmaceutical Manufacturer Orion Oy, Finland) and 25% heat-inactivated and ultrafiltrated calf serum (Grand Island Biological Co., United Kingdom). 5-bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, Mo.), thymidine (TdR) (Sigma), [6-³H]5-bromodeoxyuridine (The Radiochemical Centre, United Kingdom) (sp act 728 mCi/mmol) and adrenocorticotrophic hormone (ACTH) (Cortrophine, Organon, Holland) were added dissolved in 0.1 ml of Hanks' BSS.

Metabolism of [¹⁴C]Progesterone

[4-¹⁴C]progesterone (sp act 61 mCi/mmol) (The Radiochemical Centre) was purified on thin-layer chromatography (TLC) before use (11) and was then added to 40 μ l of propylene glycol-ethanol (vol/vol 95:5) to give a concentration 40 nCi/ml (about 0.4 μ g/ml). Basal conversion values were obtained by adding the substrate on the 16th day of cultivation and removing the medium after 24 h. Fresh medium was applied, ACTH and BrdU were added, and on the 22nd day the same substrate was added and removed after 24 h. Extraction, TLC separation, and final identification of metabolites were carried out as described previously (11).

Assay for Endogenous Steroids

This assay was carried out as described elsewhere (10). After purification by partition and Sephadex-LH-20 column chromatography deoxycorticosterone (DOC) was quantitated by radioimmunoassay and corticosterone and 18-OH-DOC by gas liquid chromatography.

Morphologic Observations

Tissue cultures were fixed *in situ* in 2.5% glutaraldehyde in Hanks' BSS, postfixed in 1% osmium tetroxide with phosphate buffer (8), and embedded *in situ* in Epon-Araldite mixture. The sections were stained with 0.2% lead citrate (15) and examined with a Hitachi-7S

electron microscope. For light microscope radioautography, 1 μm sections were cut and liquid emulsion (Ilford K2) autoradiography was done, using the "dipping method" (1). Labeling indices (percent of labeled nuclei) were based on counts of at least 1,000 cells per culture.

RESULTS

BrdU Incorporation in Cortical Cells

After the 16th day of cultivation, treatment of adrenocortical cells for 48 h with [^3H]BrdU (4 $\mu\text{Ci/ml}$) resulted in a labeling index of $19.9 \pm 2.8\%$ (mean \pm SEM, four cultures). ACTH stimulation (100 mU/ml/day) strongly reduced the number of cells incorporating [^3H]BrdU, as indicated by a corresponding index of $1.6 \pm 0.4\%$ (mean \pm SEM, four cultures). Phase-contrast and light microscope observations revealed no changes in growth or morphology with daily doses of 0.3–30 $\mu\text{g/ml}$ BrdU starting from the very beginning of the cultivation. However, when ACTH was added to the BrdU pretreated cultures, cortical cells seemed to degenerate and there was some loss if the pretreatment dose was over 1 $\mu\text{g/ml/day}$. This phenomenon was strictly confined to the cortical cell component of the cultures; fibroblasts remained in good condition. Without pretreatment cortical cells tolerated well doses up to 120 $\mu\text{g/ml/day}$ and no light microscope visible changes in growth or differentiation could be observed when BrdU was combined with ACTH.

Ultrastructure of Cortical Cells in the Presence of BrdU

Proliferative cortical cells in cultured fetal rat adrenals had the ultrastructure of zona glomerulosa cells. In the concentrations used, BrdU alone had no effect on the ultrastructure of cortical cells. ACTH induces differentiation of cortical cells and

their mitochondria from the zona glomerulosa type to the zona fasciculata type. Change in the configuration of mitochondrial inner membranes from lamellar or tubular to 600 \AA vesicles is the most prominent ultrastructural effect of ACTH (Fig. 1). Pretreatment of cortical cells with 0.5 $\mu\text{g/ml}$ BrdU/day resulted in a strong inhibition of the ACTH-induced differentiation response. Only a slight increase in the size of cortical cells was observed. The development of microvilli was scant. Smooth-surfaced endoplasmic reticulum appeared as dilated vesicles, and no increase in the number of lipid droplets was visible (Fig. 3). Mitochondria remained small in size and their inner membranes formed tubular or lamellar elements.

TdR (1 $\mu\text{g/ml/day}$) completely abolished the inhibitory effect of BrdU pretreatment. The ultrastructure of cortical cells displayed the same morphogenic differentiation as with ACTH treatment only.

If BrdU was added in doses of 30 $\mu\text{g/ml/day}$, with ACTH from the 16th to the 21st cultivation day inclusive, normal ultrastructural differentiation of cortical cells, except their mitochondria, occurred. The ultrastructure of mitochondria appeared somewhat heterogeneous. The number of their profiles decreased and they seemed larger in size than with only ACTH treatment. The inner membranes appeared lamellar or tubular (Fig. 2), and mitochondria containing only a few vesicular cristae in the empty matrix, were also observed. In the cultures to which TdR (40 $\mu\text{g/ml/day}$) had been added together with BrdU, ACTH was able to produce normal differentiation of cortical cell mitochondria.

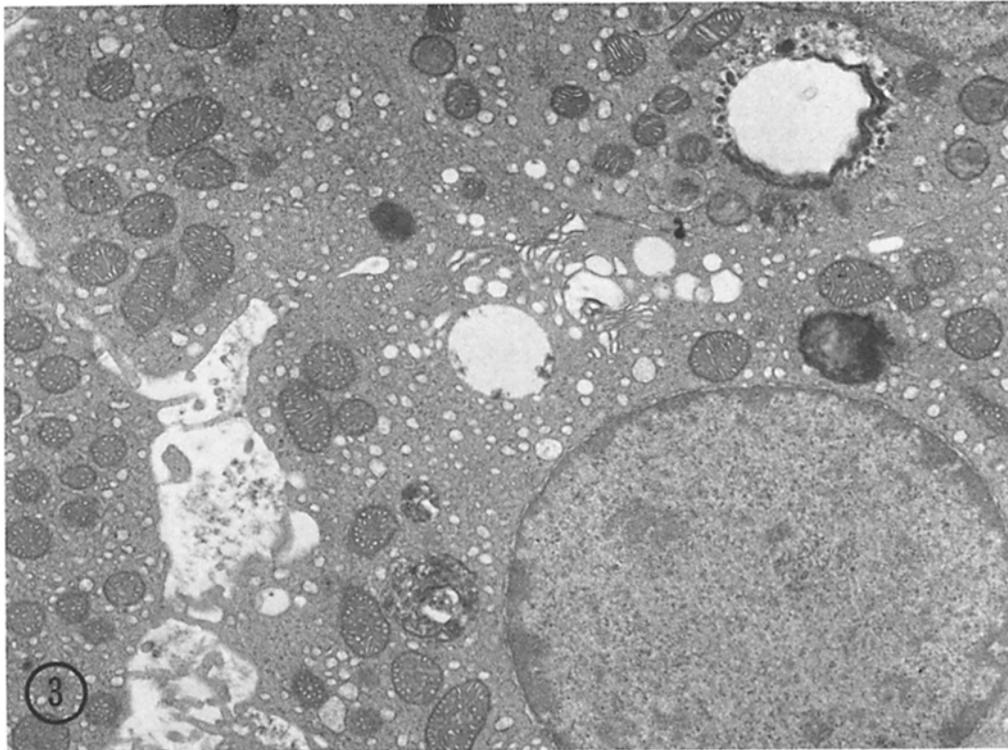
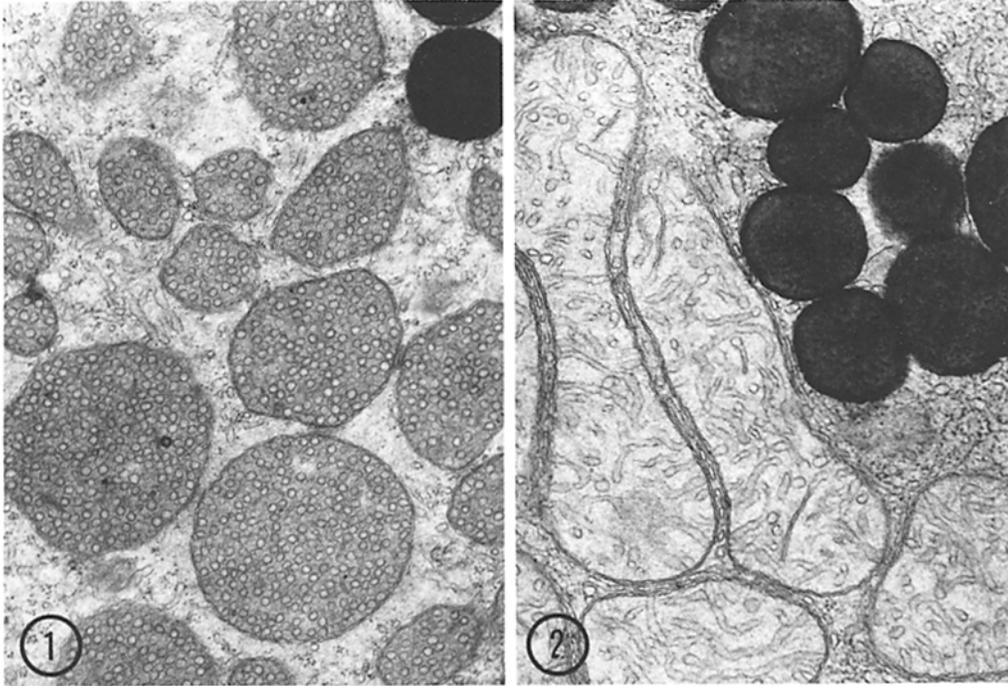
Effects of BrdU on the [^{14}C]Progesterone Metabolism

BrdU in a dose of 30 $\mu\text{g/ml/day}$ had no effect

FIGURE 1 Mitochondria in cortical cell of fetal rat adrenals in tissue culture treated with ACTH (100 mU/ml/day) from 16th cultivation day to 21st day inclusive. Note the transformation of mitochondrial inner membranes to 600 \AA vesicles. $\times 25,000$.

FIGURE 2 Mitochondria in cortical cell of fetal rat adrenals in tissue culture treated with 30 $\mu\text{g/ml/day}$ of BrdU and 100 mU/ml/day of ACTH from 16th cultivation day to 21st day inclusive. Note the well-developed tubular smooth endoplasmic reticulum and lipid droplets and inhibition of development of mitochondrial inner membranes into 600 \AA vesicles. $\times 25,000$.

FIGURE 3 Cortical cells in tissue culture of fetal rat adrenals cultivated in the presence of 0.5 $\mu\text{g/ml/day}$ of BrdU from the beginning of the cultivation and stimulated with ACTH from 16th cultivation day to 21st day inclusive. Note the absence of lipid droplets, poorly developed microvilli and smooth surfaced endoplasmic reticulum and inhibition of the differentiation of mitochondrial inner membranes. $\times 9,900$.



on the ACTH-induced cytoplasmic 21-hydroxylase dependent conversion of progesterone to DOC as found in fraction 4 (Table I). Between 80–90% of this fraction consists of DOC. Further hydroxylations in mitochondrial compartment were strongly inhibited as shown in the 11 β - and 18-hydroxylase dependent conversion to fraction 2. No effect was found on the other progesterone metabolites.

Effects of BrdU on DOC, Corticosterone, and 18-OH-DOC Secretion

There were no significant differences in the steroid output of the control cultures, 0.5 μ g/ml BrdU/day, and combined BrdU and 1 μ g/ml TdR/day treated cultures on the 15th day of cultivation (Fig. 4). Over 50% inhibition of corticosterone and 18-OH-DOC secretion was observed when the BrdU-pretreated cultures were exposed to ACTH. If TdR had been combined to pretreatment the inhibitory effect was completely abolished.

When BrdU was added in a dose of 30 μ g/ml/day from the 16th to 21st day of cultivation together with ACTH, secretion of corticosterone and 18-OH-DOC was strongly inhibited (Fig. 5). The secretion of DOC, instead, seems to stay in the levels observed in the cultures treated with ACTH only. The relation of 18-OH-DOC to corticosterone remained constant irrespective of the treatment. TdR (40 μ g/ml/day) again reversed the inhibitory effect.

DISCUSSION

With a few exceptions, the thymidine analogue, 5-bromodeoxyuridine, has been shown to influence specialized cell functions through the substitution

of thymidine and incorporation into DNA sequences involved in the control of specific proteins (9). BrdU is readily incorporated into DNA in

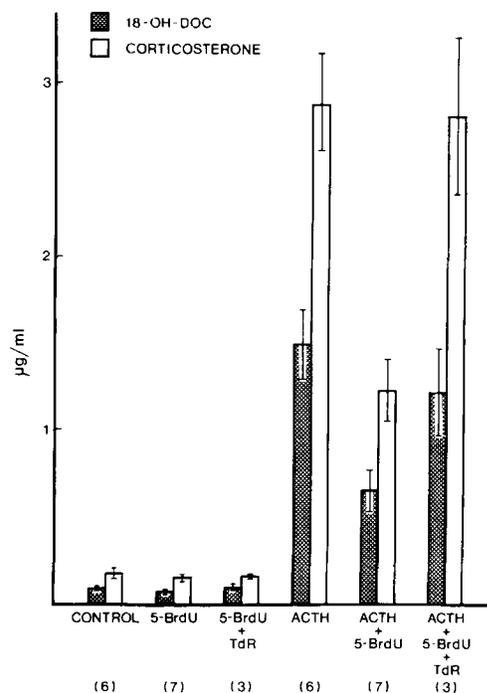


FIGURE 4 Concentrations of 18-OH-DOC and corticosterone in the tissue culture medium. The three bars in the left represent the secretion from 10th to 15th days of cultivation. The treatments (BrdU 0.5 μ g/ml/day and TdR 1 μ g/ml/day) are indicated below the bars. The three bars in the right results from the same cultures exposed to ACTH (100 mU/ml/day) from 16th day to 21st day of cultivation. Means \pm SEM are indicated. Number of the cultures per group in parentheses.

TABLE I
Percentage Incorporation of Radioactivity (\pm SEM) from [14 C]Progesterone (0.2 μ Ci) in TLC Fractions

Treatment	n	Fractions*				
		2	3	4	5	6
Control	10	5.5 \pm 0.4	4.9 \pm 0.5	5.5 \pm 0.4	16.4 \pm 3.1	59.8 \pm 3.7
ACTH (100 mU/ml/day)	5	11.1 \pm 0.6‡	5.7 \pm 1.0	8.2 \pm 0.3‡	14.4 \pm 1.8	57.2 \pm 2.9
ACTH (100 mU/ml/day) + BrdU 30 μ g/ml/day)	5	7.5 \pm 1.0§	6.2 \pm 0.5	8.0 \pm 0.8	18.4 \pm 2.2	56.9 \pm 3.8

In statistical comparisons (Student's *t* test) ACTH-treated cultures were compared with control cultures and combined ACTH- and BrdU-treated cultures were compared with ACTH-treated cultures.

* Fractions: 2 = corticosterone + 18-OH-DOC; 3 = 11 β -OH-progesterone + 5 α/β -pregnandiol; 4 = DOC + 20 α -dihydroprogesterone; 5 = 5 α/β -pregnanolone; 6 = unchanged progesterone.

‡ *P* < 0.001.

§ *P* < 0.01.

|| Nonsignificant.

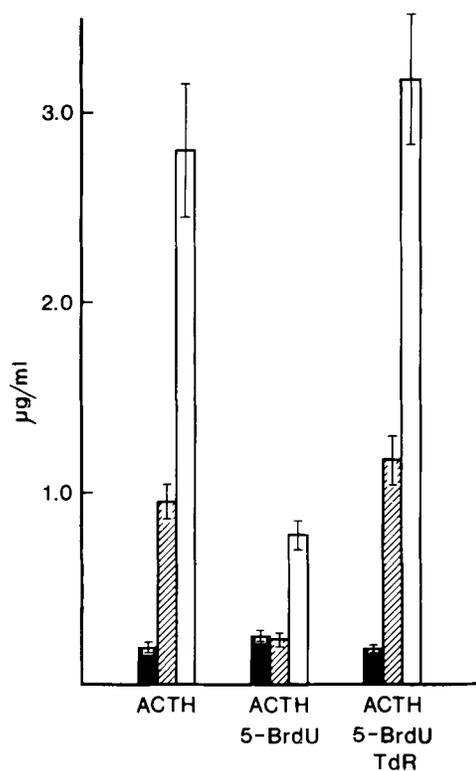


FIGURE 5 Concentrations of DOC (black bars), 18-OH-DOC (shaded bars) and corticosterone (open bars) in the tissue culture medium. The treatments (ACTH 100 mU/ml/day, BrdU 30 μ g/ml/day, and TdR 40 μ g/ml/day) are shown below the bars. Means \pm SEM of three cultures are indicated.

vitro and in vivo in prokaryotic and eukaryotic cells and it is obvious that the action of BrdU is DNA-linked (14, 16). A dose of 10^{-6} M BrdU caused about 60% inhibition of specific protein synthesis in cultures of pancreatic rudiments (14). At concentrations of 10^{-4} M BrdU 90% of the thymidine moieties are replaced by BrdU (12). Repression of morphogenic differentiation and specialized functions has been shown to occur in various tissues (7, 13, 14).

As documented in the present study, BrdU was incorporated very effectively in nuclear DNA of cortical cells in the absence of ACTH. The labeling index (19.9) after 48 h of incubation was the same as with $[^3\text{H}]\text{TdR}$.¹ It seems reasonable to

¹ Salmenperä, M., and A. I. Kahri. Studies on the dependence of mitochondrial 11 β - and 18-hydroxylation on the nuclear and mitochondrial DNA synthesis during ACTH-induced differentiation of cortical cells of rat adrenals in tissue culture. *Exp. Cell Res.* In press.

assume that the effect of adding BrdU during the proliferative phase is linked with the thymidine substitution in the nuclear DNA. Support for this in the present study is the reversal of the effect with the simultaneous thymidine addition.

The mechanism of the effect of BrdU addition during the ACTH-induced differentiation is more complicated. The substitution of thymidine by BrdU was reduced very strongly, and $[^3\text{H}]\text{BrdU}$ was found in 1.6% of the nuclei during the first two days of ACTH stimulation. So it seems quite likely that this substitution in nuclear DNA cannot be responsible for over 70% inhibition of mitochondrial 11 β - and 18-hydroxylations in a situation where the cellular viability was excellent and almost normal functional and morphological differentiation of other cytoplasmic compartments was found. Earlier observations on nuclear and cytoplasmic DNA synthesis during ACTH stimulation have revealed that ACTH inhibits strongly nuclear incorporation of $[^3\text{H}]\text{TdR}$ during the first 24 h.¹ A burst of mitochondrial incorporation of $[^3\text{H}]\text{TdR}$ occurs on the second day of stimulation as revealed by electron microscope autoradiography.¹ Also, this grain accumulation was sensitive to ethidium bromide treatment, further indicating that proliferation of circular mitochondrial DNA molecules was triggered by ACTH. In the light of these observations we assume that during this second (mitochondrial) phase of DNA synthesis BrdU is incorporated into mitochondrial DNA and that activation of the mitochondrial genome is needed for ACTH-induced differentiation of cortical cells.

SUMMARY

Cortical cells of fetal rat adrenals in tissue culture were treated with 5-bromodeoxyuridine (BrdU) during their proliferative phase and during ACTH stimulation when nuclear DNA synthesis has almost ceased. Pretreatment with 0.5 μ g/ml/day of BrdU inhibited the ACTH-induced differentiation of cortical cells as well as the secretion of corticosterone and 18-OH-deoxycorticosterone (18-OH-DOC). When nuclear DNA synthesis was suppressed and mitochondrial DNA synthesis was stimulated by ACTH BrdU addition (30 μ g/ml/day) permitted normal ultrastructural differentiation of cortical cells, except that the development of mitochondrial inner membranes was inhibited. Simultaneously mitochondrial 11 β - and 18-hydroxylations were strongly inhibited while cytoplasmic 21-hydroxylation was not affected.

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