Adrenocorticotropic (ACTH) produces dramatic changes in the ultrastructural morphology of adrenocortical cells in hypophysectomized animals (for review, see reference 8). Similar changes have been observed in adrenocortical cells maintained in primary culture. Kahri (9-11) found that fetal rat adrenal cells have ultrastructural characteristics typical of zona glomerulosa cells of the adult rat and that ACTH induced a transformation of the mitochondrial architecture to that of adult zona fasciculata cells. The reorganization of the tubulolamellar cristae of the mitochondria to vesicular cristae and the abundance of smooth-surfaced endoplasmic reticulum are the most widely recorded changes caused by ACTH (1, 9-11, 13). Milner (13) compared the effects of ACTH and cyclic AMP (cAMP) on the ultrastructure of fetal rat adrenal cells in tissue culture and concluded that cAMP induced some, but not all, of the changes normally induced by ACTH.

We have investigated the effects of ACTH on the growth, function, and morphology of normal rat adrenocortical cells in primary culture (18). In the course of these studies it was found that both ACTH and its o-nitrophenyl sulfenyl derivative (NPS-ACTH), which produces virtually no cAMP, were able to inhibit the replication of these cells with a concomitant stimulation of steroidogenesis and characteristic change in morphology. We have now examined the ultrastructural changes induced by ACTH and NPS-ACTH and compared these with the effects of dibutyryl cAMP (dbcAMP). During these studies we have also investigated the nature of the electron-opaque granules which are found in the mitochondria of adrenocortical cells and which disappear upon stimulation with ACTH.

MATERIALS AND METHODS
Highly purified ovine ACTH and NPS-ACTH were prepared as described (5). dbcAMP was purchased from Calbiochem (San Diego, Calif.). Sera, antibiotics, and medium 199 were purchased from Grand Island Biological Co. (Grand Island, N.Y.). Collagenase (209 U/mg) and DNase (2,000 U/mg) were obtained from Worthington Biochemical Corp. (Freehold, N. J.).

Cell Culture
Adrenocortical cells were isolated from the adrenal glands of adult male Sprague-Dawley rats (350-450 g) by digestion with collagenase and DNase as previously described (18). The cells were suspended in medium 199 with 10% fetal serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin, and plated at a density of 4 × 10^5 cells/cm² on 10 cm² plastic Petri dishes. The cells were maintained at 37°C in a humidified environment of 5% CO₂ in air. The medium was replaced every 2 days. For ultrastructural studies the cells were plated on plastic cover slips in plastic Petri dishes. Steroid production was measured by sulfuric acid fluorescence as described (19).

Electron Microscopy
Plastic cover slips bearing cultured adrenal cells were fixed for 1 h at room temperature in 3.0% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) buffered with 0.1 M sodium cacodylate (pH 7.4). After several rinses in the buffer, the cells were postfixed in chilled 2.0% osmium tetroxide in the same buffer for 1 h. After dehydration in ascending concentrations of ethanol, the cover slips were embedded in Epon (12). Thin sections (silver interference colors) were cut parallel to the surface of the cover slip, stained with uranyl acetate and lead citrate, and viewed in a Hitachi HS-8 electron microscope at an accelerating voltage of 50 kV.

Mitochondrial matrix granules were extracted by floating specimen support grids bearing unstained thin sections on a 0.1 M solution of ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetracetate (EGTA), pH 7.6, for 1 h at either 37°C or 60°C. Control sections were exposed to distilled water adjusted to pH 7.6 for 1 h at the same temperatures.

RESULTS
The morphology of the adrenocortical cells from
the older rats used in this study was quite similar to that of cells obtained from younger rats (15, 18). A confluent monolayer was formed 5–7 days after plating. The majority of the cells (>90%) were lipid-filled epithelial cells with rounded nuclei. ACTH (200 nM) caused a marked retraction of the cells as reported previously (15, 18). The analogue NPS-ACTH (10 μM) and dbcAMP (0.5 mM) also induced the same morphological response. ACTH caused a dose-dependent increase in steroid production. The major steroid in the medium was identified as corticosterone by thin-layer chromatography on silica gel G in the solvent chloroform-methanol (95:5 vol/vol).

The effects of ACTH, NPS-ACTH, and dbcAMP on corticosterone production were compared. These studies were performed on the same cultures used for the ultrastructural investigations described below. All three agents stimulated steroid production at similar rates over a 4-day period (Table I).

Ultrastructural Studies

Adrenocortical cells grown in the absence of exogenous ACTH for 11 days have the ultrastructure depicted in Fig. 1. The cells contain a large number of mitochondria which take on a variety of shapes. Internally, the cristae appear as lamellae extending across the long axis of the organelle. In addition, vesicular profiles are found attached to the inner mitochondrial membrane. A constant finding in these control cells is the presence of masses of circular profiles packed in a very regular array. This appearance is interpreted as a grazing section of a mitochondrion where one can see a face view of the inner mitochondrial membrane with its attached vesicular cristae. The mitochondria of these cells also contain electron-opaque granules in the matrix space. These granules vary greatly in size and number. Membranes of the smooth-surfaced endoplasmic reticulum are rarely seen in these control cells. Rough-surfaced endoplasmic reticulum is also virtually absent although numerous ribosomes are present. Other organelles are not remarkable.

By contrast, isolated adrenocortical cells cultured for 4 days in the presence of 200 nM ACTH are very similar in their fine structure to zona fasciculata cells in the intact rat (Fig. 2). The mitochondria in this group are oval or round in outline and contain numerous vesicular cristae as well as cristae which appear to be short tubules with dilated terminations. Electron-opaque matrix granules are only rarely seen. The smooth-surfaced endoplasmic reticulum is present in abundance and takes the form of long, branching tubules. Occasional short segments of rough-surfaced reticulum are seen but this organelle is never prominent in either the cultured cells or the zona fasciculata in vivo.

Cells cultured in the presence of 10 or 100 nM ACTH are similar in fine structure to the cells cultured in the presence of 200 nM ACTH. These similarities include the prominence of the smooth-surfaced endoplasmic reticulum and the paucity or absence of dense matrix granules in the mitochondria. The shape of the mitochondrial cristae appears to vary with the dose of ACTH. With the lower dose the cristae are more lamellar or tubular, while the higher dose (100 nM) produces more vesicular cristae.

Adrenocortical cells stimulated by the addition

| Hormone              | Corticosterone production, μg/ml |
|----------------------|----------------------------------|
|                      | 24 h                | 48 h                | 72 h                | 96 h                |
| None                 | 0.032 ± 0.04*       | 0.23 ± 0.03         | 0.24 ± 0.02         | 0.30 ± 0.04         |
| ACTH (10 nM)         | 2.50 ± 0.22         | 6.33 ± 0.72         | 8.32 ± 1.13         | 9.00 ± 0.36         |
| NPS-ACTH (10 μM)     | 3.20 ± 0.20         | 4.96 ± 0.24         | 9.04 ± 1.63         | 8.58 ± 0.52         |
| dbcAMP (0.5 mM)      | 4.39 ± 0.21         | 6.24 ± 0.55         | 6.76 ± 1.07         | 9.54 ± 1.01         |

Adrenal cells were plated at a density of 1 × 10⁶ cells/cm² as described under Materials and Methods and maintained in the absence of hormone for 7 days. The hormones or dbcAMP were added on the 7th day, and corticosterone production was monitored over a 4-day period. At the end of 4 days the cells were fixed for transmission electron microscopy as described under Materials and Methods.

* Mean ± SE of quadruplicate cultures.
Figure 1. Adrenocortical cell cultured for 11 days in the absence of ACTH. The mitochondria contain a mixture of lamellar and vesicular cristae. Electron-opaque matrix granules are abundant. The mass of vesicles is interpreted as a tangential section of 2 mitochondrion. × 25,000.

Figure 2. Adrenocortical cell cultured in the presence of 200 nM ACTH for 4 days. The smooth-surfaced endoplasmic reticulum is well-developed, and the mitochondria now contain tubulovesicular cristae. Matrix granules are virtually absent. × 34,000.
of NPS-ACTH (10 μM) to the medium are quite similar in ultrastructure to the adrenal cells cultured in the presence of unmodified ACTH (Fig. 3).

Addition of dbcAMP (0.5 mM) to the culture medium evoked a morphologic response which is indistinguishable from the effects of either ACTH or NPS-ACTH.

In view of the disappearance of the electron-opaque granules of the mitochondria after treatment with ACTH or NPS-ACTH, it was of interest to examine the nature of these granules. The effect of extraction with 0.1 M EGTA is shown in Figs. 4 and 5. The matrix granules are readily extracted by exposure to EGTA, suggesting that they contain calcium ions. It should be noted that the granules cannot be extracted if the section has previously been viewed in the electron microscope. This effect is probably due to either contamination of the section by residual hydrocarbons in the vacuum or to radiation damage by the beam which may render the plastic impermeable to the EGTA. It was found that if only one grid opening had been viewed before extraction was attempted, then matrix granules present in cells in adjacent grid openings, which had not been exposed to the electron beam, could be extracted with EGTA. Distilled water had no effect on the granules.

Adrenocortical cells of all experimental groups contain bundles of microfilaments beneath the plasma membrane as well as numerous microtubules. Neither of these structures is markedly altered in distribution or quantity as a result of the addition or withdrawal of trophic hormone.

DISCUSSION

The main purpose of this study was to investigate the ultrastructural changes induced by ACTH and NPS-ACTH. The latter analogue of the hormone, prepared by chemical modification of the single tryptophan residue in ACTH, has been shown to stimulate corticosterone production to the same maximal rate as ACTH in vitro (14) as well as in vivo (17). However, NPS-ACTH produced insignificant increases in cAMP synthesis compared to the 100- to 200-fold stimulation caused by ACTH in isolated adrenal cells (14) and in the adrenal glands of hypophysectomized rats (17). cAMP has
Figure 4 Higher magnification view of the mitochondria from cells cultured in the absence of ACTH. Note the prominent matrix granules. × 44,000.

Figure 5 Unstained section of cells cultured in the absence of ACTH. This section was floated on 0.1 M EGTA for 1 h at 60°C. The matrix granules have been removed. × 33,800.
been implicated as the second messenger involved in mediating both the acute and trophic effects of ACTH on the adrenal cortex (6, 7, 19). Previous studies on the regeneration of the adrenal cortex in hypophysectomized rats showed that NPS-ACTH restored the responsiveness of adrenocortical cells but not to the same extent as ACTH (17). It was hoped that comparison of the ultrastructural changes produced by ACTH, NPS-ACTH, and dbcAMP would be useful in elucidating the role of the cyclic nucleotide in the trophic actions of ACTH.

The present cytologic observations confirm and extend earlier reports that ACTH induces a morphologic transformation of cultured adrenocortical cells such that these cells resemble cells of the zona fasciculata in the adrenal of the intact animal. The results also show that NPS-ACTH and dbcAMP induce essentially the same ultrastructural changes as ACTH. This indicates that all of the cAMP produced in response to ACTH may not be necessary for producing the changes in ultrastructural morphology. Because NPS-ACTH produces only a marginal stimulation of cAMP synthesis and in fact inhibits ACTH-induced cAMP synthesis (14, 17), it may be inferred that an increase in intracellular cAMP may not be a prerequisite for the trophic actions of ACTH. The hormone may alter the availability of another factor(s) which may be necessary for the action of cAMP. In the absence of the hormone, high concentrations of the dibutyryl derivative of cAMP produce the same changes in ultrastructure. In this case the high concentration of the cyclic nucleotide either may be able to act without the other factor(s) or it may affect the other factor(s) in the same way as physiological concentrations of ACTH. Calcium ions are the most likely other factor involved in the actions of ACTH. It is well known that Ca++ is required for the steroidogenic action of both ACTH and cAMP (3, 4, 16, 20).

Among the numerous changes induced by ACTH in adrenocortical cells, the transformation of the mitochondria and the increase in smooth-surfaced endoplasmic reticulum are the most widely documented. Kahri (11) noted the presence of electron-opaque matrix granules in the cells maintained in the absence of ACTH and observed a decrease in the number of these granules after ACTH treatment. In our studies, a dramatic decrease or disappearance of these electron-opaque granules was also observed. These granules are considered to be the sites of deposition of calcium and other ions although this view has been challenged (2). Our results show that these granules can be extracted with EGTA. In view of this and the importance of Ca++ in the actions of ACTH, it is conceivable that these granules contain Ca++ which is mobilized by ACTH.

SUMMARY

The effects of ACTH, its o-nitrophenyl sulfenyl derivative (NPS-ACTH) and dibutyryl cyclic AMP (dbcAMP) on the ultrastructural morphology of adrenocortical cells of adult rats in monolayer culture have been investigated. NPS-ACTH, which has previously been shown to stimulate steroidogenesis but not cAMP synthesis in adrenal cells, induced the same characteristic transformation of mitochondrial architecture as produced by ACTH or high concentrations of dbcAMP. All three agents caused the disappearance of electron-opaque granules present in the mitochondria of unstimulated cells. It was found that these granules could be extracted with EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetate). These results are discussed in the light of the known importance of calcium ions in the actions of ACTH.

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