An Electron Microscopic Correlation of Structure with Function in the Isolated Perfused Cow Adrenal, Preliminary Observations. By JOHN LUFT*, ‡ AND OSCAR HECHTER§. (*From the Departments of Anatomy, Harvard Medical School and The University of Washington, Seattle; and The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.*)||

The isolated adrenal gland of the cow can be used in experiments devised to reveal correlations of cellular function and structure. Structure may be studied by the electron microscope. Function in the adrenal cortex appears as the synthesis of adrenocorticoid hormones, which can be separated from the effluent perfusion fluid and identified and measured quantitatively by column and paper chromatography. The hormones formed are primarily cortisol and corticosterone, with trace amounts of other substances such as aldosterone. Isolated cow adrenals perfused with homologous blood can respond to added ACTH by a manyfold acceleration of corticoid biosynthesis. Intact glands are remarkably sensitive and are maximally stimulated by only 0.1 to 1.0 I.U. of ACTH per liter of blood. In contrast, slices of the same gland require more than 38 I.U. of ACTH per liter for maximal stimulation, and tissue homogenates are totally unresponsive to ACTH, although the homogenates are still able to synthesize corticosteroids (1, 2). Thus, for the cow adrenal, functional information is available concerning: (*a*) the comparative biosynthetic capacity of perfused intact glands, slices, and homogenates, (*b*) the principal pathways of the biosynthetic sequence, (*c*) the chemistry of many of the individual reactions involved, and (*d*) the localization of certain of the specific enzymes concerned with synthesis, with respect to intracellular structures, as determined by means of the homogenate fractionation technique of Schneider and Hogeboom (2–4).

In these adrenal perfusion experiments as routinely performed, there is a considerable lapse of time between the death of the animal and the initiation of perfusion circulation. Glands are obtained at the slaughterhouse 20 to 30 minutes after the animal is killed. Transport to the laboratory in iced saline solution requires an additional hour. Immediately after initiation of perfusion with warm oxygenated beef blood, the glands do not respond to ACTH by an increase in hormone synthesis, but after 30 to 60 minutes of preliminary perfusion a good response to ACTH can be obtained (Hechter, unpublished). Some adverse biochemical change apparently takes place during the interval between the death of the donor animal and initiation of perfusion. This change is reversed during the first half-hour or hour of the perfusion period.

The functional capability of the perfused adrenal may not at first sight appear to be consistent with current concepts of cell stability based on studies with the electron microscope. Many be-

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lieve that within minutes after the cessation of circulation to a tissue, some of the intracellular structures show characteristic degenerative changes, which may contribute to the "fixation artifacts" familiar to electron microscopists. Since similar degenerative changes can be expected in the adrenal preparations prior to perfusion, the question can be raised as to whether the return of function during the first part of perfusion is associated with repair of structural damage at the subcellular level. To answer this question, as well as to determine whether ACTH action *in vitro* influences the fine structure of adrenal cortex cells, glands were examined at each of four stages of a typical perfusion experiment.

Stage 1. At the slaughter-house, about 20 minutes after the animal had been stunned and bled.

Stage 2. At the laboratory after transporting the glands in iced saline, 60 to 90 minutes after the death of the animal.

Stage 3. After 1 or 2 hours of preliminary perfusion with liver-filtered, warmed, oxygenated, citrated beef blood.

Stage 4. After a second hour of blood perfusion during which 10 I.U. of ACTH per liter was added to the blood of one gland, the other being perfused for an additional hour with the original blood as a control.

Only three pairs of glands have been studied in this manner to date, and thus the results must be regarded as somewhat preliminary. In all three cases the glands treated with ACTH showed the expected increase in corticosteroid production. Table I illustrates the data obtained for one pair of these glands.

Wedge biopsies for electron microscopy were taken of each gland at each of the four stages listed above. The wedges were about 2 mm. deep, and extended through the zona glomerulosa into the zona fasciculata. The wedges were sliced further into 1 mm. blocks, fixed 1 or 2 hours in ice cold 2.5 per cent OsO_4 at pH 7.5 in a veronal acetate buffer, dehydrated rapidly through alcohol, embedded in *n*-butyl methacrylate, sectioned with a glass knife, and examined in an RCA EMU-2e electron microscope.

The structural changes observed in the micrographs were consistent and striking. Samples taken at stages 1 and 2,

TABLE I

Final perfusion	Total perfusion		Total corticoid produced	
	Volume	Time		
	ml.	*hrs.*	*μgm.**	*μgm./gm./hr.*
No ACTH	450	2	1,530	60
ACTH 10 I.U./l	450	2	10,030	391

* Corticoid assayed by blue-tetrazolium determination of ethyl acetate extracts of perfusates fractionated by silica gel chromatography and paper chromatography.

before perfusion, whether at the slaughter-house or after transport in cold saline, revealed nearly all the cells to be atypical relative to the currently accepted standards for tissue fine structure. Fig. 1 illustrates typical appearance of the nuclei and mitochondria at stage 2, before perfusion. The nuclear chromatin is characteristically clumped (*X*) at the nuclear membrane. The mitochondria (*M*) are more or less rounded, and show many vesicles (*V*) attached to the inner bounding membrane of the mitochondrion, with only a few cristae. Only a few small dense particles (*P*) are observed in the cytoplasm of these cells. Fig. 2 shows other cells at stage 2. One can see perforations (arrows) in the endothelium

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lining the adrenal sinusoids. The cytoplasm of the endothelial cells seems either to be perforated by many irregular fenestrations or to be broken into discrete islands of cytoplasm. The basement membrane and ground substance seems to be absent at several points (*X*), permitting direct contact of the plasma membrane of the adrenal cell (*AC*) with the perfusing fluid.

Figs. 3 and 4 illustrate the appearance of glands sampled at stages 3 and 4 respectively, after perfusion with oxygenated blood. In Fig. 3 it will be seen that the nuclear chromatin is finely dispersed, and that many of the mitochondria (*M*) are elongated. The mitochondria also display the usual form, with cristae. Most of the vesicles seen in specimens sampled before perfusion have disappeared. Fig. 4 illustrates adrenal cells in a gland perfused with blood containing ACTH, as sampled at stage 4. As illustrated here, but seen equally often in stage 3, the endothelium is now found to be intact and continuous, with overlapping of endothelial cells and with normal appearing mitochondria. No endothelial perforations are evident and the basement membrane is better preserved than at stage 2. Comparison of the ACTH-treated and control glands reveals a greater density and number of cytoplasmic granules in the former (*P*). This finding is consistent with the increase in RNA of the microsomal fraction observed *in vivo* after several hours administration of ACTH (5).

The difficulties of sampling these few glands with the electron microscope are sufficiently great to discourage a statistical approach. There is considerable spread from "normal" to "damaged" structures noticeable in portions of all the glands, and, indeed, in virtually all tissue sections prepared for electron microscopy, even if no pathological complications are intended and the greatest care is exercised. However, in the authors' opinions, the changes described above are sufficiently consistent in all the material to make pure coincidence rather unlikely. Further work on this problem is desirable.

These results may have a bearing on our concepts of the mode of action of ACTH. Biochemical studies strongly suggest that ACTH accelerates an early step in conversion of cholesterol to progesterone. This is thought to be the rate-limiting reaction in the steroidogenic sequence under normal conditions (2). Moreover, it is known that the enzyme systems involved in this conversion (cholesterol → progesterone) are firmly associated with the mitochondrial fraction of cow adrenal homogenates (3, 6). In this connection, the reconstitution of normal mitochondrial structure observed after perfusion seems of special importance in view of the known sensitivity to ACTH of the whole, perfused gland, the lesser sensitivity of slices, and the complete insensitivity of homogenates. Thus the possibility exists that ACTH action requires the presence of a highly organized mitochondrion.

These findings of perforated endothelium after anoxia, with subsequent "healing" upon perfusion, may have an important bearing upon the fine structure interpretation in other tissues. It is difficult to decide which submicroscopic structures of the cells seen in micrographs are physiological features of the intact functioning cell, and which are artifacts of preparation. Independent checks upon these structures are difficult since no instrument has resolution for aperiodic, single structures equal to the electron microscope. Perforated capillary endothelia have been found and interpreted

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as physiological structures in kidney glomeruli and kidney tubular epithelial capillaries (7–9), and in choroid plexus (10). These perforations may well exist *in vivo.* Since Yamada (8) demonstrated these endothelial perforations in kidneys perfused with fixative after only very brief interruption of circulation, anoxia may not be a factor. However, it might be wise to withhold final judgment in this respect until more data are available.

It should be noted that the perfusion-analysis-biopsy technique presented here can easily be adapted to other organs. For example, we have found that under similar perfusion conditions using citrated, oxygenated beef blood, the fine structure of rat liver parenchymal cells is well maintained.

We wish to thank Mr. Morris Feinstein and Mrs. Ella Scully for invaluable technical assistance.

Note Added in Proof.—A paper has recently appeared which may have an important bearing on the interpretation of our findings relating to the development and disappearance of vesicles within mitochondria (Green, D. E., Lester, R. L., and Ziegler, D. M., *Biochim. et Biophysica Acta,* 1957, **23,** 516). These workers have isolated two types of particles from mitochondrial fractions of beef heart. One type can catalyze the oxidation of DNPH and succinate by molecular oxygen. These particles contain the full complement of cytochromes and other components for these oxidations, and have been termed electron transport particles (ETP). The second type of particles have the capabilities of the first, but in addition and at the same time carry out oxidative phosphorylations. These second more versatile particles have been termed phosphorylating electron transport particles (PETP). With Dr. Hans Ris they have examined both types of particles in the electron microscope. They find that the ETP fraction consists mainly of *vesiculated* fragments of mitochondria. The PETP fraction consists of mitochondrial fragments with *intact cristae.* They suggest that intact cristae seem to be an essential requirement for oxidative phosphorylation. The intact cristae may be either free or as intact mitochondria.

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Explanation of Plates

The line in each plate represents one micron.

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PLATE 202

FIG. 1. Electron micrograph of a segment of a single cell from the zona fasciculata of the adrenal cortex of a cow. The whole gland was obtained at the slaughter-house, and taken to the laboratory in iced saline (stage 2 in text). The nucleus (*N*) contains chromatin which is aggregated and clumped (*X*) at the nuclear membrane. Mitochondria (*M*) are more or less rounded. They contain many vesicles (*V*), although a few lamellar cristae are visible at *Cr*. A few Palade's particles are seen at *P*, apparently attached to membranes. × 22,000.

FIG. 2. Electron micrograph of portions of several adrenal cells (*AC*) under the same conditions as Fig. 1 (stage 2). Capillary lumen is seen at *CL*. Nuclear chromatin is moderately clumped and a number of vesicles are seen within the mitochondria (*M*). The thin cytoplasmic sheet of lining endothelium (*End.*) is perforated in many places (arrows). The pale ground substance interposed between the endothelium and the adrenal cells appears thinned or absent at several points (arrows, *X*), and the fluid within the capillary may have direct access to the parenchymal cells themselves. × 22,000.

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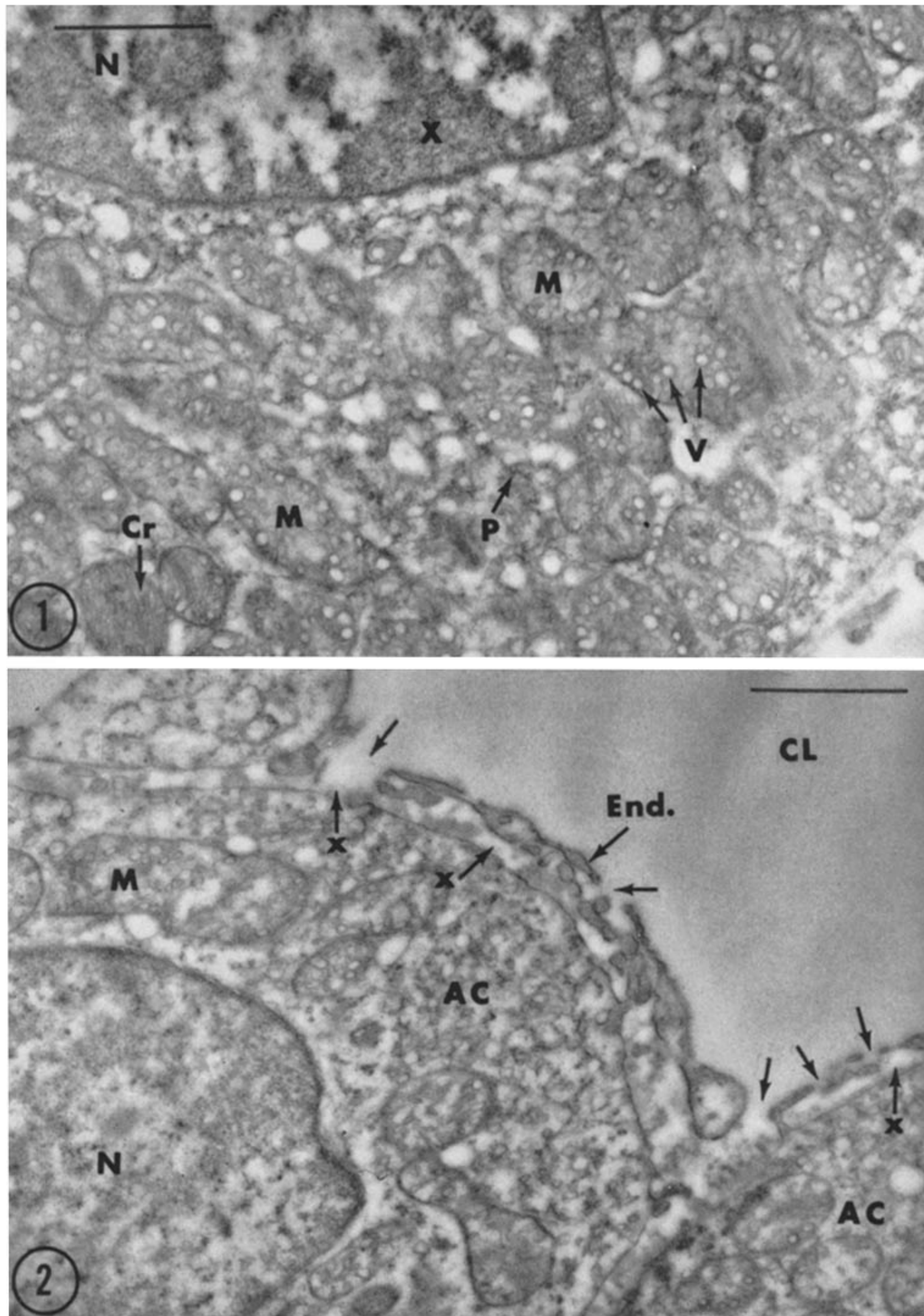

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PLATE 203

FIG. 3. Electron micrograph of portions of two adrenal cells from a gland obtained at the slaughter-house, transported in iced saline, and perfused with warm, oxygenated blood for 2 hours (stage 3). The mitochondria (*M*) now show the normal, plate-like cristae, and only an occasional vesicle. The chromatin contained within the nucleus (*N*) is evenly distributed. Palade's particles (*P*) occur either free in the cytoplasm, or attached to membranes. × 22,000.

FIG. 4. Electron micrograph of a portion of two adrenal cells from a gland which had been through the preceding stages, and then perfused with blood containing 10 I.U. ACTH per liter (stage 4). The thin sheet of endothelium (*End.*) is seen to be intact throughout its span, without perforations. The amorphous basement membrane material in the space (*X*) is moderately well preserved. The mitochondria (*M*) show the normal lamellar form of cristae, and the nuclear chromatin is evenly dispersed. Palade's particles (*P*) are of greater number and density than before. × 27,000.

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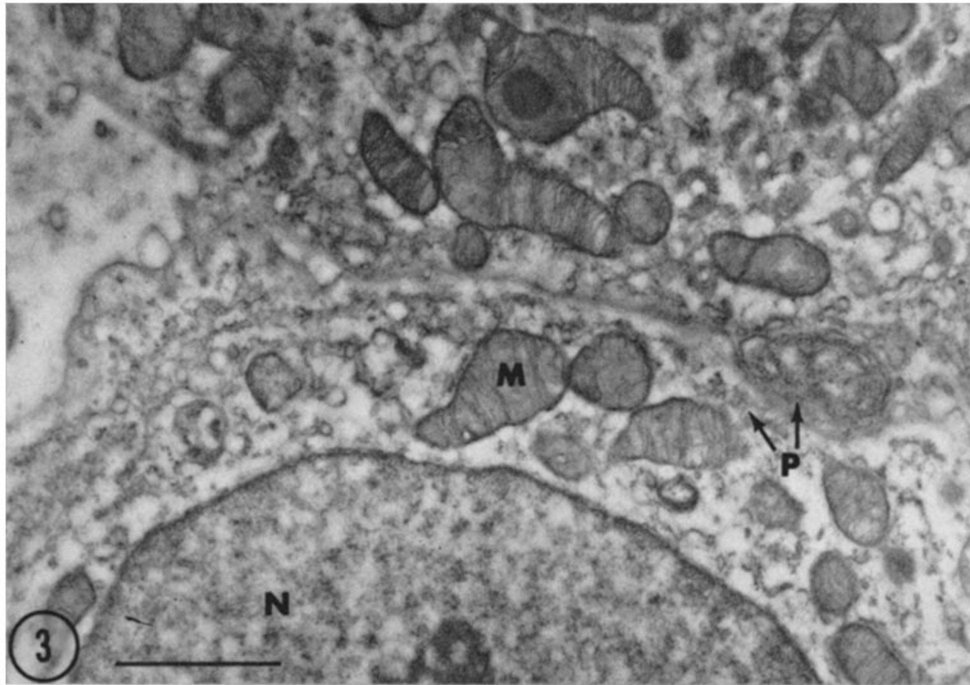

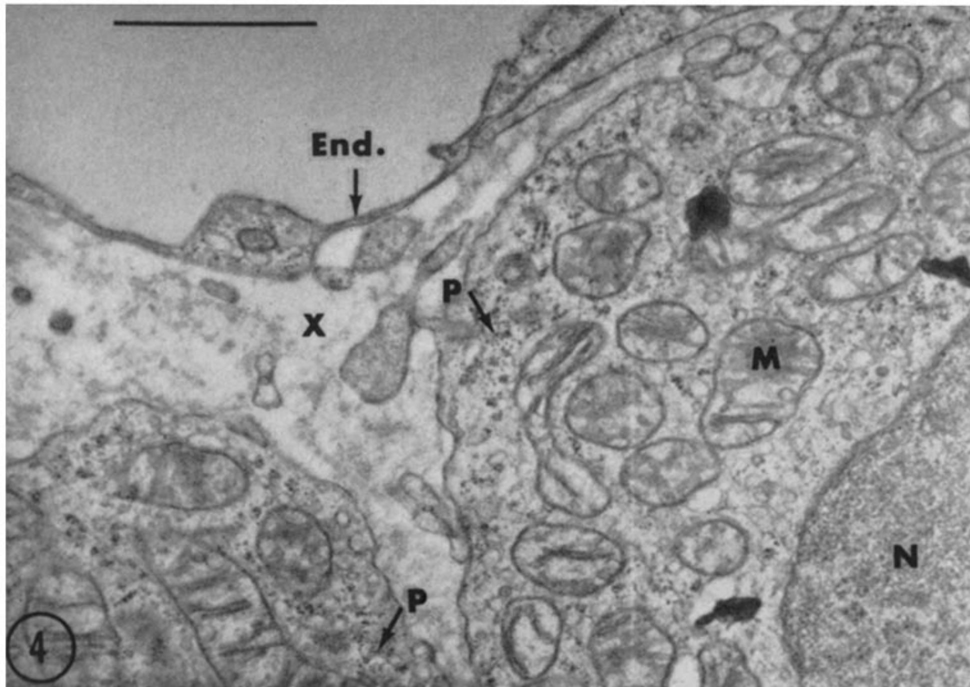

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