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Histochemical and biochemical observations on the aldosterone-stimulated urinary bladder of the toad, Bufo marinus

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HISTOCHEMICAL AND BIOCHEMICAL OBSERVATIONS ON THE ALDOSTERONE-STIMULATED URINARY BLADDER OF THE TOAD, BUFO MARINUS

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Gratis persolvere dignas

Non opis est nostrae

Virg., Aen. I, 600

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Δίδοτε καὶ δοθήσεται ὑμῖν· μέτρον καλὸν, πεπιεσμένον καὶ σεσαλευμένον καὶ υπερεκχυνόμενον δώσουσιν εἰς τὸν κόλπον ὑμῶν·

κατα Λουκαν

TABLE OF CONTENTS

INTRODUCTION

A. The toad urinary bladder and sodium transport.

The toad Bufo marinus is a ubiquitous amphibian inhabitant of the Colombian Coast of South America where it is found in brackish marshland (Gadow, '01). Among the many adaptions to terrestial life manifested by the toad, the urinary bladder has long been thought to play a prominent role in the animal's ecology as a fluid reservoir (Steen, '29). The urinary bladder reaches its largest size among the Amphibia in certain species of Bufo (Noble, '31). Leaf ('55) discovered that the urinary bladder possessed the capacity to transport sodium ions in the same manner as frog skin in the model system of Ussing and Zerahn. The latter workers (Ussing and Zerahn, '51) demonstrated that the endogenous electrical activity of the frog skin was proportional to the amount of sodium transported across the membrane, and thus ion transport could be studied electrically with the so-called "short circuit technique" in which an epithelial membrane is positioned between two chambers containing an electrolyte solution in which current can be measured. The simpler structure of the urinary bladder, with its absence of glands and thick connective tissue stroma, is even more suitable for this purpose than the frog integument.

Primarily from experiments designed after the Ussing-Zerahn model, a theory of ionic transport for the toad urinary bladder has evolved

which is felt to be applicable to a certain extent to other tissues engaged in active transport as well (see Sharp and Leaf, '66). Sodium ions are thought to move passively from the urine into the mucosal cell where by means of the "sodium pump" the ion is transported across an electrical gradient to the extracellular compartment of the animal generating an electrical potential. The process is dependent upon cellular metabolism (Leaf et al., '59) and is inhibited by cardiac glycosides. Leaf et al. ('58) have shown that the urinary bladder provides an effective reabsorption mechanism since sodium of toad urine may reach a level as low as 0.5 mEq/l with a mean value of 4.8 mEq/l, depending upon the state of hydration of the animal. This reabsorptive capacity corresponds to that of the mammalian nephron. Thus, study of the toad urinary bladder has assumed wider interest as a model for more complex tissues engaged in active transport. Caution must be exercised, however, in applying conclusions about active transport drawn from the toad bladder to other tissues. Certain differences have been discovered between the transport systems of the toad bladder and mammalian kidney; one of these is the finding that the transport mechanism in the toad bladder does not appear to carry potassium. It would seem then that the toad bladder should be regarded as a convenient model for the development of new hypotheses to be tested upon more complex tissues.

B. The effect of aldosterone on the urinary bladder.

The adrenal glands of Bufo marinus are present as scattered columns of acidophilic cells embedded within the ventral surface of the kidney (Vincent, 1898). The importance of aldosterone in phylogenetic development has been effectively reviewed by Denton ('65). While aldosterone had been purified from adrenal tissue as early as 1953 and quantified in human plasma by 1955 (Simpson and Tait, '55), Crabbé ('61a) was the first to demonstrate aldosterone in Bufo marinus. In quantitative terms, 0.68 µg aldosterone per 100 ml plasma were found in toads maintained in saline and 1.17 µg aldosterone per 100 ml plasma in those maintained in distilled water, values ten times greater than those of normal man (Peterson, '59). At the same time, Crabbé ('61a) reported that the isolated urinary bladder from toads receiving parenteral injections of aldosterone demonstrated increased sodium transport over toads not receiving aldosterone, the degree of increase being greater in toads maintained in distilled water. Subsequently, Crabbé ('61b) demonstrated that the addition of aldosterone to the media bathing the serosal surface of the membrane could stimulate transport of sodium in vitro. A delay of 60 minutes was found prior to the increase in transport, which reached a maximum within 120 minutes. The increase was much greater in toads maintained in distilled water prior to isolation of the urinary bladder.

Following the original use of the urinary bladder of the toad by Crabbé to study the physiological effects of aldosterone, this model system has contributed much to the understanding of the cellular basis for the mechanism of action of this important hormone. Use of the toad bladder has been instrumental in studies of the effective concentration and kinetics of the hormone, the role of intermediary metabolism in its function, its binding to cell components, and its relationship to cellular control mechanisms (see Sharp and Leaf, '66). The studies of Frazier ('62) demonstrated that the mucosal aspect of the membrane is responsible for sodium transport. Crabbé ('61b) established that the maximum stimulation in vitro of sodium transport by aldosterone is reached at a concentration of 10^{-7} M, beyond which very little additional stimulation was detected. The minimum effective concentration in vitro was found to be 3.3×10^{-10} M by Sharp and Leaf ('66). The hormone is nearly completely bound to the toad bladder within 30 minutes of its addition to the bathing medium, and by autoradiography using tritiated aldosterone, the hormone has been localized to the nuclei of all epithelial cells (Edelman et al., '63). Ausiello and Sharp ('68) demonstrated that the nuclear binding, presumed to represent a "receptor site", exists in at least two forms with differing dissociation constants. It is not known whether these different binding sites are present in the same nucleus or whether they represent binding by different cells.

Because aldosterone induces increased uptake of tritiated uridine simultaneously with the increased transport of sodium, and because Actinomycin D blocks the stimulation of active transport by aldosterone, Edelman et al. ('63) concluded that aldosterone acts by stimulating DNA dependent RNA synthesis to produce a messenger for the formation of a new protein. Edelman and associates ('64) further theorized that this protein might be an enzyme intimately connected with the oxidative phosphorylation of ADP to provide a high energy intermediate to drive the "sodium pump". Part of the evidence adduced for the latter theory was an increased content of ATP found by biochemical assay under conditions of increased active transport (Edelman, '64) and the finding that the substrate depleted bladder fails to respond to aldosterone with increased active transport unless supplied with a basic substrate such as pyruvate (Edelman et al., '63). Fanestil and Edelman ('66) also concluded that the mechanism for the basal rate of sodium transport differs from that of aldosterone stimulated transport since the former is not inhibited by Actinomycin D.

A contrasting theory has been proposed by Sharp and Leaf ('66) who found that under the influence of aldosterone the toad bladder displayed increased uptake of the substrates pyruvate and acetoacetate when sodium was present in the bathing medium over control tissue in which sodium was absent. This finding led them to conclude that energy metabolism was secondary to the active transport process in the toad bladder. Additional support for this concept arose from the discovery that Amphotericin B

which also stimulates sodium transport required the presence of substrate even though the antibiotic was not implicated in protein synthesis. This suggested that utilization of the mechanism of energy production is a non-specific aspect of the active transport process. They and Bonting and Canady ('64). moreover, were unable to confirm the finding of increased ATP levels under conditions of aldosterone stimulated active transport. Sharp and Leaf ('66) favor the view that a product of aldosterone induced protein synthesis, possibly an enzyme, increases the permeability of the mucosal cell membrane to sodium in a morphologically polarized manner such that sodium ions are drawn off from the serosal aspect of the mucosal cell into the extravascular space. Cuthbert ('68) however who developed an electricophysiological method to seperate permeability from active transport was not able to demonstrate increased permeability of the toad bladder after blockage of the sodium pump with ouabain and treatment with aldosterone. To date, no such permease has been isolated, and the evidence for its presence is indirect at the present time. Neither of the two major theories of aldosterone action, however, is incompatible with the other (Fanestil, '69).

C. Morphology of the toad urinary bladder.

Upon entering the abdominal cavity of the toad from the ventral aspect, the urinary bladder is easily found as a bilobed transparent

vesicle arising from the cloaca. Varying with the state of hydration, the fluid-filled bladder may occupy a large proportion of the abdomen, at times reaching the costal margin. Histologically, three major zones can be identified from the peritoneum inward to the luminal or interior surface of the bladder: first, a uniform layer of flattened squamous cells, the serosa; second, the lamina propria, composed of interwoven bands of collagen fibers containing scattered fasicles of smooth muscle, a rich vascularization and myelinated nerves; and third, the mucosal epithelium. It has been known since the late nineteenth century from standard histological observations (Schiefferdecker, 1884) and more recently from electron microscopic studies (Peachey and Rasmussin, '61; Choi, '63) that the epithelium is not a homogenous population of cells. Indeed, the mucosa is made up of several cell types: granular cells, mitochondria-rich cells, goblet cells, and basal cells. The most abundant are the granular cells, so-called because of their diastase resistant, periodic acid-Schiff (PAS) positive, subapical granules. About ten per cent of the cells are the mitochondria-rich cells, which can be identified with the phosphotungstic acid-hematoxylin (PTAH) stain for mitochondria and with histochemical methods for the dehydrogenase systems. A small percentage of the cells of the mucosa are goblet cells filled with PAS and Alcian blue positive material. A small basal cell with a bilobed nucleus is attached to the basement membrane but is not exposed to the lumen.

At the electron microscopic level (see Choi, '63), the luminal surface of all cells contains numerous microvilli. The characteristic granules of the granular cells are encased in a membrane, occasionally confluent with the plasma membrane and the endoplasmic reticulum; the granules also contain a fine fibrillar meshwork. The mitochondria-rich cell is distinguished electron microscopically by its abundant large mitochondria and its exposure to the luminal surface in the form of a peninsula of mitochondria filled cytoplasm. Both granular and mitochondria-rich cells possess numerous vesicles near the basement membrane. Both are also connected by tight junctions. The goblet cell is composed of abundant droplets containing electron-dense material. The basal cell is marked by the sparseness of its cellular components and its increased presence of filaments. DiBona et al. ('69) have demonstrated with serial electron microscopic sections that the granular and mitochondria-rich cells may lie directly upon the basement membrane of the toad bladder. This confirms the light microscopic and electrophysiological evidence that the urinary bladder may be considered as composed of a single cell layer over much of its surface.

D. Scope of the present investigation.

As reviewed above, the toad urinary bladder is felt to represent in many ways an extension of the nephron, functioning as a homeostatic

unit controlled by aldosterone. Evidence has accumulated that the epithelium of the bladder is the active component in the sodium transport mediated by aldosterone and is the target site of the hormone. While localization of aldosterone function at the subcellular level has made much progress, the methods utilized have not provided direct evidence concerning the role of the several epithelial cell types in active transport. Thus, the specific cell types in the epithelium affected by aldosterone are not definitely known. Since the epithelial cell types can be distinguished by histochemical means, which delineate morphology and to a more limited extent function, changes in staining patterns following administration of aldosterone might indicate cellular sites of action of the hormone. Thus, the present studies describe the histochemical changes observed in the mucosa following stimulation by aldosterone, under *in vitro* and *in vivo* conditions. In addition, biochemical studies on the influence of aldosterone upon oxidative phosphorylation of toad bladder mitochondria were performed and correlated with the histochemical investigations.

MATERIALS AND METHODS

A. Histochemistry

Twenty-four medium sized female toads, Bufo marinus, kept in moist terraria were employed in the following study. The protocol consisted of two parts, in vitro experiments in which aldosterone was incubated with the excised bladder, and in vivo experiments in which aldosterone in four doses was given parenterally prior to the removal of the bladder.

For the in vitro experiments, the toads were kept in 0.6% saline for 24 hours before pithing and excision of the bladder (Porter and Edelman, '64). During the entire course of the in vivo experiments, the toads were kept in 0.6% saline. The bladders were removed, bisected, immediately immersed and washed in cold Ringer's solution (made with 0.6% NaCl). In the in vitro studies, one hemibladder was incubated in a control solution of Ringer's with 2 mM pyruvate (Sharp and Leaf, '64); the other incubated in Ringer's with 2 mM pyruvate and 1×10^{-7} M aldosterone (obtained from Sigma Chemical Co. and assayed for electrical activity upon the toad bladder; see Appendix). Five mg of the hormone were dissolved in ethanol and diluted with 0.6% saline to 5 ml, from which appropriate dilutions were made. Incubations were carried out for three hours in a Dubnoff metabolic shaker at $20^{\circ}C$.

In the in vivo experiments, 1 ml of 5 X 10^{-6} M aldosterone was injected intraperitoneally every 12 hours for 48 hours, at the end of which time the animals were sacrificed. As a control, 1 ml of the excipient was injected into other toads upon the same schedule, since hemibladders could not be used for control tissue in this case.

Enzymatic histochemical reactions were carried out upon fresh tissue, while paraffin sections were made from tissue fixed in Lillie's neutral buffered formalin (Lillie, '54). Both cryostat sections (frozen in cyclohexane and cut at -20°C) and whole mounts stretched over wax impregnated corks with elastic bands were made with fresh tissue. Following incubation in the histochemical reaction media, the tissue was fixed in Lillie's neutral buffered formalin for 20 minutes, washed for ten minutes in running water and mounted in glycerine. The use of whole mounts permitted more thorough interpretation of cellular relationships and distribution.

Paraffin sections were stained with Harris hemalum and eosin (H&E), the PAS reaction with and without diastase treatment (McManus and Mowry, '60), the PTAH stain (Mallory, 1897), and the methyl green-pyronin reaction with and without ribonuclease treatment with 30 minute incubation (Pearse, '60).

The localization of adenosine 5-triphosphatase (ATPase) activity was studied on fresh and formalin fixed tissue with the lead method of

Wachstein and Meisel ('57). Fixed tissue was incubated for 60 minutes; fresh tissue, for 20 minutes. The method of Wachstein and Meisel ('56) was used for glucose 6-phosphatase activity with 20 minute incubation at 37°C.

Histochemical reactions for triphosphopyridine and diphosphopyridine nucleotide diaphorase (TPND and DPND) activity were carried out by the methods of Nachlas et al. ('58). The incubation was carried out at 37°C; 15 minutes for TPND and 30 minutes for DPND with Nitro BT (obtained from Sigma Chemical Co.). Succinic dehydrogenase (SDH) activity was localized after the method of Nachlas et al. ('57) with 30 minute incubation at 37°C. Lactic dehydrogenase (LDH), glucose 6-phosphate dehydrogenase (G6PD), and 6-phospho-glucuronate dehydrogenase (6PGD), activities were demonstrated after the methods of Hess et al. ('58) with incubation times of 20 to 30 minutes.

The G-Nadi reaction for cytochrome oxidase activity was performed according to the method of Burtner and Lillie ('53) with 20 minutes incubation. Non-specific esterase activity was studied with α-naphthol acetate and fast blue B salt after Gomori ('50).

Specificity of enzymic reactions was examined by omission of appropriate substrate from the incubation media. In all cases where quantitative observations were made, control tissues were incubated along with the experimental tissues in the same media concurrently.

B. Biochemistry -- Oxidative phosphorylation.

The oxidative phosphorylation of mitochondria under three sets of conditions was studied: mitochondria from control bladders, mitochondria treated with aldosterone without incubation after isolation from control bladders, and mitochondria from bladders incubated with aldosterone three hours prior to isolation.

Prior to pithing and excision of the bladders, toads were maintained in 0.6% saline. In the first series of experiments, mitochondria were harvested directly from the urinary bladder after washing several times in ice chilled saline. In the second series, after washing several times in saline the bladders were incubated with 10^{-7} M aldosterone and 2 mM pyruvate in 50 ml of saline for three hours at 20^{o}C. Mitochondria were harvested by scraping the mucosa with a clean glass slide; the scrapings from six bladders were pooled for each experiment. Scrapings were homogenized in .25 M sucrose with 2 mM EDTA in a Dounce type homogenizer with three strokes of the piston. The homogenate was layered over .35 M sucrose and centrifuged at 1000 X g for ten minutes to sediment nuclei and debris. The supernate was then centrifuged at 8000 X g for ten minutes to concentrate the mitochondria which were washed three times by resuspending the pellet in .35 M sucrose and centrifuging at 8000 X g for ten minutes. The mitochondrial pellet was then resuspended in 1.42 M sucrose and layered over a sucrose density gradient of 1.42 M to 1.88 M sucrose which was stored at 0^{o} C for 12 hours prior to the addition of the mitochondria.

This was centrifuged at 39,000 X g for two hours in a type 5W50L Spinco swinging bucket rotor. Allowing the gradient to leave the tube through a small perforation in the bottom, the mitochondrial fraction (located approximately 2/5 of the length of the tube measured from the top) was captured and resuspended by pipetting.

Oxygen uptake of the mitochondria was measured in a polarograph with an oxygen electrode (Clarke type) at 30°C, recording at a speed of 1 inch/ minute, each inch of deflection for O_2 uptake corresponding to 10 µatoms of oxygen. Constant agitation in the 1.5 ml cell was maintained with a magnetic micro stirring bar.

The media contained:

125 µmoles sucrose
25 µmoles Tris phosphate buffer (pH 7.4)
.75 µmoles $MgSO_4$

to which was added, first the mitochondrial suspension (3 mg protein, .1 ml); secondly, 50 µmoles glutamate-malate substrate as the potassium salt; thirdly, 460 µmoles of ADP, the Barium salt adjusted to pH 7.4 with Tris buffer. Each of the three latter additions took place after the media was allowed to equilibrate in the chamber.

RESULTS

A. Histochemistry

H & E

This stain revealed no obvious difference in the histology of control and *in vitro* treated tissue. In the *in vivo* treated tissue, approximately ten per cent of the cells were binucleated. This was especially apparent in the whole mount preparations.

PAS

Two types of staining within the epithelium are prominent with this reaction in control tissue: the opaque particles of the granular cells that are diastase resistant, and a faint diffuse tincture of the cytoplasm abolished by diastase treatment (fig. 1). Moderate increase of the latter type of staining of the cytoplasm, especially near the luminal border of the bladder, were seen with *in vitro* treatment with aldosterone (fig. 2). The increase in the cytoplasmic staining was even more marked in the *in vivo* treated material (fig. 3). Moreover, the PAS positive diastase resistant granules were smaller and less numerous among the granular cells. Goblet cells were intensely stained (figs. 1-3).

PTAH

In control tissue, the cytoplasm of the mitochondria-rich cells showed diffuse basophilic granulations characteristic of mitochondria (fig. 4). The same pattern was observed after *in vitro* treatment with aldosterone (fig. 5). Under *in vivo* conditions, more basophilic granules were seen in the cytoplasm of the granular cells, especially in the perinuclear region (fig. 6).

METHYL GREEN-PYRONIN

The cytoplasm of all cells in control tissue stained a uniform faint reddish hue (fig. 7), which was abolished by ribonuclease (RNAase) treatment of the sections. In tissues treated with aldosterone *in vitro* and *in vivo*, the staining was a distinctly deeper shade of red, pyrinophilia reaching a peak intensity at three hours (fig. 8), and remaining at the same degree under more prolonged treatment of *in vivo* conditions (fig. 9).

DPND and TPND

The diaphorase activities of the reactions which utilized Nitro BT were found to be localized diffusely in the cytoplasm of different cells as well as intensely within irregular-sized, coarse granules. Control tissue displayed its most intense reaction within mitochondria-rich cells

which were packed with reactive granules (fig. 10). Granular cells showed some variation in staining with these reactions, but were not nearly as reactive as the mitochondria-rich cells, although they too contained reactive granules (fig. 10). Few changes were observed with *in vitro* treated tissue (fig. 11), but under *in vivo* conditions, not only were the intensely reactive mitochondria-rich cells increased in number, but the granular cells contained increased numbers of reactive granules in varying degrees (fig. 12).

SDH

In control tissue, intensely reactive mitochondria-rich cells were situated against a background of less reactive granular cells (figs. 13, 15). Reaction products were localized to coarse cytoplasmic granules (figs. 13, 15). This difference between mitochondria-rich and granular cells was likewise observed under *in vitro* conditions. Granular cells did not appear to contain increased numbers of reactive granules. On the other hand, the *in vivo* experiments showed marked differences over controls: not only were the normally intensely reactive cells more numerous, but previously weakly reactive cells showed gradations in intensity of staining, some being so reactive as to make the distinction between cell types less apparent (figs. 14, 16).

CYTOCHROME OXIDASE

Results with this reaction were similar to those for SDH; mitochondria-rich cells were clearly differentiated from the sparsely staining granular cells in control tissue (fig. 17), a relationship unchanged under *in vitro* conditions. The *in vivo* treated tissue, however, showed more of the heavily reactive cells as well as increased reactivity of the granular cells (fig. 18).

LDH

This enzyme was found in control tissue to show a diffuse localization, with perhaps a slight predeliction for the mitochondria-rich cells; no cells displayed very strong activity for this enzyme. After treatment with aldosterone for three hours *in vitro*, no appreciable increase in the number of reactive granules was seen in the mucosa. A very mild increase in reactivity, primarily of the granular cells, was seen under *in vivo* conditions, however.

G6PD and 6PGD

In control tissue, activity of these enzymes was localized diffusely in the mucosal cytoplasm, as well as within small granules (figs. 19, 22). Scattered clusters of cells were intensely reactive (figs. 19, 22). After *in vitro* incubation with aldosterone for three hours, the sparse

clusters of reactive cells were found in the same distribution but were more intensely reactive in comparison to cells in control tissue (figs. 20, 23). In the in vivo experiments, the clusters of intensely reactive cells became larger and more numerous, the configurations assuming a stellate appearance (figs. 21, 24).

ATPase

The lead nitrate reaction for ATPase was carried out with both formol fixed and fresh tissue. Consistent with previous reports (Keller, '63), activity was localized to the region of the mucosa adjacent to the basement membrane in fixed tissue; in fresh tissue, activity was localized both along the entire membrane and within the cytoplasm (fig. 25). Cytoplasmic activity was particularly apparent in the mitochondria-rich cell. This pattern was found both in control and in in vivo treated tissue and repeated trials showed no increased activity over control tissue. In vivo treated tissue, however, showed more intense ATPase activity along the entire cell membrane as well as more cytoplasmic activity (fig. 26).

GLUCOSE-6-PHOSPHATASE

Control tissue showed localization of this enzyme activity within perinuclear particles and to the cell membrane of both mitochondria-rich

cells and the granular cells (fig. 27). After *in vitro* treatment, the perinuclear activity was moderately increased (fig. 28). Following aldosterone treatment *in vivo*, activity for this enzyme was found in the same location with a slightly greater intensity of reaction.

NON-SPECIFIC ESTERASE

Intense esterase activity in control tissue was found to be limited to a small population of cells, possibly the mitochondria-rich cells; in other cells it was seen within vacuoles in the periphery of the cytoplasm adjacent to the cell membrane (fig. 29). In *in vitro* and *in vivo* treated tissue, not only were these intensely staining cells even more reactive, but the peripheral cytoplasm of the remaining cells was also more reactive (fig. 30).

B. Biochemistry -- Oxidative phosphorylation.

Illustrations of several of the recordings from the polarograph are provided in figs. 31-33 in which the effect of the addition of various substrates upon the respiring mitochondria is clearly seen.

The results of the first series of experiments is summarized in Table I; here the mitochondria freshly isolated were studied when aldosterone was added without incubation. A small decrease of the P/O ratios was found in this case. It is also significant that the rate of oxygen consumption (that portion of the curve labelled State IV; compare figs. 31, 32) was greater than control mitochondria.

TABLE I

	P μmoles ADP	O μatoms	P/O	% decrease	State III / State IV
Control	460	165	2.89	9.6	5/.9 = 6
Aldo	460	175	2.61		6/5 = 1.1
Control	460	150	3.06	16.1	7/2 = 3.5
Aldo	460	180	2.55		9/7 = 1.3
Control	460	155	2.98	17.2	10/.9 = 11
Aldo	460	185	2.42		8/5 = 1.6

The results of the second series of experiments is summarized in Table II; here mitochondria were isolated from bladders which had been incubated with aldosterone for three hours and from control bladders. Moderate decreases in P/O ratios were observed for aldosterone treated tissue over controls, the per cent decreases ranging from 31 to 49.

TABLE II

	P µmoles ADP	O µatoms	P/O	% decrease	State III / State IV
Control	460	190	2.42	31	9/1.5 = 6
Aldo	460	280	1.63		4/.8 = 5
Control	460	170	2.70	37	9/1.1 = 10
Aldo	460	270	1.71		8/2.1 = 4
Control	460	145	3.20	49	4/.3 = 12
Aldo	460	285	1.62		5/2 = 2.5

DISCUSSION

The mitochondria-rich cells were found normally to have more activity for the citric acid cycle and respiratory enzymes, while granular and goblet cells were much less reactive. These results are in agreement with Keller's ('63) observations on the basic histochemistry of the toad bladder. The findings here reported show in addition that increases in the enzymes of certain metabolically significant pathways occur after exposure of the toad bladder to aldosterone. Furthermore, after prolonged *in vivo* exposure, alteration in histochemical cellular relationships could be detected.

Treatment of the toad bladder *in vitro* with aldosterone using the concentration and timespan employed by physiologists to demonstrate maximal sodium transport produces a marked pyrinophilia in all cells which is abolished with RNAase treatment. The intensity of the pyrinophilia reaches its maximum concurrently with the maximum stimulation of sodium transport, namely at 120 minutes. Pyrinophilia similarly increased following *in vivo* administration of the hormone. This finding is consistent with the increased RNA production known to occur with the induction of sodium transport (Edelman et al., '64). The histochemical studies show that the increase in RNA production following aldosterone stimulation occurs in all cell types. Thus, the evidence indicates

that aldosterone is present intracellularly in all cell types after exposure to aldosterone (see Edelman et al., '64) and that all cell types respond histochemically concurrently with the earliest biochemically detectable phase of altered metabolic function.

The enzymes of the hexosemonophosphate shunt were also found to be increased in the *in vitro* situation, and even more so *in vivo*. On the basis of the arrangement of the cells in stellate clusters, as well as the shape of the cells, these reactive cells were felt to include more than the mitochondria-rich cells. The increased activity in the hexosemonophosphate shunt, also demonstrated biochemically by Handler et al. ('69), may represent a response to increased metabolic demands upon the cells, or more likely in view of the increased RNA production noted above, may be related to increased synthesis of the precursors of nucleic acids (Pearse, '60). The finding of activity of these normally cytoplasmic enzymes both in the cytoplasm and in the form of granules approximately the size of the granular cell PAS positive diastase resistant particles raises the possibility that the enzymes may be present within these particles. The significance of the pattern of increases in the "shunt" enzymes, namely in closely adjacent cells, is unclear at present.

Leaf et al. ('59) have shown that oxidative metabolism is essential to aldosterone-stimulated transport of sodium, although baseline transport of sodium will continue under anaerobic conditions. Likewise, oxygen

consumption is increased with aldosterone induced sodium transport (Sharp and Leaf, '66). Numerous studies (Edelman et al., '63; Sharp and Leaf, '65) have demonstrated the substrate dependent nature of aldosterone action; once endogenous substrates have been depleted increased use of metabolites such as pyruvate occurs. Recently, Kirsten et al. ('68) have demonstrated significant increases in the mitochondrial tricarboxylic acid cycle enzymes after incubation with aldosterone for 15 hours. These enzymes included condensing enzyme, isocitrate dehydrogenase, glutamate dehydrogenase, and malate dehyrogenase.

Histochemically, marked increases in oxidative enzyme activity were observed after in vivo treatment with aldosterone, but not in vitro. The failure to demonstrate increased activity of the enzymes in the in vitro experiments may reflect one or more of the following: inability of the severed bladder to respond with augmented oxidative machinery, the removal of these steps of intermediary metabolism from the initial target of aldosterone action, or the failure of the histochemical methods for these enzymes to demonstrate small increases in activity, particularly in the normally intensely reactive mitochondria-rich cells. The latter possibility is made more likely by the small but significant rises in assays of the tricarboxylic acid cycle enzymes found after three hours in vitro incubation with aldosterone by Kirsten et al. ('68). Under more prolonged aldosterone stimulation, however, the diffuse histochemical

increases in the oxidative machinery reflected by SDH, DPND, TPND, and cytochrome oxidase activities is in complete agreement with the recent biochemical studies on similar enzymes. Another reflection of heightened metabolic activity is the increase in diastase labile PAS staining, presumably glycogen.

The quantitative histochemical increases then support the physiological and biochemical evidence that aldosterone stimulates oxidative metabolism. The relatively small changes seen in LDH activity after aldosterone stimulation further support the concept that this hormone primarily affects aerobic processes. This histochemical finding is also consistent with the results of Kirsten et al. ('68) who demonstrated no significant increase in LDH under in vitro conditions. The demonstrated increase in the enzymes of the tricarboxylic acid cycle may be directly related to aldosterone function. These enzymes are stimulated by aldosterone even when sodium transport is prevented by removal of sodium from the mucosal medium. Thus, aldosterone seems to independently increase the activity of these enzymes and to stimulate sodium transport (Kirsten et al., '68).

The relationship of ATPase to the mechanism of aldosterone stimulated active transport has been a focus of interest since the discovery by Skou ('62) of a sodium activated ATPase system and its linkage to active transport. Although the specificity of the ATPase reaction has been questioned (Barka and Anderson, '63), with the method employed ATPase

activity was found to be localized to the cell membrane (see also Bartoszewicz and Barrnett, '64), and to granules in the mitochondria-rich cells of control tissue. Under in vitro conditions, no histochemical alterations were observed over control tissue, a finding consistent with the biochemical assays of Sharp and Leaf ('66) and Bonting and Canady ('64). These investigators found no increases in ATPase levels under similar conditions, nor increases in ATP after blockage of the sodium pump with ouabain, results which indicated no direct association of ATPase with the active transport system. Prolonged stimulation with aldosterone, however, did result in notable histochemical increases, primarily in the cytoplasm adjacent to the cell membrane within the granular cells. Indeed, the granular cells under these conditions appeared to be nearly as reactive as mitochondria-rich cells in control tissue. Whatever the precise role of ATPase in the active transport system and its response to aldosterone may be, the nearly unvaried occurrence of this enzyme within epithelia engaged in active transport (Heinz, '67) provides a rather constant morphological characteristic of such tissue.

Several possible pathways by which aldosterone might control energy metabolism exist. Evidence has already been presented that it mediates the production of key enzymes in the tricarboxylic acid cycle. A second possibility has been shown to be less likely, namely an increase in ATPase

activity. A third possibility has received little attention: that aldosterone may affect the respiratory transport chain. Histochemically, the increases seen in cytochrome oxidase, DPND and TPND after aldosterone stimulation support this concept. In a series of experiments to test this hypothesis further the utilization of oxygen under ADP (adenosine diphosphate) controlled respiratory states was examined in mitochondria isolated from the toad urinary bladder. This method, introduced by Chance and Williams ('56) consists of adding a known amount of ADP and an excess of substrate to a suspension of mitochondria while measuring the uptake of oxygen. An assumption is made that a high energy intermediate is formed which is converted to ATP. By computing the ratio of moles of ADP to the moles of oxygen utilized (the "P/O" ratio) a value for each substrate may be arrived at: comparison with the theoretical value for this substrate allows a conclusion concerning the respiratory state of the mitochondria to be made.

The results of these experiments indicate that aldosterone does affect the electron transport chain. A mild decrease in the P/O ratio was noted when mitochondria were exposed directly to the hormone, possibly a non-specific effect of the steroid nucleus. Furthermore, a more marked decrease was consistently observed in mitochondria isolated from bladders which had been incubated with aldosterone. The latter mitochondria would be exposed to the hormone mediated metabolic control agents, unlike

those in the first series of experiments, but they would also be in contact with the hormone itself for a longer period of time. This increased utilization of oxygen per mole of ADP indicates a less tightly coupled respiratory state of the transport chain. It should be noted that the theoretical P/O ratio for the substrate is 4.0 and since the highest value in control mitochondria was 3.2, it would seem that native toad bladder mitochondria exist in a somewhat loosely coupled state also. One possible explanation of the aldosterone uncoupling mechanism is an increased catalysis of the ATP-ADP exchange reaction discovered by Wadkins and Lehninger ('63) and thought to be the terminal event in oxidative phosphorylation. Theoretically at least, increased turn-over of a high energy intermediate could contribute to the active transport process by supplying an easily accessible, if perhaps transient, energy source. Reversibility of this system would account for the failure to find increased ATP levels. Heightened activity of the electron transport chain may therefore represent yet another effect of aldosterone upon oxidative metabolism.

The histochemical approach is particularly valuable in elucidating the cellular distribution of the increase in the oxidative machinery. The results of the in vivo experiments indicate that the burden of increased sodium transport in the toad bladder is levied upon a wider population of cells than the mitochondria-rich cells, namely the granular cells. Following aldosterone administration, mitochondria-linked enzymes were

no longer neatly compartmentalized into the mitochondria-rich cells, but increased activity was found in a graded fashion in the granular cells, identified as such by their diminished but still present PAS positive granules. The indication of an increased number of mitochondria in these cells was supported by PTAH staining.

The greater number of reactive mitochondria could be related to an increase in the number or the activation of enzymes in pre-existing mitochondria. Kirsten et al. ('68) also found an increase in enzyme activity per mg of protein of the mitochondrial fraction of the cell homogenate. This finding is consistent with activation of pre-existing enzymes or an increase in concentration of enzymes within mitochondria and does not rule out simultaneous increases in the number of mitochondria.

Thus, the granular cell approaches the histochemical profile of the mitochondria-rich cell under prolonged aldosterone stimulation. If we can infer that it acquires the physiological potential as well, the granular cell may assume added responsibility for the increased sodium transport which follows aldosterone stimulation. The above conclusion is consistent with that of certain radio-kinetic studies (Hoshiko and Ussing, '60; Frazier et al., '62) indicating that the number of mitochondria-rich cells is too small to account for increased inclusion of radioactive sodium (see also Keller, '63). The histochemical studies, therefore, indicate that aldosterone has a striking influence on the morphological as well as the biochemical phenotype of the cell.

SUMMARY

Histochemical changes were observed in the epithelium of the toad urinary bladder following stimulation by aldosterone under *in vitro* and *in vivo* conditions to determine the cell types affected by this hormone. Under *in vitro* conditions, a marked pyrinophilia in comparison to control tissue affecting all cell types was observed, as well as slight increases in cytoplasmic PAS staining within the granular cells. Granular cells were likewise slightly more reactive for G6PD and 6PGD than control tissue, whereas enzymes of the oxidative pathways and ATPase were unchanged under *in vitro* conditions. In addition, oxidative phosphorylation in mitochondria isolated from bladders stimulated with aldosterone was noted to show a looser state of coupling than in that of control tissue. Under *in vivo* conditions, pyrinophilia persisted over control tissue, and PAS cytoplasmic staining was increased. Activity of ATPase was more intensely localized to the cell membrane and cytoplasm of all cells, while more cells were heavily reactive for hexosemonophosphate shunt enzymes than under *in vitro* and control conditions. The most significant changes under *in vivo* conditions appear to be the marked increases in the enzymes of the oxidative pathways and the increased number of mitochondria demonstrated with the PTAH stain within the granular cells. Thus, under

prolonged aldosterone stimulation, the granular cell approached the histochemical profile of the mitochondria-rich cell. The hormone may therefore act upon the granular cell, which comprises the largest number of cells within the epithelium, as well as upon the mitochondria-rich cell.

APPENDIX[1]

The physiological activity of the preparaton of aldosterone utilized was confirmed by the short circuit technique. A hemibladder from the same toad was mounted in each side of a Lucite dual chamber containing electrodes and toad Ringer's solution without glucose; the bladder was allowed to equilibrate within the chamber 17 hours prior to the assay. The urinary bladder employed was excised from a toad which had lived in saline for four days. Four hours before assay, fresh toad Ringer's with .1 M glucose was exchanged, and at time "0", an amount of aldosterone sufficient to bring the concentration to 10^{-7} M was added to the serosal half of the chamber. At ten minute intervals the short circuit current was recorded for the control and the aldosterone treated bladder. The results are reported in graphic form in fig. 34, where:

$$\frac{SCCt}{SCC_o} = \frac{\text{short circuit current at time x}}{\text{short circuit current at time o}} .$$

The aldosterone treated bladder showed the expected rise in current with the proper time relationship for a potent sample of the hormone. Thereafter, the same preparation, stored at 4^{o} C, was used in all studies.

1. The above work was done with the kind collaboration of Dr. Arthur Finn, Department of Physiology, Yale University School of Medicine.

BIBLIOGRAPHY

Ausiello, D. A. and G. W. G. Sharp 1968 Localization of receptor sites for aldosterone in the bladder of the toad, Bufo marinus. Endocrinol., 82: 1163-1169.

Barka, T. and P. J. Anderson 1963 Histochemistry: Theory, Practice and Bibliography, Hoeber, N. Y.

Bartoszewicz, S. L. and R. J. Barrnett 1964 Fine structural localization of nucleoside phosphatase activity in the urinary bladder of the toad. J. Ultrastruct. Res., 10: 599-609.

Bonting, S. L. and M. R. Canady 1964 Na-K activated adenosine triphosphatase and sodium transport in toad bladder. Am. J. Physiol., 207: 1005-1009.

Burtner, H. J. and R. D. Lillie 1963 Stable sudanophilia of human neutrophil leukocytes in relation to peroxidase and oxidase. J. Histochem. Cytochem., 1: 8-28.

Chance, B. and G. R. Williams 1956 The respiratory chain and oxidative phosphorylation. Advan. Enzymol., 17: 65-134.

Choi, J. K. 1963 The fine structure if the urinary bladder of the toad, Bufo marinus. J. Cell Biol., 16: 53-72.

Crabbé, J. 1961a Stimulation of active sodium transport across the isolated toad bladder after injection of aldosterone to the animal. Endocrinol., 69: 673-682.

Crabbé, J. 1961b Stimulation of active sodium transport by the isolated toad bladder with aldosterone in vitro. J. Clin. Invest., 40:2103-2110.

Cuthbert, A. W. and E. Painter 1969 Mechanism of action of aldosterone. Nature (Lond.), 222: 280-281.

Denton, D. A. 1965 Evolutionary aspects of the emergence of aldosterone secretion and salt appetite. Physiol. Rev., 45: 245-295.

DiBona,D. R., M. M. Civan and A. Leaf 1969 The anatomical site of the transepithelial permeability barriers of toad bladder. J. Cell. Biol., 40: 1-7.

Edelman, I. S. 1964 Mechanism of action of aldosterone. Proc. Intern. Congr. Endocrinol. 2nd (Lond.), 1: 60-63.

Edelman, I. S., R. Bogoroch and G. A. Porter 1963 On the mechanism of action of aldosterone on sodium transport: the role of protein synthesis. Proc. Natl. Acad. Sci. U. S., 50: 1169-1173.

Edelman, I. S., R. Bogoroch and G. A. Porter 1964 Specific action of aldosterone on RNA synthesis. Trans. Assoc. Am. Physicians, 77:307-316.

Fanestil, D. D. 1969 Mechanism of action of aldosterone. Annual Rev. Med., 20: 223-232.

Fanestil, D. D. and I. S. Edelman 1966 On the mechanism of action of aldosterone on sodium transport: effects of inhibitors of RNA and of protein synthesis. Federation Proc., 25: 912-916.

Frazier, H. S. 1962 Electrical potential profile of the isolated toad bladder. J. Gen. Physiol., 45: 515-528.

Frazier, H. S., E. Dempsey and A. Leaf 1962 Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. J. Gen. Physiol., 45: 529-543.

Gadow, H. 1901 Amphibia and Reptiles. Cambridge Nat. Hist., VIII. Cambridge University Press, Cambridge, Eng.

Gomori, G. 1950 Sources of error in enzymatic histochemistry. J. Lab. Clin. Med., 35: 802-809.

Handler, J. S., A. S. Preston and J. Orloff 1969 The effect of aldosterone on glycolysis in the urinary bladder of the toad. J. Biol. Chem., 244: 3194-3199.

Heinz, E. 1967 Transport through biological membranes. Ann. Rev. Physiol., 29: 21-58.

Hess, R., D. C. Scarpelli and A. G. E. Pearse 1958 The cytochemical localization of oxidative enzymes. II Pyridine nucleotide linked dehydrogenases. J. Biophys. Biochem. Cytol., 4: 753-760.

Hoshiko, T. and H. H. Ussing 1960 The kinetics of Na^{24} flux across the amphibian skin and bladder. Acta Physiol. Scand., 49: 74-81.

Keller, A. 1963 A histochemical study of the toad urinary bladder. Anat. Record, 147: 367-377.

Kirsten, E., R. Kirsten, A. Leaf and G. W. G. Sharp 1968 Increased activity of enzymes of the tricarboxylic acid cycle in response to aldosterone in the toad bladder. Pflueger Arch. Ges. Physiol., 300: 214-225.

Leaf, A. 1955 Ion transport of the isolated bladder of the toad. Résummés communications. 3rd Congr. Intern. Biochem., 12-4: 107.

Leaf, A., J. Anderson and L. B. Page 1958 Active Na transport by the isolated toad bladder. J. Gen. Physiol., 41: 657-668.

Leaf, A., L. B. Page, and J. Anderson 1959 Respiration and active sodium transport of the isolated toad bladder. J. Biol. Chem., 234: 1625-1628.

Lillie, R. D. 1954 Histopathologic Technic and Practical Histochemistry, 2nd ed., Blakiston, N. Y.

MacManus, J. F. and R. W. Mowry 1960 Staining Methods, Hoeber, N. Y.

Mallory, F. B. 1897 On certain improvements in histological technique. J. Exper. Med., 2: 529-534.

Nachlas, M. M., K. C. Tsou, E. DeSouza, C. S. Cheng and A. M. Seligson 1957 The cytochemical distribution of succinic dehydrogenase by the use of a new p-nitrophenyl disubstituted ditetrazole. J. Histochem. Cytochem., 5: 420-436.

Nachlas, M. M., D. G. Walker and A. M. Seligman 1958 A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. J. Biophys. Biochem. Cytol., 4: 467-474.

Noble, G. K. 1931 The Biology of the Amphibia. Dover Publications, N. Y.

Peachey, L. D. and H. Rasmussen 1961 Structure of the toad's urinary bladder as related to its physiology. J. Biophys. Biochem. Cytol., 10: 529-553.

Pearse, A. G. E. 1960 Histochemistry: Theoretical and Applied. Little, Brown, and Co., Boston.

Peterson, R. E. 1959 Metabolism of adrenocorticosteroids in man. Ann. N. Y. Acad. Sci., 82: 846-853.

Schiefferdecker, P. 1884 Zur Kenntnis des Baues der Schleimdrüsen. Arch. Mikr. Anat., 23: 382-412.

Sharp, G. W. G. and A. Leaf 1964 The central role of pyruvate in the stimulation of sodium transport by aldosterone. Proc. Natl. Acad. U. S., 52: 1114-1121.

Sharp, G. W. G. and A. Leaf 1966 Mechanism of action of aldosterone. Physiol. Rev., 46: 593-633.

Simpson, S. A. and J. F. Tait 1955 Recent progress in methods of isolation, chemistry, and physiology of aldosterone. Recent Progr. Hormone Res., 11: 183-219.

Skou, J. C. 1962 Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na and K. Biochim. Biophys. Acta, 58: 314-325.

Sobel, H. J. 1968 Enzyme cytochemistry for the pathologist -- A simple method for the ultrastructural study of tissue alterations with the light microscope. Path. Annual, 3: 57-104.

Steen, W. B. 1929 On the permeability of the frog's bladder to water. Anat. Record, 43: 215-221.

Ussing, H. H. and K. Zerahn 1951 Active transport of Na as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand., 23: 110-127.

Vincent, S. 1898 Comparative histology of the suprarenal capsules. Int. J. Anat., 15: 282-303.

Wachstein, M. and E. Meisel 1956 On the histochemical demonstration of glucose-6-phosphatase. J. Histochem. Cytochem., 4: 592.

Wachstein, M. and E. Meisel 1957 Histochemistry of hepatic phosphatase at a physiological pH. Am. J. Clin. Path., 27: 13-23.

Wadkins, C. L. and A. L. Lehninger 1963 Distribution of an oligomycin sensitive adenosine triphosphatase--adenosine diphosphatase exchange reaction and its relationship to the respiratory chain. J. Biol. Chem., 238: 2555-2563.

EXPLANATION OF FIGURES

Plate I

Figure 1. PAS stain, control, paraffin section. Goblet cells (G) are intensely stained and granular cells (Gr) contain PAS positive particles as well as faint cytoplasmic staining. (X 1175)

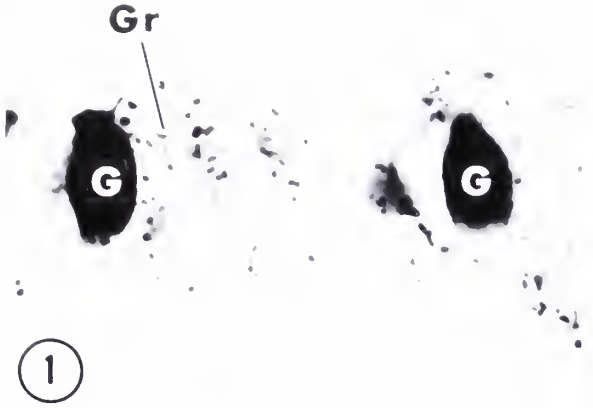
Figure 2. PAS stain, *in vitro* aldosterone, paraffin section. Cytoplasmic staining is moderately increased over control tissue, especially near the luminal border (top). (X 1500)

Figure 3. PAS stain, *in vivo* aldosterone, paraffin section. Cytoplasmic staining is more intense than in both control and *in vitro* treated tissue. Note the binucleate cell (arrow). (X 1875)

Figure 4. PTAH stain, control, paraffin section. The cytoplasm of most cells is faintly stained. At least one mitochondria-rich cell (M) can be identified by the more dense particularity of its cytoplasm. The cell with the clear cytoplasm is probably a goblet cell (G). (X 1875)

Figure 5. PTAH stain, *in vitro* aldosterone, paraffin section. No difference in staining is apparent between this and the preceding control section. (X 1875)

Figure 6. PTAH stain, *in vivo* aldosterone, paraffin section. The cytoplasm of all cells shows an increase in particulate staining. G, goblet cell. (X 1875)



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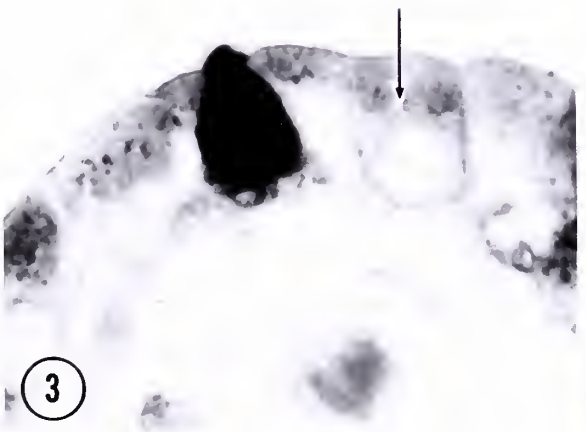

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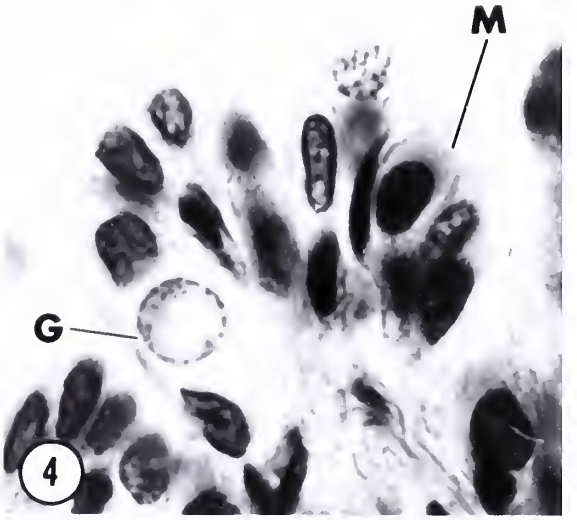

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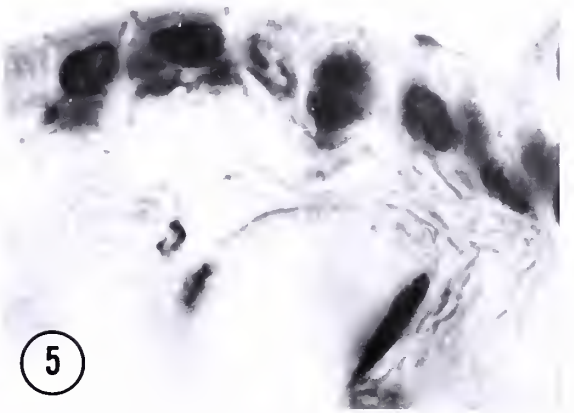

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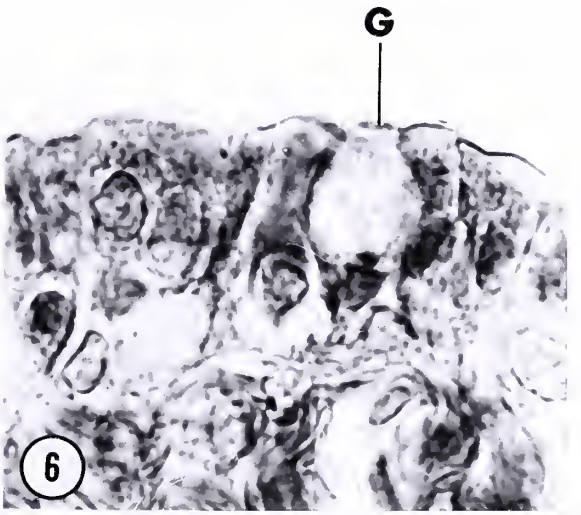

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EXPLANATION OF FIGURES

Plate II

Figure 7. Methyl green-pyronin, control, paraffin section. Uniform faint cytoplasmic staining is seen in all epithelial cells. (X 1175)

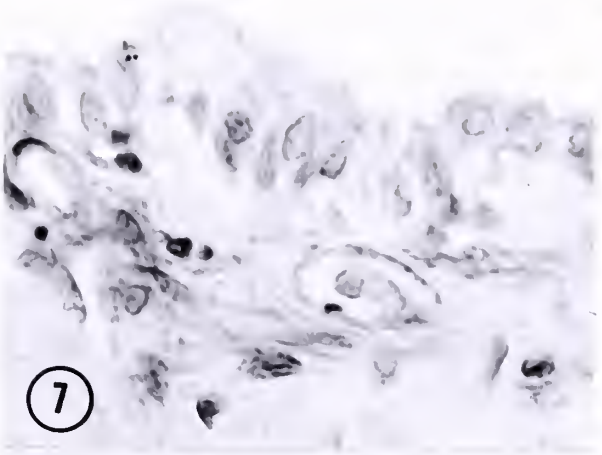
Figure 8. Methyl green-pyronin, *in vitro* aldosterone, paraffin section. Cytoplasmic staining is more intense in epithelial cells in comparison to control tissue. (X 1175)

Figure 9. Methyl green-pyronin, *in vivo* aldosterone, paraffin section. Epithelium is more intensely stained than control tissue, but similar to *in vitro* treated tissue. (X 1175)

Figure 10. DPND, control, whole mount. Intensely reactive cells (mitochondria-rich cells) are sparsely scattered among more weakly and variably reactive cells. Activity is localized to small cytoplasmic granules. (X 1175)

Figure 11. DPND, *in vitro* aldosterone, whole mount. Few differences are observed between this and control tissue. (X 1150)

Figure 12. DPND, *in vivo* aldosterone, whole mount. A greater number of intensely reactive cells can be noted over the preceding figure, as well as increased number of stained granules within the granular cells. (X 1150)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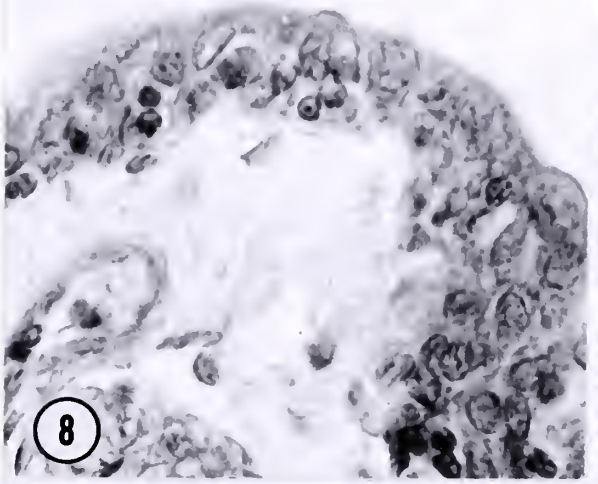
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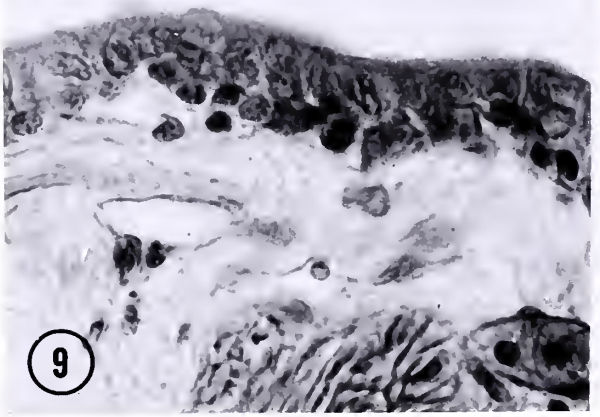
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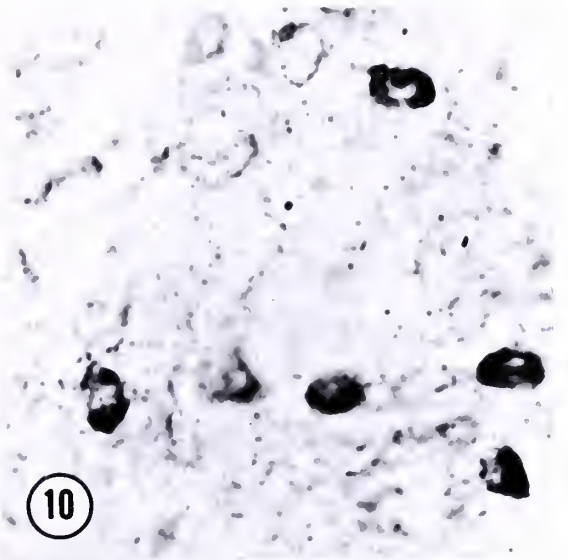
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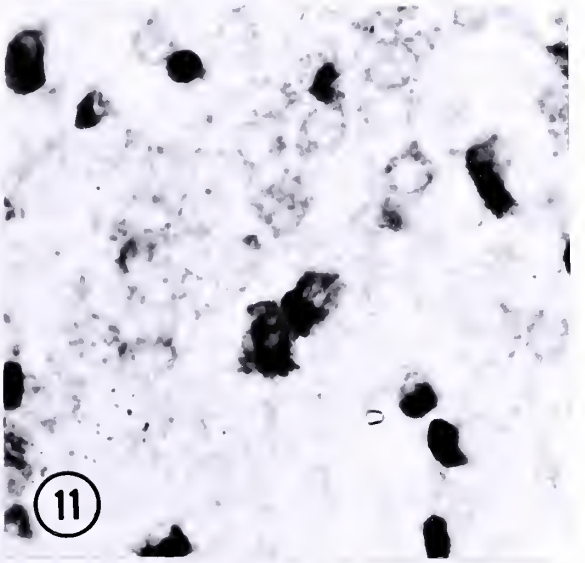
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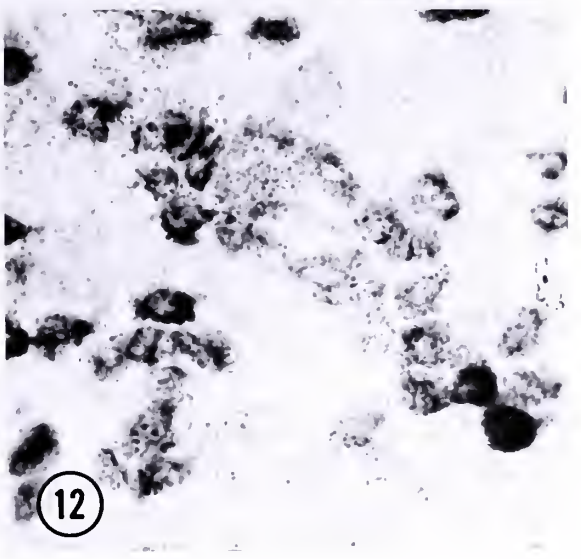
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EXPLANATION OF FIGURES

Plate III

Figure 13. SDH, control, whole mount. Mitochondria-rich cells are filled with intensely stained granules, while granular cells contain a much smaller number of stained granules. (X 2500)

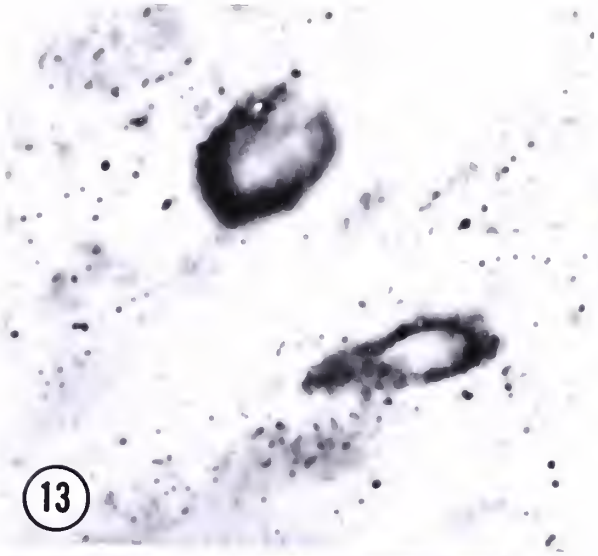
Figure 14. SDH, *in vivo* aldosterone, whole mount. Granular cells contain a larger number of stained granules than in control tissue. M, mitochondria-rich cell. (X 2500)

Figure 15. SDH, control, frozen section. Note the intensely reactive mitochondria-rich cells and their distribution in the epithelium. (X 2000)

Figure 16. SDH, *in vivo* aldosterone, frozen section. Increased numbers of intensely stained mitochondria-rich cells can be seen in comparison to control tissue. (X 1875)

Figure 17. Cytochrome oxidase, control, whole mount. Mitochondria-rich cells (M) contain large numbers of stained granules while granular cells are weakly reactive. (X 2100)

Figure 18. Cytochrome oxidase, *in vivo* aldosterone, whole mount. Granular cells are more reactive and the number of stained granules approaches the number seen in mitochondria-rich cells (M). (X 2350)



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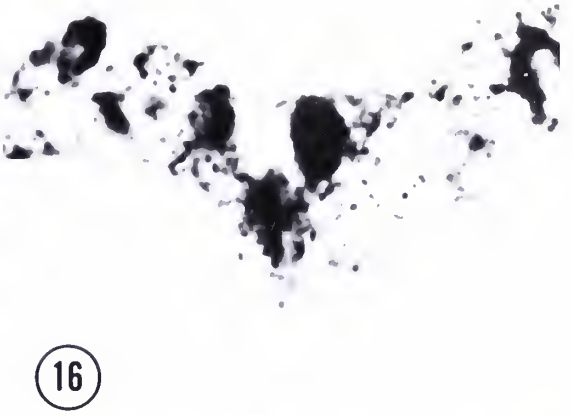

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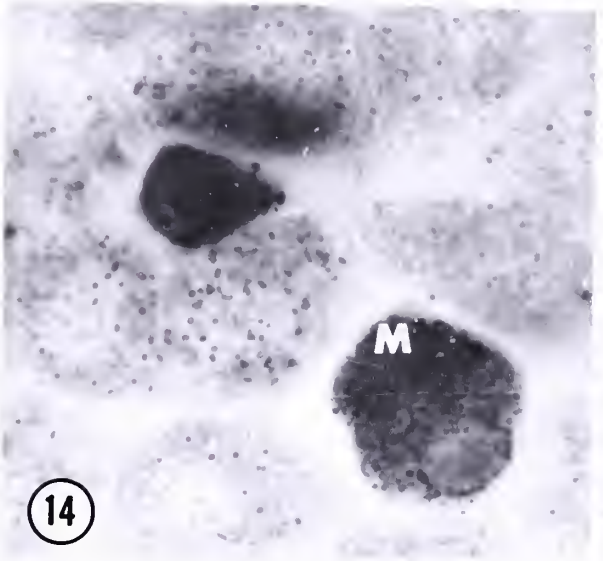

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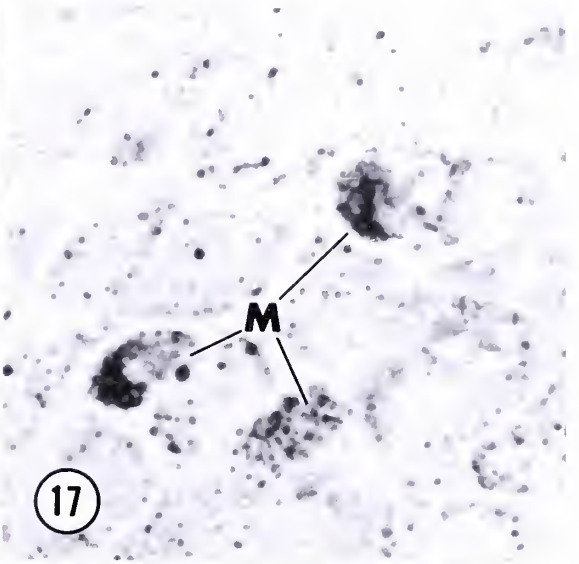

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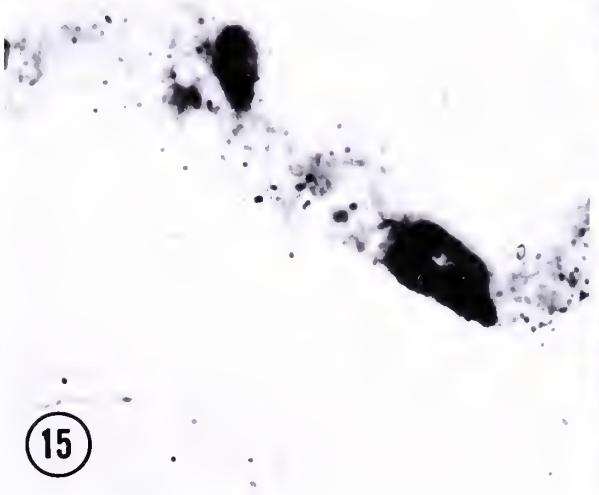

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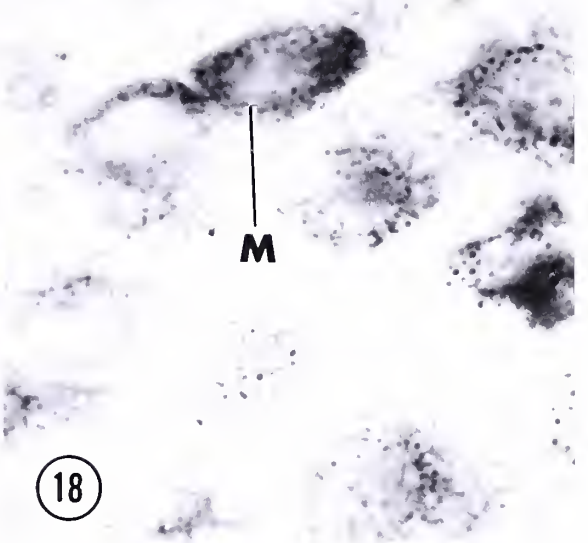

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EXPLANATION OF FIGURES

Plate IV

Figure 19. G6PD, control, frozen section. Reaction product is localized to cytoplasmic granules. Intensely reactive cells are displayed in close proximity suggesting the distribution seen in the whole mount preparation. (X 1250)

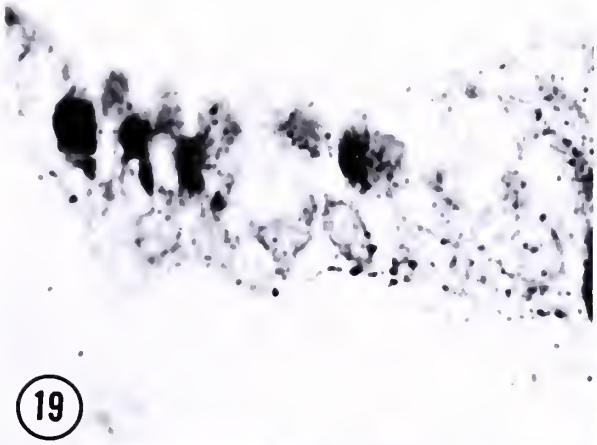
Figure 20. G6PD, *in vitro* aldosterone, frozen section. The number of reactive granules is moderately increased over control tissue. (X 1250)

Figure 21. G6PD, *in vivo* aldosterone, frozen section. In contrast to control tissue, there are many more highly reactive cells (compare with fig. 24). (X 1560)

Figure 22. G6PD, control, whole mount. The reaction is localized to cytoplasmic granules, with clear separation of the more reactive from the less reactive cells. This preparation also shows the contiguous arrangement of the highly reactive cells in a small linear cluster.(X 2300)

Figure 23. G6PD, *in vitro* aldosterone, whole mount. While the distibution of the reactive cells remains approximately the same as in control tissue, the density of the intracytoplasmic granules appears greater. (X 2300)

Figure 24. G6PD, *in vivo* aldosterone, whole mount. In comparison to control tissue, the reactive cells are more numerous and arranged in larger stellate groups. (X 2300)



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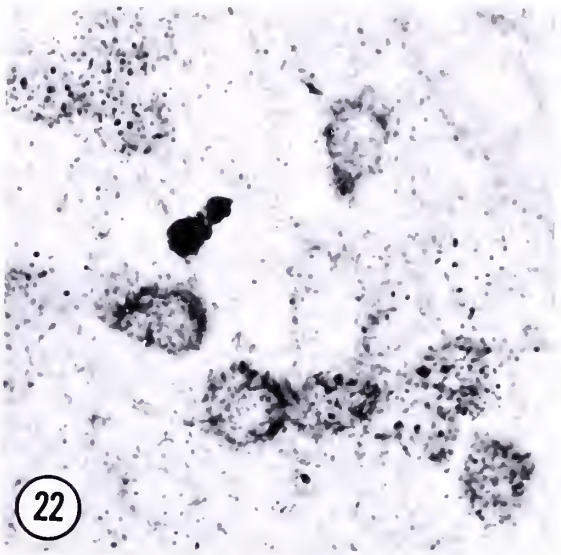

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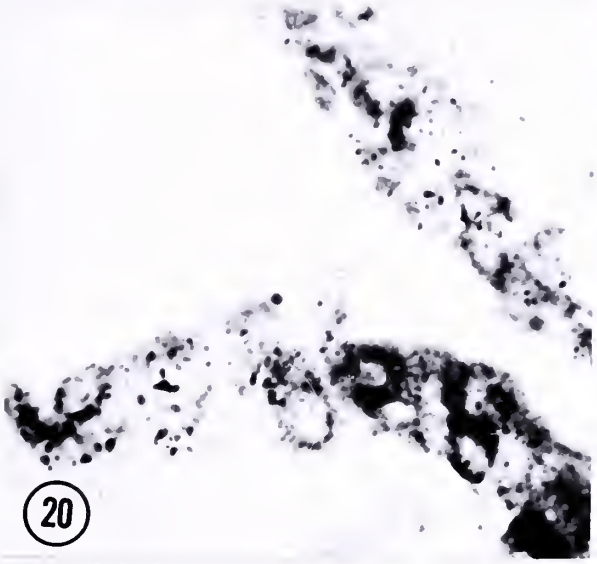

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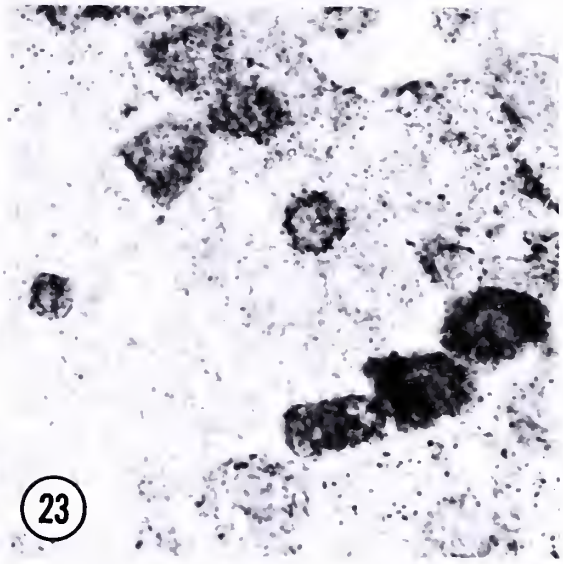

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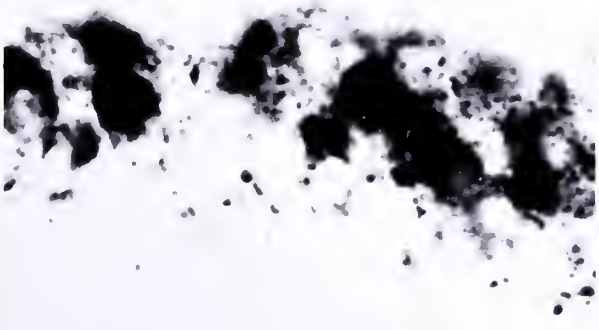

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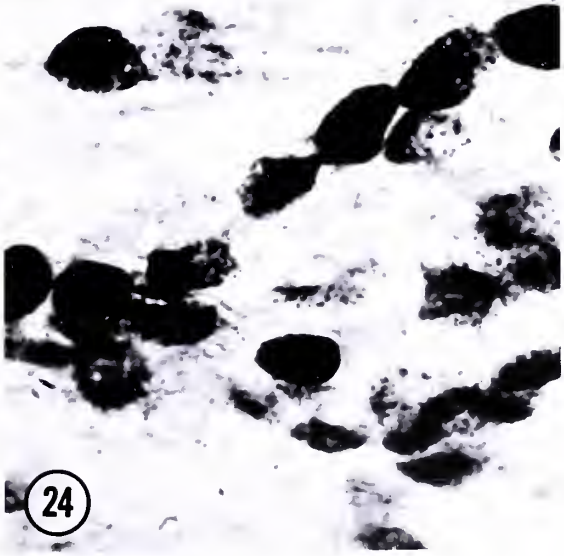


24

EXPLANATION OF FIGURES

Plate V

Figure 25. ATPase, control, frozen section. Reaction is most intense within two cells (arrow) containing enzymatic activity localized to the cytoplasm and to granules. (X 2500)

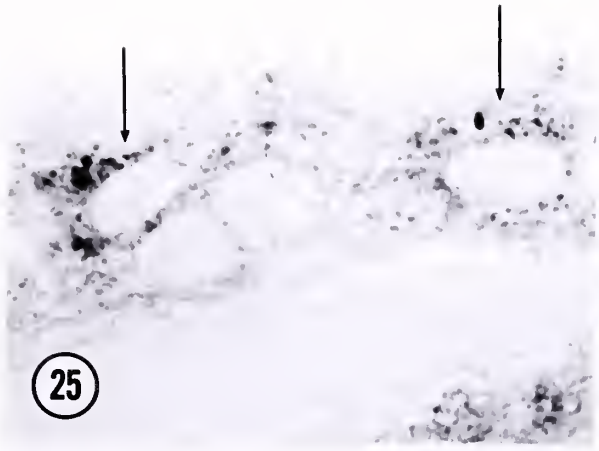
Figure 26. ATPase, *in vivo* aldosterone, frozen section. Both the cytoplasmic and granular activity is more intense than in control tissue, especially adjacent to the cell membrane. (X 2500)

Figure 27. G6Pase, control, frozen section. Enzyme activity is localized largely to perinuclear granules of the epithelial cells. (X 2000)

Figure 28. G6Pase, *in vivo* aldosterone, frozen section. While cytoplasmic localization is similar to preceding control tissue, a moderate increase in perinuclear granules can be seen. (X 2110)

Figure 29. Non-specific esterase, control, whole mount. A few cells are filled with reactive droplets, while the others show a vacuolar localization adjacent to the cell membrane. (X 1875)

Figure 30. Non-specific esterase, *in vivo* aldosterone, whole mount. The number of heavily reactive cells is greater than in control tissue. Moreover, staining of the remaining cells, as indicated by the number of vacuoles at the periphery of the cytoplasm, is more intense. (X 1875)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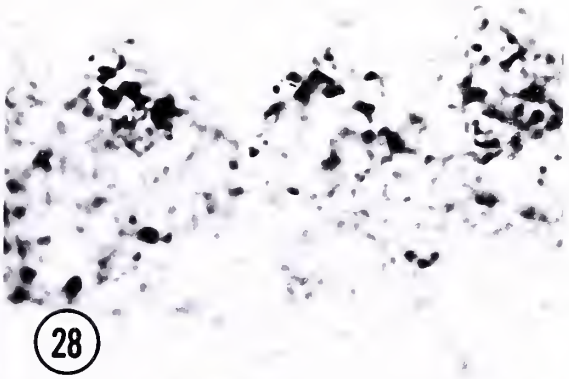
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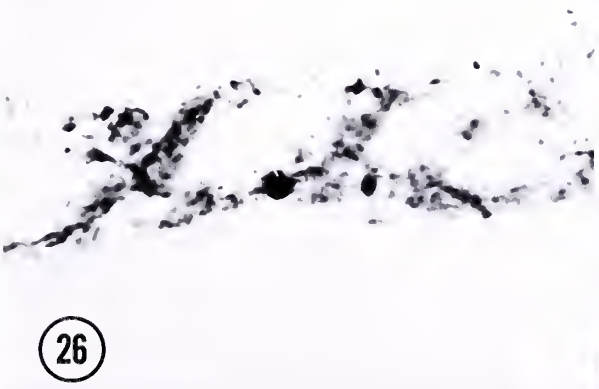
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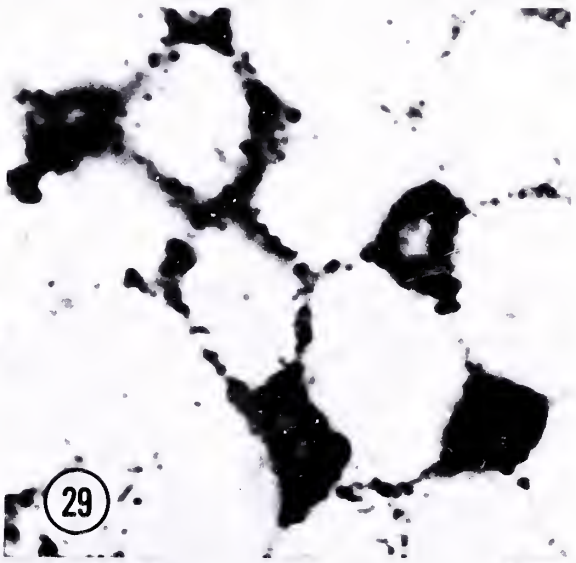
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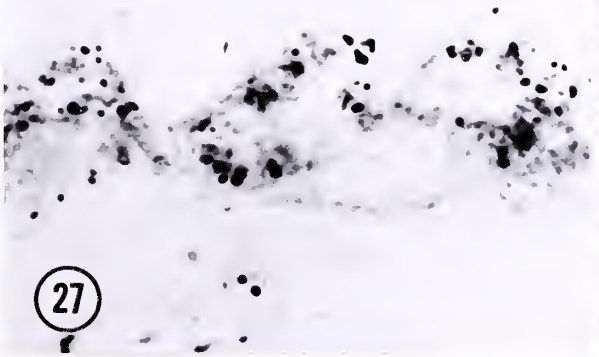
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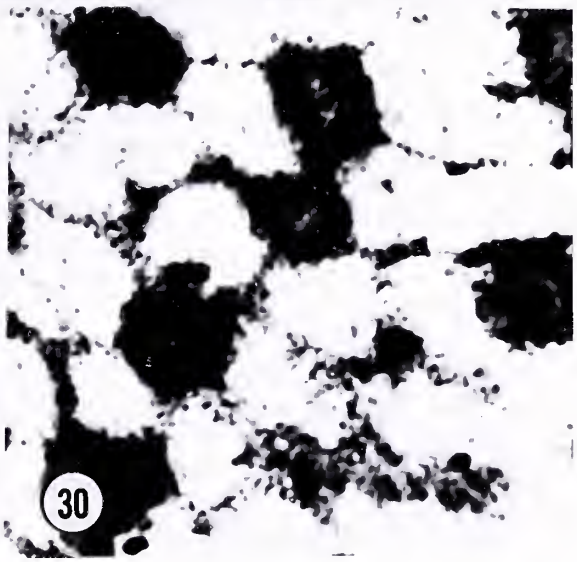
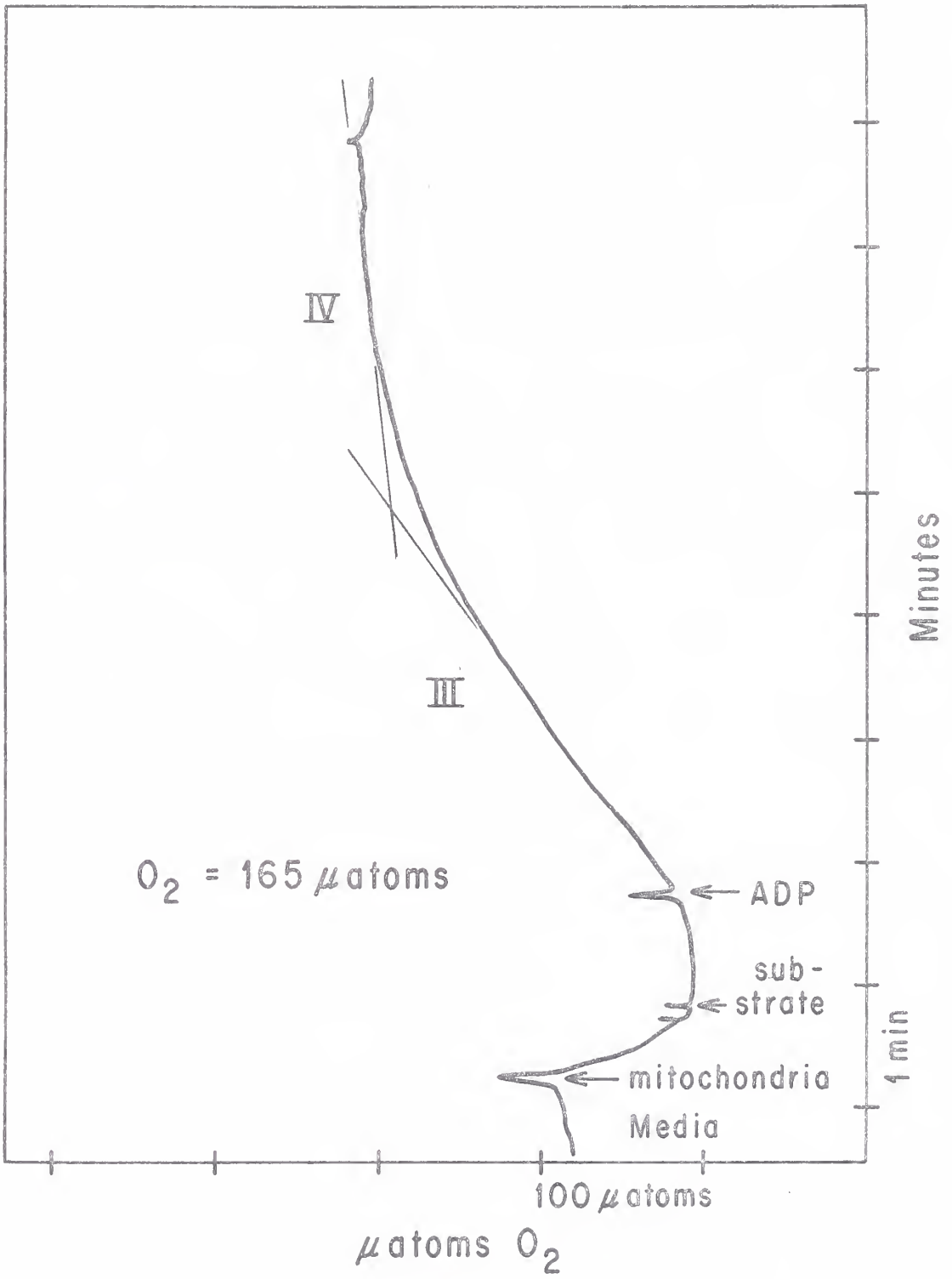

30

EXPLANATION OF FIGURE

Figure 31. This figure is a copy of a polarographic tracing of oxygen uptake in mitochondria isolated from control toad bladders. Oxygen uptake is measured along the abscissa; the ordinate represents time. The effect of the addition of substrate and ADP can be seen. A line is extended along each straight segment of the curve; the intersection of each line represents a point on the abscissa from which the amount of oxygen is determined. In this case, $P/O = \frac{460}{165} = 2.89$. The ratio of the rate of oxygen consumption in States III and IV is $5/.9 = 6$.

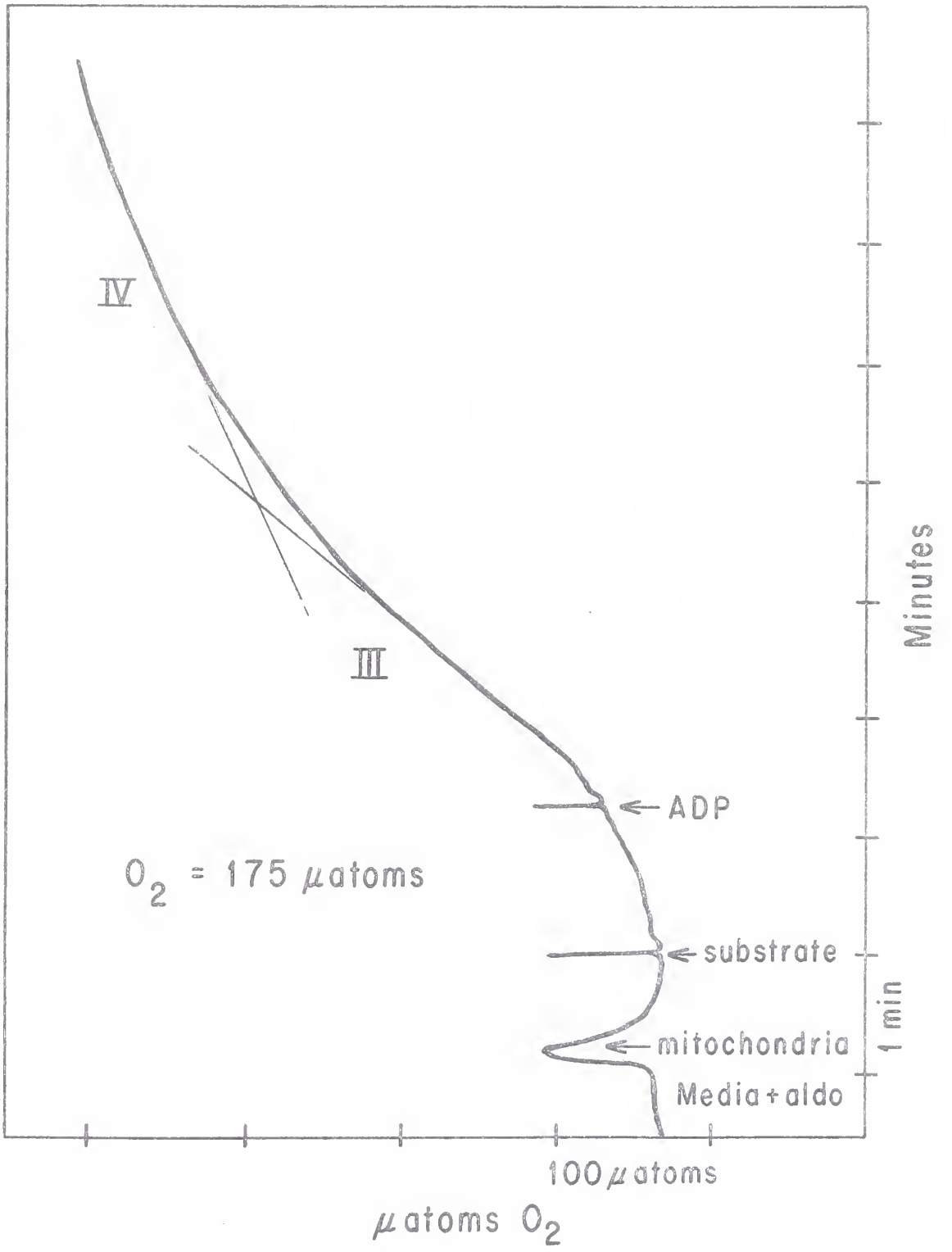
Fig. 31


IV
III
O_2 = 165 μatoms
ADP
sub-
strate
mitochondria
Media
Minutes
1 min
100 μatoms
μatoms O_2

EXPLANATION OF FIGURE

Figure 32. This figure is a copy of a polarographic tracing of oxygen uptake in mitochondria isolated from control toad bladders in which the polarographic medium contained 10^{-7} M aldosterone. Oxygen uptake is measured as in fig. 31. Here, $P/O = \frac{460}{175} = 2.61$. State III/State IV = 6/5 = 1.1. The amount and rate of oxygen consumption is greater than that of control mitochondria.

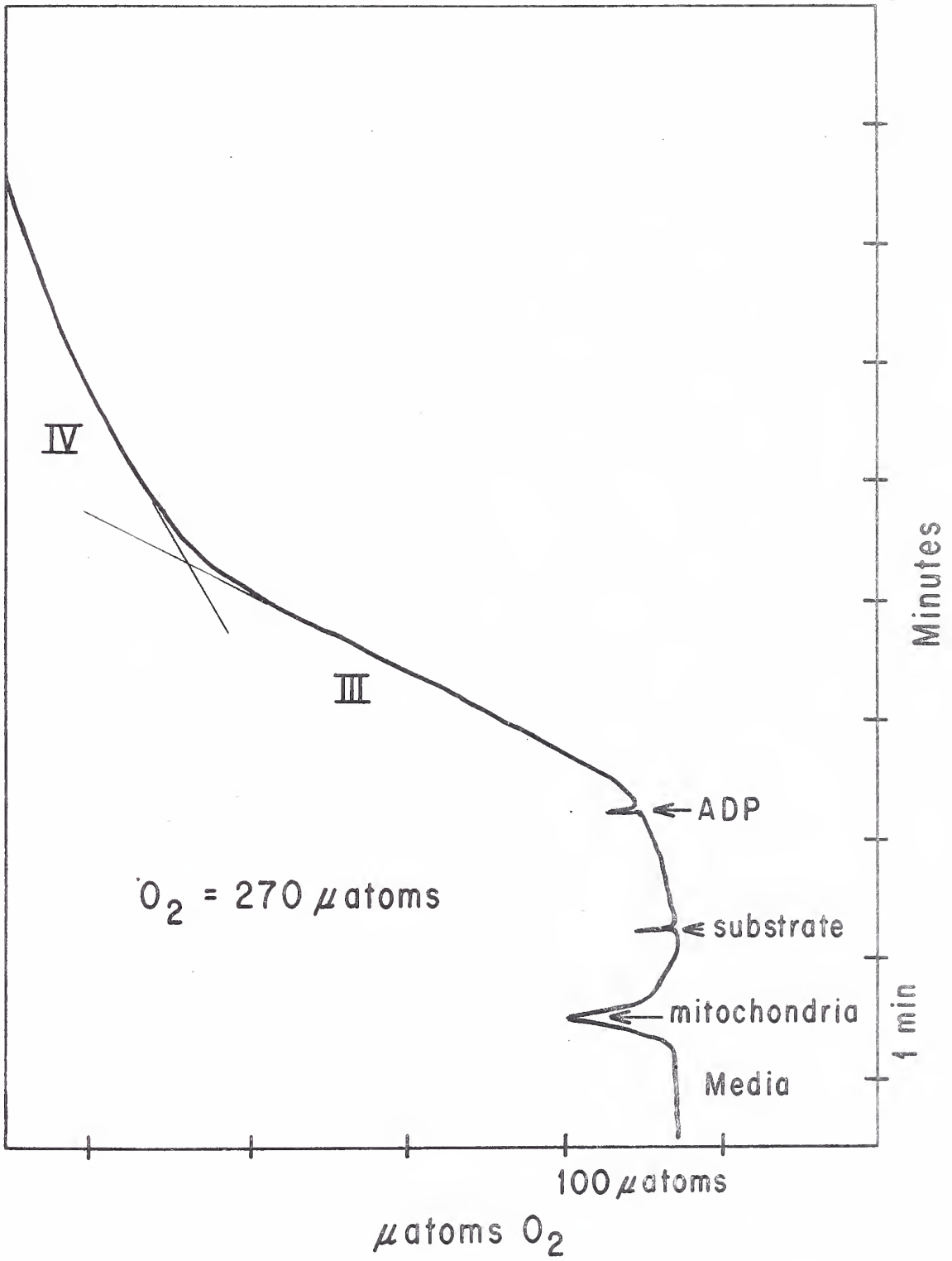
Fig. 32


IV
III
ADP
substrate
mitochondria
Media + aldo
O_2 = 175 μatoms
Minutes
1 min
100 μatoms
μatoms O_2

EXPLANATION OF FIGURE

Figure 33. This figure is a copy of a polarographic tracing of oxygen uptake in mitochondria isolated from toad bladders incubated *in vitro* with 10^{-7} M aldosterone for three hours. Oxygen consumption is measured as in fig. 31. Here, P/O = $\frac{460}{270}$ = 1.71. State III/ State IV= 8/2.1 = 4. The amount and rate of oxygen consumption is greater than both control mitochondria and mitochondria exposed to the hormone for a short period.

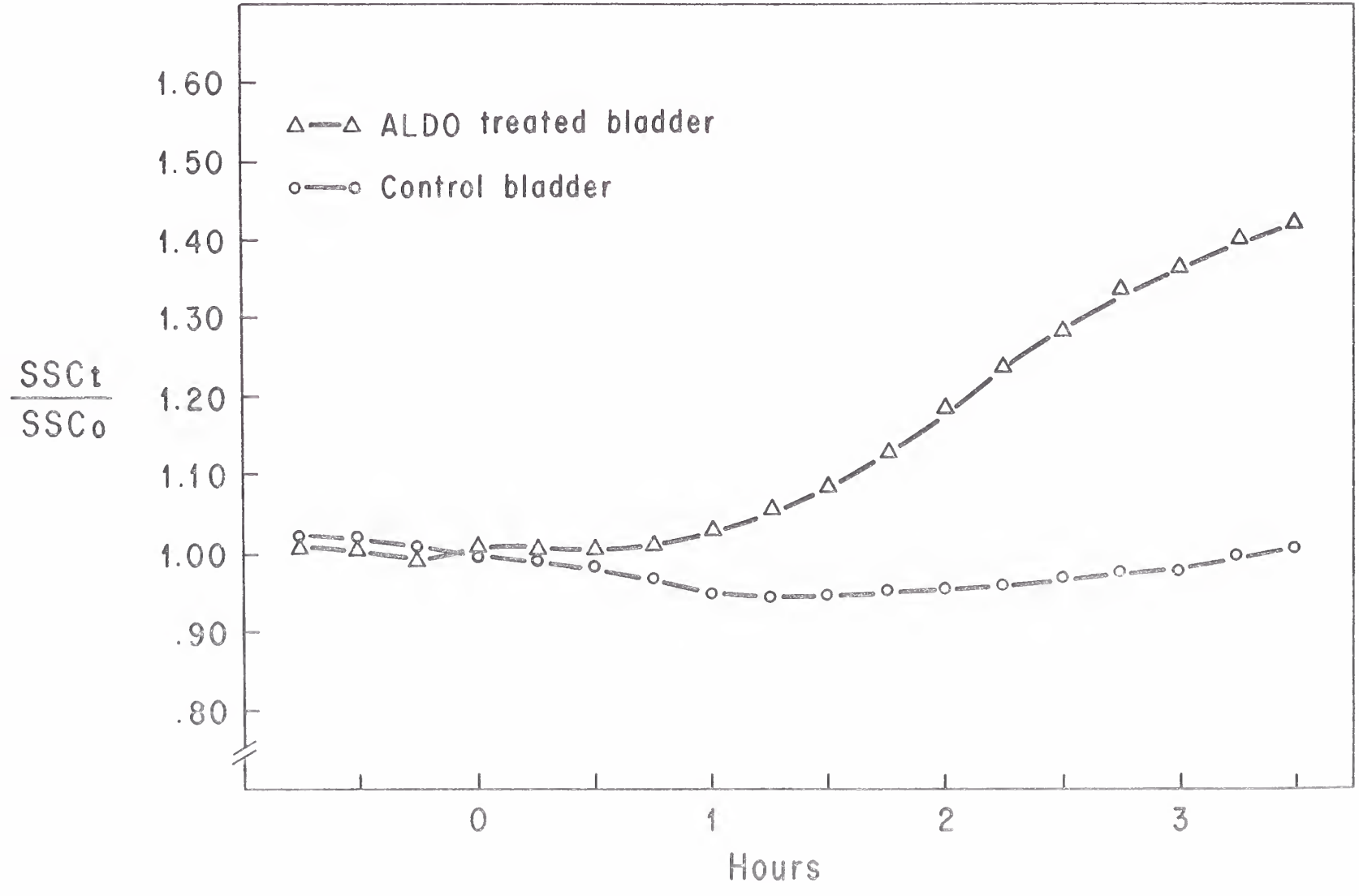
Fig. 33


IV
III
ADP
substrate
mitochondria
Media
O_2 = 270 μatoms
Minutes
1 min
100 μatoms
μatoms O_2

EXPLANATION OF FIGURE

Figure 34. This graph illustrates the degree of increase in short circuit current exhibited by the hemibladder exposed to 10^{-7} M aldosterone at the serosal aspect of the membrane. The control hemibladder showed no increase in short circuit current. This demonstrates the potency of the hormone preparation.

Fig. 34


1.60
1.50
1.40
1.30
1.20
1.10
1.00
.90
.80
ALDO treated bladder
Control bladder
SSCt / SSCo
0
1
2
3
Hours