Disorders of Cell Volume Regulation

I. Effects of Inhibition of Plasma Membrane Adenosine Triphosphatase with Ouabain

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THE ABILITY TO MAINTAIN normal intracellular volume represents a fundamental property of all living cells, a property that is often stressed when such cells are placed in unusual environments or subjected to various types of injury. Mammalian cells are assumed to be living in an isosmotic environment, which is subject to change in many disease states. At the same time, many other types of injurious situations express themselves as disorders of cell volume regulation by exerting primary effects on the cellular mechanisms for volume control, rather than on the extracellular fluid. Because of the frequency of occurrence of disorders of cell volume regulation in disease, various investigators have approached this problem in different ways—for example, the morphologist has often been content to dismiss these changes with terms such as "cloudy swelling," while the physiologist has directed his attention to disorders of ion transport and water permeability. It seems evident that more thorough understanding of this problem requires the simultaneous application of correlative approaches, enabling a more complete understanding of the phenomenon in the biologic sense. The present series of reports represents an attempt to study this problem from both the morphologic and functional standpoints.

For our studies, we are employing the isolated flounder tubule system described previously.[1] This system permits the incubation of intact nephrons for long periods of time in vitro, where they continue to function and maintain fine structure for as long as 48 hr. The advantages of this system for correlative morphologic and functional studies were discussed in detail in previous publications.[1,2]

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In a previous study concerning the cell swelling produced by replacement of extracellular sodium with potassium, a series of changes involving sequential expansions of various cell compartments was observed.[3] In that report, the hypothesis was presented that this sequence was related to the differential permeability, at differing times, of various intercellular membranes to entering solute in water. It was further argued that if sodium were the chief entering solute, the pattern of change would be the same, except that the permeability would be to sodium rather than potassium. The present paper is designed to test this hypothesis by inhibiting sodium transport at the plasma membrane by exposing flounder renal tubules to ouabain.

Materials and Methods

Collection and Maintenance of Fish

The studies were performed on isolated nephrons from the southern flounder *Paralichthys lethostigma*. The fish were collected in commercial trap nets in Back Sound near Beaufort, N. C., during their autumnal migrations to the sea, and were transported to the Duke University Marine Laboratory where they were maintained in tidally fed salt water ponds throughout the year. Immediately prior to experimentation, the fish were transferred to circulating salt water systems within the laboratory.

Preparation and Incubation of Tubules

The kidneys were removed from fish, as described previously,[1] dissected into fragments of small groups of nephrons, and incubated in the medium described by Forster.[4] The control medium had the following composition in millimoles per liter: NaCl, 135; KCl, 2.5; $CaCl_2$, 1.5; $MgCl_2$, 1.0; $NaHPO_4$, 0.5; $NaHCO_3$, 10; and chlorphenol red, 0.02. In some experiments, chlorphenol red was replaced by Diodrast ^{131}I. The osmolality of this medium was approximately 270 mosm. per kilogram.

For studies on the effects of ouabain, control mediums were prepared containing 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} moles of ouabain per liter.

Tubules were maintained in control or experimental mediums by incubating them in Petri dishes containing media gassed with 100% oxygen. All incubations were performed at approximately 23° C.

Morphologic Studies

Light Microscopy and Histochemistry. Light microscopic observations were made on unfixed living tubules at serial time intervals during the development of change. These were accomplished by mounting tubules in a drop of medium and examining them with a light microscope adjusted for partial phase effect by reducing the substage condenser apperture. Other light microscopic observations were made on the tubules within the incubation chambers utilizing a dissecting microscope.

Electron Microscopy. Tubules were fixed at serial time intervals (30 min. and 1, 2, and 4 hr.) in 1% OsO_4 buffered with s-collidine at 0–4° C. Tissues were dehydrated and prepared for electron microscopy as described previously.[1]

Functional Analyses

Active Transport. Active transport was assessed by noting the rate of dye accumulation in tubular lumens after incubation for 30 min. and 1, 2, and 4 hr. By viewing the functioning preparations by light microscopy, it is possible to judge the rate of active transport and accumulation on a semiquantitative basis by noting the intensity of dye coloration within the tubular lumens. This method was described previously in more detail.[1] Active transport was also measured by determining the rate of accumulation of Diodrast ^{131}I by the tubules. Previous investigators have shown that Diodrast is transported by a system which is very similar to or identical with that which transports organic acid dyes such as chlorphenol red.[5] These studies were performed by adding Diodrast ^{131}I (3×10^{-5} M/L.), rather than chlorphenol red to the incubation mediums. Tubules were placed on tared Millipore filters prior to placement in the incubation medium. After the desired time interval, the Millipore filters containing the tubules were removed and washed in unlabeled medium; the washings were performed in Millipore filter suction devices. Following the second wash, tubules were freed of excess medium by applying suction for an additional minute. The filter then was removed from the suction device, weighed on an analytic balance, dried for 60 min. at 100° C., and weighed again. From these measurements, the water content of the tubules was determined. After drying, the filters bearing the tubules were transferred to counting vials and counted in a manual gamma counter.

Sodium Uptake. The uptake of sodium by tubules was studied by adding ^{24}Na to the incubation mediums. The tubules were placed on Millipore filters, handled as described above, and removed from the ^{24}Na-labeled medium after 1 hr. of incubation. These samples were weighed and counted as described above.

Measurement of Protein Synthesis. In these experiments, the tubules were incubated in dishes containing 9 ml. of control or experimental medium containing 1.6 mM/L. unlabeled leucine, and 0.067 μM/L. L-leucine 4, 5-^{3}H. After 50 min., 2 hr., and 3 hr., tubules were removed from the medium and homogenized in deionized water, using a ground-glass homogenizer. Aliquots of the homogenate were analyzed for protein by the method of Lowry *et al.*[6] Other aliquots of the homogenate were pipetted onto borosilicate filter discs, and prepared by the method of Mans and Novelli,[7] modified as follows: The discs were plunged into 5% trichloracetic acid (TCA) at 4° C., washed in two changes of 5% TCA at room temperature, heated at 90° C. for 7 min. in 5% TCA, washed in two changes of 5% TCA at room temperature, washed in absolute ethanol for 1 min. at room temperature, washed in ethanol:chloroform: ether (2:2:1) for 1 min. at room temperature, and washed in two changes of ether at room temperature. Samples were air dried and placed in low-potassium glass scintillation vials. Liquid scintillation solution containing 5 gm. of 2,5-diphenyloxazole (PPO) and 0.5 gm. of 2,2-p-phenylenebis(5 phenyloxazole) (POPOP) per liter in anhydrous toluene was added to each vial. Samples were then counted in a liquid scintillation counter at an efficiency of 25%.

Measurement of Oxygen Consumption. The measurements of oxygen consumption were performed using a Clark type polarographic electrode. Suspensions of tubules were added to 3 ml. of Forster's buffer maintained at 26° C.; oxygen consumption was plotted using a recorder. Control rates were determined for 2–5 min. prior to adding ouabain at concentrations of 10^{-2} M/L. and 10^{-3} M/L.

Results

Control Preparations

The behavior of control preparations in the system has been described in detail previously.[1] The light and electron microscopic appearance of these cells was stable over the entire experimental period.

Tubules Treated with Ouabain

Our previous work with swelling induced by high extracellular levels of potassium led us to postulate the following stages of loss of cell volume regulation leading from the normal to the necrotic cell.[3]

Stage 1 (*Text-fig. 1*). This is the state of the cell and its organelles in the control preparations. (All diagrams refer to the second brush border region of the flounder nephron, which constitutes the major portion of the length of the nephron, and on which most observations have been made.)

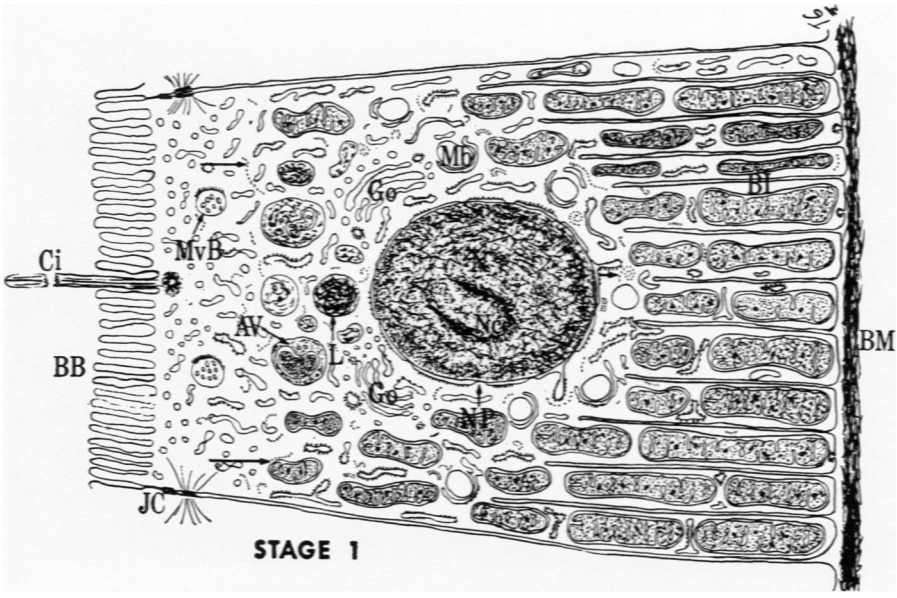
Stage 2 (*Text-fig. 2*). The only change at this stage is dilatation of cisternae of endoplasmic reticulum and of the nuclear envelope.

Stage 3 (*Text-fig. 2*). In this stage, the cell volume is expanded, with distortion of microvilli and of the infolded plasma membrane at the cell base. The expansion involves the cell sap, which is pale. In addition to the dilatation of the endoplasmic reticulum and nuclear envelope, the mitochondria are dense, with contracted matrix compartments and relative expansion of outer compartments. Abnormalities in the disposition or membrane attachment of ribosomes and polysomes were not observed.

Stage 4 (*Text-fig. 3*). In addition to the changes of Stage 3, this stage is characterized by detachment and dispersal of polysomes, membranous whorls forming from the basilar plasmalemmal invaginations, and the presence of expanded inner compartments in some mitochondria. The chromatin in the nucleus is pale, and the nucleoli are dispersed.

Stage 5 (*Text-fig. 3*). In addition to the changes of Stage 4, there were numerous interruptions in the continuity of the plasma membrane and of the nuclear envelope. The nuclear contents often were observed extending into the surrounding cytoplasm. In addition to the expanded inner compartments, the mitochondria contained flocculent, as well as microcrystalline, densities. Ribosomes were infrequent anywhere within the cytoplasm, and lysosomes could not be identified.

Although in the case of high potassium levels these five stages were thought to reflect differences in permeability of intracellular membranes to potassium, it was postulated that a similar situation would hold for loss of cell volume regulation induced by loss of sodium extrusion. The following experiments have been designed to examine this hypothesis.

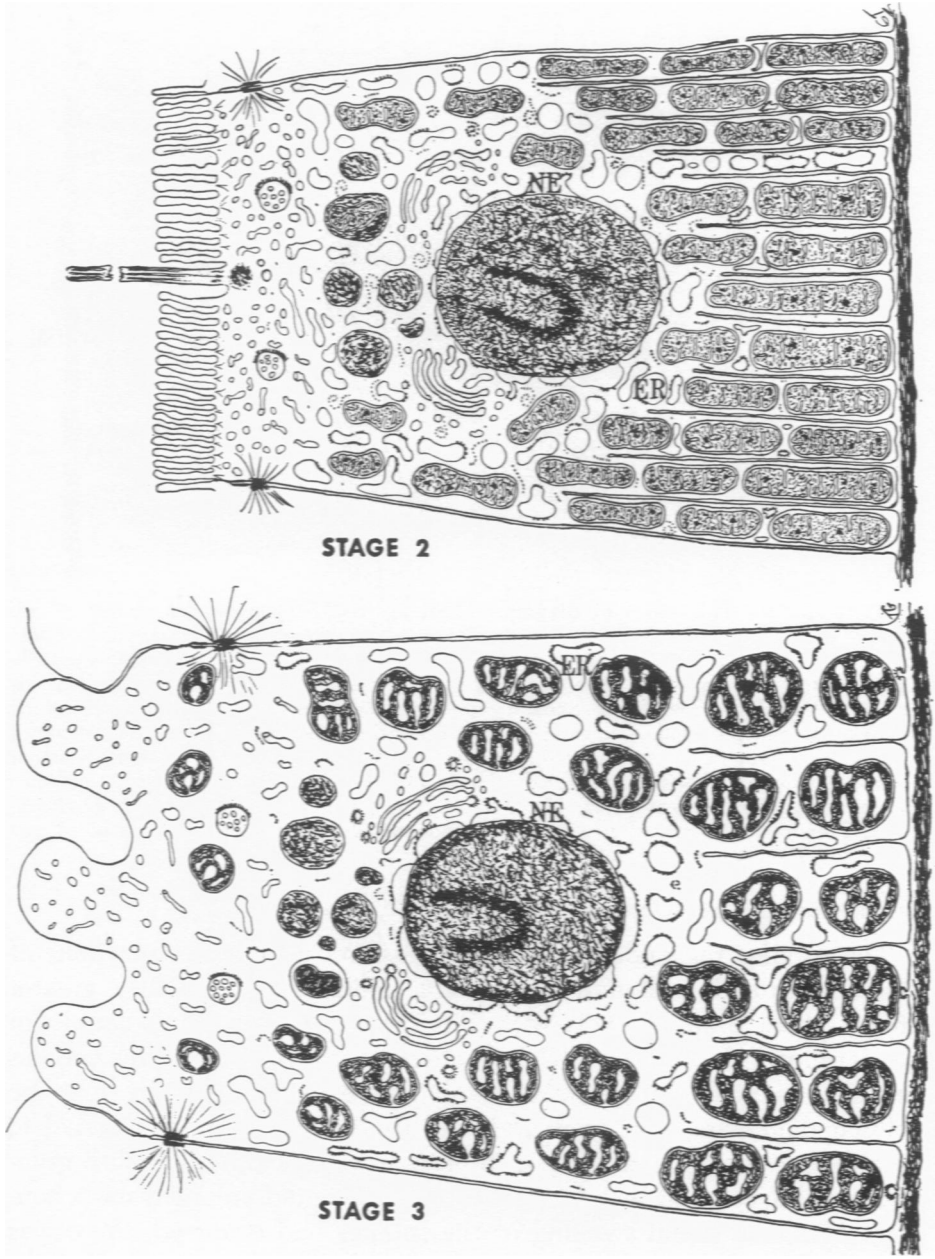


TEXT-FIG. 1. This series of diagrams (Text-fig. 1–3) depicts stages of loss of cell volume regulation leading from control (Stage 1) to necrotic cell (Stage 5). These stages were postulated on the basis of previous work;[8] the present experiments were designed to test the hypothesis that such stages could result from inhibition of sodium extrusion at the plasma membrane. *Stage 1* represents appearance of normal flounder tubule cell from second proximal segment. *Ci*, cilium; *BB*, brush border; *JC*, junctional complex composed of tight junction, intermediate junction, and desmosomes; *MvB*, multivesicular bodies; *L*, secondary lysosomes; *AV*, autophagic vacuoles; *Go*, Golgi apparatus; *N*, nucleus; *Nc*, nucleolus; *NP*, nuclear pores; *Mb*, microbodies; *free arrows*, face-on views of rough-surfaced endoplasmic reticulum showing polysomes; *BI*, basilar invaginations of plasma membrane; *BM*, basement membrane.

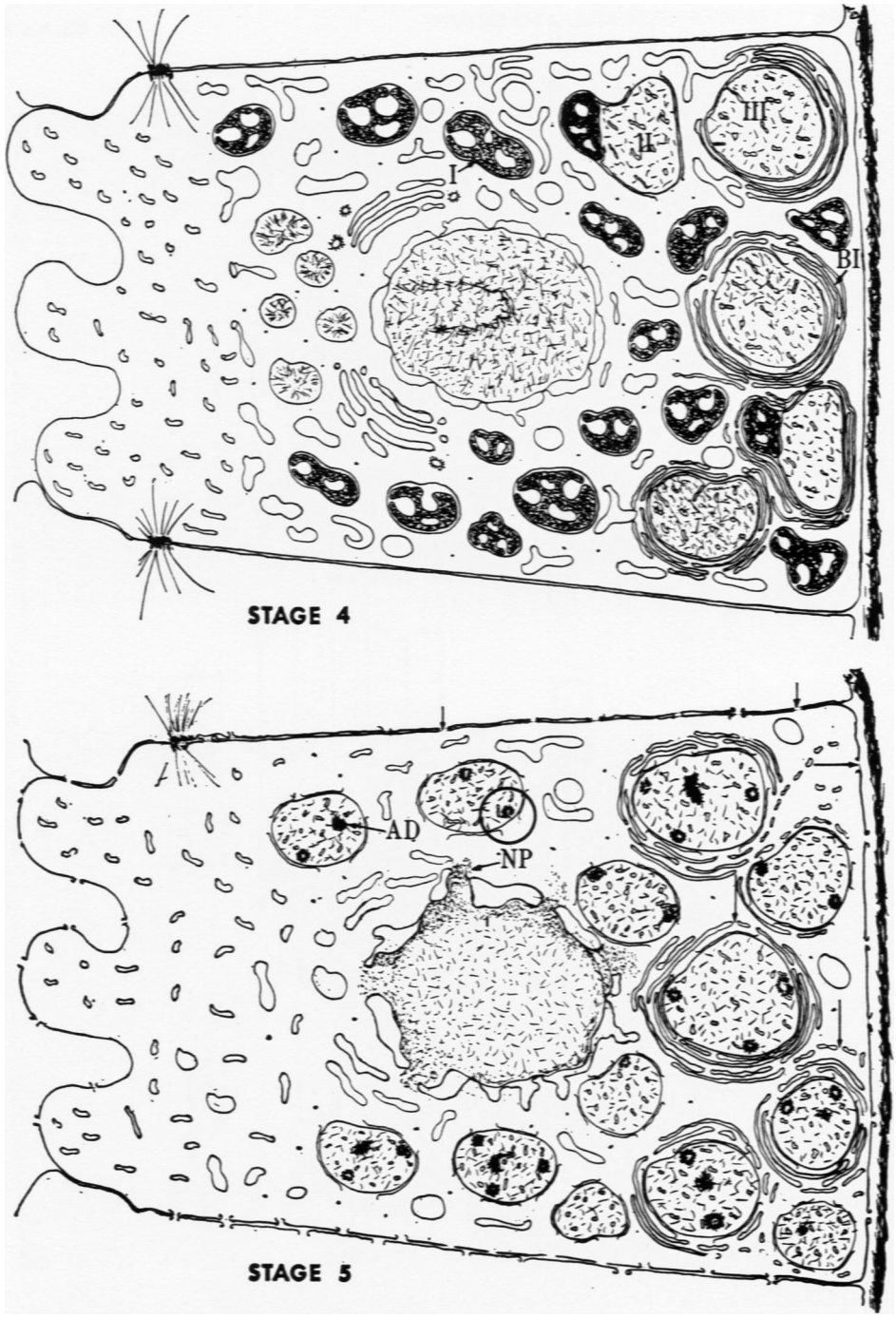
Light Microscopic and Functional Studies

The tubules responded in a similar fashion to all concentrations of ouabain studied; although there were indications of slightly greater effects at all time intervals with 10^{-3} M/L., this assertion could not be rigorously documented. Examination of the unfixed living tubules revealed marked changes in cell volume by 1 hr., at which time the cellular diameters were increased, and the luminal contours tended to be obliterated (Fig. 1 and 2). Cytoplasm was pale and filled with granular profiles, some of which apparently represented enlarged mitochondria. After the initial swelling of the tubules had occurred, there was little further change in the appearance of the tubules as seen by light microscopy.

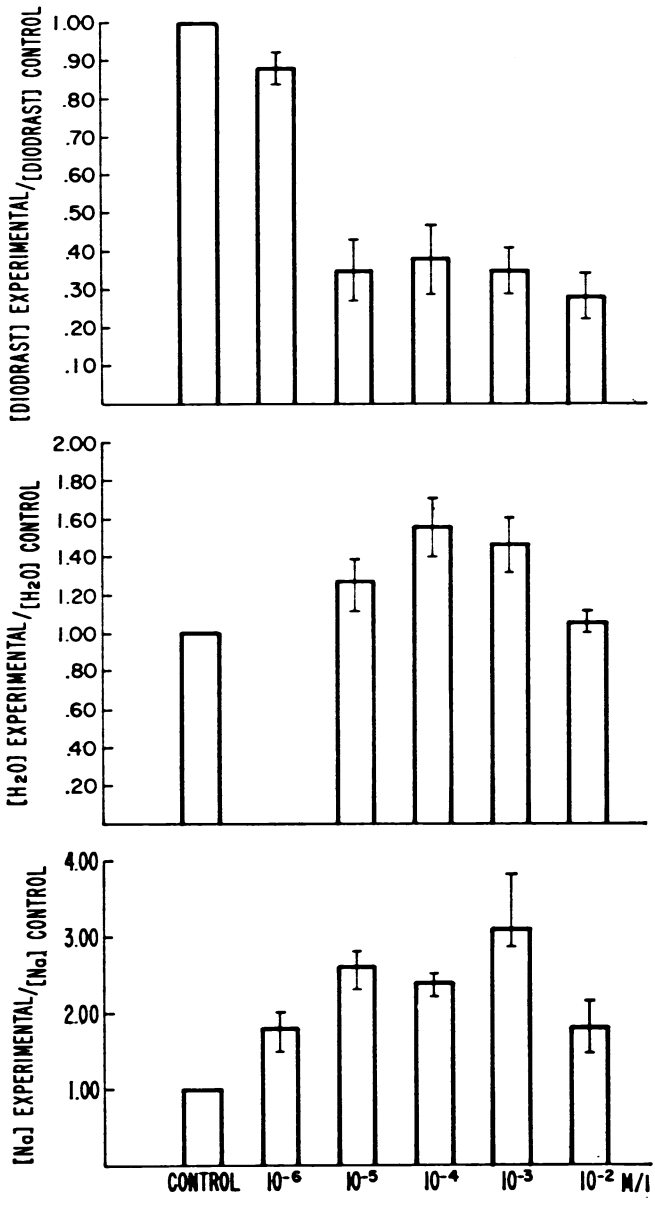
Examination of Epon sections by light microscopy permitted the easy identification of two general types of cells. One type had not deviated from normal and could include Stages 1 and 2 in the scheme presented



TEXT-FIG. 2. *Stage 2:* only change consists of dilatation of endoplasmic reticulum (*ER*) and nuclear envelope (*NE*). *Stage 3:* additional change consists of condensation of mitochondrial inner compartments with relative expansion of intracristal spaces and enlargement of cell sap, with distortion of brush border.



TEXT-FIG. 3. *Stage 4*, additional changes: Some mitochondria (*I*) are similar to those in Stage 3. Others (*II*) show condensed portion and portion in which matrix is greatly expanded. Some (*III*) show only expansion of inner compartment. Basilar infoldings often form circumferential wrappings around mitochondria. Polysomes are infrequent or absent. Lysosomes show pallor of internal content; nucleoplasm is pale and indistinct. *Stage 5:* all mitochondria show expansion of inner compartment, which contains two types of density: amorphous type (*AD*) and microcrystalline density in apposition to inner membranes (encircled areas). Interruptions occur in continuity of plasma membrane and basilar infoldings (*free arrows*). Nuclear pore (*NP*).



TEXT-FIG. 4. Water content, and uptake of Diodrast and of ^{24}Na by control and ouabain-treated tubule preparations. In each bar graph, results are expressed as ratio of experimental to control. In graphs for Diodrast uptake and water content, standard deviations are shown. In graph for ^{24}Na uptake, ranges of experimental values are shown. Values in these graphs represent typical experiments performed in triplicate.

above. The other type was distinctly abnormal, with swollen cell contours and obliterated tubular lumens. This is found in Stages 3–5, which are difficult or impossible to separate as observed by light microscopy. These studies indicated that at 15 min. most appeared unaltered; at 30 min. and 1 hr., approximately one-third of the tubules appeared swollen; and at 2 hr. or later intervals, essentially all of the tubules were swollen.

Studies of active transport using chlorphenol red indicated a depression of transport at all concentrations of ouabain examined and at all time intervals studied. Inhibition of Diodrast ^{131}I accumulation is shown in Text-fig. 4. Note the biphasic patterns of inhibition. A similar biphasic effect was noted in studies of amino acid incorporation into protein and for increased uptake of sodium and water (Text-fig. 4 and 5).

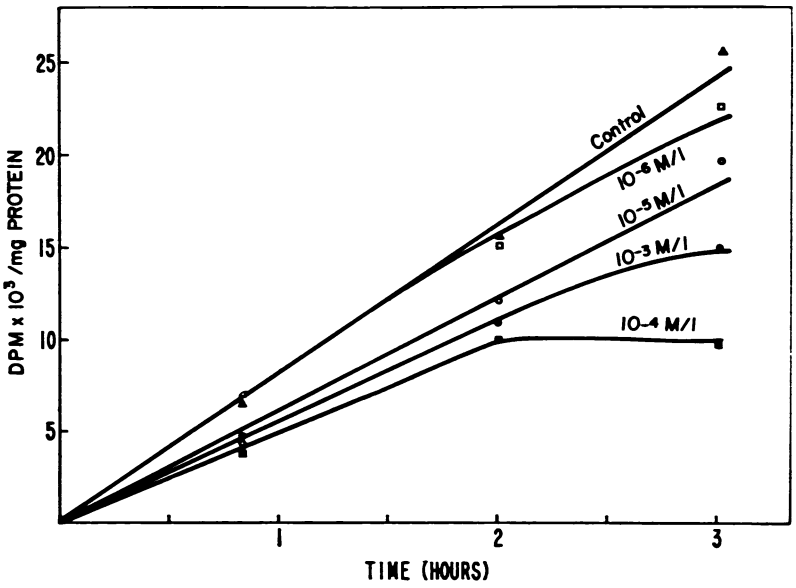
Incubation of tubules with ouabain in concentrations of 10^{-2} M/l and 10^{-3} M/l produced an immediate drop in rate of oxygen consumption to 46% and 64% of the control rate respectively (Text Fig. 6).

Electron Microscopy

The ultrastructural observations of tubules treated with ouabain are discussed in terms of the stages described above. At the completion of the electron microscopic portion of the study, it was evident that examples of tubules showing Stages 1–5 could be identified at nearly all combinations of ouabain concentrations and incubation times. It was, therefore, much more difficult to quantitate these changes in ultrastructure than the changes in functional parameters mentioned above. It was apparent from study of the electron micrographs and sections that at the initiation of incubation all tubules showed the appearance of Stage 1 (Fig. 3 and 4). Tubules incubated at the various ouabain concentrations for 1 hr. or less generally showed tubules in Stages 1, 2, and 3 (Fig. 5–7), whereas tubules incubated for 30 min. or less showed only Stages 1 and 2 (Fig. 5). Tubules incubated for 2 hr. or longer showed most tubules in Stages 4 and 5 (Fig. 8–12). It was thus evident that all changes occurring in tubules treated with ouabain could be described in terms of the above-noted stages, which are based on our previous study of cell swelling in high-potassium-containing mediums. See the figure legends for additional details of the observed ultrastructural changes.

Discussion

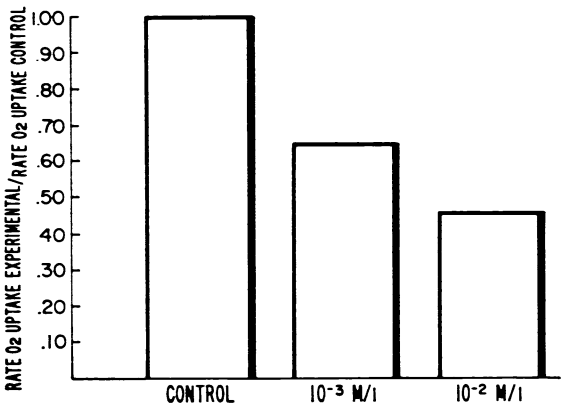
The experimental data presented above reveal the subcellular effects of inhibition of sodium extrusion at the plasma membrane. According to



TEXT-FIG. 5. Incorporation of labeled leucine into flounder tubule protein by control and ouabain-treated preparations.

current theory, cell volume regulation in vertebrates results from a balance between the rate of sodium diffusion into the cell and active sodium extrusion from the cell. Because of the presence of a higher intracellular than extracellular concentration of protein, the mammalian cell must control total cation content to regulate cell volume.[8] If such control mechanisms do not exist or are inhibited, the cells gradually increase in volume until the cell bursts or until its volume is constrained. This is the result of the Gibbs-Donnan effect.[9]

It has now been established in a variety of cell types that the cell exerts control over total cation content by possessing active transport mechanisms for sodium and potassium.[10] Such systems have vectorial properties—i.e., the sodium transport system pumps sodium from the intra- to the extracellular compartments, and the potassium transport system acts conversely. There are also good indications that the two pumps, at least in some systems, are coupled. This sodium-potassium pumping system is believed to be intimately related to, if not indentical with, a sodium-potassium-dependent, ouabain-sensitive adenosine triphosphatase (ATPase). Moreover, this sodium-potassium pumping system is evidently a rather delicate mechanism requiring a high-energy compound such as ATP or, perhaps, some other high-energy intermediate. Accordingly, the activity of the pump is highly dependent on the



TEXT-FIG. 6. Rates of oxygen consumption by control and ouabain-treated tubule preparations. Results for the two ouabain concentrations are expressed as ratio of experimental to control. Ratios represent rates of oxygen consumption during 5-min. experimental period following 2-min. control period. In other experiments, it was determined that rates of oxygen consumption for control preparations were linear for a 7-min. period.

supply of high-energy compounds resulting from synthesis during coupled respiration or glycolysis. Inhibition of the latter processes, therefore, results in inhibition of the pump. The pump is intimately associated with the plasma membrane of the cell. Accordingly, alterations which involve direct or indirect damage to the plasma membrane often result in an inefficiency of the pumping mechanism.

Diverse types of lethal and sublethal injuries in vivo result in modification of intracellular systems upon whose activity the sodium pump is dependent. Such modifications include, for example, the peroxidation of membrane lipids that occurs following the administration of certain chemical toxins,[11] the inhibition of mitochondrial function resulting from anoxia [12] or from other chemical toxins,[2] and the direct modification of the plasma membrane that occurs after chemical or physical attack.[13] It should be pointed out that most types of in-vivo injuries that occur to the cell as a part of human disease processes involve, at some stage of their development, modification of the sodium pump and, accordingly, loss of volume control, with cellular swelling occurring in an isosmotic extracellular fluid. Because of the common occurrence of such phenomena, and because many of the subcellular changes that have been observed after injuries in vivo or in vitro in both human and experimental disease involve differential expansions of intracellular compartments, it is possible that such morphologic changes in the various cell compartments might be a direct result of modification of plasmalemmal activity. More specifically, these changes may be due to loss of the cell volume control mechanisms.

We were therefore prompted to begin a rather systematic exploration of the various parameters involved in cell volume regulation and to compare the ultrastructural and functional effects observed in the experimental systems with those changes that have been well characterized as parts of in-vivo disease, both in the human and in the experimental animal.

In this report, the first of a series, we are concerned with the result of directly attacking the membrane-associated sodium-potassium-dependent ATPase by exposing cells to the cardiac glycoside ouabain.

It was striking to observe in our study that treating cells with ouabain produced a progressive pattern of change which closely mimicked the intracellular changes that occur after experimental injury in vivo. For example, following the administration of carbon tetrachloride to a rat, necrosis of the centrilobular hepatic cells occurs. The earliest lesion involves a dilatation of the endoplasmic reticulum, which is associated in that case with dispersal and detachment of polysomes.[14] This is followed at later intervals by changes in other organelles, notably the mitochondria.[15]

In our experiments, it was possible to define four stages, which began with change confined to enlargement of the endoplasmic reticulum, and ended with expansion of all cell compartments, distortion of cell membranes, and lysis of the nuclei.

We suggest that the sequence of alterations was the result of inhibition of the sodium-potassium exchange mechanism at the cell surface. This would be followed by influx of sodium, chloride, and water, and by loss of potassium. These changes would result in gradual expansion of intracellular volume. The progression of morphologic alterations suggests that this expansion occurs in different intracellular compartments at different times, resulting in the graded progression illustrated above. Because the first compartment to enlarge is the endoplasmic reticulum, it may act as a sink for the influx of sodium, chloride, and water. This concept would imply that the sodium extrusion mechanism from the lumen of the endoplasmic reticulum was less functional than those in the plasmalemma. Previous studies have shown that dilatation of the endoplasmic reticulum is a nonlethal alteration.[16] In other systems, enlargement of this compartment also occurs in conjunction with increased cell sodium and water and decreased cell potassium.

The next stage involves expansion of the cell sap and relative enlargement of the outer mitochondrial compartments. The mitochondria appear quite differently than in control tissues, having a very contracted matrix and expanded intracristal and envelope spaces. Enlargement of

the cell sap probably reflects influx of sodium and water into this compartment which, according to the hypothesis above, would imply the presence of more sodium and water than can be handled by the endoplasmic reticulum.

The striking appearance of the condensed mitochondria in Stage 3 is of interest. These mitochondria showed apparent condensation of the matrix compartment with relative expansion of the outer compartment. Our results indicate that this is a transient stage. However, as in Stages 4 and 5, mitochondria with marked expansion in the inner compartment and transitional forms were observed. The details of the relationship between the structure and functional stage of mitochondria in the intact cell has not been elucidated. There are good data indicating that ouabain in the concentration range used here depresses respiration in intact cells incubated in mediums with sodium concentrations approximating those of Forster's buffer.[17-19] This has been interpreted on the basis of the work of Chance and Williams [20] and of Chance and Hess [21] as indicative of reduced concentrations of intracellular ADP resulting from ouabain inhibition of plasmalemmal ATPase.

In our experiments, addition of ouabain resulted in rates of oxygen consumption that were from 46 to 64% that of the control, untreated tubules. Our results are similar to those of Whittam and Willis [18] on rabbit kidney slices and to those of Martin and Diamond [17] on rabbit gallbladder preparation. Whittam and Willis observed a drop in the rate of oxygen consumption following the addition of 0.625×10^{-3} molar ouabain to 58% of the control value, and Martin and Diamond observed a drop to 57% of the control value after adding 1×10^{-3} molar ouabain.

Hackenbrock [22] has studied the relationship between morphology of isolated mitochondria and the so-called respiratory steady states of Chance and Williams.[20] Hackenbrock's data indicate that mitochondria exist for relatively long periods of time with a similar condensed configuration only in respiratory Stages 2 and 3, both of which have high ADP levels. Thus, it would appear that the condensed configuration of mitochondria observed in our experiments cannot be explained by Hackenbrock's studies, even when his data are combined with the results of the effects of ouabain in intact cell respiration. It may be that the condensed configuration has additional meanings to those suggested by Hackenbrock's study—i.e., that several types of condensed mitochondria can occur, which, though structurally similar, have different functional meanings, or that additional control mechanisms not involving ADP are of importance in the intact cell. It does not appear that

ouabain itself produces these changes in mitochondria, since addition of ouabain to isolated mitochondria has little or no effect.[19] Accordingly, it would seem more likely that whatever the explanation for the condensation, it must relate in some way to changes in the intracellular environment.

As predicted by the studies on the effect of ouabain on cellular respiration, Minakami, Kakinuma, and Yoshikawa[23] observed that ouabain prevented the increase of ADP produced by potassium stimulation of respiration in brain slices.

This stage (Stage 3), with expanded cell sap and contracted mitochondria, has been observed also in other systems such as cooled rat kidney slices[24] in which sodium extrusion is also inhibited. In that study, it was noted that cells showing these changes can undergo reversible alteration and active organic acid transport.

In Stages 3 and 4, the inner mitochondrial compartments are expanded; the various cellular membranes are disrupted; and karyolysis occurs. These changes are probably irreversible. Essentially identical patterns of change have been noted after the point of no return in flounder tubules subjected to anoxia or treated with cyanide.[2,25] Essentially similar patterns were seen in liver and kidney tissues after lethal cellular injury in vivo in both human disease and in experimental situations.[25] It was interesting to note that the lysosomes within the flounder tubules did not undergo lysis until this stage, again suggesting that their lysis is a result, rather than a cause, of irreversible change within the cell.

Two types of mitochondrial deposits were observed in Stages 4 and 5. The first was a flocculent type of deposit which had been observed previously in various situations associated with lethal injury. It appears that this type of deposit occurs in irreversibly altered mitochondria; although its chemical composition is presently unknown, this deposit appears to typify the mitochondria of necrotic cells. We have given elsewhere our reasons for believing that such deposits may represent denatured mitochondrial proteins.[25] The other types of deposits consisted of microcrystalline deposition within the mitochondria. These were essentially identical with the depositions observed in calcium-loaded mitochondria in vitro[26] or in vivo,[15,27] and, accordingly, we conclude that these represent calcifications in the present situation also. It is of interest to consider the pathogenesis of these deposits which were not observed in our studies of cyanide-treated flounder tubules. While the pathogenesis of these intramitochondrial calcifications is not clear at the present time, other experiments reported in the literature

strongly indicate that treatment of kidney or brain cells with ouabain results in an intracellular influx of calcium.[28,29] Since the mitochondria have been demonstrated to be avid accumulators of calcium in vitro, and since calcification within mitochondria occurred in the present experiments following inhibition of the plasmalemma sodium pump, the mitochondria appear to be important sites for such accumulations of calcium following influx. It is also interesting to note that we did not observe such accumulations of calcium in flounder tubule mitochondria after treatment with cyanide.[2] This is in spite of the fact that cyanide treatment also is followed by inhibition of sodium extrusion. It is possible to explain this apparent discrepancy on the basis that cyanide probably also inhibits mitochondrial accumulations of calcium, as has been noted in the in-vitro loading systems.[30]

In the present experiment, treatment with ouabain was followed by inhibition of protein synthesis as measured by amino acid incorporation into protein. Because this was occurring at times when the cellular apparatus for protein synthesis was evidently intact, it would seem that this is not the result of gross modification of the system. On the other hand, there is much evidence suggesting dependence of protein synthesis on cellular potassium levels, and there are studies indicating that depletion of cell potassium is followed by inhibition of protein synthesis.[31] Because this is a well-known action of ouabain, and because the activity of the sodium pump was demonstrated to be inhibited in our experiments, this would appear to explain the protein synthesis inhibition.

The inhibition of active organic acid transport, as measured by Diodrast accumulation, may also be related to the effects on cell potassium. Burg and Orloff have noted that ouabain inhibits para-aminohippurate accumulation by rabbit kidney slices.[32] It has been demonstrated by Forster and Taggart [33] and by Puck, Wasserman, and Fishman [34] that the initial stage of organic acid transport is dependent on potassium. Incubation of cells in potassium-free medium is followed by inhibition of this first step of transport from the suspending medium into the cells. It seems possible that the inhibition of Diodrast accumulation observed here is the result of this mechanism; however, since ouabain also inhibits respiration and Diodrast accumulation is respiration dependent, the relative importance of both effects cannot be presently stated.

These arguments are strengthened by a consideration of the interesting biphasic action of ouabain in this system. Similar biphasic effects on various parameters have been noted by other investigators, who have

studied various concentrations of ouabain on both isolated and intact cellular systems.[35–37] It has been suggested that this is related to a stimulation of ATPase at the higher concentration levels. It was noted that the water content and the rate of sodium accumulation, protein synthesis, and organic acid transport all showed biphasic affects.

Summary

Normal intracellular volume control is dependent upon a sodium-potassium-dependent, ouabain-sensitive, plasmalemma-associated ATPase. The subcellular effects of inhibition of sodium extrusion are similar to those of incubating cells in mediums in which the sodium has been replaced by potassium. In cells treated with ouabain, protein synthesis is inhibited, dye and Diodrast accumulation are depressed, and intracellular sodium and water are increased.

The first ultrastructural change is dilatation of the endoplasmic reticulum. Subsequent changes involve all cellular components, including the sequential development of three types of mitochondria. In its terminal state, the cell exhibits marked disruption of its cellular organelles and their interrelations, including frequent interruptions of membranes.

These observations emphasize the role of ion and water redistributions in the pathogenesis of subcellular reaction to lethal injury and indicate that inhibition of sodium transport at the cell membrane is followed by a sequence of intracellular compartment changes resembling those that occur in lethally injured cells in vivo.

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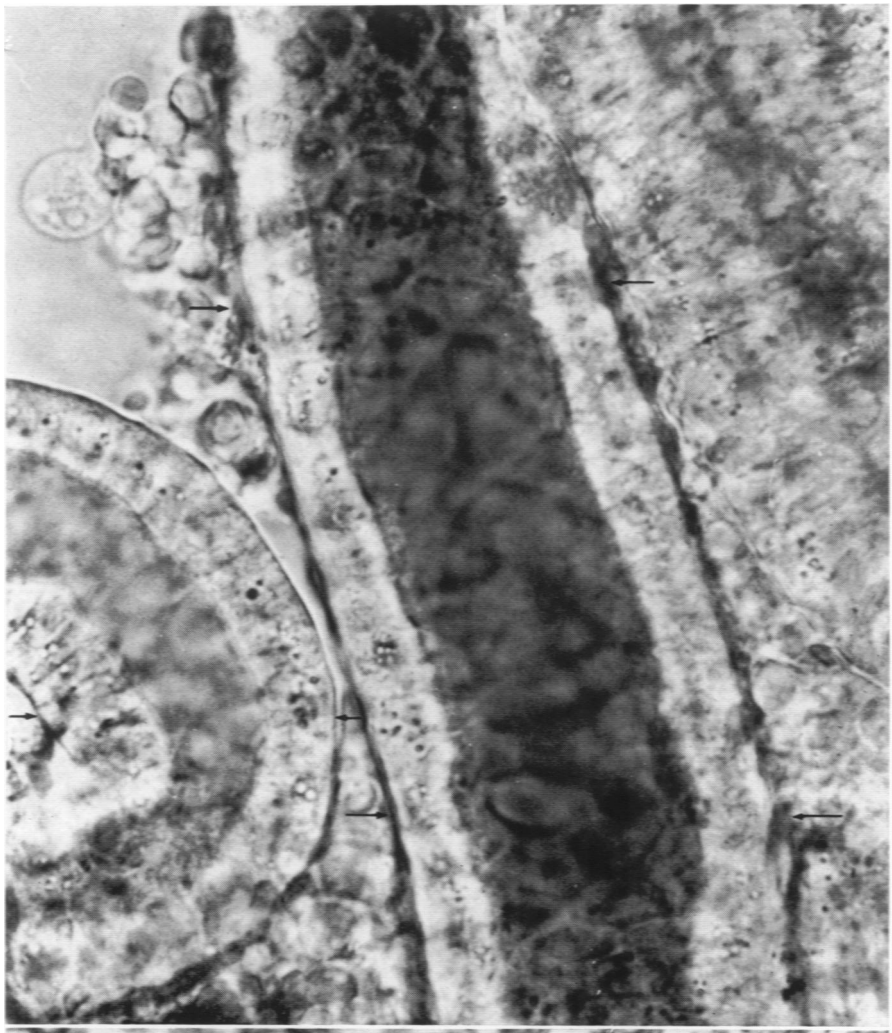
Legends for Figures

Fig. 1. Light micrograph of living control tubule incubated in dye-containing medium for 6 hr. Configuration of tubule and cells can be seen. Note high concentration of chlorphenol red in tubular lumen indicated by the dark zone, which was magenta in original preparation. Apposing arrows indicate diameters of tubules from basement membrane to basement membrane. × 960.

Fig. 2. Light micrograph of preparation similar to that in Fig. 1, in which the tubule has been incubated in ouabain (10^{-3} M/L.) for 30 min. Note that tubular lumen is obliterated, and total diameter of tubule increased. No tubular transport of chlorophenol red was noted. Luminal obliteration largely results from massive swelling of tubular epithelial cells, which have a coarsely granular apperance, in contrast to control preparation shown in Fig. 1. × 960.



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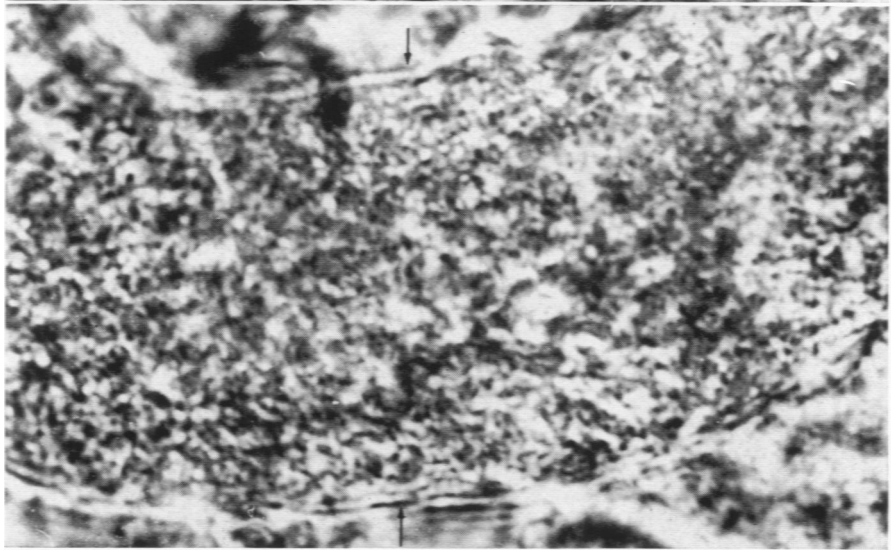

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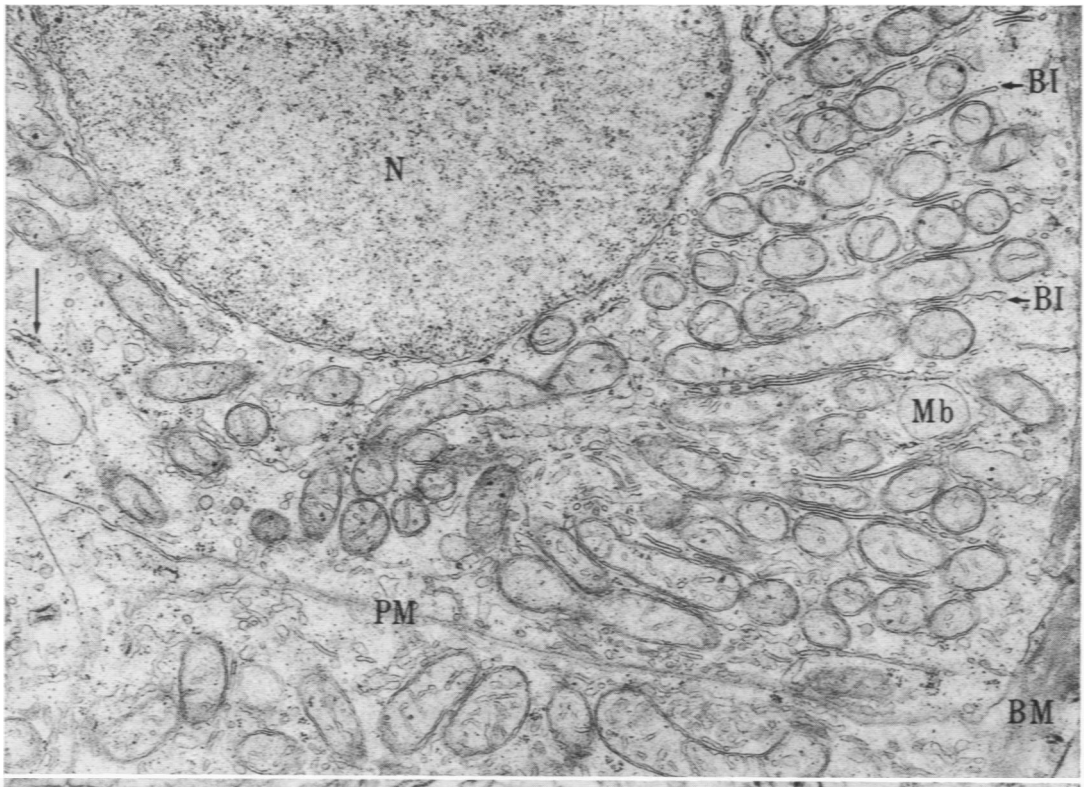
Fig. 3. Control tubule: middle and basilar portions of the cell are shown. Basement membrane *(BM)* and nucleus *(N)* are seen. Note appearance of mitochondria and endoplasmic reticulum *(free arrow)*. Note also the lateral plasma membrane *(PM)* and basilar infoldings *(BI)* of plasma membrane, which are in proximity to mitochondria. Microbody *(Mb)*. × 18,000.

Fig. 4. Higher magnification of portion of control tubular cell showing nucleus *(N)*, rough- and smooth-surfaced endoplasmic reticulum *(free arrows)*, mitochondria, and a lysosome *(L)*. × 60,000.



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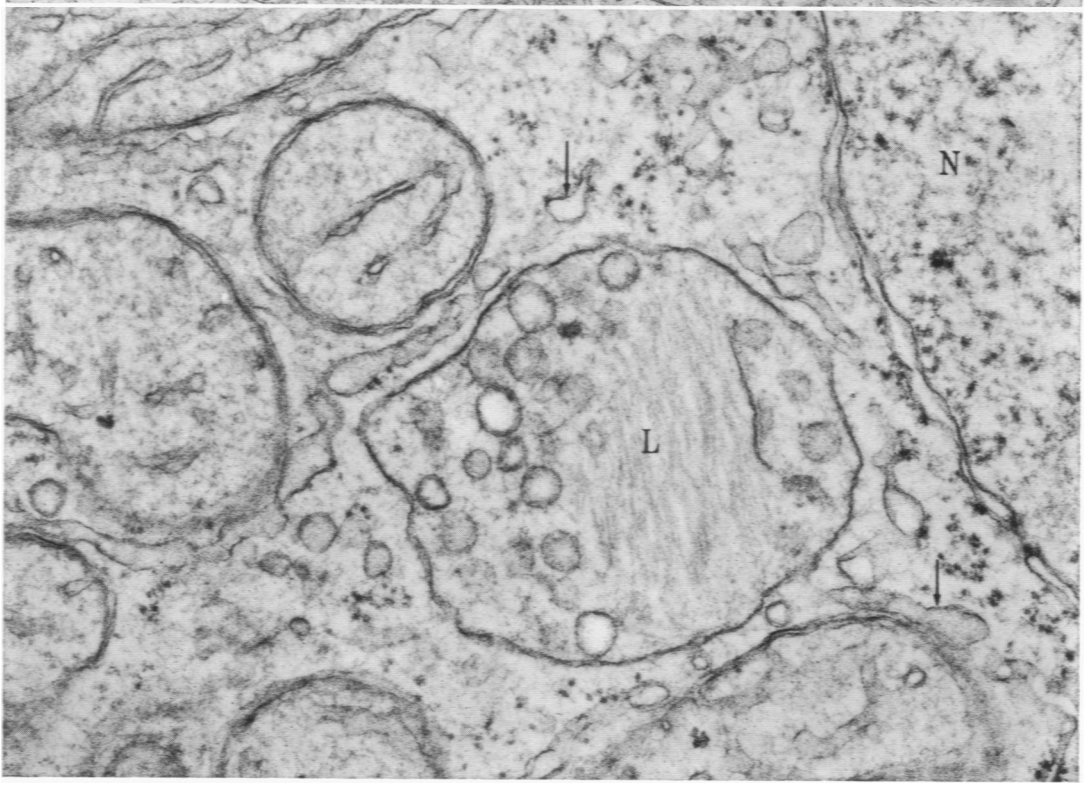

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Fig. 5. Ouabain, 10^{-4} M/L., for 30 min. Tubular cells showing changes characterized as Stage 2. Only alteration consists of dilatation of cisternae of endoplasmic reticulum *(ER)*. Basilar infoldings *(BI)*, mitochondria, polysomes *(Po)*, and cell sap appear unaltered. × 42,000.

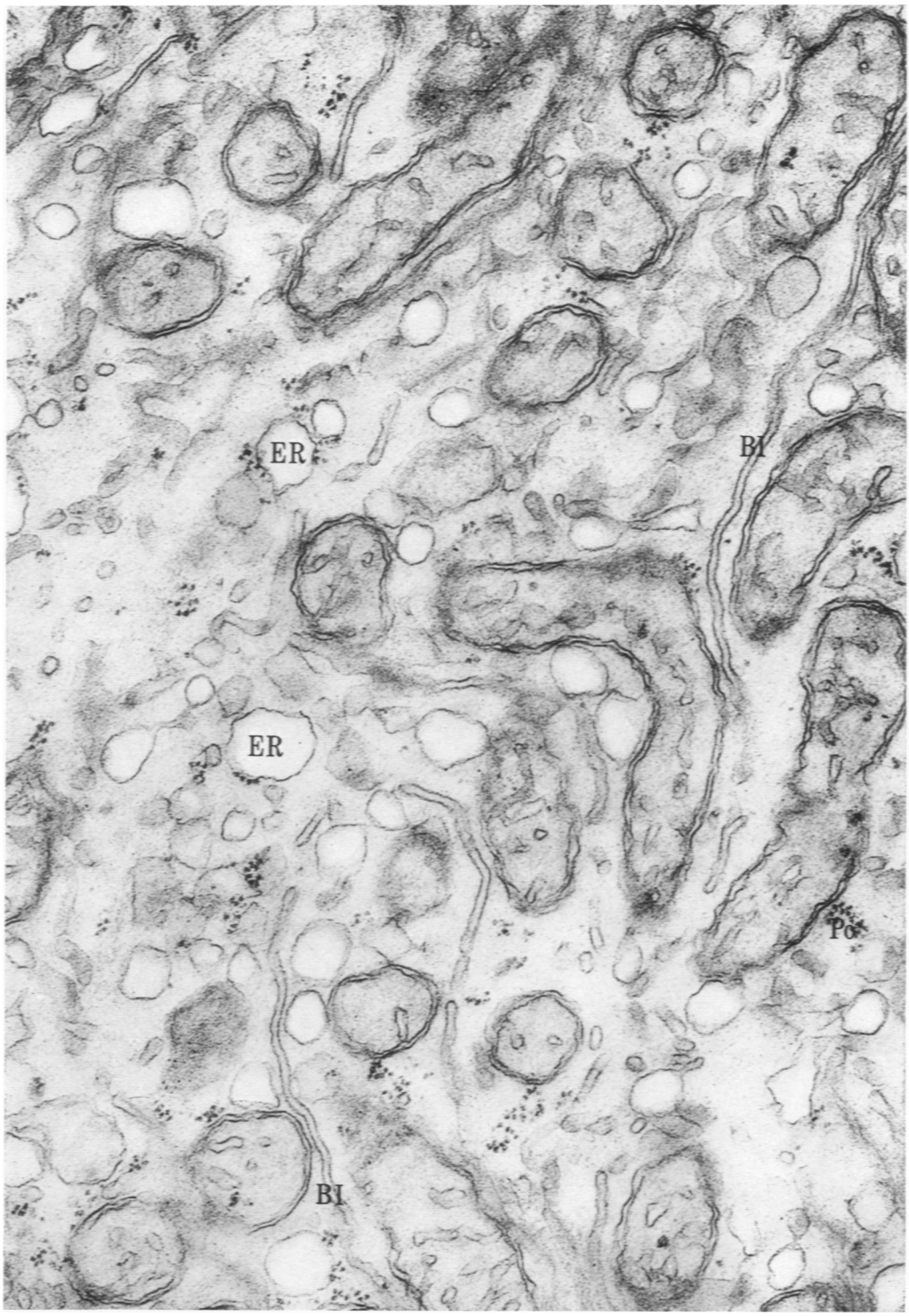

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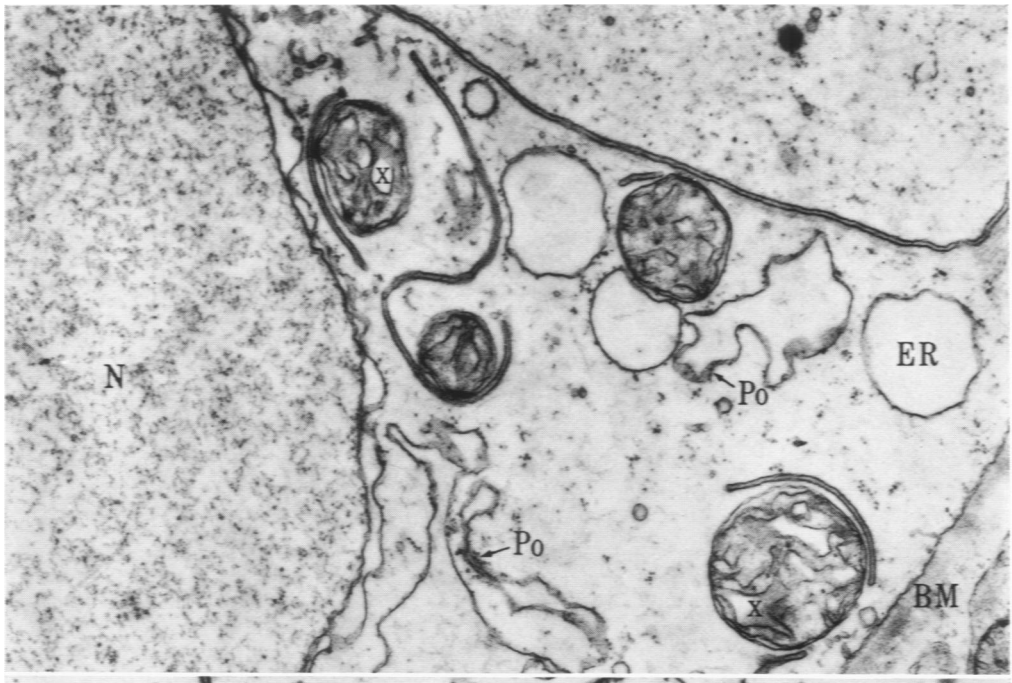
Fig. 6. Ouabain, 10^{-3} M/L., 1 hr. Tubular cells showing alterations characterized as Stage 3. Mitochondria show condensation of matrix compartment with relative enlargement of intracristal spaces *(X)*. Cisternae of endoplasmic reticulum *(ER)* are dilated, and cell sap is greatly expanded. Intact polysomes *(Po)* can still be identified. Basement membrane *(BM)*; Nucleus *(N)*. × 24,000.

Fig. 7. Ouabain, 10^{-3} M/L., 1 hr. Higher magnification of mitochondria in cells showing alterations specified as Stage 3. Note condensed matrix and expanded intracristal space. Dilated cisternae of endoplasmic reticulum *(ER)*; nucleus *(N)*. × 30,400.



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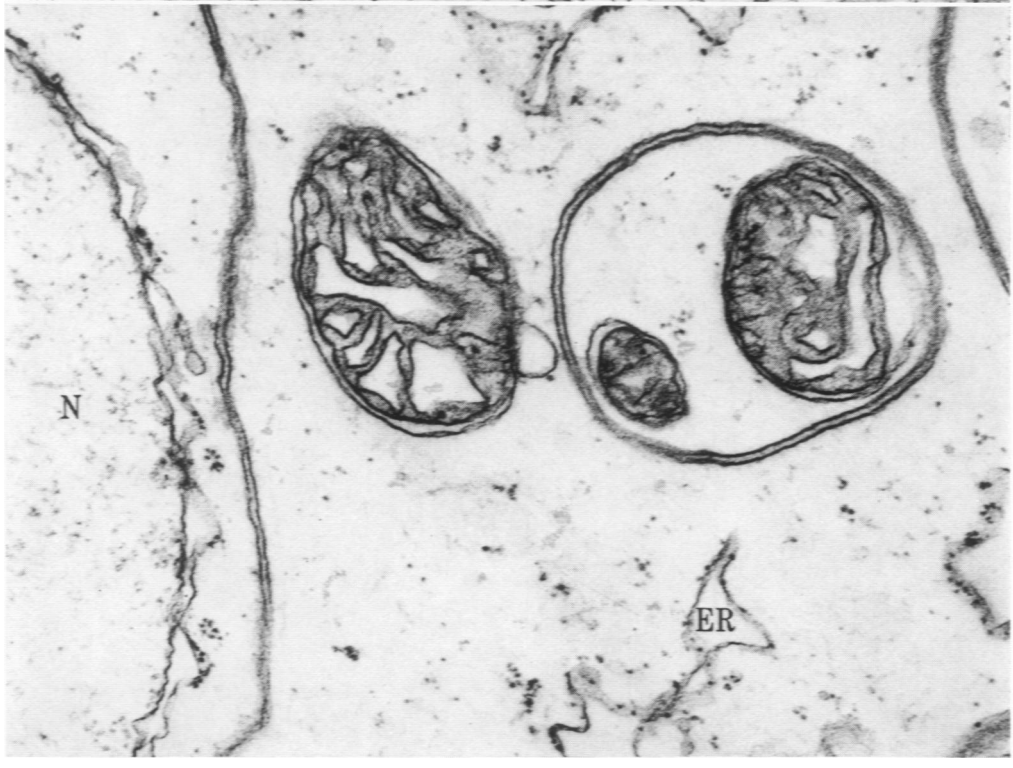

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Fig. 8. Ouabain, 10^{-5} M/L., 4 hr. This tubular cell shows changes typical of Stage 4. Alterations of all organelles are seen. The most striking feature of this stage is the three types of mitochondrial profiles observed. The first type *(I)* is similar to that observed in Stage 3, with condensation of matrix and expansion of intracristal spaces. Mitochondrial profiles of Type II show two types of matrical appearance: upper portion of mitochondrion *(II)* exhibits changes similar to that in Type I mitochondria. Most of the profile, however, shows great expansion of matrical space with inconspicuous cristae and flocculent material within the matrix, thus resembling Type III. This area is separated from contracted portion by a double-walled septum *(free arrow)* extending across the profile; this septum is continuous with inner membrane of envelope as indicated by *double arrow.* Mitochondrion of third type *(III)* demonstrates massive expansion of matrical compartment, which shows absence of matrical granules and a sparse flocculent material. Cristae appear as circular or tubular profiles and are widely separated. Nucleus *(N)* becomes very pale in this stage and shows great irregularity. At lower left is an irregular nucleus that stretches from left to right across the figure. Scattered throughout the cytoplasm are profiles of endoplasmic reticulum *(ER),* which are dilated and devoid of attached ribosomes. A few circumferential wrappings of basilar plasma membranes *(BI)* around mitochondria can be seen. × 80,600.

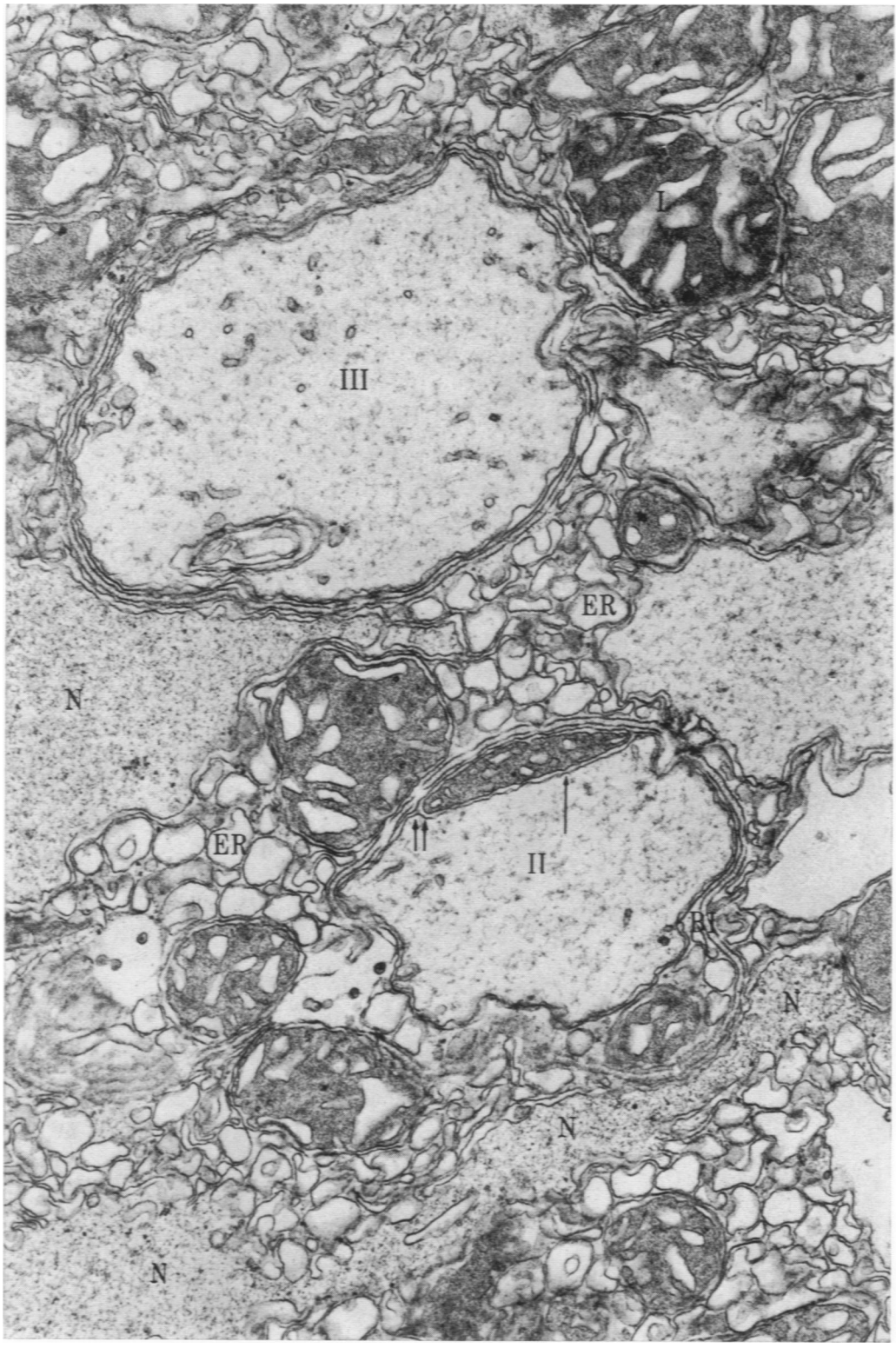

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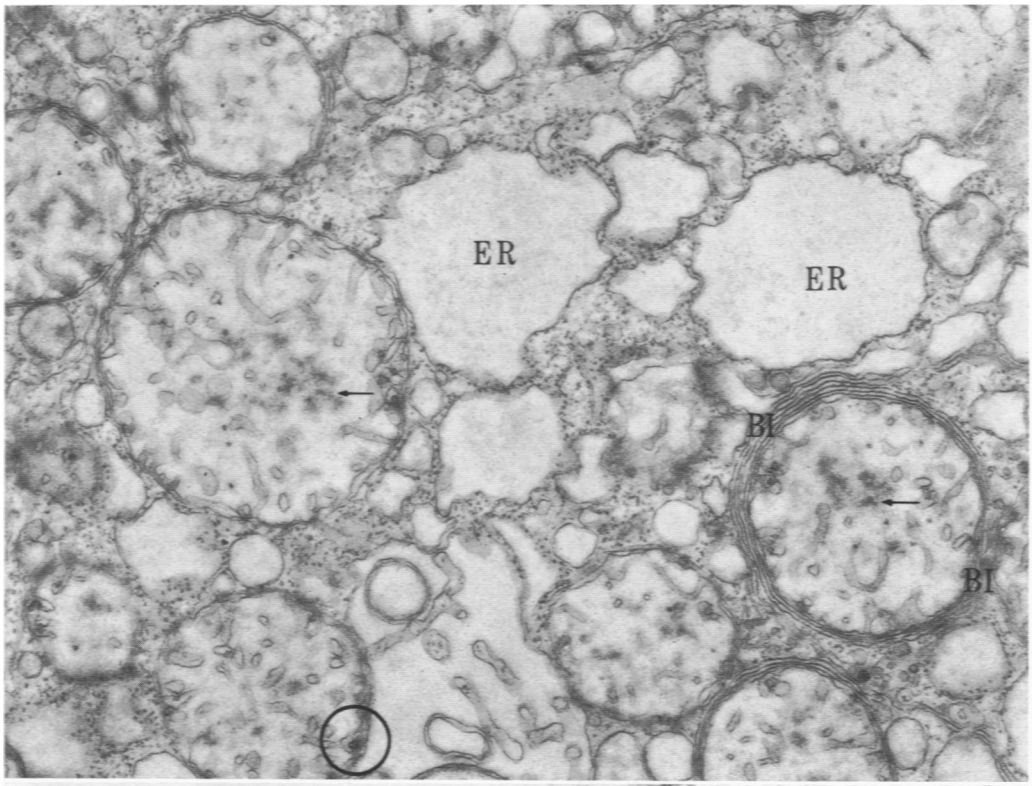
Fig. 9. Ouabain, 10^{-3} M/L., 2 hr. Portion of tubular cell showing changes corresponding to Stage 5. Mitochondria show marked swelling of matrix compartment. Cristae are widely separated and composed of irregular profiles. Matrix contains two types of deposits: (1) irregular flocculent aggregation *(free arrow)* possibly derived from flocculent material seen in Fig. 8; (2) that composed of small microcrystalline aggregates (in encircled area and elsewhere in mitochondria), which typically begin near inner membrane of envelope and always in proximity to cristae to which they are often closely applied. Cisternae of endoplasmic reticulum *(ER)* are widely dilated. Many ribosomes appear to have detached from surface of endoplasmic reticulum; those that are still attached appear to be randomly disposed. Circumferential wrappings of basilar infoldings *(BI)* surrounding mitochondria can be seen × 15,000.

Fig. 10. Ouabain, 10^{-5} M/L, 4 hr. Higher magnification of mitochondria from cells showing changes of Stage 5. Note appearance of microcrystalline aggregates *(Mc)*, composed of a series of dense particles arranged in a cluster. They are in an area bounded by inner membrane with which particles are in contact and are often embraced by cristae which are tightly apposed to the aggregates *(free arrows)*. Massive dilatation of nuclear envelope *(NE)* is also seen. × 48,500.



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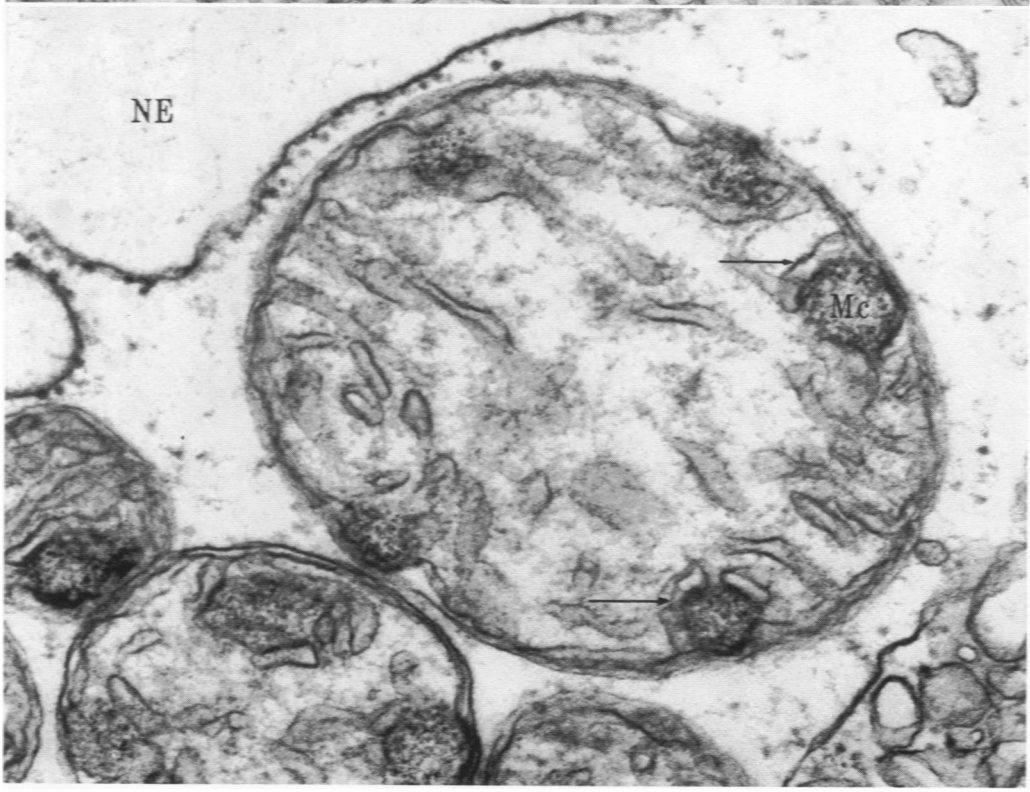

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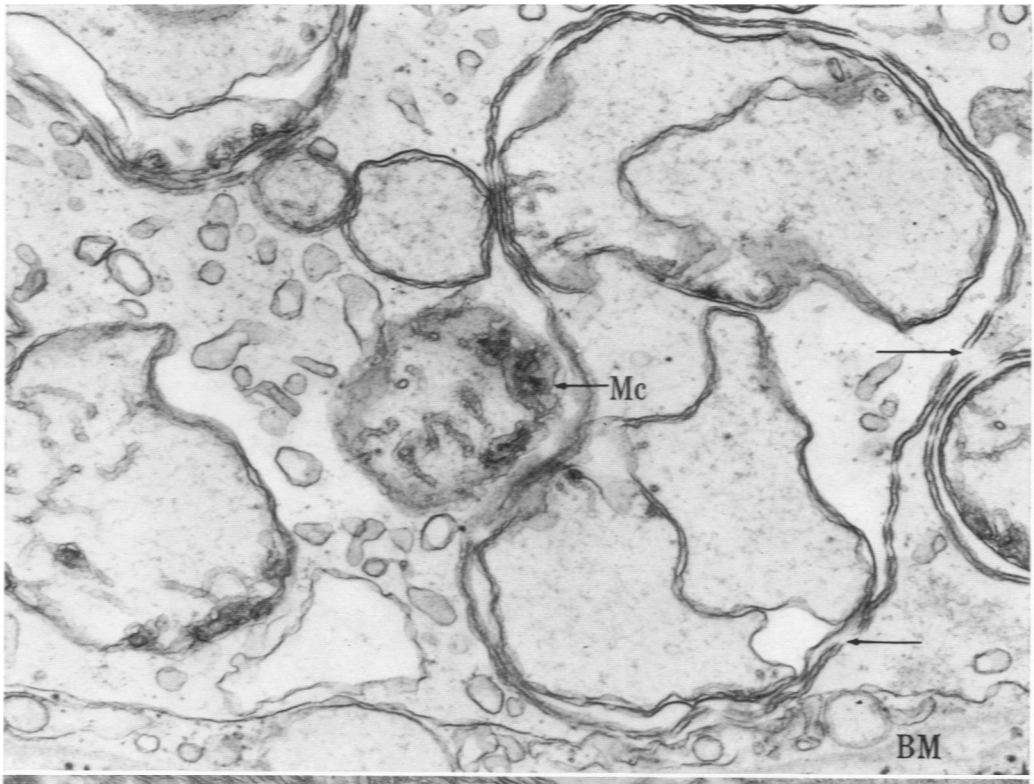
Fig. 11. Ouabain, 10^{-3} M/L., 4 hr. Base of tubular cell showing alterations characteristic of Stage 5. Mitochondria show massive enlargement of matrical compartment and a few microcrystalline densities *(Mc)*. In one mitochondrion, double-walled septum extends across profile and is continuous with inner membrane. Interruptions in continuity of basilar infoldings are indicated by *free arrows*. Cisternae of endoplasmic reticulum are dilated and are largely free of ribosomes. Cell sap is expanded and pale. Basement membrane *(BM)*. × 54,000.

Fig. 12. Ouabain, 10^{-3} M/L., 2 hr. Basilar region of tubular cell showing prominent matrical swelling of mitochondria and numerous circumferential wrappings of basilar infoldings. Nucleus *(N)*; basement membrane *(BM)*; endoplasmic reticulum *(ER)*. × 29,000.



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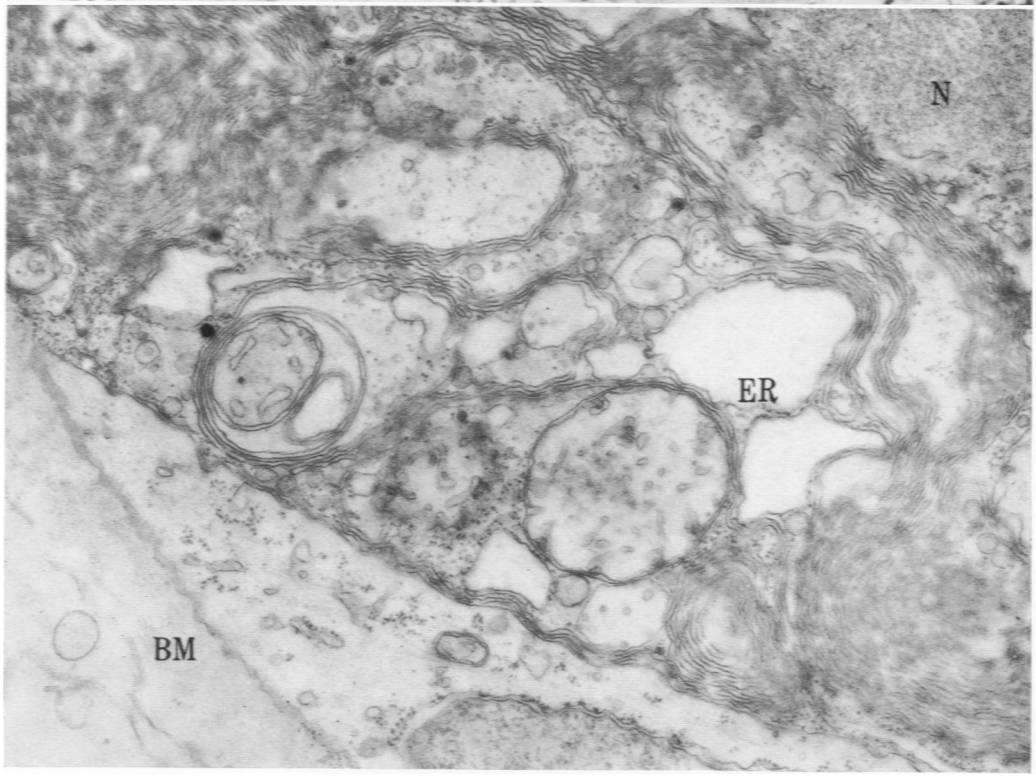

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