USE OF POTASSIUM PYROANTIMONATE IN THE LOCALIZATION OF SODIUM IONS IN RAT KIDNEY TISSUE

RUTH ELLEN BULGER

From the Department of Pathology, School of Medicine, University of Washington, Seattle, Washington 98105

ABSTRACT

Potassium pyroantimonate added to fixative solutions has been used in tissue localization of sodium ions. The distribution and specificity of the resulting precipitate in rat kidney is described in this study. Two reproducible patterns of precipitate were obtained in control tissues. The first pattern, which occurred after fixation in solutions containing aldehyde, showed the precipitate to be mainly extracellular. The second pattern, showing the precipitate in both intracellular and extracellular locations, occurred after aldehyde fixation in those experimental situations favoring cellular swelling or after fixation with solutions containing osmium tetroxide. It appeared that sodium ions could move after fixation but that sodium pyroantimonate precipitate could not. Since model systems demonstrated that dense precipitate formed when potassium pyroantimonate was added to solutions containing certain biological amines or some divalent cations, it appeared likely that the reagent did not provide specific tissue localization for sodium ions.

A reliable method for determining the location and relative concentrations of sodium ions in living tissue would be invaluable in studies of ion transport. The following characteristics would be required if such a technique were to be useful: (*a*) the capacity to precipitate sodium quantitatively and thereby fix the sodium ions in the tissue before the ions can move; (*b*) a known specificity for the sodium ion; (*c*) an ability to permeate the cell membranes and compartments; (*d*) the ability to form an electron-opaque final precipitate; and (*e*) the ability to form a precipitate that remained stationary during the subsequent preparatory techniques.

The technique for the localization of sodium ions, proposed by Komnick (8) and Komnick and Komnick (9), has been used in a variety of tissues by several authors (4, 6–9, 12, 14, 20).

This present report describes experiments designed to test various characteristics of the reaction between potassium pyroantimonate and cations in rat kidney. Considered in the investigation were the following items: (*a*) the effect of various fixatives; (*b*) the effect of blocking the ion pumping mechanism; (*c*) variations occurring with cellular swelling; (*d*) the possibility that sodium pyroantimonate precipitate could move freely in tissues; and (*e*) the effect on the potassium pyroantimonate reaction of pretreatment leaching of sodium from tissues.

MATERIALS AND METHODS

Kidneys were removed from female Sprague-Dawley rats weighing between 200 and 275 g. They were handled in one of the following two ways. (*a*) They were divided into five zones from the cortex to the papilla and chopped into small pieces; or (*b*) they were sliced on a Stadie-Riggs microtome and then

chopped into small pieces and placed in fixative solutions. The fixatives used were 2% osmium tetroxide unbuffered or buffered with potassium phosphate or Veronal-acetate, Karnovsky's fixative (5) diluted to half strength and buffered with potassium phosphate, or 4% or 6.25% glutaraldehyde buffered in 0.1 M potassium phosphate. 2% potassium pyroantimonate (K & K Laboratories, Inc., Plainview, N.Y.) was added to each of the fixatives. Some heating was required to facilitate dissolving of the compound. Adequate controls were run with fixatives free of pyroantimonate. Fixed tissues were quickly dehydrated in alcohols, embedded in Epon epoxy resin (13), cut with diamond knives, stained, and viewed in an RCA-3G or an AEI-6B electron microscope. Also, slices of kidney cortex cut on a Stadie-Riggs microtome were treated in the following ways before dehydration and embedding.

(*a*) The slices were allowed to swell for 1–4 hr in cold Robinson's buffer (17) or in cold Robinson's buffer in which all of the sodium ion had been replaced by potassium ion. (*b*) They were fixed for 4 hr in half strength Karnovsky's fixative buffered with potassium phosphate, then leached for 5 days in repeated changes of sodium-free medium, and subsequently placed in half strength Karnovsky's fixative buffered with potassium phosphate and containing 2% potassium pyroantimonate. (*c*) They were then fixed in half strength Karnovsky's fixative with potassium phosphate buffer and 2% *sodium* pyroantimonate which had been prepared from solutions containing sodium chloride and potassium pyroantimonate. (*d*) The slices were incubated at room temperature in Robinson's buffer (17) containing ouabain (1×10^{-4} M or 3×10^{-4} M) with or without oxygenation and (*e*) were then fixed for 4 hr in half strength Karnovsky's fixative and then fixed for an additional 4 hr in Karnovsky's fixative buffered with potassium phosphate also containing a 2% solution of potassium pyroantimonate.

In addition, 2% potassium pyroantimonate was added to an equal volume of the following solutions: 0.1, 0.01, 0.001, and 0.0001 M solutions of $CaCl_2$, $MgCl_2$, $BaCl_2$, and KCl; 1% histamine (Calbiochem, Los Angeles, Calif.); 1% L-histidine (Calbiochem); 1% serotonin (Calbiochem); 1% spermine (Calbiochem); and 1% L-arginine (Schwarz Bio Research Inc., Orangeburg, N.Y.).

OBSERVATIONS

Normal Kidneys Fixed with Solutions Containing 2% Potassium Pyroantimonate

Electron-opaque deposits were seen in kidney tissue after fixation in solutions containing 2% potassium pyroantimonate. Deposits varied in size and shape but were generally consistent in any given structure. The small ones were approximately 100 A, but deposits many times larger were seen. Under some circumstances the deposits formed a ring around cores of varying electron opacity. The number of electron-opaque precipitates varied from block to block and from region to region within the block.

The distribution of precipitate varied depending on whether the primary fixative contained aldehyde or osmium tetroxide. After fixation in aldehyde-containing fixative, two patterns of distribution were seen. One pattern was associated with cells which appeared to be free of cellular swelling, and the second pattern was associated with occasional cells that had morphologic evidence of cellular swelling, such as large rounded mitochondria with pale matrices or endoplasmic reticulum with expanded cisternae. After primary fixation in osmium tetroxide-containing fixatives, the deposits were similar to those seen in the regions of cellular swelling in aldehyde-fixed tissue. The pattern of distribution which will be described first will relate to areas where there were no morphologic alterations consistent with cellular swelling.

Ultrastructural preservation was similar in tissues placed in aldehyde fixative buffered with sodium phosphate buffer, potassium phosphate buffer, or potassium phosphate buffer containing 2% potassium pyroantimonate.

PROXIMAL TUBULE: Electron - opaque precipitates were seen within the lumen of the proximal tubule and along the surface of the microvilli (Figs. 1, 2). The particles were often adherent to the external surface of the plasma membrane. Precipitates were seen within the tubular invaginations at the base of the microvilli and in circular profiles in the apical cytoplasm (apical vesicles) (Fig. 2). When particles were seen within the apical vacuoles, they appeared to be associated with a layer of filaments on the luminal side of the membrane surrounding the apical vacuoles. Particles were heavily concentrated between the lateral cell membranes of adjacent cells from apex to base except for the short region of the tight junction adjacent to the tubular lumen. The amount and size of the precipitate varied even in the same tubule; some regions between the two cell membranes were filled entirely with a dense deposit. In general, deposits were larger in the lateral intercellular space than in the tubular

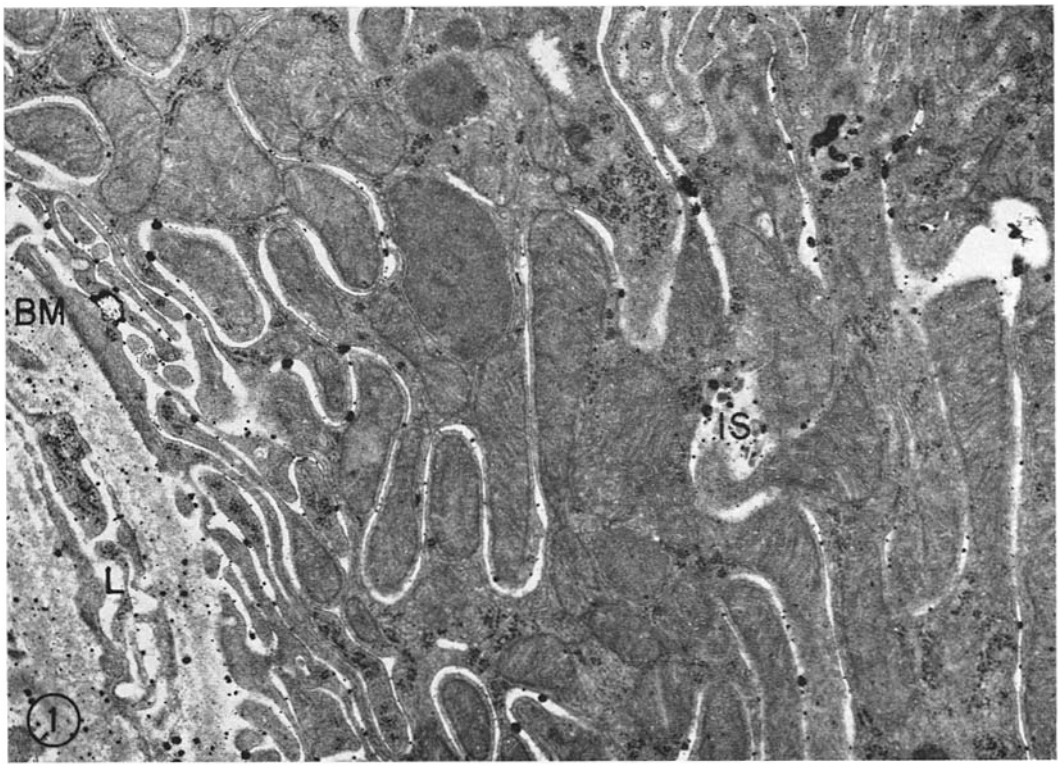


FIGURE 1 Electron micrograph of a section of proximal convoluted tubule showing the predominant pattern of distribution of the dense precipitate. Deposits can be seen between the apical microvilli, in the apical tubular invaginations, in the lateral intercellular space (*IS*), in the basement membrane (*BM*), in the interstitium, and in a capillary lumen (*L*). × 12,500.

lumen. The particles were frequently seen in close apposition to the external surface of the cell membrane, and in many instances the deposit entirely filled the area between the lateral membranes (Figs. 1–3). Particles were seen between the basal plasmalemma of the cell and the basement membrane, in addition to being concentrated within the substance of the basement membrane itself. Particles bound within the basement membrane were usually small. In most regions, the entire nucleus and cytoplasmic organelles were devoid of any dense deposits, although occasional flecks were seen.

In some tubules, evidence of swelling could be seen in occasional cells (Figs. 4, 5). The mitochondria appeared larger and rounded; cristae were rearranged, and the matrix was pale. These cells frequently showed dilated cisternae of endoplasmic reticulum. In addition to the pattern of precipitate deposition described above, these altered cells also contained precipitate in the matrix of the mitochondria, in the dilated cisternae of the rough and smooth endoplasmic reticulum, in microbodies, cytosomes, the cytoplasmic sap, and in the interchromatin space of the nucleus. These deposits, however, were never seen in the chromatin of the nucleus. Intermediate morphologic forms between the supposed nonswollen and swollen cells could occasionally be detected (Fig. 5). In these cases, the deposits were small in size and were located initially within the intracristal space, between the internal and external membranes of the mitochondria, and occasionally in microbodies. Small deposits in the intracristal space were seen in mitochondria showing only a slight decrease in matrix density. Larger intracristal deposits accompanied intracristal swelling. When the mitochondria showed matrical swelling, the deposits appeared in the matrix and consisted of a large, roughly spherical cloud of particles.

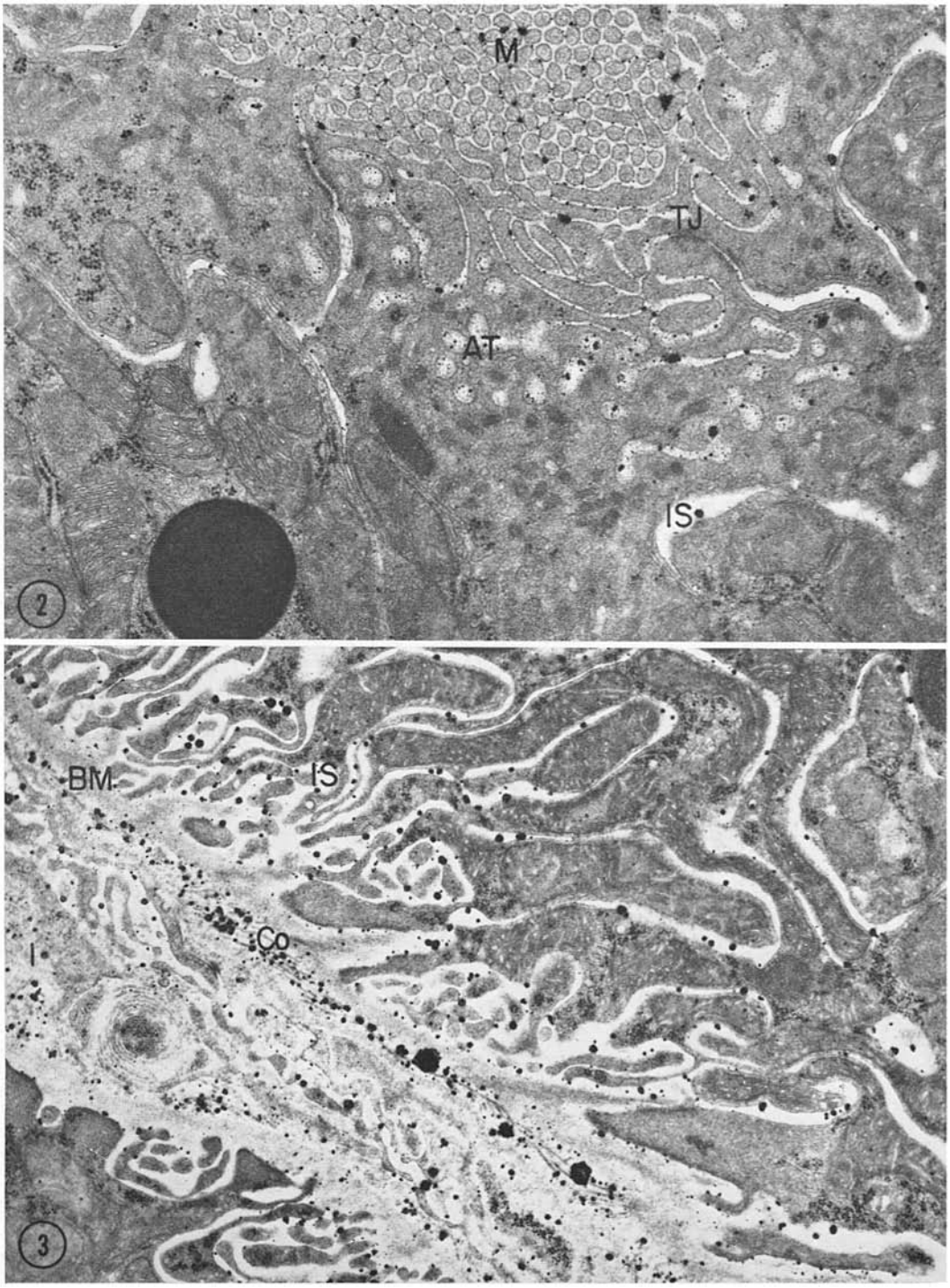


FIGURE 2 Electron micrograph showing the apical cytoplasm of cells of the proximal tubule from a normal animal fixed in Karnovsky's fixative containing 2% potassium pyroantimonate. Dense precipitate can be seen along the plasmalemma of the microvilli (*M*), in the lateral intercellular space (*IS*) below the level of the tight junction (*TJ*), and in the tubular invaginations (*AT*) and apical vesicles of the apical cytoplasm. × 25,800.

FIGURE 3 Electron micrograph showing the basal cytoplasmic region of a proximal tubular cell fixed in Karnovsky's fixative containing 2% potassium pyroantimonate. The electron-opaque precipitate can be seen in the lateral intercellular spaces (*IS*), in the basal cytoplasmic region, in the basement membrane (*BM*), and along the collagen bundles (*Co*) below the basement membrane, and in the interstitium (*I*). × 17,000.

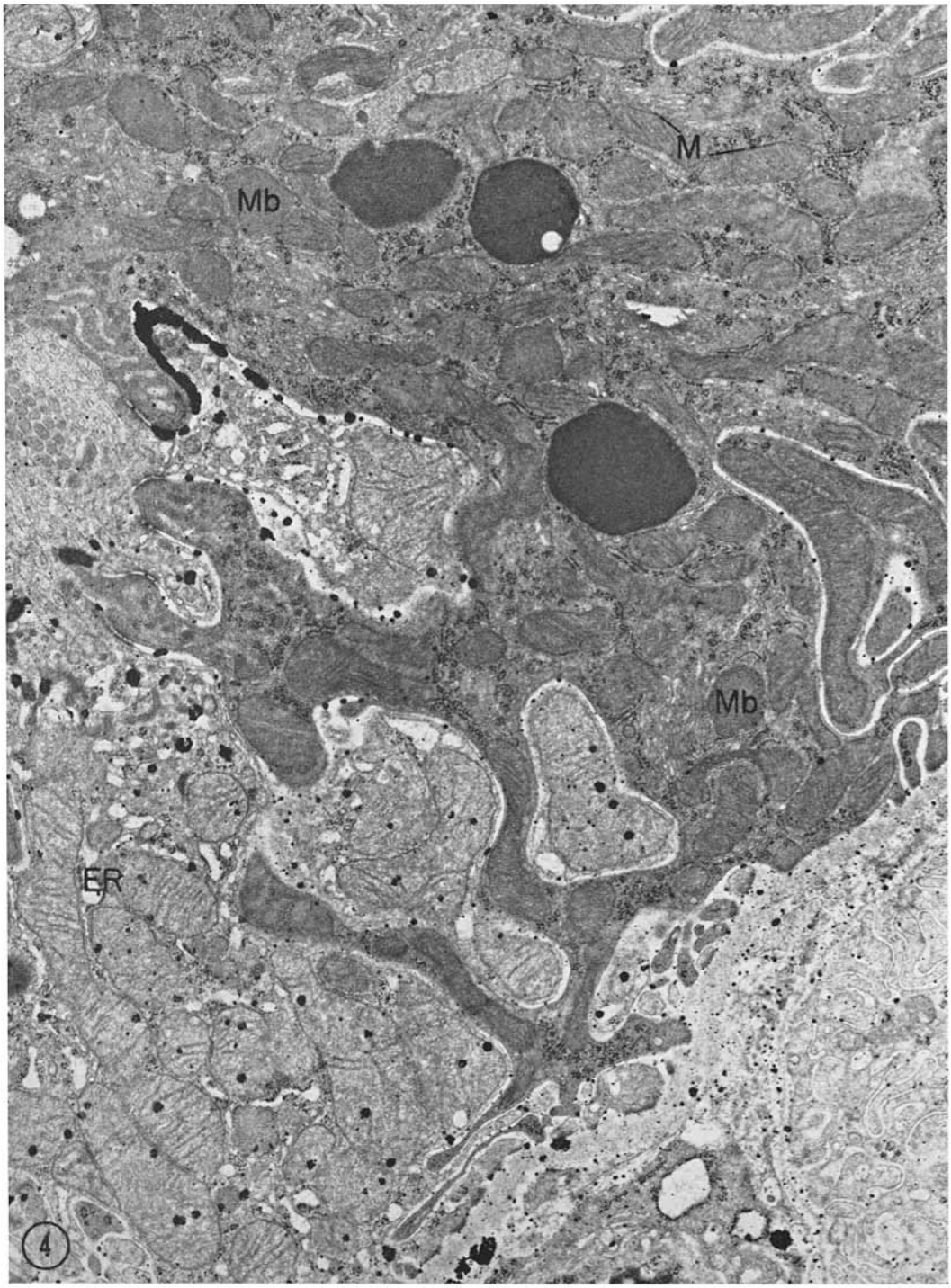
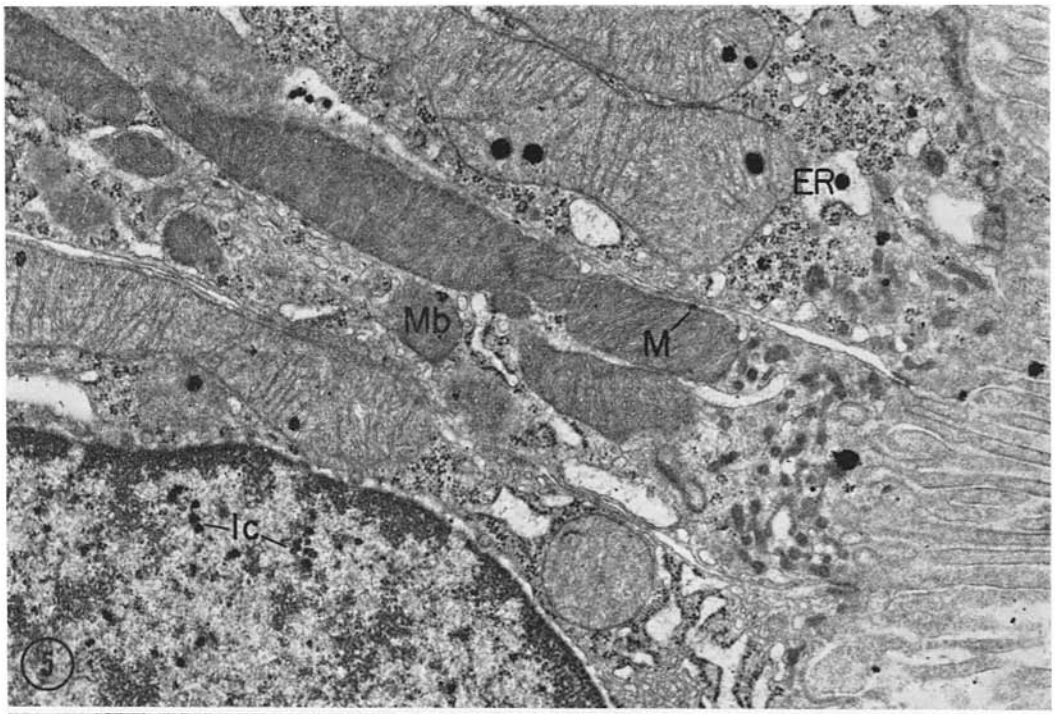


FIGURE 4 Electron micrograph showing interdigitations of adjacent proximal tubular cells from a rat kidney fixed in a solution containing 2% pyroantimonate. The cell at the lower left has large, rounded mitochondria with pale matrix material and an endoplasmic reticulum with dilated cisternae. The distribution of precipitate in this case is different from that noted in tissues which appear unswollen. This cell shows precipitate in the mitochondrial matrix, in the endoplasmic reticulum (*ER*), and in other organelles as well as in the sites found in cells showing no morphologic evidence of swelling. The cell in the middle of the field has a more normal appearance but does contain occasional dense particles between the inner and outer membranes of the mitochondria (*M*) and in microbodies (*Mb*). × 10,700.



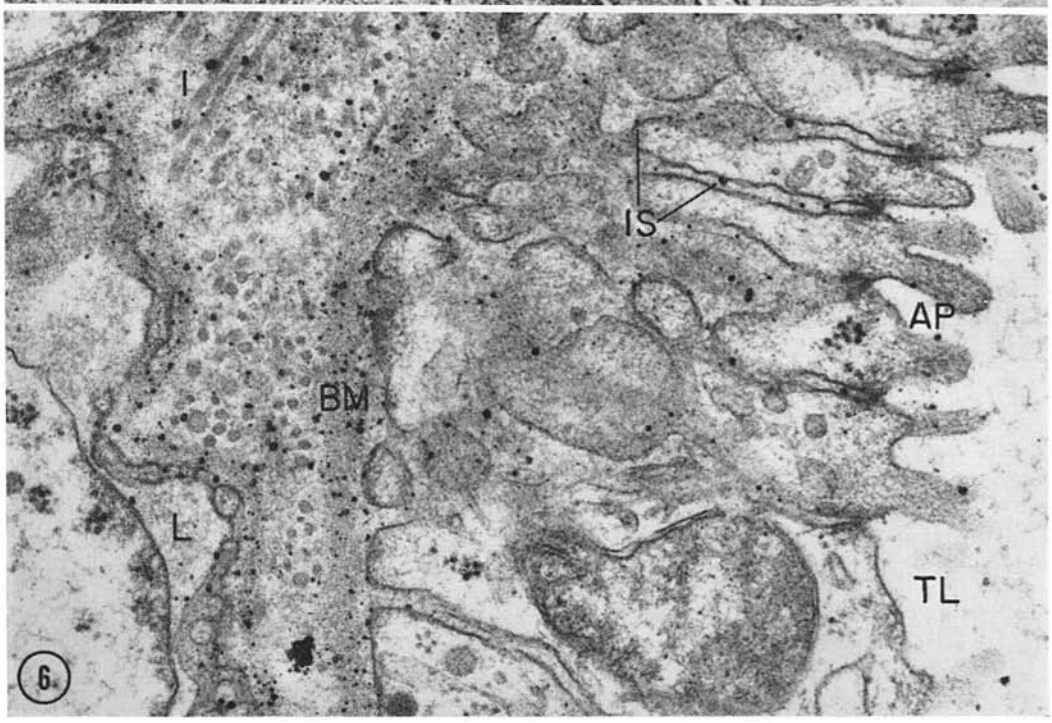


FIGURE 5 Electron micrograph showing a high magnification of the deposits in the proximal tubular mitochondria and in the endoplasmic reticulum (*ER*) of a cell which appears to be swollen. An adjacent cell has only occasional precipitates between the inner and outer membranes of the mitochondria (*M*) and in the microbody (*Mb*). Note the deposits in the interchromatin region of the nucleus (*Ic*). × 18,700.

FIGURE 6 Electron micrograph showing a region of the thin limb of Henle's loop from a normal animal fixed in Karnovsky's fixative containing 2% potassium pyroantimonate. Dense reaction products can be seen in the lumen of the tubule (*TL*), along the apical plasmalemma (*AP*), in the lateral intercellular spaces (*IS*), within the basement membrane (*BM*), in the interstitium (*I*), and in the capillary lumen (*L*). × 35,300.

Therefore, the size, configuration, and location of the deposits in mitochondria were related to the degree of morphologic change seen in the mitochondria.

When tissues were fixed in osmium tetroxide-containing solutions, localization of precipitate was similar to that seen in the swollen aldehyde-fixed cells.

THIN LIMB AND THICK ASCENDING LIMB OF HENLE'S LOOP: Although occasional dense precipitate was seen in the lumen, most of the particles appeared to be adherent to the outer layer of the plasma membrane or to the layer of filaments radiating from this membrane layer (Fig. 6). Particles were seen between the lateral plasmalemmas and between the smaller basal interdigitations. The precipitate was adjacent to the outer surface of the cell membrane in the lateral intercellular spaces. A dense precipitate could be seen between the basal plasmalemma and the basement membrane and throughout the basement membrane itself.

DISTAL TUBULE AND COLLECTING DUCT: Compared with the precipitate in the proximal tubule, larger particles of precipitate were seen in the lumen of the distal nephron (Fig. 7), usually in association with the apical cytoplasmic membrane. Although the number of electron-opaque particles was smaller than that seen in the proximal tubules, the distribution was similar. The particles were found between the lateral plasmalemmas below the region of the tight junction and between the basal interdigitations of adjacent cells. The rest of the organelles, the cytoplasmic ground substance, and the nuclei appeared to be free of any precipitate. Tubules that showed morphologic alterations consistent with cell swelling were seen rarely in our preparations, indicating that cells in this region of the nephron may be more resistant to swelling than are proximal tubular cells. Deposits were also seen within the substance of the basement membrane and between the basal plasmalemma and the basement membrane.

INTERSTITIUM: The interstitial material lying between the tubules generally appeared to contain a high concentration of electron-opaque particles (Figs. 1, 3, 6, 8). The particles were of many sizes but often were larger than those seen in the basement membranes. Circular cores rimmed with dense precipitate were seen frequently in this region as well as in the capillary lumen (Fig. 12). In the interstitium, a high concentration of large particles of precipitate often was associated with bundles of collagen (Fig. 3). Although some interstitial cells remained free of precipitate, the majority from both the cortex and the medulla showed evidence of swelling (Fig. 8). Associated with this type of cellular alteration were large numbers of electron-opaque precipitates. These cells appeared to be easily damaged by the procedures employed.

BLOOD VESSELS: The cytoplasm of endothelial cells did not show any precipitate (Figs. 1, 6). Occasional precipitate was seen adjacent to the external surface of the cell membranes of the endothelial cells. Precipitate was seen in capillary lumens, in some cases entirely filling the lumen. The smooth muscle cells in the walls of arterioles and the granulated juxtaglomerular cells also appeared free of precipitate. Occasional evidence of cellular swelling in endothelium, smooth muscle cells, and granulated juxtaglomerular cells was associated with precipitate distributed throughout the cell.

Morphology of Tissue Leached in Sodium-Free Solutions of Potassium Phosphate Buffer before Fixation with Potassium Pyroantimonate

When tissues were fixed in Karnovsky's fixative buffered with potassium phosphate, and subsequently leached for several days in potassium phosphate buffer made with ion-free water, and then refixed in Karnovsky's fixative containing 2% potassium pyroantimonate, no characteristic precipitate was seen in any of the many blocks examined (Fig. 9). A rare dense fleck of material was seen occasionally, but all spaces were completely free of typical precipitate. In general, the tissue was well fixed, and morphologic evidence of swelling was not seen with any frequency.

Tissue Fixed in Potassium Phosphate-Buffered Karnovsky's Fixative and Subsequently Refixed in Potassium Phosphate-Buffered Karnovsky's Fixative Containing Potassium Pyroantimonate

In order to determine if the substances which precipitate with the pyroantimonate are bound before or during the fixation or are free to move after fixation, tissues were fixed in potassium

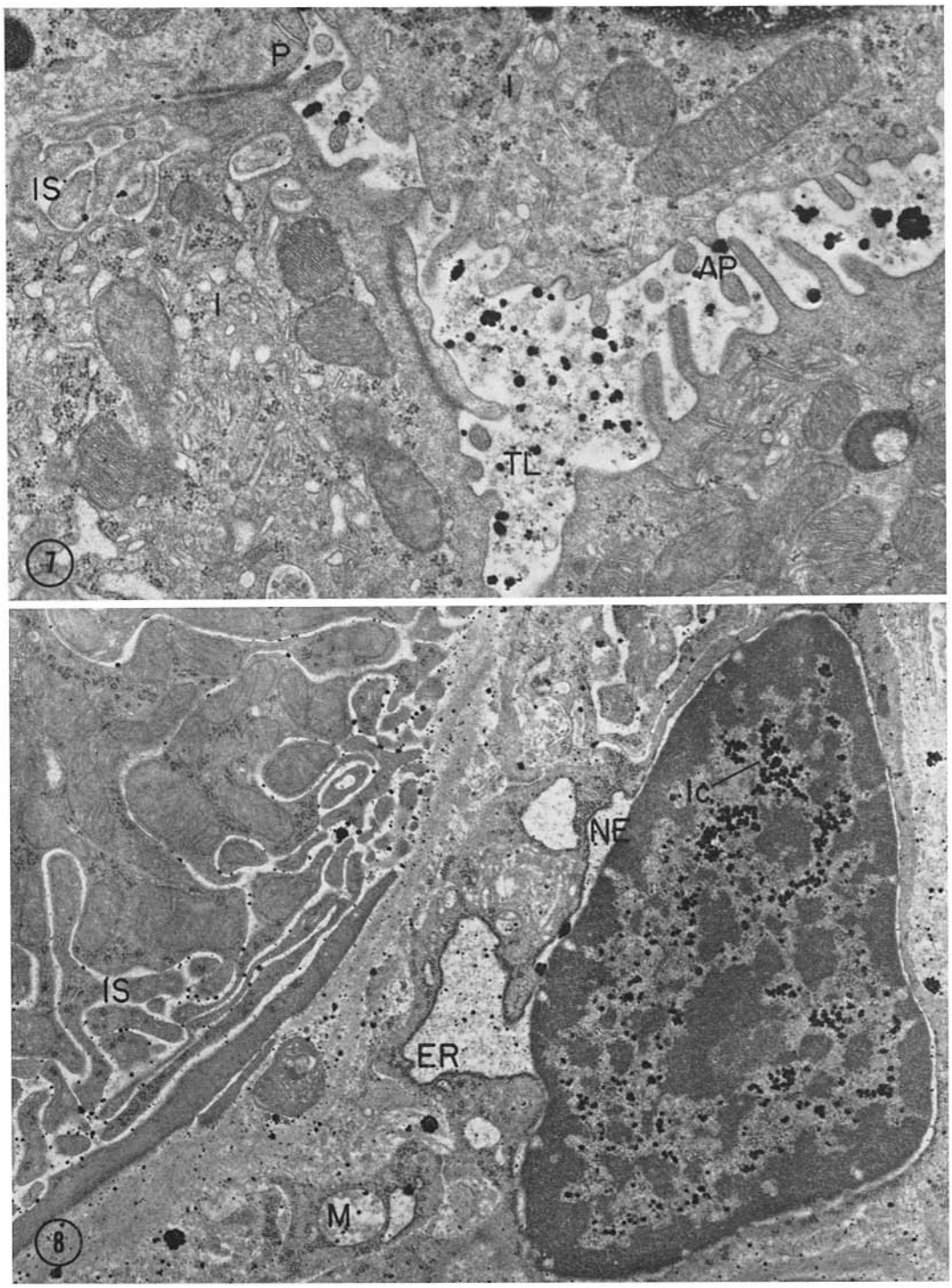


FIGURE 7 Electron micrograph showing a region of the collecting duct containing principal cells (*P*) and intercalated cells (*I*). Reaction product can be seen in the tubular lumen (*TL*), adjacent to the apical plasmalemma (*AP*), and in the lateral intercellular spaces (*IS*). The cytoplasm appears free of precipitate. × 23,300.

FIGURE 8 Electron micrograph showing a proximal tubule with dense precipitate lying in the lateral intercellular spaces (*IS*). The interstitial cell, however, shows precipitate in the interchromatin regions of the nucleus (*Ic*), in the nuclear envelope (*NE*), in the endoplasmic reticulum (*ER*), and in mitochondria (*M*), and in other cytoplasmic organelles. The decreased density of the mitochondrial matrix and the enlarged cisternae of endoplasmic reticulum of this cell are also consistent with cellular swelling. × 14,600.

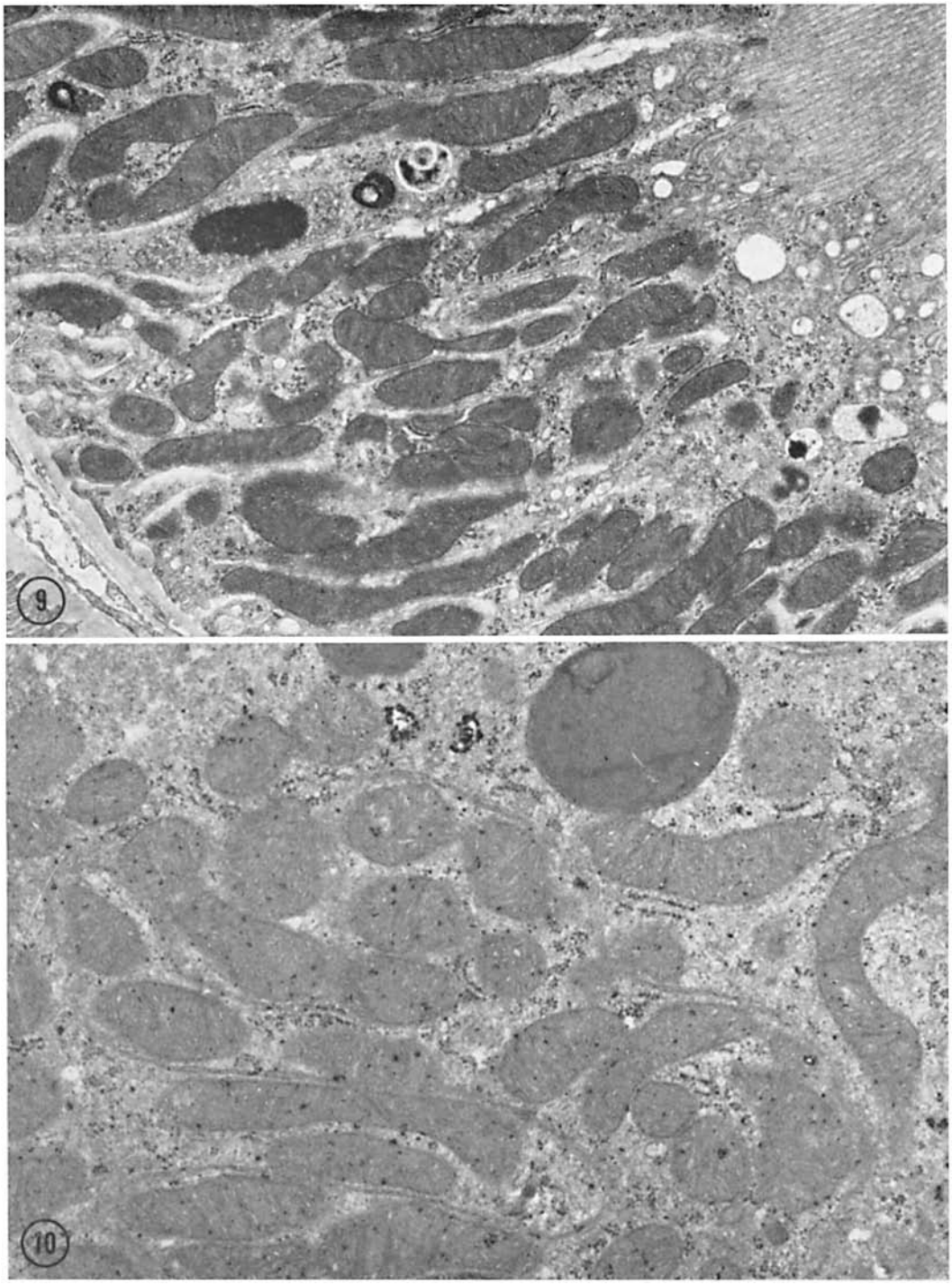


FIGURE 9 Electron micrograph of a proximal tubule taken from tissue which was fixed for 4 hr in Karnovsky's fixative and then leached for several days in potassium phosphate buffer. No precipitate was seen in any of the many blocks studied after this type of procedure. × 10,800.

FIGURE 10 Electron micrograph of a proximal tubule which was fixed without the potassium pyroantimonate for 4 hr and then refixed for 4 hr in a solution containing 2% potassium pyroantimonate. Dense precipitate is seen not only in the normal pattern but also in a somewhat random distribution over the entire cell and its contents. × 19,700.

phosphate-buffered Karnovsky's fixative and refixed later with solutions containing the pyroantimonate.

Tissues refixed with 2% potassium pyroantimonate 4 hr after initial fixation without pyroantimonate had a different pattern of precipitate distribution than tissue fixed immediately with potassium pyroantimonate. Although precipitate was seen between microvilli and between the cells in both lateral and basal regions as well as in the interstitium and blood spaces, an additional pattern of precipitation appeared superimposed on this pattern (Fig. 10). A fine precipitate was seen scattered in a somewhat random distribution over both the entire cytoplasm and nucleus. This pattern occurred in cells with morphologic evidence of swelling as well as in adjacent cells showing no evidence of swelling. With this pattern, as distinct from that seen after cellular swelling, the precipitate in the mitochondria was small and randomly distributed.

Tissue Fixed in Karnovsky's Fixative Buffered with Potassium Phosphate to Which 2% Sodium Pyroantimonate Had Been Added

Tissues were fixed in a solution to which 2% sodium pyroantimonate precipitate had been added in order to see if colloidal sodium pyroantimonate moved in tissue in a manner similar to that postulated for lanthanum nitrate solutions (16) (Fig. 11). Sections taken from these blocks, however, showed only an occasional dense fleck which appeared to be contamination. Besides these flecks, the sections showed no precipitate whatsoever in any regions examined including the surface of the tissue.

Effects of Cellular Swelling on Distribution of Precipitate

Slices of kidney tissue were allowed to swell in cold Robinson's buffer (0°–4°C) for 1–4 hr. Under these conditions, cellular respiration is significantly inhibited, and cells take up sodium chloride and water, increasing markedly in volume (2, 3, 17). After 4 hr of swelling in the cold, the slices had doubled in weight. In the deeper regions of the slice, many cells showed a normal pattern of precipitate distribution. In some regions, large numbers of proximal convoluted tubules showed marked swelling (Fig. 13). This swelling often corresponded to the edge of the slice where the cells were cut open by the knife.

Effect of Cellular Swelling in Sodium-Free Solutions on Distribution of Precipitate

Slices of kidney tissue were allowed to swell for 1–4 hr in cold Robinson's buffer (0°–4°C) in which all the sodium ions had been replaced by an equivalent molarity of potassium ions. The slices all gained weight during this time. In addition to regions showing normal morphology or swelling, a third type of cell appearance was seen that formed the most common type after swelling in sodium-free medium. This type was characterized by mitochondria with swollen intracristal spaces and relatively dense mitochondrial matrices (Fig. 14). The mitochondria were still elongate and never contained precipitate.

Effect of Incubating Rat Kidney Slices in Solutions Containing Ouabain Before Fixation in Solutions Containing Pyroantimonate

The results of incubating rat kidney slices in solutions containing 1×10^{-4} M or 3×10^{-4} M ouabain, with and without oxygenation before fixation in Karnovsky's fixative containing 2% potassium pyroantimonate, were variable and complex. Further study must be done in this area. Certain features were noted. Many cells demonstrated morphologic changes consistent with swelling and contained dense precipitate. Dense precipitate was also seen in cells showing the unique morphologic feature of large mitochondria with dense matrix material. Some cells appeared unswollen, but dense precipitate was seen in an intracellular position adjacent to the cytoplasmic leaflet of the unit membrane (Fig. 15).

Addition of 2% Potassium Pyroantimonate to Solutions of Cations and Biologic Amines

2% potassium pyroantimonate was added to 0.1, 0.01, 0.001, and 0.0001 M solutions of calcium chloride, barium chloride, magnesium chloride, and potassium chloride (Table I). Grossly visible insoluble precipitates formed with all of the divalent cations tested, but the amount of precipitate varied depending on the cation. 2% potassium pyroantimonate was also added to 1% solutions of histidine, histamine, serotonin, sper-

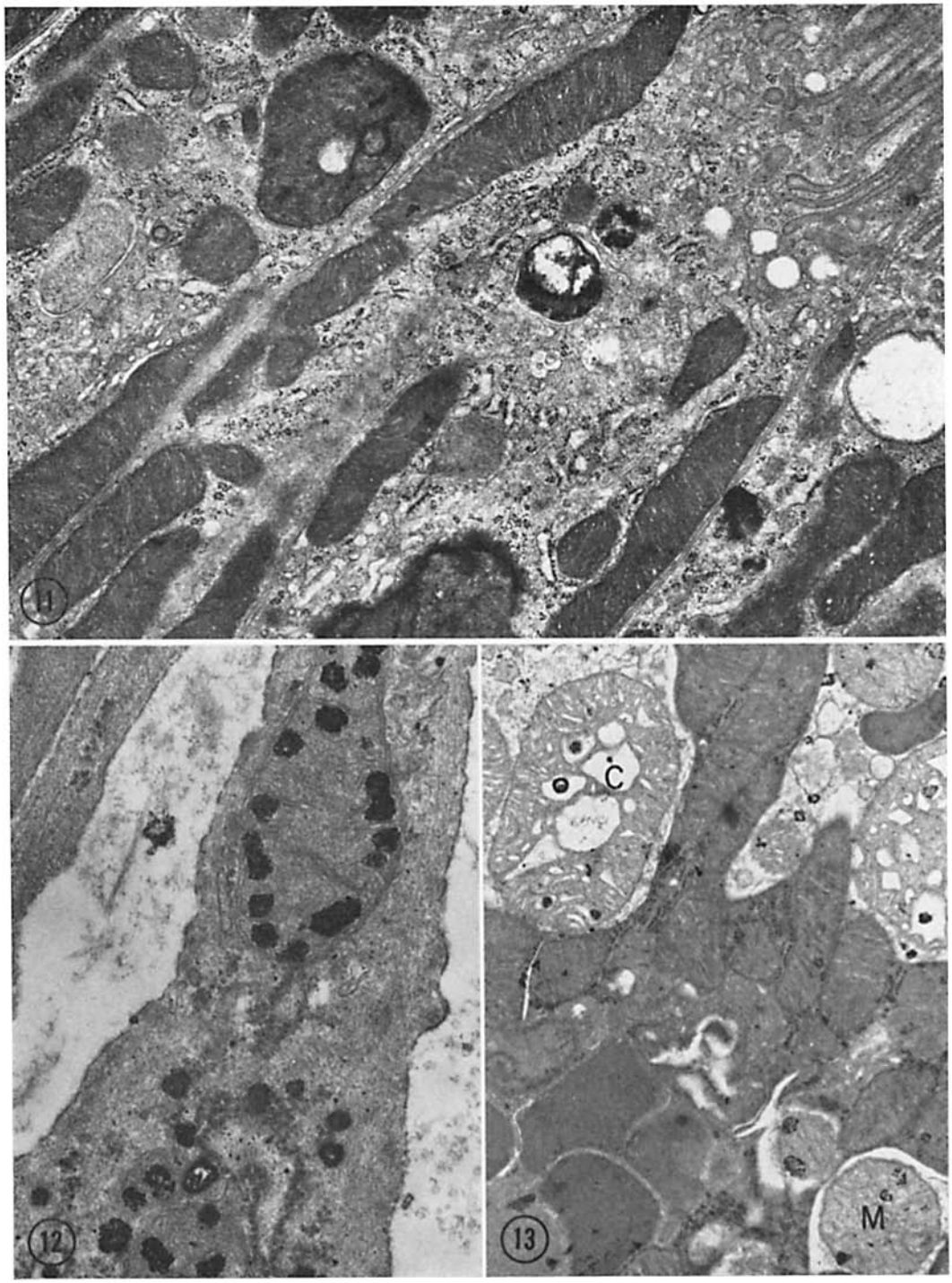


FIGURE 11 Electron micrograph showing a proximal convoluted tubule from normal tissue fixed with Karnovsky's fixative containing 2% sodium pyroantimonate. The tissue appears identical to that seen when no pyroantimonate is present in the fixing solution. × 17,300.

FIGURE 12 Electron micrograph showing a cytoplasmic region of an interstitial cell which contains large aggregates of precipitate in mitochondria, in endoplasmic reticulum, and in the cell sap. × 41,900.

FIGURE 13 Electron micrograph showing parts of cells from a tissue which was allowed to swell in Robinson's buffer in the cold for 4 hr. Some of these cells showed decreased density of the cytoplasmic sap as well as large mitochondria which show intracristal swelling or matrical swelling. Deposits of pyroantimonate precipitate are seen in the cristae (*C*) when they are swollen and in the matrix (*M*) after matrical swelling has occurred. × 14,300.

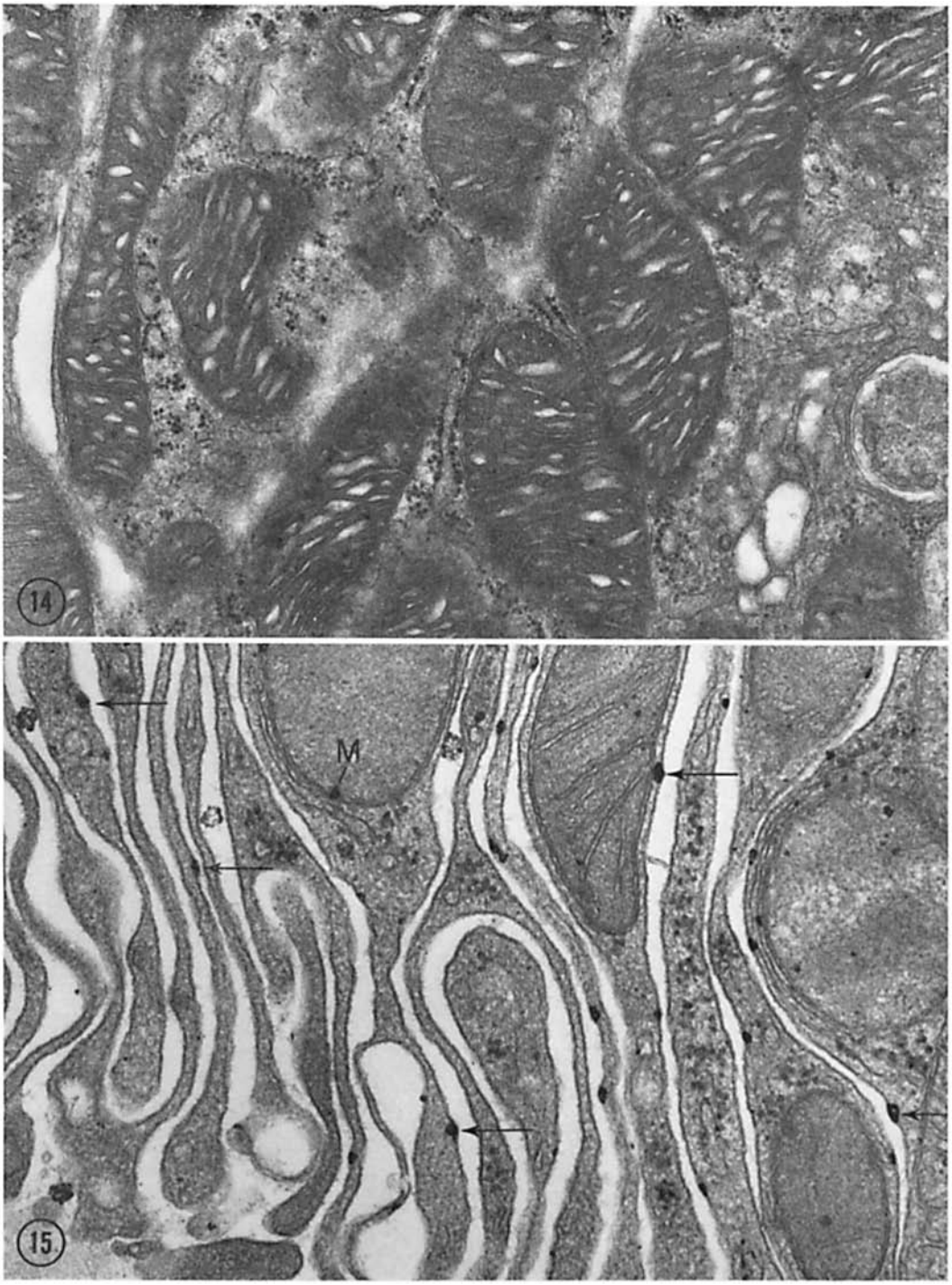


FIGURE 14 Electron micrograph taken from a slice of tissue which was allowed to swell in sodium-free Robinson's media. The majority of the proximal tubules are characterized by a lack of precipitate and by mitochondria which demonstrate the dense matrix and somewhat expanded intracristal space. × 29,800.

FIGURE 15 Electron micrograph showing the base of a proximal tubular cell from tissue which was allowed to remain in a solution containing 3×10^{-4} ouabain for 1 hr at room temperature. In this case, many of the dense deposits are seen to lie on the cytoplasmic surface of the cell membrane (arrows). Deposits were also seen between the inner and outer mitochondrial membranes (*M*). × 48,500.

TABLE I

Precipitates Formed on Addition of 2% Potassium Pyroantimonate

	0.1M	0.01M	0.001M	0.0001M	1%
$CaCl_2$	Precipitate	Precipitate	Cloudy precipitate	None immediately, faint precipitate in 24 hr	—
$BaCl_2$	Dense precipitate	Dense precipitate	Cloudy precipitate	None immediately, faint precipitate in 24 hr	—
$MgCl_2$	Cloudy precipitate	None immediately, faint precipitate in 24 hr	None immediately, faint precipitate in 24 hr	None	—
KCl	None	None	None	None	—
L-Arginine·HCl	—	—	—	—	None
Histamine·2HCl	—	—	—	—	Immediate dense precipitate
L-Histidine·HCl	—	—	—	—	None
Serotonin	—	—	—	—	None
Spermine·4HCl	—	—	—	—	Immediate moderate precipitate

mine, and arginine. Dense precipitates formed with histamine and spermine.

DISCUSSION

Potassium pyroantimonate has been used to react specifically with sodium ions as a means of quantitating sodium ions in fluids (1, 10, 11). Questions as to the specificity of this reaction have been raised (15). Bálint (1) pointed out the influence of other ions on the reaction for sodium.

The use of potassium pyroantimonate as a marker for sodium ions in tissue prepared for electron microscopy is now being attempted (4, 6–9, 12, 14, 20). The inherent usefulness of such a procedure would be great and hence makes these attempts worthwhile. Control tests of the pyroantimonate procedure for use in the precipitation of sodium pyroantimonate in tissue have included those of Komnick (8) who showed that potassium pyroantimonate formed a precipitate when reacted with 8% gelatin made in 0.9% sodium chloride and Zadunaisky (20) who demonstrated that, after treatment with 2% potassium pyroantimonate in glutaraldehyde, radioactive sodium ions showed a slower efflux than that seen in the control sartorius muscles of the frog.

This and other studies have shown that treating tissues with fixatives which contain pyroantimonate ions give consistent and reproducible patterns of precipitate distribution. In control pieces of kidney tissue the precipitate is largely extracellular. This finding is consistent with what is known about the tissue localization of large concentrations of sodium ions. However, the possibility that sodium ions can move in tissue before precipitation as sodium pyroantimonate has not been eliminated. Since the pattern of precipitate changes if the pyroantimonate is added 4 hr after the fixative, it appears that sodium ion can move in tissue. This study also showed that leaching removes all substances which cause precipitate formation. The possible formation of other pyroantimonate salts in tissues has not been tested thoroughly, however. The formation of large precipitates in the mitochondrial matrix in a pattern similar to that of the matrical

granules is suggestive that the reagents can precipitate with calcium ions.

A visible dense precipitate forms in a test tube upon the addition of 2% potassium pyroantimonate to solutions of divalent cations and to solutions of certain biologic amines. Since a dense precipitate formed when 2% pyroantimonate was added to 0.001 M calcium chloride solutions, it appears possible that the concentration in tissue of substances such as calcium ions might be large enough to cause precipitation of an insoluble compound. The studies of Komnick (8) and Zadunaisky (20) were not designed to demonstrate all the possible compounds which could be precipitated but to show that sodium ions were localized. Controls with sodium-free solutions (7) showed that a precipitate-free condition could be obtained in the absence of sodium only.

Two reproducible patterns of precipitate distribution were seen in controls. The first pattern was the predominant type seen after glutaraldehyde-formaldehyde primary fixation procedures, in which the precipitate appeared to be principally extracellular. The second pattern was occasionally seen in controls, but it appeared with greater frequency in experimental situations favoring cellular swelling or after primary fixation with osmium tetroxide-containing fixatives. This pattern was characterized by morphologic alterations consistent with cellular swelling and an intracellular distribution of precipitate. Since primary fixation of kidney tissue with solutions containing osmium tetroxide was invariably accompanied by a similar intracellular deposition of precipitate and morphology consistent with some cellular swelling, it was postulated that primary osmium tetroxide fixation was inadequate to prevent influx of sodium ions and water at some point during preparation. The use of potassium pyroantimonate could provide a sensitive marker for situations in which an increased intracellular sodium ion concentration occurs as in cell swelling. Incubation of kidney slices in medium in which the sodium ions were replaced by potassium ions produced a unique morphologic pattern of cell change which was not accompanied by deposits of precipitate. This finding indicates that sodium ions do play a role in the morphologic pattern of swelling and the distribution of precipitate.

Precipitates have been described in both extracellular and intracellular positions in rat kidney tissue after fixation in osmium tetroxide-containing solutions (12, 14). Similar results were seen during the present study with osmium tetroxide-containing fixatives but not after fixation with glutaraldehyde-formaldehyde combinations. The swelling with the osmium tetroxide fixation may be peculiar to the kidney or the buffer system used; swelling has not been noted in studies of other tissues, such as gall bladder (7).

Another problem inherent in the use of pyroantimonate as a marker for sodium ions is related to the permeability of the cell membranes to pyroantimonate anion. In kidney which shows no evidence of swelling, the precipitate generally appears to have an extracellular distribution. This could be explained by the fact that the major concentration of sodium ion is extracellular or by the inability of the pyroantimonate to permeate the cell cytoplasm. After a postulated change in the cellular membrane permeability with subsequent swelling, the precipitate can be found in the cell and particularly between the two membranes surrounding the mitochondria. In extreme swelling associated with a decrease in the density of the mitochondrial matrix, the precipitate is found in the matrices of the mitochondria, in nuclei, and in other cytoplasmic organelles. These experiments cannot determine whether the pyroantimonate anion cannot pass through the various cell membranes until some change in membrane permeability occurs, or whether the pyroantimonate can pass through the various cellular membranes but that ions in sufficient quantity to form precipitate are lacking until some permeability change occurs. Small deposits of pyroantimonate precipitate have been reported inside the cell unaccompanied by noticeable cell swelling in the ascending limb of Henle's loop of the nephron in this study and in that by Kaye et al. (6, 7). Deposits associated with the internal surface of the cell membrane have been seen after ouabain treatment in this study and in that by Kaye et al. (6) in corneal endothelium. Using a different experimental system, C. C. Tisher (personal communication) sees a pattern of nuclear precipitate in seemingly undamaged, unswollen cells. These results indicate that pyroantimonate anion seems to be able to penetrate the plasma membrane of the unswollen cell. No intracellular deposits were noted by Zadunaisky (20).

A differential degree of cell swelling is seen in the various cells of the kidney. The interstitial cells and proximal tubular cells frequently show

swelling, whereas cells of the distal convoluted tubule and collecting duct are more resistant to this process. The use of 2% potassium pyroantimonate added to fixatives appears to serve as a method to detect cell swelling and to indicate which cells are most susceptible to swelling.

After leaching the kidney blocks in sodium-free phosphate buffer, no precipitate was found. This indicates that the substances present in high enough concentration to form precipitates are either diffusible or are altered by the leaching. Tissues fixed for 4 hr and then treated with potassium pyroantimonate were characterized by a random precipitation of small particles superimposed upon the more normal pattern. The random pattern was seen intracellularly but was not accompanied by evidence of cellular swelling. Such a pattern would be consistent with the hypothesis that sodium ions can still migrate through tissue after fixation, as previously noted by Zadunaisky (20), and can react with pyroantimonate after a positional change.

After sodium ions precipitate in tissue as pyroantimonate salts, can the salts move during the subsequent preparatory procedures or can they dissolve and reprecipitate at other locations? After fixation of kidney tissue in solutions containing preprecipitated sodium pyroantimonate, no precipitate was seen even at the edge of the tissue block. This indicated that sodium pyroantimonate did not migrate through tissues in the manner proposed for colloidal lanthanum nitrate by Revel and Karnovsky (16). It also indicated that sodium pyroantimonate did not redissolve in quantities great enough to cause new precipitations.

The presence of considerable amounts of precipitate in the interchromatin region of the nucleus suggests that high concentrations of sodium ions or some other substance which causes precipitation were present in the interchromatin material. Siebert and co-workers (19) and Siebert and Humphrey (18) have shown that isolated nuclei contain a high concentration of sodium ions.

The application of potassium pyroantimonate during fixation provides reproducible patterns of precipitation, depending on several predictable parameters. Although it appears that sodium pyroantimonate may account for a large amount of the precipitation, it seems that the specificity of the reaction should be further investigated. More data are needed with respect to what substances and what concentrations of them might cause electron-opaque precipitate to form in tissues. This clarification could probably best be accomplished by the further use of model systems.

This work was supported by grant No. AM-10922 from the National Institutes of Health, United States Public Health Service. The AEI-6B electron microscope was purchased by grant No. GM-13543.

The able assistance of Miss Carlotta Minthorn and the Pathology electron microscope system technicians for technical help, of Mrs. Dorris Knibb for typing the manuscript, and of Drs. David Lagunoff and C. Craig Tisher for helpful suggestions is gratefully acknowledged.

Received for publication 19 April 1968, and in revised form 26 August 1968.

REFERENCES

1. BÁLINT, M. 1924. Eine jodometrische Mikrobestimmung des Natriums. *Biochem. Z.* **150:**424.
2. CONWAY, E. J., and H. GEOGHEGAN. 1955. Molecular concentration of kidney cortex slices. *J. Physiol. (London).* **130:**438.
3. DEYRUP, I. 1953. Reversal of fluid uptake by rat kidney slices immersed in isosmotic solutions in vitro. *Amer. J. Physiol.* **175:**349.
4. GRAND, R. J., and S. S. SPICER. 1967. Preliminary studies on the electron microscopic localization of sites of sodium transport in the human eccrine sweat gland. *Mod. Prob. Paediat.* **10:** 100.
5. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27:** 137A.
6. KAYE, G. I., J. D. COLE, and A. DONN. 1965. Electron microscopy: sodium localization in normal and ouabain-treated transporting cells. *Science.* **150:**1167.
7. KAYE, G. I., H. O. WHEELER, R. T. WHITLOCK, and N. LANE. 1966. Fluid transport in the rabbit gall bladder. *J. Cell Biol.* **30:**237.
8. KOMNICK, H. 1962. Elektronen mikroskopische Lokalisation von Na^+ und Cl^- in Zellen und Geweben. *Protoplasma.* **55:**414.
9. KOMNICK, H., and U. KOMNICK. 1963. Elektronen mikroskopische Untersuchungen zur Funktionellen Morphologie des Ionentransportes in der Salzdrüse von *Larus argentatus.* V. Experimenteller Nachweis der Transportwege. *Z. Zellforsch. Mikroskop. Anat.* **60:**163.
10. KRAMER, B., and I. GITTLEMAN. 1924. An iodo-

metric method for the determination of sodium in small amounts of serum. *J. Biol. Chem.* **62**:353.

11. KRAMER, B., AND F. TISDALL. 1921. A simple method for the direct quantitative determination of sodium in small amounts of serum. *J. Biol. Chem.* **46**:467.
12. LEE, J. C., L. JOHANSON, and J. HOPPER, JR. 1967. Compensatory renal hypertrophy; ultrastructural changes and histochemical localization of sodium ion in proximal tubules. *Amer. J. Pathol.* **50**:50a. (Abstr.)
13. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
14. MIZUHIRA, V., and T. AMAKAWA. 1966. Detection of electrolytes in tissues at the electron microscopic level with special reference to sodium ion transport mechanisms in rat kidney. *J. Histochem. Cytochem.* **14**:770.
15. PETERS, J. P., and D. D. VAN SLYKE. 1932. Quantitative Clinical Chemistry. The Williams & Wilkens Co., Baltimore, Maryland. **2**.
16. REVEL, J. P., and M. J. KARNOVSKY. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**:C7.
17. ROBINSON, J. R. 1961. Exchanges of water and ions by kidney slices determined by a balance method. *J. Physiol.* (*London*). **158**:449.
18. SIEBERT, G., and G. B. HUMPHREY. 1965. Enzymology of the nucleus. *In* Advances in Enzymology. Interscience Publishers, Inc., New York. **27**:239.
19. SIEBERT, G., H. LANGENDORF, R. HANNOVER, D. NITZ-LITZOW, B. C. PRESSMAN, and C. MOORE. 1965. Untersuchungen zur Rolle des Natrium-Stoffwechels im Zellkern der Rattenleber. *Hoppe-Seyler's Z. Physiol. Chem.* **343**:101.
20. ZADUNAISKY, J. A. 1966. The localization of sodium in the transverse tubules of skeletal muscle. *J. Cell Biol.* **31**:C11.