CELLULAR MECHANISMS IN RENAL PHARMACOLOGY

6508

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Understanding of cellular drug action in the mammalian kidney requires a firm understanding of cellular mechanisms of transport. In anticipation that several years from now these concepts will be greatly strengthened, this review examines the present beginnings of our understanding of the cellular regulation of ion flow in the kidney and the action of biologically effective chemical agents on renal function. A brief discussion of some current studies of the clinical pharmacology of newer diuretic compounds is also included at the end of this review.

General knowledge concerning cell membranes and mechanisms of transport is expanding at an impressive rate (1). The exponential growth is reminiscent of the takeoff in areas of biology such as DNA and viruses, which have vigorously altered the perspectives of medical science. This development is of obvious significance to both the general pharmacologist and the renal pharmacologist, since a large fraction of the biological activity of therapeutic agents may be exerted by direct action at the cell membrane.

Membrane structure.—Concepts of cell membrane structure have been changing in the last several years. The model proposed by Danielli & Davson (2) has been generally accepted until recently. In this model membranes consist of one or more continuous bimolecular leaflets of phospholipid covered on both sides with protein. The interaction between protein and lipid is primarily electrostatic. The proteins layered on either side of the lipid bilayer are presumably similar proteins so that the membrane is symmetrical. This model was able to account for the low surface tension and mechanical strength of the membrane.

Observations regarding the similarity of all membranes led Robertson (3) to propose the unit membrane hypothesis. The model set up by Robertson restricted the number of lipid leaflets to one bilayer and it considered the membrane to be asymmetrical, with mucoproteins on the outside and unconjugated proteins on the inner surface of the membrane. In these models, protein layers are in an extended or β-conformation and nonpolar inter-

action between protein and lipid is minimal. Direct observation of cellular membranes, however, by optical rotatory dispersion (4) or infrared absorption (5) indicates that there is random coiling and some protein in the α-helical form.

Recently it has been suggested that membranes are made up of repeating lipoprotein units (6-8). These subunits are more consistent with the current concept of organized membrane systems which require the interaction of several membrane proteins. The evidence fostering this subunit hypothesis is derived from electron microscopic examination of membranes (9, 10) or X-ray diffraction studies of membranes (11).

Newer concepts of membrane regard the structural arrangements of the membrane components as dynamic aggregates undergoing reversible structural changes (12). The observed rapid and varied turnover of membrane constituents (13) is compatible with this concept.

Proteins of membrane transport.—The most convincing evidence for the existence of specific membrane transport proteins is found in bacterial studies. These membrane systems are under genetic control and the presence or absence of these protein components can be more rigorously defined. The protein nature of these transport systems is also suggested by the necessity of protein synthesis for their induction and the inhibition of membrane transport by reagents that react specifically with protein (1).

Some representative findings in this area are cited. Neu & Heppel (14) have demostrated that a selected group of proteins is released from bacterial cell membranes when the organisms are subjected to osmotic shock. These proteins make up about five percent of the total protein. Following osmotic shock the ability of the bacteria to take up and concentrate certain compounds is impaired. The supernatant fluid obtained after the shock procedure contains proteins that bind substances whose transport had been impaired.

From shock fluid in *E. coli* there has been isolated and purified a protein binding neutral amino acids (15). Similar binding proteins for sulfate (16) and galactose (17) have been isolated and purified. Genetic studies support the concept that the binding protein and entry system are either identical or are very closely linked in their genetic control (18).

A phosphotransferase system involved in active transport of sugars has been isolated from *E. coli* and other bacterial strains (19–21). This system uses phosphoenolpyruvate as an energy source. A membrane protein confers specificity for the sugar transported.

Fox & Kennedy (22) devised a method aimed at specific labeling of membrane proteins of the lactose transport system of *E. coli*. This approach has served as a basic model for identification of membrane proteins. N-ethylmaleimide, a sulfhydryl-binding reagent, irreversibly inhibits galactoside transport. This inhibition of transport can be prevented by preincubation with thiodigalactoside for which the transport system has a high affinity.

Specific binding of radioactive N-ethylmaleimide to the transport protein is accomplished in the following way. Transport sites of the cell are protected with thiodigalactoside and nonradioactive N-ethylmaleimide is allowed to react with susceptible unprotected sulfhydryl sites. These cells are then washed to remove the protective thiodigalactoside and radioactive N-ethylmaleimide is added to label the specific transport sites that were previously protected. Since the transport system can be experimentally induced in certain strains of *E. coli,* critical evaluation of the approach is possible by working with cells in the presence and absence of the transport system.

This technique has been applied by Magour et al (23) to the mammalian kidney. By labeling the kidney tissue with dibenamine-C^{14} and using the above principals, a carrier-like protein for organic bases appears to have been partially purified.

Sodium-potassium dependent ATPase of the plasma membrane.—Cells of most mammalian tissues maintain high cellular potassium and low cellular sodium against electrochemical gradients. Sodium is extruded from the cell and potassium is accumulated in the cell by an ion pump mechanism in which the pumping of sodium and potassium is coupled. A membrane sodium-potassium dependent ATPase system is associated with the ion pumping and appears to participate in the transport process. Several papers review in detail earlier studies of this membrane enzyme system (24–26).

The activity was first described by Skou (27) in crab nerve and subsequently has been found in most tissues (28). The highest activity is found in nervous tissue and tissues concerned with secretory function.

In mammals whose erythrocytes contain high potassium levels within the cell, the erythrocyte membranes have high sodium-potassium dependent ATPase activity. Erythrocytes with low potassium levels have low membrane levels of this activity (29, 30). The enzyme activation is vectorial. Studies with erythrocyte membranes show that intracellular sodium and extracellular potassium activate the enzyme (31).

Cardiac glycosides such as ouabain inhibit the sodium-potassium dependent ATPase activity at the same levels at which they inhibit cation transport (32, 33). This site of inhibition is apparently at the external membrane surface (34). Ouabain inhibition of both the ATPase activity and active cation transport is partially reversed by high levels of potassium (33).

When ATP labeled with P^{32} in the terminal phosphate is employed, evidence is found for a phosphorylated membrane protein intermediate of the sodium-potassium dependent ATPase reaction (35–40). Hydrolysis of the ATP occurs in two steps: the first step is a rapid, sodium-dependent phosphorylation of the membrane enzyme, the second step is a potassium-dependent dephosphorylation reaction. Dephosphorylation is inhibited in vitro by ouabain and organic mercurial compounds (37, 38). Calcium blocks the sodium dependent phosphorylation (41, 42). A heavy metal, perhaps copper, is postulated to serve as a regulator of the enzyme activity (43).

Attempts at purification of the membrane sodium-potassium dependent ATPase system have met with only moderate success. Extraction of membranes with sodium iodide results in marked enhancement of the specific activity (44, 45). The extraction appears to remove low molecular weight protein unrelated to the activity (46). The system has been partially purified from guinea pig brain tissue by employing Lubrol detergent and agarose gel column chromatography (47). The enzyme activity is associated with high molecular weight components (46–48). Purification attempts are associated with rapid loss of enzyme activity. Towle & Copenhaver (48) have succeeded in partially purifying the activity from rabbit kidney. They have employed glycerol to stabilize the enzyme.

The need for a procedure specifically labeling the sodium-potassium dependent ATPase protein has been recognized. Cardiac glycosides may help since they bind in a specific fashion to the ATPase system. The binding of cardiac glycosides in presence of ATP is stimulated by sodium and is depressed by potassium (49–52). Chignell & Titus (53), employing P^{32} and disc gel electrophoresis, have apparently identified a phosphorylated intermediate of the ATPase system in rat kidney.

The role of the sodium-potassium dependent ATPase system in the function of the renal tubule is a subject of current investigation. It has been postulated that this membrane pump system participates in renal tubular reabsorption of sodium. The picture would be one of passive entry of sodium at the luminal surface and active extrusion of sodium at the peritubular surface of the renal tubule. When the ATPase activity is studied in renal tissue slices incubated in vitro, the activity level appears to correlate with the ability of the tissue to maintain normal levels of intracellular potassium (54). This is an agreement with earlier studies in the erythrocyte. However, depressed sodium-potassium dependent ATPase activity in vivo does not always appear to be associated with depressed levels of intracellular potassium (54).

Ouabain and other cardiac glycosides are potent inhibitors of the sodium-potassium dependent ATPase activity. There appears to be a correlation between natriuresis induced by ouabain administration and the effect of the ouabain on sodium-potassium dependent ATPase activity (55, 139). Digoxin administered intraarterially to a single dog kidney depresses the ATPase activity of both cortex and medulla in the infused kidney. A natriuresis is induced and renal concentrating ability is markedly reduced (56). The effect on renal concentrating ability suggests a role for the ATPase system in the ascending limb of the loop of Henle. Proximal tubules have been isolated and the effect of ouabain on sodium reabsorptive capacity in vitro has been studied (57). Ouabain has no effect on sodium reabsorption in the in vitro system although it does depress homeostasis of normal cell electrolyte content. For the present it must be concluded that the physiological action of ouabain on sodium reabsorption is related to its action on the ATPase system in the ascending limb of the loop of Henle and the more distal parts

of the renal tubule. The isoosmotic reabsorption of sodium in the proximal tubule appears to be unaffected by ouabain.

The role of cellular metabolism and hormonal regulation in renal sodium transport.—Earlier studies (58) have shown that sodium reabsorption by the renal tubule is dependent on oxidative metabolism. The link between this transport and metabolism in the kidney requires clarification. Membrane ATPase activity associated with sodium transport is coupled to cellular metabolism (59). A specific coupling has been demonstrated between cytoplasmic ATP production catalyzed by phosphoglycerate kinase and the membrane ATPase activity. Membrane ATPase activity also influences mitochondrial respiration by enhancing the availability of ADP for mitochondrial oxidative metabolism (59, 60).

When kidney slices are incubated, changes in membrane ATPase activity are associated with changes in cellular respiration secondary to the altered ATPase (54). This accounts for the observed inhibitory effects of ouabain on cellular respiration in kidney slices (61, 62).

Two patterns of cellular sodium extrusion can be demonstrated in incubated kidney slices (54, 63, 64). One is coupled to the uptake of potassium on a one for one basis and is ouabain sensitive. It is associated with the membrane ATPase activity (54). A second is ouabain insensitive (54, 63, 64). It controls sodium, chloride, and water and is clearly dependent on cellular metabolism.. It may be postulated that the isoosmotic reabsorption of sodium that occurs in the proximal tubule and is not sensitive to ouabain is in some manner associated with the second system.

The steroid hormone aldosterone regulates sodium reabsorption in the renal tubule. Cellular events associated with the action of aldosterone are complex. The use of the isolated toad bladder as a model system has provided much of our recent knowledge. The toad bladder is in large part an analog of the very distal portion of the renal tubule and is responsive to aldosterone and vasopressin. Recent studies in both the toad bladder and the mammalian kidney support the concept of aldosterone acting apparently at a gene level to induce the formation of a protein component which in turn activates the extra sodium transport associated with the hormone action.

Specific observations support this concept. Antinatriuresis or the enhanced sodium reabsorption resulting from aldosterone is preceded by a lag period, as if more than one step precedes the change in sodium reabsorption (65–69). In the toad bladder the enhanced sodium reabsorption will occur even if the aldosterone is briefly incubated with the tissue and then is removed (68, 70). If RNA synthesis is inhibited, aldosterone has no effect on sodium transport in the kidney (69, 71) or the toad bladder (68, 72). If protein synthesis is inhibited, aldosterone, similarly, has no effect on sodium transport in the kidney (73) or toad bladder (72, 74). When aldosterone is administered, synthesis of RNA is induced in mammalian kidney (71, 75, 76). All classes of kidney RNA except the soluble transfer RNA show en-

hanced formation within thirty minutes of aldosterone administration (76). Electron microscope autoradiography reveals that H^3-aldosterone is localized in plasma membranes, mitochondria, and nuclei of the kidney (77). An apparent specific uptake of aldosterone into the nucleus of the kidney cells has been described (78). The sequence of events postulated for aldosterone action is: interaction of the steroid with a specific nuclear protein, induction of specific RNA synthesis, and the formation of one or more protein components essential for the enhanced reabsorption of sodium. Aldosterone enhancement of potassium excretion in the kidney is a direct effect of the hormone. It is rapid in onset and independent of protein or RNA synthesis (69, 71, 73).

The nature of the protein thought to be induced by aldosterone has not been elucidated. Although enhanced RNA formation following aldosterone is readily observed, it has been difficult to measure directly an increase in cellular protein formation (71, 79, 80). A specific protein resulting from a specific gene activation may be involved.

Several theories have been brought forth to explain the mechanism of action of the aldosterone induced protein. They are: (*a*) enhanced energy metabolism in pathways related to ion transport (81) (*b*) induced permeability to sodium enhancing the transport (82–84) or (*c*) direct augmentation by the protein of the sodium transport system (85).

The role of steroid hormones in regulation of sodium-potassium dependent ATPase activity has been investigated. In the chronically adrenalectomized rat, the ATPase activity is considerably depressed (86, 87). This change in ATPase activity after adrenalectomy is slow in onset and reversal with steroid hormones is equally slow (86, 87). The change in this activity would appear to be secondary to an overall decrease of the plasma membrane content of the renal tubule (88). It is clear that aldosterone at physiological levels does not directly regulate sodium-potassium dependent ATPase activity (86, 87, 89).

Vasopressin (antidiuretic hormone) functions in regulation of water balance by enhancing the water permeability of the collecting duct of the renal tubule. It functions in similar fashion in the toad bladder. The peptide hormone activates the plasma membrane enzyme adenyl cyclase at the peritubular surface. The cyclic AMP formed enhances water permeability at the luminal surface of the renal tubule. Cyclic AMP in the absence of vasopressin will produce the same effect (90). Stimulation of sodium transport is seen secondary to the increase in permeability associated with an increase in the size of the pool of intracellular sodium (84).

Studies with the electron microscope show that redistribution of cellular cations occurs when sodium transport is stimulated by vasopressin (91). Sodium is mobilized from a large pool in the cell nucleus and calcium content of the mitochondria increases. This calcium change is interpreted as a mobilization of calcium from the cell membrane which in turn enhances

membrane permeability (91). Little is otherwise known about the control of membrane calcium even though calcium ions are able to modify permeability in all tissues.

Plasma membrane in the mammalian kidney.—Renal tubular cells are responsible for reabsorption of solutes from the glomerular filtrate and for secretion of solutes from the plasma into the tubular fluid. Surface membranes of these cells are specially adapted in structure to carry out the principal functions of the mammalian kidney. Peritubular membranes of proximal and distal tubule consist of surfaces greatly expanded by massive infoldings. These peritubular membrane folds are lined with mitochondria apparently providing energy for membrane transport function. A more modest expansion of membrane surface occurs at the luminal side of the proximal tubule with the characteristic features of the brush border.

Recently methods have been developed for isolation of plasma membrane from a variety of tissues and the study of membrane constituents. Cell membrane isolation began with the pioneering work of Neville employing density gradients and rat liver tissue (92). This procedure has also been applied to mammalian kidney (93). A simplified membrane isolation procedure with greater purity has been developed for rat kidney (46). Tissue is homogenized in isotonic sucrose. Nuclei and plasma membrane are sedimented. The nuclei are then sedimented in 2M sucrose. Subsequent steps eliminate those mitochondria not previously removed. When examined with the electron microscope, the preparation consists of membrane vesicles. Sodium-potassium dependent ATPase activity and adenyl cyclase are enzymatic activities specifically associated with the preparation.

Proteins of the plasma membrane may be separated in the following fashion (46). The membranes are solubilized by appropriate treatment with Lubrol W-X detergent or by sonic oscillation. Soluble proteins are passed through a gel filtration column (6% agarose). The first protein peak eluted consists of very high molecular weight complexes including sodium-potassium dependent ATPase activity. A second protein peak consists of progressively smaller protein components ranging from perhaps 200,000 to 20,000 in molecular weight. Mean molecular weight of proteins in the second peak is approximately 50,000. Polyacrylamide disc electrophoresis of the protein eluted from the column shows at least 13 electrophoretically distinct mobile components. Extraction of plasma membrane with sodium iodide eliminates much of the low molecular weight protein components and may account for the purification of sodium-potassium dependent ATPase activity achieved by the procedure. Incorporation of radioactive amino acid was utilized to measure the turnover of membrane protein (46). These results further confirmed the concept that cell membrane proteins in the mammalian kidney are heterogeneous. The membrane contains a variety of proteins varying in size, enzymatic function, turnover, and electrophoretic mo-

bility. In the kidney of the chronically adrenalectomized rat an apparent decrease of high molecular weight plasma membrane protein is seen (73). Additional separation procedures are possible and have been employed in the attempts to purify ATPase activity (48) or to isolate ATPase proteins (53).

The action of diuretic agents at a cellular level in the kidney.—A possible mechanism for the action of diuretic agents could be an alteration of the intracellular energy metabolism of the kidney associated with sodium transport. Gross disturbances of energy metabolism in the kidney will interrupt the transport of sodium (58). However, with the exception of ethacrynic acid there is presently no substantially documented evidence that commonly employed diuretic agents act directly on energy-generating systems even in model experimental systems such as kidney slices. The in vitro effects of ethacrynic acid on metabolism which are found in kidney slices are not observed in vivo and do not appear related to the diuretic mechanism.

Organic mercurial compounds have been the diuretic agents most extensively studied at a cellular level. Organic mercurial compounds with natriuretic activity do reach the intracellular cytoplasm of kidney cells (54, 94, 95). When administered in vivo they do not directly inhibit glycolytic activity of the cytoplasm or respiration of mitochondria (59, 96, 97). When added in vitro to homogenates or cell fractions they do not directly inhibit cytoplasmic glycolysis, nor do they inhibit respiration of mitochondria in the presence of cytoplasm (59). Diffusion of the mercurial through the many cytoplasmic proteins apparently limits the effectiveness of organic mercurials as an intracellular inhibitor of essential sulfhydryl groups. Evidence for an intracellular site of action of organic mercurial compounds does not exist at present.

Several investigators have studied the interaction of organic mercurial compounds with erythrocytes (98–102). Inhibition of 3% of the sulfhydryl groups on the outer surface of the cell inhibits sugar transport (99). Inhibition of a larger percentage of sulfhydryl groups increases permeability to potassium (99, 101). Another portion of the sulfhydryl groups in the membrane appears to be associated with active cation transport (98, 100, 102). Rothstein (98) concluded that the cell membrane was the first part of the erythrocyte to be inactivated by sulfhydryl agents. The cytoplasm is protected by the necessity for agents to penetrate and the presence of insensitive sites in the cytoplasm.

A comparison of organic mercurial compounds with and without natriuretic activity points to the cell membrane of the mammalian kidney as a probable locus of biological activity. When meralluride is administered to rats at 4 mg Hg/Kg a rapid natriuresis is obtained and the urinary excretion of potassium is depressed. Renal sodium-potassium dependent ATPase activity is inhibited (96). If these kidneys are removed and tissue slices prepared from them, the rate of passive outflow of potassium from the tis-

sue is depressed (54, 59). The nondiuretic mercurial, p-chloromercuribenzoate, does not produce natriuresis and has no effect on potassium permeability or on the ATPase activity (54). Both p-chloromercuribenzoate and meralluride cause swelling of tissue slices in vitro (54, 103) but have little effect in vivo on tissue water content (54).

The binding of organic mercurials in vivo to the plasma membranes of mammalian kidney has been measured (104). Substantial binding of organic mercurial compounds to rat kidney plasma membrane is found two hours after IM administration. This is the time interval normally associated with a peak natriuretic response. Two hours after administration most of the binding is found to be that of free mercury detached from the organic moiety. When plasma membrane is fractionated on agarose gel columns after use of the diuretic chlormerodrin there are found high and low molecular weight proteins of the plasma membrane. The nondiuretic mercurial p-chloromercuribenzoate is almost exclusively associated with high molecular weight protein of the membrane.

The same investigators have more recently examined the in vivo binding of organic mercurials to dog kidney plasma membrane (105). Organic mercurial compounds radioactive in the mercury moiety were infused directly into the renal artery. Chlormerodrin radioactivity persisted at substantial levels in both low and high molecular weight protein components associated with the plasma membrane preparation. Radioactivity of p-chloromercuribenzoate is lost progressively from low molecular weight protein components over a two-hour period. Renal cortical plasma membrane binding of both compounds is considerably higher than that of the outer medulla in the dog kidney.

Since the finding that ouabain induced natriuresis is associated with the inhibition of membrane sodium-potassium dependent ATPase activity (55, 56, 106), the relationship of natriuretic agents to the activity of this membrane system has been studied in several laboratories. Organic mercurial diuretics, when administered in vivo to rats, inhibit this ATPase activity in the kidney (96). Nondiuretic mercurials do not inhibit this ATPase activity in the rat kidney. Organic mercurial diuretics, when administered in vivo to dogs, have no effect on the sodium-potassium dependent ATPase activity of the renal cortex (105, 106) and outer medulla (105). Organic mercurial diuretics incubated with rat kidney slices inhibit the membrane sodium-potassium dependent ATPase activity (54). Organic mercurial diuretics incubated with dog kidney cortex slices do not inhibit this activity. When rat kidney slices are incubated with organic mercurial diuretics, the fall in ATPase activity is associated with a fall in intracellular potassium (54, 59) and a fall in that portion of cellular respiration and glycolysis associated with the ATPase activity (54, 59, 62).

Several other diuretic agents that have been tested do not appear to affect sodium-potassium dependent ATPase activity either in vivo or in kidney slice incubations. Furosemide and ethacrynic acid administered in vivo

do not inhibit sodium-potassium dependent ATPase activity of rat kidney plasma membrane (107). A 10 mg/kg dose of ethacrynic acid administered in vivo to rabbits does not inhibit the ATPase activity of rabbit kidney (105). Ethacrynic acid (5×10^{-4}M) when incubated with rabbit kidney cortex slices does not alter the sodium-potassium dependent ATPase activity measured in these slices (105). Furosemide and hydrochlorothiazide incubated in vitro with rabbit and rat kidney slices are without effect on intracellular potassium, glycolysis, or cellular respiration (59). A significant inhibition of sodium-potassium dependent ATPase activity in these slices would diminish all of these parameters.

Membrane binding of C^{14}-ethacrynic acid has been measured in the rat kidney following in vivo administration (59). Ethacrynic acid is associated almost exclusively with the low molecular weight membrane protein components, whereas the ATPase activity is associated with high molecular weight protein complexes. Nechay et al (106) measured the binding of ethacrynic acid to subcellular components of dog kidney following in vivo administration. The binding of ethacrynic acid was very much lower than would appear to be compatible with ATPase inhibition.

In contrast to some other diuretics, ethacrynic acid does have a direct effect on pathways of energy metabolism in kidney cells incubated in vitro. When slices of rat, rabbit, or dog kidney are incubated with 0.1 mM ethacrynic acid, tissue respiration is depressed (59, 62) and intracellular potassium levels are decreased (59). Macknight reported similar effects on tissue respiration and intracellular potassium of rat kidney slices working with 1 mM levels of ethacrynic acid (108). Inhibition of cellular respiration by ethacrynic acid is also seen with incubated Ehrlich ascites tumor cells (109). At 1 mM levels ethacrynic acid inhibits the sodium transport of red cells by an undetermined mechanism (110, 111).

Ethacrynic acid is an inhibitor of the respiration of isolated mitochondria (59, 60, 112). In contrast to organic mercurials, this respiratory inhibition occurs even when cytoplasmic protein is present in the incubation medium (59). When the respiration of isolated mitochondria is stimulated by the addition of a membrane fraction, this membrane ATPase stimulated respiration is largely abolished by .05 mM ethacrynic acid (59, 60). This level of ethacrynic acid has no inhibitory effect on membrane ATPase activity in vitro. The inhibition of membrane stimulated respiration by ethacrynic acid is reversible by an increase in the ADP level of the incubation (59). ADP does not reverse ethacrynic acid inhibition of mitochondrial respiration in absence of the membrane fraction. Possibly membrane binding of ethacrynic acid is a factor in the dynamics of this experimental situation.

Fulgraff and collaborators have carried out studies in which renal oxygen consumption, tubular sodium reabsorption and renal blood flow are measured following administration of diuretic agents in the dog (113–115). Ouabain infusion (113) depresses sodium reabsorption and oxygen consumption in parallel fashion in dog kidney. This suggests that ouabain

effects a sodium transport system coupled to oxidative metabolism. Ethacrynic acid (114) and furosemide (115) have no effect on renal oxygen consumption but do depress tubular sodium reabsorption. This observation appears to indicate that the effects of ethacrynic acid on tissue respiration in vitro are not encountered in the in vivo situation. The effects obtained with furosemide and ethacrynic acid are interpreted as an effect on permeability of the renal tubule (possibly an enhanced back leak) rather than an effect on a transport system. An alternative possibility suggested is an uncoupling of oxidative metabolism from renal sodium transport.

It is appropriate to place cellular mechanisms of diuretic agents in some perspective. The evidence is rather consistent that ouabain and related cardiac glycoside compounds cause a natriuresis by the inhibition of a sodium transport system of the plasma membrane. Ouabain when administered in vivo appears to be associated with high molecular weight protein components of the renal plasma membrane (104). Kleinzeller & Cort (116) observed many years ago that high levels of organic mercurials (1 mM) increase the permeability of kidney cortex slices. Kleinzeller and coworkers also observed that when mercuric chloride is administered there is plasma membrane localization mostly at the peritubular side (117). Organic mercurials also enhance in vitro the permeability of erythrocytes (99, 101). In contrast following in vivo administration of meralluride and mercaptomerin at a diuretic dose (presumably a lower level) membrane permeability is depressed (54). Diuretic mercurials appear to differ from the nondiuretic mercurials in the persistent association of the mercury moiety with low molecular weight plasma membrane protein (104). Ethacrynic acid is also found associated only with low molecular weight components of the plasma membrane protein. Chlorothiazide and furosemide interact with renal plasma membrane in vitro to depress titratable sulfhydryl groups (59). In the erythrocyte membrane, sulfhydryl groups appear to augment permeability in the reduced state (118). In the toad bladder epithelium, increases in sodium transport caused by a variety of compounds (aldosterone, vasopressin, etc.) are associated with a decrease in protein bound disulfide of the tissue (140). It is suggested that diuretic agents modify the plasma membrane of the renal tubule and regulate ion movements by their interaction with sulfhydryl groups of low molecular weight proteins governing ion movements (59). Recent studies with macrolide antibiotics (119) suggest the potential of cyclic peptide compounds as carriers of ions through membrane barriers.

A postulate of long standing concerning the mode of action of organic mercurial diuretics is the mercuric ion hypothesis (120). According to the postulate, organic mercurial compounds reach the vicinity of the receptor site and here the carbon-mercury bond is broken allowing reaction with the receptor. Organic mercurial compounds with highly stabilized carbon-mercury bonding will not function as diuretics. The controversy surrounding this hypothesis is comprehensively reviewed by Cafruny (121). Present studies

of chlormerodrin and p-chloromercuribenzoate binding to renal plasma membrane provide a measure of support for the mercuric ion hypothesis, if one accepts the plasma membrane as the active drug receptor site. In rat kidney, two hours after administration at the time of peak natriuresis, approximately 90% of the apparent chlormerodrin binding to renal plasma membrane represents mercury separated from the organic moiety (104). When studied in dog kidney (105), chlormerodrin-Hg^{203} rapidly labels the renal plasma membrane, and this label persists in the renal plasma membrane over a two-hour period. Presumably as in the rat this is mostly mercuric ion. The nondiuretic p-chloromercuribenzoate labeled with Hg^{203} is distributed in the plasma membrane like chlormerodrin ten minutes after administration. During the following two hours it is progressively lost from the renal plasma membrane (105). If the initial labeling of membrane by p-chloromercuribenzoate had been in the form of the mercuric ion, no subsequent difference from the labeling by chlormerodrin would be expected to occur.

The mode of action of furosemide is currently under active investigation. Furosemide abolishes sodium and osmolal concentration gradients in the renal medulla (122, 123). Renal blood flow is increased with the administration of furosemide and has been postulated as a mechanism for the above effect. When renal blood flow is experimentally held constant, a rapid effect of furosemide on the corticomedullary gradient is still readily observed (122). Perfusion of segments of the renal tubule with furosemide clearly indicates that furosemide has a direct cellular action on the loop of Henle (123) and it is, therefore, unlikely that redistribution of renal blood flow in the medulla is an important part of the diuretic effect. A proximal tubular effect of furosemide on sodium reabsorption is also demonstrable in the above microperfusion experiments (123).

Amiloride and triamterene (124), which are relatively weak compounds, are purposely discussed separately from other natriuretic agents. Their unique property is a reduction of urinary potassium (a potassium-sparing action) which can offset the kaliuresis induced by more potent diuretics. These compounds also reduce hydrogen ion secretion in the distal tubule by acting mainly on the distal part of the nephron (125, 126).

Studies of amiloride in the toad bladder (127, 128) are suggestive of the nature of the mode of action of these compounds. Amiloride blocks sodium transport in the toad bladder at very low (10^{-6}M) levels. It is effective only at the mucosal surface of the toad bladder cell. The mucosal surface is equivalent to the luminal surface of the renal tubule. Since active transport of sodium occurs primarily at the serosal surface, a sodium pump would appear to be ruled out as a primary site of action. Sodium pool size of the toad bladder cells is decreased by amiloride (127). Amphotericin B, a polyene antibiotic, enhances permeability of the mucosal surface and abolishes the action of amiloride on sodium transport (127). These observations implicate an inhibitory effect on permeability of the luminal membrane.

Hypercapnea, or infusion of sodium bicarbonate, reverses the natriuretic and antiacidifying effect of triamterene and amiloride in the rat kidney (124). The potassium-sparing action remains unchanged. The interpretation offered for the existence of two apparently independent actions is as follows (124). Both amiloride and triamterene decrease the permeability to sodium and potassium at the luminal surface of the distal tubule. The change in permeability raises the potential difference across the luminal membrane. Potassium movements are inhibited by a decrease in both transtubular potential and membrane permeability. Hydrogen ion active secretion is intimately linked with a passive influx of sodium ions. An increase in bicarbonate greatly increases the availability of hydrogen ions, which in turn overcomes the antiacidifying effects of triamterene or amiloride. It is also postulated that drug modified permeability to potassium may be greater than that to sodium (124).

Physiological substances are found with apparent natriuretic action and have been investigated with newer experimental techniques. Prostaglandins have been found in significant concentration in the renal medulla but are absent in the renal cortex (129). These compounds are vasodepressor in action and stimulate smooth muscle (130). A role for prostaglandins has been postulated in the modulation of membrane adenyl cyclase activity (130). Prostaglandins will antagonize vasopressin induced adenyl cyclase activity (131). The mild natriuretic effects seen with administration of prostaglandins could result from vasopressin antagonism or from modulation of blood flow in the renal medulla. Vander (132) infused prostaglandin E_1 directly into the dog renal artery, and interpreted the resulting natriuresis as a direct action of the compound on renal tubular sodium reabsorption and not secondary to effects on renal blood flow or vasopressin.

The octapeptide angiotensin II has natriuretic activity most readily seen under conditions of expanded extracellular space (133). Angiotensin II inhibits sodium ion movements in kidney slices (134) but does not inhibit renal sodium-potassium dependent ATPase activity (135). Recent micropuncture studies indicate that it acts at the serosal surface of the distal tubule (but not on the proximal tubule) to inhibit renal tubular sodium reabsorption (136).

Efforts are currently being made to identify additional substances in the plasma with natriuretic activity that may be secreted in or become active in the salt loaded animal (137). In progressive renal disease the sodium excretion per nephron may be increased. The possibility has been raised that here too a humoral-like substance in the plasma may be present with natriuretic activity (138).

Some practical principles guided the selection in this portion of the review covering diuretics and cellular mechanisms in the kidney. Preference has been given in general to experimental observations following in vivo administration of a drug. When model systems such as tissue slices or isolated toad bladder have been studied, low dose drug effects (e.g. 10^{-6}M) are

more impressive than effects at high levels (e.g. 1 mM). In rare instances the specific methodology employed appeared to be faulty in the eyes of the reviewer.

Clinical pharmacology of newer diuretic agents.—Amiloride (MK-870) has been the subject of several clinical studies. In human subjects amiloride demonstrates a dose response relationship between 5 and 40 mg with a plateau above 40 mg (141). Osmolar clearance is increased, but no change is seen in free water clearance. In single dose studies (30 mg) amiloride causes potassium retention (142). The diuretic activity of amiloride has been compared in man with the activity of hydrochlorothiazide. Amiloride combined in treatment with hydrochlorothiazide reduces the thiazide induced loss of potassium. Both diuretics produce an antihypertensive effect. The natriuresis obtained with amiloride plus hydrochlorothiazide is greater than with either single drug (143). Hydrochlorothiazide is the more potent antihypertensive agent. Amiloride increased sodium, chloride, and water excretion in thirty-four patients with edema (144). Combination of amiloride and ethacrynic acid in six patients with edema resulting from liver cirrhosis and congestive heart failure has shown a significant reduction in the kaliuresis produced by the ethacrynic acid (144). Amiloride alone produced no significant natriuresis in these patients.

Clinical studies with clopamide (Brinaldex) demonstrated significant diuretic-natriuretic activity in six patients with congestive heart failure (145). After three months therapy a slight decrease in serum potassium was observed. Serum urate levels tended to rise, but no effect on blood glucose levels was seen. Clopamide was also found to possess antihypertensive activity (146).

Several carbonic anhydrase inhibitors have been studied in man. Benzolamide (CL 11,366) produced an increase in renal excretion of sodium, potassium, and bicarbonate (147). Plasma pH and bicarbonate levels decreased but no inhibition of red cell carbonic anhydrase was found. Clinical studies with mefruside (148) have demonstrated marked natriuretic activity with small increases in potassium excretion. No hyperglycemia was observed. Clorexolone was studied in essential hypertension (149). It reduced recumbent and standing blood pressure, plasma potassium, and chloride to an extent similar to that produced by hydrochlorothiazide, with which it was compared. Chlorexolone increased blood sugar but this was ameliorated by a potassium supplement.

Azido pyrimidine compounds are of interest because they are potent diuretics in rats but not in dogs. One such compound, 5-ethoxy ethyl-2-amino-4-amido-6-phenylpyrimidine (SC-16102), was studied in normal human volunteers (150). When given in a 100 mg oral dose a maximum diuretic effect was obtained in two hours. The pattern of diuresis is similar to that of hydrochlorothiazide. In the water loaded state the free water clearance in-

creased and this was interpreted as indicating that the site of drug action is predominantly in the proximal tubule.

Several other clinical studies are noted. Oral administration of hydrochlorothiazide in edematous patients has been compared with intramuscular meralluride for relative efficacy (151). The diuresis potential of hydrochlorothiazide is approximately one-half that of meralluride. The relation between the amount of edema and magnitude of diuretic response has also been investigated (152). With thiazide diuretics the degree of edema and the magnitude of diuresis appear to be unrelated. Diminished response with serial dosage is considered to be due to release of antidiuretic factors. The antihypertensive activity of furosemide on chronic administration in a study of twenty patients demonstrated significantly reduced supine and erect systolic and erect diastolic blood pressure with a 40 mg BID dose of the drug (153). No significant toxicities were encountered.

The nicotinic acid derivative, triflocin [4-(α,α,α-trifluoro-m-toluidino)-nicotinic acid] has been shown to possess potent diuretic activity in experimental animals (154). Triflocin, which represents a new chemical class of diuretics, was discovered during the testing of flufenamic acid derivatives for antiinflammatory activity. The diuresis-natriuresis produced by triflocin is similar in magnitude to that of furosemide. The diuretic is effective in the rat, rabbit, guinea pig, dog, and monkey. Diuretic activity remains in the face of metabolic acidosis or alkalosis.

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