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The Stimulation of Sodium Transport by Aldosterone

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The stimulation of sodium transport by aldosterone

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Aldosterone, the major sodium retaining hormone in man, will stimulate active transport of sodium across the urinary bladder of the toad, *Bufo marinus in vitro*, at physiological concentrations of the hormone. The *in vitro* action of aldosterone is mimicked by steroid hormones with known mineralocorticoid properties and it is competitively inhibited by other analogues, e.g. spironolactone and cortisone. Aldosterone is bound to physiological receptor sites within the transporting epithelial cells, chiefly within the nuclei, and is displaced from these binding sites specifically by structural analogues including other mineralocorticoids. Effects of aldosterone are dependent upon availability of metabolizable substrates to support the active transport of sodium. Although the stimulation of sodium transport by aldosterone can be specifically inhibited by actinomycin D, an inhibitor of RNA synthesis, and by puromycin, an inhibitor of protein synthesis, direct evidence of stimulation of new RNA and protein synthesis during the latent period with physiological concentrations of aldosterone is still lacking. It is possible, however, that the amounts of RNA and protein that are involved are too small to be detected by available techniques.

Evidence is summarized which leads us to conclude that the increased sodium transport induced by aldosterone is the consequence of a reduced resistance of the apical plasma membrane of the transporting epithelia to the entry of sodium into the transport pathway.

Introduction

Present developments in mineralocorticoid physiology stem from the late 1920s when the value in adrenal insufficiency of replacement therapy with sodium salts was first recognized by Marine & Bauman (1927). In 1930 adrenal extracts with the ability to prolong life in patients with Addison's disease or adrenalectomized animals were prepared by Hartman, Brownell & Hartman (1930), and by Swingle & Pfiffner (1930) and shortly afterwards Loeb (1932, 1933) and Harrop *et al.* (1933*a*, *b*, *c*) defined the urinary sodium wasting which occurs in adrenalectomized animals and in Addisonian patients. Aldosterone, the most active sodium retaining factor from the adrenal cortex, was discovered by Simpson *et al.* in 1953 and also by Mattox, Mason & Albert (1953*a*, *b*) and Leutscher & Johnson (1954).

In the mammal direct physiological effects of aldosterone have been established in the kidneys, intestines, sweat glands and salivary glands, all of which reabsorb sodium salts. Although such oriented transtubular or transepithelial transport of sodium may utilize the same active transport mechanisms that are continuously functioning to extrude sodium from all cells to maintain normal low concentrations of intracellular sodium, little evidence is available that aldosterone affects this universal cellular transport mechanism. A limitation of the action of aldosterone to oriented transepithelial transport systems may indicate that aldosterone does not act on the active component of the transport system, which is probably uniformly similar, but rather on those modifications responsible for the directed transepithelial transport—a thesis we will try to support.

Although there have been many informative studies on the action of aldosterone on kidneys, intestine, sweat and salivary glands of the whole animal, impetus to examine the mechanism of action of this hormone came with the demonstration by Crabbé (1961) of an effect of aldosterone *in vitro* on sodium transport in the toad bladder.

The urinary bladder of the toad

In the toad the urinary bladder actively reabsorbs sodium from the urine. The functional epithelium is a single layer of cells lining the urinary surface. Two major cell types are present; granular cells and mitochondrial-rich cells; the former comprising some 85 % and the latter, 10 % of the cells of this layer. Basal cells, intercalated between the granular cells and thin basement membrane, and scattered goblet cells are also present (Choi 1963; Keller 1963). Histological studies (DiBona, Civan & Leaf 1969) have demonstrated that every cell exposed to the urinary or mucosal surface also touches the basement membrane so that functionally this mucosal epithelium is a single layer of cells. This mucosa is supported on a loose submucosa of connective tissue containing bundles of smooth muscle and capillaries and lined on its contramucosal surface by a fine serosa. Sheets of this tissue may be obtained for transport studies using the short circuit technique of Ussing & Zerahn (1951) or each hemibladder may be studied as a closed bag (Bentley (1958).

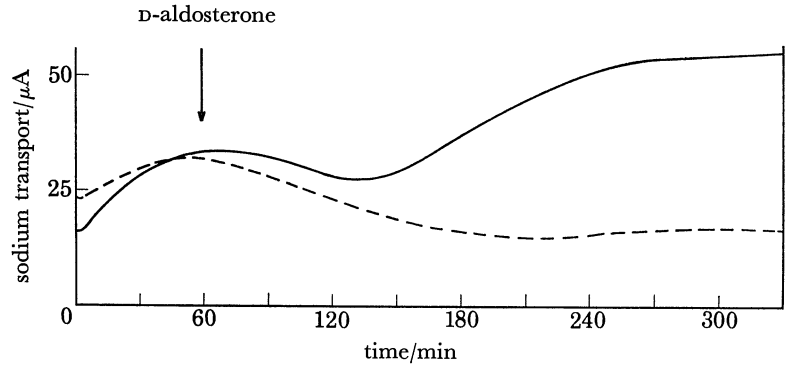

Figure 1. Stimulation of sodium transport by aldosterone at 3.6×10^{-9} mol/l (Sharp & Leaf 1964).

Stimulation of sodium transport by aldosterone

Figure 1 shows a typical response of the toad bladder to aldosterone added *in vitro* to the medium bathing the bladder. After a latent period of 30 to 120 min a gradual increase in short-circuit current occurs in the tissue exposed to aldosterone. This increase of short-circuit current after aldosterone has been shown to result from an equivalent increase in net sodium transport from mucosal to serosal surfaces of the bladder (Crabbe 1961).

The lowest concentration at which aldosterone has been found effective is 3.3×10^{-10} mol/l and maximal responses are obtained with concentrations of D-aldosterone close to 10^{-7} mol/l. Other related sterols have been found to stimulate sodium transport in this tissue: cortisol, corticosterone, cortexolone, 18-hydroxycorticosterone, 9α-fluorocortisol, deoxycorticosterone, prednisolone, 2-methyl-9α-fluorocortisol and 2-methyl cortisol. Progesterone, testosterone, 17β-estradiol and iso-aldosterone are without stimulatory effect. Cortisone was without effect on sodium transport even at concentrations as high as 10^{-4} mol/l (Sharp & Leaf 1964). That cortisone itself is inactive supports the view that cortisone must be converted to cortisol to have biological activity. Certain analogues of aldosterone, such as progesterone and spironolactone, were found to antagonize the action of aldosterone. This antagonism is shown in figure 2.

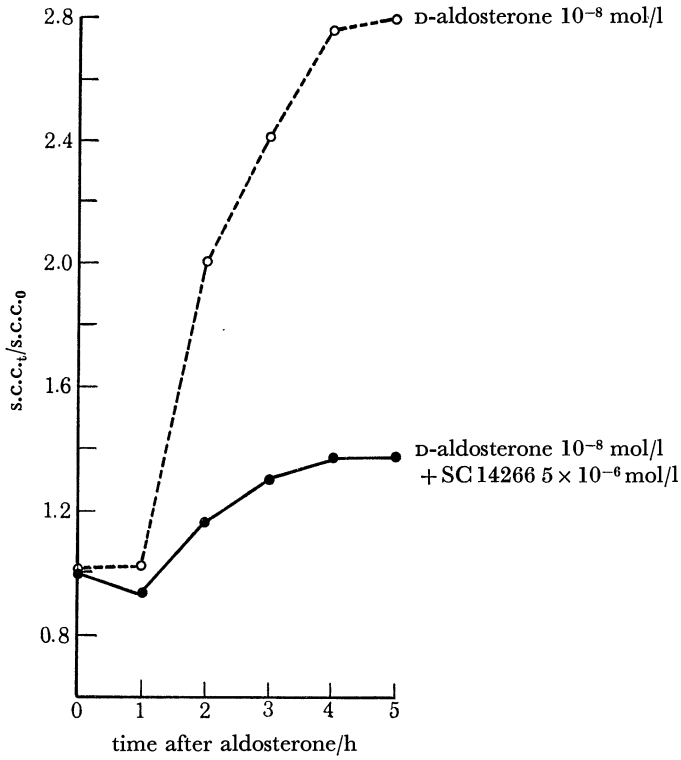

FIGURE 2. Effect of spironolactone on stimulation of sodium transport by aldosterone. Results are plotted as the short-circuit current at any particular time (s.c.c.$_t$) divided by the short-circuit current at time zero when the aldosterone was added. $n = 5$; $p < 0.05$ at 3, 4 and 5 h. (Alberti & Sharp 1970.)

Role of substrates

Since the action of aldosterone is to stimulate the active transport of sodium it might be expected that its effect would be dependent upon the availability of metabolizable substrates to supply the energy. When aldosterone is added to a bladder shortly after its removal from the animal, the expected stimulation of sodium transport occurs after the usual latent period. Addition of substrate; glucose, pyruvate or acetoacetate, to such fresh tissue has no effect on the hormonal response (Sharp & Leaf 1965). On the other hand, if the tissue is incubated in the absence of substrate for 12 to 24 h the subsequent addition of aldosterone will cause little or no stimulation of sodium transport until substrate is added to the incubating medium (Edelman, Bogoroch & Porter 1963). Sodium transport will then increase almost immediately and the hormonal effect is evident, tissue similarly incubated but in the absence of aldosterone shows no response to the addition of substrate alone (Sharp, Lichtenstein & Leaf 1965). Thus either the absence of aldosterone or the restricted availability of substrate as an energy source may be rate limiting on sodium transport.

Any of those substrates which readily yield acetyl-coenzyme A in the course of their metabolism serve as effective energy sources to support the aldosterone stimulation of sodium transport. Thus glucose, lactate, pyruvate, acetoacetate, and β-hydroxybutyrate are effective while most other substrates including acetate and members of the tricarboxylic acid cycle prove ineffective. In the case of the latter low rates of penetration into the cells of the bladder is the probable reason for their ineffectiveness since the mitochondria isolated from the mucosal

epithelial cells can readily oxidize succinate despite the inability of this substrate to support sodium transport when added to the medium bathing the intact tissue.

That these substrates act solely by supplying energy for sodium transport is indicated by two kinds of observations: (1) Removal of sodium from the mucosal surface of the aldosterone-treated bladder will, of course, stop sodium transport and simultaneously prevents the expected increase in metabolism of substrates. (2) Using ^{14}C-labelled pyruvate and acetoacetate it has been shown that the ratio of the increment in sodium transport to the increased substrate utilized yields a value of 2.7 sodium ions transported per high energy phosphate bond generated; a figure in good agreement with the energy requirements for sodium transport previously obtained from measurements of oxygen consumption by this tissue (Sharp & Leaf 1965).

Binding of aldosterone

The uptake of a steroid hormone by a tissue is likely to be a complex process. In its simplest analysis the uptake must consist of the hormone that is bound or adsorbed in the tissue and the hormone that accumulates because of favourable solubility conditions within certain phases of the tissue. Since not all the hormone taken up by the tissue is likely to be physiologically active, measurement simply of the total uptake could be misleading. If tissue is first equilibrated with low concentrations of [^{3}H]D-aldosterone and then exposed to a large excess of nonradioactive d-aldosterone a small amount of the bound [^{3}H]D-aldosterone is displaced from the tissue (Sharp, Komack & Leaf 1966). Actually two binding sites are revealed but one of high affinity which binds only 9×10^{-14} mol of aldosterone per gram of tissue seems unrelated to the physiologic effects of the hormone since it is fully saturated at concentrations of aldosterone which are too low to elicit a hormonal effect. A set of binding sites with lower affinity binds 3×10^{-12} mol of aldosterone per gram of tissue and seems to be the physiological binding sites for the hormone for the following reasons: (1) Bladder mucosal cells contain these binding sites but they are not demonstrable in tissues which are not engaged in transepithelial transport of sodium, i.e. skeletal muscle, myocardium, serosa, lung. (2) The correspondence of the physiological concentration range of the hormone with the degree of saturation of the binding sites is satisfactory. (3) Mineralocorticoids, in addition to aldosterone, and certain structural analogues of aldosterone competitively displace [^{3}H]D-aldosterone from the sites whereas inactive steroids do not. Aldosterone, deoxycorticosterone, cortisol, spironolactone, and progesterone all displace aldosterone, and their displacing ability is of the order to be expected from their activity in the tissue. Cholesterol and testosterone which have no detectable effect on sodium transport were without effect on binding.

Once the binding of aldosterone in the tissue was established, the rate of interaction of the hormone with the binding sites could be determined. The results indicate that within 30 min the binding was complete for each concentration of aldosterone tested. Thus the latent period between addition of aldosterone and onset of the hormonal effect on sodium transport cannot be entirely accounted for on the basis of the time required for the hormone to reach its binding sites.

The location of the receptor sites for the hormone was sought by Fanestil & Edelman (1966*a*) who found that 15 min following the injection of [^{3}H]D-aldosterone into the rat the nuclear fraction contained the most label and the binding in the nuclear fraction was saturable. They showed the physiological significance of the nuclear receptors by demonstrating that

9α-fluorocortisol, a mineralocorticoid, displaced [^{3}H]D-aldosterone from the nuclear receptors whereas 17β-oestradiol, which is inactive on sodium transport, did not. Further studies showed that treatment of prelabelled nuclei with proteolytic enzymes, but not with deoxyribonuclease, ribonuclease or lipase, released [^{3}H]D-aldosterone from the nuclei and suggested that the receptor sites were protein.

More recently, [^{3}H]D-aldosterone bound as a macromolecular complex has been isolated by Herman, Fimognari & Edelman (1968) from rat kidney nuclei. The macromolecular complex appears to be protein and the stability of the complex seems to be dependent upon the receptor-SH groups as the complex is dissociated by treatment with *p*-hydroxy mercuribenzoate.

In experiments to determine the site of aldosterone binding in toad bladder, 'displaceable' aldosterone binding was not found in the mitochondria or microsomes. Bound aldosterone, as defined by the competitive displacement technique, was detected in purified nuclei (Ausiello & Sharp 1968). 6.5×10^{-14} mol of [^{3}H]D-aldosterone per 100 μg DNA were calculated to be bound in the nuclei.

Further studies (Alberti & Sharp 1969) showed that the bound aldosterone could be extracted from the nuclei by sonication and then exists in the supernatant of the sonicated material as macromolecular complexes. Susceptibility to destruction by proteolytic enzymes suggest that these macromolecular complexes are primarily protein. The supernatant from the sonicate of nuclei reveal at least three sets of sites as judged by their rates of dissociation, by displacement studies and by agarose gel chromatography. A rapidly dissociating fraction and a stable fraction can be detected by time course studies with the stable fraction separable into two components by agarose column chromatography. The mineralocorticoid binding sites were attributable on the basis of displacement studies to the rapidly dissociating complex; the binding to the stable fractions failed to show saturability or displaceability.

In a separate study the binding of [^{3}H]cortisol was compared with that of [^{3}H]D-aldosterone. As aldosterone is thought to stimulate sodium transport maximally in toad bladder at 10^{-7} mol/l while cortisol is approximately 50 times less active, cortisol would be expected to occupy only a small percentage of the sites at 10^{-7} mol/l. The results showed that little [^{3}H]cortisol was bound to the rapidly dissociating complex, but did show binding to the non-displaceable non-mineralocorticoid sites. [^{3}H]D-Aldosterone, on the other hand, was bound to both the stable and unstable components. Interestingly aldosterone can be bound to the macromolecular complex in the supernatant fraction of toad bladder homogenates *in vitro* but this binding is non-displaceable, dissociates rapidly on removal of the free [^{3}H]D-aldosterone, and does not show saturation at aldosterone concentrations up to 10^{-5} mol/l. To obtain binding to the physiologic sites aldosterone apparently must be added while the responsive cells are still intact.

Aldosterone and RNA synthesis

Knowing that aldosterone binds primarily in the nuclei of responsive cells still leaves unanswered how it effects its physiological action which is presumed to be on the transfer of sodium across the plasma membranes of these responsive cells. Edelman *et al.* (1963) and Williamson (1963) have postulated an affect of this hormone to stimulate DNA-dependent synthesis of ribonucleic acids (RNA). This hypothesis was initially suggested by the seemingly specific inhibition of the aldosterone effect by actinomycin D, a known inhibitor of DNA-mediated RNA synthesis. This effect of actinomycin D is illustrated in figure 3. This evidence for an

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action of aldosterone via RNA synthesis has been supported by reports of increased labelling of RNA in toad bladder and rat kidney with radioactive precursors following application of aldosterone to these tissues. (DeWeer & Crabbe 1968; Edelman *et al.* 1963; Fimognari, Porter & Edelman 1967; Forte & Landon 1968; Rousseau & Crabbe 1968). A more recent study of this subject by Vancura, Sharp & Malt (1971), however, has failed to substantiate earlier findings of enhanced synthesis of RNA in response to aldosterone. This latest study utilizes refinements in isolation, separation and quantitation of RNA and its labelled moieties from

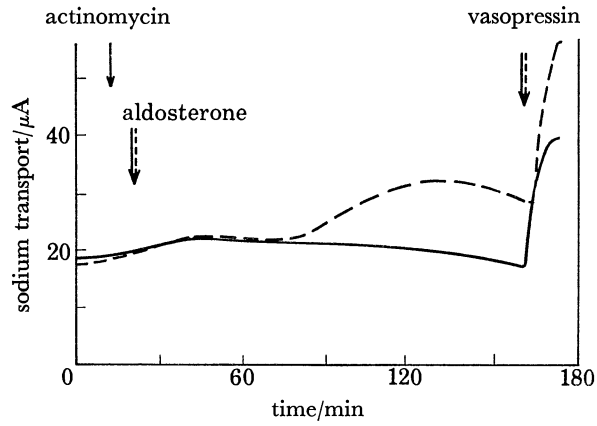

FIGURE 3. Effect of actinomycin D (10^{-6} mol/l) on the stimulation of sodium transport by aldosterone. It can be seen that the response to aldosterone (1.8×10^{-7} mol/l) is eliminated whereas the effect of vasopressin (0.5 unit) to stimulate sodium transport is unaffected by the actinomycin D (Sharp & Leaf 1966.)

toad bladder and yet failed to find any increase in RNA or its synthesis during the latent period, in any fraction of RNA isolated from toad bladder following exposure to physiological amounts of aldosterone either *in vitro* or *in vivo*. The earlier reports they attribute to: (1) excessive concentrations of aldosterone which might have stimulated other processes than those responsible for enhanced sodium transport; (2) secondary effects resulting from the stimulation of sodium transport after the latent period; (3) contamination with bacterial RNA when adequate precautions to avoid growth of bacteria during the *in vitro* incubation of tissue with aldosterone were neglected. The hypothesis that aldosterone acts via stimulation of DNA-dependent RNA synthesis thus still rests uneasily on its conceptual attractiveness and on the uncertain interpretation of the effects of inhibitors. It is still possible, however, that aldosterone could have an action on RNA synthesis that is small and below the sensitivity of current techniques of detection.

ALDOSTERONE AND PROTEIN SYNTHESIS

It was postulated by Porter, Bogoroch & Edelman (1964) that the RNA produced in response to aldosterone in turn stimulated synthesis of protein which mediated the physiological action of the hormone. Again this hypothesis can be attractively supported with inhibitors of protein synthesis (Fanestil & Edelman 1966*b*). Figure 4 shows the seemingly specific inhibition by puromycin of the stimulation of sodium transport by aldosterone. Other known inhibitors of protein synthesis have a similar effect. However, attempts to demonstrate directly new synthesis of protein or increased incorporation of amino acids into proteins in response to aldosterone have thus far been unsuccessful. The attractiveness of this hypothesis through

analogy with the mechanism of action of other steroid hormones is considerable. Although it lacks direct proof the possibility remains that the amount of protein involved or the kinetics of its synthesis are such as to make its presence undetectable by present techniques in the small amounts of tissue available. There is as yet no information from which we can estimate the quantity of a protein that might be needed to enhance sodium transport by 30 to 100 % as occurs with aldosterone. In fact how the hypothetical protein or proteins might function in the transport mechanism for sodium remains a controversial issue.

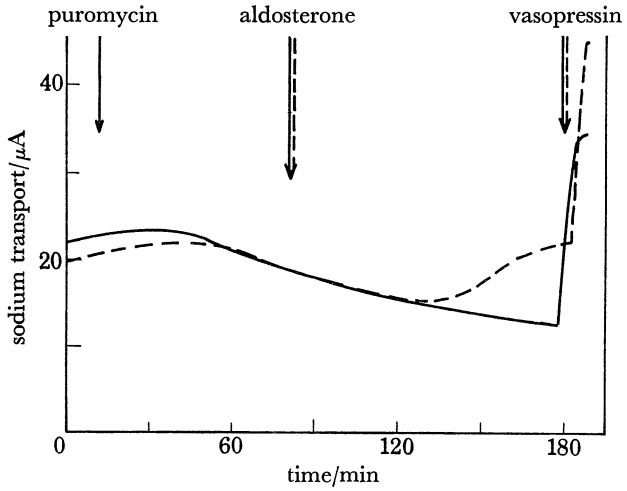

FIGURE 4. Effect of puromycin (9×10^{-6} mol/l) on the stimulation of sodium transport by aldosterone. It can be seen that the response to aldosterone (1.8×10^{-7}mol/l) is eliminated whereas the effect of vasopressin (0.5 unit) to stimulate sodium transport is unaffected by the puromycin (Sharp & Leaf 1966).

The possible role of a protein in the stimulation of sodium transport

Until the mechanism for active sodium transport is fully understood, it may be an idle exercise to speculate at what step aldosterone acts to enhance the overall process. However, a description of transepithelial transport of sodium can be provided although only in its gross aspects. This is depicted schematically in figure 5. Sodium is shown to move passively down its electrochemical gradient from urine or luminal medium across the apical plasma membrane of the transporting cells into the cells. Though this translocation of sodium is thought to be passive it is not by simple diffusion but requires specific interaction with a component or components of the apical plasma membrane (Leaf 1965). The sodium within the cell must then be pumped from cell interior across the lateral-basal plasma membranes out into the serosal medium. Since sodium apparently moves against both a chemical and electrical gradient in accomplishing this step of transport, it is here that the energy derived from metabolism is expended on sodium transport.

Aldosterone could affect the initial entry step or the extrusion process of sodium transport according to this formulation. If the extrusion process were the site of hormonal action there are at least two ways in which this could be enhanced: provision of more energy for the transport process or an increased number or activity of the transport mechanisms located at the basal and lateral borders of the cells.

The first attempts to measure the concentrations of high energy intermediates in the aldosterone-treated toad bladder failed to show changes in adenosine triphosphate or creatine

phosphate (Sharp & Leaf 1966). This has been reinvestigated by Handler, Preston & Orloff (1969*a*) with an improved method of extracting the high energy intermediates and they found a statistically significant reduction in creatine phosphate in the aldosterone treated as compared with the control tissues. This finding would exclude a primary effect of the hormone to provide more energy for the transport process. It is consistent with a primary enhanced activity of the transport process secondarily requiring more energy, thus causing a decrease in high energy intermediates which in turn stimulates energy metabolism.

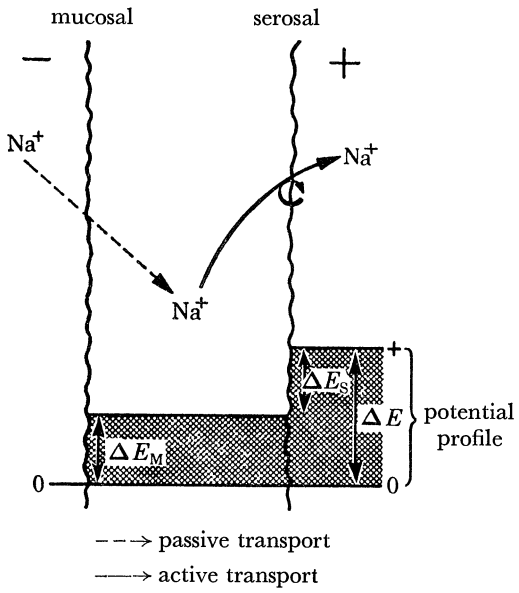

FIGURE 5. The current model of the mechanism of transepithelial transport of sodium by toad bladder 'electrogenic' sodium transport (Leaf 1964).

Whether the stimulation of the transport process results from an enhancement of the initial mucosal entry step in sodium transport or the final serosal extrusion step is still not established. Since aldosterone affects sodium transport only in cells engaged in transepithelial transport it seems likely that aldosterone modifies the properties of the epithelium which relate to the oriented transport. It is possible that it is the unidirectional facilitated transport of sodium from urine across the apical plasma membrane of the bladder that determines the orientation of sodium transport by this tissue. The serosal extrusion of sodium may partake of a process common to all animal cells. Sharp & Leaf (1966) have summarized their evidence that aldosterone acts primarily by enhancing the mucosal entry of sodium into the transporting cells. The evidence though indirect may be briefly stated as follows:

(1) When radioactive sodium is added to the medium bathing the mucosa of the bladder, there is an increase in radioactive sodium in the tissue concommitant with the stimulation of sodium transport across the bladder by aldosterone. A rise in sodium transport with an increase in the content of sodium in the tissue (if the tissue radioactivity in fact measures intracellular sodium) indicates an increased entry of sodium into the tissue as the primary effect of the hormone.

(2) The stimulation of substrate utilization (pyruvate) by aldosterone is entirely dependent upon availability of sodium in the mucosal medium.

(3) This last point is further strengthened by the demonstration that another agent, amphotericin B which allows sodium from the mucosal medium to enter the tissue by producing a nonspecific increase of permeability of the apical plasma membrane, enhances sodium transport and also the utilization of just the substrates (pyruvate, lactate, glucose, acetoacetate and β-hydroxybutyrate) which are effective in increasing sodium transport in the presence of aldosterone.

(4) Hoffman & Civan (1971) recently observed that a fall in electrical resistance across the bladder, averaging 18 %, regularly accompanies the increase in sodium transport stimulated by aldosterone. The increase in sodium transport that follows addition of pyruvate to the medium of aldosterone treated, but substrate depleted bladders, on the other hand, was not accompanied by a detectable change in electrical resistance of the tissue. These measurements of electrical resistance are most readily explained by an action of aldosterone to increase the rate of sodium movement across the apical surface of the epithelial cells.

Other metabolic effects of aldosterone

One intriguing effect of aldosterone is the inhibition of $^{14}CO_2$ release from [1-^{14}C]glucose. This inhibition of the hexose monophosphate shunt pathway was demonstrated in toad bladder at a time when the activity of the Embden–Meyerhof pathway was increased due to the raised energy requirement for stimulated sodium transport (Kirchberger, Martin, Leaf & Sharp 1968) Unlike the effects of aldosterone on other parameters of metabolism which have been examined, i.e. oxygen consumption, glucose, pyruvate and acetoacetate utilization, inhibition was not secondary to the increased rate of transport as it occurs even in the absence of sodium in the mucosal medium. Because of the primary nature of this effect, studies have been performed to test the relationship between the effects of aldosterone on glucose metabolism and sodium transport, respectively. Both effects have the same time course, concentration dependence, and steroid specificity. Both are abolished by actinomycin D. Aldosterone and deoxycorticosterone are more potent in both effects than hydrocortisone so that the inhibition of the shunt pathway appears to be related to the stimulation of sodium transport. Spirolactones, thought to be relatively specific antagonists of mineralocorticoids inhibit this effect on glucose metabolism (Sharp, unpublished observations), although in one report (Handler, Preston & Orloff 1969*b*) inhibition of the pentose cycle by dexamethasone was thought to be blocked by spironolactone; a finding which we have not been able to confirm.

Recently it has been shown that an inhibitor of sodium transport, amiloride, which blocks entry of sodium into the transporting cells when applied to the mucosal medium reverses the effect on glucose metabolism of aldosterone (Rebecca Leaf, unpublished data). Ouabain, as an example of an inhibitor of sodium transport which exerts its effect at the serosal surface of the epithelial cells, also abolished the effect of aldosterone on sodium transport but without affecting the $^{14}CO_2$ released from [1-^{14}C]glucose. This again suggests that aldosterone affects both sodium transport and glucose metabolism at a site near or in the apical plasma membrane of the transporting mucosal epithelial cells.

One explanation of the similarity of effects of mineralocorticoids on sodium transport and on the hexose monophosphate shunt pathway is that a common precursor exists which is involved in both actions. Identification of such a precursor would be a valuable addition to our knowledge.

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