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REVIEW

Microscopical methods for the localization of Na^+, K^+-ATPase

STEPHEN A. ERNST and SETH R. HOOTMAN

Department of Anatomy, University of Michigan, Medical Sciences II, Ann Arbor, MI 48109, U.S.A.

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Summary

Na^+, K^+-ATPase plays a central role in the ionic and osmotic homeostasis of cells and in the movements of electrolytes and water across epithelial boundaries. Microscopic localization of the enzyme is, therefore, of crucial importance in establishing the subcellular routes of electrolyte flow across structurally complex and functionally polarized epithelia. Recently developed approaches to the localization of Na^+, K^+-ATPase are reviewed. These methods rely on different properties of the enzyme and encompass cytochemical localization of the K^+-dependent nitrophenylphosphatase component of the enzyme, autoradiographic localization of tritiated ouabain binding sites, and immunocytochemical localization of the holoenzyme and of its catalytic subunit. The rationales for each of these techniques are outlined as are the critieria that have been established to validate each method. The observed

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localization of Na^+, K^+-ATPase in various tissues is discussed, particularly as it relates to putative and hypothetical mechanisms that are currently thought to mediate reabsorptive and secretory electrolyte transport.

Introduction

Among the many adenosine triphosphatase (ATPase) activities described in the literature, sodium- and potassium-activated ATPase (Na^+, K^+-ATPase; EC 3.6.1.3) has received considerable attention since its initial characterization in crab nerve homogenates (Skou, 1957). It is now established that this enzyme provides the catalytic machinery for the coupling of the energy released by the hydrolysis of ATP to the exchange of Na^+ for K^+ across plasmalemmal surfaces against their respective concentration gradients. The enzyme thus plays a key role in the maintenance and regulation of a constant internal milieu in eukaryotic cells and, in many epithelia, has the additional function of driving transepithelial movements of electrolytes and, secondarily, water. The enzyme also has been shown to provide a potential energy store within the cell in the form of a low intracellular Na^+ concentration which can be used to facilitate the uphill transport of certain solutes (for example, amino acids, glucose, Cl^-) by coupling their movement to the downhill Na^+ diffusion gradient. Finally, the enzyme plays an essential role in propagation of action potentials in contractile and excitable tissues.

With the foregoing in mind, it is not surprising that considerable effort has been devoted to developing methods for the localization of the enzyme in intact tissues. Such methods have the potential advantage over cell fractionation techniques of showing not only cellular distribution and relative amounts of the enzyme within tissues, an objective of particular importance in epithelia composed of heterogenous cell populations, but also of selectively localizing sites of Na^+, K^+-ATPase activity to specific plasmalemmal surfaces in functionally polarized cell types. A further objective, which to date has not been obtained, is the mapping of intracellular pathways involved in the translocation of newly synthesized enzyme to its insertion in the plasma membrane as an integral protein.

Cytochemical localization of Na^+, K^+-ATPase

The Wachstein & Meisel procedure and its modifications

Initial attempts to localize Na^+ pumps cytochemically centred about the Wachstein & Meisel (1957) technique, which adapted the classical Gomori-type heavy metal capture reaction (Gomori, 1952). Using ATP as substrate and lead as the precipitating ion, this technique often showed distributions of reaction product (lead phosphate) that were superficially compatible with the distribution of Na^+, K^+-ATPase as expected on the basis of physiological and biochemical data (for example, Novikoff *et al.*, 1962; Ashworth *et al.*, 1963; Farquhar & Palade, 1966;

Ernst & Philpott, 1970; Majack *et al.*, 1979). However, despite the fortuitous nature of these localizations relative to putative sites of Na^+ pump activity, results from further biochemical investigations showed that this plasmalemmal ATPase and Na^+, K^+-ATPase were separate entities since it did not co-purify with Na^+, K^+-ATPase in cell fractionation studies (Hokin *et al.*, 1973). In addition, the two enzymes displayed different kinetic properties (Ernst *et al.*, 1967) and sensitivity to fixatives (Ernst & Philpott, 1970), and in cytochemical studies, reaction product deposition in tissue incubated in the Wachstein & Meisel medium was not affected by the addition of ouabain or by omission of Na^+ or K^+ (Novikoff *et al.*, 1961; Farquhar & Palade, 1966; Tormey, 1966). Moreover, precipitates attributable to Mg^{2+}-ATPase activity were invariably localized to the external surface of the plasma membrane, the side opposite to that of Na^+, K^+-ATP phosphohydrolase activity (Ernst, 1972b, 1975). The function of this background ATPase, let alone its specificity, remains unclear. For example, whereas kidney distal and collecting tubular ATPase required the presence of a divalent cation, staining of microvilli and, to a lesser extent, basolateral plasma membrane folds of proximal tubules with ATP as substrate appeared independent of Mg^{2+} (Ernst, 1975). Ca^{2+} was shown to stimulate the proximal tubule staining reaction and to restore the distal and collecting tubule staining in the absence of Mg^{2+} (Novikoff *et al.*, 1961; Ernst, 1975). Haussler *et al.* (1970) have suggested on the basis of biochemical data that alkaline phosphatase, Ca^{2+}-ATPase, and much of the Mg^{2+}-ATPase of proximal tubular microvilli are manifestations of the same enzyme. Such a situation may obtain along other plasma membrane surfaces as well. The apparent absence of specific divalent cation requirements thus further complicates interpretation of the functional role(s) of this enzymatic activity (see Firth, 1978 and 1980, for further critical discussion).

Attempts at modifying the Wachstein–Meisel technique in order to demonstrate sites of Na^+, K^+-ATPase activity have centred on alteration of lead and ATP concentrations and ratios in the incubation medium, and addition of either activating cations or ouabain or both to resolve the monovalent cation-activated component. Results gained from this approach have been either equivocal or unsuccessful (Farquhar & Palade, 1966; Tormey, 1966; Jacobsen & Jorgensen, 1969), a situation that has accordingly prompted critical review of the technique itself (see Ernst, 1972b for pertinent references and discussion). Although the outcome of this controversy seems to be a cautious acceptance of the procedure for demonstrating Mg^{2+}-ATPase activity under well-controlled conditions (Novikoff, 1970; Rosenthal *et al.*, 1970; Pearse, 1972), reliable localization of Na^+, K^+-ATPase by a suitable modification of the method appears to be precluded by the potent inhibitory effect of lead on the enzyme's activity (K_i = 0.1 mM; Jacobsen & Jorgensen, 1969), and by the necessity of resolving cytochemically a Na^+- and K^+-activated component separate from the Mg^{2+}-dependent background ATPase in the face of as much as a six-fold higher sensitivity of Na^+, K^+-ATPase to lead (Jacobsen & Jorgensen, 1969). The situation is further complicated by the absence of a suitable specific inhibitor of Mg^{2+}-ATPase

activity. With these considerations in mind, suggestive localizations of Na^+, K^+-ATPase by modifications of the Wachstein–Meisel procedure, even when carefully performed as in the studies of Tervo *et al.* (1977) and Palva *et al.* (1978), must be interpreted with caution (see, for example, Tormey, 1966). Minimally, biochemical assays of enzymatic activity under cytochemical conditions are required to demonstrate a residual Na^+, K^+-ATPase of sufficient amount to account for cytochemical depositions that are interpreted as demonstrating sites of ouabain-sensitive and monovalent cation-activated ATPase activity.

K^+-NPPase cytochemistry

Recognition of the problems inherent in use of the Wachstein–Meisel technique for localization of Na^+, K^+-ATPase activity led to the development of a new, methodological approach (Ernst, 1972a,b). The enzymatic basis for this technique was patterned after the initial biochemical characterization of a K^+-dependent, ouabain-sensitive phosphatase activity in erythrocyte ghosts by Judah *et al.* (1962). Numerous subsequent studies have established that this activity represents a partial reaction that is basically equivalent to the K^+-dependent dephosphorylation step of the complete Na^+, K^+-ATPase reaction sequence (see Ernst, 1972a, for pertinent references). Although the identity of this activity with the Na^+, K^+-ATPase complex is established, the actual reaction mechanism is not fully understood (see reviews by Glynn & Karlish, 1975, and Robinson & Flashner, 1979). The cytochemical method was designed initially to provide quantitative data on enzymatic activity under varying cytochemical conditions simultaneously with *in situ* localization of the enzyme. For this purpose, *p*-nitrophenylphosphate (NPP) was chosen as substrate, since hydrolysis of the phosphate ester bond in this compound yields both inorganic phosphate, which can be precipitated by heavy metal capture ions, and the coloured nitrophenolate ion, which can be assayed spectrophotometrically. Strontium was chosen as a suitable capture ion since its inhibitory effect on the transport enzyme was much less than that of lead at equivalent concentrations, and since it proved to precipitate the released phosphate ions efficiently at alkaline pH. Seminal studies with the NPPase procedure were carried out on tissue sections of avian salt gland (Ernst, 1972a,b), an organ that is notable for its very high level of Na^+, K^+-ATPase activity (Ernst *et al.*, 1967). These investigations established a kinetically sound cytochemical medium in which the specificity of the enzymatic reaction for K^+- and Mg^{2+}-activation and for ouabain inhibition was demonstrated by close correlation of spectrophotometric and cytochemical data. The studies were carried out with tissue fixed with 2 or 3% formaldehyde, prepared from paraformaldehyde, since previous biochemical studies had showed that Na^+, K^+-ATPase was not unduly inhibited by this monoaldehyde (Ernst & Philpott, 1970). Subsequent studies (Ernst, 1975) have demonstrated that inclusion of low concentrations of glutaraldehyde (for example 0.25%) in 1% formaldehyde fixative solutions results in superior morphological preservation while maintaining adequate enzymatic activity.

Localization of Na^+, K^+-ATPase activity via the enzyme's K^+-NPPase partial reaction has now been accomplished in a wide variety of tissues (see Table 1 in Ernst & Mills, 1980, for list and references). The cytochemical results in these studies have been basically consistent, localizing enzymatic activity in most cases to basolateral plasma membrane surfaces. Moroever, identical distributions of the enzyme have been demonstrated in several tissues using alternative methods (see below), thereby lending further credence to the validity of the technique.

Despite the apparent success of the K^+-NPPase procedure in localizing sites of Na^+, K^+-ATPase activity, this cytochemical technique is not without its drawbacks. One of the more notable problems inherent in the method is the strong inhibitory effect of Sr^{2+} on enzymatic activity (Ernst, 1972a; Firth & Marland, 1975), although this heavy metal is much less inhibitory than Pb^{2+} or Ca^{2+} (Jacobsen & Jorgensen, 1969; Ernst, 1972a). A related problem is that the alkaline pH required for efficient precipitation of phosphate is sub-optimal for enzymatic activity (Ernst, 1972a). To some extent, both of these reductions in activity may be compensated for by the amplifying nature of the reaction in which precipitated phosphate is allowed to accumulate during the incubation. Thus, abundant reaction product could be localized in salt gland tissue after 20 min of incubation (Ernst, 1972b), despite nearly 85% inhibition of K^+-NPPase activity by 20 mM Sr^{2+} (Ernst, 1972a). The net effect, however, is to reduce the general sensitivity of the method, which may limit its utility to those tissues that exhibit relatively high Na^+, K^+-ATPase activities. A second concern is also related to the need to carry out the NPPase reaction at an alkaline pH in order to assure efficient trapping of released phosphate. At pH 8 and above, NPP is readily hydrolysed by non-specific alkaline phosphatase. This potential problem did not surface in the initial study with the avian salt gland (Ernst, 1972b), since the tissue exhibits negligible levels of alkaline phosphatase as judged by the absence of reaction product deposition when β-glycerophosphate (a substrate for alkaline phosphatase but not for K^+-NPPase) was substituted for NPP in the incubation medium. In other tissues such as mammalian kidney cortex (Firth, 1974; Ernst, 1975) and teleost branchial epithelium (Hootman & Philpott, 1979), however, alkaline phosphatase activity is abundant and presents a potentially substantial source of competition for substrate. Fortunately, alkaline phosphatase activity is subject to inhibition by several compounds that have minimal inhibitory effects on K^+-NPPase. These include cysteine (Ernst, 1975) and the family of anthelmintic drugs such as tetramisole and its analogues (Borgers, 1973; Firth & Marland, 1975; Hootman & Philpott, 1979). Alternatively, alkaline phosphatase can be differentiated from K^+-NPPase by its K^+-independence and ouabain insensitivity (Ernst, 1975). Although alkaline phosphatase and K^+-NPPase activities are localized to different plasmalemmal surfaces in kidney cortical tubules (Firth, 1974; Ernst, 1975), this is not necessarily the case in all tissues. The inclusion of appropriate alkaline phosphatase inhibitors in reaction media when attempting to localize sites of K^+-NPPase activity is, therefore, considered a prudent control. In general, higher

concentrations of cysteine (5–20 mM) than of tetramisole and its analogues (0.1–1 mM) appear to be necessary to achieve maximal inhibitory potency, although the use of cysteine may have the advantage of actually stimulating Na^+, K^+-ATPase activity, as has been reported in partially purified rabbit kidney enzyme preparations (Specht & Robinson, 1973). Independent studies on the localization of K^+-NPPase in liver have shown moderate enhancement of reaction product deposition in the presence of cysteine (Blitzer & Boyer, 1978; Latham & Kashagarian, 1979), although whether this results from a direct activation of the enzyme by the amino acid (possibly as a result of heavy metal chelation) or as a secondary consequence of increased substrate availability following alkaline phosphatase inhibition is at present unclear.

A final limitation of the nitrophenylphosphatase method for Na^+, K^+-ATPase localization is that the nature of the staining reaction precludes accurate quantification of enzyme sites. An interesting modification of the phosphatase method was recently introduced by Guth & Albers (1974), however, that has allowed enzyme activity to be assessed more quantitatively. In their studies, dimethylsulphoxide was included in the incubation medium, a procedure that shifts the pH optimum of the K^+-NPPase reaction to 9.0. Under these conditions, phosphate hydrolysed during the reaction remains within reactive cells without the necessity of an added heavy metal capture ion and may be visualized at the light microscopic level by post-incubatory treatment of tissue sections with $CoCl_2$. Beeuwkes & Rosen (1975) showed by electron probe microanalysis of sections of kidney incubated in the Guth & Albers medium that the phosphate liberated by K^+-NPPase was precipitated as a mixture of $KMgPO_4$ and $Mg(PO_4)_2$ and could be analysed with the microprobe to estimate enzymatic activity in a semi-quantitive fashion (see also Rosen & Beeuwkes, 1979). This approach, although promising, has only been reported in studies on the kidney and activity has only been resolved (visually or with the microprobe) in segments of the distal tubule where specific activity is high relative to other nephron segments (see Schmidt & Dubach, 1969; Ernst, 1975). Accordingly, the sensitivity of the method may be low and, despite the advantages of semi-quantification, morphological preservation is poor and the nature of the phosphate precipitation process precludes subcellular definition of reaction product distribution.

In a recent paper, Mayahara *et al.* (1981) presented a modification of the NPPase procedure wherein lead citrate was substituted for strontium as the capture reagent and dimethyl sulphoxide was included in the reaction medium to increase K^+-NPPase activity. A subsequent study (Mayahara & Ogawa, 1980) demonstrated K^+-dependent deposition of reaction product in the rat kidney with the distribution of reactive sites paralleling that resolved in the kidney with the original method (Firth, 1974; Ernst, 1975). This variation thus seems to provide a valid alternative technique for the localization of the transport enzyme, its advantage being one of slightly greater methodological simplicity. Since both the original procedure and this modification appear to localize sites of ouabain-sensitive, K^+-NPPase activity

adequately, the choice of procedure may rest on the relative sensitivity of the two methods. Studies comparing both the intensity and specificity of reaction product deposition in tissues fixed and incubated under identical cytochemical conditions in the strontium and lead citrate variations would be of obvious interest in this regard.

Experience gained in this laboratory as well as that garnered from several others where the K^+-NPPase procedure has been employed under carefully controlled conditions has suggested the following criteria for valid localization of Na^+, K^+-ATPase activity by this cytochemical method.

(1) Deposition of cytochemical reaction product must show an obligatory requirement for the presence of Mg^{2+} and K^+ and should be significantly inhibited by ouabain or other cardiac glycosides that are established inhibitors of Na^+, K^+-ATPase activity. Demonstration of reduction in K^+-dependent reaction product deposition by ouabain may be difficult, however, in certain rodent tissues, where the inherent sensitivity of Na^+, K^+-ATPase to ouabain is low (Allen & Schwartz, 1969). This problem may be compounded, additionally, by the reduced affinity of the enzyme for ouabain in the presence of Sr^{2+} (Ernst, 1972a). The species- and tissue-specific sensitivity of Na^+, K^+-ATPase to ouabain inhibition was illustrated in an earlier study of K^+-NPPase activity in rat kidney cortex (Ernst, 1975), where K^+-dependent reaction products were not reduced by ouabain, although identical localizations in the more sensitive rabbit kidneys were inhibited markedly.

(2) In addition to ion specificity and glycoside sensitivity, K^+-NPPase activity should be distinguishable from non-specific alkaline phosphatase which also hydrolyses NPP by the use of inhibitors such as cysteine (Ernst, 1975) or tetramisole and its analogues (Firth, 1974; Hootman & Philpott, 1979).

(3) Unlike precipitates visualized after application of the Wachstein–Meisel technique and its various modifications, reaction products attributable to K^+-NPPase activity should be localized to the cytoplasmic side of the plasma membrane in order to be consistent with biochemical data showing internal release of phosphate hydrolysed by Na^+, K^+-ATPase (Sen & Post, 1964). Most localizations with the method have shown this orientation of reaction product, although Leuenberger & Novikoff (1974) in rat cornea, and Milhorat *et al.* (1975) in choroid plexus resolved activity only on the extracellular side of the plasmalemma. In the latter case, the localization was different from that obtained with [^{3}H]ouabain (Quinton *et al.*, 1973) and in conflict with physiological data (Wright, 1972). Although these observed localizations may be due to a different enzymatic activity, reaction product deposition in both cases was K^+-dependent and ouabain-sensitive. Leuenberger & Novikoff (1974) suggested that the extracellular localization of reaction products in the cornea might be due to the presence of sites on the surface of the plasma membrane that 'seeded' precipitate formation. Why the sidedness of such a mechanism should differ from most other tissues is not apparent.

(4) The pattern of localization should be distinct from that obtained by

non-enzymatic means (Ernst, 1972b, 1975), since inefficient trapping of hydrolysed phosphate and non-specific binding of heavy metals to tissue components are notorious artefacts accompanying heavy metal-capture phosphatase cytochemistry.

(5) Preservation of tissue ultrastructure must be accomplished without excessive loss of K^+-NPPase activity. In general, good preservation of tissue cytoarchitecture results in a more clearly defined and membrane-specific localization of enzymatic activity. Although fixatives containing even moderate amounts of glutaraldehyde should be avoided, low concentrations (0.1–0.5%), particularly when added to formaldehyde fixative, have been quite successful in preserving both cellular morphology and enzymatic activity (Ernst, 1975; Hootman & Philpott, 1979). Surprisingly good results can be obtained with vascular perfusion of tissues with such a fixative, even for short time intervals (2–10 min).

Autoradiographic localization of Na^+, K^+-ATPase

Specificity of ouabain binding to Na^+, K^+-ATPase

The cardiotonic steroid, ouabain, is a highly specific inhibitor of Na^+, K^+-ATPase activity and has been shown to bind with very high affinity to Na^+, K^+-ATPase-enriched membrane fractions in a ligand-specific manner (Schwartz *et al.*, 1975; Akera, 1977). Binding of the glycoside to partially purified Na^+, K^+-ATPase preparations is supported by Na^+, Mg^{2+} and ATP, ligands that favour formation of the phosphorylated enzyme intermediate (Albers *et al.*, 1968; Matsui & Schwartz, 1968; Schwartz *et al.*, 1968). The requirement for these physiological ligands in promoting formation of the steroid–enzyme complex *in vitro* suggests that enzyme turnover is a prerequisite for binding. The ligand specificity of the ouabain–enzyme interaction extends to ouabain binding to intact cells (Ernst & Mills, 1980). Incubation of single cells, epithelial sheets, or tissue slices in physiological salt solutions under conditions that decrease intracellular ATP levels (for example, anoxia, cyanide, 2,4-dinitrophenol) significantly depresses binding of the tritium-labelled glycoside (Baker & Willis, 1972; Clausen & Hansen, 1974; Mills & Ernst, 1975; Mills *et al.*, 1977). Similarly, omission of Na^+ from the incubation medium, thereby reducing intracellular Na^+, reduces binding (Baker & Willis, 1972; Clausen & Hansen, 1974; Gardner & Frantz, 1974), as does exposure of cells to [^{3}H]ouabain at temperatures low enough to inhibit enzymatic hydrolysis of ATP (Hossler *et al.*, 1978; Shaver & Stirling, 1978). Specificity of binding is demonstrated further by showing that K^+, which is known to retard ouabain binding to Na^+,K^+-ATPase preparations, also depresses binding to intact cells (Quinton *et al.*, 1973; Ernst & Mills, 1977; Mills & Ernst, 1975; Mills *et al.*, 1977). Binding to intact cells in the presence of high concentrations of K^+ should not be considered non-specific, however, since the effect of K^+ is primarily on the rate of binding and not on the absolute amount bound at equilibrium (Gardner & Conlon, 1972; Ernst & Mills, 1977; Mills *et al.*, 1977). That ouabain specifically binds to and inhibits the

functioning enzyme in intact cells is supported additionally by the observation that the degree of inhibition of electrical transport parameters in frog skin (Mills *et al.*, 1977; Cala *et al.*, 1978), or K^+ influx in Hela cells (Baker & Willis, 1970) and red cells (Joiner & Lauf, 1978a,b), correlated closely with the extent of ouabain binding.

Autoradiography of [^{3}H]ouabain binding sites

The observations discussed above suggest that ouabain is ideally suited as a probe for the localization of functioning Na^+, K^+-ATPase complexes in intact cells and tissues. Visualization of sites of ouabain binding has been approached in two ways. Most studies have utilized the tritium-labelled glycoside in the autoradiographic procedure introduced and developed by Stirling (1972, 1976), although a promising cytochemical method that employs a haempeptide derivative of ouabain also has been reported (Mazurkiewicz *et al.*, 1978). In the following discussion, we will evaluate the advantages and cautions inherent in [^{3}H]-ouabain autoradiography before returning to this latter method and other potential means by which ouabain may be modified in order to facilitate its visual identification.

Ouabain, like most other steroids, is highly soluble in both acetone and ethanol, a circumstance that prevents the use of standard procedures for tissue dehydration prior to embedment. Stirling (1972, 1976) was able to circumvent this problem by freeze-drying tissue previously exposed to [^{3}H]ouabain solutions and then embedding them directly in epoxy resins. The technique itself and the criteria for establishing its specificity were reviewed in detail recently (Ernst & Mills, 1980) and will be covered only briefly here. Unfixed tissue slices (100 μm thick) or intact epithelial sheets are incubated in Ringer's solutions containing [^{3}H]ouabain at micromolar concentrations. Unbound labelled glycoside is extracted after suitable incubation periods by washing with label-free Ringer's and the tissue is then frozen in barely melted Freon, pre-cooled to -180° C in liquid nitrogen. The tissue is freeze-dried under vacuum at -70° C, with the sublimated water being trapped by a cold finger containing molecular sieve cooled in liquid nitrogen. The dried tissue is subsequently vapour-fixed with osmium tetroxide under vacuum and embedded, usually with Spurr low viscosity embedding medium. Autoradiographs are obtained in the routine manner employing 1 μm-thick sections and commercial liquid emulsions. With rapid freezing, tissue disruption due to ice crystal formation appears minimal when viewed at the light microscopic level.

The Stirling technique has been used to localize ouabain-binding sites in a wide variety of epithelial tissues (see Table 1 in Ernst & Mills, 1980). The autoradiographic data obtained has complemented physiological data and paralleled enzymatic distributions determined by biochemical and cytochemical analyses. For example, studies in the avian salt gland (Ernst, 1972b; Ernst & Mills, 1977) and in teleost gill (Hootman & Philpott, 1979) have demonstrated that the cell-specific and plasma membrane-specific localization of K^+-NPPase activity is identical to the distribution of [^{3}H]ouabain-binding sites in autoradiographs (Ernst & Mills, 1977, and Karnaky

et al., 1976, respectively), while in kidney tubules, the distribution of [^{3}H]ouabain-binding sites (Shaver & Stirling, 1978) is coincident with sites of Na^+, K^+-ATPase localization determined both by immunocytochemical methods (Kyte, 1976a,b) and by phosphatase cytochemistry (Firth, 1974; Ernst, 1975).

While the technique appears to be successful in faithfully localizing sites of Na^+, K^+-ATPase activity at the light microscopical level in most tissues, problems in demonstrating unequivocal localization may arise under some circumstances. The method requires that the glycoside be tightly bound to receptors so that unbound label may be extracted by washing. Although this requirement is met by most tissues, caution should be exercised since some tissues such as toad urinary bladder (Mills & Ernst, 1975) and rat kidney (Allen & Schwartz, 1969) bind ouabain in a relatively reversible manner. These same tissues in the frog and rabbit, respectively, exhibit correspondingly low dissociation constants and are thus amenable to autoradiographic analysis (Mills & Ernst, 1975; Shaver & Stirling, 1978). A second caution relating to the concentration of the glycoside employed is also of importance. Depending on the specific activity of Na^+, K^+-ATPase in the tissue under investigation, incubations generally are performed in Ringer's solutions containing 1–5 μCi/ml [^{3}H]ouabain and sufficient unlabelled inhibitor to reach concentrations in the range of 0.5–2.0 μM. Under these conditions, ouabain binding exhibits saturation kinetics as expected for a single class of high affinity binding sites (Ernst & Mills, 1977; Mills *et al.*, 1977; Shaver & Stirling, 1978). Higher concentrations of ouabain should be avoided, however, since a non-specific linear uptake of glycoside by cells has been reported to contribute significantly to total uptake and binding at elevated ouabain concentrations (Baker & Willis, 1972; Mills *et al.*, 1977; Widdicombe *et al.*, 1979). Such an uptake would presumably contribute little toward total binding at low inhibitor concentrations where the saturable binding component predominates (see Baker & Willis, 1970, 1972; Mills *et al.*, 1977). Providing that ouabain is tightly bound to its Na^+ pump receptor in the particular tissue of interest, non-specific uptake, if present, may be reduced to minimal levels by adequate washing of tissue samples (Ernst & Mills, 1977).

A major advantage of the [^{3}H]ouabain binding method for Na^+, K^+-ATPase localization that is not enjoyed by the K^+-NPPase procedure is the ease with which enzyme sites may be quantitated. Processing of paired tissue samples for autoradiography and liquid scintillation counting yields parallel data on Na^+ pump density and distribution, as well as information on Na^+ pump turnover under varying conditions of incubation (Karnaky *et al.*, 1976; Mills & DiBona, 1977, 1978; Eveloff *et al.*, 1979; DiBona & Mills, 1979). Morphometric analysis of grain distributions in autoradiographs coupled with biochemical determination of [^{3}H]ouabain bound may thus yield information not only on the relative density of Na^+ pump sites associated with specific cell types, but on their distribution to specific membrane surfaces as well. This advantage has been of particular importance in assessing the role of Na^+, K^+-ATPase in transepithelial electrolyte movements in

tissues such as frog skin (Mills *et al.*, 1977), teleost branchial epithelium (Karnaky *et al.*, 1976), and mammalian kidney (Shaver & Stirling, 1978) that are composed of a heterogeneous assortment of cell types.

Other methods for localizing ouabain binding sites

As mentioned previously, another method has been recently introduced that exploits the pharmacological specificity of ouabain for Na^+, K^+-ATPase (Mazurkiewicz *et al.*, 1978). This study employed a haempeptide derivative of ouabain, produced by conjugating haemundecapeptide to the oxidized rhamnose component of the parent glycoside. The peroxidatic activity of the peptide was used to localize binding sites for the altered glycoside to avian salt gland tissue slices at the electron microscopic level. Reaction product deposition, which appeared in the form of a slight enhancement of plasma membrane density, was distributed along the basolateral plasma membranes of secretory cells, a localization identical to that previously determined by the K^+-NPPase (Ernst, 1972b) and [^{3}H]ouabain (Ernst & Mills, 1977) procedures. This technique, therefore, seems to have extended the use of ouabain as a probe for Na^+ pump sites to the ultrastructural level, although the use of an amplifying peroxidative reaction for visualization of glycoside binding sites prevents their accurate quantitation.

Another analogue of ouabain that has, to our knowledge, not as yet been exploited for visualization of binding to intact cells, yet holds great promise in that regard, is the fluorescent derivative, anthroylouabain. This compound, synthesized by Fortes (1977), undergoes a significant enhancement of fluorescence at 470 nm during binding to purified preparations of Na^+, K^+-ATPase. The compound retains the inhibitory specificity and ligand requirements of the parent glycoside (Fortes, 1977; Moczydlowski & Fortes, 1980) and thus provides a means by which cardiotonic steroid interaction with the transport enzyme can be measured directly by fluorescence spectroscopy. The possibility, therefore, arises of continuously monitoring binding of the probe to the surfaces of single intact cells or epithelial sheets mounted in chambers designed for fluorescence microscopy. Although this technique would be confined to the light microscopical level, the advantages of direct and simultaneous visual and spectrophotometric analysis of binding kinetics to intact cells are obvious.

Immunocytochemical localization of Na^+, K^+-ATPase

An immunocytochemical approach to the localization of Na^+, K^+-ATPase requires preparation of highly purified enzyme. Biochemical techniques designed for this purpose have been developed in several laboratories (reviewed in detail by Schwartz *et al.*, 1975). The purified holoenzyme is a complex of two polypeptide chains, a large molecular weight catalytic subunit (84 000–110 000) which is phosphorylated by ATP in the native state in the presence of Na^+ plus Mg^{2+} (Kyte, 1972) and which binds ouabain (Ruoho & Kyte, 1974), and a low molecular weight sialoglycoprotein

(44 000–60 000), the function of which remains unclear. In an excellent series of studies, Kyte demonstrated the ultrastructural localization of the enzyme by immunochemical staining of the enzyme in Na^+, K^+-ATPase-rich vesicular fractions (Kyte, 1974) and in ultrathin frozen sections (Kyte, 1976a,b) from canine kidney cortex. Rabbit antibodies specific for either the holoenzyme or the catalytic subunit were demonstrated along peritubular surfaces of proximal and distal tubules with ferritin conjugates of goat anti-rabbit γ-globulin. A weak reaction was resolved along the luminal borders with the conjugated antibody raised against the holoenzyme, but not with that raised against the catalytic polypeptide. With the exception of the apical localization, these results are complementary to those obtained with the K^+-NPPase procedure (Firth, 1974; Ernst, 1975). A limitation of the immunocytochemical method is that unlike the [^{3}H]ouabain binding and K^+-NPPase procedures, this technique depends only on enzyme antigenicity and does not indicate, therefore, whether the localized antigen represents active enzyme. Accordingly, the luminal localization with holoenzyme antisera in renal proximal and distal tubules, in the absence of staining with anti-catalytic protein serum, suggests only that antigenic determinants associated with the sialoglycoprotein subunit, which itself does not exhibit enzymatic activity, are present in comparatively small amounts along the apical surface.

An alternative immunocytochemical approach was exploited by Wood *et al.* (1977). Antisera to eel electroplax Na^+, K^+-ATPase was used to localize the enzyme in a heterologous tissue, the brain of the knifefish, using horseradish peroxidase substituted for ferritin in the goat anti-rabbit IgG conjugate. The advantage of this approach is that antigenic sites are localized with an enzyme marker which increases the sensitivity of the technique, since peroxidase activity provides for an amplification of the immunological reaction by accumulation of electron-dense reaction product at sites of antigen–antibody interaction. For the same reason, however, peroxidase conjugates, unlike those with ferritin, cannot be used for quantitative analysis. Of considerable interest in the study of Wood *et al.* (1977) was the observation that although the enzyme was distributed along the plasmalemma of somata and dendrites of neurons, and somata and cellular processes of glia, it was restricted to nodes of Ranvier in myelinated axons. To our knowledge, this is the only case of an unambiguous asymmetric distribution of this enzyme along an uninterrupted plasmalemmal surface. The observed localization is, of course, consonant with the saltatory nature of conduction in myelinated nerves.

The observations of Wood *et al.* (1977) on the nodal distribution of Na^+, K^+-ATPase were confirmed recently in studies on goldfish optic nerve (Schwartz *et al.*, 1980a,b). Denatured catalytic polypeptide from goldfish brain was purified to provide a source of Na^+, K^+-ATPase antigen (Schwartz *et al.*, 1980a), and rabbit antisera raised against the denatured catalytic protein were used to localize Na^+, K^+-ATPase by the peroxidase–antiperoxidase technique (Schwartz *et al.*, 1980a,b). In addition to confirming the nodal distribution of the enzyme in

myelinated nerve, we showed that the enzyme is distributed uniformly along the glia-free neurites and their growth cones in cultured retinal explants (Schwartz *et al.*, 1980b). The methods used in these studies to prepare purified Na^+, K^+-ATPase antigen are relatively simple and should be applicable for similar studies with other tissues.

Significance of Na^+, K^+-ATPase localization

Following the discovery of an alkali metal cation-activated adenosine triphosphatase (Skou, 1957), it quickly became apparent that this enzyme was widely distributed among many tissues and was most probably a ubiquitous component of eukaryotic cell plasma membranes. Of particular importance to the functional significance of the enzyme was the observation by Bonting and his co-workers (Bonting, 1970) that its activity was relatively high in epithelial tissues, and particularly so in those that separated fluid compartments of markedly different compositions and tonicities. At the same time, morphological studies aided by the increasingly widespread use of the electron microscope established an underlying unity of structure among transporting epithelia that transcended phylogenetic bounds. Specifically, most epithelia that could be shown to effect net fluxes of ions from one epithelial surface to the other, either in a secretory or an absorptive mode, were composed of cells that contained relatively large populations of mitochondria and exhibited amplified basal and lateral plasma membranes (Fawcett, 1962). These extensions of the cell surface ranged in form from the simple plate-like folds of the lateral plasmalemma in the absorptive cells of the vertebrate gallbladder (Tormey & Diamond, 1967) to the elaborate labyrinthine networks in chloride-secreting cells of the teleost gill (Philpott & Copeland, 1963). In many transporting epithelia, moreover, adjacent cells were seen to be separated by long and narrow channels that extended from the basal lamina of the epithelium to the tight junctions which circumscribe the luminal compartment (Berridge & Oschman, 1972).

On the basis of voltage clamp studies with frog skin, Koefoed-Johnson and Ussing (1958) suggested a model for Na^+ transport in which Na^+/K^+-exchange pumps were restricted to inward-facing cell surfaces, thereby providing an energy-dependent driving force for passive uptake and transepithelial transport of Na^+ from the free surface. Autoradiographic localization of [^{3}H]ouabain-binding sites in frog skin (Mills & DiBona, 1977; Mills *et al.*, 1977) directly confirmed the premise on which this model was based. The Koefoed-Johnson & Ussing model was later extended by Diamond & Bossert (1967) in their formulation of the standing gradient osmotic flow model for coupling ion transport to water flow across epithelia. In this model, Na^+ is extruded actively into the intercellular spaces by Na^+ pumps located along the amplified basolateral cell surface. The constant influx of solute into these narrow channels establishes local solute gradients and, consequently, a concurrent flow of water which sweeps the solute-laden fluid toward the open (basal) end of the intercellular channel, thus effecting the net transepithelial passage of ions and water.

Although this generalized model of absorptive ion and water transport has been challenged in several specialized cases (see review by Diamond, 1979, for discussion) and modified by recent findings relating to such factors as junctional permeabilities, continual reassessment has not disproven the generalized tenets on which the theory is based. One prediction that has been supported almost without exception in this natriocentric view of epithelial absorptive transport is the location of Na^+ pumps. In all of the hypertonic and isotonic absorptive epithelia that have been examined, either with the K^+-NPPase procedure or by [^{3}H]ouabain autoradiography (see listings in DiBona & Mills, 1979, and Ernst & Mills, 1980), Na^+ pumps sites have been localized exclusively to the basolateral plasma membranes facing the intercellular spaces. Of particular importance with regard to the standing gradient model has been the observation that pump distribution along these membranes is relatively uniform from the apical to basal poles of the epithelia, pumps being concentrated only where extensive membrane folding and packing occurs (Mills & DiBona, 1978). This suggests that factors other than a suggested (Diamond & Bossert, 1967) asymmetric distribution of pumps toward the closed end of the channel must be present in order to account for isotonic reabsorption. Such parameters as narrow channels and high channel membrane water permeabilities could allow for sufficient osmotic equilibration to produce near isotonic absorbates without Na^+ pump polarization (Diamond, 1979). Cytochemical and autoradiographic localizations of Na^+ pump sites have, therefore, provided clear and consistent evidence for a mechanistic uniformity among absorptive epithelia in general and have demonstrated how a specific enzymatic active transport element may be integrated within the cytoarchitectural framework of epithelia to effect efficient movements of solutes and water.

Concurrent with investigations of Na^+ pump site localization in epithelia where the direction of net ion and water transport is from the luminal or free to serosal surface were studies localizing the transport enzyme in hypertonic and isotonic secretory epithelia. The tissue with which the K^+-NPPase procedure was developed, the avian salt gland (Ernst, 1972a,b; Ernst & Mills, 1977) is, for example, one of the best known hypertonic secretory tissues. The distribution of Na^+ pump sites in several other hypertonic secretory epithelia, including the elasmobranch rectal gland (Goertemiller & Ellis, 1976; Eveloff *et al.*, 1979) and teleost gill (Karnaky *et al.*, 1976; Hootman & Philpott, 1979) and operculum (Ernst *et al.*, 1980a), have also been characterized. Isotonic secretory epithelia have been less extensively studied, although Na^+ pump site localization has been established by [^{3}H]ouabain autoradiography in the cat submandibular gland (Bundgaard *et al.*, 1977) and human sweat gland (Quinton & Tormey, 1976). In both hypertonic and isotonic secretory epithelia, localization of Na^+ pump sites is identical to that determined in absorptive epithelia, that is, along basolateral cell surfaces. The demonstrated uniformity of Na^+, K^+-ATPase localization in epithelia, irrespective of net solute transport

vectoriality, has led to a rethinking of postulated modes of ion secretion. Previous models emphasizing the presence of Na^+ pumps on apical cell surfaces, or 'backwards oriented' solute pumps along basolateral cell membranes, are not supported by currently available data. Instead, emerging evidence linking Na^+, K^+-ATPase activity in an obligatory fashion to electrogenic Cl^- secretion, and the growing appreciation of paracellular pathways as channels for ionic conductance have led to the development of a new model incorporating these observations (Ernst & Mills, 1977; Silva *et al.*, 1977a,b). In this model, Na^+ pumped into the lateral intercellular spaces gains the free or luminal epithelial surface of the epithelium by traversing the zonula occludens, while Cl^- follows a transcellular route, both processes being driven ultimately by basolateral plasmalemmal Na^+, K^+-ATPase. The details of this model and evidence for postulated roles of various transport processes were reviewed recently (Field, 1978; Frizzell *et al.*, 1979; Ernst *et al.*, 1980b), and will not be further discussed here. It should be noted, however, that the initially perplexing cytochemical and autoradiographic localization of Na^+ pumps to basolateral cell surfaces in ion secretory epithelia provided a critical stimulus to the development of this model.

A further point regarding the significance of Na^+, K^+-ATPase localizations to epithelial physiology has been its utility in identifying sites of transport activity in epithelia that are composed of heterogeneous cell populations. In the teleost branchial epithelium, for example, [^{3}H]ouabain autoradiography (Karnaky *et al.*, 1976) and K^+-NPPase cytochemistry (Hootman & Philpott, 1979) demonstrated the presence of Na^+, K^+-ATPase activity in abundance in chloride cells, a localization previously suggested by cell isolation studies (Sargent *et al.*, 1975; Hootman & Philpott, 1978). These studies, coupled with the observed sensitivity of branchial NaCl secretion to ouabain (Maetz & Bornancin, 1975) provided a convincing argument for assigning a major role in ion secretion to these cells. Cell-specific differences in Na^+ pump density within a single epithelium have also been noted in frog urinary bladder (Mills & Ernst, 1975) and frog skin (Mills *et al.*, 1977), results that have also had important subsequent effects on prevailing views of ion transport in these tissues. In recent studies on the outer stripe of rat kidney medulla, we have shown that descending thin limbs of juxtamedullary long loop nephrons, in addition to ascending thick limbs, are sites of appreciable K^+-NPPase activity (unpublished results). In contrast, the descending thin limbs of short loop nephrons, which are less structurally specialized in terms of basolateral membrane amplification than those of long loops, exhibit little, if any, activity. Similarly, thin descending limbs in rabbit outer medulla, all of which are similar morphologically to the rat short loop segments, are unreactive. Such data have particular relevance to models for urinary concentration, particularly with respect to the mechanisms of solute concentration in the thin limbs of rats and rabbits, and illustrate the value of morphological methods for localizing Na^+ transport sites in structurally and functionally complex tissues.

The repeated demonstration that Na^+ pumps are restricted primarily to contraluminal surfaces in ion transporting epithelia has led to an additional application for Na^+, K^+-ATPase localization procedures: recognition of emergent functional polarity *in vitro* in artificially formed epithelia. Several cultured cell lines, most notably those derived from canine (MDCK) or porcine (LLC–PK_1) kidney, form confluent monolayers when plated at appropriate densities, and display both physiological and structural hallmarks of functionally polarized epithelia (see review by Handler *et al.*, 1980). These include the establishment of occluding junctions between adjacent cells and the subsequent ability of these polarized monolayers to effect net transepithelial ion and water movements, thus segregating extracellular compartments of differing ionic composition (Misfeldt *et al*, 1976; Cereijido *et al.*, 1978; Rabito *et al.*, 1978; Mills *et al.*, 1979). Mills and his co-workers (1979) showed that [^{3}H]ouabain-binding sites are localized exclusively to the basal surfaces of confluent LLC–PK_1 monolayers, as more recently have Cereijido *et al.* (1980) in monolayers of MDCK cells. In addition, Rabito & Tchao (1980) have demonstrated that MDCK cells in suspension have only one-third as many ouabain binding sites as the cells in confluent monolayers and that the increase in Na^+ pumps is coincident with the establishment of electrical resistance after the cells reach confluency. These results suggest that *de novo* synthesis of Na^+, K^+-ATPase may constitute a required event in re-organization of the plasma membrane preparatory to assembly of the cells into functionally competent epithelial sheets. Localization of this transport enzyme in re-organizing epithelia *in vitro* may, therefore, mark the differentiation of true basolateral cell surface definable on the basis of both physiological and cytochemical criteria.

Because of the demonstrated abundance of Na^+, K^+-ATPase in epithelia and its obligatory role in ion and water transport, localization of the enzyme in these tissues has been most intensively pursued. Several recent studies, however, have also localized Na^+ pumps in nervous tissue (Stahl & Broderson, 1976; Wood *et al.*, 1977; Broderson *et al.*, 1978; Schwartz *et al.*, 1980a, b). An observation of particular importance in immunocytochemical studies was the limitation of staining to nodes of Ranvier in myelinated axons (Wood *et al.*, 1977; Schwartz *et al.*, 1980b). This observation has particular relevance to questions regarding the way in which specific integral membrane proteins are integrated within functionally and spacially restricted domains of the cell surface.

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