# LOCALIZATION OF SODIUM PUMPS IN THE CHOROID PLEXUS EPITHELIUM

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## INTRODUCTION

The ability of epithelial membranes to absorb and secrete salt and water is a subject of considerable physiological importance. Although the detailed mechanisms involved are not understood, it is generally accepted that active sodium transport is the driving force for fluid movement across the intestine, gall bladder, renal tubule, and choroid plexus, and that sodium pumping is linked to the activity of membrane-bound Na/K ATPases (see Bonting, 1970). Furthermore, it is well established that cardiac glycosides, like ouabain, are potent and specific inhibitors of these enzymes and sodium transport. Thus, cytological localization of the site of ouabain action should provide strong evidence as to the location of sodium pumps in epithelia.

This report describes a combined biochemical and autoradiographic approach to localization of ouabain-binding sites in the frog choroid plexus. This tissue is known to secrete sodium by a ouabain-sensitive pump, which is thought to reside on the apical surface of the epithelium (Wright, 1972 a). Since ouabain is only effective in the solution bathing the apical surface of the epithelium, the cerebrospinal fluid (CSF) side, we anticipated that ouabain should bind to the apical (brush border) membrane, if indeed this membrane participates in active sodium secretion.

## METHODS

The frog posterior choroid plexuses were mounted in flux chambers as described previously by Wright (1972 a). Tritiated ouabain was added to the solution bathing either the CSF or blood side of the epithelium and, in most experiments, [$^{14}$C]sucrose was added as an extracellular marker. We monitored the

flux of [$^3$H]ouabain and [$^{14}$C]sucrose across the tissue. and at the termination of the experiment the amount of the two isotopes in the tissue was assayed by conventional liquid scintillation counting techniques as described earlier for amino acids (Wright, 1972 b) and lysergic acid diethylamide (Wright, 1972 c).

Autoradiographic localization of the tritiated ouabain in the plexus was carried out in a manner similar to that described by Stirling (1972) for the rabbit ileum. Briefly, at the termination of experiments, where ouabain was added to either one or both sides of the plexus, the tissue was frozen at −155°C in Genatron 23, freeze-dried at −70°C for 7 days, exposed to osmium tetroxide vapor for 2 h at 22°C, vacuum embedded in an Araldite 502 plastic mixture, sectioned (1 μm) onto water with a glass knife, collected on microscope slides, coated with Kodak NTB-2 emulsion (2 μm), and finally, exposed for 1 wk at 4°C before photographic development. Chemographic effects were ruled out by suitable controls.

All experiments were carried out at room temperature (22°-24°C) in a Ringer solution containing 85 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM $NaHCO_3$ gassed with 95% $O_2$/5% $CO_2$. In the experiments designed to characterize ouabain binding we varied the Na, K, Ca, and Mg concentrations in the solutions. Isethionate was used as an impermeable anion to maintain constant cell volume while we varied the potassium concentration. [$^3$H]Ouabain was obtained from New England Nuclear, Boston, Mass., at a specific activity of 13 Ci/mmol, and cold ouabain from Calbiochem, San Diego, Calif. [$^{14}$C]Sucrose was supplied by ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.

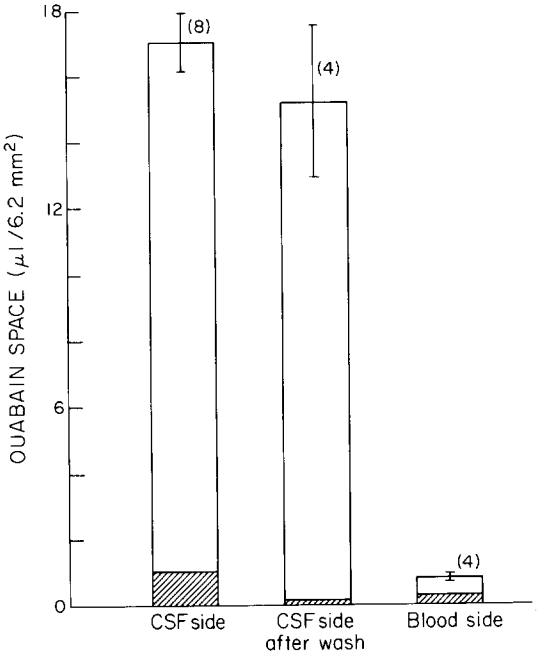


FIGURE 1 Ouabain vs. sucrose spaces in frog choroid plexus. Open bars correspond to ouabain spaces and cross-hatched bars correspond to sucrose spaces for each condition: space on the CSF side, space on the CSF side after washing 2.5 h, space on the blood side. The number of experiments appears in parentheses near the vertical lines indicating the standard error of the mean for ouabain spaces. Standard errors for sucrose were all less than 10% of the mean. (Vertical scale: calculated volume of solution (microliter) required to bring ouabain in the tissue to a specific activity equal to its activity in the bathing media per area [6.2 mm$^2$] of epithelia; 6.2 mm$^2$ represents the cross-sectional area of mounting chamber.)

## RESULTS

Preliminary experiments showed that both the flux of ouabain across the plexus and ouabain

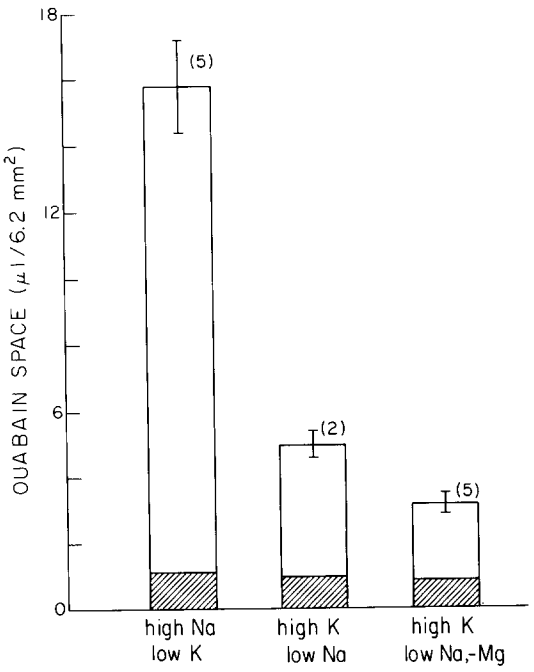


FIGURE 2 Ionic dependence of ouabain binding on apical surface of the choroid plexus. Open bars represent ouabain spaces and cross-hatched bars represent sucrose spaces under the indicated conditions. Specifically, high Na, low K = 85 mM Na isethionate, no K, 2.0 mM $MgSO_4$; high K, low Na = 85 mM K isethionate, no Na, 2.0 mM $MgSO_4$; high K, low Na, −Mg = 85 mM K isethionate, no Na, no Mg. Otherwise, all solutions contained 0.1 mM sodium EDTA, no $Ca^{++}$, 25 mM $NaHCO_3$, and were gassed with 95% $O_2$/5% $CO_2$. Number of experiments, standard errors, and vertical scale units are presented as in Fig. 1.

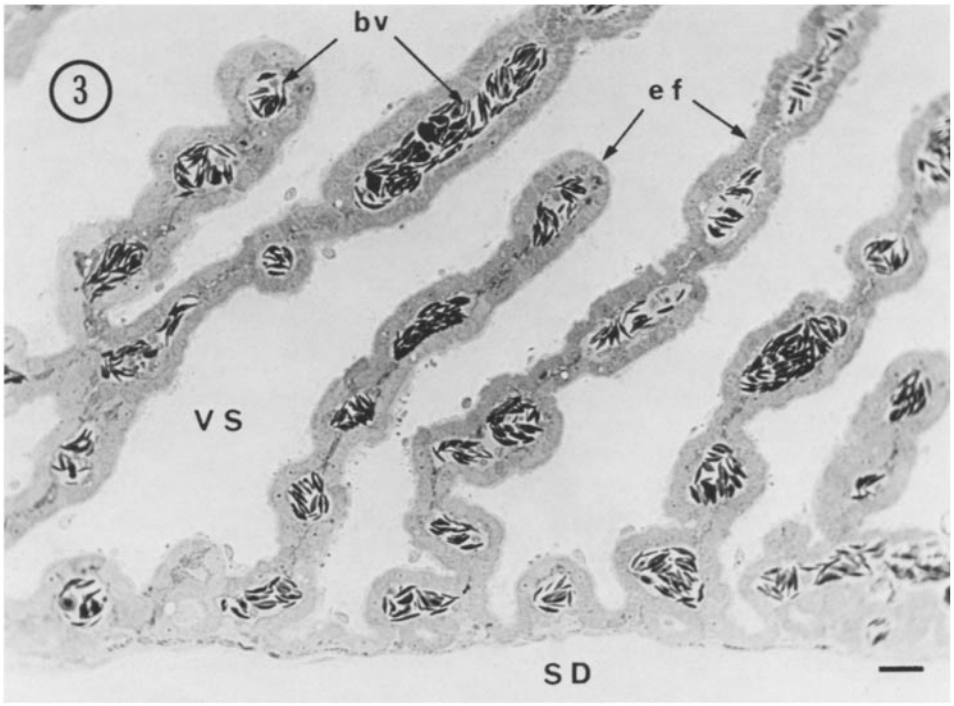


FIGURE 3 Low magnification photomicrograph of frog choroid plexus. Complex epithelial folds into the the ventricular space are prominent. An intricate bed of blood vessels resides in a thin matrix of loose connective tissue which separates the epithelium from the subdural space. Magnification, 170 diameters. Scale, 40 $\mu$m. (*ef*, epithelial folds; *VS*, ventricular space; *bv*, blood vessels; *SD*, subdural space).

binding to the tissue reached a steady value some 60–90 min after addition of the isotope. (The time-course of ouabain binding follows closely the inhibition of active sodium [Wright, 1972 a] and iodide[1] transport across this epithelium.) In seven experiments the permeability coefficients for ouabain and sucrose were $7.6 \pm 0.7 \times 10^{-7}$ and $1.4 \pm 0.1 \times 10^{-6}$ cm/s, respectively. We noted no variation in permeability when the isotopes were added to either side of the plexus and when the ouabain concentration was increased from $5 \times 10^{-7}$ to $1 \times 10^{-4}$ M. On the basis of these experiments it was concluded that there was no significant leak of [$^3$H]ouabain across the plexus when the isotope was added to either side of the epithelium. In all subsequent experiments the plexus was exposed to 10 $\mu$Ci/1.5 ml of [$^3$H]ouabain (i.e., $\sim 5 \times 10^{-7}$ M) for 2.5 h.

The results of the binding studies are summarized in Figs. 1 and 2. In Fig. 1 it is shown that the ouabain space on the CSF side of the plexus was about 17 times greater than the sucrose space, and that washing the plexus with ouabain-free solutions failed to reduce the amount of ouabain in the tissue even though 90% of the sucrose was washed out. (It has been noted that the inhibition of transport by ouabain is also irreversible.[1]) Increasing the ouabain concentration to $1 \times 10^{-4}$ M, by adding cold ouabain, eliminated the difference between the ouabain and sucrose spaces. This confirmed that it was tritiated ouabain that was bound to the tissue. On the blood side of the epithelium, even though the ouabain was significantly greater than the sucrose space, the amount of ouabain in the tissue was less than $\frac{1}{17}$th of that on the CSF side. In this case prolonged washing reduced the ouabain and sucrose by 60 and 90%, respectively. Thus, there was 40 times more ouabain bound to the ventricular surface than the serosal surface.

The ionic dependence of ouabain binding to the ventricular surface is shown in Fig. 2. These experiments demonstrated that replacing 85 meq/liter of sodium in the Ringer's solution with potassium reduced ouabain binding by 60%, and that

[1] Wright, E. M. 1973. Manuscript in preparation.

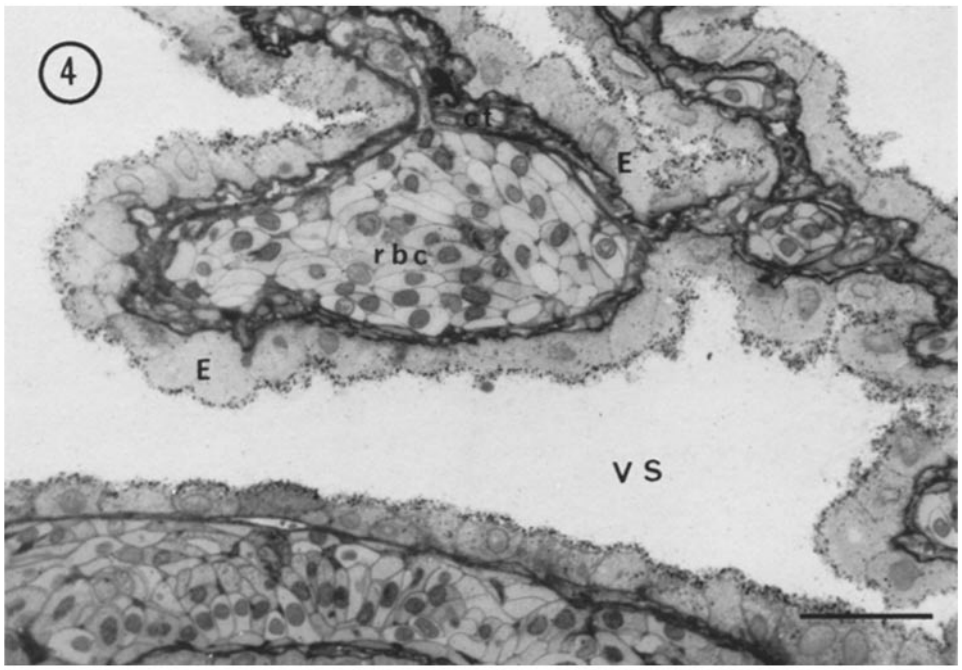


FIGURE 4 Autoradiogram of parts of two folds of choroid plexus exposed on both sides to $5 \times 10^{-7}$ M [$^3$H]ouabain and subsequently washed. Silver grain density occurs uniformly over the entire ventricular surface of the epithelium. Magnification, 420 diameters. Scale, 40 $\mu$m (*E*, epithelium; *VS*, ventricular space; *rbc*, nucleated red blood cells; *ct*, connective tissue).

binding was reduced a further 20% upon the omission of Mg from the saline. (Similar experiments carried out at lower ouabain concentrations [$1 \times 10^{-7}$ M], showed that binding was even more sensitive to the ionic composition of the solutions.) This Na-, K-, and Mg-sensitive binding of ouabain to the frog choroid plexus is very similar to that observed previously for cardiac muscle ATPase (Matsui and Schwartz, 1968), human red blood cells (Ellory and Keynes, 1969; Gardner and Conlon, 1972), and sheep red blood cells (Dunham and Hoffman, 1971).

We then turned to autoradiography to learn the precise localization of the binding sites. The fact that ouabain exerts its physiological action and is bound to the plexus only when applied to the CSF side suggests binding to sites on the apical membrane of the epithelium. However, if ouabain permeates through the apical membranes, part, or even all, of the binding could be on the cytoplasmic side of the basal and/or lateral membranes.

In these autoradiographic studies, plexuses were exposed to $5 \times 10^{-7}$ M [$^3$H]ouabain for 2.5 h and were then washed repeatedly in ouabain-free saline for another 2.5 h. Fig. 3 (a conventionally prepared section) has been included to illustrate the overall morphology of the frog choroid plexus. The ventricular side is seen to consist of complex folds of highly vascularized tissue covered by a continuous layer of cuboidal epithelial cells. Fig. 4 depicts parts of several folds and shows that ouabain is bound to the brush border region (apical membrane) of the epithelium. Scattered grains are seen elsewhere, but only at background levels (seven counts/$\mu$m$^2$). More specifically, the frequency of grains over the lateral and basal cell membranes does not rise above background. The specificity of localization is demonstrated more clearly in Fig. 5, where dark-field and bright-field illumination of a small region are compared. All regions of all the folds examined appeared to be equally heavily labeled. No differences were observed between tissues in which ouabain was applied to both sides and those in which it was applied to the ventricular side only. These results are complementary to those of Stirling (1972) in that ouabain was bound only to that side of the epithelium on which the glycoside blocks active transport.

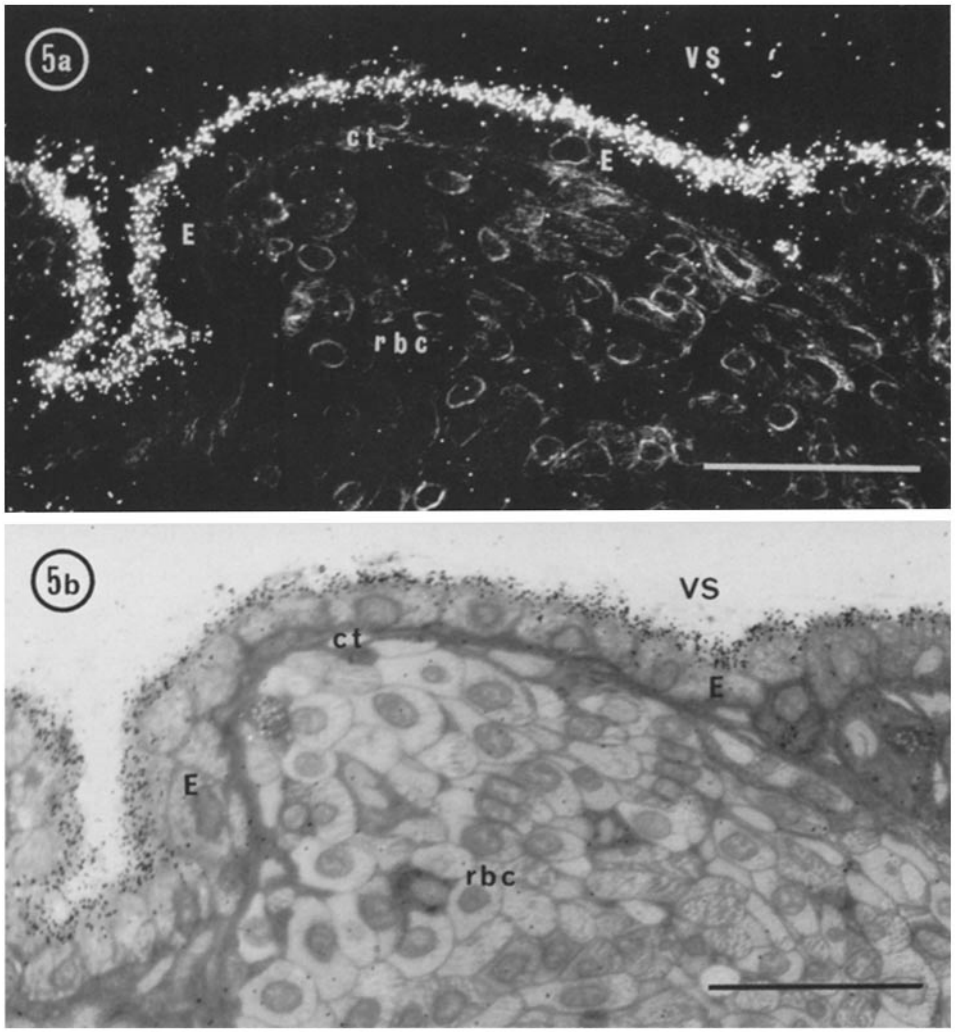


FIGURE 5 (*a*) Dark-field photomicrograph part of a fold exposed to $5 \times 10^{-7}$ M [$^3$H]ouabain as in Fig. 4. Grain densities occur exclusively over the apical membrane region of the epithelial cells. Grain counts over basolateral membrane regions of the epithelium and over the underlying blood and connective tissue are not distinguishable from background. (*b*) Bright-field illumination of same field as Fig. 5 *a*. Magnification, 725 diameters. Scale, 40 $\mu$m (*E*, epithelium; *ct*, connective tissue layer; *rbc*, nucleated red blood cells; *VS*, ventricular space).

At present it is not possible to give an accurate estimate of the number of ouabain sites on the apical surface of the choroidal epithelium as we do not know the effective area of the tissue. However, assuming one site for each bound ouabain molecule there are $1.5 \times 10^{12}$ sites/mg wet weight at a ouabain concentration of $5 \times 10^{-7}$ M. (The weight of the tissue exposed in the flux chambers was about 3 mg.) This estimate is very close to that obtained by Baker and Willis (1969) for slices of guinea pig liver and kidney. Assuming that the surface area of the plexus is amplified 100 times by folding, it can be estimated that there are about $10^4$ sites/$\mu$m$^2$. Compared with red blood cells (approximately 1 site/$\mu$m$^2$) this high site density is not unreasonable in view of the specialized secretory function of the choroidal epithelium.

## DISCUSSION

Two facets of this report are of primary significance: the first concerns the possibility of studying the location of Na/K ATPase by the ouabain-

binding techniques, and the second bears on the implications of these studies on the mechanism of ion transport across epithelia. Understanding the physiology of epithelia demands the specific description of the localization and orientation of ion pumps. To date two approaches have been attempted, namely, cytochemical localization of Na/K ATPases by some application and/or modification of the Gomori technique to detect $PO_4$ liberated by the action of the enzyme, and biochemical techniques for the localization and characterization of plasma membranes. Much criticism is directed towards the histochemical approach due to the inability to demonstrate substrate specificity (e.g., Tormey, 1966), and, at best, the technique is tedious and complex. With regard to the separation of basolateral and brush border membranes by biochemical technique, there are serious questions yet to be answered about the true identity of many of the so-called plasma membrane fractions (see DePierre and Karnovsky, 1973).

The approach of localizing bound ouabain by autoradiographic techniques such as offered by Stirling (1972), coupled with tests of binding characteristics demonstrated here open broad new possibilities for the definitive localization of sodium and potassium transport sites.

In this study we have demonstrated by autoradiography that [$^3$H]ouabain is bound to the apical membrane of the choroid plexus epithelium. Our data indicate that this binding is associated with the Na/K ATPase in the membrane, since the ionic requirements of ouabain binding closely resemble that reported for binding to Na/K ATPases in cardiac muscle (Matsui and Schwartz, 1968), red blood cells (Ellory and Keynes, 1969; Dunham and Hoffman, 1971; Gardner and Conlon, 1972), and nerve (Landowne and Ritchie, 1970). The presumed identity between these ouabain-binding sites and a Na/K pump involved in transport across the epithelium is further strengthened by the observations that (*a*) the time-course of ouabain binding in the plexus closely follows the time-course of ouabain inhibition of sodium and iodide transport, and that (*b*) both binding and the effects on transport are irreversible.

The localization of ouabain binding on the brush border membrane supports the model put forward by Wright (1972 a) to account for sodium secretion by the frog choroid plexus. Briefly, it was proposed that sodium entered the epithelial cell from the blood down the electrochemical gradient, and was subsequently pumped across the brush border of the cell into the CSF by a ouabain-sensitive pump. However, we cannot conclude from the present work that sodium pumps are present or absent from the basolateral membranes since it is generally accepted that ouabain only inhibits sodium pumps on the side of the membrane to which sodium is pumped. The absence of appreciable ouabain binding to the lateral membranes indicates that if sodium pumps are present in these membranes they are inaccessible to ouabain on the blood side of the epithelium. In view of a recent report of histochemical localization of Na/K ATPase on the basolateral membranes of a secretory epithelium, the avian salt gland (Ernst, 1972), we await further experiments to determine whether, in the choroid plexus, basolateral membrane pump sites are absent, inaccessible, or different from brush border-binding sites.

Finally, as discussed by Wright (1972 a) the structural organization of the epithelium, the location of the sodium pumps, and the presence of cilia on the brush border membrane raise serious questions as to whether current models of solute-solvent coupling can explain how active salt transport produces isotonic water flow across this epithelium. This problem is under current investigation.

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