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Ethacrynic Acid and Furosemide Alter Cl, K, and Na Distribution Between Blood, Choroid Plexus, CSF, and Brain

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Can loop diuretics like ethacrynic acid and furosemide, when administered intravenously, significantly alter ion transport and fluid dynamics in CNS? To shed light on this unresolved issue, we tested the ability of these agents to effect redistribution of Na, K and Cl in adult rat brain. Cl penetration into various CNS regions was assessed as the volume of distribution, i.e., uptake, of ^{36}Cl from blood. Ethacrynic acid and furosemide (50 mg/kg IV) reduced by 20-30% the rate of permeation of ^{36}Cl across the blood-CSF barrier, and they elevated [K] and [Cl] in choroid plexus (CP) by 15-25%. The loop diuretic-induced buildup of K and Cl in CP (lateral and 4th ventricle) was likely a reflection of decreased movement of these ions across the apical membrane into CSF. ^{36}Cl activity in parietal cortex and pons-medulla decreased in treatment with furosemide and ethacrynic acid, due to slowing of Cl transport across blood-brain and/or blood-CSF barriers. Our inhibitory findings in intact rats are consistent with those from previous in vitro experiments demonstrating diminution by loop diuretics of Na, K and Cl transport across isolated CP membranes.

KEY WORDS: Choroid plexus epithelium; cerebrospinal fluid; loop diuretics; ethacrynic acid; furosemide.

INTRODUCTION

Loop diuretic agents and ion substitution procedures with artificial CSF have been useful tools for elucidating the presence in isolated choroid plexus (CP) of a protein that cotransports Na, K and Cl into the choroidal epithelium (1,2). Both furosemide and bumetanide reduced the rate of transport of Na, K and Cl into the in vitro CP. The effects of loop diuretics on the CP content of tracers have not been analyzed, however, in intact animals where the choroidal tissue is bathed basolaterally by interstitial fluid (ISF) and apically by cerebrospinal fluid (CSF). Such in vivo analyses permit characterization of choroid cellular composition, and they deserve attention because of implications for vectorial transport and the regulation of cell volume (3), acid-base balance (4), and secretion of CSF (5). Javaheri (6) has recently reviewed the need for additional studies to clarify the role, if any, of NaCl cotransport in CSF secretion.

Furosemide has been the most extensively analyzed loop diuretic in studies of mammalian CP-CSF systems (7–15). Furosemide effects have been difficult to interpret because this drug inhibits carbonic anhydrase as well as Na-K-Cl cotransport, and not all investigators have nephrectomized their in vivo preparations to prevent systemic volume depletion. Other loop agents, like bumetanide and ethacrynic acid, inhibit cotransport but not carbonic anhydrase. Ethacrynic acid has been shown to suppress ^{22}Na uptake into CSF (9), but direct effects of this organic acid on CP Cl transport mechanisms and CSF composition have not been analyzed. In the present

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investigation we sought to determine if the cotransport inhibitors bring about alterations in CP and CSF ions similar to those caused by acetazolamide and amiloride, i.e., inhibitors of Na-H exchange. We found that CSF inhibitors generally promote retention of Cl and K by CP.

EXPERIMENTAL PROCEDURE

Adult rats, 200 to 350 g, were anesthetized with ether for surgical procedures associated with functional nephrectomy, i.e., ligation of the hillock (pedicles) in both kidneys. Furosemide, ethacrynic acid or saline vehicle was administered intravenously immediately after the blood flow to the kidneys was stopped. The purpose of the nephrectomy was to prevent a non-steady state condition caused by continual washout of ions, water, drug and tracer into the urinary outflow.

Ninety min was allowed for attainment of steady-state effects of the drug on CP function. Then the animals were sampled for electrolyte or pH measurements, or in other experiments, were given ^{36}Cl intravenously and sacrificed 15, 30, or 45 min later under ketamine (80 mg/kg) anesthesia. The ketamine was administered approximately 10 min prior to the end of the experiment when the tissues and CSF were collected. Following removal of the terminal arterial sample, the residual blood within the CNS vasculature was displaced by perfusing isotonic sucrose through the brain.

At the conclusion of experimentation, fluids and tissues were removed from the following sites: blood (abdominal aorta), CSF (cisterna magna), choroid plexuses (lateral and 4th ventricles) and brain (parietal cortex and pons-medulla). Choroid plexuses and brain tissues were desiccated for determination of dry weights. Gases and pH were measured on an IL 213 Blood Gas Analyzer, Na and K on an IL 143 Flame Photometer, and tracer activity on a Beckman LS 7500 liquid scintillation counter.

In another set of experiments, the femoral arterial pressure was continuously monitored in nine animals (n = 3 for each of the 3 treatments) throughout the experiment by means of a transducer and recorder from the Gould Co. The right femoral vessels were cannulated under ketamine anesthesia, and arterial blood was subsequently sampled at 2.5, 5, 10, 15, 30, and 45 min after femoral vein administration of ^{36}Cl for determination of the shape of the arterial curve. Each of the femoral samples was generally between 0.06 and 0.09 ml. The average blood pressure for a given experiment was calculated from six determinations, each of which was taken just before arterial sampling.

Formulas and assumptions for quantitating tracer uptake from blood by various regions of the CNS have been previously described in detail (3,5). Briefly, the volume of distribution (V_d) of tracer was taken as the ratio, at the time of sacrifice, of the following activities: dpm/g tissue (or CSF) ÷ dpm/g (ml) plasma H_2O. Because of the known ability of loop diuretics to alter CP water content (2), we have expressed the V_d values per weight of dry rather than wet tissue. Statistical testing of the data is described in tables and figures.

Materials. Ethacrynic acid and furosemide were obtained from Sigma Co. The source of ^{36}Cl was the New England Nuclear Co. Ketamine was from Parke-Davis, Morris Plains, NJ.

RESULTS

Stability of Plasma Ions and pCO_2. Due to the nature of these transport experiments, it was essential to have stability in the ionic composition of the plasma. Neither furosemide nor ethacrynic acid, each at 50 mg/kg for 90 min, disrupted plasma [Na] or [K] (Table I). Furthermore, the arterial pH, pCO_2 and $[HCO_3]$ remained stable (Table I).

The choroid plexus contents of Na and K were altered by ethacrynic acid, which markedly decreased [Na] in 4VCP and elevated [K] in LVCP as well as 4VCP. Furosemide induced smaller effects. On the other hand, [Na] and [K] in cerebral cortex and pons-medulla tissues were not altered by loop diuretic treatment (Table II).

Arterial Tracer Levels and Blood Pressure. The desired stability of plasma [^{36}Cl] was rapidly attained by the nephrectomy procedure. Representative findings are presented in Figure 1. Animals in all groups displayed an initial small transient rise of comparable magnitude at 2.5 min, and a steady-state plateau thereafter. Plasma [^{36}Cl] was stable by 5 min after the start of tracer infusion. Neither furosemide nor ethacrynic acid, compared to vehicle control, significantly altered the shape or the area of the arterial curve of ^{36}Cl activity vs. time. The comparable magnitudes of the respective area under the arterial curves for the three groups indicates similar exposures of the tissues to ^{36}Cl in blood. Thus the same apparent times (5) justify a comparison, control vs. drug, of the V_d values obtained for the treatments. Mean arterial blood pressure with 50 mg/kg of ethacrynic acid (90 ± 5 torr) or furosemide (80 ± 10) was not signif-

Table I. Electrolyte and Acid-Base Measurements for Fluids in Adult Rats Treated with Loop Diuretics

	Control Saline vehicle	Furosemide (50 mg/kg)	Ethacrynic Acid (50 mg/kg)
A. Electrolytes (mEq/L)			
Plasma [Na]	139 ± 1.8	140 ± 1.5	137 ± 0.8
CSF [Na]	153 ± 1.5	151 ± 1.5	155 ± 2.1
Plasma [K]	4.57 ± .14	4.58 ± .17	4.46 ± .22
CSF [K]	2.92 ± .12	2.81 ± .08	2.87 ± .05
B. Acid-base and gas			
pH	7.42 ± .02	7.42 ± .01	7.42 ± .02
pCO_2	29.8 ± 1.7	33.3 ± 2.0	31.5 ± 0.8
$[HCO_3]$	19.1 ± .72	21.4 ± .82	20.2 ± .31

Means ± SEM for 4 animals. Drug treatment was for 90 min. Blood was collected from the abdominal aorta and CSF from the cisterna magna. Kidneys were isolated from the circulation, by ligation of the renal hillock, to prevent diuresis.

Table II. Sodium and Potassium Concentrations in Different Regions of Choroid Plexus and Brain of Rats Treated with Loop Diuretic Agents

	Control Saline vehicle	Furosemide (50 mg/kg)	Ethacrynic Acid (50 mg/kg)
A. Sodium (mEq/kg dry)			
LVCP [Na]	238 ± 14	245 ± 17	210 ± 11
4VCP [Na]	314 ± 30	270 ± 16	235 ± 12[a]
Cerebral cortex	194 ± 2.2	192 ± 2.4	200 ± 1.0
Pons-medulla	196 ± 5.9	192 ± 4.0	187 ± 5.0
B. Potassium (mEq/kg dry)			
LVCP [K]	518 ± 9.3	542 ± 23	585 ± 17[a]
4VCP [K]	510 ± 11	515 ± 5.5[b]	550 ± 7.5[a]
Cerebral cortex	540 ± 8.4	527 ± 8.8	553 ± 3.8
Pons-medulla	512 ± 15	483 ± 7.5	495 ± 5.5

Means ± SEM for 4 animals. Drug treatment was for 90 min. LVCP and 4VCP refer respectively to lateral and 4th ventricle choroid plexus.
[a] $P < 0.05$, drug vs. control, by Dunnett's test.
[b] $P < 0.05$, furosemide vs. ethacrynic acid.

icantly different from that of controls (91 ± 8 torr) ($P > 0.05$, by Dunnett's *t* test).

^{36}Cl uptake by Choroid Plexuses and Other Regions. In LVCP and 4VCP there was an augmentation of ^{36}Cl activity from 20–30% resulting from treatment with furosemide or ethacrynic acid, the maximal effect occurring at 45 min (Figure 2). In contrast, in CSF and in the two regions of brain analyzed, there were significant reductions (by 15–20%) in the ^{36}Cl concentrations measured at 30 to 45 min after isotope exposure (Figures 3 and 4).

DISCUSSION

Correlation of In Vivo and In Vitro Findings. Previous investigations of CP isolated in artificial CSF established the presence of NaCl cotransport in rat choroidal epithelium (1,2). There are two likely functions of the NaCl cotransporter, i.e., regulation of choroid cell volume and participation in CSF secretion. Because CSF secretion stems mainly from the activity of the CP transporters of Na and Cl, we postulated that loop diuretics which directly interfere with NaCl cotransport (16) would inhibit the net penetration of Cl from blood to CSF. Our ability to demonstrate this in intact animals strongly implicates a secretory role for the NaCl cotransporter in choroid plexus.

Control of Factors Potentially Complicating The Interpretation of CSF Experiments. Javaheri (6) summarized several conditions not always adequately controlled in previous CSF research protocols with loop diuretics: *i)* systemic volume depletion, *ii)* arterial acid-base status, *iii)* selection of drugs to evaluate an indirect effect on ion transport caused by inhibition of carbonic anhydrase, and *iv)* drug bioavailability. *i)* Bilateral nephrectomy was done to prevent salturesis and urinary water excretion. By avoiding distortions in plasma chemistry and volume, the disequilibration of such systemic factors can be ruled out as the cause of the altered Cl transport

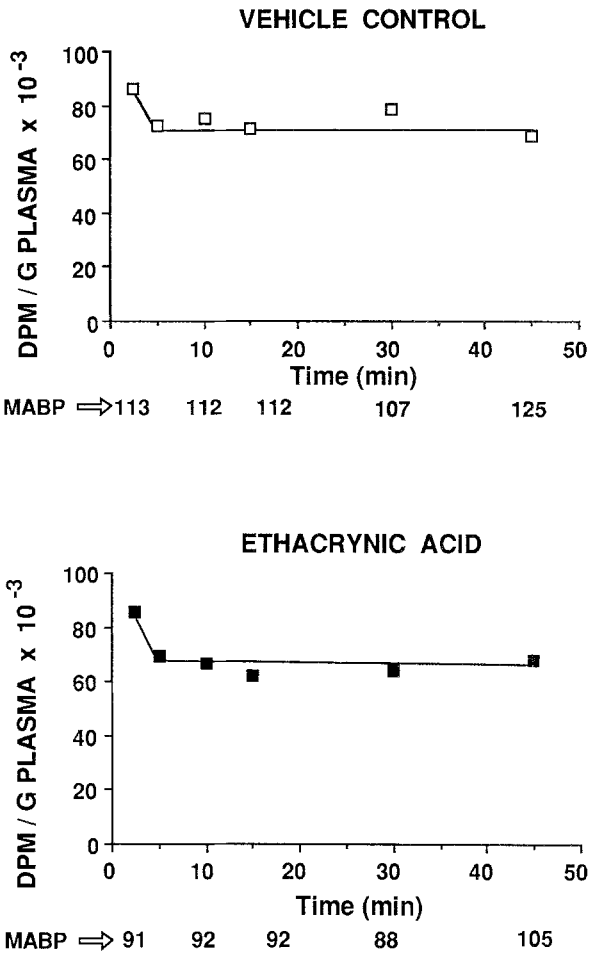

Fig. 1. Time course of ^{36}Cl activity in arterial blood of adult rats treated with ethacrynic acid (bottom panel) or with vehicle control (top). The animals were paired for body weight, anesthetized with ketamine, bilaterally nephrectomized and immediately injected IV with saline vehicle or drug. Ninety min later, 5 μCi of ^{36}Cl was infused (time 0) over 1 min into the femoral vein. The first sample was collected at 2.5 min. The ^{36}Cl activities for the 6 samples were plotted to determine the shape of the arterial curve. See Experimental Procedure section for protocol description. The furosemide curves (not shown) were indistinguishable from those for ethacrynic acid. Mean arterial blood pressure (MABP) values in torr, given at the bottom of each panel, correspond to the specified times on the abcissa above.

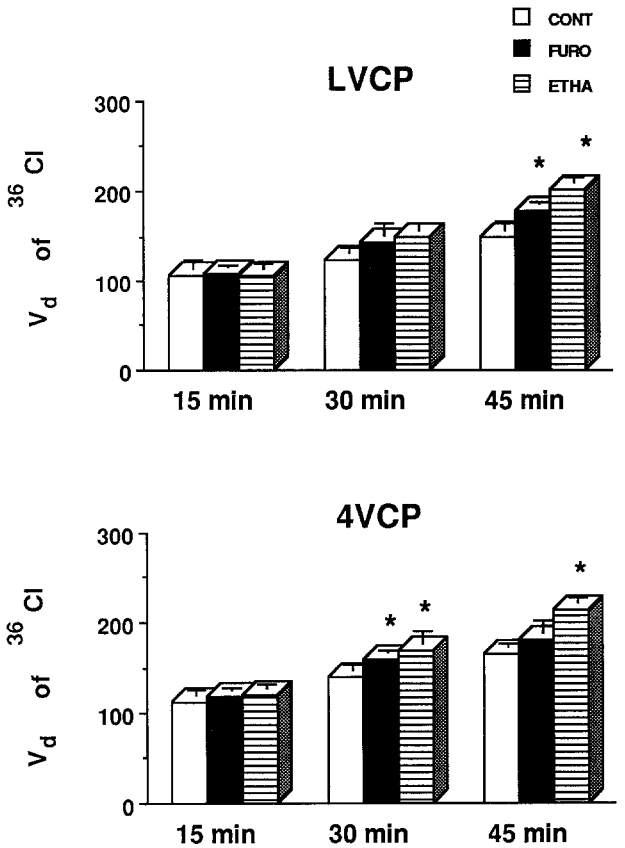

Fig. 2. Uptake of ^{36}Cl from blood by choroid plexuses in nephrectomized adult rats treated with 50 mg/kg of furosemide (FURO) or ethacrynic acid (ETHA). V_d = volume of distribution, in ml/g dry tissue (defined in Methods). Each bar is the mean ± SEM for 4 animals. LVCP and 4VCP refer to lateral and fourth ventricle choroid plexuses, respectively. *P < 0.05, drug treatment vs. control (CONT), by Dunnett's test.

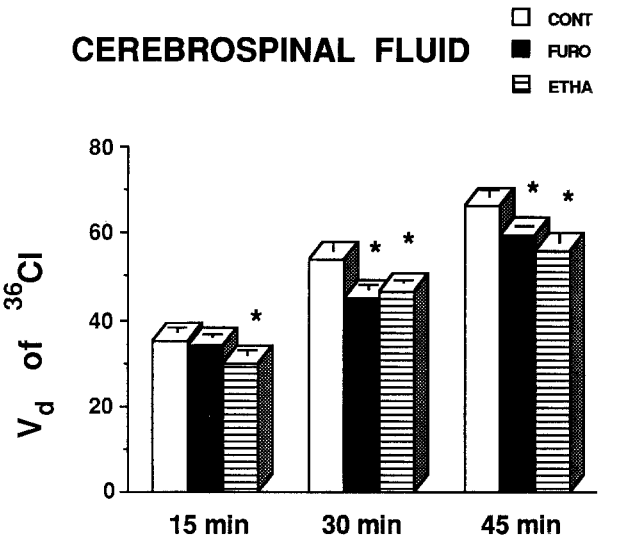

Fig. 3. Uptake of ^{36}Cl by cerebrospinal fluid, sampled from cisterna magna, in rats administered saline vehicle or 50 mg/kg of loop diuretic. V_d (ml/g) and drug abbreviations are defined in Figure 2. Means ± SEM for 4 animals. *P < 0.05, drug vs. control (CONT), by Dunnett's test.

into the CP-CSF system. *ii)* Cl transport from blood to CSF is affected by the pH and CO_2 tension of blood (unpublished data). Moreover, the CP content of K and Na is a sensitive function of blood acidity (21,22). We measured arterial pH and pCO_2 to assure that these parameters were stable (Table I). It was established that blood [H] and CO_2 tension were not disturbed by furosemide or ethacrynic acid. *iii)* Furosemide at high concentrations can inhibit carbonic anhydrase (c.a.), therefore we also used ethacrynic acid to compare effects of an agent that does not inhibit c.a. If anything, the inhibitory effects of ethacrynic acid were greater than those of furosemide (Figures 2–4), indicating that the loop diuretic inhibition of Cl transport was not necessarily linked to c.a. inhibition. *iv)* Drugs were given IV rather than IP to prevent incomplete absorption possibly caused by precipitation or deposition at peritoneal sites.

Yet another in vivo factor potentially capable of modifying CP transport and CSF secretion is arterial blood pressure. Severe arterial hypotension (28) or hypertension (29) can affect transport at the blood-CSF barrier. However, neither furosemide nor ethacrynic acid significantly altered MABP. Furosemide tended to reduce MABP, but the mean value of 80 torr is well above the threshold level below which the slower flow of blood would limit CP secretion (28).

Furosemide vs. Ethacrynic acid vs. Bumetanide. Loop diuretic doses assuring maximal inhibition in vivo were utilized. Other investigators have used 50 mg/kg of ethacrynic acid (9) or furosemide (11,17,18) to attain maximal reduction in CSF formation or CP ion transport. The 50 mg/kg dose that we used should give plasma levels of about 10^{-5} to 10^{-4}M (4). Bumetanide has been used at 1 mg/kg (19), a dose equivalent to about 40 mg/kg furosemide (11).

Furosemide and bumetanide have been compared for their ability to inhibit Na and Cl uptake by isolated CP (1); the maximal degree of inhibition of in vitro uptake of ^{22}Na and ^{36}Cl that could be achieved was 30–45%, and this occurred at 10^{-5} to 10^{-4} M in the extracellular medium. Bumetanide reduced by 30% the transport of ^{22}Na from blood into CSF dialysate (19). Overall there is substantial evidence that furosemide, ethacrynic acid and bumetanide inhibit Na and Cl uptake by both the in vitro CP and the in vivo CSF system. This is

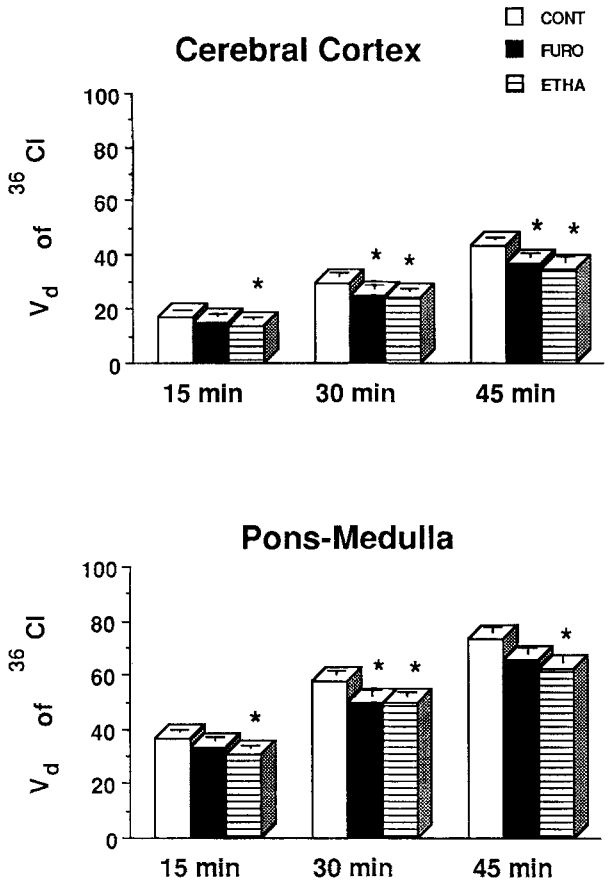

Fig. 4. Time course of penetration of ^{36}Cl into two regions of brain, in adult rats treated for 90 min with 50 mg/kg of furosemide (FURO) or ethacrynic acid (ETHA). V_d is in units of ml/g dry tissue (see Methods). Mean ± SEM for 4 animals. *P < 0.05, drug vs. control, by Dunnett's test.

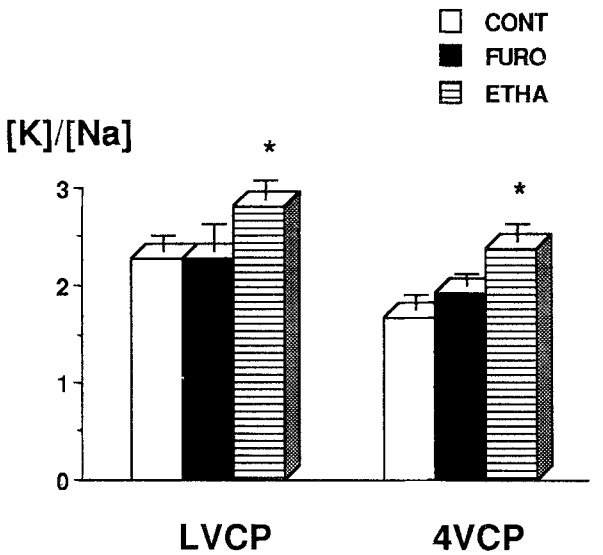

Fig. 5. Ratio of K to Na concentrations in lateral (LVCP) and fourth (4VCP) ventricle choroidal tissues. Na and K were determined by flame photometry. Prior to sampling of choroid plexuses, the residual blood in the tissue was removed by displacement with isotonic sucrose solution. Data for Na and K concentrations individually are presented in Table II. Each bar represents a mean for 4 rats. Limits are SEM. *Dunnett's t test was used to ascertain significant differences between the control (CONT), 50 mg/kg furosemide (FURO) and 50 mg/kg ethacrynic acid (ETHA) groups.

consistent with a basolateral localization of NaCl co-transport in rat CP, as originally proposed by Smith et al. (20).

Why Do CSF Formation Inhibitors Cause CP to Retain KCl? Furosemide and ethacrynic acid treatments led to an accumulation of K and Cl in CP. This was not entirely unexpected because in previous experiments with CSF-inhibiting agents from other pharmacologic classes (acetazolamide and amiloride (3)), there was also a buildup of K and Cl in CP. The concentration of K and Cl in CP epithelium is the complex resultant of ion movements via transporters and channels in the external limiting membranes of CP. Figure 6 summarizes knowledge of basolateral and apical systems in CP mediating transfer of ions between blood and CSF, and the most likely sites of inhibition by ethacrynic acid and furosemide.

Diuretic agents generally reduce CSF secretion by decreasing basolateral Na uptake from CP interstitial fluid (Figure 6). Following drug-induced reduction in Na uptake, with a resultant lowering of tissue and cell [Na], there is consequently redistribution of K and Cl between choroid cellular and extracellular fluids (see Figs. 7 and 8 in ref. 3). Parenchymal cell retention of K relative to Na (Figure 5) likely results from suppressed K efflux concurrent with diminished Na uptake. The rise in CP [K]/[Na] need not be explained by stimulation of Na-K pumping (21,22). We postulate that augmented CP [K] helps to balance the diminished [Na] in cells caused by diuretic-induced slowing of inward basolateral Na transport. The changes in CP ion distribution do not significantly alter CSF [Na] and [K] after treatment with loop diuretics (Table I) or with carbonic anhydrase inhibitors (23), probably because the volume of ventriculocisternal CSF (diluting effect) is considerably greater than that of CP epithelium where modified ion transport occurs.

Elevated [K] and [Cl] in CP tissue, during drug-induced curtailment of CSF formation, is presumably due to diminished efflux of K and Cl from cells rather than to accelerated uptake of these ions. For example, furosemide, bumetanide and acetazolamide decreased by 30–40% the Cl efflux from in vitro CP (15,24), and they also slowed K (Rb) efflux from isolated CP (unpublished data). Zeuthen (30), using salamander CP, similarly found

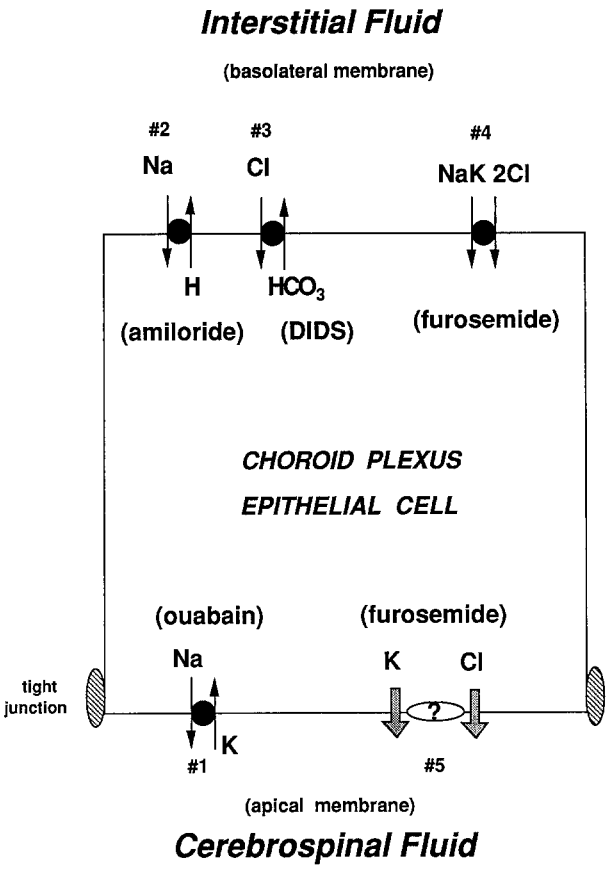

Fig. 6. Ion transporters in external limiting membranes of rat CP. Arrows depict vectorial transport. Drugs that inhibit transporters are indicated in parentheses. Primary active extrusion of Na from cell by Na-K pump (#1) keeps intracellular Na, i.e. $[Na]_i$, low relative to extracellular [Na]. Thus, the inwardly-directed transmembrane gradient for Na drives the secondary active transport of Na into cell by Na-H exchange (#2) or NaK2Cl cotransport (#4). Na-H exchange is inhibited directly by amiloride and indirectly by acetazolamide which, by lowering cell [H], reduces availability of H ion for exchange with extracellular Na (3). Cl is actively transported into cell by Cl-HCO_3 exchange (#3), and by cotransport (#4) (see refs. 27 and 31). K is actively transported into cell mainly (>90%) by Na-K pump (#1), and to a much lesser extent by the cotransporter (#4) (see ref. 2). K and Cl exit the cell apically either by diffusion through their channels (thick strippled arrows, #5) or by a cotransport protein sensitive to furosemide (see ref. 30). In *in vitro* studies loop diuretics inhibit uptake of Na, K and Cl (presumably at #4) (refs. 1 & 2), and they also inhibit efflux of K and Cl (probably at #5) (refs. 15 & 24). Therefore loop diuretics may exert direct inhibitory effects at both poles of the cell, at #4 and #5. We postulate that as basolateral Na uptake is slowed by furosemide, there is concurrently a marked suppression of K and Cl efflux across the apical membrane which promotes retention of KCl by CP (ref. 3). Evidences for the various transporters and channels in choroid plexus are summarized in refs. 1-3, 5, 6, 10, 13, 15, 20-24, 27, 30, 31.

that furosemide inhibited outward transport of K and Cl across the ventricular (apical) membrane; such efflux of K and Cl by a cotransport mechanism was coupled to the movement of water out of the choroid cell. Diuretic drug-induced retention of K and Cl in CP has been associated with altered cell volume (3), which may be linked to the disruption of CP ion transport and CSF secretion.

Loop Diuretic Effects on Brain vs. Choroid Plexus. In both the cerebral cortex and pons-medulla, the tissue concentrations of Na and K were remarkably stable in all experiments. This is in contrast to the choroid plexus, eg. 4VCP, in which ethacrynic acid decreased [Na] by 25% and increased [K] by 8%. It would be interesting to know if the endothelial cells of the blood-brain barrier undergo diuretic-induced changes in cell ions to an extent similar to those found in the counterpart epithelial cells of the choroidal blood-CSF barrier. Alterations in brain capillary endothelial cell ions could not be determined because only 1–2% of brain tissue is endothelium.

In those regions of brain sampled, parietal cortex and pons-medulla, there were significant reductions in the V_d of ^{36}Cl brought about by treatment with both loop diuretics (Figure 4). This decrease in ^{36}Cl uptake by brain (Figure 4) may have resulted from inhibition of ^{36}Cl entry via NaCl cotransport in the luminal membrane of brain capillary endothelium (25–27). However, brain regions near the ventricles, eg. medulla, may take up tracer simultaneously from CSF as well as blood. This is particular relevant to tracers like ^{36}Cl which are rapidly transported across CP into CSF. Thus, CSF could act as a source of ^{36}Cl for brain regions close to the ventricles, and inhibition of ^{36}Cl movement across the CP from blood would diminish this source.

It was previously considered that ^{22}Na uptake into pons-medulla was significantly affected by ^{22}Na transfer across CP into CSF (5). Net diffusion of ^{22}Na into brain from CSF occurred because during the initial entry of ^{22}Na there was presumably an activity gradient from ventricular CSF to brain interstitial fluid. Thus, consistent with the idea of CSF acting as a source of tracer also in the present study, is our finding that the decreases in ^{36}Cl V_d observed in CSF (Figure 3) closely match those seen in brain (Figure 4). In other words, when brain uptake is mirrored by CSF uptake, the tight correlation suggests tracer permeation across the CSF-brain interface.

Summary. Under conditions of normal blood pressure and stable composition of arterial Na, K, pH, PCO_2 and tracer Cl activity, we demonstrated that both ethacrynic acid and furosemide inhibited by 20–30% the penetration of Cl from blood to CSF in adult rats. In these and in other acute experiments (1 to 2 hr), the CSF Cl transport inhibitors did not significantly alter CSF [Na] and [K]. However, the NaCl cotransport-inhibitor etha-

crynic acid caused a retention of Cl and K in CP, just as do acetazolamide and amiloride which interfere with basolateral Na-H exchange (3). Therefore, during inhibition of CSF formation, the KCl accumulation by CP presumably occurs to counter the depletion of cell Na, caused by drug-induced slowing of basolateral uptake of Na from interstitial fluid.

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