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Studies of Drug Elimination From the Cerebrospinal Fluid:
Cimetidine Transport in Choroid Plexus Epithelium

by

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DISSERTATION

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To my mother, Lafadie Belle Whittico, whose loving support and constant encouragement sustained me during this endeavor; and in memory of my father, James Malachi Whittico Sr.

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Abstract

Studies of Drug Elimination from the Cerebrospinal Fluid: Cimetidine Transport in Choroid Plexus Epithelium

Mathew Thomas Whittico

Many exogenous substances including drugs may accumulate in the central nervous system (CNS) to toxic concentrations. This accumulation may be caused by impaired cerebrospinal fluid (CSF) elimination processes. The H_2-receptor antagonist, cimetidine, has been observed to accumulate in the CSF of certain patient populations and the accumulation correlates well with symptoms of CNS toxicity. Although the processes by which substances may be eliminated from the CSF are known, the mechanisms by which these processes mediate CSF elimination has not been well characterized. The overall goal of this dissertation was to elucidate the mechanisms involved in the elimination of basic drugs from the CSF using cimetidine as a model compound. First the question of whether the elimination of cimetidine from the CSF involves pathways other than the normal bulk flow turnover of CSF was addressed. We studied the clearance of cimetidine from the ventricular CSF of the rat in vivo, employing a ventricular bolus injection technique and applying pharmacokinetic principles. The clearance of cimetidine from the CSF following a low dose was 11.8 ± 3 μl/min. We demonstrated that cimetidine is cleared from the CSF via pathways in addition to the bulk flow pathway. In addition, following the administration of a high dose of cimetidine the CSF clearance decreased to 8.04 ± 2.07 μl/min which is evidence that the clearance involves a saturable process. These results demonstrate that cimetidine is eliminated from the CSF of the rat by a pathway in addition to the bulk flow pathway and that at least one pathway is saturable and therefore possibly inhibitable.

A possible location for the saturable cimetidine CSF elimination process is the choroid plexus in the cerebral ventricles. Therefore, the second part of this dissertation was designed to elucidate the mechanisms of cimetidine transport at the apical membrane of the choroid plexus epithelial cell. We established a procedure for the separation and isolation of vesicles formed from the apical membrane of bovine choroid plexus epithelium. The effectiveness of the procedure to separate and isolate apical membrane vesicles was validated by monitoring the specific enhancement of the brush-border and basolateral membrane enzyme markers Na^+/K^+ATPase (12-fold enhancement) and alkaline phosphatase (3-fold enhancement), respectively, and by demonstrating the presence of a sodium-sensitive and concentrative L-proline transport system. Transport studies were conducted to determine if cimetidine transport at the apical membrane is via a carrier-mediated process. We studied the effect of the concentration of cimetidine on the initial rate of transport of cimetidine in the vesicles. A computer fit of the data showed that cimetidine transport in the vesicles occurs via saturable and nonsaturable processes. The estimated value for the saturable transport parameters, K_m and V_{max}, were 53.1μM and 16.7 pmol/sec/mg protein, respectively, and the nonsaturable parameter, K_{ns}, was 0.023 μl/sec/mg protein. Cimetidine uptake was inhibitable only by the hydrophobic organic cation, quinidine. Neither the hydrophilic organic cations, tetraethylammonium and ranitidine, nor the organic anions, p-aminohippuric acid and salicylic acid, were effective inhibitors of cimetidine transport. Evidence for the involvement of a mobile membrane protein carrier in the mediation of cimetidine transport was obtained from counter-flux studies. The transport was insensitive to chemical gradients of Na^+, HCO_3^-, and Cl^- and was accelerated by an outwardly-directed H^+ gradient. The results of these studies suggest that cimetidine is transported across the apical membrane of bovine

choroid plexus epithelium via a saturable, carrier-mediated process which is stimulated by an outwardly directed H^+ gradient and is insensitive to chemical gradients of Na^+, HCO_3^-, and Cl^- across the membrane. The transporter appears not to be selective for hydrophilic organic cations or organic anions. Based on these data we propose that cimetidine is transported across the apical membrane of choroid plexus epithelium by a facilitated active transport system.

Kathleen M. Giacomini

CHAPTER 1

Introduction - Cimetidine Cerebrospinal Fluid Elimination and the Role of the Choroid Plexus

Overall Goal

The goal of this dissertation is to elucidate the mechanisms involved in the elimination of basic drugs from the central nervous system (CNS). The studies are divided into two groups : (1) in vivo studies of the elimination of cimetidine from the cerebrospinal fluid (CSF) of the rat; and (2) in vitro studies of cimetidine transport at the apical (brush-border) membrane of the bovine choroid plexus. The objectives of the first group of studies were to address questions about the accumulation of cimetidine in the CSF of the intact animal and to determine specifically whether a saturable and inhibitable process is involved in its elimination from the CSF. The objectives of the second group of studies were to answer questions concerning the mechanisms of cimetidine transport in the choroid plexus epithelium, and in particular, the mechanisms at the brush-border membrane of the epithelial cell. In this chapter, both groups of studies will be introduced. In introducing the first section, particular attention will be placed on whole animal and clinical studies. In the second section, more detailed information about the choroid plexus and mechanisms of membrane transport will be presented.

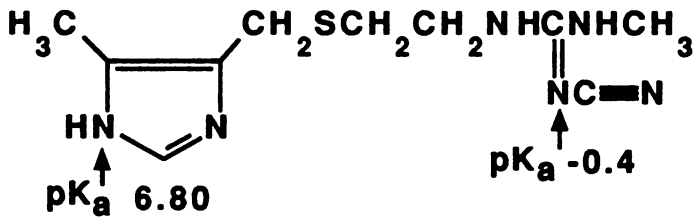
Cimetidine and Central Nervous System Toxicity

Cimetidine is a histamine H_2-receptor antagonist whose principal action is on parietal cells. It inhibits the secretion of gastric hydrochloric acid and reduces the output of pepsin by these cells stimulated by histamine, pentagastrin,

acetylcholine, insulin, and food (Bavin and Zarembo, 1984; Somogyi and Gugler, 1983). Chemically, the drug is classified as a substituted imidazole the structure of which is presented in Figure 1. Cimetidine has the following physiochemical properties: it is a weak base (pK_a =6.8); the melting point is between 140 and 143.5°; it decomposes above 175-185°; it is soluble in water and very soluble in ethanol and methanol; the apparent partition coefficient in an octanol/pH 7.4 phosphate buffer system at 25° is 2.0 (Bavin and Zarembo, 1984).

Molecular Weight 252.352

Empirical Formula $C_{10}H_{16}N_6S$



Common Name: Cimetidine

Chemical Name:
N"-cyano-N-methyl-N'-[2-(5-methyl-imidazol-4-yl)-methyl]thio]ethyl]guanidine

Trade Name: **Tagamet**

Figure 1 Chemical structure of cimetidine (From Bavin and Zarembo, 1984)

The pharmacokinetics of cimetidine in humans have been reviewed by Somogyi and Gugler (1983) and a brief summary of the pharmacokinetic parameters obtained in healthy volunteers is presented here. Following intravenous administration, the cimetidine plasma concentration verses time

profile exhibits multicompartmental characteristics. The total systemic clearance is 500-600 ml/min, approximately two thirds of which is renal clearance. Since the renal clearance is more than 3-fold higher than the GFR, net secretion of cimetidine into the urine occurs. The steady state volume of distribution is approximately 1 l/kg body weight. The elimination half-life is approximately 2 hr. Two peaks, which may be attributable to discontinuous absorption, are often seen in the plasma concentration profile following an oral dose . The absolute bioavailability is 60% in healthy subjects and increases to 70% with high variability in patients with peptic ulcer disease. Absorption and clearance of the drug are linear in the normal dosing range and steady state plasma concentrations range from 0.64 to 1.64 μg/ml following a 1 g/day dosing regimen. Following intravenous administration 50-80% of the dose is recovered unchanged in urine. Three known metabolites have been identified in human urine and the percentage recovery of the administered dosefor each are: cimetidine-sulfoxide (30%), 5-hydroxymethylcimetidine (5-8%), and guanylcimetidine (2%). Binding of cimetidine to plasma proteins is between 18 and 26% at plasma concentrations between 0.05 and 50 μg/ml. Red blood cell/plasma concentration ratios are close to unity.

Cimetidine was introduced in Great Britain in 1976 and in the United States in 1977 and is marketed by Smith Kline and French Laboratories under the trade name Tagamet. The drug has been widely accepted by the medical profession for its indicated uses in the treatment of duodenal ulcers and gastric acid hypersecretory states including the Zollinger-Ellison syndrome, systemic mastocytosis, and multiple endocrine adenoma syndrome (McGuigan, 1981). The wide acceptance of cimetidine is largely due to its efficacy in gastric acid secretion disorders and because of its relative safety. Adverse effects are few

and infrequent and serious toxicity is rare (Somogyi and Gugler, 1983; McGuigan, 1981). However, serious toxicity does occur because cimetidine can alter the hepatic metabolism of certain drugs through its interaction with the hepatic microsomal cytochrome P_{450} system. Other side effects have been attributed to the multireceptor action of the drug, acting at not only H_2-receptors but at androgen receptors, and receptors in the brain (Debas et al., 1986). Responses elicited as a result of this polyreceptor action include increased prolactin levels, gynecomastia, and symptoms of central nervous system (CNS) toxicity in certain patient populations. Consequently, two new H_2-receptor antagonist, ranitidine and famotidine, have been introduced. Possibly because of differences in their ring structures these agents do not appear to interact with pharmacological receptors other than the H_2-receptor and therefore do not exhibit the adverse effects as cimetidine.

Since its introduction in 1977 reports of CNS toxicity associated with the use of cimetidine have appeared in the medical literature (Table 1). The effects appear to be dose related and are most often seen in patients who are either very young (2 months) or elderly (> 55 years old) and in patients who have hepatic or renal dysfunction (McGuigan, 1981; Russell and Lopez, 1980; Weddington et al., 1982).

Table 1 Published Reports of Cimetidine-Associated Toxicity

Age (yr)	Dosage	Mental Syndrome	Onset[1]	Duration[2]	Reference
50	200 mg tid 400 mg p.o. hs (double dose for 24 hrs)	delirium	48 hr	24 hr	Grimson, 1977
34	200 mg tid 400 mg p.o. hs (double dose for 24 hrs)	delirium	24 hr	24 hr	Grimson, 1977
81**	200 mg i.v. q 4 hr	delirium	24 hr	24 hr	Grave et al., 1977
78	not given	delirium	48 hr	24 hr	Menzies-Gow and Nelson, 1977
25	12 g (od)	delirium	9 hr	24 hr	Menzies-Gow and Nelson, 1977
74	200 mg p.o. tid 400 mg p.o. hs	delirium	several days	few days	Delaney and Ravey, 1977
55	200 mg p.o. tid 400 mg p.o. hs X 10 wk 200 mg tid X 4 wk	confusion	3 wk	_	Robinson and Mulligan, 1977
65	_	confusion	_	_	Robinson and Mulligan, 1977
72	300 mg i.v. q 6 hr	delirium	10 hr	40 hr	Agarwal, 1978
11	20 mg/kg slow i.v. infusion	coma	15 min after 2nd dose	2 hr	Bacigalupo et al., 1978
53*	300 mg i.v. q 6 hr	coma	24 hr	24 hr	Levine, 1978
71	200 mg p.o. tid 400 mg p.o. hs	delirium	48 hr	16 days	McMillian et al., 1978
56	300 mg i.v. q 6 hr then 600 mg q 6 hr	delirium followed by coma	24 hr	96 hr	McMillian et al., 1978

Table 1 (continued)

Age (yr)	Dosage	Mental Syndrome	Onset[1]	Duration[2]	Reference
72	200 mg i.v. q 6 hr	delirium	3 days	_	Pomare, 1978
53**	300 mg i.v. q 6 hr	delirium	11 days	48 hr	Wood et al., 1978
54	200 mg p.o. tid 400 mg p.o. hs	delirium	48 hr	16 days	Cumming and Foster, 1978
49	300 mg i.v. q 6 hr	delirium	24	24	Arneson, 1979
4	15 mg/kg/day (75 mg i.v. qid)	delirium	10 days	3 days	Thompson et al., 1979
2 mo	20 mg/kg/day X 10 days, then 40 mg/kg/day	obtundation	48 hr	16 hr	Thompson et al., 1979
58	300 mg i.v. over 6 hr then 300 mg p.o qid	delirium	4 hr	96 hr	Barnhart and Bowden, 1979
58	300 mg p.o. tid	delirium	24 hr	48 hr	Barnhart and Bowden, 1979
58**	200 mg i.v. q 8 hr	delirium, status epilepticus	3 wk	few days	Edmonds et al, 1979
72**	200 mg i.v. q 8 hr	delirium	24 hr	24 hr	Edmonds et al, 1979
62	200 mg i.v. q 8 hr	convulsions	24 hr	_	Edmonds et al, 1979
81	300 mg p.o. qid	depression	months	months	Jefferson, 1979
37	200 mg p.o.tid 400 mg p.o. hs	anxiety- depression	3 wk	6 mo	Johnson and Bailey, 1979
76	1 g daily	delirium	5 days	_	Kinnel and Webb, 1979
80	450 mg i.v., then 150 mg i.v. q 8 hr	delirium	24 hr	24 hr	Schentag et al., 1979
73*	300 mg i.v. q 12	delirium obtundation	24 hr	48 hr	Schentag et al., 1979

Table 1 (continued)

Age (yr)	Dosage	Mental Syndrome	Onset[1]	Duration[2]	Reference
58**	300 mg i.v. q 6 hr	obtundation	36 hr	few days	Mogelnicki et al., 1979
60**	300 mg i.v. q 6 hr	obtundation	24 hr	few days	Mogelnicki et al., 1979
51	300 mg p.o. tid	paranoia	24 hr	72 hr	Adler et al., 1980
48	300 mg qid	depression	days	7-12 mo	Crowder and Pate, 1980
56*	30 mg i.v. q 12 hr	delirium	4 days	24 hr	Kimelblatt et.al., 1980
64	300 mg i.v. q 6 hr	delirium	24 hr	days	Weddington et al., 1981
49**	300 mg tid X 18 mo	visual hallucinations	6 hr following 0.375 mg triazolam	12 hr after d.c. both drugs	Britton and Waller, 1985
36*	300 mg i.v. q 6 hr	general disorientation	within 24 hr	hrs	Rosse, 1986
70	1 g/day i.v.	delusions	48 hr	24 hr	Yaron et al., 1986

[1] Onset, time between last dose.and start of mental status chances.

[2] Duration, time between onset and cessation of mental status changes.

* concomitant liver disease.

** concomitant renal dysfunction

(modified from Weddington et al. 1982)

Schentag et al. (1981) and Kimelblatt et al. (1980) studied the relationships between CNS toxic symptoms associated with the use of cimetidine and various patient factors including dose and serum concentrations, age, and concomitant disease state. A striking result of these studies was the apparent accumulation of cimetidine in the CSF of patients with liver disease. The CSF/plasma

cimetidine concentration ($\frac{C_{CSF}}{C_p}$) ratio in healthy volunteers was 0.2 whereas that in patients with liver was as high as 0.5. In these studies 300 mg of cimetidine was usually infused over 5-20 minutes every 6,8, or 12 hours. Steady state plasma concentrations of cimetidine were present in all subjects. Blood and urine samples were collected between two doses at 3 hours after a dose and 30 minutes prior to a dose. Samples were analyzed by high performance liquid chromatography (HPLC). The patients either had no impairment of renal or hepatic function, renal disease, liver disease, or both renal and liver diseases. It had previously been suggested that mental status changes correlated with elevated cimetidine trough concentrations (Schentag et al., 1979), however this correlation did not hold in these studies and systemic clearance values were not significantly increased in patients with liver disease. One conclusion drawn from these studies was that the blood-brain barrier permeability to cimetidine increases in the disease state. Ziemniak et al. (1984) studied the distribution of cimetidine into the CSF of mongrel dogs after intravenous administration. CSF concentrations peaked within 1 hour and declined in parallel with plasma concentrations. The cimetidine CSF partitioning was comparable to that seen in healthy human subjects ($\frac{C_{CSF}}{C_p}$ = 0.125) and the sulfoxide metabolite was not found in CSF or brain. Therefore, it was concluded was that cimetidine appears to enter the CSF by passive diffusion and that brain metabolism of cimetidine is unlikely.

While the mechanisms involved in the manifestations of CNS toxicity associated with the use of cimetidine is an interesting area and poses many intriguing questions, an equally interesting and perhaps more important question, in terms of its implications to other basic drugs, concerns the

mechanisms involved in the accumulation of cimetidine in the CSF. Various aspects of this question are addressed in this dissertation.

As discussed in Chapter 3, the apparent accumulation of cimetidine in the CSF of patients with hepatic dysfunction could be explained as follows: (1) the permeability of the blood-brain barrier for cimetidine is increased in patients with liver disease as proposed by Schentag et al. (1981); and (2) the clearance of cimetidine from the CSF to blood is impaired in patients with liver disease. In these studies we investigated the possibility of impaired cimetidine CSF clearance and the mechanisms of transport of cimetidine from the CSF. In order to conduct studies of CSF clearance under relatively physiological conditions we modified existing experimental techniques and applied pharmacokinetic principles. These methods are presented in Chapter 2.

The Role of the Choroid Plexus as an Organ of Elimination

In Chapter 3 evidence is presented for the apparent involvement of a saturable process in the elimination of cimetidine from the CSF. As will be discussed in Chapter 5, Suzuki et al. (1985) demonstrated a saturable uptake process for cimetidine in isolated rat choroid plexus. The objectives of the second portion of this dissertation were to elucidate the mechanisms involved in the uptake of cimetidine at the apical (CSF-facing) membrane of the choroid plexus epithelium.

Choroid Plexus Review

A historical review of the embryology, macrostructure, microstructure, fine structure, function, and pathology of the choroid plexus has been presented by Dohrmann (1970), The physiology of the choroid plexus in terms of bulk fluid

production, composition of CSF secretion, absorptive processes, and function has been reviewed by Cserr, (1971 and 1975). Netsky and Shuangshoti (1975) in collaboration with others have provided a thorough description of the literature on the choroid plexus in both normal and pathological conditions. Masuzawa and Sato (1983) have provided an excellent historical account of the study of choroid plexus histochemistry along with their findings on the localization of enzyme activity in the choroid plexus and the function of various enzymes in the tissue in terms of those findings. The purpose of this discussion is to provide an overview of the anatomy and physiology of the choroid plexus in order that an appreciation for the questions being addressed in this dissertation may be obtained and to summarize our present knowledge of the transport of cimetidine from the CSF via the choroid plexus.

The choroid plexus is recognized as the major site of CSF production (Cserr, 1975; Wright, 1978a) however, certain aspects of the mechanisms of CSF secretion and other function of the tissue are still unexplained. Therefore, most nonpathological studies of the choroid plexus are related to the anatomy, physiology, histochemistry, and absorptive properties of the tissue in hopes of correlating structure and activity to facilitate a better understanding of the mechanisms involved in the function of this complex organ. Histologically, the choroid is unique among epithelia which have secretory function in that the localization of the enzyme Na^+/K^+ATPase is in the apical membrane and alkaline phosphatase is localized in the basal membrane. The reverse is true in intestinal and renal epithelia. This location of Na^+/K^+ATPase at the brush-border membrane provides the unique secretory mechanism of fluid production in response to ion secretion as opposed to flow-dependent ion flux (Cserr, 1971).

Anatomy and Location of the Choroid Plexus

Netsky and Shuangshoti (1975) and Nolte (1981) have described the anatomical features and location of the choroid plexus. In humans the choroid plexus is present in each lateral, third and fourth ventricle of the brain (Figure 2).

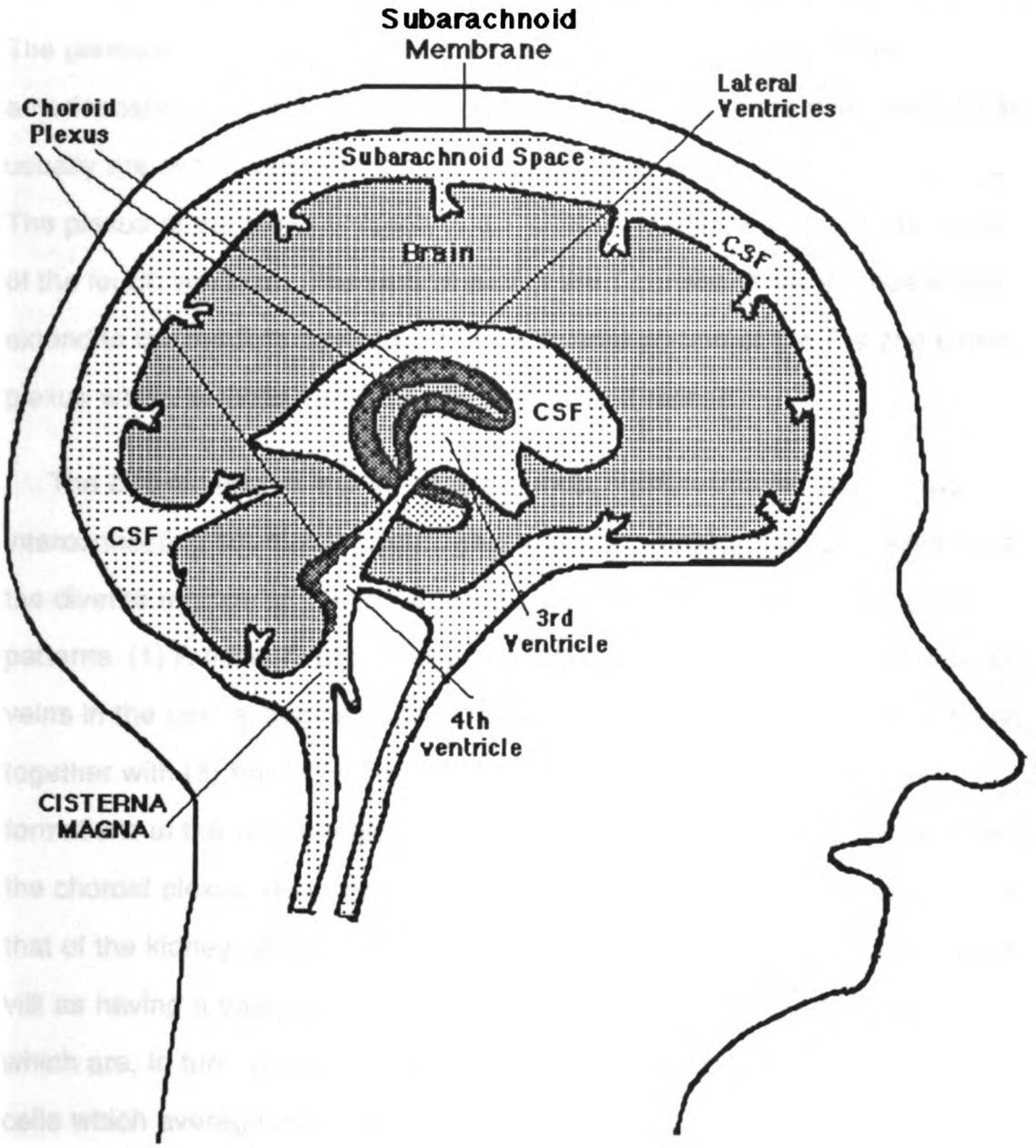


Figure 2 Sagital view of the choroid plexus in the ventricles of the brain (modified from Nolte, 1981)

The plexus of the lateral ventricle follows the C-shaped choroidal fissure which begins at the interventricular foramen. It passes along the floor of the ventricle posteriorly and laterally appearing as a long mesh or net-like structure along the surface of the thalmus and fornix and becomes enlarged as it turns and descends laterally and anteriorly into the temporal horn where it terminates. The plexuses of the third ventricle are two parallel strips lying in the anterioposterior direction along the midline of the roof of the third ventricle and usually are continuous with the plexus of the lateral ventricles of the same side. The plexus of the fourth ventricle is a T-shaped structure which arises in the roof of the fourth ventricle. The vertical part of the T consist of two strands which extend to the medium aperture. The transverse portion of the T is one strand of plexus which extends vertically into each lateral recess.

The choroid plexus is a mesh of tortuous, highly vascular, and villous interconnecting structures (Masuzawa and Sato, 1983). The capillary bed in the diverse regions of the tissue in the lateral ventricles belong to one of 3 patterns: (1) A network of capillary meshes that envelop the large arteries and veins in the central segment; (2) A leaf-like organization of sinusoids is found together with (3) fronds, infoldings of villous processes, of "glomerular" formations in the villous regions of the plexus (Motti et al.,1986). Blood flow to the choroid plexus measures approximately 3 ml/min/g which is about 2-fold that of the kidney (Wright, 1978a). Brightman (1975) described the choroidal villi as having a vascular core surrounded by loosely arranged stromal cells which are, in turn, completely encased by a sleeve of simple, cuboidal, epithelia cells which average approximately 15 μm in height. These cuboidal epithelia cells, first studied by Purkinje (as cited by Dohrmann, 1970), are

morphologically polar, having a microvillous apical (brush-border) membrane and a basal membrane (Figure3).

The apical membrane has numerous microvilli which greatly enhance its surface area. The basal membrane also has infoldings typical of other secreting epithelia (Cserr, 1971). The epithelia cells are encircled at the apical membrane by strands of fibrils which form zonulae occludentes (tight junctions) between adjacent cells (Brightman and Reese, 1969). Voetmann (1949, as cited by Dohrmann, 1970) separated the epithelial cells from the stroma by maceration in distilled water and determined the total number of cells from all four plexuses. In the choroid plexuses from six men the total number of cells ranged from 91.1 to 130.2 millions and in 14 women the range was from 76.8 to 116.2 millions. The mean value was 107 and 96.3 millions for men and women, respectively, and the mean for both sexes was 100.6 millions. The mean surface area of the individual cells was 213 square microns, therefore, the total surface area of all four plexuses approximates 213 cm^2 which equals about two thirds of the total ventricular surface area.

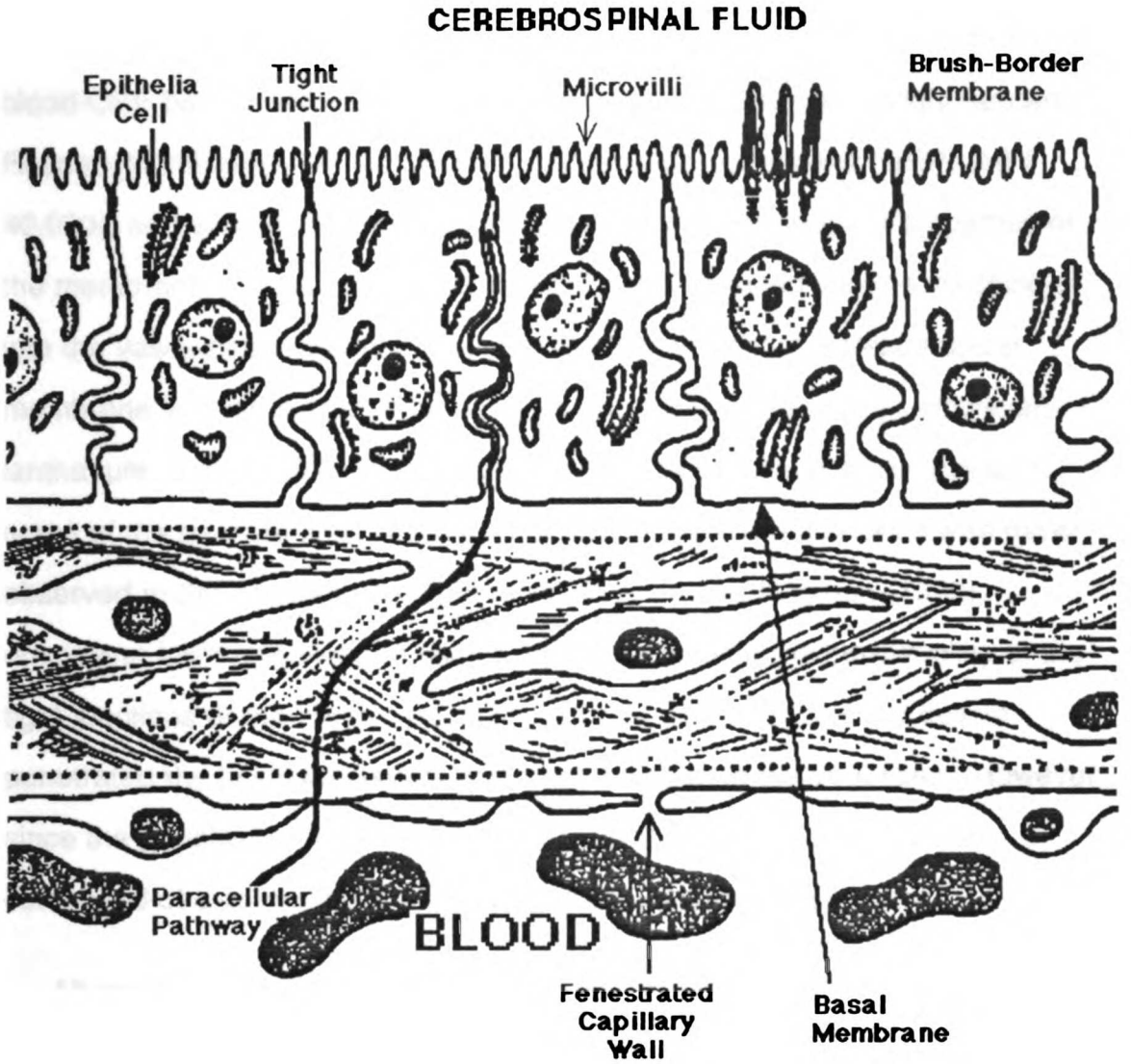


Figure 3 Microstructure view of choroid plexus cells (from Nolte, 1981)

Tight Junctions - a Paracellular Flux Pathway

An anatomical feature of the choroid plexus which has been a target of investigation for the past 20 years is the so called tight junction between the epithelia cells. Insight into the function of these specialized structures may lead to a better understanding of the mechanisms of transcellular fluxes at this interface between the blood and the CSF. It is thought that these structures,

which cause the cells to abut at the apical membrane, constitute part of the blood-CSF barrier, the counterpart of the blood-brain barrier. Brightman and Reese, (1969) used the microprotein, horseradish peroxidase (HRP) (MW 40,000), and electron microscopy to determine the permeability properties of the membrane in mice. In their study HRP, when injected into the ventricle or into the vasculature, was not observed to cross the choroid plexus apical membrane into the extracellular clefts or ventricles, respectively. However, lanthanum, a colloidal ion (MW 139), was able to penetrate a few layers of some of the pentalaminar junctions, but according to the authors it was never observed in the opposing compartment. Bouldin and Krigman (1975), employing La^{3+} and electron microscopy to determine the permeability of the tight junctions of the choroid epithelium of rats, observed that the colloid penetrated the junctional layers and could be seen in the ventricles. However, since the penetration was focal it was concluded that the structures are composed of a combination of "very tight" and "leaky" tight junctions.

Alternative to tracer proteins and colloids, the freeze-fracture technique has been used to characterize the structural features of tight junctions. The method involves fixing the tissue with glutaraldehyde followed by freezing. The fracture process yields a plate with material from the protoplasmic side (P-face), where the tight junctions usually appear as ridges, and a plate with material from the exoplasmic side (E-face) where evidence of tight junctions appears as grooves. Problems arise in interpretation of electronmicrographs of the plates because, oftentimes there are discontinuities in the ridges of the P-face which can not be accounted for by particles in the grooves of the E-faces. This has lead some investigators to conclude that these discontinuities are leaks in the tight junctions and contribute to the low resistance across the membrane. However,

assessment of tight junctions by freeze-fracture is qualitative at best. Geometric relationships of contiguous cells and the physiochemical properties of the junctional ridges are the factors which would ultimately influence the permeability of these structures (Bouldin and Krigman, 1975). Mollgard et al. (1979) used freeze-fracture along with double replica techniques to analyze tight junctions in fetal sheep choroid plexus. In their studies all P-face discontinuities could be accounted for by particles in E-face grooves. However, complex strands were identified which contained rows of particles between two parallel ridges. Also gap junction formations were observed within tight junction structures. The authors propose that the discontinuities in tight junctions cited by others were artifacts of the process. Claude and Goodenough (1973) had proposed that the number of junctional strands determine the tightness of the tight junction (< 4 = "leaky", > 5 = "very tight"). Van Deurs and Koehler (1979), using the freeze-fracture technique, determined that there is an average of 7-8 junctional strands comprising the tight junctions of the choroid plexus of the rat, 25% of which have 7-8 nm discontinuities. This contrasts sharply with "very tight" tight junction epithelia (e.g., intestine) which have 5% discontinuities. The authors concluded that these discontinuities are pores which account for the low resistance and high membrane permeability observed by others. The number of tight junctional strands in frog choroid plexus epithelium was determined by von Bulow et al. (1984) who concluded that the high number (5-6) did not fit the previously proposed model of Claude and Goodenough (1973) for correlation of morphology and physiology (i.e.; "leaky" tight junctions should have fewer junctional strands than "very tighter" tight junctions). Because of this discrepancy between morphology and permeability, the authors suggested that the present concept of the role of tight junctions may be oversimplified.

Microenvironment and Physiology of the Choroid Plexus Epithelia Cell

A related subject of interest to the issue of the structure and function of the tight junctions of the choroid plexus epithelium is the nature of the secretory process involved in the elaboration of CSF. Considerable insights into the mechanisms of inorganic ion fluxes and CSF formation have been elucidated over the past two decades. The unidirectional fluxes of solutes and water, transepithelial electrical potential, currents and resistances, intracellular pH, transmembrane electrical potentials, and intracellular ion activities including K^+, Na^+, Cl^- and HCO_3^- have been studied in in vitro preparations of frog choroid plexus mounted in Ussing-type flux chambers (Wright, 1972a, 1972b, 1974, 1977a, 1977b, 1978a, 1978b, 1978c, 1982, 1984, 1987; Quinton et al., 1973; Zeuthen, 1979; Zeuthen and Wright, 1978, 1981; Ehrlich and Wright, 1982; Saito and Wright, 1982, 1983, 1985, 1987; Wright and Saito, 1987) and in isolated rat choroid plexus tissue uptake preparations (Johanson et al., 1974; Smith and Johanson, 1980; Johanson, 1984; Smith and Johanson, 1985; Johanson et al., 1985). A graphic description of the pH, chemical potential, and electrical potential gradients across the choroid cell of the bullfrog is presented in Figure 4.

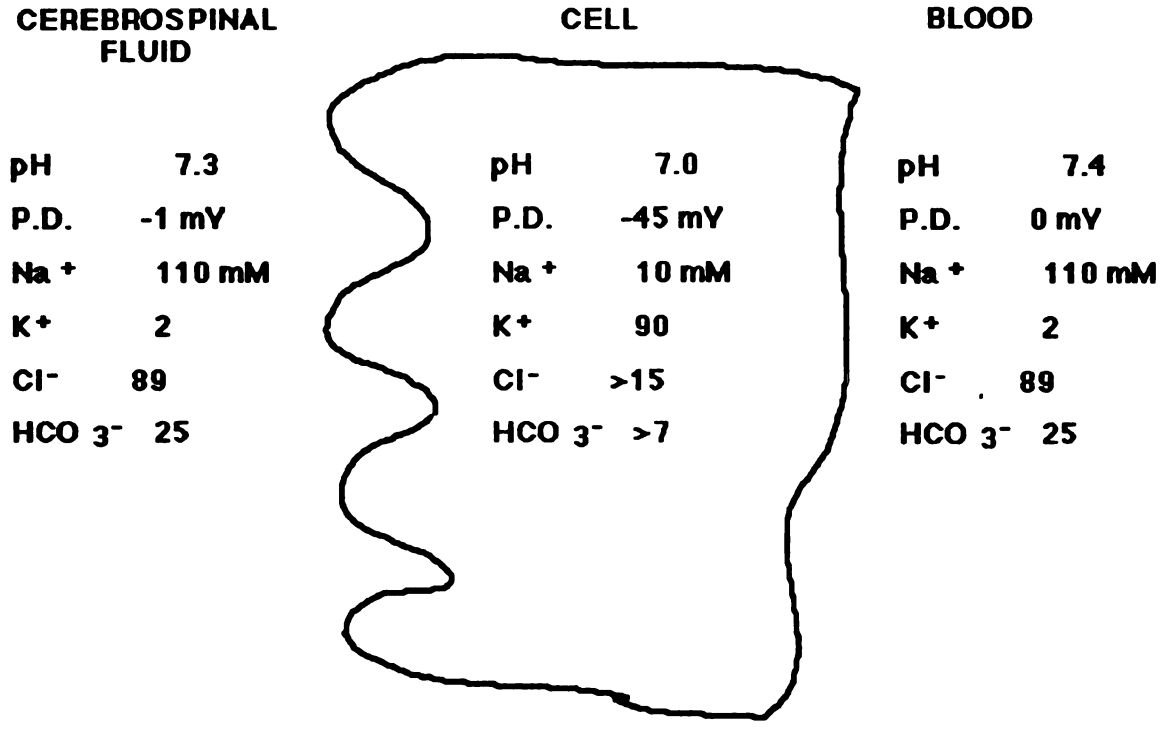


Figure 4 Electrical potential, chemical potential, and pH gradients across the choroid plexus epithelia cell of the bullfrog (after Johanson,1984; Wright and Saito, 1987)

From these studies models of ion transport across the choroid plexus epithelium have been developed (Saito and Wright, 1987; Johanson, C.E., 1984, respectively).

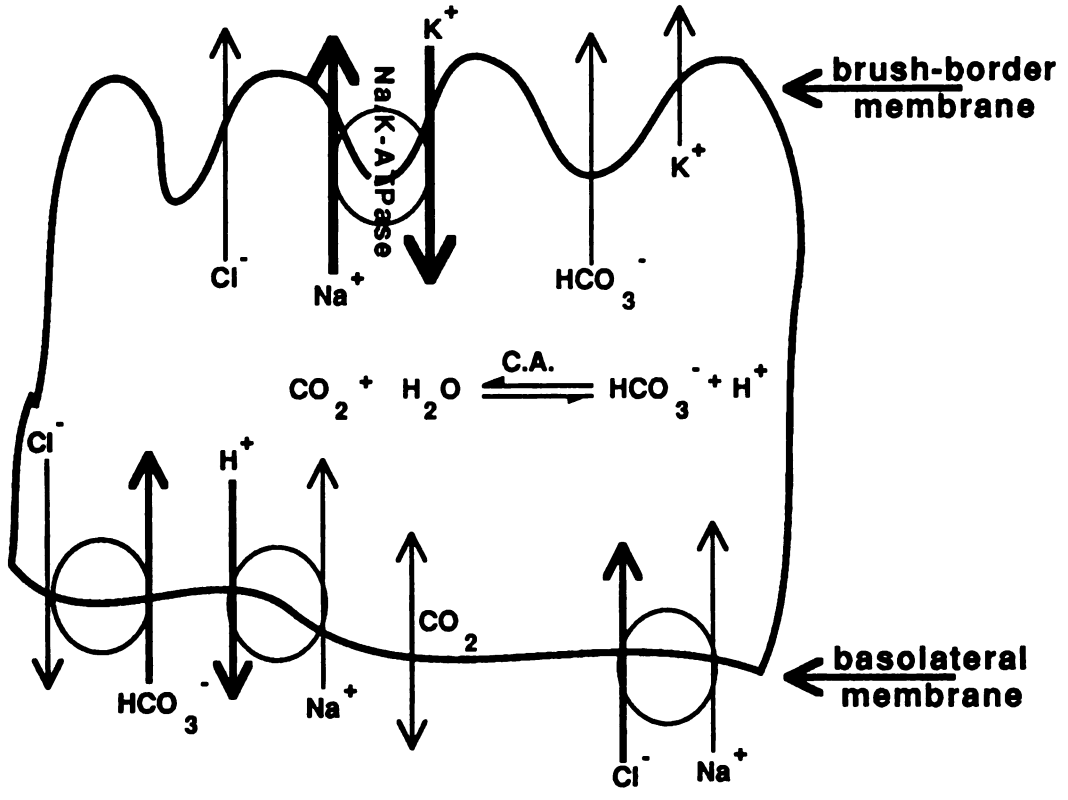


Figure 5 A model of ion transport mechanisms across the choroid plexus epithelium (from Wright and Saito, 1983).

In the model developed by Wright and Saito (1983) (Figure 5) there is net transport of Na^+, Cl^-, HCO_3^- and fluid toward the CSF and a small net K^+ transport toward the blood across the choroid plexus epithelium. The Na-K pump in the brush-border membrane creates steep Na^+ and K^+ electrochemical potential gradients across the membranes. The inside negative membrane potential of 45 mV is explained in terms of K^+ diffusing back into the CSF and the electrogenicity of the Na-K pump. Under the favorable Na^+ gradient, Na^+

enters the epithelial cells across the serosal border via Na-H exchange and NaCl cotransport mechanisms. Intracellular HCO_3^- is supplied through CO_2 hydration and Cl-HCO_3 exchange mechanisms. Intracellular activities of both HCO_3^- and Cl^- are higher than that predicted for passive distribution, and these anions diffuse into CSF across the brush-border down the electrochemical potential gradients. Since the presence of HCO_3^- stimulates the ouabain-sensitive flux of both Na^+ and Cl^- across the brush-border membrane, the effect of HCO_3^- is either on the pump itself or it creates a favorable potential across the membrane which enhances cation flux in either direction. HCO_3^- transport into CSF was stimulated either by intracellular concentrations of c-AMP which were increased either by hormones or by phosphodiesterase inhibitors, or by increased conductance of HCO_3^- across the brush-border membrane. The neuronal influence on CSF production is in agreement with the results of Lindvall and Owman (1978) and Lindvall et al. (1981) who observed a sympathetic neurogenic influence of the production even in early postnatal development in the rabbit and a sympathetic influence on the choroid accumulation of cations and anions in the rat and the rabbit. Zeuthen and Wright (1981) were able to show that the membrane permeability for K^+ is much greater in the brush-border membrane than in the basal membrane, that resistance at the serosal side is 9-fold greater than at the ventricular side, and that 96% of the transmural current due to K^+ is paracellular in this preparation.

From the work done by Johanson's group (Johanson et al., 1974; Smith and Johanson, 1980; Johanson, 1984; Smith and Johanson, 1985; Johanson et al., 1985), studying the effects of alterations in the concentrations of HCO_3^- and Cl^- both intra-and extracellular on intracellular pH and Na^+ concentrations, a model

was developed to describe intracellular and transcellular events during the elaboration of CSF in the rat (Figure 6)

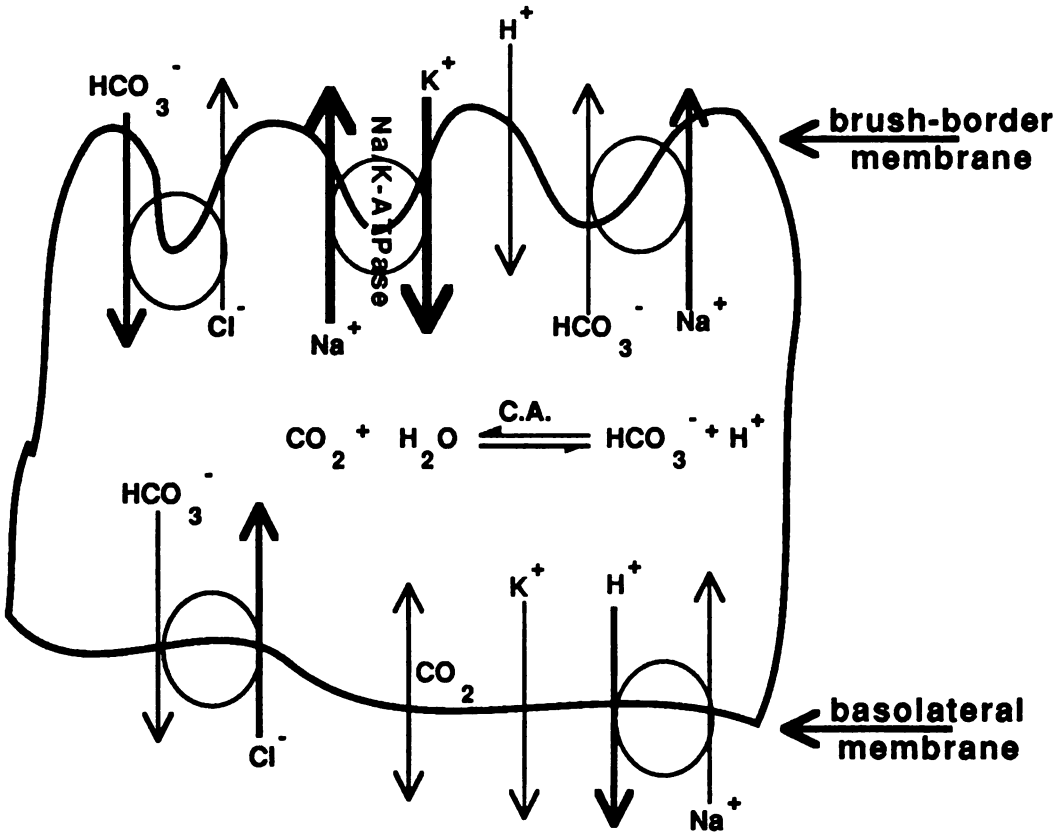


Figure 6 A model of ion transport mechanisms across the choroid plexus epithelium (from Johanson, 1984).

The model is not significantly different from that of Wright and Saito (1983). There is net secretion of Na^+, Cl^-, HCO_3^- and water from blood to CSF, net reabsorption of K^+ and H^+ from CSF to blood. Na^+ enters the cell via a Na-H exchanger in the basal membrane and exits via the Na/K pump and a proposed Na-HCO_3 cotransport system, both in the brush-border membrane. The transcellular flux of Cl^- is mediated by HCO_3-Cl exchangers at both membranes.

In both models the formation and composition of CSF by the plexus may be influenced by anion conductances across the epithelial cells and the intracellular concentration of HCO_3^- is significant to the overall process. The major difference between the two models is the mechanism of Cl^- and Na^+ entry at the basal membrane. Wright's group found a Na^+-dependence in the Cl^- flux at this membrane in bullfrogs, whereas the Johanson group observed the flux to be dependent only on HCO_3^- in rats. These differences may be species related.

These models can be used to gain insights into the possible mechanisms by which other molecules including drugs may be transported across the choroid plexus when one considers that the transport of many solutes across epithelia is coupled to the flow of inorganic ions down their electrochemical gradient. Since cimetidine exists partially as an organic cation at the physiological pH of CSF, in this dissertation several models of the mechanisms underlying active organic cation transport in the choroid plexus were tested to determine the mechanisms of transport for cimetidine at the brush-border membrane. These models were based on information obtained about ion gradients across the choroid plexus epithelium as well as on results from studies showing an influence of ion gradients on cimetidine transport in renal epithelium.

Common Organic Cation Transport System in the Choroid Plexus

Evidence that a transport system which is selective for organic cations may exist in the choroid plexus was provided by Schanker et al. (1962) who demonstrated that the organic cations NMN, hexamethonium and decamethonium were eliminated from the CSF by pathways in addition to bulk flow and that the elimination of both hexamethonium and decamethonium could be inhibited by NMN. That study also provided the first evidence that organic

cations may be eliminated from the CSF by pathways which appear to involve a saturable transport mechanism in addition to bulk flow. Although the study gave no indication of the location of the transport mechanism, subsequent studies by Tochino and Schanker (1965a, 1966) demonstrated that the choroid plexus may be the site of active organic cation transport. Further studies by Tochino and Schanker (1965b) demonstrated that the primary amines, serotonin and norepinephrine, as well as the secondary amine, epinephrine, appeared to share the same uptake process since these compounds inhibited the uptake of the quaternary amines and vice versa. Since these early studies a number of studies have documented that a variety of basic drugs and endogenous amines actively accumulate in whole choroid plexus of the rat and the rabbit (Aquilonius and Winbladh, 1972; Cserr, 1971; Hug, 1967; Eriksson and Winbladh, 1971; Spector and Goldberg, 1982; Lindvall-Axelsson et al., 1985; Lindvall et al., 1981; Goldberg et al., 1987; Spector, 1980; Miller and Ross, 1976; and Suzuki et al., 1986). These compounds include morphine, dihydromorphine, nalorphine, codeine, atropine, nicotine, lidocaine, diphenhydramine, and cimetidine. Methods used in most of these studies have been similar to those of Tochino and Schanker (1965a). Tissue to media ratios for a number of basic compounds in whole choroid plexus from rabbit brain have ranged from 5 to 540 (Cserr, 1971). Metabolic inhibitors have been employed to ascertain whether accumulation is dependent upon oxidative metabolism, and competitive inhibitors have been used to elucidate the specificity of the transport. In general these studies have corroborated the early studies (Tochino and Schanker, 1965a; Schanker, 1962; and Tochino and Schanker, 1966) and suggest that an active transport system for organic cations of diverse chemical structures exists in the choroid plexus. Because these studies have been carried out in whole tissue preparations, the site of organic cation

transport is unknown. Transport in these preparations may occur in capillary endothelial cells or in the epithelium. In contrast to the studies mentioned above, Ehrlich and Wright (1982) observed in the bullfrog choroid plexus that the organic cation, choline, is transported from CSF to blood by Na-choline cotransport in the arachnoid membrane. Choline accumulated in the choroid plexus by a ouabain sensitive mechanism but net flux across the plexus was small and ouabain insensitive. Both ouabain and probenecid were effective inhibitors of choline transport and Li^+ greatly stimulated choline transport across the arachnoid membrane suggesting a Li^+-Na^+ counterport system. The organic anion, p-aminohippuric acid (PAH), actively accumulated in the choroid plexus but not in the arachnoid membrane and there was net transport from CSF to blood which was sensitive to probenecid and ouabain, but insensitive to Li^+.

The organic cation transport system in the choroid plexus is clearly different from the system in the renal proximal tubule in the direction of net transport (Rennick, 1981; Miller and Ross, 1976; Schanker et al., 1962). Moreover, as described below, recent studies with cimetidine in isolated choroid plexus of the rat have demonstrated that there may be important differences in the structural selectivities of the systems in the two epithelia (Suzuki et al., 1986a, 1986b). Thus the organic cation transport mechanism in the choroid plexus may exclude cimetidine and the system involved in cimetidine transport may exclude hydrophilic organic cations.

Notable differences in choroid plexus transport mechanisms in comparison to transport mechanisms in other epithelia have been reported for glucose and amino acids (Deane and Segal, 1984; Ross and Wright, 1984; Wright, 1972a and 1972b; Wright, 1978; Wright and Saito, 1987). In brush-border membrane

vesicles isolated from rabbit and bovine choroid plexus, Ross and Wright (1984) reported that the well-characterized Na^+-glucose cotransporter which exists in the renal and intestinal brush-border membrane was absent in the choroid plexus brush-border membrane. Moreover, phenylalanine, which inhibits Na^+-driven proline and methylaminobutyric acid transport in the brush-border membrane of the kidney and intestine, did not ,even at high concentrations, inhibit their transport in the plexus brush-border membrane.These data suggest that the choroid plexus Na^+/amino acid cotransport system has a unique structural specificity. Deane and Segal (1984), in experiments in which the vasculature of the choroid plexus from the lateral ventricle of the sheep was perfused, reported that there was a net entry of sugars from blood to CSF at all concentrations of sugars and the order of affinity of the transporting system for the sugars studied was 2-deoxy-D-glucose >> D-glucose > 3-O-methyl-D-glucose >> D-galactose. The transport from blood to CSF and from CSF to blood both consisted of a saturable and a nonsaturable component. However, the blood to CSF transport involved a low affinity and high capacity system, whereas the CSF to blood transport involved a high affinity and low capacity system. The concentration of glucose in newly formed CSF was only 45-60% of that in plasma. The Na^+-dependence of the transport in these systems was not tested. Another unique feature of the choroid plexus epithelium, is that in contrast to epithelium of the renal tubule and intestine, the sodium pump is on the brush-border, rather than the basolateral membrane (Quinton et al., 1973).

Studies of the Mechanisms of Cimetidine Transport in the Choroid Plexus

The studies of organic cation transport and the noted apparent differences in transport properties for different classes of compounds in the choroid plexus

to those observed in other epithelia emphasizes the uniqueness of this specialized tissue and its role in regulating the microenvironment of the brain. It appears that this uniqueness in transport characteristics extends to the transport of cimetidine as well.

Suzuki et al. (1985) studied the elimination of cimetidine from the CSF in the rat. They employed the technique of ventriculocisternal perfusion to determine whether a saturable pathway is involved in cimetidine CSF elimination. The cerebral ventricles of the rat were perfused from the lateral ventricle to the cisterna magna at a rate of 17.4 µl/min with a perfusate containing 3H-cimetidine (14 nM or 4 mM) and 14C-sucrose as a marker of bulk flow of CSF. Steady-state concentration levels were achieved within 80 min and concentrations of cimetidine and sucrose were determined from the radioactivity in samples of the outflow perfusate by scintillation counting. The extraction ratio of cimetidine and sucrose from the perfusate was determine from the ratios of the outflow and inflow concentrations. At perfusate concentrations of 14 nM the extraction ratio of cimetidine was 0.19 and decreased to 0.09 at perfusate concentrations of 4 mM. This result was evidence that a saturable process may be involved in the elimination of cimetidine from the CSF.

Suzuki et al. (1986a) have studied the uptake of cimetidine into isolated whole choroid plexus tissue from the rat. The lateral ventricle choroid plexuses of the rat were each divided into two parts and incubated in an artificial CSF medium containing 0.22 µM ^{3}H-cimetidine with and without various inhibitors. Tissue to medium cimetidine concentrations were determined following a 3 min incubation period. The effect of various inhibitors and other perturbations were determined by comparing initial rates of uptake. The uptake of cimetidine was almost completely inhibited by the metabolic inhibitors 2,4-dinitrophenol (1mM)

and KCN (1mM). A 10° reduction in the temperature of the incubation medium decreased cimetidine uptake by 78%. These findings suggest that cimetidine accumulation in the tissue occurs via an energy-dependent process. Protein modifying agents had mixed effects on cimetidine uptake. p-Hydroxy-mercuribenzoate (0.1 mM) reduced the uptake while N-ethylmaleimide (0.1 mM) did not. Na^+/K^+ATPase inhibition by ouabain had no effect on cimetidine uptake nor did replacement of Na^+ with Li^+. These results suggest that the uptake of cimetidine into choroid plexus tissue is sodium independent. The effect of cimetidine concentrations in the medium on the initial rate of uptake reflected a saturable process. Kinetic parameters for cimetidine transport were 53 μM and 12 nmol/min/ml for K_m and V_{max}, respectively. When the pH of the medium was varied between 4 and 9 the uptake of cimetidine was inhibited at pH values less than 7. From these data the investigators concluded that the accumulative uptake of cimetidine could not be explained solely on the basis of partition theory. The hydrophilic organic cations tetraethylammonium (TEA), [1]N-methylnicotinamide (NMN), and choline and the bases histamine, creatinine, and procainamide were ineffective as inhibitors at 1 mM concentrations. TEA, NMN, and choline were also ineffective at 20 mM concentrations, but histamine and creatinine inhibited cimetidine uptake at this concentration. The hydrophobic organic cations, quinine and quinidine inhibited cimetidine uptake at 0.1 mM concentrations. These findings imply that the involvement of a common organic cation transport system in the mediation of cimetidine transport in isolated choroid plexus is limited. The organic anions, 5-hydroxyindoleacetic acid, p-aminohippuric acid, 4-hydroxy-3-methoxyphenylacetic acid, salicylic acid, and benzylpenicillin all inhibited cimetidine uptake by more than 50% at 1 mM concentrations with benzylpenicillin having the greatest inhibitory effect, suggesting that cimetidine

shares a common transport pathway with organic anions. There was approximately 50% inhibition of the uptake by oligopeptides Gly-Gly (20 mM) and Gly-Gly-His (20 mM) and to a lesser extent by Gly (40 mM) and His (20 mM), but not by ß-alanylhistidine (20 mM). The apparent K_m for cimetidine transport increased 2-fold (100 μM vs 53 μM) as a function of Gly-Gly concentrations and the K_i for Gly-Gly inhibition was 23 mM while the V_{max} was unchanged. Cimetidine (1 mM) inhibited the uptake of benzylpenicillin by 65% compared to a 66% reduction by unlabeled benzylpenicillin, and salicylic acid uptake was inhibited by 32%. TEA did not accumulate in the tissue against a concentration gradient, nor was it significantly inhibited by cimetidine. The results of this study are evidence for active cimetidine transport in the choroid plexus of the rat. The data suggest that transport occurs via an energy-dependent, Na^+-independent process which may be common to organic anions, imidazoles, and oligopeptides.

Suzuki et al. (1986b) continued their previous study (Suzuki et al., 1986a) by determining whether there is a difference in the specificity of cimetidine transport between the lateral and the 4th ventricles choroid plexuses of the rat employing the same isolated whole choroid plexus technique. Briefly, the investigators examined the effects of the organic cation inhibitor, TEA and the organic anion inhibitor benzylpenicillin on the uptake of cimetidine on choroid plexus from the lateral and 4th ventricles. Benzylpenicillin (1 mM) inhibited the uptake of cimetidine whereas 1 mM TEA did not. The authors concluded that there is no difference in cimetidine transport in the lateral and the 4th ventricle choroid plexuses.

Suzuki et al. (1988) investigated the efflux of cimetidine from the CSF of the rat by the method of lateral ventricle injection and cisterna sampling. A bolus

injection of a solution containing ^{3}H-cimetidine (25.5 mg or 45.7 mg) and ^{14}C-inulin (1.7μg) with and without benzylpencillin or NMN was administered into the lateral ventricle. Samples were drawn from the cisterna and assayed for cimetidine and inulin concentration by scintillation counting. CSF concentration data were fit by computer to an equation for a 2-compartment model with an estimated volume of distribution of 400 μl. CSF clearances were estimated from the dose and the AUC. The AUC was calculated from the estimated parameters obtained from the fit of the data to the 2-compartment model equation and the estimated volume of distribution (400 μl). The CSF clearances of cimetidine following the 25.5 mg dose and the 45.7 mg dose were 72 μl/min and 24 μl/min, respectively. Cimetidine CSF clearances in the presence of 80 μmol benzylpencillin or NMN were 26 μl/min and 72 μl/ min, respectively. These results suggest that the clearance of cimetidine from CSF to blood in the rat involves a saturable pathway which is inhibited by the organic anion benzylpenicillin, but not by the organic cation TEA. The values obtained for cimetidine CSF clearance in this study are 22-fold higher than those calculated from the data from the previous study (Suzuki et al., 1985). However, the experimental design, as stated by the authors assumes uniform distribution of the drug in the total estimated CSF volume of 400 μl within a few minutes after administration. This may be an overestimation of the distribution rate. The rate of CSF production in the rat is 2.2 μl/min (Cserr, 1971,1975). The flow of CSF through the ventricles and out into the entire 400 μl CSF space probably requires more than a few minutes. It is more likely that the reference volume should be that of the ventricles, since a cannula place in the cisterna collects fluid which is immediately exiting the apertures of the ventricular cavity.

A single methodological advancement is primarily responsible for the greatly enhanced understanding of the mechanisms involved in the renal transport of organic cations, namely, the development of methods used to separate and purify the polar membranes of epithelia (Schmitz et al., 1973; Booth and Kenny, 1974). Application of isolated membrane vesicles to investigations of the mechanisms involved in organic cation transport in the kidney has allowed the dissection of the events occurring at the brush-border and the basolateral membrane surfaces. The recently developed methodological advances for the isolation and separation of brush-border and basolateral membranes have not been applied to the study of organic cation transport in choroid plexus. Thus we know nothing about the molecular events involved in the transport of organic cations in each membrane, the structural specificity of the transporter in each membrane, and importantly, the driving forces and coupled diffusion pathways for organic cation transport. The application of isolated membrane vesicles in this disseration to investigate the mechanisms of cimetidine at the brush-border of the choroid plexus epithelia provides a model for studying the mechanisms of the transport of basic drugs in this tissue.

The choroid plexus transport of basic drugs is of considerable biological significance. Presumably, the system functions as part of the blood-CSF barrier in protecting the CNS from biologically potent amines including a number of basic drugs and their metabolites. Therefore, transport systems in the choroid plexus may control the exposure of the CSF and the brain to specific agents including toxic compounds. Knowledge of the mechanisms involved in the transport of basic drugs may lead to an enhanced awareness of the mechanisms involved in CNS toxicities as well as facilitate the design of future compounds specifically targeted to or away from the CSF. The work presented

in this dissertation provides further insight into the overall process of drug interactions with the blood-CSF interface.

CHAPTER 2

Development of a Bolus Ventricular Injection Model

Introduction

The translocation of substances from the CNS, and especially from the CSF, to the systemic circulation has long been of interest to many neurophysiologists and pharmacologists. The principle methods used to study this process have involved: (1) continuous ventriculocisternal perfusion, first used extensively by Leusen (1948 and 1950) and modified by Pappenheimer et al. along with a mathematical model for assessing clearances of perfused substances from the CSF (Pappenheimer et al.,1962 and Heisey et al., 1962); and (2) slow micro-infusion or bolus injection of compounds into the ventricles or subarachnoid space of the brain. Although the technique of continuous ventriculocisternal perfusion is particularly advantageous in quantitating clearances from the CSF under steady-state conditions, it involves the use of artificial perfusates and flows which might obscure real changes in the elimination of compounds from the CSF. Such changes may result from the accumulation of endogenous inhibitors in the CSF or alterations in bulk flow of CSF. Cannula implantation for bolus injection of compounds into the ventricles was first employed by Feldberg (1953,1954,1957,and 1959) in studies of the behavioral effects of ions, drugs and hormones injected into the CSF. However, a technique for determining the fate of substances injected into the ventricular CSF was first described by Prockop and Schanker (1962). This technique, which is particularly useful in small animals, has been used to study the disposition of a wide variety of endogenous (e.g., neurotransmitters, amino acids, hormones, and ions) and exogenous (e.g., neutral, acidic, and basic drugs) substances in

the CSF. A major drawback of these studies is that the disposition of compounds in the CSF has been quantitated in terms of half-life, a secondary parameter which is dependent on the clearance and volume of distribution of the compound in the CSF. Accordingly, a major goal of this study was to establish an animal model which could be used to quantitatively assess the clearance of compounds from the CSF under relatively physiologic conditions.

We have applied model-independent clearance concepts to a modification of existing methods of bolus ventricular injection and cisterna magna sampling. Our study demonstrates that the clearance of compounds from the CSF can be accurately quantitated following a single bolus injection of the substance into the lateral ventricle of the brain of a rat followed by sampling of CSF from the cisterna magna. Using this model, the pathways involved in the elimination of cimetidine from the CSF were characterized (see Chapter 3).

Methods

Cannulae Implantation

Male Sprague-Dawley rats (250-325 g) were anesthetized with an intramuscular dose of a mixture of ketamine (130 mg/kg) and acepromazine (1.6 mg/kg) in the right hind limb (ca 1 cm above the knee joint). Hair on the head was trimmed with scissors and the head was fixed in a stereotaxic device (Figure 7). The procedure for fixing the head in the stereotaxic device involves placement of ear bars in the ear cannels and alignment of the head so that the incline of the head is consistent with that used in the stereotaxic atlas (Myers et al., 1972). Surgical and cannulae implantation methods as described by Myers et al., (1972) and Bouman et al., (1979) for ventriculocisterna studies were used. In detail, a midline scalp incision was made from the neck to a point

between the eyes, and the skull was exposed by removal of the periosteum. Coordinates obtained from a stereotaxic atlas of the rat brain as illustrated in Figure 8 (Pellegrino et al., 1979) were used to locate the lateral ventricle (ca 1.8 mm rostal and 3.8 mm lateral of bregma and 4 mm below the skull as shown in Figure 9a) and to determine cannula placement. A Dremble Tool® variable speed drill with a # 56 size drill bit, purchased from a local hobby shop, was used to drill a hole into the frontal bone of the skull for placement of a cannula-guide for injection into the lateral ventricle (Figure 9b). A second hole was drilled just anterior to the interparietal-occipital bone suture line

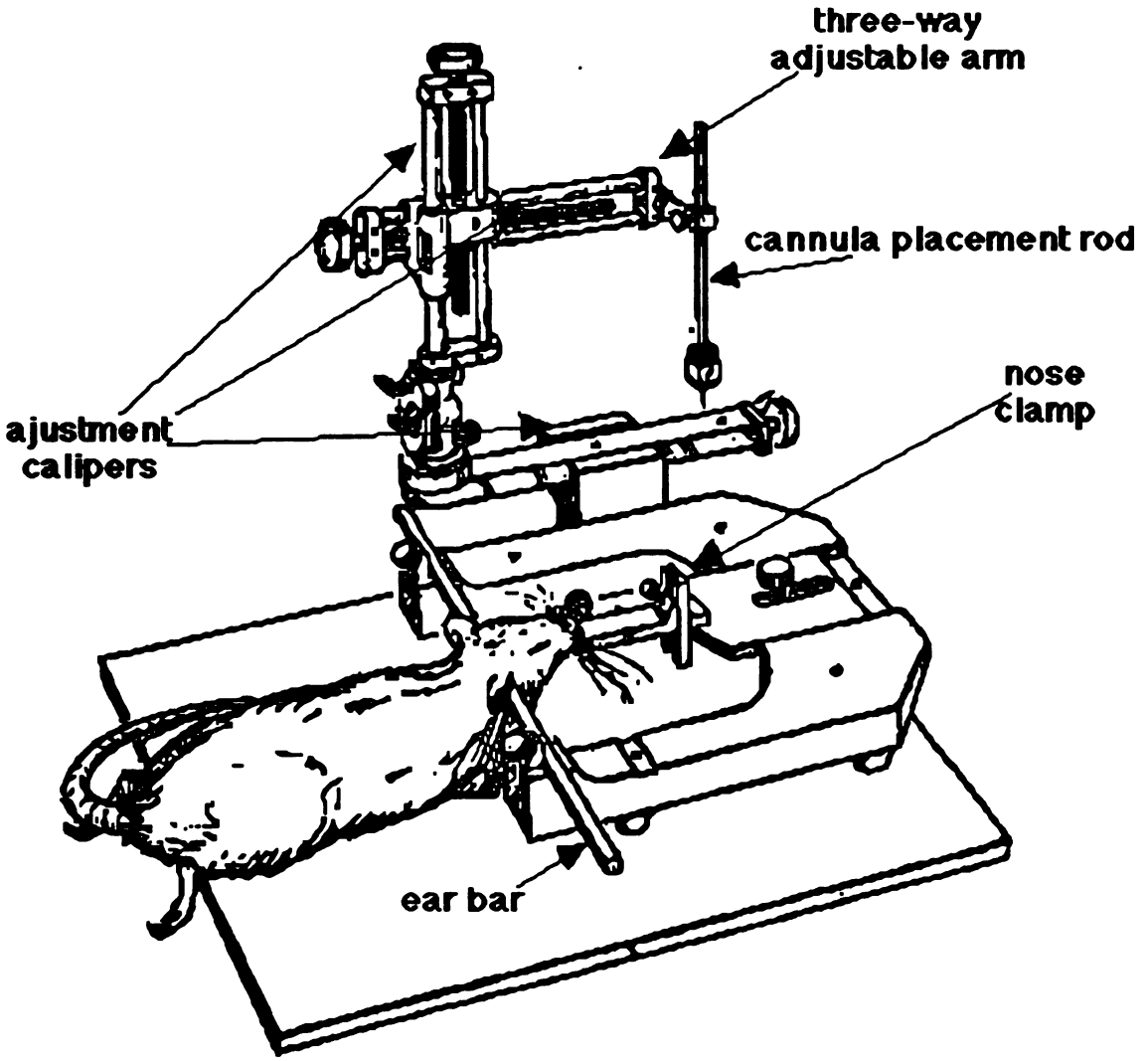


Figure 7 An illustration of a rat in a stereotaxic device. The head is fixed in place with the nose clamp and ear bars. (modified from: Hart and Hoebel, 1969)

(Figure 9a). This hole was drilled at an angle so that the occipital bone could be used as a guide while lowering a cannula-guide into the cisterna magna (Bouman and van Wimersma Greidanus, 1979). Three additional holes were drilled for placement of 0-80 stainless steel screws (0.031 inch length) to anchor the cannula-guides to the skull (Figure 9c) The three screws were mounted first and then a stainless steel cannula-guide (6.5 mm length) with a threaded teflon pedestal (Figure 10a) was placed into the cisterna magna by hand and the

space between cannula and skull was sealed with super glue. A cannula-guide was then lowered into the brain to a point just above the right lateral ventricle using the electrode manipulator of the stereotaxic device (Figure 7) and super glue was applied as a seal. Dental cement was applied to anchor the cannula-guides to the skull and the anchor screws. Screw-capped cannula-dummy wires (Figure 10a) were screwed into the cannula-guides to maintain a closed system, as well as to keep the cannula-guides patent. The preparation was equilibrated for 45 minutes to allow CSF pressure to normalize.

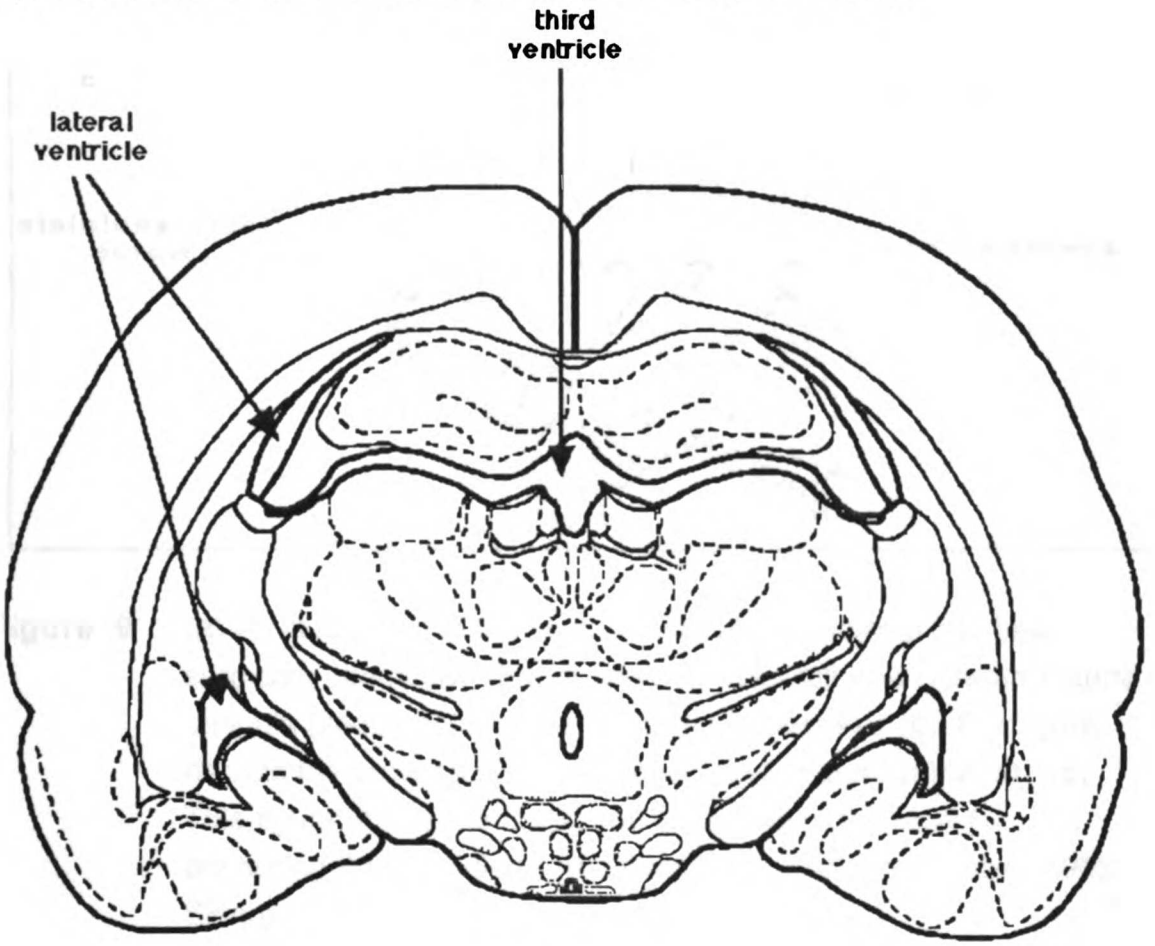


Figure 8 An illustration of a frontal view of a transverse section of the rat brain as it appears in a stereotaxic atlas. Areas surrounded by dark borders and indicated by arrows represent ventricular spaces. (modified from: Pellegrino et al., 1979)

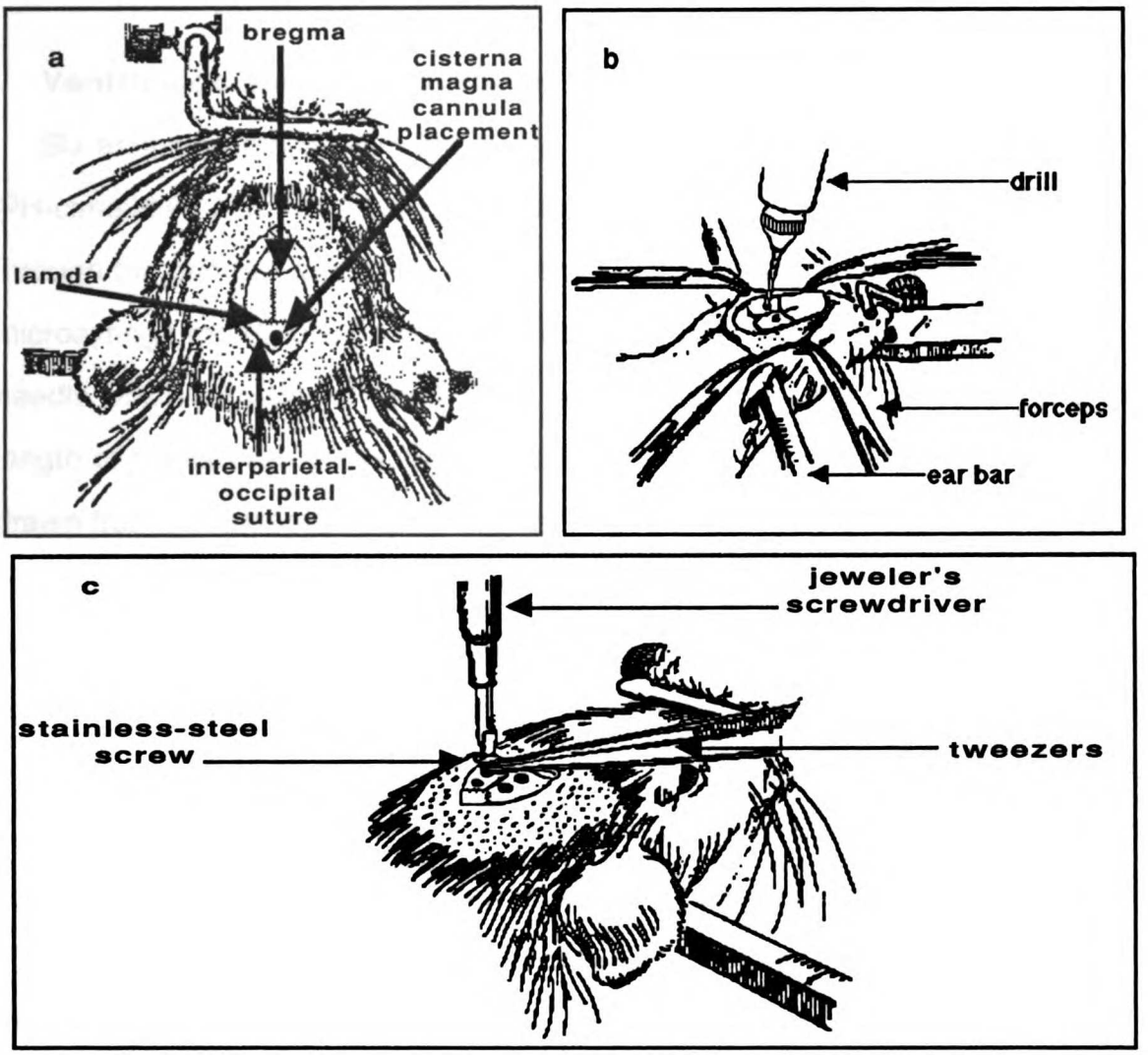


Figure 9 (**a**) An illustration of the skull of a rat after the scalp has been surgically removed. Suture lines on the skull and cisterna magna cannula placement site are indicated by arrows. (**b**) The figure depicts the process of drilling holes into the skull of the rat. (**c**) This figure shows the instillation of screws in the skull for anchoring the cannulae. (modified from: Hart and Hoebel, 1969; Skinner, 1971)

Ventriculocisternal Procedure

Six animals were used in this study. Solutions containing test compounds (^{3}H-cimetidine and ^{14}C-inulin), described in detail in Chapter 3, were injected through the cannula-guide into the lateral ventricle using a Hamilton microsyringe modified with a piece of stainless steel tubing soldered to the needle which allowed the bevel of the needle to extend only 1.5 mm beyond the length of the cannula-guide (Figure 10b). Five microliter samples of CSF were drawn from the cisterna magna, at 0, 2, 5, 10, 20, 40, 60, 90, 120, 150, and 180 minutes after injection using another Hamilton microsyringe modified with a piece of stainless steel tubing soldered to the needle so that the needle tip reached only to the end of the cannula-guide in the cisterna magna (Figure 10b). The dose of cimetidine and inulin injected into the lateral ventricle were 4-8 ng and 53-78 μg, respectively. CSF samples were placed into vials containing 10 ml of aqueous based scintillant for assay by scintillation counting. The data were analyzed to determine clearance values for the test compounds from the CSF as described in Chapter 3.

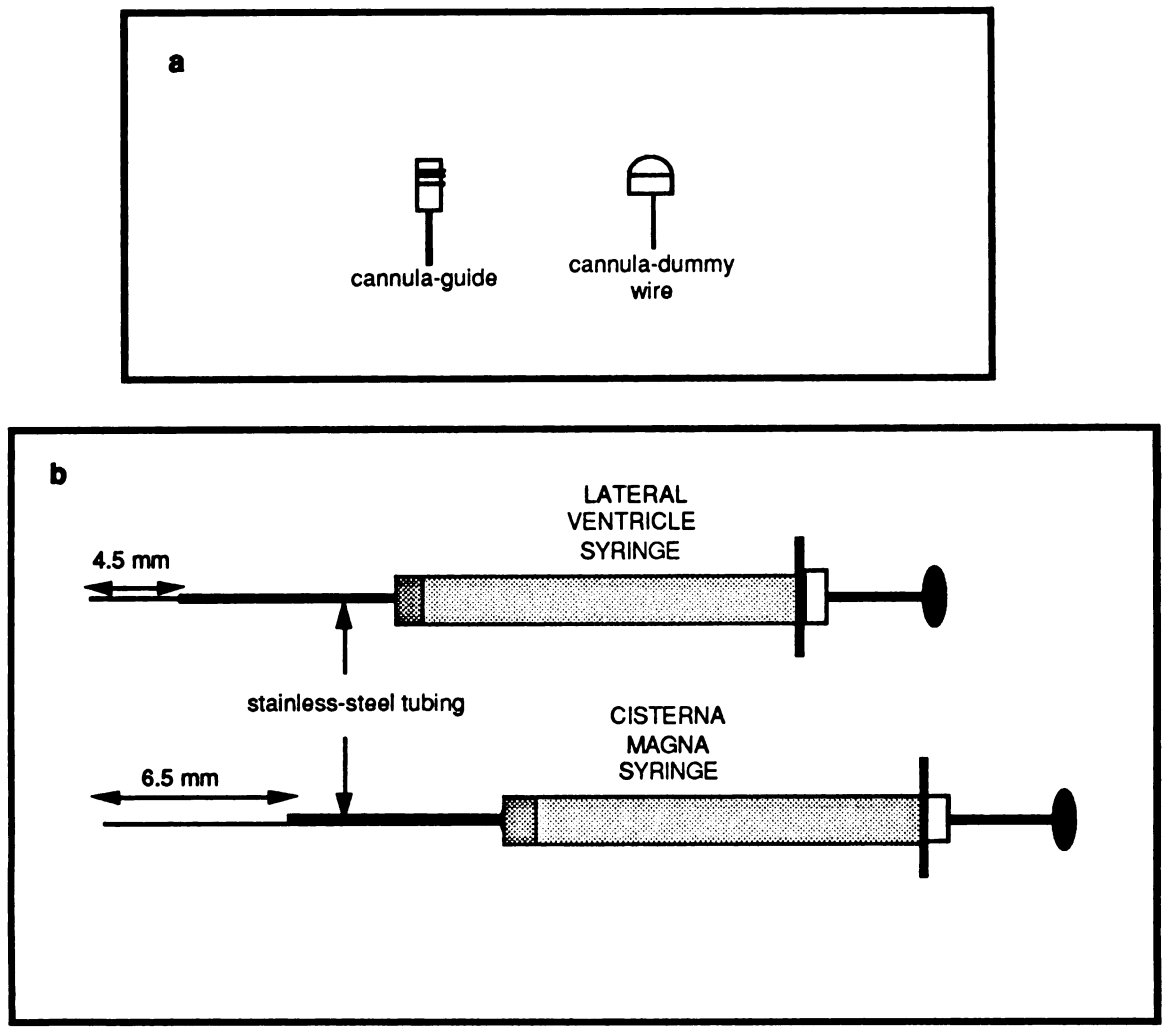


Figure 10 (**a**) An illustration of a cannula and its companion cannula dummy-wire which will be placed in either the lateral ventricle or the cisterna magna. (**b**) An illustration of the modified syringes used for injection into the lateral ventricle or for sampling from the cisterna magna.

Materials

Sprague-Dawley rats were obtained from Batin and Kingman Inc., Fremont CA. Ketaset® and acepromazine were products of Bristol Laboratories, Syracuse, NY and TechAmerica, Elwood, KS, respectively. The stereotaxic device is a product of David Kopf Instruments, Tujunga, CA. Stainless steel

screws, cannula-guides, and cannula-guide dummy wires were purchased from Plastic Products Co., Inc., Roanoke, VA. QuickGel® super glue is a product of Loctite Corporation, Cleveland, OH. Dental cement was purchased from Lang Dental Mfg. Co. Inc., Chicago, IL. The scintillant, ScintiVerse II® was purchased from Fisher Scientific Co., Fairlawn, NJ. The scintillation counter used in this study was a model LS7800, a product of Beckman Instruments, Fullerton, CA. Hamilton syringe needles were modified by the Research and Development Department of the University of California, San Francisco

Results

Semilogarithmic plots of the CSF concentrations of inulin and cimetidine versus time from experiments in a representative rat are shown in Figure 11. The CSF concentrations of inulin declined in an apparent log linear fashion in this animal and in the other animals. Peak concentrations of inulin were achieved at about 10 minutes after injection. Cimetidine concentrations in the CSF declined biexponentially in this animal as well as in the other animals with peak concentrations occurring at about 5 minutes.

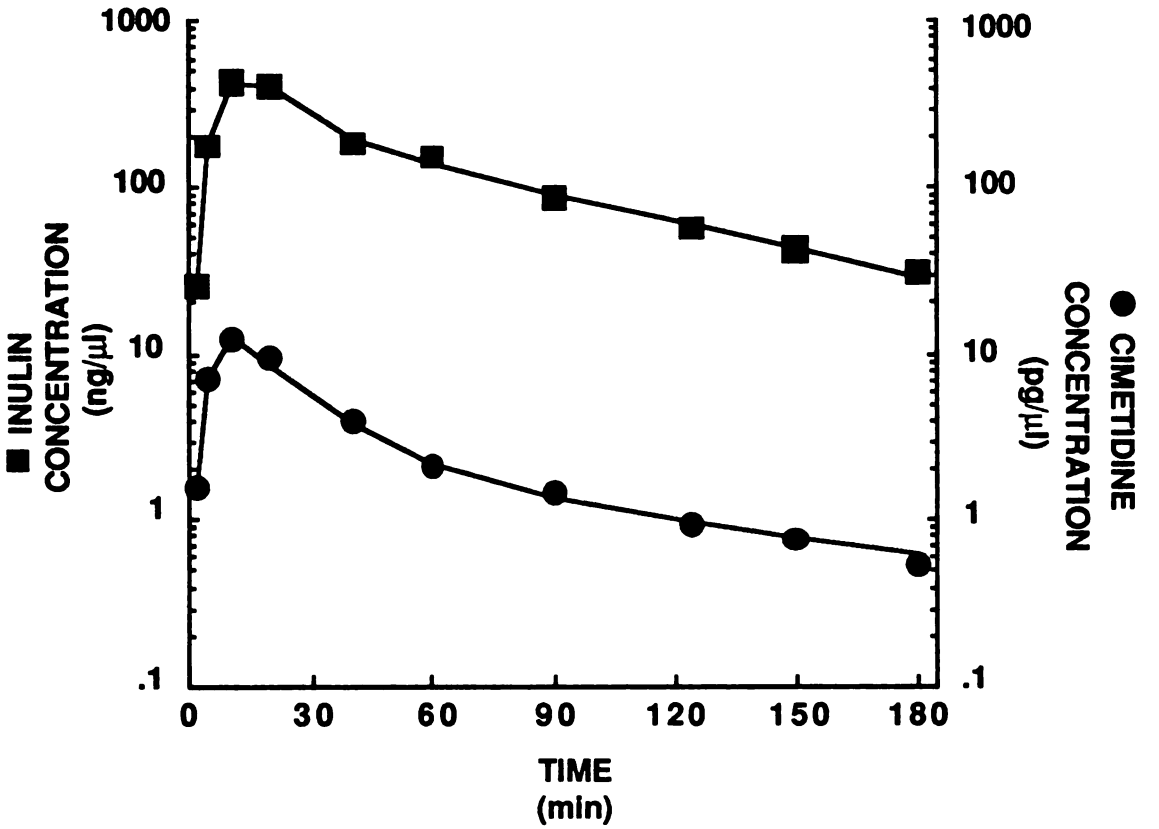


Figure 11 Inulin (■) and cimetidine (●) concentrations in the CSF in a single animal that received ^{14}C-inulin and a low dose of ^{3}H-cimetidine. Cimetidine and inulin concentrations differ by an order of magnitude as indicated in the axis labels.

Figure 12 depicts a plot of cimetidine AUC versus dose. AUC increased linearly with dose ($r = 0.98$; $P < 0.001$), suggesting that linear pharmacokinetic principles are applicable to this model. The clearance of cimetidine, calculated as the reciprocal of the slope of the regression line which was forced through zero, was 10.7 µl/min.

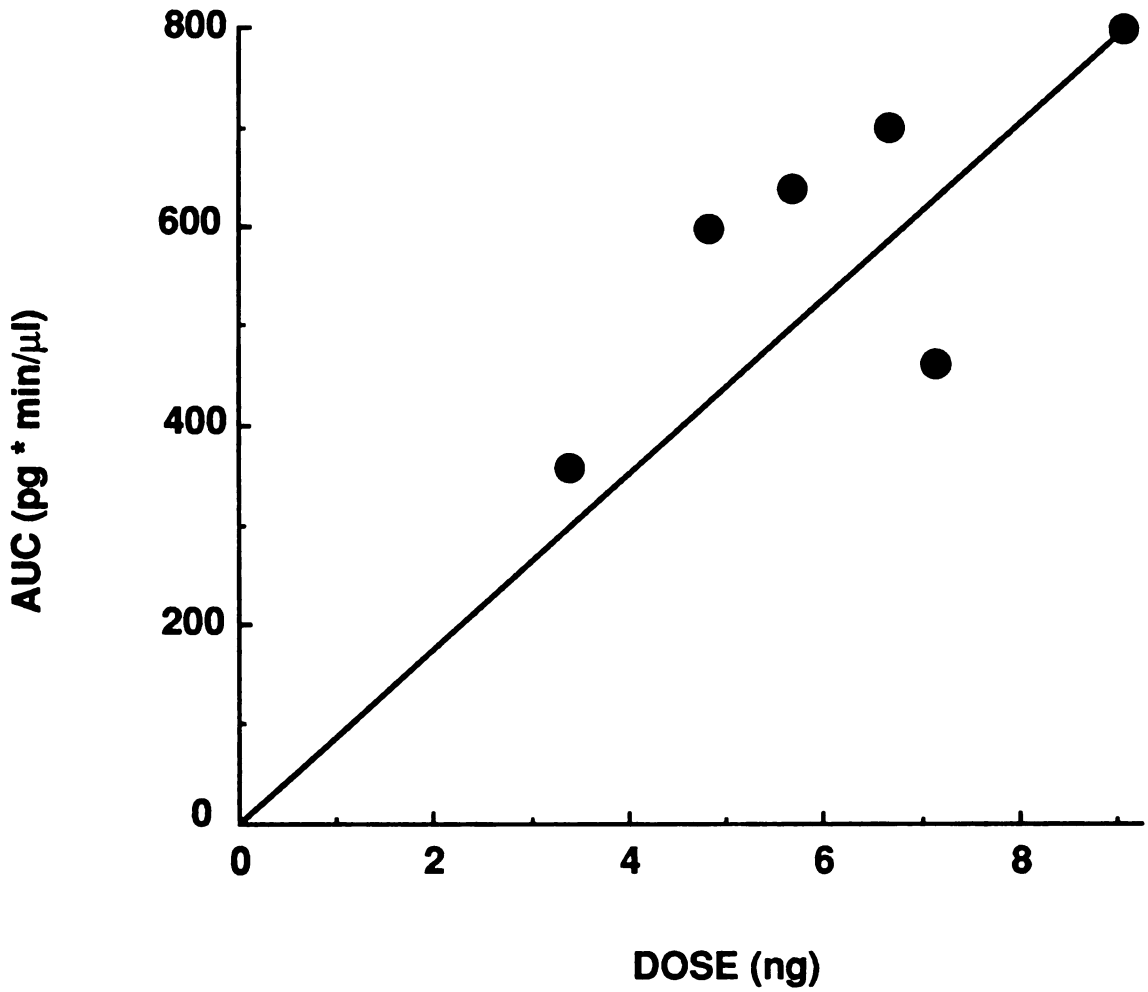


Figure 12 A plot of the area under the CSF concentration versus time curve (AUC) versus dose in six rats that received a low dose of cimetidine. The slope of the regression line is 0.0935 min/µl ($r=0.98$; $P<0.001$).

In Table 2 the clearances of cimetidine and inulin obtained in six rats are shown. The mean clearance values for cimetidine and inulin were 11.8 µl/min and 2.04 µl/min, respectively.

Table 2. CSF Clearances of Cimetidine and Inulin in the Rat

Animal Number	Dose (ng)	Clearance (µl/min) Cimetidine	Inulin
1	7.6	10.34	1.7
2	8.2	18.03	2.17
3	7.7	11.15	2.20
4	3.9	11.17	1.86
5	6.5	10.46	2.27
6	5.7	9.46	2.02
Mean		**11.77**	**2.04**
S.D.		**3.13**	**0.22**

Discussion

A major goal of this study was to establish an animal model that could be used to quantitatively assess clearances of compounds from the CSF under relatively unperturbed physiologic conditions. Although the technique of ventriculocisternal perfusion has been widely used to quantitate clearances of compounds from the CSF (Cserr, 1971; Heisey et al, 1962), we felt it had particular limitations related to the use of artificial perfusates and flow rates. Accordingly, we used an animal model which involved the administration of the

compound via a bolus injection into the lateral ventricle of an anesthetized rat followed by sampling of CSF from the cisterna magna. Clearance of the compound from the CSF was then determined by applying pharmacokinetic principles of linear systems. This method for ventriculocisternal study of the distribution and elimination of compounds from the CSF has previously been described.(Prockop and Schanker, 1962; Schanker et al., 1962; Levin et al., 1983,1984,1985a,1985b,and 1985c; Sarna et al., 1984). Clearance concepts have also been applied to this method by Levin et al. (1983,1984,1985a,1985b,and 1985c) who performed studies of drug distribution and elimination from the CSF in beagle dogs. Previous studies in smaller animals have relied solely on half-life determination to assess CSF elimination (Prockop and Schanker, 1962; Schanker et al., 1962). Clearances have not been determined.

In this study we used three criteria to determine whether clearances could be accurately quantitated in the rat model. First, a basic assumption in using equation 4 (Chapter 3), $CL = \frac{Dose}{AUC}$, is that AUC increases linearly with dose, i.e., the system is linear. In six animals, a range of low doses of cimetidine was used. When AUC was plotted against Dose and the data were analyzed by linear regression analysis, a good linear relationship resulted (Figure 12) suggesting that the principles of linearity were valid for this model.

As a second criteria, we compared the inulin clearances obtained in this study to the rate of CSF production in the rat. Inulin is a large (MW 5000 daltons) polar compound. Elimination of this compound from the CSF is therefore presumed to occur almost exclusively by the bulk flow pathway (Prockop and Schanker, 1962). Presumably, under normal physiologic

conditions the rate of CSF production should approximate the rate of bulk flow of CSF through the ventricular system. The mean inulin clearance obtained in this study was 2.04 µl/min (Table 2) which is in excellent agreement with the value of CSF production in the anesthetized rat of 2.2 µl/min obtained from ventriculocisternal perfusion studies (Cserr, 1971). A similar relationship between inulin clearance and CSF production rate was observed in the dog (Levin et al., 1983,1984,1985a,1985b, and 1985c).

Third, the clearance of cimetidine from the CSF obtained in this study was compared to that calculated from a ventriculocisternal perfusion study of Suzuki et al. (1985). Using a perfusion flow rate of 17.4 µl/min Suzuki et al. (1985) obtained an extraction ratio of 0.354 for cimetidine. Since in their system, clearance is equal to the product of flow rate and extraction ratio, a cimetidine clearance of 6.2 µl/min can be calculated from their data. The difference between this value and the clearance value of 11.8 µl/min obtained in our study may reflect the possible artifacts introduced by the use of an artificially high flow rate and the need to determine small concentration differences in large volumes in their study.

In conclusion, the technique of bolus injection into the lateral ventricle and sampling from the cisterna magna used in these experiments provides a method for studying elimination of substances from the CSF of the rat. The model has the advantage of allowing CSF clearances to be ascertained under relatively physiologic conditions. Artificial perfusates and flow rates are eliminated. Application of the pharmacokinetic principles of linear systems to quantitate clearances appears to be valid for cimetidine and inulin.

CHAPTER 3

Cimetidine CSF Clearance Studies

Introduction

The concentration of cimetidine in the CSF relative to its concentration in plasma appears to be elevated in certain disease states (Schentag et al. ,1979 & 1981). In normal patients, the CSF:plasma concentration ratio for cimetidine at steady-state is 0.18. Ratios as high as 0.5 have been observed in patients with liver disease. About 20% of the total cimetidine in plasma is bound to plasma-proteins. Therefore, the unbound fraction, f_u, is 0.8. Since only the unbound drug crosses the blood-brain barrier (Ziemniak et al., 1984), the CSF:plasma cimetidine concentration ratio ($\frac{C_{CSF}}{C_p}$) based on this criteria alone would be expected to be approximately 0.8 at steady-state,

$$\frac{C_{CSF}}{C_p} = \frac{C_u}{C_T} = f_u \qquad \textbf{(Equation 1)}$$

where C_u and C_T are the unbound and total drug concentrations in plasma, respectively, and C_T and C_p are synonymous. Moreover, cimetidine has a pK_a of 6.8 and at the pH of blood (7.4) and CSF (7.3) it is unionized approximately 79 and 77%, respectively (Bavin and Zarembo, 1984). Assuming that only the unionized species would cross the blood-brain barrier, barring any transport processes, the steady-state ratio of concentrations based solely on pH partitioning theory would be

$$\frac{C_{CSF}}{C_p} = \frac{1 + 10^{6.8-7.3}}{1 + 10^{6.8-7.4}} = 1.05, \qquad \textbf{(Equation 2)}$$

and may be referred to as the partition factor (P_f). Therefore, the highest expected CSF:plasma cimetidine concentration ratio can be estimated from the

product of Equations 1 and 2 as

$$\frac{C_{CSF}}{C_p} = \frac{C_u}{C_T} \cdot P_f = 0.8 * 1.05 = 0.84. \qquad \textbf{(Equation 3)}$$

The fact that the value in normal patients is 0.18, which is about 5-fold lower than expected from equation 3, suggests either that the blood-brain barrier permeability is less than would be predicted based of the physicochemical properties of the drug or that "sink conditions" exist for the elimination of cimetidine from the CSF and may suggest that a saturable transport process exist.

A 3-fold increase in the CSF:plasma cimetidine concentration ratio in certain patient populations suggests that the drug accumulates in the CNS of these patients. Two hypotheses may explain this apparent accumulation: an enhanced penetration of the drug into the CNS; or an impaired elimination from the CSF. Both explanations are equally as valid, and both may in fact occur simultaneously. Jonsson et al. (1982,1984) and Ziemniak et al. (1984) have studied the penetration of cimetidine into the CSF in humans and dogs, respectively. We chose to investigate whether the elimination of cimetidine from the CSF is impaired in patients with liver disease. It was first necessary to demonstrate the existence of an inhibitable or saturable process involved in the elimination of cimetidine from the CSF.

A goal of this project was to determine whether cimetidine CSF elimination involves a saturable process. The following mathematical relationship describes the CSF:plasma ratio of a substance in terms of clearances;

$$\frac{C_{CSF}}{C_p} = \frac{CL_{p \rightarrow CSF}}{CL_{CSF \rightarrow p}} \qquad \textbf{(Equation 4)}$$

where $CL_{p \rightarrow CSF}$ and $CL_{CSF \rightarrow p}$ are the clearances from plasma to CSF and from CSF to plasma, respectively. Assuming $CL_{p \rightarrow CSF}$ to be constant, $\frac{C_{CSF}}{C_p}$ is inversely proportional to $CL_{CSF \rightarrow p}$. Compounds may be cleared from the CSF by bulk flow of CSF through the arachnoid villi, passive diffusion, biotransformation, and transport systems located in the choroid plexus and other tissue (Figure 13) (Cserr, 1971)

Suzuki et al. (1985), using a ventriculocisternal perfusion technique in the rat, demonstrated that cimetidine was transported from CSF to blood by a saturable transport system as evidenced by decreasing extraction ratios with increasing cimetidine concentrations in the perfusate. As discussed in Chapter 2, the ventriculocisternal perfusion method may introduce artifactual errors into the data. Accordingly, to quantitate cimetidine CSF elimination in terms of the physiologically relevant parameter, clearance, we employed the methods discussed in Chapter 2 to address the following questions: 1) Is cimetidine eliminated from the CSF by pathways other than via bulk flow? 2) Is cimetidine biotransformed in the CSF? 3) Does the clearance of cimetidine from the CSF involve a saturable process?

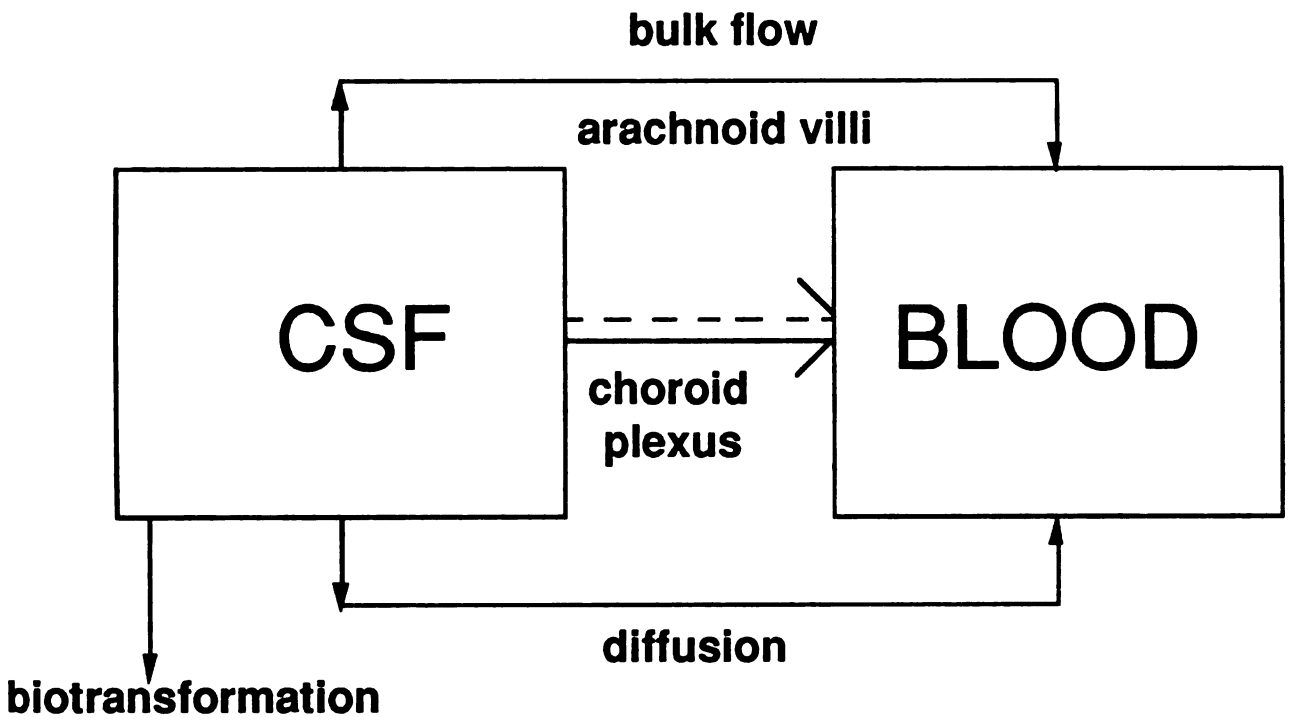


Figure 13 A schematic drawing of the routes by which a compound may be eliminated from the cerebrospinal fluid (CSF).

Methods

Ventriculocisternal Procedure

Following surgical implantation of cannulae into the lateral ventricle and the cisterna magna as described in Chapter 2, six rats received a high dose and six rats received a low dose of cimetidine. Five microliters of a solution containing ^{3}H-cimetidine (4-8 ng) ,^{14}C-inulin (53-78 µg), which is a marker of bulk CSF flow, and unlabeled cimetidine (22.7 µg) in mock CSF (127.6 mM Na^{+}, 2.5 mM K^{+}, 1.3 mM Ca^{2+}, 1 mM Mg^{2+}, and 134.7 mM Cl^{-}, pH 7.33) (Cserr, 1971; Myers, 1972) was administered to six animals, while six animals received 5 µl of a solution containing ^{3}H-cimetidine (4-8 ng) ,^{14}C-inulin (53-78 µg) and mock CSF without unlabeled cimetidine. Solutions were administered and CSF samples were collected as described in Chapter 2. ^{3}H and ^{14}C in the samples were

assayed as DPM/5μl by liquid scintillation counting using dual isotope monitoring. Counting efficiencies for ^{3}H and ^{14}C were approximately 33% and 50%, respectively.

Data Analysis

^{3}H and ^{14}C activities in the CSF samples were converted to cimetidine and inulin CSF concentrations, respectively, using the specific activities of the compounds. To ascertain the clearances of cimetidine (CL_{CIM}) and inulin (CL_{IN}) from CSF we used a model-independent method which employs the equation

$$CL = \frac{Dose}{AUC_{t_0 \rightarrow t_\infty}} \qquad \textbf{(Equation 5)}$$

where Dose represents the dose of the compound administered and $AUC_{t_0 \rightarrow t_\infty}$ represents the area under the CSF concentration versus time curve (Figure 11) from time zero, t_0, to time infinity, t_∞. $AUC_{t_0 \rightarrow t_z}$, from time zero to the last sample time, t_z, was calculated from the data by the trapezoidal rule (Gibaldi and Perrier, 1982) and extrapolated to time infinity by addition of $AUC_{t_z \rightarrow t_\infty}$ which was calculated as,

$$AUC_{t_z \rightarrow t_\infty} = \frac{C_z}{\lambda_z} \qquad \textbf{(Equation 6)}$$

where C_z is the computer generated estimate of the concentration of the last sample and λ_z is the terminal elimination rate constant. To obtain λ_z the CSF concentration versus time data were fit to a mono- or bi- exponential equation using DRUGMODEL on the PROPHET computer system. In these studies the extrapolated $AUC_{t_z \rightarrow t_\infty}$ was less than 20% of the total $AUC_{t_0 \rightarrow t_\infty}$. CL_{IN}, CL_{CIM}, and CL_{CIM^*} , the CSF clearance of cimetidine when the dose of unlabeled drug was administered, were compared statistically by groups. Statistical analysis

for differences between cimetidine and inulin clearances within each group was performed using Student's paired *t*- test. Intergroup differences between CL_{CIM} and CL_{CIM^*} were evaluated using Student's unpaired *t*- test. A p-value of less than 0.05 was considered statistically significant.

Cimetidine Metabolism Studies

To study the extent of cimetidine metabolism in the CSF of in vivo preparations, 5 µl of a solution containing only ^{3}H-cimetidine (4-8 ng) in mock CSF was injected into the lateral ventricle of a single rat. Five microliter samples were drawn from the cisterna magna at various times after administration of the drug. Two and one-half microliters of each sample was spotted onto a thin layer chromatography (TLC) plate previously spotted with about 8 µg of unlabeled cimetidine. The remaining 2.5 µl of each sample was placed in 10 ml of scintillant. The TLC separation procedure was performed using an ethylacetate:methanol:NH_4OH (5:1:1) solvent system, a modification of the assay method described by Bavin et al. (1984). Following development, the entire plate was scraped in sections. Sections associated with cimetidine (R_f = 0.65) and with its sulfoxide metabolite (R_f = 0.40) were 2 cm in length and other sections were 1 cm. R_f values for cimetidine and cimetidine sulfoxide were empirically determined using pure compounds and development in a tank with iodine crystals. The scraped sections were placed in vials containing 1 ml of water, to elute radioactive material from the silica, and 10 ml of scintillant. The radioactivity in the samples was quantitated by single label scintillation counting. Experiments designed to determine the extent of sulfoxide formation in in vitro preparations were conducted. Samples of CSF were drawn from a rat which had not received an injection of ^{3}H-cimetidine. The samples were spiked with ^{3}H-cimetidine (2,500 DPM/µl) and allowed to incubate. At different time

periods during incubation, 2 µl aliquots of spiked sample were spotted on a TLC plate and assayed as described above. To determine if cimetidine sulfoxide was formed as an artifact of the assay, an aliquot of mock CSF was spiked with ^{3}H-cimetidine and a 2 µl sample was spotted on a TLC plate and developed as described above. Following this first development, the plate was allowed to dry and then placed in the developing solvent, rotated 90° so that the migration of the solvent was in a lateral direction to that in the first development. The plate was then dried and assayed as described above.

Materials

^{3}H-Cimetidine (specific activity 10.6 Ci/mmole) and ^{14}C-inulin (specific activity 6.88 mCi/mmole) were purchased from Amersham Corp., Arlington Hts, IL. Unlabeled cimetidine was purchased from the Sigma Chemical Company, St. Louis, MO. Cimetidine sulfoxide was generously provided by Smith Kline & French (Philadelphia, PA). The scintillant, ScintiVerse II®, and EM Reagents (silica gel 60 precoated, nonfluorescent, 0.2 mm layer thickness, aluminum backing) TLC support, and all solvents, which were of analytical grade, were purchased from Fisher Scientific Co., Fairlawn, NJ.

Results

Figures 14A and 14B are representative semilogarthmic plots of the cimetidine and inulin CSF concentrations versus time profiles in single experiments where the rat received an intraventricular injection of a solution containing a low dose and a high dose of cimetidine, respectively. Concentrations of cimetidine in the samples taken from the cisterna magna reached a maximum generally within 5 min in all animals. Peak concentrations of inulin were usually attained at 10 min. The decline of inulin concentrations fit

well to a monoexponential mathematical equation while the decline of cimetidine concentrations fit better to a biexponential equation.

Figure 15A depicts the paired clearances of cimetidine and inulin obtained in the six rats that received a low dose of cimetidine. Cimetidine clearance in each animal was greater than that of inulin (mean ± SD; 11.8 ± 3.1 μl/min vs 2.04 ± 0.22 μl/min; $P = 0.001$). Figure 15B shows the paired clearances of cimetidine and inulin in the six animals that received a high dose of cimetidine. Again, the clearance of cimetidine was significantly greater than that of inulin.(mean ± SD; 8.04 ± 2.07 μl/min vs 2.01 ± 0.23 μl/min; $P = 0.001$). These results suggest that cimetidine may be eliminated from CSF by routes which are supplementary to bulk flow. Cimetidine clearance was significantly lower in the animals that received the high dose of cimetidine ($P < 0.05$), whereas inulin clearances were not affected by the high dose of cimetidine ($P > 0.05$).

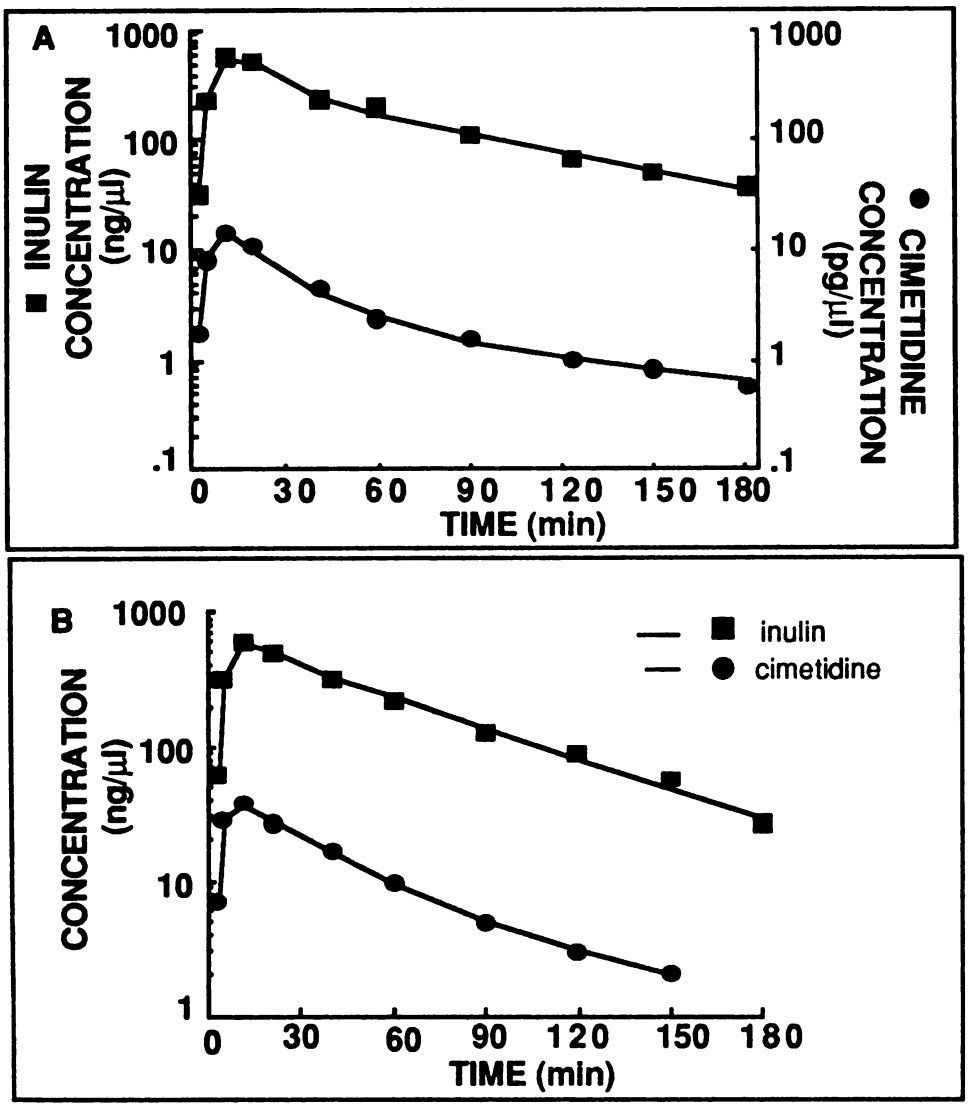


Figure 14 (A) Inulin (■) and cimetidine (●) concentrations in CSF in a single animal that received inulin and a low dose of cimetidine. Cimetidine and inulin concentrations differ by and order of magnitude as indicated in the axis labels. (B) Inulin (■) and cimetidine (●) concentrations in the CSF in a single animal that received inulin and a high dose of cimetidine. Cimetidine and inulin concentrations are the same order of magnitude.

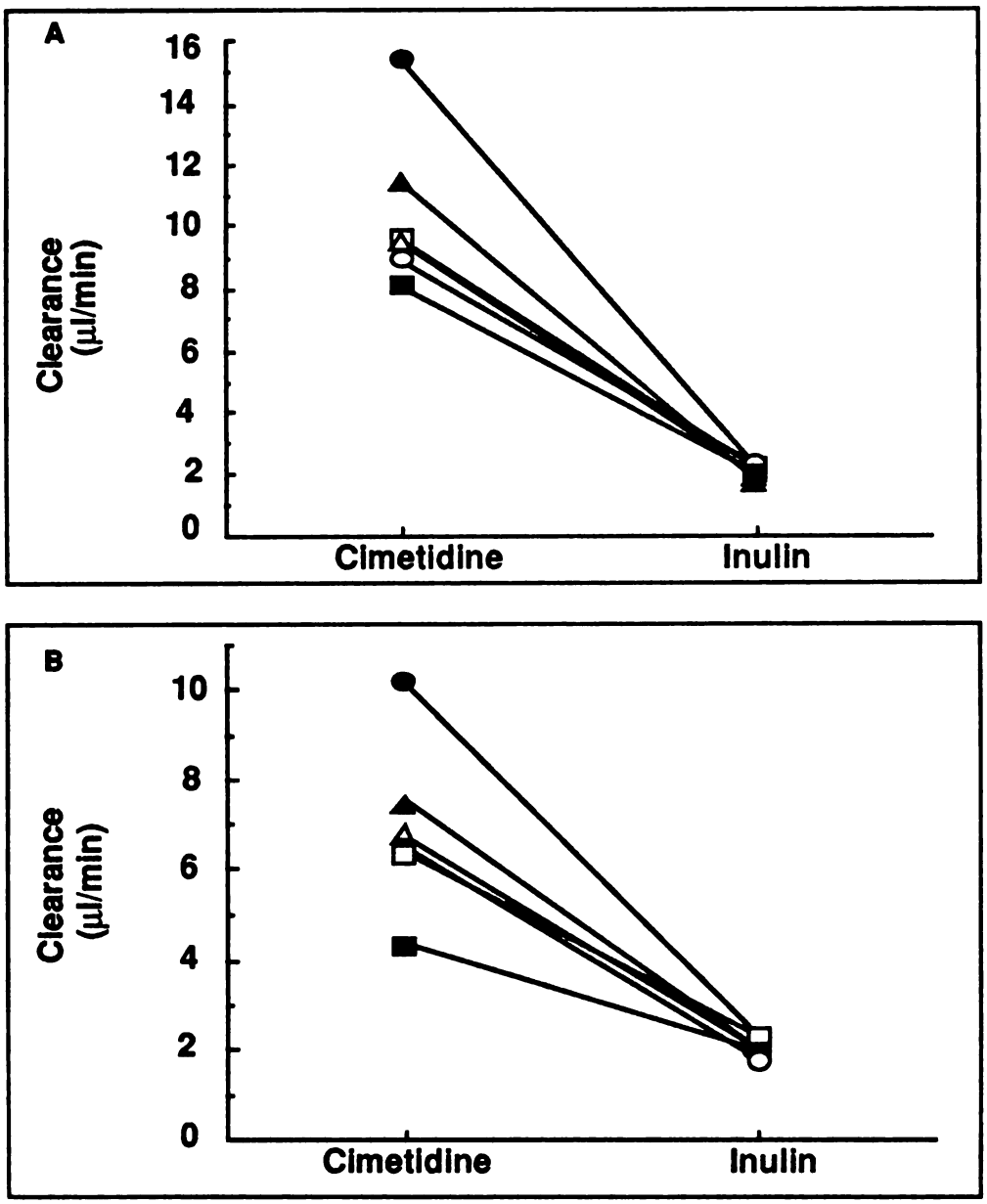


Figure 15 (A) Paired relationships between cimetidine and inulin CSF clearances in the six animals that received a low dose of cimetidine. (B) Paired relationships between cimetidine and inulin CSF clearances in the six animals that received a high dose of cimetidine.

These results suggest that at least one route by which cimetidine is eliminated from the CSF is saturable.

The extent of biotransformation of cimetidine to its sulfoxide metabolite in the CSF, both in vitro and in vivo was studied using thin layer chromatography. CSF samples were spiked with ^{3}H-cimetidine to determine whether cimetidine is converted to its sulfoxide metabolite in vitro. The data in Figure 16 shows that the amount of sulfoxide associated with a spot on a TLC plate remained constant with increasing time indicating that cimetidine is not metabolized to the sulfoxide in CSF. A second in vitro experiment was designed to determine whether the appearance of cimetidine sulfoxide in the CSF metabolism experiments was an artifact of the assay procedure. Figure 17 simulates the events when an ethanol solution of the pure ^{3}H-cimetidine was spotted on a TLC plate and was subjected to separation analysis. Migration of the solvent system produced a separation of two compounds at locations corresponding to the R_f values of cimetidine and cimetidine sulfoxide. When the plate was allowed to dry and then placed in a solvent system, but rotated 90° so that the direction of solvent migration was acutely divergent from that of the first, a second apparent cimetidine sulfoxide spot was separated from the migrated cimetidine spot, suggesting that there is formation of cimetidine sulfoxide during the assay procedure. Figure 18 is a representative graph of the contribution of cimetidine and cimetidine-sulfoxide to the ^{3}H-cimetidine radioactivity in the CSF of a single rat. The top curve represents the data when a 2 µl sample of CSF was assayed directly by scintillation counting. The middle curve represents the data from the TLC separation assay corresponding to the R_f value for cimetidine as described under Methods. The lower curve represents the data from the TLC

separation assay corresponding to the R_f value for cimetidine-sulfoxide. The

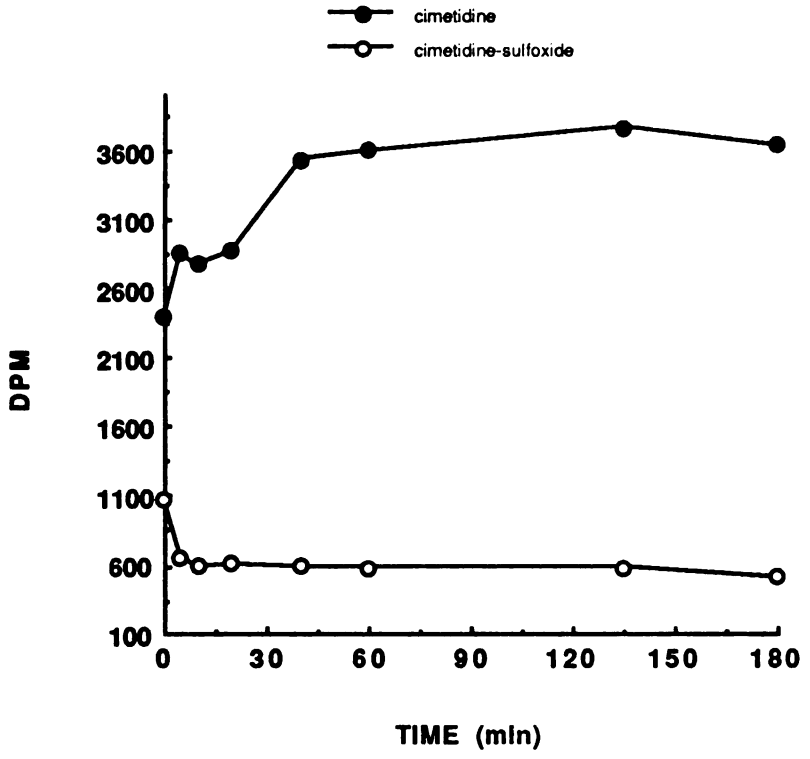


Figure 16 The amount of radioactivity in a CSF sample spiked with ^3H-cimetidine as a function of time. The amounts of radioactivity associated with the R_f values of cimetidine (●) and cimetidine-sulfoxide (○) are depicted

cimetidine-sulfoxide to cimetidine ratio in all animals was very low and the AUC of the sulfoxide contributed less than 10% to the AUC calculated from total radioactivity. The sulfoxide formation in these assays was determined to be largely an artifact of the assay and since the percentage of apparent cimetidine sulfoxide formation is the same for in vivo and the two in vitro experiments, these results suggest that cimetidine conversion to its sulfoxide metabolite in the CSF in vivo is negligible.

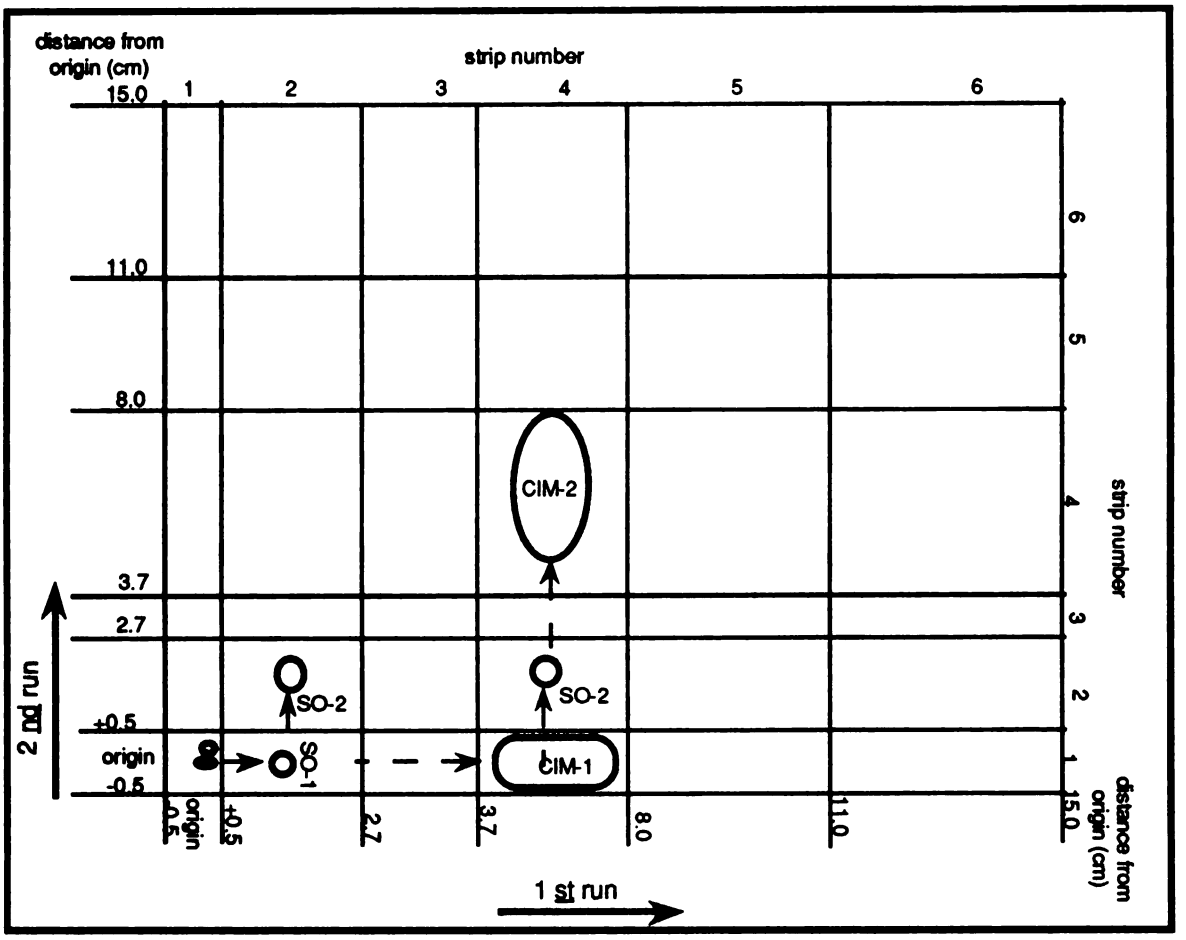


Figure 17 A schematic drawing of the events which occurred during two successive TLC separations of a sample of pure ^{3}H-cimetidine at 90° divergent solvent migrations. The labels SO-1 and CIM-1 refer to the radioactivity associated with the R_f value of cimetidine-sulfoxide and cimetidine following the first solvent migration (1st run), respectively. The labels SO-2 and CIM-2 refer to the radioactivity associated with the R_f value of cimetidine-sulfoxide and cimetidine following the second solvent migration (2nd run), respectively.

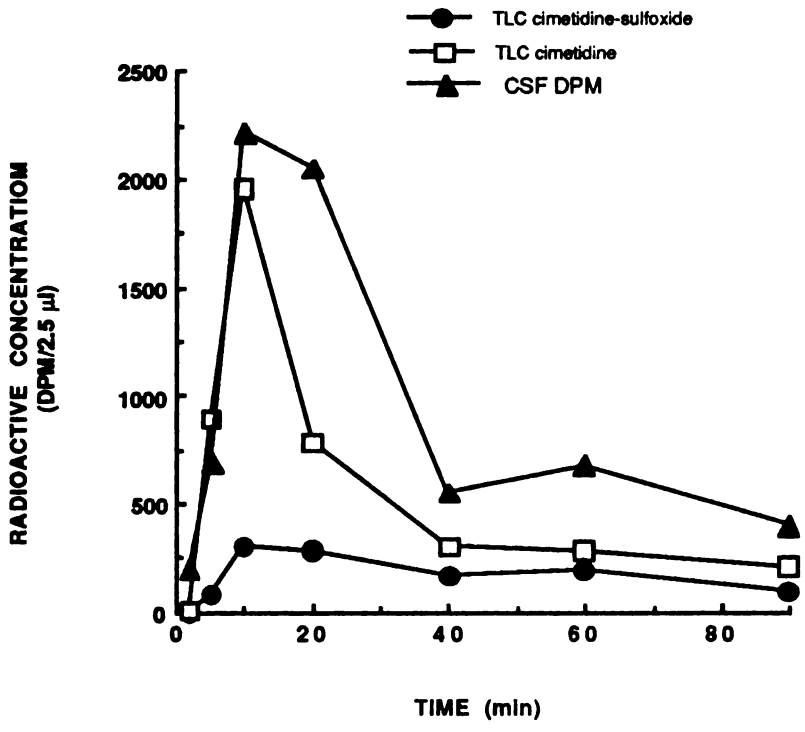


Figure 18 Concentration of ^{3}H-cimetidine in the CSF following intraventricular injection. The solid triangles (▲) represents the data when 2.5 µl of the sample was assayed directly by scintillation counting. The open rectangles (□) and closed circles (●) represent the data associated with the R_f value of cimetidine and cimetidine-sulfoxide, respectively, following TLC separation analysis of a 2.5 µl sample.

Discussion

An alteration in the elimination of cimetidine from the CSF could be responsible for the increased CSF:P ratios observed in patients with liver disease. Figure 13 illustrates the pathways available for the elimination of compounds from CSF. Any substance which is soluble in CSF will be eliminated with the normal turnover of CSF via bulk flow passage through the arachnoid villi. Additionally, given the correct concentration differential,

substances possessing the necessary permeability properties will leave the CSF by passive diffusion. Facilitated or active transport systems located in the choroid plexus epithelium of the ventricles will contribute to the elimination of substances with specific physical and chemical properties (Cserr, 1971; Ross and Wright, 1984; Lorenzo and Spector, 1973; Huang, 1982)

A major goal of this study was to address specific questions related to the pathways of elimination of cimetidine from the CSF. Four characteristics of the elimination of cimetidine from the CSF were examined in this study. First, we examined whether cimetidine is eliminated from the CSF by pathways in addition to bulk flow. Since cimetidine and inulin clearances were determined in each animal, it was possible to compare these clearances within animals. In each animal the clearance of cimetidine was significantly greater than that of inulin (Figures 15A and 15B). Assuming that inulin is cleared almost exclusively by bulk flow, these data suggest that cimetidine is eliminated from the CSF via a pathway (s) in addition to the bulk flow pathway.

Second, we observed that the clearance of cimetidine, but not of inulin, was significantly decreased after administration of the high dose of cimetidine (Figure 15B) suggesting that cimetidine is eliminated from the CSF by a saturable pathway or pathways. These results are consistant with results from previous studies demonstrating that cimetidine actively accumulates in choroid plexus tissue (Suzuki et al.,1986). As discussed in Chapter 2, the clearance of cimetidine from the CSF obtained in this study was compared to that calculated from the data of Suzuki et al. (1985). From their data a CL_{CIM} of 6.2 µl/min is obtained, which differs from the value of 11.8 µl/min obtained in this study. However, the value from our study represents total cimetidine clearance (CL_T) as depicted in Figure 13, i.e.,

$$CL_T = CL_{bf} + CL_o \qquad \textbf{(Equation 7)}$$

where CL_{bf} is clearance by bulk flow of CSF and CL_o is clearance by processes other than by bulk flow and may be referred to as an intrinsic clearance (CL_i), the clearance which relates the innate ability of the process to clear the compound to the unbound concentration at the clearance site (Rowland and Tozer, 1989). Rearranging equation 7 gives $CL_o = CL_T - CL_{bf}$ and substituting values of 11.77 µl/min and 2.04 µl/min for CL_T, and CL_{bf}, respectively, the intrinsic clearance in this study calculated from equation 7 is 9.73 µl/min. A physiologic well-stirred mathematical model for clearance is

$$CL = \frac{Q \cdot CL_i \cdot f_u}{Q + CL_i \cdot f_u} \qquad \textbf{(Equation 8)}$$

where Q is the flow through the system, f_u is the fraction unbound in CSF. Rearranging Equation 8 to solve for CL_i gives

$$CL_i \cdot f_u = \frac{Q \cdot CL}{Q - CL} \qquad \textbf{(Equation 9)}$$

Using the clearance value of 6.2 µl/min and flow of 17.4 µl/min obtained from the data of Suzuki et al. (1985) above to calculate $CL_i \cdot f_u$ from equation 9 results in an intrinsic clearance value of 9.63 µl/min, assuming f_u to be unity in CSF, which is in excellent agreement with the value of 9.73 µl/min obtained in this study. In this study, we did not ascertain the nature of the transport process, nor did we determine whether one or more transport systems were involved in the elimination of cimetidine from the CSF of the rat. The notion of multiple transport pathways for cimetidine is consistent with the results of Gisclon et al. (1987) in renal tissue, and those of Suzuki et al, (1986) which demonstrated inhibition of cimetidine uptake into choroid plexus tissue by organic anions, organic cations, and oligopeptides (discussed in Chapter 5).

After a high dose of cimetidine, the clearance of cimetidine (8.04 ± 2.07 µl/min) was significantly greater than that of inulin (2.04 ± 0.22 µl/min) suggesting that there may

be an additional unsaturable pathway for the elimination of cimetidine from the CSF. Cimetidine (pKa 6.80) has an oil:pH 7.4 phosphate buffer partition coefficient of 2.0 (Bavin and Zarembo, 1984) and is about 77% unionized at the pH of the CSF (7.33). The compound might therefore be expected to be eliminated from the CSF via lipophillic diffusion. This pathway could account for the difference between the decreased cimetidine clearance and the clearance of inulin. The disparity can also be explained by assuming that saturation of the transport system was incomplete. Incomplete saturation of cimetidine transport is arguable on the grounds that the CSF concentrations of cimetidine achieved in these experiments may not have been high enough to completely saturate the transport process.

An additional pathway for the elimination of a drug from the CSF is biotransformation. We studied the formation of the major metabolite of cimetidine, cimetidine-sulfoxide, in the CSF and observed only small amounts which may have been an artifact of the assay method. Thus, in agreement with the results in the CSF of dogs of Ziemniak et al. (1984), our data suggest that there is no significant formation of this metabolite in the CSF of the rat.

In conclusion, the data suggest that cimetidine is eliminated from the CSF of the rat by pathways in addition to the bulk flow of CSF through the arachnoid villi. A saturable transport process appears to be involved and there is evidence that diffusion of the compound from the CSF may occur. Further studies are necessary to address questions related to the elimination of cimetidine from the CSF in disease states.

CHAPTER 4

Preparation of Brush-Border Membrane Vesicles from Bovine Choroid Plexus

Introduction

In the past two decades, techniques have been developed for the isolation and separation of the polar membranes of renal and intestinal epithelium (Booth and Kenny, 1974; Biber et al., 1981). The membranes spontaneously vesiculate and the formed vesicles can be readily used to study the mechanisms of solute transport. Studies in isolated brush-border membrane and basolateral membrane vesicles have dramatically enhanced our understanding of the mechanisms involved in the transport of a number of endogenous inorganic and organic compounds in epithelia of the kidney and the intestine (Aronson, 1981; Sachs et al., 1980). In contrast there have been only two such studies in brush-border membrane vesicles from choroid plexus (Langenbeck and Kinne, 1980; Ross and Wright, 1984), both examining amino acid transport, and no studies in basolateral membrane vesicles. As a result we have very little knowledge of the mechanisms involved in the transport of substances in the choroid plexus.

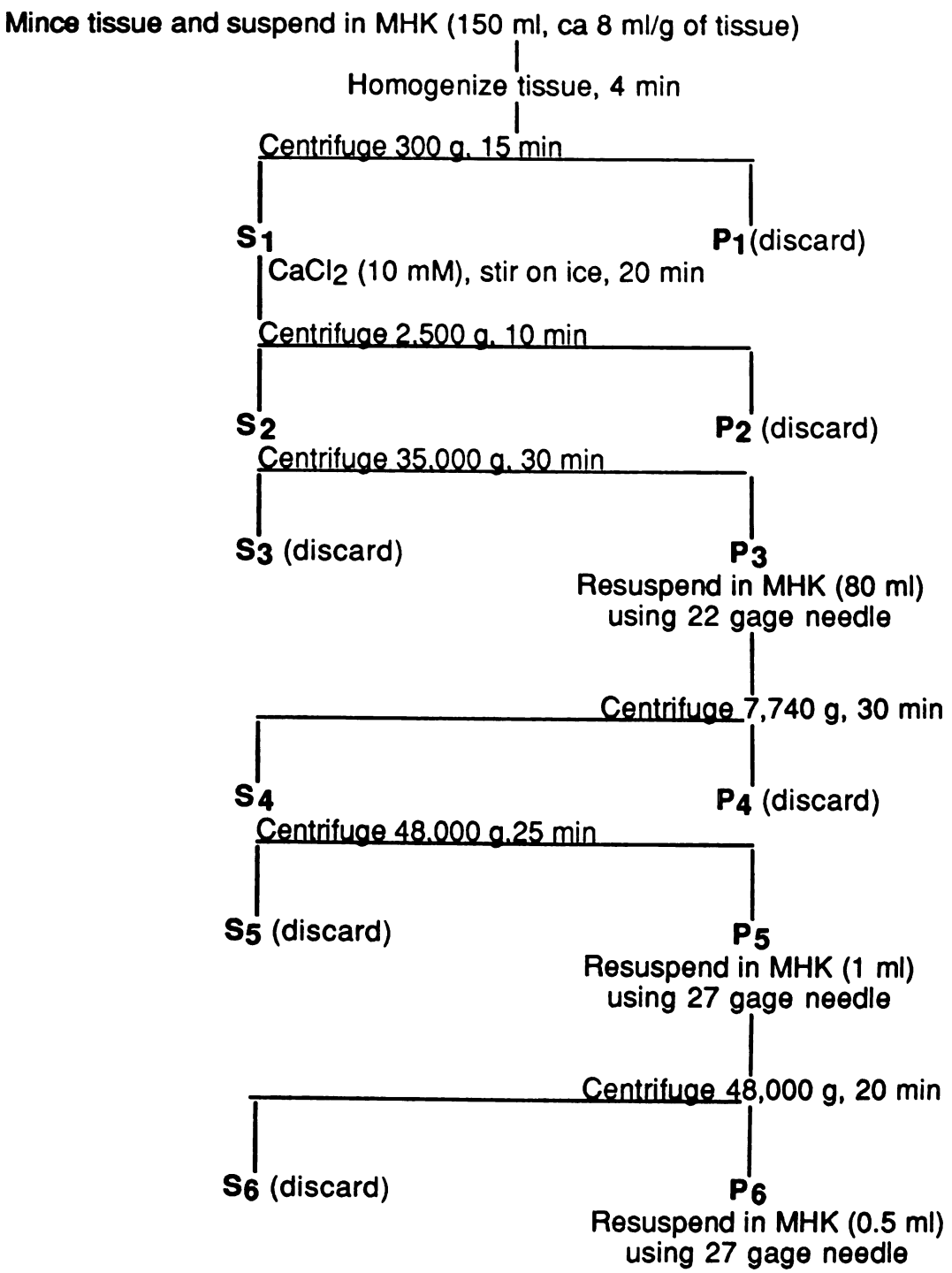
We wished to study the events of translocation of cimetidine across the cells of the choroid plexus epithelium. Accordingly, a major goal of this study was to establish a method for preparing isolated brush-border membrane vesicles from choroid plexus. We have modified a method described by Ross and Wright (1984) for the isolation and separation of brush-border membrane vesicles from the choroid plexus of the cow. The brain of the cow is sufficiently large to obtain enough tissue for transport studies. The tissue can be obtained from a local

abattoir and does not require animals to be sacrificed for the purpose of research. The method has the following attributes: (1) the protein yield is sufficiently high so as to be able to carry out detailed transport studies; (2) the preparation is reasonably pure in membranes from the brush-border of the choroid plexus epithelium, demonstrable by enhancement of specific enzymes as membrane markers; (3) the procedure is reasonably facile; (4) preparation of the vesicles requires less than 6 hr; and (5) the procedure is inexpensive.

Methods

Vesicle Preparation

Bovine choroid plexus tissue was obtained from a local abattoir (Ferrara Meat Co., San Jose, CA). Within 10 min after an animal was killed by intrusion of a rod into the brain, the skull was cleaved and the brain was removed. The brain was then dissected and the choroid plexus was harvested from the lateral and third ventricles and placed in ice-cold normal saline. In detail, an incision was made into the corpus callosum of the superior cerebral cortex which effectively bisected the cerebral hemispheres to the midbrain. A second incision was made into the medial margin of the corpus callosum of each hemisphere on a line from the Foremen of Monroe to the temporal lobe thereby exposing the entire lateral ventricle in both hemispheres as well as the third ventricle. In this way the complete choroid plexus could be harvested intact as it exists in the ventricles of the brain. The tissue from 40-50 brains was harvested in this manner and then transported to the laboratory in iced saline where vesicles were prepared using a modification of a procedure described by Ross and Wright (1986). The membrane isolation procedure in this study differs from that



Scheme.1 Vesicle Preparation Protocol

MHK = 100 mM mannitol, 100 mM KCl, 10 mM HEPES titrated to desired pH with KOH.

described by Ross and Wright (1984) in that the homogenization buffer composition and pH were changed slightly and additional centrifugation steps were incorporated in an attempt to further purify the preparation. In addition, in our procedure pellets are resuspended by forcing them through small gage needles instead of by using a glass/Teflon homogenizer. In detail, clotted blood, large blood vessels, and connective tissue were manually removed from the choroid plexus. Preliminary experiments revealed that excessive washing of the tissue in saline in an attempt to remove all of the red blood cells resulted in a significant reduction of the protein yield in the final preparation. Therefore no additional effort was made to remove red blood cells from the tissue. Excess water was removed by allowing the tissue to drain in the dry palm of the hand. This process was repeated until most of the excess water was drained from the tissue. The wet weight was determined and the tissue was coarsely minced with stainless-steel surgical scissors. The minced tissue was washed into a stainless-steel canister with 150 ml of a buffer solution containing 100 mM mannitol, 100 mM KCl, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), adjusted to pH 7.33 with KOH, called MHK buffer. Cells were disrupted by homogenization on ice in the MHK buffer (approximately 125 mg tissue/ml) using a Sorvall model 17105 Omni Mixer (DuPont Company, Newtown, CT) set at maximum speed for 4 min. The separation procedure was as follows and is diagramed in Scheme 1. The homogenate was centrifuged at 300 g for 15 min in a Beckman J2-21 refrigerated centrifuge with a JA-20 fixed-angle rotor (Beckman Instruments, Palo Alto, CA). All centrifugation steps were performed at a rotor temperature of 4°. The pellet (P_1) was discarded and the supernatant (S_1) was added to $CaCl_2$ (100 mM) to yield a final concentration of 10 mM $CaCl_2$ and stirred on ice for 20 min. The $CaCl_2$ containing suspension was then centrifuged at 2,500 g for 10 min and the pellet (P_2) was discarded.

The supernatant (S_2) was centrifuged at 35,000 g for 30 min. The resulting pellet (P_3) was resuspended in MHK buffer (80 ml) using a 35 cc Monoject syringe and forced through a 22 gage needle. This suspension was centrifuged at 7,740 g for 30 min and the resulting pellet (P_4) was discarded. The supernatant (S_4) was centrifuged at 48,000 g for 25 min. The supernatant (S_5) was discarded and the pellet (P_5) was resuspended in 1 ml MHK buffer using a 3 cc Monoject syringe and forced through a 22 gage needle and then through a 27 gage needle. This suspension was centrifuged at 48,000 g for 20 min. The supernatant (S_6) was discarded and the pellet (P_6) was resuspended in 0.5 ml of the buffer to be used in the uptake experiment using a 3 cc Monoject syringe and forced through a 22 gage needle and then through a 27 gage needle. This final suspension was placed in a refrigerator on ice and uptake experiments were performed the next day.

Protein and Enzyme Assays

Protein concentrations were determined by the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard and the amount of protein in a sample was determined optically from the absorbance at 650 nm. The standard curves ranged between 5 and 50 μg BSA and correlation coefficients ranged between 0.998 and 1.0.

Na^+/K^+ATPase (EC 3.6.1.3) activities were determined by the optical assay method of Schoner et al. (1965) and by the method of Mamelok et al. (1982). This enzyme marks the brush-border membrane of the choroid plexus and is particularly localized in the microvillus portions of the membrane (Masuzawa and Sato,1983). The method described by Schoner et al.(1965) involves a coupled reaction in which ATP is dephosphorylated by ATPases and the ADP product is rephosphorylated by pyruvate kinase which transfers inorganic

phosphate from phosphoenolpyruvate producing pyruvate and ATP. Pyruvate is then reduced to lactate in the presence of NADH by lactate dehydrogenase. The rate of NADH oxidation is measured at 37° and 340 nm using an Apple IIe computer with a software package from LKB Instruments. The assay method described by Mamelok et al. (1982) involves a single reaction in which the rate of dephosphorylation of ATP is measured at 37° and 340 nm. A detailed description of these assays are presented in Appendix A.

The activities of alkaline phosphatase (EC 3.1.3.1), a marker enzyme for basolateral membranes (Masuzawa and Sato,1983) were determined by a modification of the method described by Linhardt and Walter (1965). The rate of dephosphorylation of 4-nitrophenyl phosphate at pH 9.5 was monitored at 37° and 410 nm. A detailed description of the assay is presented in Appendix A.

Acid phosphatase (EC 3.1.3.2), as in most other tissue, is a marker of lysosomal membranes in the choroid plexus epithelia cell (Masuzawa and Sato,1983). The activities of this enzyme were monitored using the fixed-time method described by Moss (1984). The rate of 4-nitrophenyl phosphate dephosphorylation at pH 4.9 was measured at 37° and 405 nm. A detailed description of the assay is presented in Appendix A.

The enzyme glucose-6-phosphatase (EC 3.1.3.9) is a marker of endoplasmic reticulum (Masuzawa and Sato,1983). The activity of this enzyme was monitored by determining the rate of release of inorganic phosphate from glucose-6-phosphate using the method of Aronson and Touster (1974). The reaction was carried out at 37° and the optical density of the solution was read at 660 nm against a water blank. A detailed description of the assay is presented in Appendix A.

The absorbance was measured in all assays with a Ultrospec spectrophotometer model 4050 equipped with a model 4070 Autofill temperature controller (LKB Instruments, Gaithersburg, MD).

Membrane Orientation Experiments

The method described by Kinsella et al. (1979) was used to determine the orientation of the vesicles in the P_6 fraction. The method involves determining the specific activity of Na^+/K^+ATPase in lysed and unlysed vesicles. Briefly, the vesicles were divided into two suspensions. The unlysed fraction remained suspended in the original MHK buffer as described in the vesicle preparation protocol. The lysed fraction was resuspended in 0.1% deoxycholic acid and frozen at -20° for 2 hr before the assay and then allowed to thaw at room temperature. The Na^+/K^+ATPase activity in each fraction was determined by the method of Schoner et al. (1965) as described in Appendix A.

Uptake Experiments

Methods for L-proline uptake and osmolarity sensitivity reaction experiments are described in detail in Chapter 5.

Chemicals

All chemicals except for ^{14}C-L-proline and ^{3}H-cimetidine were purchased from either Sigma, Fisher Scientific, or Aldrich.

Results

The relative specific activities of Na^+/K^+ATPase, alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase in fractions P_4 and P_6 using the separation procedure in Scheme 1 are depicted in Figure 19. The activity of each membrane marker enzymes in each fraction is relative to the activity of that

enzyme in the homogenate. The activity of Na^+/K^+ATPase in fractions P_4 and P_6 was enhanced greater than 12-fold and 15-fold, respectively, when compared with its specific activity in the homogenate. The specific activities of glucose-6-phosphatase, acid phosphatase, and alkaline phosphatase were enhanced 1, 2, and 3-fold respectively, in the P_4 fraction and 2, 3, and 3-fold, respectively, in the P_6 fraction. These results suggest that the population of brush-border membranes relative to the populations of other membranes in the homogenate has been enhanced in the final preparation. The data are presented as the mean ± SD of 6 preparations for Na^+/K^+ATPase and alkaline phosphatase and the mean ± SD of 3 preparations for acid phosphatase and glucose-6-phosphatase specific activities.

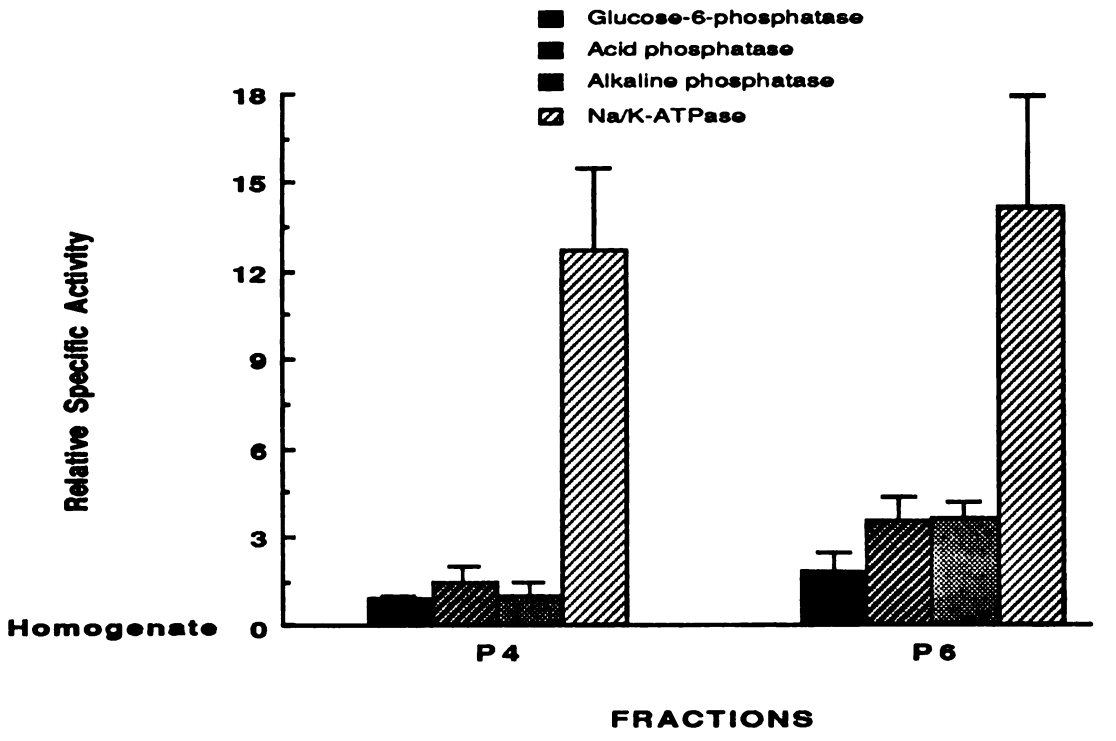


Figure 19 The relative enhancement of membrane enzyme markers in the isolated fractions P_4 and P_6 from several preparations is depicted. Enhancement values are relative to the activity of the homogenate in Scheme 1. (Values are presented as mean ± SD; n = 6 for Na^+/K^+ATPase and alkaline phosphatase, n = 3 for acid phosphatase and glucose-6-phosphatase)

The effectiveness of the isolation procedure and the purity of the final vesicle preparation were determined by monitoring the percentage of protein recovered and the specific activities of the four membrane-marker enzymes in the fractions obtained from each step in the procedure in Scheme 1. The data are presented in Table 3 as the mean ± SD in three separate preparations. The protein recovery data represents the percentage of protein recovered in a fraction from the two succeeding fractions. The data for the amount of protein in each fraction is provided in Appendix D. The recovery of protein was close to 100% at most steps. From the starting material (i.e., the whole choroid plexus tissue, the wet

weight of which averaged approximately 21 g), 6% was assayed in the homogenate as protein while only 0.7% appeared in P_6 as protein. The amount of protein in P_6 was 1.25% of that in the original homogenate. The specific activity of Na^+/K^+ATPase was observed to increase progressively in the fractions which were used for isolation of the membranes (P_3, S_4, P_5, and P_6). Alkaline phosphatase (basolateral membranes) and to a lesser extent acid phosphatase (lysosomes), and glucose-6-phosphatase (endoplasmic reticulum) activities remained relatively low in earlier fractions (P_3 and S4) but then appeared to be elevated in the final product (P_6). These results suggest that brush-border membranes are isolated in each step and that there may be some co-isolation of other membranes as well.

Table 3 Specific Acitivity of Membrane Marker Enzymes and %Recovery of Protein

Fractions	%Recovery Protein	Specific Activities (U/g protein) Na+/K+ ATPase	Acid Phosphatase	Alkaline Phosphatase	Glucose-6-Phosphatase
H	98.8±13.2	32.5±3.2	22.1±3.6	54.7±4.2	21.0±3.5
P_1	___	25.7±6.8	___	67.1±23.0	___
S_1	96.7±2.6	22.9±4.6	___	47.0±4.4	___
P_2	___	167.3±28.5	___	165.7±31.1	___
S_2	110.0±8.0	8.9±5.3	___	44.4±6.0	___
P_3	71.8±18.4	146.9±8.5	___	65.9±3.9	___
S_3	___	4.4±2.5	___	29.7±3.4	___
P_4	___	336.9±58.1	53.9±7.7	163.0±27.1	41.1±5.7
S_4	95.1±14.4	120.8±1.4	___	71.8±11.5	___
P_5	79.9±37.2	353.8±85.3	___	213.1±46.2	___
S_5	___	148.1±127.0	___	29.8±7.3	___
P_6	___	357.3±46.9	101.0±21.2	214.9±38.2	58.9±13.8
S_6	___	87.9±15.7	___	168.4±29.0	___

The values represent the mean±SD in three preparations. The precentage recovery of protein for a fraction is based on the amount of protein in the fractions derived from that fraction. e.g., $\%H_1 = \frac{P_1+S_1}{H_1} \times 100$, where H_1, P_1, and S_1 are the total amount of protein recovered in those fractions, respectively.

The orientation of the membrane vesicles was determined by a method described by Kinsella et al. (1979) in which the activity of Na^+/K^+ATPase was monitored in intact vesicles and in the membranes of vesicles which had been lysed by incubation in 0.1% deoxycholate and freezing and thawing. The total ATPase activity in unlysed vesicles was 17.63 ± 1.12 U/mg protein and the Na^+/K^+ATPase accounted for 5.14 ± 0.63 of this activity. After vesicle disruption the total ATPase activity increased to 30.1 ± 1.8 U/mg protein and the Na^+/K^+ATPase to 22.44 ± 0.78 U/mg protein. The proportion of Na^+/K^+ATPase in unlysed vesicles compared to that in lysed vesicles reflects the presence of sheets of membranes. Therefore about 23% ($\frac{5.14}{22.44}$ X 100%) occurred as sheets. The total ATPase in unlysed vesicles (17.63) is equivalent to ouabain-sensitive and -insensitive ATPase in the sheets and in inside-out membrane vesicles. The total ATPase in sheets is 6.89 U/mg protein (0.229 X 30.1). Since the total ATPase in unlysed vesicles represents total ATPase activity in sheets and inside-out vesicles, the total ATPase activity in inside-out vesicles is 10.74 (17.63-6.89). Therefore 35.68% of the membranes are inside-out vesicles ($\frac{10.74}{30.1}$ X 100%) and 41.42% (100-58.58) are rightside-out vesicles.

Figure 20 is an electron micrograph of the vesicles in an aliquot from P_6 suspension .

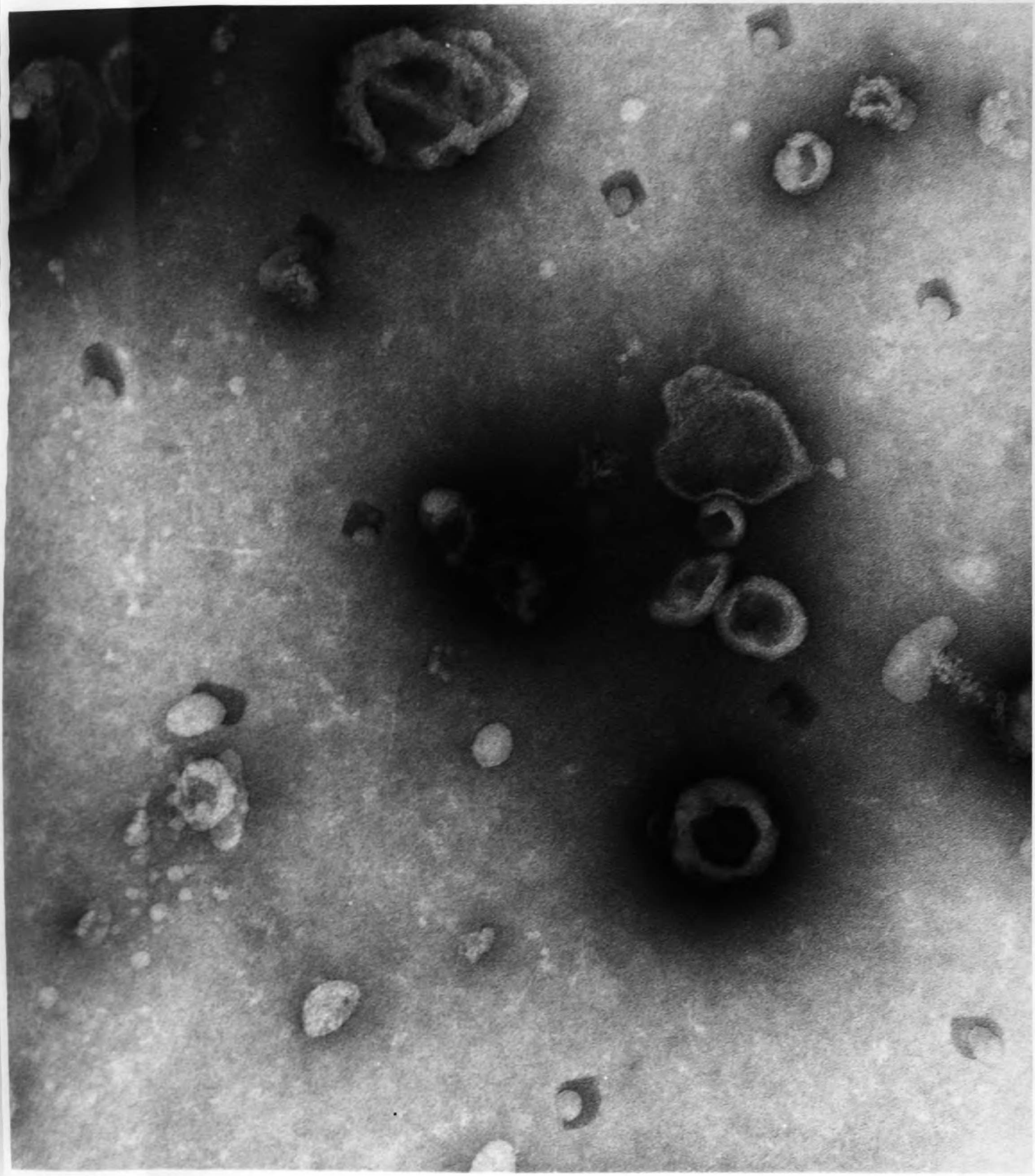


Figure 20 Electronmicrograph of brush-border membrane vesicle suspension. (previous page) (maginification X 55,000)

L-proline is transported into vesicles prepared from choroid plexus via a sodium sensitive process and accumulates against a concentration gradient as demonstrated by an "overshoot" phenomenon (Ross and Wright, 1984). The accumulation of ^{14}C-L-proline in bovine choroid plexus brush-border membrane vesicles with time using the P_6 fraction is depicted in Figure 21. An equilibrium was reached within 2 hr in the presence of an initial inwardly-directed potassium gradient (100 mM). When sodium (100 mM) was present initially in the extravesicular medium, the initial rate of uptake of L-proline was accelerated and the amount of proline taken up reached a maximum which exceeded the 2 hr equilibrium value. These results are similar to those reported by Ross and Wright (1984) and suggest that the preparation consists of functional brush-border membrane vesicles. When vesicles from the P_4 fraction were used in L-proline uptake experiments there was an acceleration of the initial rate of uptake, however, no "overshoot" phenomenon was observed (data not shown).

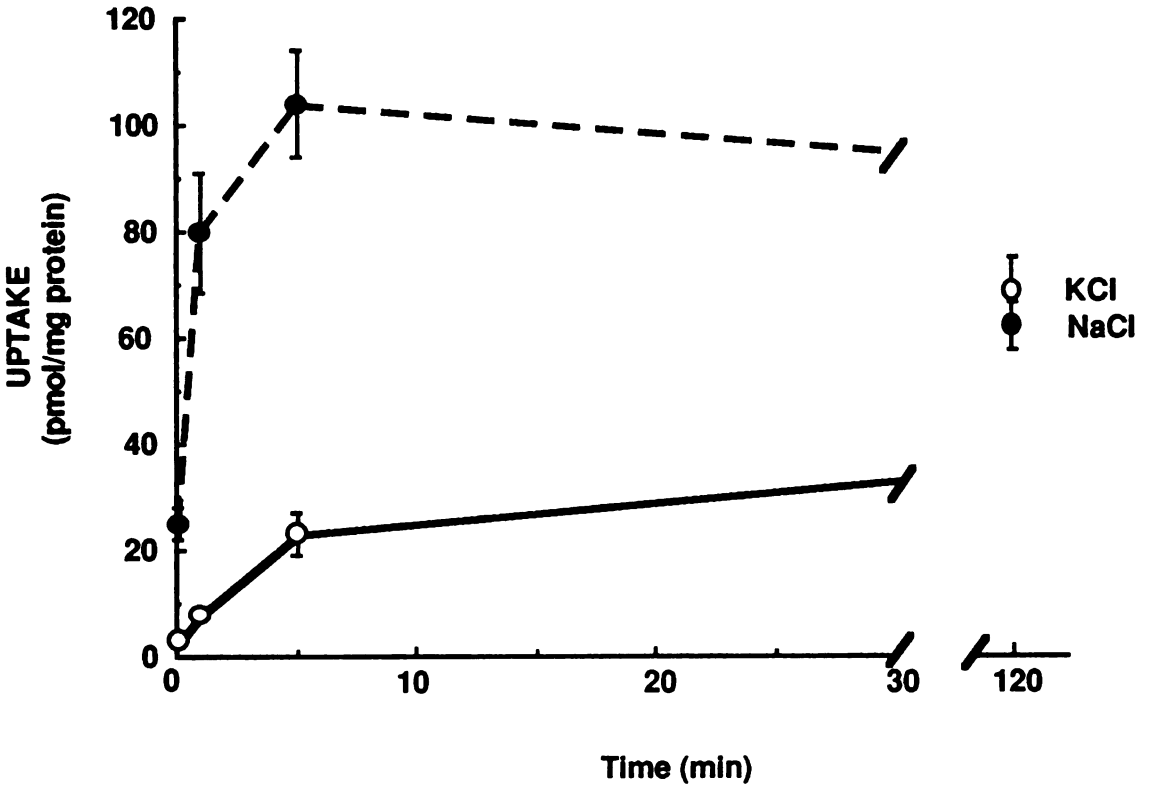


Figure 21 The uptake of [^{14}C]-L-proline (50 μM) in brush-border membrane vesicles from bovine choroid plexus. The solid circles (●) and the open circles (○) represent the data (mean ± S.E.; n=3) in the presence and absence of an initial inwardly-directed sodium gradient (100 mM), respectively.

The osmotic sensitivity of the vesicles is demonstrated in Figure 22. When the vesicles were subjected to extravesicular media of increasing osmolarities the amount of ^{3}H-cimetidine taken up by the vesicles at equilibrium decreased in a linear fashion as determined by linear regression analysis suggesting that cimetidine is taken up into an intravesicular space which is osmotically reactive. Cimetidine binding to the membrane was estimated from these data by dividing the value of the intercept of the regression ordinate by the uptake value obtained in the medium of the least osmolarity. Using this algorithm, the binding of cimetidine to the membrane was estimated to be less that 18%.

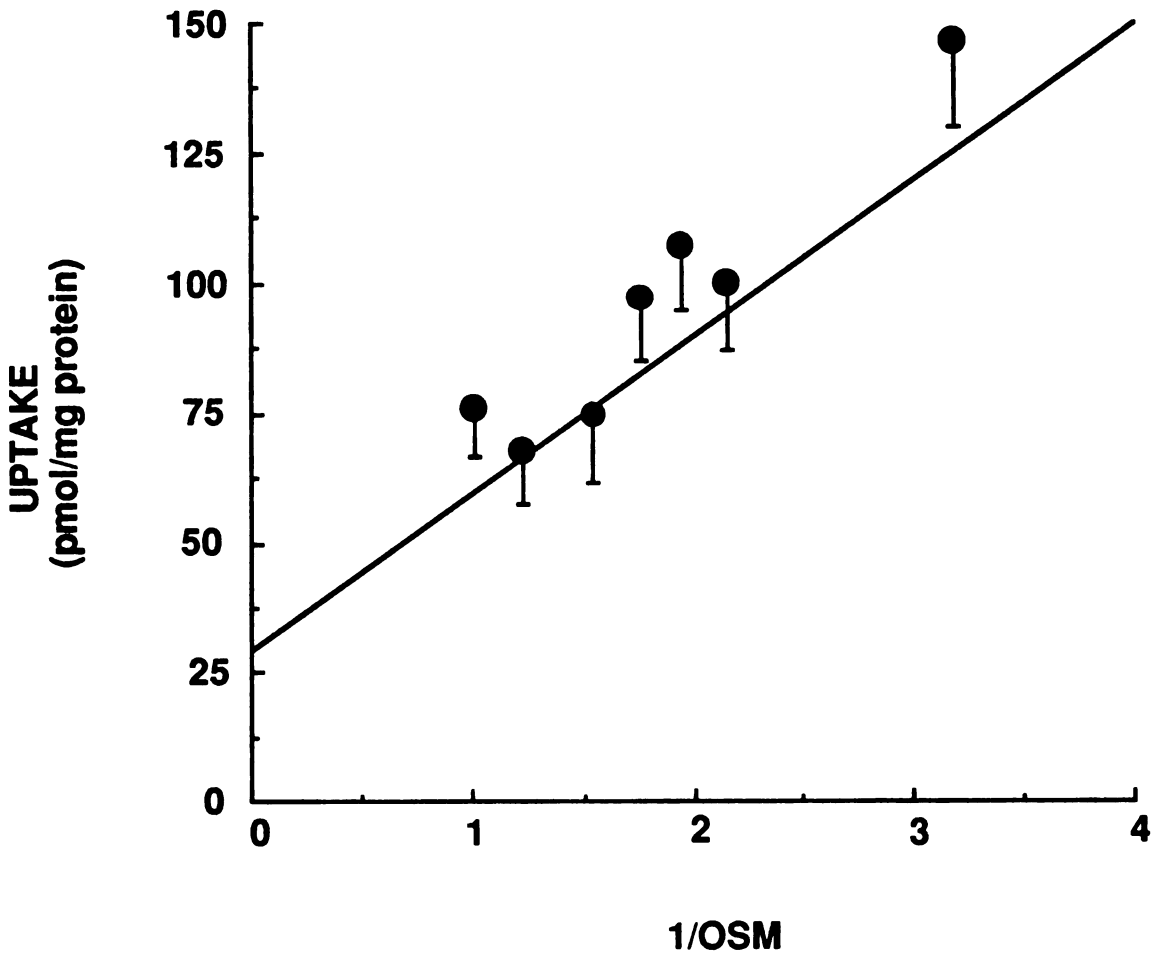


Figure 22 The 2 hr (equilibrium) uptake of ^{3}H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus as a function of the inverse osmolarity of the medium. The line through the data points represents the best fit of the data by linear regression analysis (r=0.954). The data represent the mean ± S.E. of three experiments.

Discussion

In this study we have established a method for preparing isolated brush-border membrane vesicles from bovine choroid plexus. The method involves a

modification of the procedure described by Ross and Wright (1984) and is based upon the method of Booth and Kenny (1974) which was developed to separate the brush-border from the basolateral membrane of renal epithelia. The procedure involves aggregation of the basolateral membrane and membranes of intracellular organelles with the divalent cation, calcium. Brush-border membranes, having a greater surface charge density than other membranes of the choroid plexus cell due to the high density of negative charges, contributed mostly by sialic acid of the glycocalyx on the microvillus membrane (Booth and Kenny, 1974), are separated by a series of differential centrifugations.

Histochemical and immunocytochemical localization studies have shown that the membrane-bound enzymes, Na^+/K^+ATPase and alkaline phosphatase, are localized at the brush-border and basal membranes of the choroid plexus epithelia cell, respectively, (Masuzawa and Sato, 1983; Ernst et al., 1986) and therefore can be used as membrane markers to determine the purity of the isolation preparation. In our final preparation (P_6) the specific activity of Na^+/K^+ATPase was enhanced greater than 15-fold when compared with its specific activity in the homogenate, while the specific activity of alkaline phosphatase was enhanced 3-fold (Figure 19). These values are in good agreement with those of Ross and Wright (1984) who reported enhancements of 9 to 14-fold for Na^+/K^+ATPase and 3-fold for alkaline phosphatase. We have also monitored enzyme markers for endoplasmic reticulum (glucose-6-phosphatase) and lysozomal (acid phosphatase) membranes and found that these enzymes are slightly enriched in the final membrane preparations (Figure 19). As discussed by Booth and Kenny (1974) lysozomes have the same high density of negative charges as do brush-border microvillus membranes.

Therefore it is expected that acid phosphatase activity would be enhanced in our preparation. Alternatively, acid phosphatase enhancement could result from disruption of the lysosomal membrane causing the free water-soluble enzyme to be released into the suspension (Booth and Kenny,1974). In the choroid plexus brush-border membrane vesicle preparations both we and Ross and Wright (1984) obtained some co-purification of alkaline phosphatase, presumably reflecting co-isolation of basolateral membranes. Alternatively, alkaline phosphatase is an ubiquitous enzyme in biological tissues, therefore, the enhancement in these studies may reflect its presence on the brush-border membrane.

In addition to monitoring specific enzyme markers, we have determined the uptake of the neutral amino acid, L-proline in the presence of an initial inwardly directed Na^+ gradient. Previously, Ross and Wright (1984) have demonstrated that L-proline is transported across the brush-border membrane of the choroid plexus by a Na^+ co-transport mechanism. The data shown in Figure 21 are in excellent agreement with the data obtained by Ross and Wright (1984) and demonstrate that the preparation consists, at least in part, of functional, tightly-sealed brush-border membrane vesicles.

Because of the possible co-isolation of membranes we examined other centrifugation fractions for enhancement of Na^+/K^+ATPase activity without co-enhancement of alkaline phosphatase activity. We observed that the P_4 fraction of our procedure had a relatively high enhancement of Na^+/K^+ATPase although lower than that of the P_6 fraction. However, the alkaline phosphatase was less enhanced in P_4 in comparison to P_6 (Figure 19 and Table 3). The activities of other membrane marker enzymes relative to that of Na^+/K^+ATPase in P_4 were similar to what was observed in P_6. However, the sodium-sensitive,

concentrative L-proline transport observed in the P_6 fraction was not present in P_4 (data not shown). Therefore, the P_6 fraction was used for cimetidine transport studies.

The data in Figure 22 represent the effects of shrinking the vesicles, by incrementally increasing the osmolarity of the extravesicular medium, on the equilibrium value of cimetidine uptake. As discussed in Chapter 5, estimates of membrane binding from these experiments were consistent with binding to plasma proteins. Therefore these data provide further evidence that the isolated membranes form tightly sealed vesicles which are selectively-permeable and contain an intravesicular space which is sensitive to an osmotic gradient across the membrane.

The question of orientation of the membrane vesicles was addressed by a method described by Kinsella et al. (1979) in which the activity of Na^+/K^+ATPase in lysed and unlysed vesicles is determined. The activity of this enzyme should be very low in intact right-side-out vesicles because of the lack of accessibility of ATP to its binding site inside the vesicle. The activity of Na^+/K^+ATPase in lysed and unlysed vesicles was compared to determine what percentage of the membranes can be attributed to sheets in the unlysed suspension and what percentage can be attributed to right-side out orientated vesicles. In these experiments it was determined that approximately 23% of the brush-border membranes exist as sheets and approximately 41% exist as right-side out orientated vesicles.

In summary, a bovine choroid plexus brush-border membrane vesicle preparation procedure has been established. The method has the advantages of being relatively facile, short in duration, and provides a sufficiently high

protein yield to perform transport studies. The vesicles prepared in this manner are enriched in Na^+/K^+ATPase, an enzyme marker specifically localized on the brush-border of the choroid plexus epithelia cell, however, there may be some contamination by co-isolation of other membranes. The vesicle preparation exhibited an osmotically reactive intravesicular space and the ability to take up L-proline via a concentrative and sodium sensitive process. Bovine choroid plexus brush-border membrane vesicles prepared in this manner appear to be suitable for studies designed to elucidate the mechanisms of transport of substances at the brush-border membrane of this epithelium.

CHAPTER 5

Cimetidine Transport Studies in Bovine Choroid Plexus Brush-Border Membrane Vesicles

Introduction

The CNS is unique in that it has the ability to selectively control its macro-environment. The blood-brain barrier, which is largely the function of the CNS microvasculature endothelium, selectively restricts the passage of substances into and out of this compartment on the basis of their physicochemical properties (Rapoport, 1976; Rapoport et al., 1979; Bradbury, 1985). Juxtaposed with the discriminatory activity of the microvasculature is the blood-CSF barrier which appears to maintain homeostasis mostly through passive and active elimination processes (Cserr, 1971). These two interfaces with the blood compartment can not only selectively control admittance to the CNS environment, but can maintain low concentrations of substances using a "frontdoor-backdoor" system which may create a "sink" condition in the CNS and thereby avoid the accumulation of undesirable compounds. A substance which has the required physicochemical properties may readily cross the blood-brain barrier from the arterial blood supply (frontdoor). At steady-state the CSF concentration (C_{CSF}) of the substance would be expected to approach the unbound concentration in plasma (C_u). However, because specific active transport processes may expedite expulsion to the venous vasculature (backdoor), the rate of elimination of the substance from the CNS can exceed its rate of entry and C_{CSF} may be far lower than expected.

As discussed in Chapter 3, in healthy human volunteers the steady-state CSF to plasma concentration ratio (C_{CSF}:C_P) of the H_2-receptor antagonist

cimetidine is approximately 0.2 (Schentag et al., 1979 and 1981). Because cimetidine has a octanol:pH 7.4 phosphate buffer partition coefficient of 2.0, and about 80% of the compound is present in the unionized form at pH 7.4 (Bavin and Zarembo, 1984), its penetration into CSF should be rapid (Rapoport, 1976). Therefore, the ratio of cimetidine concentrations in the CSF to its unbound concentration in plasma should be close to unity. Since very little cimetidine in plasma is bound to plasma proteins, and the pH of the CSF (7.33) would favor preferential partitioning into the CSF, the substantially lower C_{CSF}:C_P suggests that the drug may be either metabolized in the CSF or actively absorbed from the CSF. We (Whittico and Giacomini, 1988; Chapter 3) and others (Suzuki et al., 1985 & 1988) have demonstrated in experimental animals that cimetidine is eliminated from the CSF, in part, via a saturable process (see Chapter 3 for a detailed discussion). Cimetidine has been demonstrated to accumulate in whole choroid plexus tissue preparations isolated from the rat brain (Suzuki et al.,1986). The accumulation occurred against a concentration gradient and was saturable, temperature dependent, sodium insensitive, and inhibitable by various structurally heterogeneous compounds. Collectively these results suggest that cimetidine may be eliminated from the CSF by a saturable and concentrative transport process which appears to be localized in the choroid plexus.

A common organic cation transporter analogous to the well-described organic cation transporter in renal epithelium (Cacini et al., 1982; McKinney and Kunnemann, 1986; McKinney et al., 1981; McKinney and Speeg, 1982; Rennick et al., 1984; Sewing and Kaplowitz, 1979; Somogyi et al., 1983; Takano et al., 1985; and Weiner and Roth, 1981; Gisclon et al., 1987) is thought to exist in choroid plexus epithelium. However, a notable difference between the two

transporters is that in the kidney, the transporter functions in the secretory direction whereas in the choroid plexus, the transporter functions in the absorptive direction. Since cimetidine appears to share the common organic cation transporter in the proximal tubule, it is reasonable to hypothesize that the drug may share the analogous system in the choroid plexus. However, recent studies in isolated whole choroid plexus from rats have demonstrated that cimetidine uptake is not affected by hydrophilic organic cations such as N^1-methylnicotinamide (NMN) or tetraethylammonium (TEA), but is affected by organic anions, oligopeptides and hydrophobic organic cations including quinine and quinidine. These data suggest that the characteristics of cimetidine transport in the choroid plexus are different from those in renal epithelium and that the compound may be transported by an entirely different mechanism in the choroid plexus.

The choroid plexus epithelia cell is similar in morphological features to cells of other epithelia known to contain transport proteins such as the renal proximal tubule and the small intestine. The plasma membranes of the cell are quite polar. The membrane which faces CSF is called the brush-border membrane because it has numerous infoldings called villi and microvilli. These structures have the effect of increasing the surface area of the cell at this membrane. Other features localized at this membrane include tight junctions which occlude the intercellular space, thereby restricting the movement of substances between CSF and the fenestrated blood vessels; and numerous mitochondria which usually indicate the existence of energy requiring systems such as active transport systems. In contrast, the membrane at the opposite side of the cell is in close proximity to the vasculature which supplies the tissue. This membrane, called the basal membrane has relatively few infoldings and mitochondria. For

vectorial transport, mechanisms of transport in the brush-border and basolateral membranes must necessarily differ.

A major advancement in studying transport mechanisms in epithelia has been the development of techniques to isolate and separate the brush-border and basolateral membranes of the cells. The isolated membranes vesiculate and the formed vesicles can be used to study transport mechanisms. The transport of many compounds including cimetidine has been well characterized in isolated brush-border membrane vesicles prepared from renal cortex. Surprisingly except for two studies, both examining amino acid transport (Ross and Wright, 1984; Langenbeck and Kinne, 1980), isolated plasma membrane vesicles have not been used to study the mechanisms of solute transport across choroid plexus epithelium. The overall goal of this study was to elucidate the mechanisms of cimetidine transport in the brush-border membrane of choroid plexus epithelium. Since data are available from studies in isolated brush-border membrane vesicles prepared from renal cortex, we were particularly interested in learning if the mechanisms for organic cation transport across the brush-border membrane of choroid plexus epithelium were similar to those of the brush-border membrane of the proximal tubule. We addressed the following specific questions: (a) Does cimetidine accumulate in an intravesicular space of isolated brush-border membrane vesicles of choroid plexus epithelium? (b) Is cimetidine transported across the brush-border membrane via a saturable mechanism? (c) What compounds inhibit its transport? (d) Can a driving force be identified for cimetidine transport across the brush-border membrane? (e) Does the transport involve a mobile membrane-carrier?

Our data demonstrate that cimetidine was transported into an intravesicular space in part via a saturable, inhibitable, and sodium insensitive process. An outwardly directed proton gradient stimulated the transport but accumulation against a concentration gradient did not occur. Evidence for the involvement of a membrane-protein carrier was demonstrated.

Methods

Vesicle Preparation

Bovine choroid plexus tissue was obtained from a local abattoir (Ferrara Meat Co., San Jose, CA) and brush-border membrane vesicles were prepared as described in Chapter 4. In general, choroid plexus from approximately 50 cows were required to obtain sufficient protein yield for transport studies for one day (ca 18-20 g wet weight). This required about 3 hr of brain dissection and tissue harvesting.

Uptake Experiments

All uptake experiments were performed at 25° and, in general, were conducted as follows except where stated. Reactions were initiated by the addition of 40 µl of the reaction mixture to 10 µl of the vesicle suspension (7.5-15 µg protein/µl) in a 16 X 100 mm borosilcate disposable culture tube (Fisher Scientific Company, Fairlawn, NJ). After incubation for a desired length of time the reaction was quenched by the addition of 5 ml of ice-cold stop buffer consisting of 100 mM mannitol, 100 mM KCl, and 10 mM HEPES, pH 7.33, and immediately filtered under vacuum (ca 15 in Hg) through a nitrocellulose membrane filter (0.3 µm, type PH, Millipore, Bedford, MA) on a Hoefer FH225V ten-place filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). The filters were washed twice with 5 ml ice-cold stop buffer and placed in vials

containing scintillant (ScintiVerse II, Fisher Scientific Company, Fairlawn, NJ). The amount of radioactivity remaining on the filter was determined in a Beckman LS 1801 scintillation counter (Beckman Instruments, San Jose, CA). The radioactivity on blank filters was determined as above except that the 10 µl of vesicle suspension was excluded. The radioactivity associated with the vesicles was determined by subtracting the radioactivity of the blank filters from total radioactivity (vesicles + filters). The counting efficiency ranged between 49 and 50%.

L-Proline Uptake Experiments

The reaction mixture for studies of time-dependent uptake of cimetidine contained 66.67 µM ^{14}C-L-proline, 10 mM HEPES, 100 mM of either NaCl or KCl and 100 mM mannitol, pH 7.33. The vesicles were suspended in a buffer which contained 10 mM HEPES and 300 mM mannitol, pH 7.33.

Cimetidine Uptake Experiments

The experiments which monitored cimetidine uptake as a function of time were performed as described above. The reaction mixture consisted of 62.5 µM ^{3}H-cimetidine, 10 mM HEPES, 100 mM KCl, and 100 mM mannitol, pH 7.33. The vesicles were suspended in a buffer containing 10 mM HEPES, 100 mM KCl and 100 mM mannitol, pH 7.33.

Osmolarity Experiments

Osmotic sensitivity experiments were performed by incubating the vesicles for 2 hr in reaction mixtures which were the same as for time-dependent studies except for containing various concentrations of sucrose. Osmolarities of the reaction mixtures were 0.98, 0.813, 0.645, 0.565, 0.513, 0.463, and 0.315 osmol/l.

Michaelis-Menten Studies

For studies examining the effect of concentration on cimetidine uptake, incubation times of 5 s were used. Final concentrations of cimetidine in the incubation suspension were 0.25, 0.5, 1.0, 2.0, 10, 25, 50, 100, 600, and 1200 μM.

Counter-flux Experiments

Counter-flux experiments were performed by pre-incubating the vesicles for at least 2 hr in a solution of ^{3}H-cimetidine, such that the final cimetidine concentration of the suspension was less than 1 μM. The reaction was initiated as described above. The reaction mixture was the same as for the time-dependent experiments except that the unlabeled cimetidine concentration was 500 μM. The radioactive concentration of the vesicle suspension was unchanged by the dilution with the reaction mixture.

Transport Inhibition Experiments

Experiments to assess the affect of various compounds on cimetidine uptake were performed at incubation times of 5 s. The reaction mixtures were the same as for time-dependent experiments except for the addition of of either TEA (6.25 mM), ranitidine (6.25 mM), PAH (6.25 mM), salicylic acid (6.25 mM), or quinidine (0.125 mM), and the mannitol concentration was adjusted to maintain osmotic balance.

Driving Force Experiments

To determine whether an outwardly-directed H^+ gradient, or an inwardly-directed gradient of Na^+, HCO_3^-, or Cl^- could drive the transport of cimetidine into the vesicles, the vesicles were loaded by resuspension and pre-incubated for at least 2 hr in a buffer at either pH 6.0 (for H^+ gradient countertransport) or

7.5 (for cotransport and controls). The uptake of cimetidine as a function of time was monitored in vesicles from these two preparations as described previously. The reaction mixtures were the same as that for the time-dependent cimetidine uptake experiments except that 100 mM KCl or 190 mM of either NaCl, $NaHCO_3$, or LiCl was present and the pH was 7.5. The vesicles were suspended in the same buffer as was used in the time-dependent experiments. Differences in cimetidine uptake at each time point were analyzed using Student's *t* test.

The extent of metabolism of cimetidine by membrane-bound enzymes and its possible contribution to the uptake process was investigated using thin-layer chromatography (TLC) as previously described (Chapter 3, Methods). The reaction mix was the same as for time-dependent uptake experiments. Reactions were initiated as described above and incubation times were for 2 hr. Following incubation, 2 µl of the incubating vesicle suspension was pipetted onto a (silica gel 60 precoated, 0.2 mm layer thickness, aluminum backing) TLC plate and the amounts of cimetidine and cimetidine-sulfoxide associated with the spot were quantitated, following chromatographic separation in a ethylacetate:NH_4OH:methanol (5:1:1) mobile phase solvent system, by scintillation counting.

Data Analysis

The amount of radioactivity on the filters was quantitated by scintillation counting as DPM as described above. DPM was converted to pmol/mg protein using the specific activity of the radiolabeled compound, the volumes and concentrations of the reaction mixtures, and the protein concentration of the vesicle suspension. The data from studies investigating the effect of concentration on cimetidine uptake were transformed to rates by dividing the

amount of cimetidine uptake at each concentration by 5 s. The rate vs concentration data were then fit to a Michaelis-Menten mathematical model as

$$v_0 = \frac{V_{max} * C}{K_m + C} + K_{ns} * C \qquad \textbf{(Equation 10)}$$

where v_0 is the initial rate of transport, V_{max} is the maximal transport rate, K_m is the cimetidine concentration when the initial rate is at one-half the maximal, K_{ns} is the nonsaturable term normally taken as the permeability coefficient (Hofer, 1981), and C is the extravesicular cimetidine concentration. The nonlinear, least-squares regression,and iteration-based computer procedure, FIT FUNCTION, on the National Institute of Health computer system, PROPHET, was used for the fitting of the data.

All data points were determined as the mean of three replicates and at least three experiments were performed for each study using separate membrane vesicle preparations. Data are presented as mean±S.E. of three replicate experiments except where stated in the text. Statistical differences were determined by either Student's *t* test or analysis of variances, ANOVA, as appropriate. Probability (P) below the 0.05 level was considered significant.

Chemicals

^{14}C-L-proline (285 mCi/mmol) and ^{3}H-cimetidine (10-28.7 μC/mmol) were purchased from Amersham Corporation, Arlington Hts, Ill. All other chemicals were purchased from either Sigma, Fisher Scientific or Aldrich. All solvents were of analytical grade.

Results

The uptake of ^{3}H-cimetidine in the vesicles as a function of time is depicted in Figure 23A. Cimetidine accumulated in the vesicles with time and reached equilibrium within 2 hr. Linear regression of the first 3 time points demonstrated that the rate of uptake was linear in this portion of the curve, therefore we elected to use the uptake rate at 5 s in subsequent studies of the affect of concentration on the uptake of cimetidine. Extrapolation of the regression line to time zero in Figure 23B yields a value which reflects the initial adsorption of cimetidine to the membrane, approximately 15% (Suzuki et al.,1987)

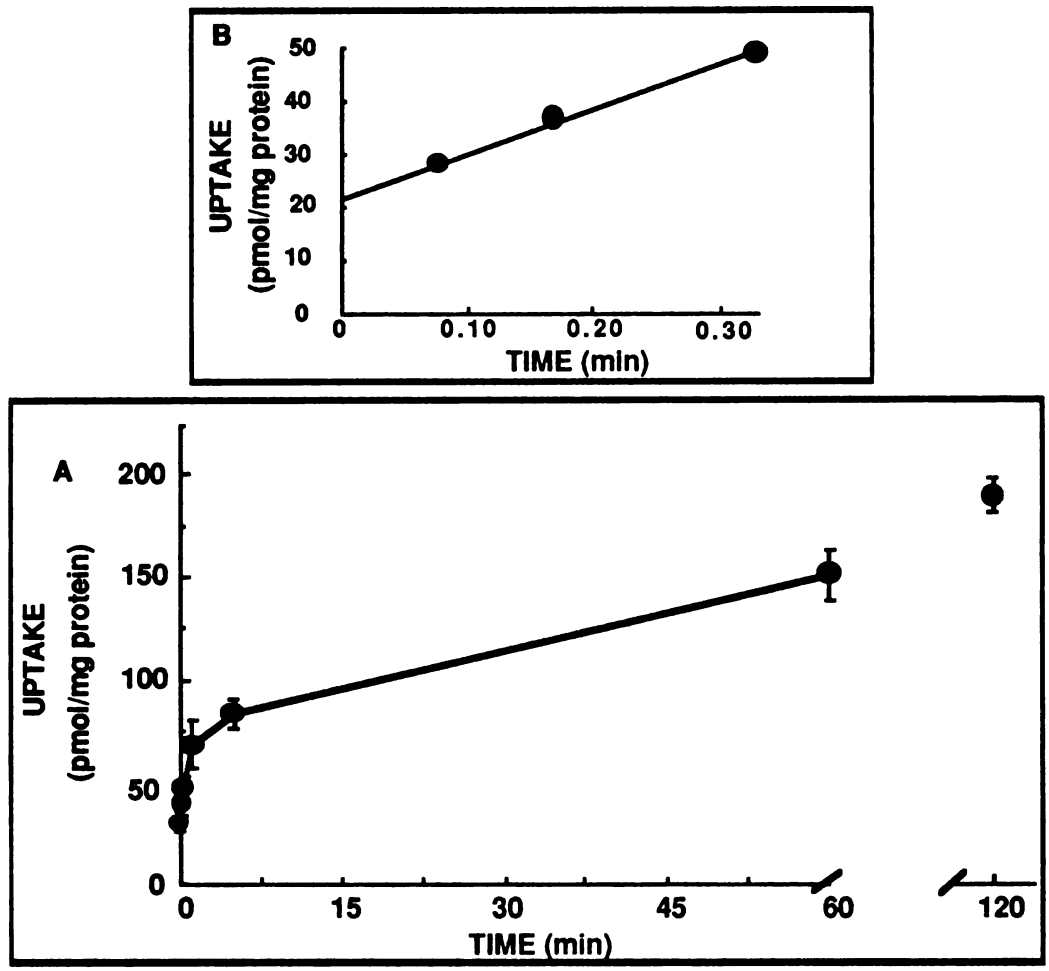


Figure 23 (**A**) The uptake of ^{3}H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus. The data represent the mean ± S.E. of at least 3 experiments. (**B**) The uptake of ^{3}H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus during the initial uptake period. The line through the data points represents the best fit of the data by linear regression analysis (r=0.988).

The initial rate of ^{3}H-cimetidine uptake into the vesicles as a function of cimetidine concentration in the extravesicular medium is shown in Figure 24A. The uptake rate was linear at concentrations between 0.25 and 10 μM and became curvilinear at higher concentrations. The data were fit to the equivalent Michaelis-Menten type equation for transport, equation 9, as described in the

Data Analysis section, and the estimates obtained for the parameters V_{max}, K_m, and K_{ns} were 16.7 ± 5.9 pmol/s/mg protein, 58.1 ± 3.06 μM, and 0.023 ± 0.0035 μl/s//mg protein (mean ± S.E.), respectively, as shown in Table 4.

Table 4. Transport Parameters

	Vmax (pmol/sec * mg protein)	Km (μM)	Kns (μl/sec * mg protein)
experiment number			
1	13.5	52.4	0.027
2	8.4	57.2	0.027
3	28.1	63.8	0.016
AVERAGE	**16.7**	**58.1**	**0.023**
S.D.	**10.2**	**5.30**	**0.0061**

The solid line labeled in TOTAL in Figure 24A represents the computer fit of the data to equation 10, containing both saturable and nonsaturable components. The dashed-line labeled SATURABLE was generated using the equation

$$v_0 = \frac{V_{max} * C}{K_m + C} \qquad \textbf{(Equation 11)}$$

where the estimates of the parameters V_{max} and K_m were used to estimate the rate of uptake at each concentration. The dashed-line labeled NONSATURABLE resulted when the nonsaturable estimated parameter, K_{ns}, was used to estimate the uptake rate as a function of cimetidine concentration as

$$v_0 = K_{ns} * C. \qquad \textbf{(Equation 12)}$$

From these estimates of the individual components of the transport process it could be further estimated that at initial rates, the nonsaturable component accounted for less than 12% of the total transport process at concentrations up

to 50 μM as can be seen in Figure 24B. At higher concentrations nonsaturable transport contributed increasingly to the overall transport process.

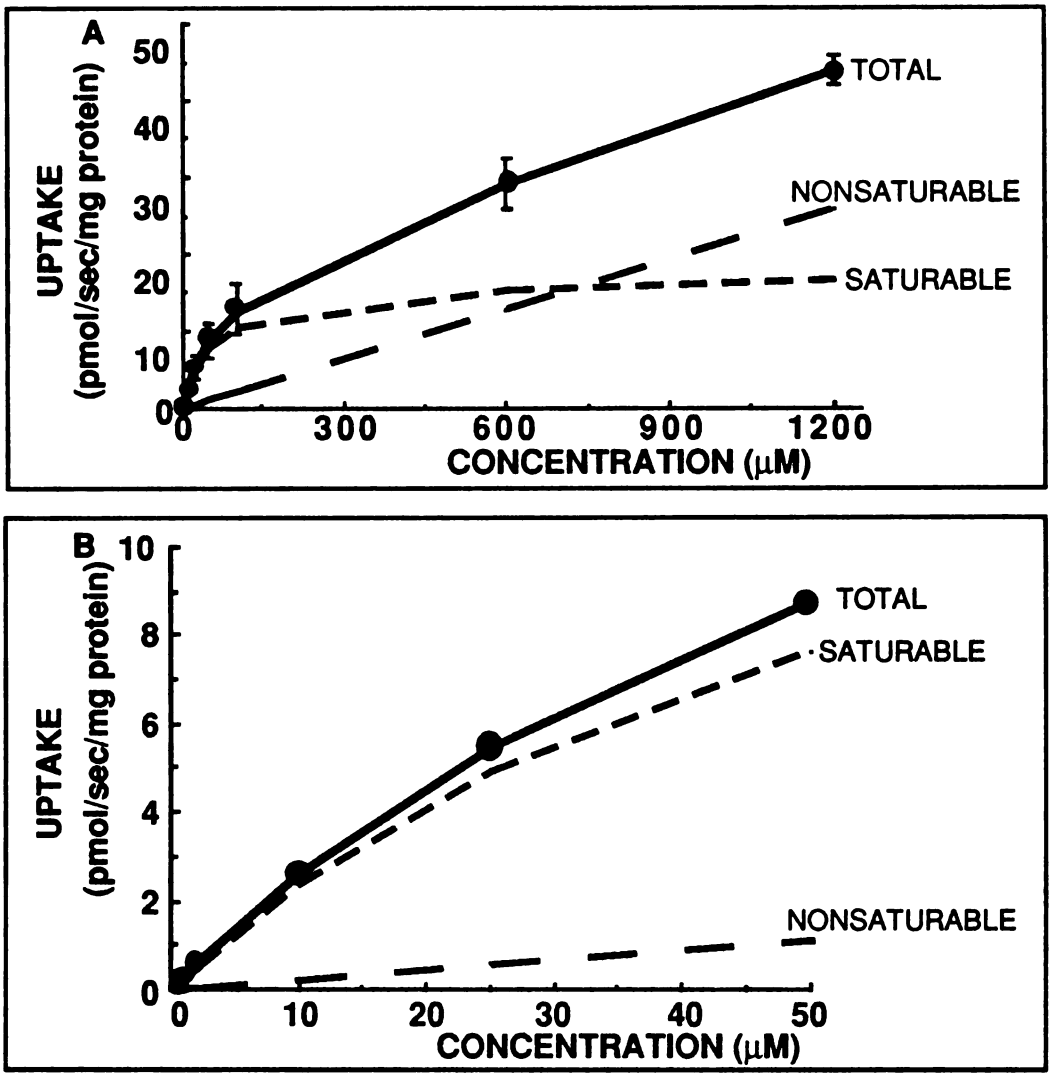


Figure 24 (**A**) The initial uptake rate (5 s) of ^{3}H-cimetidine in brush-border membrane vesicles from bovine choroid plexus as a function of cimetidine concentration. The data are presented as mean ± S.E. of 3 experiments. The solid line represents a computer fit of the data to an equation incorporating a saturable and a nonsaturable component. The dashed lines were generated using estimates of the transport parameters obtained from the computer fit (see text for details). (**B**) The same curve as shown in **A** except that cimetidine concentrations now range between 0.25 and 50 μM.

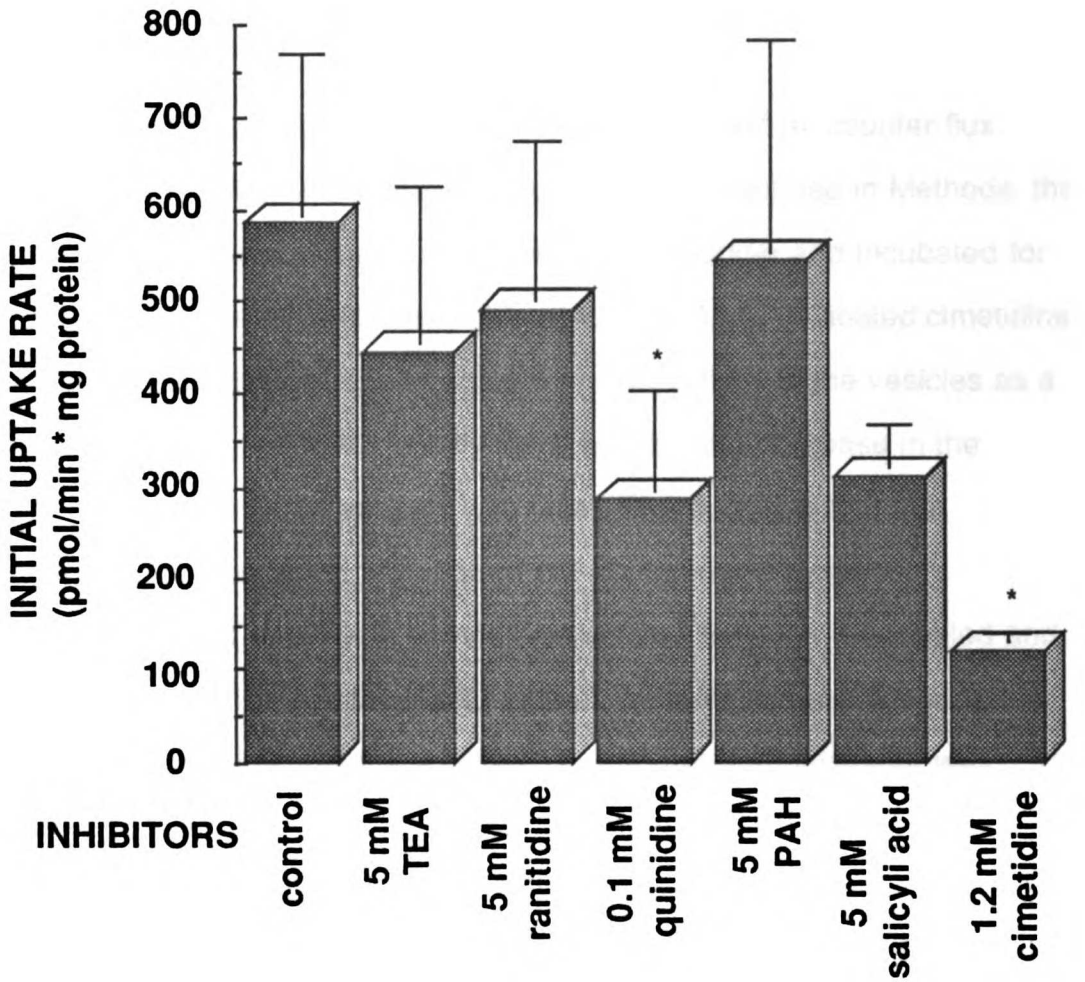


Figure 25 The effect of various inhibitors on the initial rate (5 s) of uptake of ^{3}H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus. The data represent the mean ± S.E. of a least 3 experiments.

The effects of the organic cations TEA, ranitidine, cimetidine, and quinidine and the organic anions PAH and salicylic acid on the uptake of ^{3}H-cimetidine in choroid plexus brush-border membrane vesicles are shown in Figure 25. The uptake was not significantly altered by TEA (5 mM), ranitidine (5 mM), or PAH (5 mM). Cimetidine (1.2 mM), and quinidine (0.1 mM) significantly reduced the uptake of 50 μM ^{3}H-cimetidine in the vesicles when the initial uptake rates were

compared by ANOVA. Salicylic acid (5 mM) reduced cimetidine uptake by 60% but not significantly at the ANOVA 95% level.

The mobile membrane-protein carrier model was tested by counter-flux experiments and the data are shown in Figure 26. As described in Methods, the vesicles were suspended in buffer containing ^{3}H-cimetidine and incubated for at least 2 hr prior to the experiments. The effect of 500 μM unlabeled cimetidine in the extravesicular medium on the amount of radioactivity in the vesicles as a function of time was monitored. Initially there was a rapid decrease in the amount of radioactivity in the vesicles with time. This suggests that the radioactive species was being transported out of the vesicles against a concentration gradient and that a competition existed between the labeled and unlabeled species on the extravesicular side of the membrane which reduced the influx of ^{3}H-cimetidine into the vesicles. A "counter-flux" minimum was reached at 1 min, indicating a static head (Friedman,1986), followed by a gradual return to the equilibrium value within 1 hr as the gradients dissipated. The results suggest that a mobile protein carrier is involved in the transport of cimetidine at the brush-border membrane.

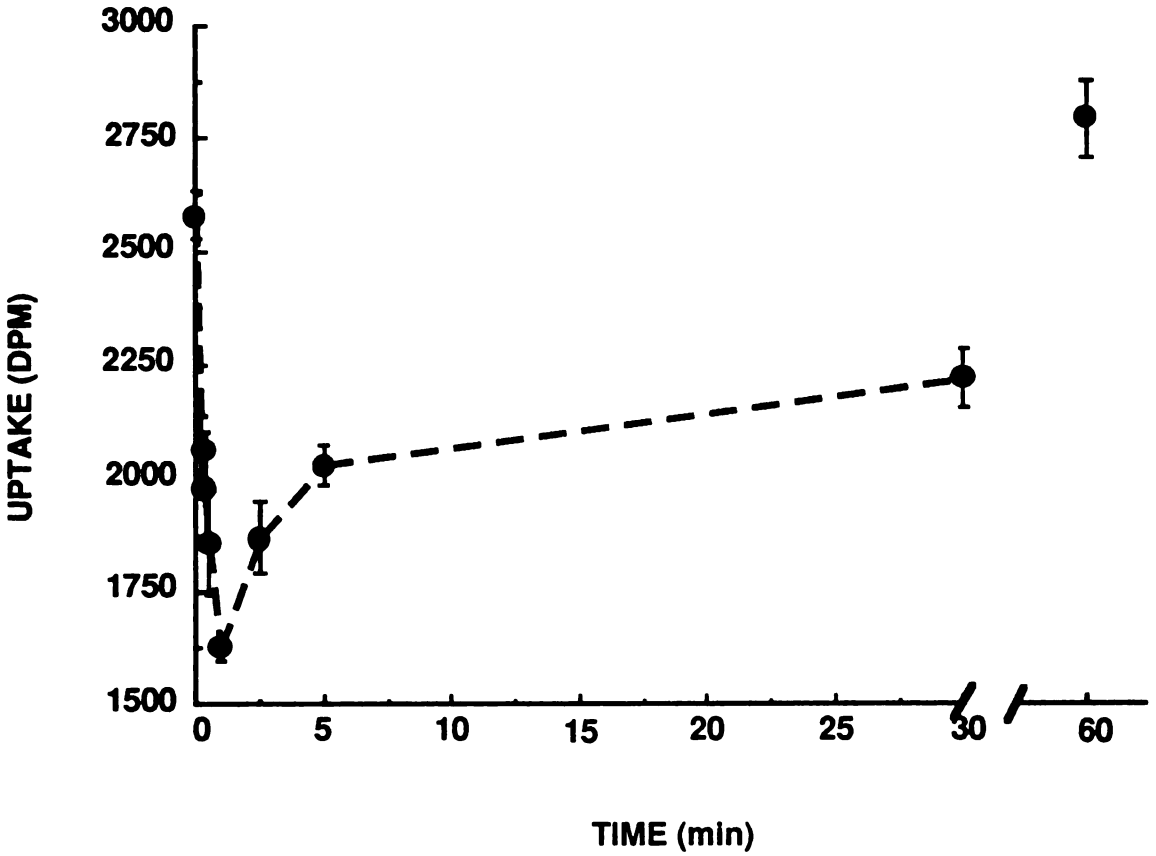


Figure 26 The radioactivity in brush-border membrane vesicles from bovine choroid plexus following the addition of unlabeled cimetidine (500 μM) to vesicles that had been preincubated in ^{3}H-cimetidine (1 μM) for at least 2 hr. The data are presented as mean ± S.E. of 3 experiments.

Figure 27 represents the data when a pH gradient was imposed across the vesicle membrane. After 10 s cimetidine uptake in the vesicles was significantly enhanced in the presence of a proton gradient. The equilibrium uptake values were not significantly different.

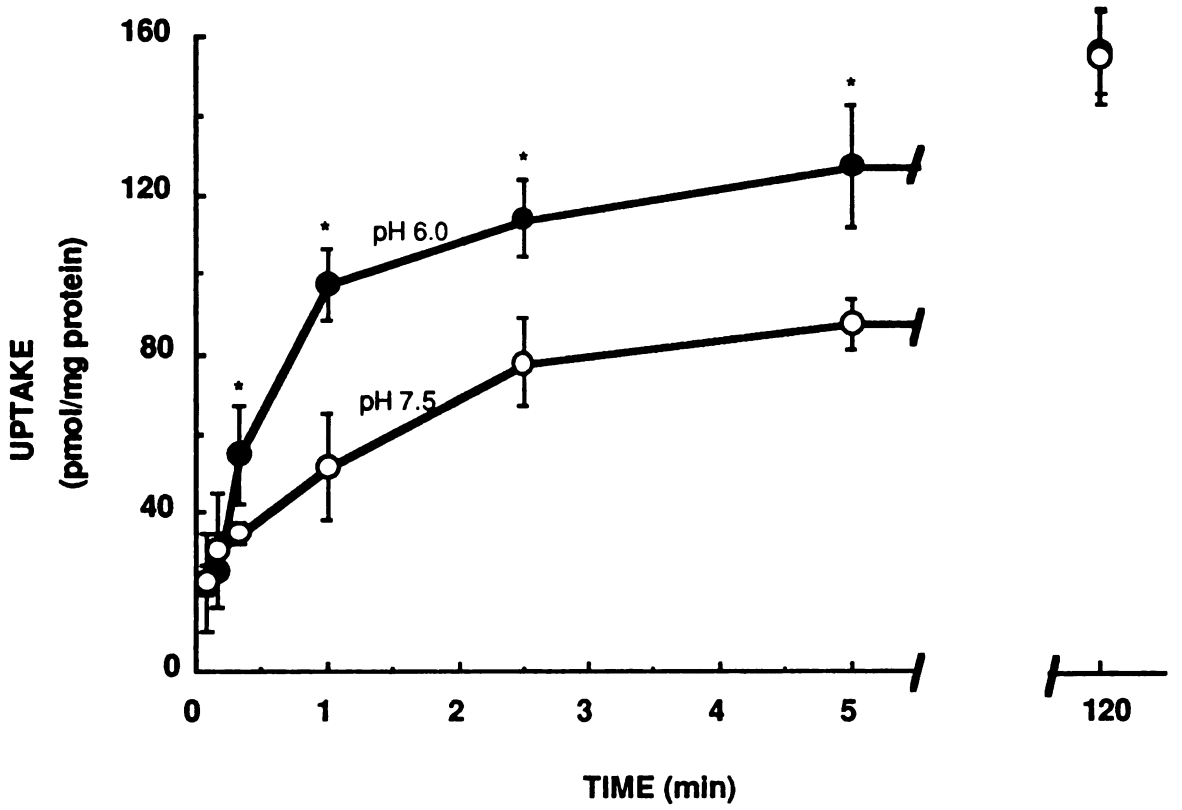


Figure 27 The effect of an outwardly-directed H^+ gradient on the uptake of ^{3}H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus. The solid circles (●) and the open circles (○) represent the data when the intravesicular pH was 6.0 and 7.5, respectively. The extravesicular pH was 7.5. The data are presented as mean ± S.E. of 3 experiments (* = $P < 0.05$; Student's t-test).

The affect of a 500 mM sodium gradient, from out to in, on the time course of ^{3}H-cimetidine uptake in choroid plexus vesicles is shown in Figure 28. The presence of an inwardly-directed sodium gradient had no significant effect on cimetidine uptake. Analysis using Student's *t* test revealed no significant difference in cimetidine uptake under these two conditions at any time point.

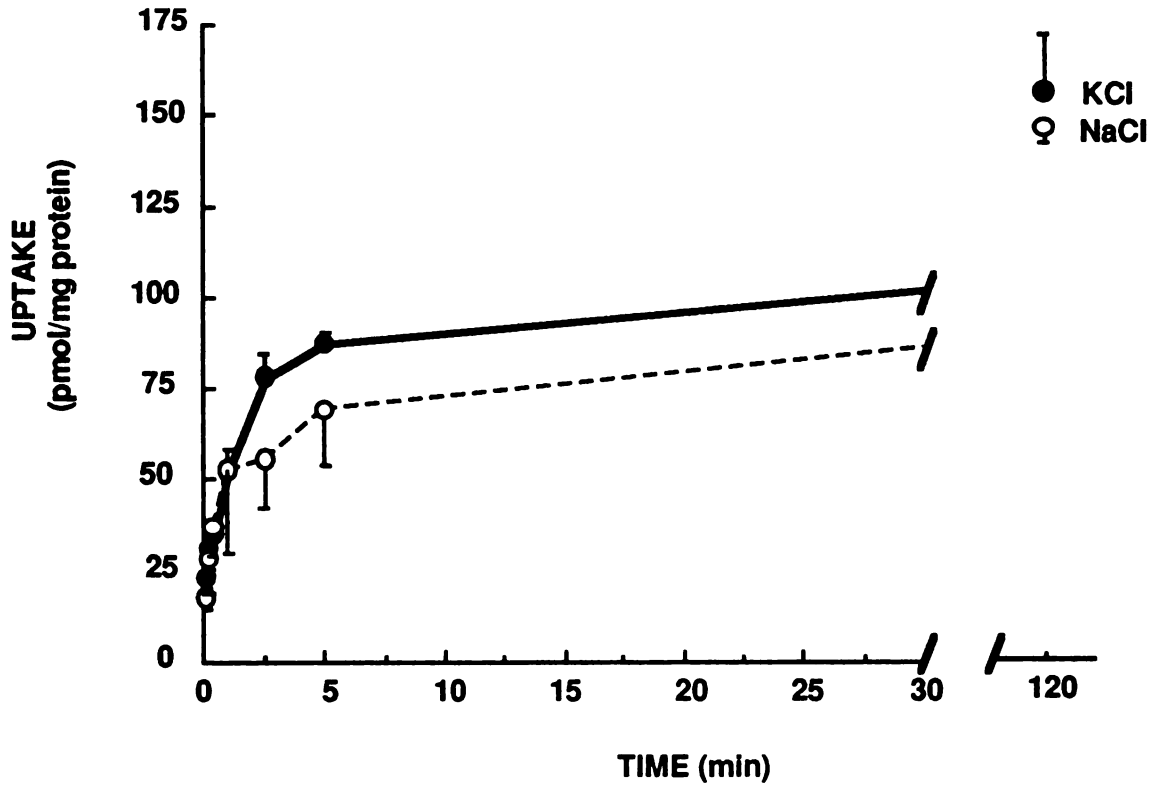


Figure 28 The effect of an inwardly-directed Na^+ gradient on the uptake of ^3H-cimetidine (50 μM) in brush-border membrane vesicles from bovine choroid plexus. The solid circles (●) and the open circles (○) represent the data when a 500 μM Na^+ gradient was present in or absent from the extravesicular medium, respectively. The data are presented as mean ± S.E. of 3 experiments.

We investigated the possibility that cimetidine uptake in these experiments could be the result of metabolism in the vesicle preparation. Data from TLC separation and analysis to determine the amount of cimetidine and its major metabolite, cimetidine-sulfoxide, present in the vesicle reaction mixture following 2 hr incubation indicate that no cimetidine-sulfoxide was formed during the reaction (data not shown).

Discussion

Suzuki et al. (1986) have shown that cimetidine accumulates in whole choroid plexus tissue isolated from the brain of the rat. The accumulation occurred against a concentration gradient and the process was saturable. Estimates of the transport parameters K_m and V_{max} were 53 μM and 12 nmol/ml/min, respectively. The organic anions p-aminohippuric acid (PAH), homovanillic acid (HIAA), salicylic acid, and benzylpenicillin, the sulfhydryl reagent p-hydroxymercuribenzoate (PHMB), the metabolic inhibitors KCN and 2,4-dinitrophenol (2,4-DNP), and the organic cations quinine and quinidine as well as some oligopeptides, glycylglycine and glycylglycylhistidine, were able to inhibit the uptake of cimetidine into the tissue. The organic cations NMN, TEA, choline, histamine, and creatinine had no inhibitory affect on cimetidine accumulation at low concentrations (1 mM). However, histamine, creatinine, glycine, and histidine significantly inhibited the process at higher concentrations (20 mM). The uptake was insensitive to an inwardly directed sodium gradient but was sensitive to changes in temperature. These findings suggest that cimetidine is transported in choroid plexus, at least in part, via carrier-mediated active transport process. It is not clear however, whether these results describe events at a single membrane or are the composite of events occurring at both the apical and basal membranes. Whether the transport occurs via a single transport system, inhibitable by a wide range of heterogeneous classes of compounds, or by several different transporters (a family of transporters) each contributing a small but significant part to the total process is also unknown.

In this study we wished to elucidate the mechanisms of cimetidine transport across the brush-border membrane of choroid plexus epithelium. We were particularly interested in learning if the mechanisms were similar to those that

had been observed for cimetidine transport in the renal brush-border membrane (McKinney and Kunnemann, 1986; Takano et al., 1985; Gisclon et al., 1987). Cimetidine transport across the brush-border membrane of the proximal tubule involves both saturable and non-saturable mechanisms and is inhibitable by both hydrophobic and hydrophilic organic cations including TEA, NMN, and quinine. In addition cimetidine transport across the renal brush-border membrane appears to be driven by a proton gradient which exists across the luminal membrane of the proximal tubule from lumen to cell (Gisclon et al., 1987). We hypothesized that its transport across the choroid plexus epithelium, which occurs in the absorptive direction (CSF to blood), was driven by a proton exchange system which may exists at the brush-border and basolateral membranes (Wright and Saito, 1987). Hence, a proton flux, directed differently across renal (passive) and choroid plexus epithelia (active), may be responsible for active cimetidine transport, and moreover, may be the determinant of the direction of transport across the two epithelia.

We addressed the question of whether a transport system for cimetidine was present at this membrane by first demonstrating that cimetidine accumulates in the vesicles with time as shown in Figures 23A and 23B. The accumulation was very rapid during the first 60 seconds and reached equilibrium within 2 hours. To determine whether the uptake was a membrane binding phenomena or represented accumulation into an intravesicular space, the vesicles were subjected to conditions which caused an increased osmotic pressure across the membrane by incrementally increasing the osmolarity of the extravesicular medium as discussed in Chapter 4. The data, shown in Figure 22 (Chapter 4) suggest that cimetidine accumulation in the vesicles is into an osmotically reactive intravesicular space. In addition, the contribution of membrane binding

of the drug to the total amount taken up by the vesicles can be estimated from the linear regression analysis. The intercept value given by extrapolation of the regression line to the ordinate represents uptake at infinitely high osmolarity, and if one assumes that the vesicles are completely shrunken, i.e., there is no intravesicular space, the value also represents the amount bound to the membrane of the vesicles. The percentage of membrane binding is estimated by relating the amount bound to the equilibrium uptake value when no osmotic gradient exists across the membrane. The value obtained in these experiments was approximately 18%. This value is consistent with values obtained in renal brush-border membrane vesicles (Gisclon et al., 1987; McKinney et al., 1987) and with the values for binding of cimetidine to plasma proteins (Somogyi and Gugler, 1983), as well as the value obtained from the experiments monitoring the uptake of cimetidine as a function of time (Figure 23B).

We investigated the concentration-dependence of the rate of cimetidine uptake in the vesicles to determine if the accumulation in choroid plexus brush-border membrane vesicles satisfies criteria for carrier-mediated transport processes (Hofer, 1981). The initial rate of uptake was linear at low cimetidine concentrations and became curvilinear at high concentrations as shown in Figure 24A. Best-fit analysis indicates that the data represents a process consisting of a saturable and a non-saturable component. The K_m for cimetidine transport was 58.1 μM. This value is an order of magnitude greater than the apparent K_m (4.6 μM) for cimetidine transport in the brush-border membrane of the proximal tubule (Gisclon et al., 1987). However, the value agrees well with the Km (53 μM) obtained by Suzuki et al.(1986) in isolated whole choroid plexus from the rat. The V_{max} of cimetidine transport (16.7 pmol/s•mg protein) obtained in this study is higher than the value obtained for

cimetidine transport in brush-border membrane vesicles prepared from rabbit renal cortex (6.8 pmol/s/mg protein) by Gisclon et al. (1987). Thus, in comparison to the renal brush-border membrane, cimetidine is transported across the brush-border membrane of choroid plexus epithelium with a lower affinity and a higher maximum rate. These differences may reflect either epithelia or species differences since the renal brush-border membranes were from rabbits and the choroid plexus brush-border membranes were from cows. In both renal and choroid plexus brush-border membrane vesicles, there was a non-saturable transport component which may reflect diffusion of the unionized species.

The effect of various inhibitors on cimetidine transport was studied. The compounds tested as potential inhibitors were the hydrophilic organic cation, TEA, the hydrophobic organic cation, quinidine, the H_2-receptor antagonist, ranitidine and the organic anions, salicylate and PAH. With respect to the organic cations, our data agree well with data obtained in isolated whole choroid plexus demonstrating that hydrophobic organic cations inhibit cimetidine transport whereas hydrophilic organic cations do not (Suzuki et al., 1986). However, both salicylate (5 mM) and PAH (5 mM) inhibited cimetidine transport in isolated whole choroid plexus from rat ventricle whereas these compounds did not significantly affect cimetidine transport in isolated brush-border membrane vesicles from bovine choroid plexus epithelium (Figure 25). These data may reflect species differences. Alternatively, one needs to consider that in whole choroid plexus, uptake may reflect transport across capillary endothelium, transport across the basolateral membrane, non-specific binding to the membrane, or metabolism. The inhibitory effect of a compound may be on any of these processes.

In brush-border membrane vesicles prepared from rabbit renal cortex cimetidine transport was inhibited by both hydrophobic and hydrophilic organic cations (Gisclon et al., 1987). However, in this study, cimetidine transport in brush-border membrane vesicles from bovine choroid plexus was inhibited only by quinidine and not by either TEA or ranitidine (5 mM). These data suggest that there may be important differences in the specificity of the transporter for cimetidine between the two epithelia.

The data from the counter-flux experiments suggest that cimetidine transport at the brush-border membrane of the choroid plexus epithelium is mediated by a mobile protein carrier in the membrane. As discussed by Hofer (1981), this phenomena can be explained as resulting from competition for the same transport site on the carrier between two different species of the same substrate (^{3}H-cimetidine and unlabeled cimetidine) that have the same binding affinity for the transporter. Initially, the concentration of ^{3}H-cimetidine is the same on both sides of the vesicle membranes (equilibrium conditions) and although unidirectional fluxes across the membrane continue, there is no net flux of ^{3}H-cimetidine. Upon addition of unlabeled cimetidine at a molar concentration more than 500-fold higher than that of ^{3}H-cimetidine, but at the same radioactive concentration so that the radioactive concentration equilibrium is not disturbed, a competition is created for the transport site on the carrier. The initial result is that the unidirectional flux of ^{3}H-cimetidine into the vesicles is impeded while its unidirectional flux out of the vesicles is not since it is the only species present inside the vesicles. This is observed as an initial rapid decrease in the amount of ^{3}H-cimetidine in the vesicles with time (Figure 26). As the concentration gradients dissipate and there is competition for the carrier transport site on both sides of the membrane, a "counter-flux minimum" is

reached at "static head", the opposing chemical potential difference that reduces the flux of ^{3}H-cimetidine to zero (Friedman, 1986), followed by a slow return to the initial equilibrium value of ^{3}H-cimetidine in the vesicles. The initial unidirectional flux of ^{3}H-cimetidine out of the vesicles constitutes transport against a concentration gradient and therefore suggests active transport. The data provide supportive evidence for the presence of a mobile protein-carrier in the membrane which is able to mediate the transport of substrate in both directions simultaneously, a property not attributable to simple pores nor to a diffusional process (Hofer, 1981). These data agree with those of Suzuki et al. (1986) from isolated choroid plexus and support their conclusion that cimetidine transport in the choroid plexus occurs via a carrier-mediated process.

An important goal of this study was to identify a driving force for cimetidine transport across the brush-border membrane of the choroid plexus. As discussed previously, since a proton antiport system appears to be the driving force for cimetidine transport in the renal brush-border membrane, we wanted to determine whether a proton antiport system may drive cimetidine transport across the choroid plexus brush-border membrane. However it is important to point out that, although an outwardly directed proton chemical gradient exists across the brush-border membrane of the choroid plexus epithelium (intracellular pH < extracellular pH), passive (downhill) flux of protons would necessarily be inward because of the membrane potential difference which causes the proton electrochemical gradient to be inwardly directed (see Chapter 1, Figure 4). Therefore, if cimetidine transport is coupled to the expulsion of protons across the brush-border membrane, one would have to postulate that the exchange mechanism is electroneutral. Our data, depicted in Figure 27, demonstrate that an outwardly directed proton gradient can

accelerate cimetidine influx. However, no "overshoot phenomenon" was observed. These data demonstrate that the outwardly directed proton gradient could not drive the transport of cimetidine against a concentration gradient under the conditions in these experiments. The data may suggest that a proton exchange system, is in fact, the driving force for cimetidine transport across the brush-border membrane, but that alternative proton shunt pathways are present in the vesicles. Protons could escape through such pathways and not be present in sufficient concentrations to mediate the uphill transport of cimetidine. Alternatively the data are consistent with pH partitioning and non-ionic diffusion. Since cimetidine is a base and our data suggest that it is transported in the brush-border membrane vesicles in part by non-ionic diffusion, such partitioning could occur. Another possibility is that the proton gradient created a favorable membrane potential difference (inside negative) which accelerated cimetidine influx. Further studies will be needed to clarify the mechanisms involved in this phenomenon and the possible role of protons in cimetidine transport.

In renal brush-border membrane vesicles cimetidine transport is driven against a concentration gradient by an outwardly directed proton gradient (Gisclon et al., 1987). The apparent overshoot phenomenon is about three times the equilibrium value. We do not know whether the differences between the data obtained in brush-border membrane vesicles from choroid plexus epithelium and renal epithelium are merely quantitative, i.e., a proton exchange drives cimetidine transport across both membranes, or the differences reflect real differences in the mechanisms of transport between the two membranes. We attempted to drive cimetidine transport with other ion gradients including Na^+ (Figure 28), HCO_3^-, and Cl^- (data not shown). None of the gradients altered cimetidine uptake. Thus, a driving force for concentrative uptake of

cimetidine in the brush-border membrane vesicles from bovine choroid plexus could not be elucidated in this study.

In summary, the results of this study suggest that a carrier-mediated process is involved in cimetidine transport across the brush-border membrane of bovine choroid plexus. The transport is inhibited by the hydrophobic organic cation, quinidine, but not by the organic cations, TEA and ranitidine. These data suggest that the specificity of the transporter for cimetidine in the brush-border membrane of choroid plexus epithelium differs from the specificity in the renal brush-border membrane in which cimetidine appears to share a common organic cation transporter. Because an ion gradient driving force was not identified, the data may indicate that (1) the driving force for cimetidine transport in the choroid plexus is at the basolateral membrane; (2) transport of cimetidine across the brush-border membrane is primarily active (directly linked to ATP hydrolysis); or (3) the proton gradient may actually drive cimetidine transport, but leak pathways prevent the occurrence of an overshoot phenomenon. Thus, some of the mechanisms of cimetidine transport in the brush-border membrane of choroid plexus epithelium have been elucidated, however, it is clear that further studies of cimetidine transport in choroid plexus epithelium are needed, particularly studies at the basolateral membrane, to obtain an overall view of the mechanisms involved in the transcellular movement of cimetidine in this tissue.

CHAPTER 6

Summary and Conclusions

Summary

Cimetidine is an H_2-receptor antagonist used principally in hypergastric acid secretion disorders such as peptic ulcer disease and Zollinger-Ellison Syndrome. Elevated cimetidine CSF concentrations as great as 2.5-fold that of normal have been observed in certain patient populations. Hypotheses have been put forth to explain the accumulation of the drug in the CSF. The leading hypothesis is that the blood-brain barrier permeability for cimetidine increases in the disease state similar to what is observed in hepatic encephalophathy. The physicochemical properties of cimetidine are such that it is unlikely that a leakiness of the blood-brain barrier would enhance CSF concentrations by 2.5-fold without concomitant impairment of the CSF elimination process given its documented rapid entry into the CNS under normal conditions (Jonsson et al., 1982, 1984). We hypothesize that a saturable and inhibitable process is involved in the elimination of cimetidine from the CSF and that in the disease state this process is impaired. Impaired CSF elimination may be a result of primary or secondary mechanisms. A primary mechanism could involve saturation of transporter sites if only a small increase in concentration is needed to saturate the system. A secondary mechanism could involve increased CSF concentrations of an endogenous substance creating competition for a transporter involved in the elimination of cimetidine from the CSF. In the first part of this dissertation studies were carried out to address the question of whether the contribution of a saturable process for the elimination of cimetidine from the CSF is significant under physiological conditions.

For the second part of this dissertation questions related to the mechanisms involved in the transport of cimetidine across the brush-border membrane of choroid plexus epithelium were addressed. Studies have shown that transport systems exist in the choroid plexus of the cerebral ventricles for a number of different classes of compounds. Evidence for a saturable transport process for cimetidine has been obtained in whole choroid plexus uptake studies by Suzuki et al. (1986). To date there have been no studies addressing questions regarding the mechanisms of epithelia transport of cimetidine in this tissue.

In the following sections a summary of the major conclusions of this dissertation is presented.

Lateral Ventricle Bolus Injection - Small Animal Model

The question of whether significant cimetidine elimination from the CSF occurs via a saturable process under physiological conditions was addressed. Conventionally the ventriculocisternal perfusion method has been employed to study the disposition of substances during passage through the ventricular system. This method has certain deficiencies related to the use of perfusion rates which are higher than the normal flow of CSF through the ventricles and the necessity to discern small concentration changes in large volumes in order to obtain clearance information. We developed a procedure in a rat model which involved administration of radiolabeled cimetidine into the CSF of the animal via a bolus injection into the lateral ventricle. CSF samples were collected from the cisterna magna at various times during the procedure and CSF cimetidine concentrations were quantitated from radioactive concentration data. The clearance of cimetidine from the CSF was determined using the model independent method which relates the area under the cimetidine CSF

concentration versus time curve (AUC) to the dose of cimetidine administered. The method was used by Levin et al. (1983,1984,1985a,1985b,1985c) to study the distribution and elimination of chemotherapeutic agents from the CSF of beagle dogs.

Three criteria were met for acceptance of the method as a model for physiological CSF cimetidine clearance determinations. First, there was a good linear relationship between the dose of cimetidine ranging between 3 and 10 ng and the AUC as determined by linear regression analysis. Second, the clearance of inulin (a compound which should be cleared from the CSF principally by bulk flow; Prockop and Schanker, 1962) from the CSF was similar to the CSF production rate. Third, the clearance of cimetidine from the CSF obtained in this study was compared with that calculated from the data obtained in the rat by Suzuki et al. (1985) using a ventriculocisternal perfusion method. At low doses of cimetidine we obtained a mean cimetidine clearance which was in reasonable agreement with that calculated from the data of Suzuki et al. (1985). The different methodologies employed may account for the differences in CSF clearance of cimetidine between the two studies. When one takes into account that in the study of Suzuki et al. (1985) extraction ratios were determined at steady-state, a situation in which distribution into the interstitial fluid space is not expected to significantly contribute to the overall clearance value but may have been of major significance in the present study, and the criticisms of the perfusion method discussed previously, the difference in the two values is acceptable.

The technique of bolus injection into the lateral ventricle and sampling from the cisterna magna in the rat and then determining the CSF clearance by the

model independent method established in this study provides a physiological model for studying the elimination of drugs from the CSF in small animals.

Cimetidine Clearance from the CSF

Cimetidine concentrations in the CSF may be elevated in patients with hepatic dysfunction due to an alteration in the elimination of cimetidine from the CSF. Pathways available for the elimination of substances from the CSF are: (1) with the bulk flow of CSF through the arachnoid villi and granulations in its return to the systemic circulation; (2) by simple diffusional processes across membranes; (3) via facilitated or active transport systems located in the choroid plexus; and (4) by biotransformation.

In this dissertation we examined the involvement of these pathways in the elimination of cimetidine from the CSF. Cimetidine and inulin clearances were determined in each animal and compared within animals to assess the contribution of bulk flow to the overall clearance process. In each animal the clearance of cimetidine was significantly greater than that of inulin suggesting that cimetidine is cleared from the CSF via a pathway(s) in addition to the bulk flow pathway.

Upon administration of a high dose of cimetidine the clearance of cimetidine, but not of inulin, was significantly decreased (32%), suggesting that cimetidine is eliminated from the CSF by a saturable pathway or pathways. These results are consistent with those reported by Suzuki et al. (1985). Interestingly, manipulation of the data produced very similar intrinsic clearance values.

At high doses of cimetidine the clearance of cimetidine was still 4-fold greater than that of inulin, suggesting that unsaturable pathways other than the

bulk flow pathway exist for the elimination of cimetidine from the CSF. In this study a concentration gradient was always present from the CSF across other compartment membranes. Distribution of the drug from CSF into the interstitial fluid compartment of the brain or diffusion across the membranes of the cerebral vasculature may have occurred. A distribution phenomena could also explain the difference between the clearance value obtained in this study and that calculated from the data of Suzuki et al. (1985).

Results of TLC experiments designed to address questions regarding biotransformation of cimetidine in the CSF were in agreement with the findings of Ziemniak et al. (1984) in dogs suggesting that there is no significant conversion to the major metabolite, cimetidine-sulfoxide, in the CSF of the rat.

Thus, cimetidine appears to be eliminated from the CSF of the rat by pathways in addition to the bulk flow of CSF through the arachnoid villi. At least one saturable transport process appears to be involved, and in this study there was evidence that diffusion from the CSF may occur. It is now possible to use this model to address questions regarding the contribution of these CSF elimination processes to the accumulation of cimetidine in the CSF in liver disease.

Separation and Isolation of Bovine Choroid Plexus Brush-Border Membrane Vesicles

The demonstration of a saturable process for the elimination of cimetidine from the CSF has given impetus to studies to determine the location of the process and the mechanisms involved. The choroid plexus located in the cerebral ventricles have been demonstrated to possess transport systems for a number of endogenous and exogenous substances. Suzuki et al. (1986a)

demonstrated the involvement of a saturable and inhibitable process in the uptake of cimetidine into whole choroid plexus of the rat. To date no studies have been reported on the mechanisms involved in the process.

Methods for the separation and isolation of the individual membranes of a cell have been employed to characterize mechanisms of membrane transport and transcellular movement of a number of substances including cimetidine in the proximal tubule of the kidney as well as in the small intestine. Only two studies employing this technique to examine membrane transport mechanisms in the choroid plexus have been reported, both of which examined the transport of neutral amino acids at the brush-border membrane. In this dissertation we established a technique for the separation and isolation of brush-border membrane vesicles from the choroid plexus of the cow using a modification of the method described by Booth and Kenny (1974) as modified by Ross and Wright (1984). The preparation exhibited very similar enhancement of membrane marker enzymes to that reported by Ross and Wright (1984). This group also reported that an L-proline transport system which is sodium-sensitive and concentrative ("overshoot" phenomena) is present in the vesicles. We were able to demonstrate such a system in the vesicles prepared in this study. The question of vesicle integrity was addressed by demonstrating that cimetidine is taken up into an intravesicular space which is osmotically reactive and that binding of the drug to the membrane is similar to that observed in renal brush-border membrane vesicles (Gisclon et al., 1987) as well as to binding to plasma proteins (Somogyi and Gugler, 1983). Studies show that the majority of the vesicles are orientated rightside-out.

Although questions of membrane purity need to be further addressed this preparation provides vesicles with the similar characteristics to those reported

by others. The preparation is suitable for conducting membrane transport studies in the choroid plexus of the cow.

Cimetidine Transport in Bovine Choroid Plexus Brush-Border Membrane Vesicles

To address questions regarding the mechanisms of transcellular movement of cimetidine in the choroid plexus we studied the uptake of cimetidine in brush-border membrane vesicles prepared from bovine choroid plexus. Specifically we wished to determine whether the uptake of cimetidine in the vesicles satisfied criteria for carrier mediated transport; i.e., is the transport process saturable, inhibitable, and can it mediate the transport of substrate in both directions simultaneously? The saturable properties of cimetidine transport in the vesicles were characterized by observing the effect of the concentration of cimetidine on the initial rate of cimetidine uptake. Computer analysis and fit of the data to mathematical models for saturable transport processes indicate that cimetidine transport in brush-border membrane vesicles involves a process with a saturable and a nonsaturable component and that the values for the transport parameters K_m, V_{max}, and K_{ns} are 58.1 μM, 16.7 pmol/s/mg protein, and 0.023 μl/s/mg protein respectively. The K_m value in this study is an order of magnitude greater than that reported by Gisclon et al. (1987) in vesicles from rabbit renal brush-border membrane vesicles but very similar to that reported by Suzuki et al. (1986a) in rat choroid plexus whole tissue uptake studies. The V_{max} of cimetidine transport in this study was more than 2-fold greater than that reported by Gisclon et al. (1987) in renal brush-border membrane vesicles from the rabbit. Thus it appears that in comparison to the renal brush-border membrane cimetidine is transported with a lower affinity and at a higher maximum rate, though these differences may reflect differences in species.

We hypothesized that cimetidine transport in the choroid plexus brush-border membrane may involve an organic cation transport system analogous to that described in renal brush-border membrane vesicles (Gisclon et al., 1987). Accordingly, known inhibitors of organic cation transport systems in renal proximal tubule were employed to address questions regarding cimetidine transport inhibition. Only the hydrophobic organic cation, quinidine, was able to significantly inhibit the uptake of cimetidine. The hydrophilic organic cations TEA and ranitidine were not effective inhibitors. These results agree with those of Suzuki et al. (1986a) in whole choroid plexus uptake studies. However, this group also reported significant inhibition of cimetidine uptake by the organic anions, PAH and salicylate, an effect not observed in this study. These differences in inhibition of cimetidine transport may reflect differences between transport studies carried out in whole tissue and those carried out in isolated membrane vesicles, since results in whole tissue uptake studies represent uptake into capillaries and connective tissue, as well as uptake at both surfaces of the epithelium.

The involvement of a mobile membrane protein in the mediation of cimetidine transport in brush-border membrane vesicles was investigated in counter-flux experiments. Following the attainment of equilibrium, addition of a high concentration of unlabeled cimetidine to the extravesicular medium created a competition for the carrier causing net flux of radiolabeled cimetidine out of the vesicles. A counter-flux minimum was obtained followed by a gradual return to the equilibrium value. These results are evidence for the participation of a mobile membrane protein in the mediation of cimetidine transport.

We attempted to identify a driving force for the transport of cimetidine across the brush-border membrane of choroid plexus epithelium. In renal epithelium a proton gradient exist from lumen to cell and an inwardly-directed proton gradient drives cimetidine transport in renal brush-border membrane vesicles. We hypothesized that, since in the choroid plexus epithelium protons may be expelled from cell to ventricle, an outwardly-directed proton gradient would drive cimetidine transport in choroid plexus brush-border membrane vesicles. In these studies an outwardly-directed proton gradient accelerated cimetidine influx but no "overshoot phenomenon" was observed. These results suggest that cimetidine may be driven into the vesicles by a proton gradient but that the gradient across the membrane achieved in this study was not sufficient for accumulation against a concentration gradient. Alternatively, the data may reflect real differences between the mechanisms of cimetidine transport in choroid plexus and renal epithelia. Attempts to drive cimetidine with other ions including Na^+, HCO_3^-, and Cl^- were also unsuccessful. The inability to elucidate a driving force for cimetidine transport in brush-border membrane vesicles in this study may indicate that (1) the driving force for cimetidine transport in the choroid plexus is at the basolateral membrane; (2) transport of cimetidine across the brush-border membrane is primarily active (directly linked to ATP hydrolysis); or (3) proton exchange may actually drive cimetidine transport, but leak pathways rapidly dissipate the proton gradient in these experiments and prevented the occurrence of an "overshoot phenomenon".

Thus cimetidine transport in the brush-border membrane of the choroid plexus appears to be very different from that in the renal brush-border membrane. Notable differences include the following: (1) The vectorial direction of cimetidine transport in the choroid plexus is towards the blood

compartment, whereas, in the kidney, cimetidine is secreted from the blood into the urine. (2) The choroid plexus cimetidine transporter displays kinetics of a system with a lower affinity and a higher capacity than in the kidney. (3) The systems involved in cimetidine transport in the two tissues appear to have different substrate selectivities. In particular, there is evidence that cimetidine transport at the renal brush-border membrane is mediated by a general organic cation transport system whereas there is no evidence for the involvement of a general organic cation transport system at the brush-border membrane of the choroid plexus. (4) The proton-organic cation exchange transport system observed in the renal brush-border membrane could not be demonstrated in the choroid plexus brush-border membrane. These differences in transport may reflect either the morphological differences between the two tissues, species differences, or entirely different transport processes.

The data presented in this dissertation suggest that cimetidine is transported across the brush-border membrane of the bovine choroid plexus epithelia via a saturable, carrier-mediated process which is insensitive to inwardly-directed gradients of Na^+, HCO_3^-, and Cl^-. The selectivity of the transporter could not be established; neither a hydrophilic organic cation nor an organic anion transporter appears to be involved. Data from the studies of Suzuki and coworkers (1986a) in isolated whole choroid plexus from the rat suggest that cimetidine is transported into choroid plexus tissue by a concentrative, energy-dependent, sodium insensitive process which appears to be in common with the transport system for organic anions and oligopeptides but not with organic cations. The differences in the results obtained in these two studies may be attributable to differences in species or experimental methods.

Models for Cimetidine Transport in the Choroid Plexus Epithelium

Results from both studies indicate that the transport of cimetidine is via saturable, carrier-mediated process which is sodium-insensitive and not exclusive to organic cations. Possible driving forces for cimetidine transport at the brush-border membrane include (1) a metabolic energy source other than Na^+/K^+ATPase and (2) a proton exchange transport system. Specific information has not been obtained about the mechanisms of cimetidine transport at the basolateral membrane. A model for the transport of cimetidine across the choroid plexus epithelium can be proposed from this evidence and from the models proposed by Wright and Saito (1987) and by Johanson (1984) (see Chapter 1).

Model 1 for the transport of cimetidine (Figure 29) is based on the results of studies in this dissertation and the model for ion fluxes proposed by Wright and Saito (1987). In accordance with the results from transport inhibition experiments, cimetidine crosses the brush-border membrane via a facilitated transport process down its electrochemical gradient. Presumably the transport across the basolateral membrane is active since flux from the cell is against the electrochemical gradient. Therefore, cimetidine transport from cell to blood could occur either as cotransport via HCO_3^- electrodiffusion, Na-cimetidine exchange, or via some other, more complex, energy transducing system.

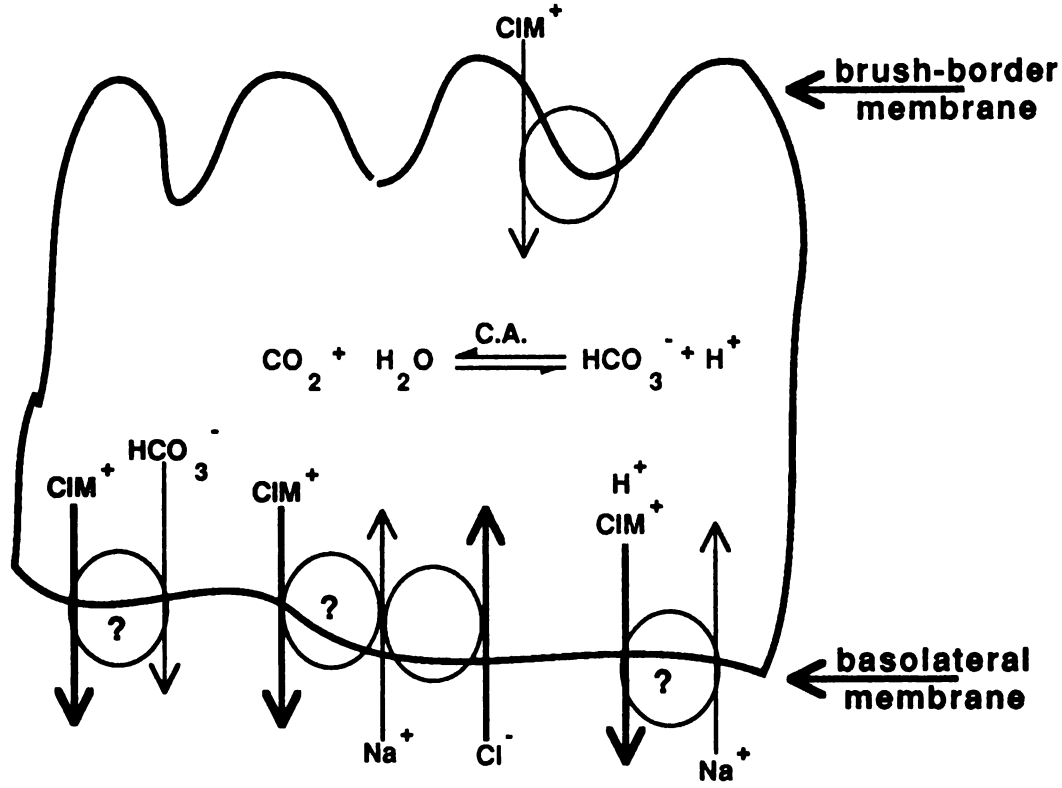


Figure 29 Model 1. Cimetidine transport across the choroid plexus cell (after Wright and Saito, 1987). cim^+ = cimetidine; C.A. = carbonic anhydrase; heavy arrows and light arrows, respectively, indicate transport against and with an electrochemical gradient.

Model 2 (Figure 30) is based on the experimental results of this dissertation and the model proposed for ion fluxes across the choroid plexus epithelia cell of the rat from the studies of Johanson (1984). Since in our studies, an outwardly directed proton gradient was able to accelerate the uptake of cimetidine, a proton exchange system may drive the transport of cimetidine at the brush-border membrane. The studies of Johanson (1984) concluded that Cl^- transport across the cell from blood to CSF is mediated by a Cl^--HCO_3^- exchange transporter in both membranes and the expulsion of H^+ from the cell is mediated by a Na^+-H^+ exchange transporter at the basal membrane. HCO_3^- and H^+ are generated intracellularly by the carbonic anhydrase catalyzed

hydration of CO_2 and by dissociation of H_2CO_3 to HCO_3^- and H^+. We propose that the expulsion of H^+ from the cell could be augmented by electroneutral H^+ exchange for cimetidine which could offset the electrical potential gradient for protons. Cimetidine could be transported from cell to blood either by faciliated diffusion or coupled to the flux of ions across the membrane as discussed for model 1.

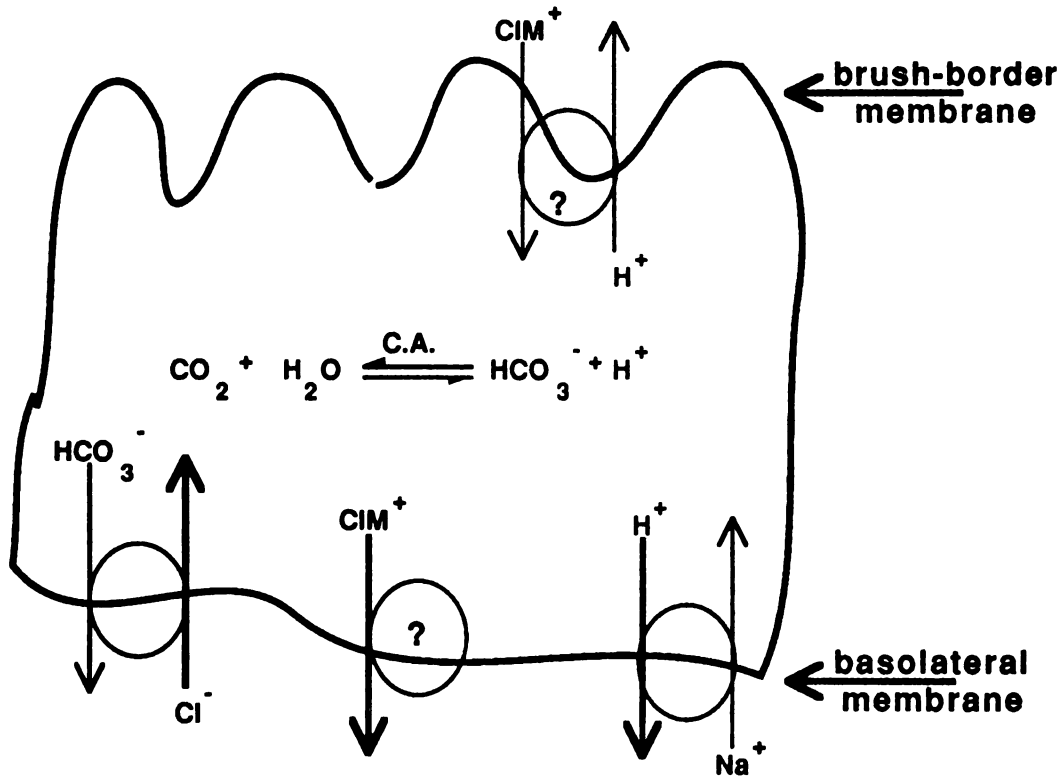


Figure 30 Model 2. Cimetidine transport across the choroid plexus cell (after Johanson, 1984). cim^+ = cimetidine; C.A. = carbonic anhydrase; heavy arrows and light arrows, respectively,indicate transport against and with an electrochemical gradient.

Conclusions

In conclusion, these studies provide further insight into the mechanisms of elimination of cimetidine from the CSF in experimental animals with implications to human subjects. The principle mechanisms involved are both passive (bulk flow and diffusion) and facilitated (saturable processes in the choroid plexus). Additionally, these studies establish a model for studying drug elimination from the CSF in small animals as well as a basic procedure for preparing brush-border membrane vesicles from bovine choroid plexus. Both of these tools have implications for studies of drug transport from the CSF. Future studies employing the methods developed and the conclusion drawn from these studies may address the possible influences of disease states on these systems.

References

Adler, A.E., Sadja, L., Wilets, G. Cimetidine toxicity manifested as paranoia and hallucinations. Am. J. Psychiatry **137**: 1112-1113, 1980.

Agarwal, S.K. Cimetidine and visual hallucinations. JAMA **240**: 214, 1978

Aquilonius, S.-M. and Winbladh, B. Cerbrospinal fluid clearance of choline and some other amines. Acta Physiol. Scand. **85**: 78-90, 1972.

Arneson, G.A. More on toxic psychosis with cimetidine. Am. J. Psychiatry **136**: 1348-1349, 1979.

Aronson, N.N.,Jr. and Touster, O. Isolation of rat liver plasma membrane fragments in isotonic sucrose: in Methods in Enzymology; Biomembranes, S. Fleischer and L. Packer (eds.), vol. XXXI, Part A. Academic Press, New York and San Francisco, 1974.

Aronson, P. Identifying secondary active solute transport in epithelia. Am. J. Physiol. **240**: F1-F11, 1981.

Bacigalupo, A., Van Lint, M.T., and Marmont, A.M. Cimetidine-induced coma. Lancet **2**: 46-46, 1978.

Barnhart, C.C, and Bowden, C.L. Toxic psychosis with cimetidine. Am. J. Psychiatry **136**:725-726, 1979.

Bavin, P. M. G., Post, A., and Zarembo, J. E. Cimetidine.: in Analytical Profiles of Drug Substances K. Florey (ed.), vol. 13, Academic Press, New York, San Francisco, and London, 1984, pp. 127-182.

Biber, J., Stieger, B., Hase, W., and Murer, H.. A high yield preparation for rat kidney brush border membranes: different behavior of lysosomal markers. Biochim. Biophys. Acta **647**: 169-176, 1981.

Bouldin, T.W. and Krigman, M.R. Different permeability of cerebral capillary and choroid plexus to lanthanum ion. Brain Res. **99**: 444-448, 1975.

Booth, A.G., and Kenny, A.J. A rapid method for the preparation of microvilli from rabbit kidney. Biochem. J. **142**: 575-581, 1974.

Bouman, H. J., and van Wimersma Greidanus, T. B. A rapid and simple cannulation technique for repeated sampling of cerebrospinal fluid in freely moving rats. Brain Res. Bull. **4**:575-577, 1979.

Bradbury, M. W.B. The blood-brain barrier: Transport across the cerebral endothelium. Circulation Res. **57**: 213-222, 1985.

Brightman, M.W. Ultrastructural characteristics of adult choroid plexus: Relation to the blood-cerebrosponal fluid barrier to proteins: in The Choroid Plexus in Health and Disease, University Press of Virginia, Charlottesville, 1975, p. 86-112.

Brightman, M. W. and Reese, T.S. Junctions between intimately apposed cell membranes in the vertebrate brain. J. Cell Biol. **40**: 649-677, 1969.

Britton, M.L. and Waller, E.S. Central nervous system toxicity associated with concurrent use of triazolam and cimetidine. Drug Intell. Clin. Pharm. **19**: 666-668, 1985.

Cacini, W., Keller, M.B., and Grund, V.R. Accumulation of cimetidine by kidney cortex slices. J. Pharmacol. Exp. Ther. **221**: 342-346, 1982.

Claude, P. and Goodenough, D.A. Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. J. Cell Biol. **58**: 390-400, 1973.

Crowder, M.K. and Pate, J.K. A case report of cimetidine-induced depressive syndrome. Am. J. Psychiatry **137**: 1451, 1980.

Cserr, H. F. Physiology of the choroid plexus. Physiol. Rev. **51**: 273-311, 1971.

Cserr, H.F. Physiology of the choroid plexus: in The Choroid Plexus in Health and Disease, University Press of Virginia, Charlottesville, 1975, p. 175-193.

Cserr, H.F. and Bundgaard, M. Blood-brain interfaces in vertebrates: A comparative approach. Am. J. Physiol. 246: R277-R288, 1984.

Cumming, W.J.K., and Foster, J.B. Cimetidine-induced brainstem dysfunction. Lancet **2**: 1096, 1978.

Deane, R. and Segal, M.B. The transport of sugars across the perfused choroid plexus of the sheep. J. Physiol. **362**: 245-260, 1984.

Debas, H.R. and Mulholland, M.W. New horizons in the pharmacologic management of peptic ulceration. Am. J. Surg. **151**: 422-430, 1986.

Delaney, J.C. and Ravey, M. Cimetidine and mental confusion. Lancet **2**: 512, 1977.

Dohrmann, G. J. The choroid plexus: A historical review. Brain Res. **18**: 197-218, 1970.

Edmonds, M.E., Ashford, R.F.U., Brenner, A. Cimetidine: Does neurotoxicity occur? Report of three cases. J. Royal Soc. Med. **72**: 172-175, 1979.

Ehrlich, B.E. and Wright, E.M. Choline and PAH transport across blood-CSF barriers: The effect of lithium. Brain Res. **250**: 245-249, 1982.

Eriksson,K.-H. and Winbladh, B. Choroid plexus uptake of atropine and methylatropine in vitro. Acta Physiol. Scand. 83: 300-308, 1971.

Ernst, S.A., Palacios, J.R.,II, and Siegel, G.J. Immunocytochemical localization of Na^+,K^+-ATPase catalytic polypeptide in mouse choroid plexus. J. Histochem. Cytochem. **34**: 189-195, 1986.

Feldberg, W., and Sherwood, S.L. A permanent cannula for intraventricular injection in cats. J. Physiol. **120**: 3P-4P, 1953.

Feldberg, W. and Sherwood, S.L. Injection of drugs into the lateral ventricle of the cat. J. Physiol., London **123**: 148-154, 1954.

Feldberg, W., and Sherwood, S.L. Effects of calcium and potassium injected into the cerebral ventricles of the cat. J. Physiol., London **139**: 408-418, 1957.

Feldberg, W., and Malcolm, J.L. Experiments on the site of action of tubocurarine when applied via the cerebral ventricles. J. Physiol., London **149**: 58-67, 1959.

Gisclon, L., Wong, F.M., and Giacomini, K.M. Cimetidine transport in isolated luminal membrane vesicles from rabbit kidney. Am. J. Physiol. **253:** F141-F150, 1987.

Goldberg, M., Spector, R., and Chiang, C.-k. Transport of diphenhydramine in the central nervous system. J. Pharmacol. Exp. Ther. 240: 717-722, 1987.

Grave,W., Nadorp, J.H.S.M., and Rutten, J.J.M.H. Cimetidine and renal failure. Lancet **2**: 719-720, 1977.

Grimson, T.A. Reactions to cimetidine. Lancet **1**: 858, 1977.

Hart, B.L. and Hoebel, B.G. Stereotaxic surgery. in: Experimental Psychobiology A Laboratory Manual. B.L. Hart (ed.) W.H. Freeman and Co. San Francisco, 1969.

Heisey, S. R., Held, D., and Pappenheimer, J. R. Bulk flow and diffusion in the cerebrospinal fluid system of the goat. Am. J. Physiol. **203**: 775-781, 1962.

Hofer, M.: Transport Across Biological Membranes, Pitman Publishing Inc., Marshfield, Massachesetts, 1981, pp 26-31.

Hug, C.C. Transport of narcotic analgesics by choroid plexus and kidney tissue in vitro. Biochem. Pharmacol. **16**: 345-359, 1967.

Jefferson, J.W. Central nervous system toxicity of cimetidine: A case of depression. Am. J. Psychiatry **136**: 346, 1979.

Johanson, C.E., Reed, D.J., and Woodbury, D.M. Active transport of sodium and potassium by the choroid plexus of the rat. J. Physiol. **241**: 359-372, 1974.

Johanson, C.E. Differential effects of acetazolamide, benzolamide and systemic acidosis on hydrogen and bicarbonate gradients across the apical membranes of the choroid plexus. J. Pharmacol. Exp. Ther. **231**: 502-511, 1984.

Johanson, C.E., Parandoosh, Z., and Smith, Q.R. Cl-HCO_3 exchange in choroid plexus: analysis by the DMO method for cell pH. Am. J. Physiol. **249**: F478-F484, 1985.

Johnson, J. and Bailey, S. Cimetidine and psychiatric complications. Br. J. Psychiatry **134**: 315-316, 1979.

Jonsson, K-A., Eriksson, S-E., Kagevi, I., Norlander, B., Bodemar, G. and Walan, A. Cimetidine, but not oxmetidine, penetrates into the cerebrospinal fluid after a single intravenous dose. Br. J. Clin. Pharmacol. **14**: 815-819, 1982.

Jonsson, K-A., Eriksson, S-E., Kagevi, I., Norlander, B., Bodemar, G. and Walan, A. No detectable concentrations of oxmetidine but measurable concentrations of cimetidine in cerebrospinal fluid (CSF) during multiple dose treatment. Br. J. Clin. Pharmacol. **17**: 781-782, 1984.

Kimelblatt, M.J., Cerra, F.B., Calleri, G., Berg, M.J., McMillen, M.A., and Schentag, J.J. Dose and serum concentration relationships in cimetidine-associated mental confusion. Gastroenterology **78**: 791-795, 1980, .

Kinnel, H.G. and Webb, A. Confusion associated with cimetidine. Br. Med. J. **2**: 1438, 1979.

Kinsella, J.L., Holohan, P.D., Pessah, N.I., and Ross. C.R. Isolation of luminal and antiluminal membranes from dog kidney cortex. Biochim. Biophys. Acta **522**: 468-477, 1979.

Langenbeck, U., and Kinne, R. Enrichment and preliminary characterization of a plasma membrane fraction from hog choroid plexus. Geminsame Herbstagung **361**: 311, 1980.

Leusen, I. Influences des modifications simultanees de la concentration en ions K en Ca du liquide cephalorachidien sur le system vasomoteur. Experientia **4**: 154, 1948.

Leusen, I. The influence of Calcium, potassium and magnesium ions in cerebrospinal fluid on vasomotor system. J. Physiol., London. **110**: 319-329, 1950.

Levin, V. A., Csejtey, J., Byrd, D. J. Brain, CSF, and tumor pharmacokinetics of α-difluoromethylornithine in rats and dogs. Cancer Chemother. Pharmacol. **10**:196-199, 1983.

Levin, V. A., Byrd, D., Campbell, J., Davis, R. L., and Borcich, J. K. CNS toxicity and CSF pharmacokinetics of intraventricular DFMO and MGBG in beagle dogs. Cancer Chemother. Pharmacol. **13**: 200-205, 1984.

Levin, V. A., Byrd, D., Campbell, J., Giannini, D. D., Borcich, J. K., and Davis, R. L. Central nervous system toxicity and cerebrospinal fluid pharmacokinetics of intraventricular 3-[(4-amino-2-methyl-5-pyrimidinyl)ethyl]-1-(2-chloroethyl)-1-nitrosourea and other nitrosoureas in beagles. Cancer Res. **45**: 3803-3809, 1985a.

Levin, V. A., Byrd, D., Sikic, B. I., Etiz, B. B., Campbell, J., Borcich, J. K., and Davis, R. L. Central nervous system toxicity and cerebrospinal fluid pharmacokinetics of intraventricularly administered bleomycin in beagles. Cancer Res. **45**: 3810-3815, 1985b.

Levin, V. A. and Landahl, H. D. Pharmacokinetic approaches to drug distribution in the cerebrospinal fluid based on ventricular administration in beagle dogs. J.Pharmacokinet. Biopharm. **13**: 387-403, 1985c.

Levine, M.L. Cimetidine-induced coma in cirrhosis of the liver. Lancet **2**: 45-46, 1978.

Lindvall, M., Owman, Ch. and Winbladh, B. Sympathetic influence on transport function in the choroid plexus of rabbit and rat. Brain Res. **223**: 460-464, 1981.

Lindvall, M. and Owman, Ch. Early development of noradrenaline-containing sympathetic nerves in the choroid plexus system of the rabbit. Cell Tiss. Res. **192**: 195-203, 1978.

Lindvall-Axelsson, M., Owman, Ch. and Winbladh, B. Early postnatal development of transport functions in the rabbit choroid plexus. J. Cerebral Blood Flow and Metab. **5**: 560-565 , 1985.

Linhardt, K. and Walter, K. : in Methods of Enzymatic Analysis, H.-U. Bergmeyer (ed.), Academic Press, New York and London 1965, p.783

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265-275, 1951.

Mamelok, R.D., Tse, S.S., Newcomb, K., Bildstein, C.L., and Liu, D. Basal-lateral membranes from rabbit renal cortex prepared on a large scale in a zonal rotor. Biochim. Biophys. Acta **692**: 115-125, 1982.

Masuzawa, T. and Sato, F. The enzyme histochemistry of the choroid plexus. Brain **106**: 55-99, 1983.

Menzies-Gow, N. and Nelson, P.H. Cimetidine and mental confusion. Lancet **2**: 512, 1977.

McGuigan, M.E. A consideration of the adverse effects of cimetidine. Gastroenterology **80**: 181-192, 1981.

McKinney, T.D., Myers, P., and Speeg, K.V., Jr. Cimetidine secretion by rabbit renal tubules in vitro. Am. J. Physiol. **241**: F69-F76, 1981.

McKinney, T.D. and Speeg, K.V., Jr. Cimetidine and procainamide secretion by proximal tubules in vitro. Am. J. Physiol. **242**: F672-F680, 1982.

McKinney, T.D., and Kunnemann, M.E. Cimetidine uptake by rabbit renal cortical brush border membrane vesicles (Abstract). Kidney Int. **29**: 420, 1986.

McKinney, T.D. and Kunnemann, M.E. Cimetidine transport in rabbit renal cortical brush-border membrane vesicles. Am. J. Physiol. **252**: F525-F535, 1987.

McMillian, M.A., Amclis, D., and Siegel, J.H. Cimetidine and mental confusion. N. Engl. J. Med. **298**: 284-285, 1978.

Miller, T.B. and Ross, C.R. Transport of organic cations and anions by choroid plexus. J. Pharmacol. Exp. Ther. **196**: 771-777, 1976.

Mogelnicki, S.R., Walter, J.L., and Finlayson, D.C. Physostigmine reversal of cimetidine-induced mental confusion. JAMA **241**: 826-827, 1979.

Mollgard, K. Lauritzen, B., and Saunders, N.R. Double replica technique applied to choroid plexus from early foetal sheep: completeness and complexity of tight junctions. Neurocytology. **8**: 139-149, 1979.

Moss, D.W. Acid phosphatases. : in Methods of Enzymatic Analysis. Third edition. J. Bergmeyer and M. Graβl (eds.) vol. IV. Enzymes 2, Verlag Chemie, Weinheim, Deerfield Beach, Florida, and Basel, 1984.

Motti, E.D.F., Imhof, H.-G., Janzer, R.C., Marquardt, K., and Yasargil, G.M. The capillary bed in the choroid plexus of the lateral ventricles: A study of luminal casts. Scanning Electron Microsc. **IV**: 1501-1513, 1986.

Myers, R.D. : in Methods in Psychobiology, R.D. Myers (ed.), Academic Press, New York, 1972, pp 169-221.

Netsky, M.G. and Shuangshoti, S. Normal structure of choroid plexus: in The Choroid Plexus in Health and Disease, University Press of Virginia, Charlottesville, 1975, p. 151-160.

Nolte, J. : in The Human Brain: An Introduction To Its Functional Anatomy, The C.V. Mosby Company, St. Louis, 1981.

Pappenheimer, J.R., Heisey, S.R., Jordan, E. F., and Downer, deC. Perfusion of the cerebral ventricular system in unanesthetized goats. Am. J. Physiol. **203**: 763-774, 1962.

Pomare, E.W. Hyperosmolar non-ketonic diabetes and cimetidine. Lancet **1**: 1202, 1978.

Pellegrino, L.J., Pellegrino, A.S., and Cushman, A.J. : in A Stereotaxic Atlas of the Rat Brain, Plenum Press, New York, 1979.

Prockop, L.D. and Schanker, L.S. On the mode of exit of substances from cerebrospinal fluid. Life Sci. **4**: 141-149, 1962.

Quinton, P.M., Wright, E.M., and Tormey, J.M^{c}D. Localization of sodium pumps in the choroid plexus epithelium. J. Cell Biol. **58**: 724-730, 1973.

Rapoport, S.I. Blood-Brain Barrier :in Physiology and Medicine. Racin Press, New York, 1976.

Rapoport, S.I., Ohno, K., and Pettigrew, K.D. Drug entry into the brain. Brain Res. **172**: 354-359, 1979.

Rennick, B.R. Renal transport of organic cations. Am. J. Physiol. **24**: F83-F89, 1981.

Rennick, B., Ziemniak, J., Smith, I., Taylor, M., and Acara, M. Tubular transport and metabolism of cimetidine in chicken kidneys. J. Pharmacol. Exp. Ther. **228**: 387-392, 1984.

Robinson, T.J. and Mulligan, T.O. Cimetidine and mental confusion. Lancet **2**: 719-720, 1977.

Ross, H.J. and Wright, E.M. Neutral amino acid transport by plasma membrane vesicles of the rabbit choroid plexus. Brain Res. **295:** 155-160, 1984.

Rosse, R.B. Cimetidine and mental status changes. J. Clin. Psychiatry **47**: 99-100, 1986.

Russell, W.L. and Lopez, L.M. Cimetidine-induced mental status changes: Case report and literature review. Am. J. Hosp. Pharm. **37**: 1667-1671, 1980.

Sachs, G., Jackson, R.J., and Rabon, E.C. Use of plasma membrane vesicles. Am. J. Physiol. **238**: G151-G164, 1980.

Sarna, G., Hutson, P. H., and Curzon, G. A technique for repeated sampling of cerebrospinal fluid in freely moving rats and its uses. J. Physiol., Paris **79**: 536-537, 1984.

Saito, Y. and Wright, E.M. Kinetics of the sodium pump in the frog choroid plexus. J. Physiol. **328**: 229-243, 1982.

Saito, Y. and Wright, E.M. Bicarbonate transport across the frog choroid plexus and its control by cyclic nucleotides. J. Physiol. **336**: 635-648, 1983.

Saito, Y. and Wright, E.M. Cl potential profile across choroid plexus epithelium. Fed. Proc. **44**: 1746, 1985.

Saito, Y. and Wright, E.M. Regulation of intracellular chloride in bullfrog choroid plexus. Brain Res. **417**: 267-272, 1987.

Schanker, L.S., Prockop, L. D., Schou, J., and Sisodia, P. Rapid efflux of some ammonium compounds from cerebrospinal fluid. Life Sci. **10**: 515-521, 1962.

Schentag, J.J., Cerra, F.B., Calleri, G., DeFlopper, E,. Rose, J.Q. and Bernhard, H. Pharmacokinetic and clinical studies in patients with cimetidine-associated mental confusion. Lancet **1:** 177-181, 1979.

Schentag, J.J., Cerra, F.B., Calleri, G.M., Leising, M.E., French, M.A. and Bernhard, H. Age, disease, and cimetidine disposition in healthy subjects and chronically ill patients. Clin. Pharmacol. Ther. **29:** 737-743,1981.

Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J., and Crane, R.K. Purification of the human intestinal brush-border membrane. Biochim. Biophys. Acta **323**: 98-112, 1973.

Schoner, W., Ilberg, C.V., Kramer, R., and Seubert, W. On the mechanism of Na^+ - and K^+-stimulated hydrolysis of adenosine triphosphate. I. Purification and properties of Na^+- and K^+-activated ATPase from ox brain. Eur. J. Biochem. **1:** 334-343, 1967.

Sewing, K., and Kaplowitz, N. Drugs affecting the pharmacokinetics of ^{14}C-cimetidine in rats (Abstract). Gastroenterology **76**: 1242, 1979.

Skinner, James E. Stereotaxic surgery and histological examination of the brain: in Neuroscience: A laboratory manual. R.D. Singer (ed.). W.B. Saunders Company. Philadelphia, London, and Toronto, 1971.

Smith, Q.R. and Johanson, C.E. Effect of ouabain and potassium on ion concentrations in the choroidal epithelium. Am. J. Physiol. **238**: F399-F406, 1980.

Smith, Q.R. and Johanson, C.E. Active transport of chloride by lateral ventricle choroid plexus of the rat. Am. J. Physiol. **249**: F740-F477, 1985.

Somogyi, A., Gugler, R. Clinical pharmacokinetics of cimetidine. Clin. Pharmacokinet. **8**: 463-495, 1983.

Somogyi, A., McLean, A., and Heinzow, B. Cimetidine-procainamide pharmacokinetic interaction in man: evidence of competition for tubular secretion of basic drugs. Eur. J. Clin. Pharmacol. **25**: 339-345, 1983.

Spector, R. Transport of lignocaine by rabbit choroid plexus in virto. Clin. Sci. **58**: 107-109, 1980.

Spector, R. and Goldberg, M.J. Active transport of nicotine by the isolated choroid plexus in vitro. J. Neurochem. **38**: 594-596, 1982.

Suzuki, J. Sawada, Y., Sugiyama, Y., Iga, T., and Hanano, M. Saturable transport of cimetidine from cerebrospinal fluid to blood in rats. J. Pharmacobio.-Dyn. **8:** 73-76, 1985.

Suzuki, J. Sawada, Y., Sugiyama, Y., Iga, T., and Hanano, M. Transport of cimetidine by rat choroid plexus in vitro. J. Pharmacol. Exper. Ther. **239:** 927-935, 1986a.

Suzuki, J. Sawada, Y., Sugiyama, Y., Iga, T., and Hanano, M. Comparative uptake of cimetidine by the rat choroid plexus between the lateral and the 4th ventricles. J. Pharmacobio-Dyn. **9**: 327-329, 1986b.

Suzuki, H., Sawada, Y., Sugiyama, Y., and Hanano, M. Transport of benzylpenicillin by the rat choroid plexus in vitro. J. Pharmacol. Exp. Ther. **242:** 660-665, 1987.

Suzuki, J. Sawada, Y., Sugiyama, Y., Iga, T., and Hanano, M. Efflux of cimetidine from rat cerebrospinal fluid. Drug Metab. Dispos. **16:** 328-330, 1988.

Takano, M., Inui, K., Okano, T., and Hori, R. Cimetidine transport in rat renal brush border and basolateral membrane vesicles. Life Sci. **37**: 1579-1585, 1985.

Tochino, Y. and Schanker, L.S. Active transport of quaternary ammonium compounds by the choroid plexus in vitro. Am. J. Physiol. **208**: 666-673, 1965a.

Tochino, Y. and Schanker, L.S. Transport of serotonin and norepinephrine by the rabbit choroid plexus in vitro. Biochem. Pharmacol. **14**: 1557-1566, 1965b.

Tochino, Y. and Schanker, L.S. Serum and tissue factors that inhibit amine transport by the choroid plexus in vitro. Am. J. Physiol. **210**: 1229-1233, 1966.

Thompson, J. Lilly, J.,and Bale, J.F. Cimetidine-induced cerebral toxicity in children. Lancet **1**: 725,1979.

Van Deurs, B. and Koehler, J.K. Tight junctions in the choroid plexus epithelium. J. Cell Biol. **80**: 662-673, 1979.

von Bulow, F., Mollgard, K., and Van Derus, B. Tight junction structure in relation to transepithelial resistance in frog choroid plexus. Eur. J. Cell Biol. **33**: 90-94, 1984.

Weddington, W.W., Mueling, A.E., and Moosa, H.H. Cimetidine toxic reactions masquerading as delirium tremens. JAMA **245**: 1058-1059, 1981.

Weddington, W.W.,Jr., Muelling, A.E., and Mossa, H.H. Adverse neuropsychiatric reactions to cimetidine. Psychosomatics **23**: 49-53, 1982.

Weiner, I.M., and Roth, L. Renal excretion of cimetidine. J. Pharmacol Exp. Ther. **216**: 516-520, 1981.

Whittico, M.T., and Giacomini, K.M. Cimetidine elimination from the cerebrospinal fluid of the rat. Pharm. Res. **5:** 628-633, 1988.

Wright, E.M. Accumulation and transport of amino acids by the frog choroid plexus. Brain Res. **44**: 207-219, 1972a.

Wright, E.M. Mechanisms of ion transport across the choroid plexus. J. Physiol. **226**: 545-571, 1972b.

Wright, E.M. Active transport of iodide and other anions across the choroid plexus. J. Phyysiol. **240**: 535-566, 1974.

Wright, E.M. Effects of bicarbonate and buffers of choroid plexus Na/K pump. Biochim. Biophys. Acta **468**: 486-489, 1977a.

Wright, E.M. Fluid secretion by the frog choroid plexus. Expl. Eye Res. **25**: Suppl. 149-155, 1977b.

Wright, E.M. Transport processes in the formation of the cerebrospinal fluid. Rev. Physiol. Biochem. Pharmacol. **83**: 1-31, 1978a.

Wright, E.M. Ion transport across the choroid plexus: in Membrane Transport in Biology, G. Giebesch, D.C. Tosteson, and H.H. Ussing (eds), Springer-Verlag, New York, 1978b, Vol. 3, Chap. 12, p. 355-376.

Wright, E.M. Anion transport by choroid plexus epithelium.: in Membrane Transport Processes, J.F. Hoffman (ed.), Raven Press, New York, 1978c, Vol. 1, p. 293.

Wright, E.M. Secretion and circulation of the cerebrospinal fluid. Front. Horm. Res. **9**: 4-14, 1982.

Wright, E.M. Electrophysiology of plasma membrane vesiclel. Am. J. Physiol. **246**: F363-F372, 1984.

Wright, E.M. and Saito, Y. The choroid plexus as a route from blood to brain. Annals of the New York Academy of Sciences **481**: 214-220, 1987.

Wood, M.A., Isaacson, M.L., and Hibbs, M.S. Cimetidine and mental confusion. JAMA **239**: 2550-2551, 1978.

Yaron, B., Zlatkis, L., and Kosakov, K. Cimetidine and encephalopathy. Ann. Intern. Med. **105**: 977, 1986.

Zeuthen, T. Epithelial potassium transport in the choroid plexus of the bullfrog. J. Physiol. (London) **291**: P68, 1979.

Zeuthen, T. and Wright, E.M. An electrogenic Na/K pump in the choroid plexus. Biochim. Biophys. Acta **511**: 517-522, 1978.

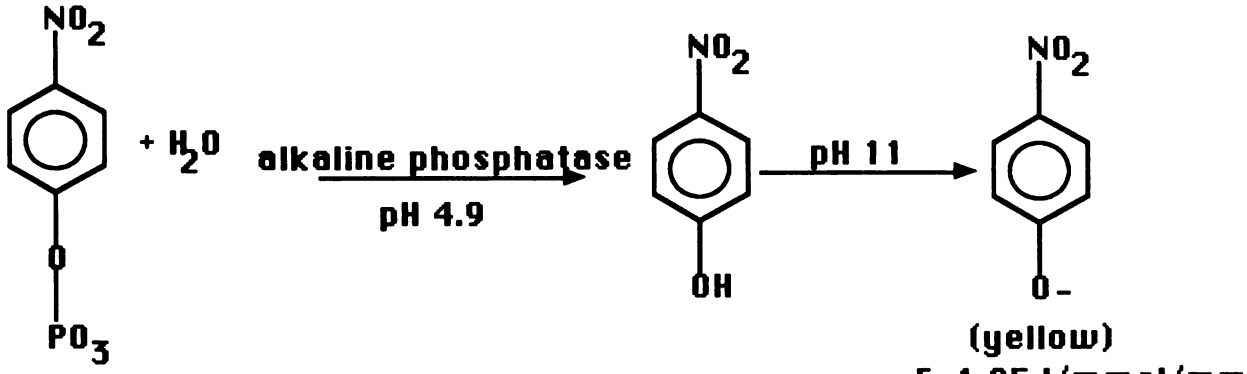
Zeuthen, Y. and Wright, E.M. Epithelial potassium transport: Tracer and electrophysiological studies in choroid plexus. J. Membrane Biol. **60**: 105-128, 1981.

Ziemniak, J.A., Shank, R.G., and Schentag, J.J. The partitioning of cimetidine into canine cerbrospinal fluid. Drug Metab. Dispos. **12**: 217-221, 1984.

Appendix A

Enzyme Assays

Acid Phosphatase (Moss, 1984)



Solutions:

A. 45.9 mM trisodium citrate, pH 4.9

B. 7.6 mM 4-nitrophenyl phosphate
(144 mg Na_2-4-nitrophenyl phosphate•$6H_2O$ in 50 ml solution A q.s. to 51 ml with H_2O) store at 4°

C. 100 mM NaOH

Reaction:

1. Pipette 1 ml of solution B into test tube and sit in water-bath for 10 min, 37°
2. Add 200 µl of diluted enzyme mixture or sample plus enough water to make 200 µl
3. Vortex and incubate for exactly 30 min in 37° water-bath
4. Stop reaction with 4 ml solution C and vortex
5. Read absorbance at 405 nm against blank or water. E_{405} = 1.85 l X $mmol^{-1}$ X mm^{-1}

Appendix A (continued)

Acid Phosphatase (Moss, 1984) (continued)

Blank:

same as above except sample is added after addition of solution C

Appendix A (continued)

Alkaline Phosphatase Assay (Linhardt and Walter,1965)

Reaction mixture:

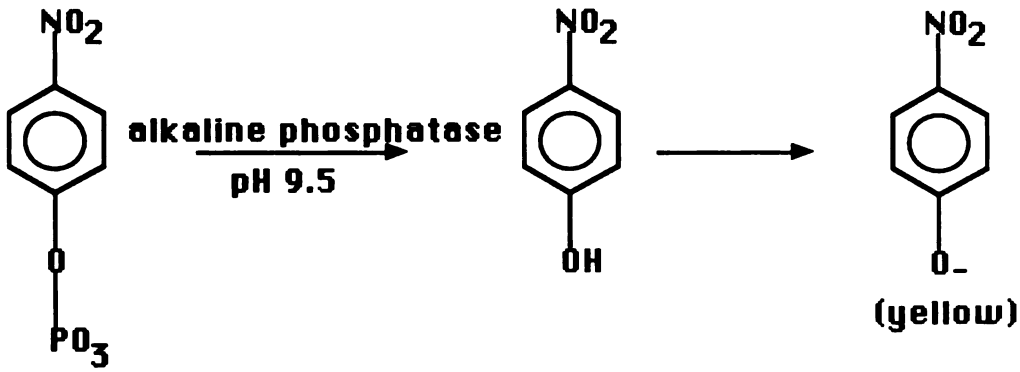
90 mM Tris

2 mM $MgCl_2$

10 mM 4-nitro phenyl phosphate

pH to 9.5 with 1N HCl

Rection:



1. Add 0.1 ml (enzyme sample (ca 75 µg protein) + water) to 0.9 ml reaction mixture
2. Incubate exactly 10 min, 37°
3. Add 2 ml 0.1N NaOH to stop reaction
4. Run blank by above steps except add NaOH solution before adding enzyme sample
5. Read sample at 410 nm against blank

   E = 16.2 mM

Appendix A (continued)

Glucose-6-Phosphatase Assay

(Aronson and Touster, !974)

Reagents for substrate mixture:

a. 100 mM Na-glucose-6-phosphate, pH 6.5

b. 35 mM Histidine, pH 6.5

c. 10 mM Na-EDTA, pH 7.0

Substrate Mixture (SM):

Combine solutions a,b,c and water in volume ratios 2:5:1:1 (mixture can be stored frozen in aliquots for 1 yr)

Ames Reagent (AR):

A. 0.42% ammonium molybdate$\cdot 4H_2O$ in 1N H_2SO_4 (store in dark bottle,4°)

B. 10% ascorbic acid in water (prepare fresh daily,may store frozen 1 month)

Combine solutions A and B (6:1)

Stop Solution Tetrachloracetic acid (TCA):

8% TCA (store at 4°)

Phosphate Standard Solution (PSS):

8 mM K_2PO_4

Appendix A (continued)

Glucose-6-Phosphatase Assay (continued)

(Aronson and Touster, !974)

Reaction:

Recommended protein concentration of sample: ca 2 µl/µl

1. Add 50 µl sample to 450 µl substrate mixture
2. Incubate for exactly 30 min, 37°
3. Add 2.5 ml iced stop solution and place in ice.
4. Centrifuge 3,000 g, 5 min, 5°
5. Place on ice for 10 min
6. Read optical density at 660 nm against water blank.

Standard Curve:

Pipette the following volumes (µl) in a test tube:

PSS	H_2O	SM	TCA
50	0	450	2500
35	15	450	2500
25	15	450	2500
10	40	450	2500
5	45	450	2500
0	50	450	2500

1. Add 2 ml from each test tube to 4 ml (AR)
2. Incubate 5 min, 45°
3. Place in ice for 10 min
4. Read absorbance at 660 nm against water blank

Appendix A (continued)

Na^+/K^+ATPase Assay (Mamelok et al.,1982)

$$ATP + H_2O \xrightleftharpoons[Mg^{2+}]{ATPase} ADP + Pi$$

Solutions:

Reaction mix (RM):

A. 280 mM NaCl
11 mM $MgCl_2$
40 mM KCl
6 mM EGTA
5 mM Na_3N
40 mM Tris

B. 10 mM ATP in 1:2 dilution of solution A in water, pH 7.4 (calculate volume needed for 2 X all samples)

C. 5 mM ouabain in one-half of solution B

10% Tetrachloracetic Acid (TCA)

Phosphate Standard Stock (PSS):

8 mM K_2HPO_4

make dilutions of 0.96, 0.48, 0.24, 0.12, 0.06, and 0.03 mM

Ames Reagent (AR):

A. 0.42% ammonium molybdate-$4H_2O$ in 1N H_2SO_4 (store in dark bottle,4°)

B. 10% ascorbic acid in water (prepare fresh daily, may store frozen 1 month)

Appendix A (continued)

Na^+/K^+ATPase Assay (Mamelok et al.,1982) (continued)

AR = A + B (6:1)

Reaction with sample:

1. Add 300 µl of sample diluted to 67-267 µg/ml (or 20-80 µg sample q.s to 300 µl) to 200 µl solution RM-B (for reaction without ouabain) or solution RM-C (for reaction with ouabain)
2. Incubate 10 min, 37°
3. Stop reaction with 500 µl TCA and place in ice for 15 min
4. centrifuge 3000g, 10 min, 4°
5. Remove 800 µl and add to 1.6 ml AR solution
6. Incubate exactly 5 min, 45°
7. Place immediately in ice for 10 min
8. Read absorbance at 660 nm or 820 nm

Standard Curve:

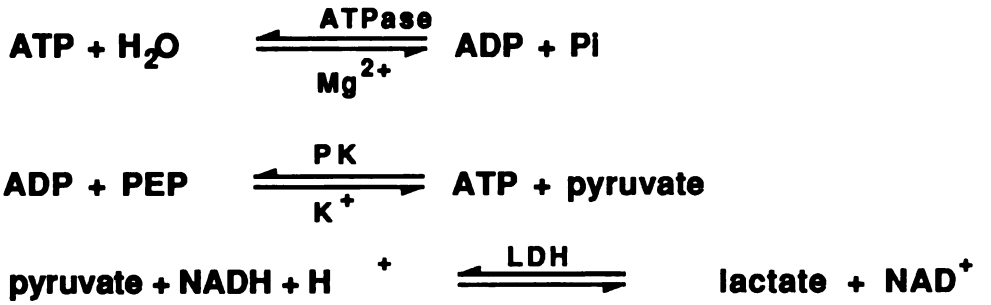
Place the following volumes (µl) in duplicate test tubes (PSS volumes 100-25 µl are from 8 mM PSS; all others are from dilutions):

Source (mM)	H_2O	PSS	PO_4^{2-} (nmol)	Reaction mix A	TCA
8	200	100	800	200	500
8	225	75	600	200	500
8	250	50	400	200	500
8	275	25	200	200	500
0.96	100	200	192	200	500
0.48	100	200	96	200	500
0.24	100	200	48	200	500
0.12	100	200	24	200	500
0.06	100	200	12	200	500
0.03	100	200	6	200	500

1. Add 1.6 ml of AR solution to each tube
2. Incubate exactly 5 min, 45°
3. Place tubes in ice for 10 min
4. Read absorbance at 660 nm or 820 nm

Appendix A (continued)

Na^+/K^+ATPase Assay (Schoner et al., 1965)



Solutions:

Buffer A:

62.5 mM Imidazole 425.6 mg/100 ml

62.5 mM NH_4Cl 334.3 mg/100 ml

3.125 mM $MgCl_2 \cdot 6H_2O$ 63.5 mg/100 ml

1.25 mM EGTA 1.25 ml of 0.1 M solution, pH 7.5

Dissolve in water, pH to 7.3 with HCl

Buffer B:

50 mM Imidazole 340.5 mg/100 ml (pH 7.3)

C:

1 M NaCl 5.84 g/100 ml

Reaction Mixture:

18.75 ml buffer A

2.8 ml buffer C

q.s. to 30 ml with water

Divide into 15 ml volumes and 13.7 mg ouabain (1.25 mM) to one volume and label (+) oubain (poorly soluble, requires vigirous mixing). Label the other volume (-) oubain. Let stand at room temperature or 37°

Appendix A (continued)

Na^+/K^+ATPase Assay (Schoner et al., 1965) (continued)

Cofactor Mixture (must be prepared fresh each time):

Compound	wt (mg)	μmoles	stock conc.	Final conc.
NADH	7.92	10.13	3.06	0.306
PEP	3.13	6.8	2.06	.206
PK	0.66	330 U	100 U/ml	10U/ml
LDH	0.44	363 U	110 U/ml	11U/ml
ATP	30.26	50	15.15	1.51

Weigh all ingredients into graduate cylinder, q.s. to 3.3 ml with buffer A. Vortex to mix well, keep in ice.

Assay Procedure:

1. All reactions are at 37°; set Autofill temperature to 37°; calibrate sipper time to collect 1 ml sample (ca 3 s)
2. Wave length setting is 340 nm; deuterium lamp on
3. Use Enzyme Kinetics Program # 7 on Apple IIe computer disk, Reaction Rate @ Autofill [Epson]
4. Turn on computer and set parameters 5.
5. Place 800 μl of (+) oubain in small test tube (13X75 mm)
6. Add 100 μl of cofactor mix
7. Add volume of solution C which would be required to q.s. to 1000 μl after adding sample
8. Add 5-100 μl of sample, vortex and suckup with Autofill sipper
9. Follow reaction for about 5 min

Appendix A (continued)

Na^+/K^+ATPase Assay (Schoner et al., 1965) (continued)

Auto mode calculation setting in computer program will use most linear and steepest part of reaction for rate caculation. If rate is to fast, decrease sample volume or dilute enzyme sample.

Abreviations:

PK = pyruvate kinase

ATP = adenosinetriphosphate

PEP = phosphoenolpyruvate

LDH = lactate dehydrogenase

NADH = reduced nicotinimide adenine dinucleotide

Appendix B

Cimetidine Binding to Nitrocellulose Filters

A critical factor in any assay procedure is its sensitivity. In order to discern real differences at low levels it is essential that the noise to signal ratio be as low as possible, i.e., the background signal should be at an absolute minimum. This is especially critical in membrane vesicle transport studies where most of the experiments are designed with the assumption that unidirectional fluxes are being measured and are therefore conducted at very short time intervals, i.e., 2-5 seconds. Consequently, the amount of substrate taken up by the vesicles at these early time points is very small, and is difficult to discern with high background levels. High noise to signal ratios in rapid filtration uptake experiments are caused by adsorption of the radiolabeled substrate to the filters in control (blank) experiments. The average value of the blanks is subtracted from the value obtained in experiments with vesicles present to obtain the amount of radioactivity associated with the vesicles alone. High blanks will also have high variability, therefore, subtracting high blank values to obtain small uptake values often yields highly varible and unreliable results.

^{3}H-Cimetidine is particularly problematic in this regard. With the nitrocellulose filters used in the experiments in this dissertation the background signal typically ranged between 800 and 1500 DPM. Several experiments were conducted to determine the optimum soaking conditions, filter type, and possible binding inhibitor in order to maintain the lowest possible background signal. Figure B-1 depicts the results of experiments designed to determine the difference in filter binding between two types of filters as well as to identify possible competitive inhibitors of filter binding.

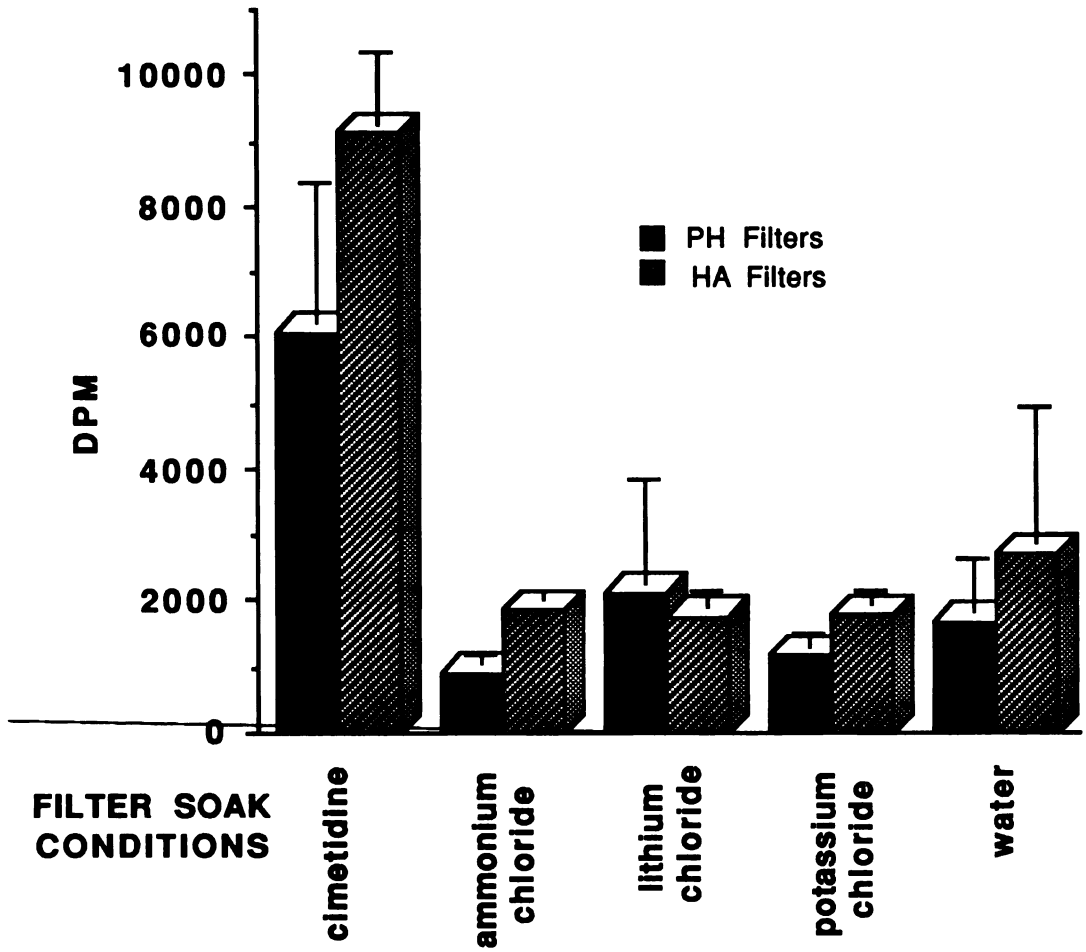


Figure B-1 Data from cimetidine membrane filter-binding inhibitor study. See text for details

The filters used were nitrocellulose type HA, 0.45 mm and nitrocellulose type PH, 0.3 mm. Batches of the two filter types were soaked in buffer solutions containing either (10 mM) NH_4Cl, LiCl, cimetidine, (300 mM) KCl, or double deionized distilled water. The filters were allowed to soak in the solutions for at least 2 hr. The results show that in this study type PH filters bind less ^3H-cimetidine than do type HA filters. None of the potential binding inhibitors were effective when the type HA filters were used, while NH_4Cl was most effective in reducing ^3H-cimetidine binding to the PH filters. An unexplained phenomenon

was observed when 10 mM unlabeled cimetidine was used in an attempt to saturate the binding sites of the filters. The binding of ^{3}H-cimetidine was enhanced 3-fold on type PH filters and 5-fold on type HA filters.

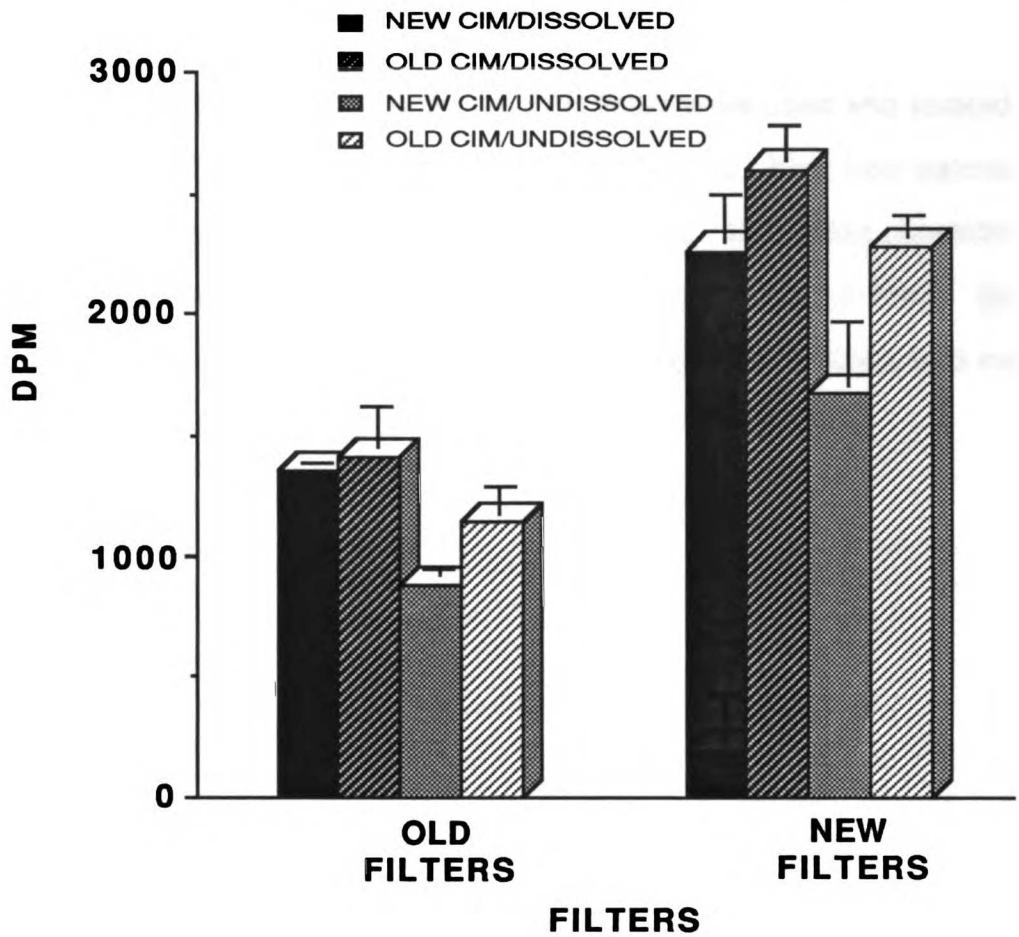


Figure B-2 Data from experiments to determine the effects of different batches of filters, cimetidine, and of filter dissolution time on the binding of cimetidine to membrane filters. See text for details.

Figure B-2 depicts the results of experiments designed to determine whether there was a difference in ^{3}H-cimetidine filter binding between different manufacture filter batches (old and new filters), batches of ^{3}H-cimetidine (old and new CIM), and the time that the filters are allowed to dissolve in the

scintillant before counting (dissolved, overnight; and undissolved, 2 hr or less in scintillant before counting). These results show that there was a significant difference in filter binding between the two batches of filters irrespective of the batch of ^{3}H-cimetidine used or the dissolution time.

Based of these experiments type PH filters were used and soaked in 10 mM NH_4Cl/HEPES solution in subsequent experiments. New filter batches were tested to determine optimum soaking conditions. Unlabeled cimetidine can not be used to displace ^{3}H-cimetidine from binding sites on the filters. Samples should sit overnight before scintillation counting to allow filters and membranes to dissolve in the scintillant.

Appendix C

Cimetidine and Inulin Concentration Data and Parameters from CSF Clearance Experiments

MTWDL1 (7/28/86)

	Cimetidine (S.A.10 μCi/nmol)		Inulin (S.A.6.88 μCi/μmol)	
Time (min)	DPM/5 μl	Concentration (pg/μl)	DPM/5 μl	Concentration (ng/μl)
3	10130.70	23.15	6937.40	455.21
6	10998.30	25.14	10820.90	711.78
10	9045.90	20.67	12002.90	789.87
20	5894.30	13.44	9428.00	619.75
40	2373.40	5.36	3780.40	246.63
60	1101.50	2.44	2093.70	135.19
90	639.60	1.38	1286.40	81.86
120	434.10	0.91	907.50	56.82
150	301.30	0.61	765.40	47.44
180			617.40	37.66
Dose (pg)		7588.00	Dose (ng)	70124.00
AUC (pg•min/μl)		733.85	AUC (ng•min/μl)	41214.19
Clearance (μl/min)		10.34	Clearance (μl/min)	1.70

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC1 (7/29/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine Concentration (ng/μl)	Inulin (S.A.6.87 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
3	1695.80	6.79	973.30	61.17
5	6976.80	28.40	4914.60	321.56
11	9363.70	38.17	8907.70	585.38
21	6486.80	26.40	7446.10	488.81
40	4056.90	16.45	4988.20	326.43
60	2356.40	9.49	3358.10	218.73
90	1237.80	4.91	2008.20	129.55
120	740.60	2.88	1376.80	87.83
150	512.70	1.95	900.20	56.34
180			432.30	25.43
Dose (ng)		17065.55	Dose (ng)	70124.00
AUC (ng•min/μl)		2161.20	AUC (ng•min/μl)	35017.09
Clearance (μl/min)		7.90	Clearance (μl/min)	2.00

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDL2 (8/4/86)

Time (min)	Cimetidine (S.A.10 μCi/nmol) DPM/5 μl	Cimetidine (S.A.10 μCi/nmol) Concentration (pg/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin (S.A.6.88 μCi/μmol) Concentration (ng/μl)
2	3401.40	7.80	3496.90	227.90
5	6825.20	15.66	10257.60	674.56
10	6038.60	13.85	11562.00	760.74
20	3304.50	7.58	7497.90	492.24
40	1421.30	3.26	3666.50	239.11
60	766.70	1.76	2426.60	157.19
90	453.20	1.04	1578.40	101.15
120	307.50	0.71	1265.60	80.48
150	146.40	0.34	867.30	54.17
180	118.00	0.27	620.30	37.85
Dose (pg)		8189.46	Dose (ng)	78139.01
AUC (pg·min/μl)		454.21	AUC (ng·min/μl)	35966.59
Clearance (μl/min)		18.03	Clearance(μl/min)	2.17

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC2 (7/30/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine Concentration (ng/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
2	184.10	0.60	118.00	4.66
7	2025.70	8.14	1133.70	71.77
12	10642.60	43.42	8954.00	588.44
20	10340.50	42.18	10288.40	676.60
40	4680.10	19.01	5939.50	389.28
60	3361.80	13.61	4489.50	293.48
97	1308.20	5.20	2604.90	168.97
121	681.30	2.64	1406.10	89.77
150	394.90	1.46	741.60	45.86
181			629.60	38.46
Dose (ng)		16675.55	Dose (ng)	70124.00
AUC (ng·min/μl)		2208.45	AUC (ng·min/μl)	40882.00
Clearance (μl/min)		7.55	Clearance (μl/min)	1.72

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDL3 (8/5/86)

Time (min)	Cimetidine (S.A.10 μCi/nmol) DPM/5 μl	Cimetidine Concentration (pg/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
2	6215.20	14.26	5285.50	346.07
5	14541.00	33.36	16689.30	1099.49
10	11142.90	25.56	17664.10	1163.89
20	5023.50	11.52	8421.80	553.28
40	1573.70	3.61	2741.30	177.98
60	697.60	1.60	1803.20	116.00
90	382.20	0.88	769.20	47.69
120	271.60	0.62	558.10	33.74
153	194.60	0.45	471.20	28.00
182	114.00	0.26	316.20	17.76
	Dose (pg)	7671.11	Dose (ng)	72803.65
	AUC (pg·min/μl)	687.84	AUC (ng·min/μl)	33036.89
	Clearance (μl/min)	11.15	Clearance (μl/min)	2.20

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC3 (7/31/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine Concentration (ng/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
2	5889.00	23.99	3187.80	207.48
5	13252.40	54.17	8764.20	575.90
10	17580.30	71.92	15048.10	991.06
20	5337.60	21.73	11233.90	739.07
42	2945.90	11.92	3739.20	243.91
60	1726.60	6.93	2414.30	156.38
90	865.90	3.40	1385.40	88.40
120	416.60	1.56	777.30	48.22
154	201.20	0.67	617.30	37.65
182			370.30	21.33
	Dose (ng)	**16625.55**	**Dose (ng)**	**68718.78**
	AUC (ng•min/μl)	**1924.83**	**AUC (ng•min/μl)**	**36819.47**
	Clearance (μl/min)	**8.64**	**Clearance (μl/min)**	**1.87**

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDL4 (8/14/86)

Time (min)	Cimetidine (S.A.10 μCi/nmol) DPM/5 μl	Cimetidine (S.A.10 μCi/nmol) Concentration (pg/μl)	Inulin (S.A.6.88 nCi/nmol) DPM/5 μl	Inulin (S.A.6.88 nCi/nmol) Concentration (ng/μl)
2	949.10	2.18	1660.80	106.59
5	3994.20	9.16	9559.90	628.47
10	5266.50	12.08	15715.90	1035.18
21	3195.10	7.33	10056.60	661.28
41	937.00	2.15	3648.30	237.90
60	559.80	1.28	2148.80	138.83
90	307.50	0.71	1462.30	93.48
120	180.00	0.41	696.10	42.86
152	128.50	0.29	569.30	34.48
180	119.00	0.27	477.90	28.44
Dose (pg)		3898.33	Dose (ng)	68914.90
AUC (pg•min/μl)		349.00	AUC (ng•min/μl)	36969.81
Clearance (μl/min)		11.17	Clearance (μl/min)	1.86

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC4 (8/8/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine Concentration (ng/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
2	4939.00	20.09	6828.20	447.99
5	4952.20	20.14	8766.80	576.07
10	3411.50	13.83	8921.20	586.27
20	1464.70	5.85	4890.80	319.99
40	839.20	3.29	3104.50	201.98
60	518.50	1.97	2592.90	168.18
90	857.40	3.36	1673.20	107.41
120	429.20	1.61	1359.10	86.66
150	339.10	1.24	904.10	56.60
180	253.60	0.89	772.10	47.88
Dose (ng)		4585.55	Dose (ng)	63192.92
AUC (ng•min/μl)		878.64	AUC (ng•min/μl)	33594.54
Clearance (μl/min)		5.22	Clearance (μl/min)	1.88

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDL5 (8/18/86)

Time (min)	Cimetidine (S.A.10 μCi/nmol) DPM/5 μl	Concentration (pg/μl)	Inulin (S.A.6.88 nCi/nmol) DPM/5 μl	Concentration (ng/μl)
2	757.10	1.74	504.70	30.21
5	3501.00	8.03	3531.00	230.15
11	6062.60	13.91	3468.70	226.04
20	4852.70	11.13	7965.00	523.10
41	1906.30	4.37	3662.80	238.86
60	1026.50	2.35	3006.50	195.50
90	695.80	1.60	1716.20	110.25
124	461.90	1.06	1085.20	68.57
152	378.40	0.87	795.50	49.43
180	268.40	0.62	599.80	36.50
Dose (pg)		6535.72	Dose (ng)	73737.55
AUC (pg·min/μl)		625.08	AUC (ng·min/μl)	32481.08
Clearance (μl/min)		10.46	Clearance (μl/min)	2.27

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC5 (8/28/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine Concentration (ng/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin Concentration (ng/μl)
2	6792.80	27.69	3438.20	224.02
5	4973.00	20.23	6711.80	440.30
10	10368.50	42.34	8079.40	530.66
20	2323.20	9.37	5074.60	332.14
40	2270.20	9.15	2531.00	164.09
60	1245.80	4.95	1513.70	96.88
90	648.40	2.51	749.40	46.38
120	412.20	1.54	507.70	30.41
150	259.80	0.91	401.10	23.37
180	206.50	0.69	360.30	20.67
Dose (ng)		13635.55	Dose (ng)	57764.80
AUC (ng•min/μl)		1180.09	AUC (ng•min/μl)	25205.85
Clearance (μl/min)		11.55	Clearance (μl/min)	2.29

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDL6 (8/26/86)

Time (min)	Cimetidine (S.A.10 μCi/nmol) DPM/5 μl	Cimetidine (S.A.10 μCi/nmol) Concentration (pg/μl)	Inulin (S.A.6.88 nCi/nmol) DPM/5 μl	Inulin (S.A.6.88 nCi/nmol) Concentration (ng/μl)
2	343.10	0.79	193.30	9.64
5	2686.30	6.16	2231.50	144.30
10	5551.00	12.73	6315.10	414.09
20	6011.40	13.79	8830.30	580.27
40	2258.10	5.18	3523.10	229.63
60	1171.10	2.69	2221.60	143.64
90	614.20	1.41	1230.30	78.15
120	386.70	0.89	838.60	52.27
150	278.50	0.64	665.70	40.85
180	226.20	0.52	503.40	30.13
Dose (pg)		5749.55	Dose (ng)	58162.06
AUC (pg•min/μl)		607.78	AUC (ng•min/μl)	28820.30
Clearance (μl/min)		9.46	Clearance (μl/min)	2.02

S.A. = specific activity

APPENDIX C (continued)

Cimetidine and Inulin Concentration Data and Parameters From CSF Clearance Experiments

MTWDLPC6 (8/19/86)

Time (min)	Cimetidine (S.A.5.6 μCi/μmol) DPM/5 μl	Cimetidine (S.A.5.6 μCi/μmol) Concentration (ng/μl)	Inulin (S.A.6.88 μCi/μmol) DPM/5 μl	Inulin (S.A.6.88 μCi/μmol) Concentration (ng/μl)
2	19374.10	79.27	6575.60	431.30
5	13636.70	55.75	6562.50	430.44
10	9056.80	36.98	4820.10	315.32
20	6849.90	27.93	4148.20	270.93
40	3602.70	14.62	2998.80	194.99
60	1610.10	6.45	1842.10	118.57
90	707.40	2.75	1051.00	66.31
120	424.90	1.59	690.70	42.50
150	280.80	1.00	487.40	29.07
180	194.70	0.65	423.50	24.85
	Dose (ng)	14485.55	Dose (ng)	52885.00
	AUC (ng•min/μl)	1968.47	AUC (ng•min/μl)	23389.46
	Clearance (μl/min)	7.36	Clearance (μl/min)	2.26

S.A. = specific activity

Appendix D

Choroid Plexus Protein Recovery in Fractions from Brush-border Membrane Isolation Procedure

9/19/88

Fraction	H1	P1	S1	P2	S2
Conc. (g/L)	6.61	3.42	5.26	13.3	4.4
Volume (L)	0.167	0.07	0.131	0.0033	0.141
Amount (mg)	1103.87	239.4	689.06	43.89	620.4
Recovery (mg)	928.46		664.29		735
%Recovery	84.11		96.41		118.6

Fraction	P3	S3	P4	S4	P5
Conc. (g/L)	2.5	3.76	14.84	0.7	11.3
Volume (L)	0.082	0.141	0.0033	0.082	0.0012
Amount (mg)	205	530.16	48.97	57.07	13.67
Recovery (mg)	106.04			45.18	13.53
%Recovery	51.73			79.16	99

Fraction	S5	P6	S6
Conc. (g/L)	0.39	18.94	1.22
Volume (L)	0.081	0.0007	0.001
Amount (mg)	31.51	12.31	1.22
Recovery (mg)			
%Recovery			

APPENDIX D (continued)

Choroid Plexus Protein Recovery in Fractions from Brush-border Membrane Isolation Procedure

11/30/88

Fraction	H1	P1	S1	P2	S2
Conc. (g/L)	7	4.99	7.24	3.75	6.05
Volume (L)	0.176	0.055	0.149	0.013	0.16
Amount (mg)	1232	274.45	1078.8	48.75	968
Recovery (mg)	1353.21		1016.8		1050.7
%Recovery	109.84		94.25		108.5

Fraction	P3	S3	P4	S4	P5
Conc. (g/L)	2.44	5.42	10.1	1.43	19.89
Volume (L)	0.083	0.1565	0.0042	0.078	0.0025
Amount (mg)	202.52	848.23	42.42	111.54	49.73
Recovery (mg)	153.96			119.48	18.45
%Recovery	76.02			107.11	37.1

Fraction	S5	P6	S6
Conc. (g/L)	0.93	20.5	2.68
Volume (L)	0.075	0.0007	0.0013
Amount (mg)	69.75	14.97	3.48
Recovery (mg)			
%Recovery			

APPENDIX D (continued)

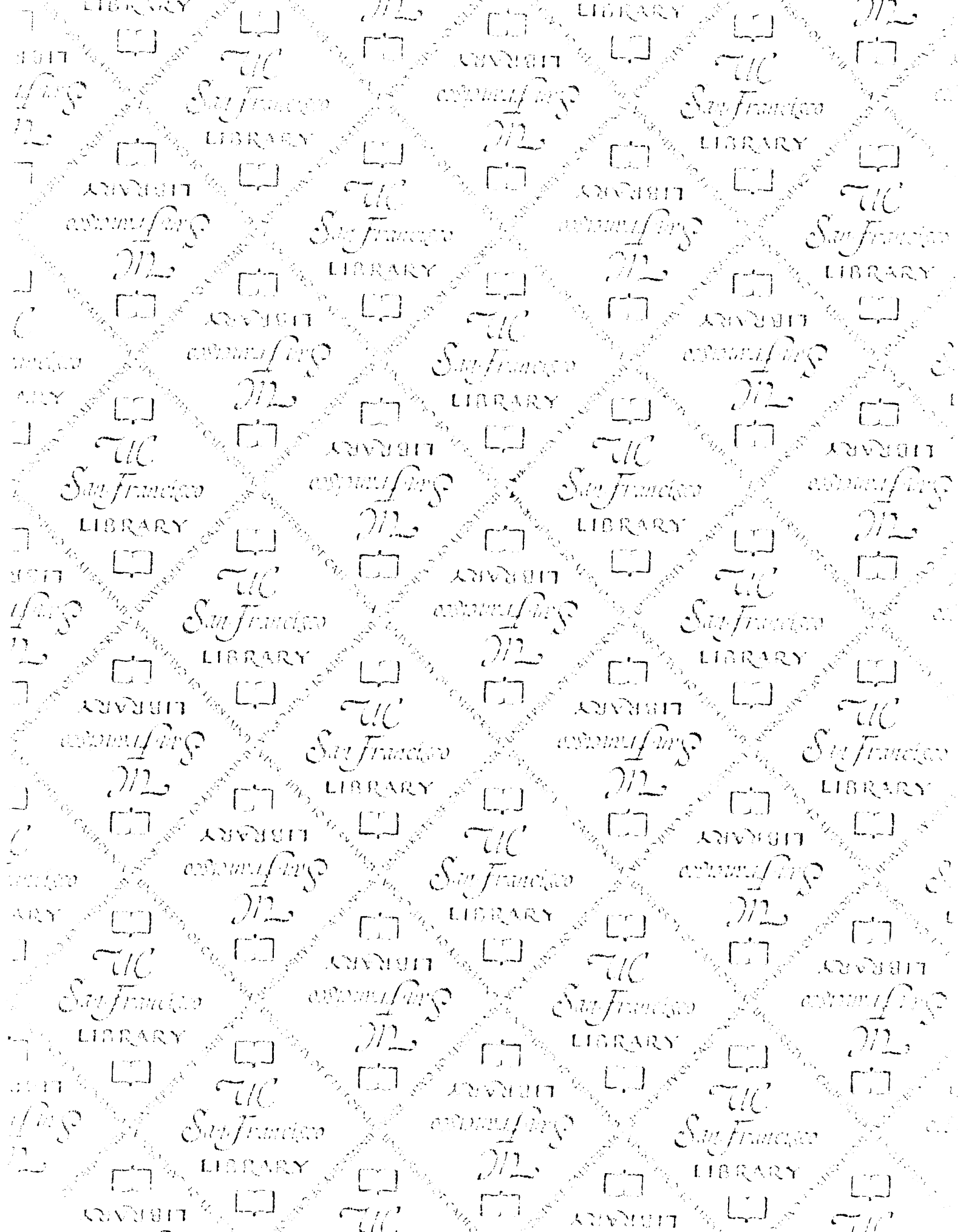
Choroid Plexus Protein Recovery in Fractions from Brush-border Membrane Isolation Procedure

12/5/88

Fraction	H1	P1	S1	P2	S2
Conc. (g/L)	8.04	8.41	7.35	14.7	6.17
Volume (L)	0.1596	0.053	0.118	0.0028	0.133
Amount (mg)	1283.18	445.73	867.3	41.16	820.61
Recovery (mg)	1313.03		861.77		843.8
%Recovery	102.33		99.36		102.8

Fraction	P3	S3	P4	S4	P5
Conc. (g/L)	2.18	5.23	15.6	1.25	12.9
Volume (L)	0.08	0.128	0.0038	0.075	0.0015
Amount (mg)	174.4	669.44	59.28	93.75	19.35
Recovery (mg)	153.03			92.79	20.06
%Recovery	87.75			98.98	103.66

Fraction	S5	P6	S6
Conc. (g/L)	1.02	24.6	1.91
Volume (L)	0.072	0.0007	0.0011
Amount (mg)	73.44	17.96	2.1
Recovery (mg)			
%Recovery			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