ACTIVE TRANSPORT OF ORGANIC ANIONS FROM THE BRAIN VENTRICLES OF THE DOG

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ABSTRACT

ACTIVE TRANSPORT OF ORGANIC ANIONS
FROM THE BRAIN VENTRICLES
OF THE DOG

By

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The development of an organic anion transport mechanism in the choroid plexus was studied in vitro in 1- to 4-week-old and adult dogs. Lateral ventricular choroid plexuses (LVCP), fourth ventricular choroid plexuses (FVCP) and diaphragm muscle were incubated for 1 hour at 37° C in a buffered salt solution containing p-aminohippuric acid (PAH) and mannitol. Accumulation of PAH and mannitol by the plexuses was expressed as tissue:medium concentration ratios (T/M) and comparisons made of the ratios of PAH and mannitol. T/M mannitol ratios of the adult LVCP and FVCP are significantly less ($p < 0.05$) than for animals 1 week old; T/M mannitol ratios in diaphragm muscle did not change with age. The active accumulation of PAH for both plexuses was indicated by accumulation against a concentration gradient, transport saturation, inhibition by organic anions and a metabolic dependence. PAH

transport is poorly developed in the LVCP and FVCP (T/M = 2.12; T/M = 1.97, respectively) of animals 1 week old, but is highly developed in the adult (T/M = 4.04; T/M = 3.84, respectively). Maximum accumulation of PAH by the LVCP occurs in 2 week animals whereas maximum accumulation by the FVCP occurs in the adult.

Prior administration of procaine penicillin G in 1- and 2-week-old dogs induced in vitro development of the organic anion transport system. LVCP's from 1-week-old dogs previously treated with 300,000 IU penicillin showed a significant increase ($p < 0.05$) in PAH accumulation compared to saline-treated animals. No induction of PAH accumulation was produced by treatment with 600,000 IU in 1-week-old dogs or 120,000 IU in 2-week-old dogs. FVCP's from 1-week-old dogs treated with 300,000 or 600,000 IU penicillin showed no increase ($p > 0.05$) in PAH uptake but FVCP's from 2-week old animals treated with 120,000 IU showed significantly greater ($p < 0.05$) PAH accumulation than controls. The presence of substrate (penicillin) during development significantly enhanced the rate of maturation of the organic anion transport mechanism.

The brain ventricular system of the adult dog was perfused with an artificial cerebrospinal fluid (CSF) containing inulin, creatinine and radioactively labeled PAH and mannitol. Measurements

were made of steady-state rates at which inulin, mannitol and PAH were removed from the ventricular system. Clearance of inulin represents bulk absorption of fluid occurring distal to the fourth ventricle and varied linearly with intraventricular pressure. The efflux coefficient represents clearance of a molecule by means other than bulk absorption and for mannitol, a passively diffusing molecule, efflux is independent of intraventricular pressure. The efflux of PAH is pressure dependent; PAH efflux increasing over the range -15 to +15 cm HOH pressure indicating that PAH efflux may be a function of the perfused surface area of the FVCP. Active transport of PAH was indicated by competitive inhibition with other organic anions (Diodrast and penicillin) and self-saturation (150-800 μg/ml PAH). Efflux coefficients of creatinine and PAH (46 ± 4 μl/min; 34 ± 4 μl/min, respectively) are significantly greater ($p < 0.05$) than mannitol (16 ± 8 μl/min), suggesting that creatinine and PAH leave CSF by an active process in addition to passive diffusion.

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INTRODUCTION

The development of the mammalian blood-brain and blood-CSF barriers is of considerable importance in determining the composition of the cerebrospinal fluid (CSF) and thereby the maintenance of central nervous system activity and function. In adults, lipid-soluble basic and acidic dyes, injected intravenously, do not cross the capillary walls and enter into brain or CSF (Davson, 1967). However, some molecules such as creatinine, urea, K and Na when perfused into the brain ventricles leave CSF and enter into blood and brain (Heisey et al., 1962). This restriction on the movement of some molecules between blood and brain or blood and CSF has led to the concept of blood-brain and blood-CSF barriers.

In the past decade active transport from CSF to blood of primary, tertiary and quarternary amines (Tochino and Schanker, 1965a; Takemori and Stenwick, 1966; Hug, 1967), inorganic ions (Becker, 1961; Welch, 1962; Robinson et al., 1968) and amino acids (Lorenzo and Cutler, 1969) has been demonstrated. Pappenheimer et al. (1961) showed an active transport mechanism for Diodrast, p-aminohippuric acid (PAH) and phenolsulphonphthalein

from CSF. The presence of these transport mechanisms from CSF or brain into blood suggests that the blood-brain and blood-CSF barriers may consist of a series of active transport mechanisms.

In the fetus and neonate there is evidence that the blood-brain barrier is not completely developed. In 1927, Stern and Peyrot reported that the blood-brain barrier to ferrocyanide was not fully developed in the neonate but developed a few days after birth. In human fetuses and newborns with kernicterus, bilirubin passed freely from blood into brain or CSF (Bakay, 1956). Asghar and Way (1970) demonstrated that intravenous injection of morphine resulted in higher brain concentrations of morphine in animals less than 10 days old than in adults. Thus the development of organic ion transport systems is of biological importance since the removal of organic ions from CSF and brain extracellular fluid provides a mechanism for protecting the central nervous system from potentially toxic compounds and for maintaining homeostasis.

It is the aim of this study to determine whether PAH, an organic anion, is actively transported from CSF in vivo and actively accumulated by the isolated choroid plexus and to study the postnatal development of this organic anion transport mechanism.

LITERATURE REVIEW

The cerebrospinal fluid (CSF) is a colorless fluid derived from blood which fills the brain ventricular cavities and subdural spaces of the central nervous system (CNS). Although there are no ions found in CSF which are not also found in blood, CSF, unlike blood, contains no cells and only a very small amount of protein (Millen and Wollam, 1962; Davson, 1967). Investigators have disagreed about the formation of CSF: some contend CSF is an ultrafiltrate or dialysate of blood whereas others believe CSF is formed by active secretion from the cells of the choroid plexus (Flexner, 1938; Cserr, 1971). If CSF were an ultrafiltrate of blood, then the ionic composition of CSF would be the same as a dialysate of plasma. Flexner (1938) reported that in the pig, Na and Cl concentrations were greater in CSF than in a dialysate of plasma and urea concentration was lower in CSF than in a plasma dialysate. Dog CSF is similar to that of the pig but the Na concentration in CSF is the same as that of a plasma dialysate while Mg concentration is higher in CSF than in a plasma dialysate (Davson, 1967). Therefore ultrafiltration is not an adequate explanation of CSF

formation since an energy source other than hydrostatic pressure of the blood must be supplied to give the observed ionic concentrations. Flexner (1938) suggests that this extra energy must be supplied by the source of the CSF, the cells of the choroid plexus.

Ehrlich first observed that with intravenous injection of acid analine dyes, practically all tissues of the body were stained with the exception of the CNS (Davson, 1967; Grazer and Clemente, 1958). Grazer and Clemente (1958) reported that Trypan blue injected into developing rat embryos, ranging in age from 10 days prenatal to gestation, did not stain the CNS and this impermeability of the developing CNS exists at 10 days prenatal when the blood vessels begin to invade the brain. In icteric adults with high blood bilirubin concentrations, no bilirubin was found in the CSF or grey matter of the brain (Bakay, 1956). This restriction on the movement of molecules between blood and CSF and blood and brain has led to the terms blood-CSF and blood-brain barriers. Bakay (1956) points out that the blood-brain and blood-CSF barriers may not be a simple screening agent but a complex mechanism which is functionally adapted to the needs of the CNS.

Some molecules such as creatinine, K and Na, when added to CSF, leave the CSF and enter into blood and brain (Heisey et al., 1962). Two techniques have been used in vivo to measure the rate

at which molecules are removed from CSF: 1) intracisternal injection of test substances; and 2) perfusion of the brain ventricular system with an artificial CSF containing test substances.

Davson et al. (1962) observed that ^{24}Na, p-aminohippuric acid (PAH), sucrose or inulin injected into the cisterna magna of anesthetized rabbits were removed from CSF at different rates. One hour after injection a large sample of CSF was withdrawn and analyzed. The rate at which test substances left the CSF was estimated from concentration differences between injected and withdrawn fluid. Loss of test substances from CSF, expressed as a percentage relative to the loss of Na (100%), was always in the same order: ^{24}Na (100) > PAH (82 ± 2) > sucrose (63 ± 3.5) > inulin (48 ± 8). Davson assumed that inulin was removed from CSF only by bulk absorption from the subarachnoid space; sucrose was removed at a higher rate because in addition to bulk absorption, it diffused into the extracellular space of the brain. Although the transfer rate of PAH from blood to CSF or brain was less than sucrose (Davson, 1955; Davson and Spaziani, 1959), PAH removal from CSF was always greater than sucrose. Davson suggested that PAH might be removed from CSF by a process requiring metabolic energy.

By perfusing the brain ventricular system of goats, Pappenheimer et al. (1961, 1962) estimated rates of bulk absorption

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of CSF from the subarachnoid spaces, diffusion of molecules through the ependymal linings and active transport of molecules out of CSF. Cannulas were implanted in the lateral ventricle and cisterna magna and an artificial goat CSF containing test molecules infused into the lateral ventricle and collected from the cisterna magna. Both the volume and steady-state concentrations of molecules in the inflow and outflow CSF were measured and the clearance of test molecules calculated by a formula analogous to that used to calculate renal clearance. At intraventricular pressures of -15 cm HOH, all inulin (M.W. = 5000) entering the lateral ventricles was recovered in the cisternal outflow, indicating that inulin does not diffuse from the ventricles at any detectable rate. Inulin is assumed to be removed mainly by bulk absorption from the subarachnoid spaces distal to the fourth ventricle and inulin clearance was used as a measure of bulk absorption. Bulk absorption was found to be dependent on intraventricular pressure since the clearance of inulin increased linearly with intraventricular pressures greater than -15 cm HOH. The diffusional loss of molecules ranging in weight from labeled water (^{3}HOH; M.W. = 20) to fructose (M.W. = 180) were estimated. As the ventricular pressure was increased, clearance of water, creatinine or fructose increased proportionally with the increase in inulin clearance. The amount of clearance due

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to diffusion could be estimated and was found to be independent of intraventricular pressure.

Diodrast was transported from CSF to blood or brain 3 times more rapidly than creatinine. If only molecular size is considered, creatinine (M.W. = 131) should leave CSF more rapidly than Diodrast (M.W. = 405). Transfer rates of Diodrast from CSF to blood were 15 times greater than from blood to CSF; elevation of plasma Diodrast concentration to twice that in the perfusate did not alter the transfer rate of Diodrast from CSF to blood. At elevated CSF concentrations of Diodrast, Pappenheimer et al. (1961) demonstrated saturation of the transport mechanism. The transfer rate of Diodrast from CSF plotted as a function of increasing perfusate concentration showed a 2-component curve: an active absorption component and a passive diffusion component. The amount of Diodrast diffusing from CSF increased linearly with concentration; the active absorption component was saturated at high Diodrast concentrations with a transport maximum (Tm) of 2.5 μg/min.

The ventriculocisternal perfusion technique has been used to demonstrate active transport of other molecules from CSF, namely: sulphate and iodide (Cutler et al., 1968), pertechnetate and iodide (Oldendorf et al., 1970), methotrexate (Rubin et al.,

1968) and morphine (Asghar and Way, 1970). Flux coefficients of sulphate out of CSF were 8 times greater than influx coefficients and plasma sulphate concentrations greater than those in CSF did not alter the efflux coefficient. Sulphate transport satisfied other criteria of active transport (Cutler et al., 1968): saturation of the transport mechanism at elevated CSF sulphate concentrations (4 mM/l) and reduction of the outflux coefficient by the addition of a competitive inhibitor (4 mM/1 thiosulphate) to the perfusate. The addition of 2, 4 dinitrophenol did not inhibit transport of sulphate, indicating that the transport process may not be dependent on oxidative energy.

Many functional and morphological changes occur in the brain with age. In the developing rat cerebral cortex, total brain water is inversely proportional to increases in protein and lipid concentrations due to the deposition of myelin and increase of cellular proteins (Vernadakis and Woodbury, 1962). Until 12 days postnatal, rat brain extraneuronal space consists mostly of interstitial fluid and a few glial cells. As brain weight increases, total brain chloride (Cl) concentration decreases and glial cell density doubles. Since Cl concentration inside the glial cell is less than Cl concentration of the extraneuronal space (Tasaki and Chang, 1958), replacement of extraneuronal space by glial cells could produce the observed decrease in total brain Cl concentration.

There is little information concerning the exchange of solutes between blood and CSF in the newborn animal. In contrast to adults, newborns show an increased permeability of certain molecules and dyes between CSF and blood or blood and brain. In kernicteric infants bilirubin in the blood penetrates the grey nuclear matter of the brain and enters the CSF (Bakay, 1956). Fries and Chaikoff (1941a, 1941b) injected ^{32}P subcutaneously in the developing rat and found that uptake by the liver, kidney, skeletal muscle and blood remained constant or increased slightly with age, while the uptake by the brain decreased. At birth ^{32}P uptake by all parts of the CNS was maximal but rapidly declined until animals weighed 50 g.

Cutler et al. (1968) observed that the sulphate carrier mechanism was poorly developed in the kitten but highly developed in the adult. In kittens and adult cats injected intravenously with 0.5 mC/kg sulphate-(^{35}S), steady-state CSF:plasma sulphate ratios were greater in the 2-week-old kitten (0.28 ± 0.02) than in the adult (0.07 ± 0.005). Perfusion of the ventricular system of adult cats and kittens with ^{35}S showed that influx coefficients for both kittens and adult cats were the same (0.003 ± 0.001 ml/min); however, efflux rates in adult cats were 3 times greater than efflux rates of the 2-week-old kitten. Cutler and his colleagues suggest

that higher CSF:plasma sulfate ratios in kittens may not be the result of an increased blood-CSF permeability but are due to decreased CSF efflux of sulphate.

The choroid plexus is a modification of the pia mater, located along the walls of the lateral cerebral ventricles and roof of the third and fourth ventricles, and is composed of both neuro-epithelial and vascular elements. The choroidal epithelium is modified cuboidal or columnar ependymal cells arranged in villi or folds around a core of highly vascularized connective tissue (Davson, 1967). Blood supply to the lateral ventricular choroid plexus (LVCP) is supplied by the anterior choroidal artery which is a branch of the internal carotid artery. The fourth ventricular choroid plexus (FVCP) is supplied by the posterior choroidal artery; a branch of the posterior cerebellar artery. The body or glomus of the plexus is supplied by a large capillary network (Crosby et al., 1962). Both the LVCP and FVCP are innervated by sympathetic nerves which terminate on the vascular smooth muscle and by sensory nerves which arise from the dorsolateral medulla (Kappers et al., 1960). Galen is reported to credit the discovery of the LVCP to Herophilus (c. 335-280 B.C.) (Dohrmann, 1970).

The cells of the choroid plexus are structurally similar to those of other actively secreting tissues of the body such as the

renal proximal tubule, intestinal villus and the ciliary body of the eye. The brush border of the choroidal epithelium is composed of microvilli as in both renal proximal tubule cells and cells on the surface of intestinal villi. Similar to the tubule epithelium of the kidney, the basilar membrane of the choroid plexus has many infoldings which interdigitate with those of adjacent cells (Davson, 1967).

Many morphological changes occur in the choroid plexus with age. In humans the primordial choroid plexus appears during the second month of gestation. At 6 weeks the primordial myelencephalic choroid plexus (FVCP) invaginates at the roof of the fourth ventricle and the telencephalic plexus (LVCP) projects into the lateral ventricles (Truex and Carpenter, 1969). Meningeal mesenchyme forms the blood and vascular system as well as the stroma of the plexus. At 6 weeks the epithelium is pseudostratified columnar. At 8 weeks the telencephalic plexus becomes enlarged and lobular and fills almost 75% of the ventricular cavity. Surface epithelium changes from pseudostratified to low columnar and the stroma appears as loosely organized gelatinous connective tissue containing an amorphous, mucoid ground substance. Few capillaries are present in the stroma and are parallel to and distant from the epithelium. By 4 months gestation age the size of the plexus

decreases and the stroma is reduced due to formation of fibrils which replace the gelatinous connective tissue. Increases in the size of the capillary bed and reduction of the stroma brings capillaries closer to the epithelial surface. At birth the choroid plexus is a large leaf-like process containing many capillaries which produce elevations in the epithelium resembling villi (Kaplan and Ford, 1966; Kappers, 1958).

Smith (1966) reported that the LVCP from 8- to 20-day-old chick embryos showed 2 morphological alterations: a change in the epithelium, at 9-16 days, from pseudostratified columnar to columnar; and a concomitant change in the number and position of mitochondria within the epithelial cells. She suggests that epithelial cell transformations may be caused by the LVCP descending from the cerebral hemispheres into the lateral ventricles by the twelfth day. At 8-9 days mitochondria appeared spherical and randomly distributed throughout the cytoplasm. As the plexus matured mitochondria became rod-shaped with well-developed cristae and they migrated to the brush border of the epithelial cell. Smith suggests that the location of mitochondria along the brush border could facilitate ATP production necessary for active carrier mechanisms between CSF and blood.

The choroid plexus has been suggested as one site for the production and regulation of CSF composition (de Rougement et al.,

1960; Welch et al., 1963). A comparison of hematocrits from choroidal arterial blood and venous blood draining the choroid plexus showed that as blood passed through the choroid plexus, plasma volume was reduced. From venous-arterial hematocrit ratios and venous blood flow, calculated from the velocity of a spherule of 1-octanol in the main choroidal vein and venous cross-sectional area, the CSF production for one lateral ventricular plexus was estimated at 2.6 μl/min (Welch et al., 1963). De Rougement et al. (1960) observed that the ionic composition of fluid collected from the surface of the cat choroid plexus closely resembled the ionic composition of CSF from the cisterna magna and the cisterna pericallosa. Cl concentrations were higher and K concentrations lower in fluid collected from the choroid plexus and cisterna magna than the concentrations measured in a dialysate of plasma.

The choroid plexus has been suggested as a site for the regulation of electrolyte and metabolite movement between blood and CSF. Increased or decreased plasma K concentration does not significantly alter the CSF K concentration (3.14 mM/kg HOH) (Bekaert and Demeester, 1954; Ames et al., 1965). Newly formed CSF from the LVCP showed a damped response to changes in plasma K concentrations below 3 mM/kg HOH: a 39% decrease in

plasma K concentration caused a 21% decrease in CSF K concentration. However, doubling plasma K concentration caused only a 9% increase in the K concentration of the newly formed fluid. Ames et al. (1965) suggest a transport mechanism for the regulation of CSF K concentration.

Coben (1969) reported active transport of iodide from CSF by the dog LVCP. Sodium iodide was injected intraperitoneally and after 2 hours, when blood iodide reached steady-state concentrations, the animals were sacrificed and the LVCP's removed. Iodide concentration, measured in the CSF and LVCP, was expressed as milligrams iodide per milliliter of CSF or plexus water. As plasma iodide concentrations increased, CSF iodide concentrations increased linearly. However, iodide uptake by the LVCP showed both a diffusion and active transport component and became saturated at plasma iodide concentrations of 0.5 mg/ml or greater. The diffusion component was calculated from the permeability coefficient of iodide and CSF iodide concentrations. The active component was calculated from the difference between the total uptake by the plexus and the passive diffusion component. The transport maximum for the active component was 0.99 mM/hr.

Table 1 lists molecules which are actively accumulated by the choroid plexus in vitro. The LVCP's and FVCP's were

incubated for 30 minutes to 2 hours at 37° C in an artificial CSF solution containing the test molecule and glucose as an energy source. Accumulation of the radioactively labeled test molecule is expressed as a tissue:medium concentration ratio:

$$\frac{T}{M} = \frac{\text{counts (CPM)/g tissue (wet wt.)}}{\text{counts (CPM)/ml medium}}$$

The tissue:medium ratio (T/M) is a measure of the concentration gradient established between the tissue and medium. If a molecule were distributed between the tissue and medium by passive diffusion alone, then the expected T:M ratio would indicate distribution in tissue water and would be less than 1.0 due to proteins and salts present within the tissue. Ratios greater than 1 indicate active accumulation against a concentration gradient (Cross and Tagart, 1950. T:M ratios of all molecules in Table 1 are greater than 1.0. Test molecules listed in Table 1 fulfill other criteria of active transport: metabolic dependency and competitive inhibition by structural analogues.

Choroid plexuses from adult rabbits, dogs and cats have been used to demonstrate active accumulation by this tissue of a wide variety of compounds ranging from small monovalent ions to relatively large compounds (Table 1). Active accumulation of

organic anions such as PAH, phenolsulfonphthalein, Diodrast and penicillin have not been studied in the in vitro choroid plexus, although active transport of certain organic anions have been reported in vivo (Pappenheimer et al., 1961). In a few studies, T:M ratios of the rabbit choroid plexus are higher than T:M ratios of dog plexuses for the same molecule (Hug, 1967; Tochino and Schanker, 1965a). Only in the rabbit and with only a few molecules has active accumulation of the FVCP been studied. With the exception of 1 molecule (5-hydroxyindoleacetic acid) T:M ratios in the FVCP are less than ratios in the LVCP. Within and among groups of molecules there is a wide range of T:M ratios. For example, 2 tertiary amines, morphine and dextrophan, have T:M ratios which are 10 fold different (37.2 and 3.1, respectively; Table 1). Differences in T:M ratios may be due to more transport sites available to certain molecules, different transport rates for different molecules or different affinities of molecules for the same transport mechanism. The T:M ratio is a function of medium concentration; and since the T:M ratios are not reported either at saturation concentration or even at the same medium concentration, T:M ratios of different molecules are not comparable.

The in vitro choroid plexus shows a development of active transport mechanisms with age. LVCP's and FVCP's (from rabbits

at various ages incubated for 1 hour in medium containing 1 mM radioactively labeled morphine) actively accumulate morphine (Asghar and Way, 1970). The FVCP T:M ratio decreased with increasing age (T/M = 4.3, newborn; T/M = 2.6, adult); the LVCP T:M ratio also decreased with age (T/M = 4.9, newborn; T/M = 3.1, adult), but the 15-day LVCP accumulated more morphine (T/M = 9) than plexuses at other ages. In contrast, uptake of sulphate by isolated LVCP of fetal rabbits (T/M = 1.7) was significantly lower than adult LVCP's (T/M = 2.5) but maximal at 3-10 days postnatal (T/M = 3.2) (Robinson et al., 1968). Decreased transport of sulphate in the immature animal could lead to higher sulphate concentrations in brain extracellular fluid. This may be advantageous to the immature animal since myelination requires sulphate for synthesis of sulphatides. Robinson et al. (1968) reported iodide transport was well developed in the fetus (T/M = 60, rabbits; T/M = 45, cats) and significantly lower in the adult (T/M = 25, rabbits, cats). These adult iodide T:M ratios correspond to those reported by Becker (1961) (Table 1). Iodide transport in the fetal choroid plexus appears to be better developed than sulphate transport.

Many functions of the liver and kidney are not fully developed at birth. In the liver, activity of glycolytic and drug-metabolizing

enzymes is low at birth but reaches adult activity in the neonate. Increased enzymatic activity after birth could be cause by substrate-induced stimulation of enzymes (Dawkins, 1966). By challenging the kidney with penicillin, an organic anion, during the period of development, Hirsch and Hook (1969a) stimulated maturation of PAH transport. In littermate rabbits, 60,000 IU procaine penicillin G was administered subcutaneously bidaily for 3 days to 2- to 4-week-old animals. Kidney cortical slices were incubated in vitro with PAH in the medium and PAH accumulation by the tissue expressed as T:M PAH concentration ratios. In 2-week-old treated animals, PAH accumulation was increased 4 times over nontreated animals but in 4-week-old animals there was no difference between control and treated animals. Apparently transport development was complete by 4 weeks of age. Other organic anions, folic acid and triiodothyronine stimulated PAH accumulation by cortical slices in young rats and rabbits (Hirsch and Hook. 1969a, 1969b, 1969c). Presumably stimulation of organic anion transport was selective since the uptake of N-methylnicotinamide, an organic base, was not increased by prior treatment with penicillin (Hirsch and Hook, 1970).

Table 1. --Molecules actively transported by the choroid plexus.

Compound	T/M Ratio		Animal (Adults)	Reference
	LVCP	FVCP		
Purines				
Xanthine	2.81 ± 0.31		Rabbit	Berlin, 1969
Amines: Primary				
Serotonin	14.4 ± 2.4	9.0 ± 2.1	Rabbit	Tochino & Schanker, 1965b
Norepinephrine	16.1 ± 1.6	10.1 ± 2.4	Rabbit	Tochino & Schanker, 1965b
Amines: Tertiary				
Dihydromorphine	6.6 ± 1.4		Rabbit	Hug, 1967
	3.0		Dog	Hug, 1967
Morphine ^{14}C	5.1 ± 0.6		Rabbit	Hug, 1967
	3.1 ± 0.4	2.6 ± 0.25	Rabbit	Asghar & Way, 1970
Codeine ^{3}H	16.6 ± 1.3		Rabbit	Asghar & Way, 1970
Dextrophan ^{3}H	37.2 ± 7.8		Rabbit	Asghar & Way, 1970
Levorphan ^{3}H	12.7 ± 1.8		Rabbit	Asghar & Way, 1970

Quarternary Ammonium				
Hexamethonium	25.4 ± 0.2		Rabbit	Tochino & Schanker, 1965a
	3.3 ± 0.4		Dog	Tochino & Schanker, 1965a
Decamethonium	7.1 ± 0.5		Rabbit	Tochino & Schanker, 1965a
N' -methlynicotinamide	6.2 ± 0.5		Rabbit	Tochino & Schanker, 1965a
Organic Acids				
5' hydroxyindole-acetic acid	8.2 ± 0.8	11.7 ± 0.9	Rabbit	Cserr & Van Dyke, 1971
Amino Acids				
AIB (Aminoisobutyric acid)	3.0 ± 0.2		Cat	Lorenzo & Cutler, 1969
Anions: Monovalent				
Iodide	25.6 ± 6.2		Rabbit	Becker, 1961
	25		Rabbit	Robinson et al., 1968
Anions: Divalent				
Sulphate	2.5		Rabbit	Robinson et al., 1968
	2.5		Cat	Robinson et al., 1968

METHODS

Organic Anion Accumulation by the Choroid Plexus In Vitro

A. Experimental preparation

1. Animals and tissue removal

All animals were obtained from the Michigan State University Center for Laboratory Animal Resources (C.L.A.R.). Dogs 1 and 2 weeks old were decapitated; dogs older than 3 weeks were anesthetized with either chloroform (inhalation) or sodium pentobarbital (60 mg/kg i.v.; Abbott Laboratories, North Chicago, Ill.) and decapitated. The skin and muscles of the head and neck were retracted, the calvaria removed and the dura mater retracted. Two incisions in the cerebral hemispheres were made 5 mm lateral and on each side of the midline exposing the left and right lateral ventricles; the cerebellum was retracted exposing the fourth ventricle. Both the lateral and fourth ventricular choroid plexuses were excised and immediately placed into separate beakers containing 3 ml of incubation medium. Diaphragm muscle (approximately

10 mg) obtained from a midline incision, caudal to the rib cage, was removed and placed into incubation medium to serve as a control tissue.

2. Incubation medium composition

The incubation medium consisted of a buffered salt solution (artificial CSF; Appendix 1) containing 0.5 mg/ml mannitol, p-aminohippuric acid (PAH; 2-20 μg/ml), 0.1 μc/ml PAH (glycyl-2-^{3}H) and 0.01 μc/ml D-mannitol-1-^{14}C (New England Nuclear Corp., Boston, Mass.).

3. Incubation procedure and preparation for analysis

Incubations were performed by shaking (50 times/min) in a water bath at 37° C for 60 minutes in an atmosphere of 95% oxygen and 5% carbon dioxide. At the end of the incubation period the tissue was removed, dipped into non-radioactive artificial CSF and excess fluid removed by blotting on absorbent paper. Each choroid plexus and piece of diaphragm muscle was weighted on a Mettler balance (sensitivity = ± 0.01 mg; model B6; Mettler Instrument Co., Princeton, New Jersey) and homogenized in 0.5 ml of 10% trichloroacetic acid using a glass rod in a 3 ml centrifuge tube and placed on a vortex mixer (model S8220; Scientific Products,

Allen Park, Mich.). The homogenate was centrifuged at 2000g for 10 minutes and duplicate 0.1 ml samples of supernatant and incubation medium were added to 10 ml of scintillation fluid (Aquasol; New England Nuclear Corp., Boston, Mass.). Tritium and carbon-14 were counted differentially in a trichannel liquid scintillation spectrometer (model Mark I; Nuclear Chicago Corp., Des Plaines, Ill.) (Appendix 6). Non-radioactive PAH concentrations were determined colorimetrically (Appendix 2).

4. Tissue:medium ratio

Accumulation of mannitol or PAH by the choroid plexus or diaphragm muscle is expressed as a tissue:medium concentration ratio (T/M) where:

$$\frac{T}{M} = \frac{\text{dpm/g tissue (wet wt.)}}{\text{dpm/ml medium}}$$

Numerical calculations of T:M ratios were performed on the Michigan State University CDC 6500 computer (Appendix 7).

B. Effect of metabolic inhibitors on PAH accumulation

Sodium cyanide or iodoacetic acid was added to the incubation medium to determine whether metabolic energy is required for the accumulation of PAH by the choroid plexus. Medium concentrations

of PAH and mannitol were 20.6 μM/l and 2.7 mM/l respectively. Inhibitor concentrations in the medium were 1×10^{-3} and 5×10^{-4} M/l sodium cyanide and 1×10^{-2} M/l iodoacetic acid.

C. Effect of competitive inhibitors on PAH accumulation

Competitive inhibition of PAH accumulation by the choroid plexus was studied by incubating the tissue in incubation medium to which was added 2, 4 dinitrophenol (1×10^{-4} M/l; DNP), iodopyracet (7×10^{-4} M/l; Diodrast; Winthrop Laboratories, Inc., New York, New York), PAH (30-295 μM/l) or crystalline penicillin G (12,500-50,000 international units (IU)/l; potassium salt; Parke Davis Co., Detroit, Mich.). Medium concentrations of PAH and mannitol were 20.6 μM/l and 2.7 mM/l respectively. Since 50,000 IU penicillin G contains 79 meq/l K, KCl equivalent to the amount of K present in the penicillin preparation was added to the control incubation medium.

D. Effect of penicillin treatment on PAH accumulation

PAH accumulation was studied in choroid plexuses taken from 1- and 2-week-old littermate dogs previously injected with penicillin. Young animals were housed in C.L.A.R. facilities with their mother. From 12 mongrel pups, 4 animals were injected

intramuscularly (i.m.) with 75,000 IU/kg procaine penicillin G (Duracillin; Eli Lilly and Co., Indianapolis, Ind.) bidaily and 4 animals were injected with 150,000 IU/kg bidaily beginning on the fourth postnatal and continuing through 7 days of age (1 week); the other 4 animals served as controls, receiving an equivalent volume of saline intramuscularly. Six Labrador retriever pups were injected with procaine penicillin G (60,000 IU/kg i.m.; bidaily) beginning on the eighth day postnatal and continuing for 7 days (2 weeks) and another 6 Labrador pups received an equivalent volume of saline i.m. (controls). Animals were sacrificed 24 hours after the last injection and the choroid plexus' ability to accumulate PAH was compared in the 2 groups of animals (penicillin-treated and controls).

Organic Anion Transport from the Brain Ventricular System

A. Experimental preparation

1. Surgical procedure

Mongrel adult dogs (approximately 5-12 kg) of either sex were obtained from the Michigan State University C.L.A.R. and anesthetized with sodium pentobarbital (60 mg/kg i.v.; Abbott Laboratories, North Chicago, Ill.) or intraperitoneally with Dial urethane (0.6 mg/kg; Appendix 5). Femoral arterial pressure was

monitored using a Statham pressure transducer (model P23 DC; Grass Instrument Co., Quincy, Mass.) and a Grass model 5D polygraph (Grass Instrument Co., Quincy, Mass.). The arterial pressure transducer was calibrated using a mercury manometer; the response to pressure was linear over the range 0-200 mmHg. The animal's ventilation was controlled throughout the experiment by means of a respiratory pump (model 607; Harvard Apparatus Co., Dover, Mass.) connected to a plastic "Y" tube in the trachea; the third arm was open to the air and fitted with a clamp for adjusting lung inflation. The respiratory pump was set to cycle 10-12 times/min with a stroke volume of 200-250 ml.

The dog's head was secured in a model 1504 stereotaxic frame (David Kopf Instrument Co., Tujunga, Calif.) by means of ear bars (inserted into the external auditory meatus) and a clamp which secured the dog's snout. The animal's neck was flexed so that the parietal surface of the head was tilted downward at an angle of 30° from the horizontal position. A midline skin incision was made extending from a point 5 cm caudal to the orbital sockets to the second cervical vertebra. Muscles were retracted with cauterization and the parietal and occipital bones exposed. A $\frac{1}{4}$-inch trephine hole in the skull, at a point 5 mm rostral and 5 mm lateral to the junction of the central saggital and coronal sutures, exposed the dura mater overlying the left cerebral hemisphere.

2. Implantation of Cannulas

The cisterna magna was penetrated with a 20-gauge, 2-inch, short bevel pointed tube (Vita Needle Co., Needham, Mass.) held in a micromanipulator (model MM-3; Eric Sobotka, Inc., Farmingdale, New York) and directed rostrally and parallel to the top of the skull at the midline. A piece of PE-90 tubing led from the needle to a syringe. The needle was first quickly lowered to a depth 5-8 mm beneath the atlantooccipital membrane and then slowly withdrawn until cerebrospinal fluid could be withdrawn. Collodion (Mallinckrodt Chemical Co., St. Louis, Missouri) or dental acrylic (Hygienic Dental Mfg. Co., Akron, Ohio) was used to seal any leaks around the cisternal needle.

A 22-gauge, $1\frac{1}{2}$-inch, thin wall disposable needle held in a model 1270 standard electrode holder (David Kopf Instrument Co., Tujunga, Calif.) was used to penetrate the lateral cerebral ventricle. The needle was directed ventrally and normal to the dura mater. Anterior-posterior and lateral coordinates for the puncture (referenced to the junction of the saggital and coronal sutures) were 5 mm caudal and 5 mm lateral. The ventricular needle was connected by a male "T" adaptor and PE-50 tubing to a constant syringe drive pump (model 975; Harvard Apparatus Co., Dover, Mass.) and to a Statham pressure transducer (model P23 AC; Grass Instrument Co.,

Quincy, Mass.). The pressure transducer was calibrated using a water reservoir; output was linear over the range 0-40 cm HOH; zero pressure was referenced to the level of the stereotaxic earbars. While artificial CSF was pumped through the ventricular needle, pressure (representing the resistance of the inflow needle and tubing) was recorded with the tip of the needle on the dura mater. As the needle was lowered slowly through the dura mater and brain tissue, perfusion pressure rose; with the puncture of the lateral ventricular cavity, there was an abrupt fall in pressure. Injection and withdrawal of fluid from the syringe connected to the cisternal cannula resulted in pressure increases and decreases respectively and indicated connection between the ventricular and cisternal cannulas. The PE tubing from the outflow cannula was connected to a photoelectric drop recorder (model PTT1; Grass Instrument Co., Quincy, Mass.) for monitoring outflow rate. Intraventricular pressure was calculated by subtracting the pressure with the needle tip on the dura from that with the needle tip in the lateral ventricle. Intraventricular pressure could be varied by adjusting the height of the outflow tubing.

3. Perfusion fluid composition

The perfusion fluid consisted of an artificial dog CSF (Appendix 1) containing: inulin (1.0 mg/ml), mannitol (0.5 mg/ml),

carbon-14 labeled mannitol (D-mannitol-1-^{14}C; 0.01 μc/ml; New England Nuclear Corp., Boston, Mass.), PAH (2-80 μg/ml), tritiated PAH (glycyl-2-^{3}H; 0.1 μc/ml; New England Nuclear Corp., Boston, Mass.) and in some experiments creatinine (1.0-2.0 mg/ml).

4. Perfusion technique

Perfusion fluid was pumped at rates of 170-240 μl/min into the lateral cerebral ventricle. Perfusion rate varied between experiments but was constant in any one experiment. Inflow rate was determined gravimetrically by collecting fluid from the perfusion syringe over timed periods (10-20 minutes) in tared vials at the beginning and end of the experiment. Outflow rate was determined by collecting effluent from the cisternal cannula over 3 timed periods (10-20 minutes) in tared vials. Inflow and outflow concentrations were determined on aliquots from the inflow syringe and from outflow vials, respectively.

B. Effect of inhibitors on PAH transport

Inhibition of PAH transport from the ventriculocisternal system was studied by using perfusion fluid containing: iodopyracet (0.07-7.0 mg/ml; Diodrast); PAH (80.0-8000 μg/ml) or crystalline penicillin G (10,000 IU/ml; potassium salt).

C. Transport of molecules from CSF

1. Clearance

The clearance of large molecules such as inulin from CSF is calculated by an equation described by Heisey et al. (1962):

$$C_{in} = \frac{\dot{V}_i c_i - \dot{V}_o c_o}{c_o} \qquad (1)$$

where: $\dot{V}$ = rate of flow, μl/min

c = concentration, quantity/ml

i, o = subscripts refer to inflow and outflow

C_{in} = clearance of inulin, μl/min

For small molecules which leave the ventricular system by diffusion or active transport, clearance is calculated by:

$$C_x = \frac{\dot{V}_i c_i - \dot{V}_o c_o}{\bar{c}} \qquad (2)$$

where: C_x = clearance of the molecule, μl/min

$\bar{c}$ = mean ventricular concentration;

estimated by: $\bar{c} = \frac{c_i - c_o}{\ln(c_i/c_o)}$

2. Efflux coefficient

Molecules smaller than inulin leave the ventricular system by active transport or passive diffusion in addition to bulk absorption. The outflux coefficient represents the clearance of any molecule from CSF by means other than bulk absorption. The bulk absorption component is estimated by the clearance of inulin times the outflow concentration of the test molecule divided by its mean ventricular concentration $\left[\frac{C_{in}c_o}{\bar{c}}\right]$. The efflux coefficient is calculated by an equation described by Heisey et al. (1962):

$$K_o = \frac{\dot{V}_i c_i - \dot{V}_o c_o - C_{in} c_o}{\bar{c}} \qquad (3)$$

where: K_o = outflux coefficient, μl/min

c = concentration of the small molecule

C_{in} = clearance of inulin

Statistical Analysis

All data obtained were subjected to statistical analysis using analysis of variance, Student-Newman-Keul's test, student's "t," paired or group comparisons. The 0.05 level of probability was used as the criteria of significance in all statistical tests.

RESULTS

A. In vitro studies

Accumulation of PAH and mannitol was studied in the lateral ventricular choroid plexus (LVCP) and fourth ventricular choroid plexus (FVCP) from 1-week, 2-week, 3- to 4-week postnatal and adult dogs and in diaphragm muscle from 1- to 4-week dogs (Table 2). Medium concentrations were 2.7 mM/l mannitol and 9.7 μM/l PAH. For all ages diaphragm PAH and mannitol T:M ratios are less than 1.0 (Table 2). Within each age group there is no difference ($p < 0.05$) between T:M PAH and T:M mannitol ratios and there is no change in either ratio with age.

The LVCP T:M mannitol ratios are not significantly greater ($P > 0.05$) than 1.0. Shown graphically in Figure 1, the LVCP T/M mannitol decreases with increasing age; the 3- to 4-week and adult T/M mannitol are significantly less ($p < 0.05$) than those at 1 week postnatal. For all ages LVCP T:M PAH ratios are significantly greater ($p < 0.05$) than 1.0 and greater than those of mannitol. At 1 week postnatal the LVCP T/M PAH is approximately 2.0, increases to 6.0 at 2 weeks and then decreases

to 2.5 in the adult. The adult T/M PAH is not significantly greater ($p > 0.05$) than the ratio at 1 week postnatal.

The FVCP T:M mannitol ratio is not significantly greater ($p > 0.05$) than 1.0 (Table 2; Figure 2) and shows an apparent decrease with increasing age, although only the adult T/M mannitol is significantly less ($p < 0.05$) than the ratio at 1 week. The FVCP T:M PAH ratio does not change significantly ($p > 0.05$) with age and remains at approximately 1.8. The LVCP T/M PAH at 2-4 weeks is greater than those of the FVCP. At 1 week postnatal and in the adult LVCP and FVCP T/M PAH are not significantly different ($p > 0.05$) from each other and there is no difference ($p > 0.05$) in T/M PAH between the 1-week-old plexus and the adult.

Histological sections of choroid plexuses from 1-day-old and adult dogs showed structural changes with age in both the LVCP and FVCP. Figure 3 is a photograph (magnification = 287×) of a histological section of the FVCP from 1-day postnatal (left) and adult (right) dogs. The epithelium of the 1-day FVCP (left) is composed of columnar epithelium, approximately 15 μ in height with a large, centrally located nucleus. The majority of the plexus is composed of a loosely organized, gelatinous connective tissue with few fibroblasts and fibrocytes present (16-24/100 μ^2). Blood vessels are few and are distant from the epithelial surface (8-100 μ).

In contrast there are an increased number of nuclei in the adult FVCP (right), indicating an increased number of cells. The height of the epithelial cells is reduced (7-8 μ) and the cells are low columnar or cuboidal epithelium. The epithelial cell nuclei are located near the base of the cell, leaving more cytoplasm apically: a feature similar to the proximal tubule cell of the kidney (Davson, 1967). The stromal volume is greatly reduced and is composed of densely packed connective tissue. Compared to the 1-day-old plexus, the vacuolated appearance of the stroma is absent and there are large numbers of fibroblasts and fibrocytes (40/100 μ^2) which indicates proliferation and production of collagen and other components of connective tissue (Greep, 1966). In the adult, blood capillaries are more numerous and lie closer to the epithelial surface; the distance between the capillary endothelium and epithelial basement membrane being only 3-5 μ.

These histological changes with age suggest that as the plexus matures, the space into which mannitol can diffuse decreases. A decreasing T/M mannitol in both the LVCP and FVCP (Figures 1 and 2) presumably represents a decreasing extracellular space in the maturing plexus. In order to compare PAH accumulation between plexuses of different ages, PAH accumulation will be expressed as T/M PAH/T/M mannitol (PAH/MAN).

Data in Table 3, shown graphically in Figure 4, shows PAH accumulation (PAH/MAN) of both the LVCP and FVCP for different aged dogs. Medium concentration of mannitol and PAH were 2.7 mM/l and 9.7 μM/l respectively. Although the FVCP shows an apparent increase in PAH accumulation with increasing age, only the adult PAH/MAN is significantly greater ($p < 0.05$) than at 1 week postnatal. The 2-, 3- to 4-week and adult PAH/MAN of the LVCP are significantly greater ($p < 0.05$) than those at 1 week and accumulation appears to be well developed at 2 and 3-4 weeks (PAH/MAN = 6.0). There is no difference ($p > 0.05$) between the PAH/MAN of the FVCP and LVCP in either the adult or 1-week plexus. However, the adult LVCP and FVCP PAH/MAN are significantly greater ($p < 0.05$) than those at 1 week postnatal.

T:M ratios greater than 1.0 are suggestive of active transport. Further evidence for transport of PAH by the choroid plexus was: 1) saturation at high medium concentrations; 2) competitive inhibition and 3) inhibition by metabolic blocking agents. Data in Table 4 shows effect of medium concentration on PAH accumulation (T/M PAH) of the LVCP and FVCP from littermate 3-week-old dogs. T/M mannitol values were low, resulting in high PAH:MAN ratios (Figure 5) but T/M mannitol remained constant (T/M = 0.42) over the range of PAH concentrations used. As the medium

concentration of PAH increased, PAH/MAN of the LVCP decreased approximately from 20 at 29 μM PAH/l to 3.2 at 294 μM PAH/l (Figure 5). PAH uptake by the FVCP, although less than that of the LVCP, also decreased from approximately 8 to 2.8 over the same range of medium PAH concentrations. This decrease in PAH accumulation is non-linear and at high PAH concentrations (> 200 μM/l) transport appears saturated and the amount of accumulation by the LVCP and FVCP is approximately equal.

Results of adding metabolic and competitive inhibitors to the incubation medium are shown in Table 5. The dogs were 3 weeks, 6 weeks postnatal or older. The medium PAH concentration was 20.1 μM/l and mannitol concentration was 2.7 mM/l. Competitive inhibition of PAH transport was demonstrated using two organic anions (Diodrast and penicillin G) and 2, 4 dinitrophenol (DNP). Diodrast (7×10^{-4}M) completely inhibited PAH accumulation in both the LVCP and FVCP, decreasing the PAH:MAN ratio to 1.0. Penicillin G was also an effective inhibitor over a wide dosage range (12,500-50,000 IU) and appeared to inhibit completely both the LVCP and FVCP. Large variations in penicillin control PAH:MAN ratios are possible caused by high medium concentrations of potassium. An uncoupler of oxidative phosphorylation, 2, 4 DNP has been shown to be a competitive inhibitor of PAH

transport in the kidney (Huang and Lin, 1965) and at a medium concentration of 1×10^{-4}M appeared to inhibit the FVCP (96% inhibition) to a greater extent than the LVCP (74% inhibition). Two metabolic inhibitors produced different effects on PAH accumulation: sodium cyanide, an inhibitor of the electron transport system, produced no inhibition of PAH accumulation in either the LVCP or FVCP at medium concentrations of 5×10^{-4}M or 1×10^{-3}M; iodoacetic acid, an inhibitor of glycolysis, decreased the LVCP PAH:MAN ratio from 7.2 to 1.3 and the FVCP PAH:MAN from 4.2 to 1.0. Iodoacetic acid is also an organic acid and may be acting as a competitive inhibitor of PAH transport.

Hirsch and Hook (1969a, 1969b, 1969c, 1970) showed that organic anion transport in renal cortical slices could be induced in immature kidneys by prior treatment of the animal with penicillin. A similar study was performed using the choroid plexus to determine if PAH accumulation could be induced by prior treatment of animals with penicillin G (Table 6). Incubation medium concentration was 9.7 μM/l PAH and 2.7 mM/l mannitol. LVCP's from dogs 1 week old, previously treated with 300,000 IU penicillin G/kg for 7 days, showed a significant increase ($p < 0.05$) in PAH accumulation (PAH/MAN) over saline injected animals. However, at twice the dosage (600,000 IU/kg) there was no difference ($p > 0.05$) in the

PAH/MAN between control and penicillin-treated animals. FVCP's from the same animals showed no increase in PAH uptake by treatment with either 300,000 or 600,000 IU/kg. Two-week-old dogs, previously injected bidaily with 60,000 IU penicillin G/kg for 7 days, showed no significant increase ($p > 0.05$) of PAH accumulation by the LVCP. However the PAH/MAN of the penicillin-treated FVCP (2.76 ± 0.31) was significantly greater than the control FVCP PAH/MAN (1.80 ± 0.37) ($p < 0.05$).

B. In vivo studies

Intraventricular pressure is an important determinant of the clearance of any molecule from CSF since it determines the rate at which a molecule is removed from the ventricular system by bulk absorption. The importance of intraventricular pressure on PAH clearance will be shown later. Intraventricular pressure was varied by adjusting the height of the cannula draining the cisterna magna. Data obtained from 27 animals and shown in Figure 6 show a linear relationship between calculated intraventricular pressure and outflow cannula height. The slope of the line is 0.65 cm HOH pressure/cm change in outflow cannula height, indicating the magnitude of error associated with estimating intraventricular pressure. Intraventricular pressures shown in Table 7 were estimated from this figure.

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The brain ventricular system of the dog was perfused with an artificial dog CSF containing inulin, mannitol, creatinine and PAH in order to compare the relative rates of removal of these substances from the CSF. Table 7 contains measured and calculated quantities from ventriculocisternal perfusions in 10 adult dogs. Radioactive counts of mannitol correspond to 0.25 mg/ml mannitol; the first 4 experiments ($\alpha 2 - \beta 4$) contained no radioactive material. Clearance of inulin, calculated using Equation 1 (Methods); mannitol, creatinine and PAH, calculated using Equation 2 (Methods) and outflux coefficients (K_o) of mannitol, creatinine and PAH were calculated using Equation 3 (Methods) between 30 - 90 minutes perfusion time.

Figure 7 illustrates calculated inulin, mannitol and PAH clearances as a function of intraventricular pressure. Lines through these points were fitted by the method of linear regression and the slopes of all 3 lines are significantly greater ($p < 0.05$) than zero. The clearance of inulin decreases linearly from 85 μl/min at 10 cm HOH pressure to almost zero at -15 cm HOH. Although mannitol clearance is always greater than inulin clearance, the slope of the regression lines for mannitol and inulin are not significantly different from each other ($p > 0.05$). Over the pressure range -15 cm HOH to +15 cm HOH, PAH clearance is always greater than that of inulin or mannitol and the slope of PAH clearance is significantly greater

($p < 0.05$) than that of inulin, decreasing from 150 μl/min at 10 cm HOH to 15 μl/min at -15 cm HOH.

Mannitol and PAH efflux coefficients (K_o; Table 7; representing movement from the ventricular system by means other than bulk absorption) are plotted as a function of intraventricular pressure (Figure 8). Efflux coefficients were calculated between 30 - 90 minutes perfusion time. The slope of the mannitol efflux is not significantly greater ($p > 0.05$) than zero, indicating that the removal of mannitol is independent of intraventricular pressure. However, the slope of PAH efflux is significantly greater ($p < 0.05$) than zero and increases linearly from 10 μl/min at -15 cm HOH intraventricular pressure to 87 μl/min at 10 cm HOH. These data indicate that the efflux of mannitol, a passively diffusing molecule, from CSF is independent of intraventricular pressure, whereas the outflux of PAH in low concentrations (< 10 μg/ml) is pressure dependent.

In 5 experiments (γ-3, γ-4, γ-6, Δ-12, Δ-17; Table 7) the ventricular system was perfused simultaneously with inulin, mannitol, creatinine and PAH. Clearance of these molecules was always in the same order: Creatinine > PAH > mannitol > inulin. Efflux coefficients of these molecules were: Creatinine (46 ± 4 μl/min) > PAH (34 ± 4 μl/min) > mannitol (16 ± 8 μl/min) (Figure 9).

Efflux coefficients of both creatinine and PAH are significantly greater than that of mannitol ($p < 0.05$), suggesting some mechanisms other than passive diffusion for their removal.

Outflux coefficients of PAH were always greater than those of mannitol, suggesting active transport. To test for an active process, various competitive inhibitors were added to the perfusion: PAH, Diodrast and penicillin G. Table 8 contains measured and calculated quantities from ventriculocisternal perfusions in 4 adult dogs in which inhibitors were added to perfusion fluid. Radioactive concentrations of mannitol correspond to 0.25 mg/ml mannitol. Clearance of inulin and outflux coefficients of mannitol and PAH were calculated by Equations 1 and 3 respectively (Methods). The efflux coefficient of mannitol, a passively diffusing molecule, does not remain constant between periods. In order to compare PAH efflux between periods, PAH efflux was expressed as a ratio to that of mannitol (K_oPAH/K_oMAN).

The addition of increasing amounts of PAH (5 - 8,000 μg/ml) to the perfusion fluid inhibited transport of PAH out of CSF. At PAH concentrations less than 88 μg/ml (dog, Δ12) PAH efflux coefficients are always greater than those of mannitol and K_oPAH:K_oMAN ratios are approximately 2.0. At a perfusion concentration of 150 μg/ml PAH, the ratio decreased to 1.7. PAH concentrations

greater than 80 μg/ml (dog, sat. 2) showed a large decrease in the K_oPAH:K_oMAN ratio, PAH outflux approaching that of mannitol. The ratio decreased from 8.2 at 80 μg/ml PAH to 0.7 at 800 μg/ml and 1.0 at 8,000 μg/ml. Thus PAH transport is probably saturated between 150 μg/ml and 800 μg/ml PAH since K_oPAH/K_oMAN between these 2 concentrations approaches 1.0.

With perfusion concentrations of mannitol and PAH remaining constant throughout the experiment (0.25 mg/ml and 10 μg/ml, respectively), a series of 10 fold increases in Diodrast concentration were added to the perfusion fluid (Table 8; Figure 10). With the addition of 0.07 mg/ml Diodrast, PAH outflux decreased from 83 μl/min during the control period to 62 μl/min in period II. Diodrast concentrations of 0.7 and 7.0 mg/ml result in PAH outfluxes of 67 μl/min and 42 μl/min respectively. K_oPAH/K_oMAN also decreased from 4.9 to 1.6 upon the addition of 0.07 mg/ml Diodrast and with the addition of greater concentrations of Diodrast this ratio approached 1.0, indicating inhibition of PAH transport.

The addition of 10,000 IU penicillin G/ml to the perfusion fluid produced only slight inhibition of PAH transport (Table 8; dog △16). Perfusion fluid concentrations of mannitol (0.05 mg/ml) and PAH (10 μg/ml) remained constant throughout the experiment. Both mannitol and PAH outfluxes increased slightly on the addition

of penicillin to the perfusion fluid; K_oPAH/K_oMAN decreased from 1.45 to 1.24.

Table 2. --T/M ratios of PAH and mannitol of choroid plexuses and diaphragm from different aged dogs.

Age	LVCP		FVCP		Diaphragm	
	T/M PAH	T/M Mannitol	T/M PAH	T/M Mannitol	T/M PAH	T/M Mannitol
1 week	2.08 ± 0.28(20)	1.20 ± 0.14(20)	1.71 ± 0.26(10)	1.23 ± 0.37(10)	0.75 ± 0.07(10)	0.88 ± 0.05(10)
2 weeks	6.19 ± 0.75(12)	1.02 ± 0.08(12)	1.58 ± 0.14(6)	1.07 ± 0.27(6)	0.87 ± 0.00(6)	0.90 ± 0.03(6)
3-4 weeks	4.80 ± 0.91(18)	0.84 ± 0.16(18)	1.95 ± 0.22(9)	1.00 ± 0.18(9)	0.68 ± 0.04(9)	0.75 ± 0.06(9)
Adult	2.47 ± 0.25(10)	0.68 ± 0.07(10)	1.91 ± 0.48(6)	0.51 ± 0.04(6)		

T/M values are expressed as the mean ± S.E.M.; number of observations in parentheses.

Table 3. --PAH/MAN of choroid plexuses from different aged dogs.

Age	PAH/MAN*	
	LVCP	FVCP
1 week	2.12 ± 0.37 (20)	1.97 ± 0.47 (9)
2 weeks	6.26 ± 0.71 (12)	1.80 ± 0.37 (6)
3-4 weeks	6.34 ± 1.00 (18)	2.44 ± 0.46 (9)
Adult	4.04 ± 0.55 (10)	3.84 ± 1.00 (6)

* PAH/MAN values expressed as mean ± S.E.M.; number of observations in parentheses.

Table 4. --Effect of medium PAH concentration on PAH accumulation by the 3-week-old dog choroid plexus.

Medium PAH Concentration (μM/l)	LVCP		FVCP	
	T/M PAH	T/M MAN	T/M PAH	T/M MAN
28.8	11.81	0.48	2.41	0.45
	7.21	0.34		
	11.38	0.59	3.90	0.42
	6.87	0.44		
57.7	6.53	0.30	1.86	0.41
	5.04	0.32		
87.5	4.53	0.36	1.32	0.27
	3.94	0.31		
146.7	3.51	0.44	1.72	0.57
	3.75	0.49		
293.6	1.65	0.46	1.35	0.49
	1.32	0.45		

Table 5. --Inhibition of PAH accumulation in the dog choroid plexus.

Inhibitor (n)	Concentration	Age of Dog	PAH/MAN (mean ± S. E. M.)		Inhibition (%)
			Control	Experimental	
Diodrast					
LVCP (3)	7×10^{-4} M	3 weeks	4.98 ± 1.68	1.22 ± 0.09	95
FVCP (3)	7×10^{-4} M	3 weeks	3.13 ± 0.62	0.85 ± 0.18	100
Penicillin G					
LVCP (1)	50,000 IU	6 weeks	18.2	0.5	100
(1)	25,000 IU	6 weeks	16.9	1.0	100
(1)	12,500 IU	6 weeks	5.8	0.5	100
FVCP (1)	50,000 IU	6 weeks	2.4	0.8	100
(1)	25,000 IU	6 weeks	2.2	0.7	100
(1)	12,500 IU	6 weeks	1.8	0.5	100
Dinitrophenol					
LVCP (4)	1×10^{-4} M	> 6 weeks	7.60 ± 1.46	2.74 ± 0.96	74
FVCP (4)	1×10^{-4} M	> 6 weeks	5.39 ± 1.17	1.36 ± 0.36	91
Sodium Cyanide					
LVCP (4)	5×10^{-4} M	3-4 weeks	6.45 ± 1.48	11.18 ± 1.37	0
(4)	1×10^{-3} M	Adult	11.45 ± 1.53	12.73 ± 2.50	0
FVCP (4)	5×10^{-4} M	3-4 weeks	3.10 ± 0.49	3.83 ± 0.63	0
(4)	1×10^{-3} M	Adult	6.80 ± 1.81	5.47 ± 0.89	33
Iodoacetic Acid					
LVCP (4)	1×10^{-2} M	> 6 weeks	7.16 ± 1.57	1.26 ± 0.03	96
FVCP (4)	1×10^{-2} M	> 6 weeks	4.25 ± 1.04	1.04 ± 0.04	99

Table 6. --Accumulation of PAH by choroid plexuses from animals treated with procaine penicillin G.

Age	Treatment (IU penicillin/ kg/day)	PAH/MAN*	
		LVCP	FVCP
1 week	Saline	8.33 ± 1.94 (4)	3.73 ± 1.06 (4)
	300,000	14.38 ± 0.97 (4)	4.04 ± 0.18 (4)
	600,000	8.70 ± 2.83 (4)	5.40 ± 1.64 (4)
2 weeks	Saline	6.23 ± 0.84 (6)	1.80 ± 0.37 (6)
	120,000	6.70 ± 1.00 (6)	2.76 ± 0.31 (4)

* PAH/MAN values are expressed as mean ± S.E.M.; number of observations in parentheses.

Table 7. -- Effects of intraventricular pressure on the rate of removal of substances from CSF in the dog.

Dog #	Intra-ventricular Pressure (cm HOH)	Measured Quantities												Calculated Quantities						
		Flow Rate (μl/min)		Inulin (mg/ml)		Mannitol (dpm/ml)		Creatinine (mg/ml)		PAH (μg/ml)		PAH (dpm/ml)		Clearance (μl/min)				K_o (μl/min)		
		$\dot{V}_i$	$\dot{V}_o$	C_i	C_o	C_i	C_o	C_i	C_o	C_i	C_o	C_i	C_o	Inulin	Mannitol	Creatinine	PAH	Mannitol	Creatinine	PAH
α-2	-5	220	260	1.18	0.76					8.12	4.85			75.0			76.0			19.0
α-3	-5	220	210	0.86	0.77					8.42	5.93			35.0			86.0			56.0
β-3	6	180	220	0.86	0.62					4.04	1.92			29.0			107.0			87.0
β-4	-15	190	200	1.02	0.97					5.18	4.66			0.0			10.0			10.0
γ-3	-12	190	200	0.94	0.78	982	776	2.31	1.51	2.94		35355	25277	30.0	36.0	73.0	55.0	9.0	49.0	30.0
γ-4	-12	190	280	1.08	0.74	1076	749	1.75	0.99	7.23		31908	19420	0.0	0.0	42.0	24.0	-6.0	42.0	25.0
γ-6	-5	190	180	0.97	0.95	2027	1375	1.78	1.34	2.31		36452	18678	14.0	57.0	63.0	62.0	45.0	50.0	49.0
Δ-12	-8	190	230	1.09	0.87	2938	2221	0.89	0.54	4.60		49314	33963	3.0	18.0	58.0	38.0	16.0	56.0	35.0
Δ-17	-3	190	200	1.06	0.85	3448	2590	0.94	0.65	10.00		51515	35452	36.0	47.0	64.0	62.0	16.0	34.0	33.0
Dio. I	12	170	110	0.74	0.57	2027	1375			10.50		49993	24894	123.0	107.0		163.0	17.0		83.0

Table 8. --Inhibition of PAH transport from CSF in the adult dog.

Dog	(Period)	Inhibitor	Experimental Time (min)	Measured Quantities								Calculated Quantities			
				Flow Rate (μl/min)		Inulin (mg/ml)		Mannitol (dpm/ml)		PAH (dpm/ml)		Clearance (μl/min)	K_o (μl/min)		$\frac{K_o PAH}{K_o MAN}$
				$\dot{V}_i$	$\dot{V}_o$	C_i	C_o	C_i	C_o	C_i	C_o	Inulin	Mannitol	PAH	
Δ-12	(I)	4.6 μg/ml PAH	31-75	190	230	0.87	0.54	2938	2221	49394	33963	3	16	35	2.19
	(II)	20.8 μg/ml PAH	100-130	190	210	0.88	0.80	2310	1845	49504	37653	0	30	34	1.13
	(III)	88.0 μg/ml PAH	168-200	190	210	0.99	0.92	2058	1681	50150	36318	0	20	44	2.20
	(IV)	150.0 μg/ml PAH	251-267	190	200	1.04	0.86	1486	1070	58836	38244	29	20	50	1.73
Sat-2	(II)	80.0 μg/ml PAH	120-170	244	250	0.99	0.93	2286	2122	64396	53170	9	4	33	8.25
	(III)	800.0 μg/ml PAH	200-245	244	183	0.99	0.90	2546	2053	56399	47040	84	33	24	0.73
	(IV)	8000.0 μg/ml PAH	280-305	244	165	1.00	0.86	1980	1340	59447	39844	117	64	67	1.05
Dio.I	(Control)		31-91	174	112	0.74	0.55	2027	1475	49993	24894	123	17	83	4.88
	(II)	0.07 mg/ml Diodrast	129-177	174	134	0.96	0.78	2348	1554	51719	30258	81	39	62	1.53
	(III)	0.7 mg/ml Diodrast	210-255	174	156	0.90	0.83	2588	1715	59916	37942	34	59	67	1.13
	(IV)	7.0 mg/ml Diodrast	286-330	174	144	0.97	0.84	1892	1338	44738	30774	58	37	42	1.14
Δ-16	(Control)		30-64	190	200	0.99	0.75	3262	2192	48546	30608	45	31	45	1.45
	(II)	10,000 IU/ml Pen. G	116-145	200	130	1.00	0.81	2665	1860	52501	34922	106	42	52	1.24

Figure 1. --Accumulation of PAH and mannitol by the in vitro lateral ventricular choroid plexus (LVCP) in the developing dog. Accumulation of PAH and mannitol (ordinate) expressed as tissue:medium concentration ratios (T/M). Medium concentration: mannitol, 2.7 mM/l; PAH, 9.7 μM/l. Data expressed as mean ± S.E.M.; numbers of observations indicated in parentheses.

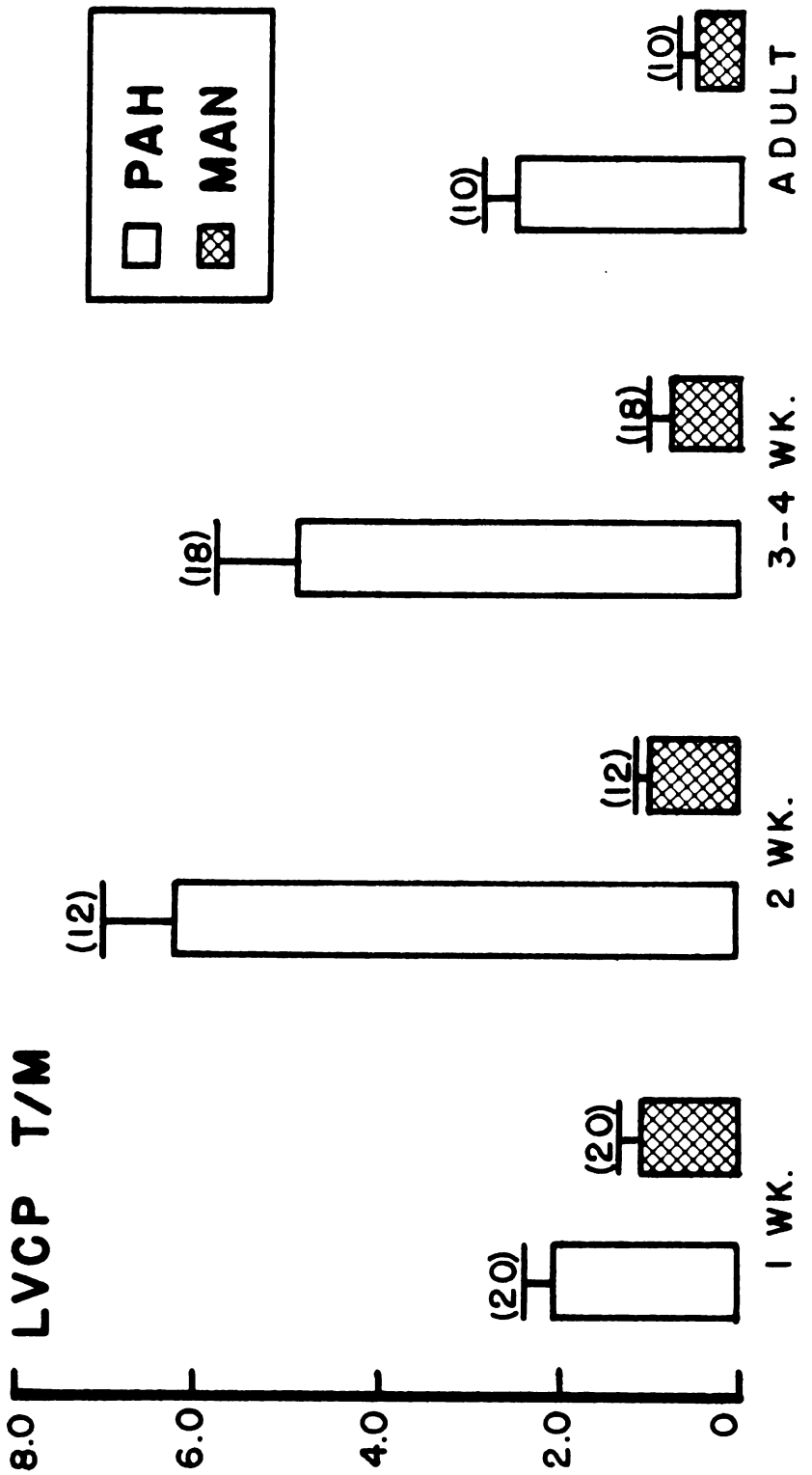

LVCP T/M
8.0
6.0
4.0
2.0
0
PAH
MAN
(20)
(20)
(12)
(12)
(18)
(18)
(10)
(10)
1 WK.
2 WK.
3-4 WK.
ADULT
AGE

Figure 1

Figure 2. --Accumulation of PAH and mannitol by the in vitro fourth ventricular choroid plexus (FVCP) in the developing dog. Accumulation of PAH and mannitol (ordinate) expressed as tissue:medium concentration ratios (T/M). Medium concentration: mannitol, 2.7 mM/l; PAH, 9.7 μM/l. Data expressed as mean ± S.E.M.; numbers of observations indicated in parentheses.

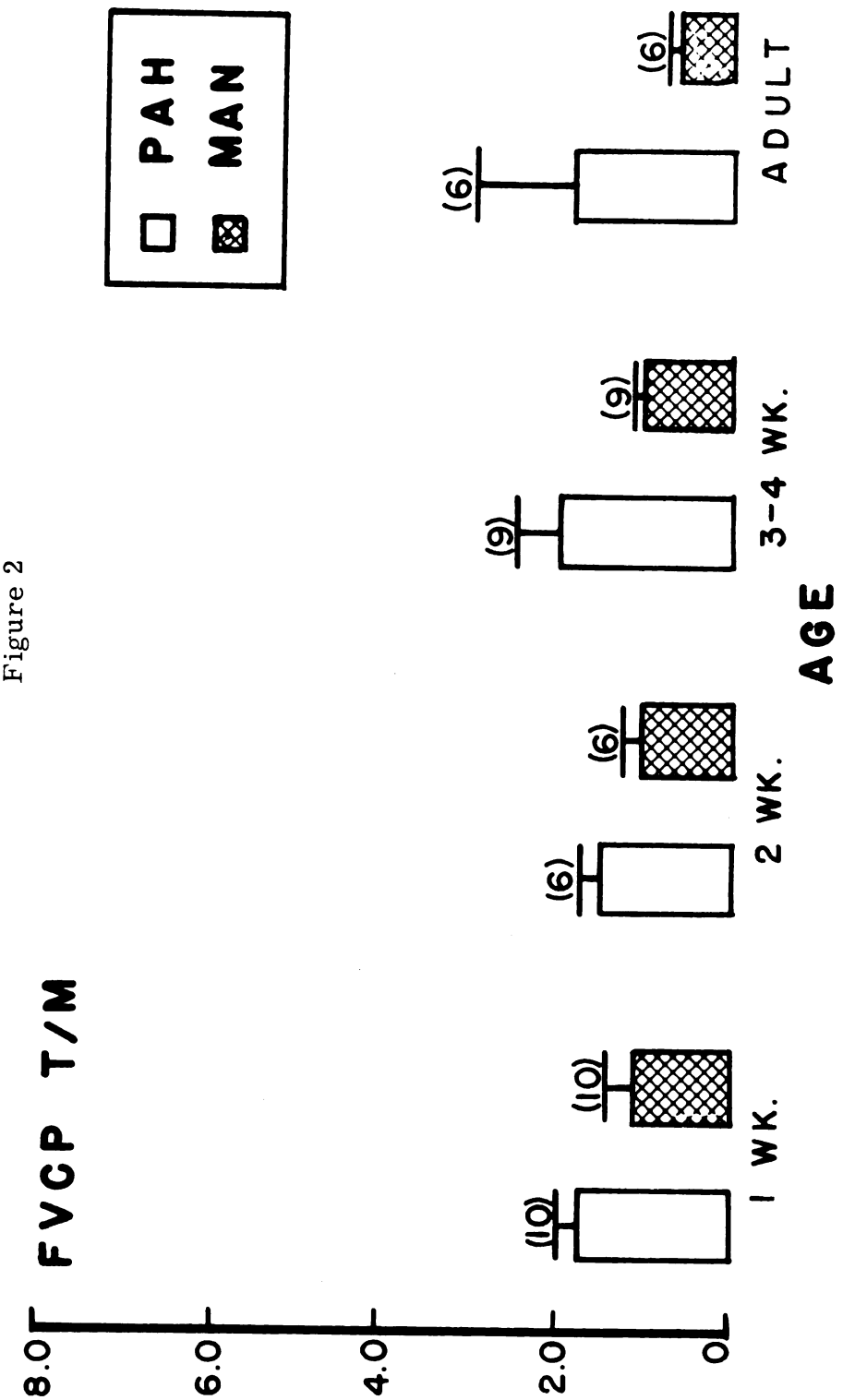


Figure 2

Figure 3.--Histological sections (287×) of FVCP from 1-day-old and adult dogs. A. One-day-old FVCP illustrating columnar epithelium and large stromal volume containing few fibroblasts and fibrocytes. B. Adult FVCP contrasting the epithelium (cuboidal), decreased stromal volume and increased number of fibroblasts and fibrocytes. Epithelium, Ep; stroma, St; fibroblasts and fibrocytes, fb; artery, a. Sections: 6 μ thick; Hematoxin and eosin stain.

Figure 3

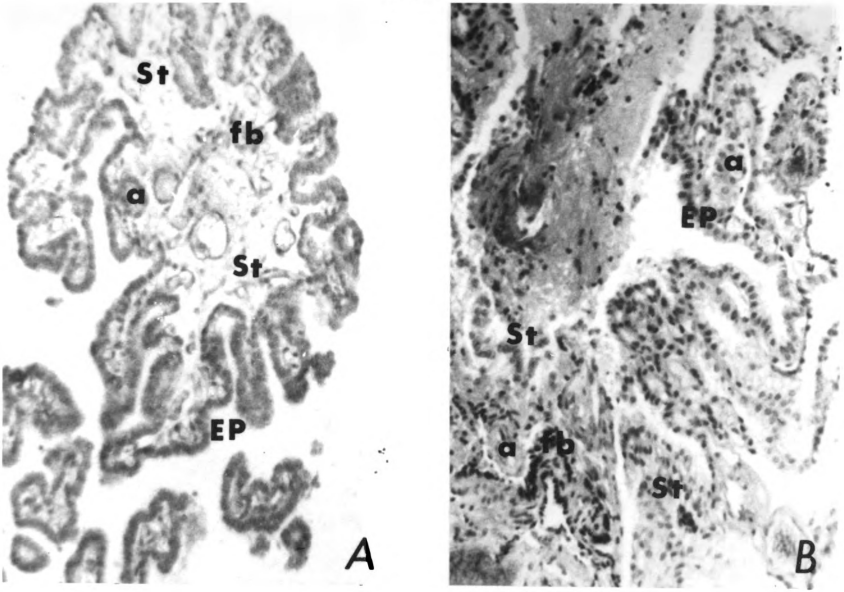


Figure 4. --Accumulation of PAH by isolated LVCP and FVCP in the developing dog. Medium concentrations: mannitol, 2.7 mM/l; PAH, 9.7 μM/l. Uptake of PAH (ordinate) is expressed as T/M PAH:T/M mannitol ratios. Each bar represents mean ± S.E.M.; number of animals indicated parenthetically.

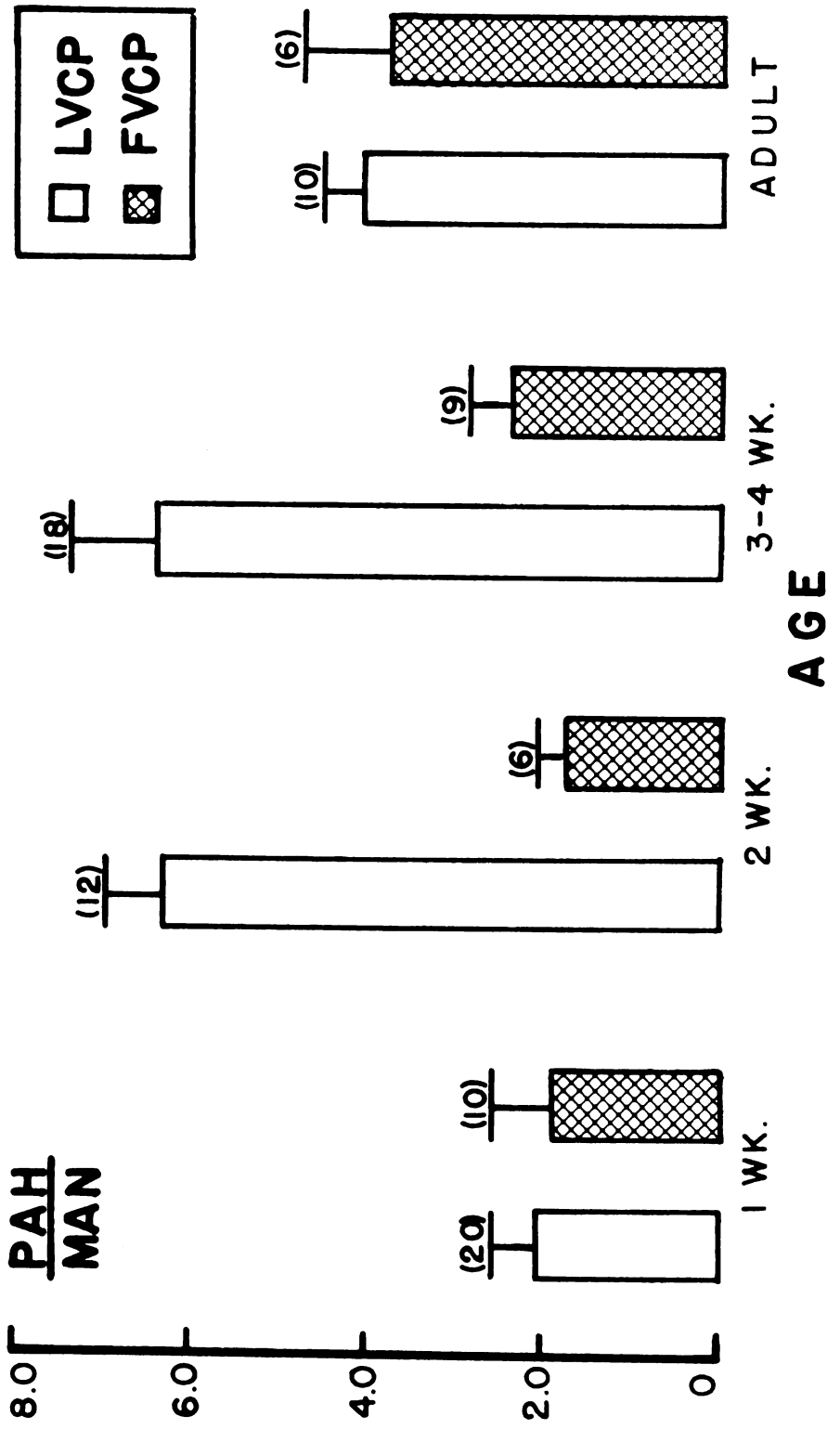

LVCP
FVCP
PAH
MAN
8.0
6.0
4.0
2.0
0
(20)
(10)
(12)
(6)
(18)
(9)
(10)
(6)
1 WK.
2 WK.
3-4 WK.
ADULT
AGE

Figure 4

Figure 5.--Saturation curve of PAH transport by the LVCP (0——0) and FVCP (●——●) of littermate 3-week-old dogs. $\frac{PAH}{MAN}$ is plotted on the ordinate as a function of medium PAH concentration (mM/l). Each point represents 1 observation. Lines connecting data points were fitted by eye. T/M PAH ratios were calculated as $\frac{\text{mM PAH/gm of wet tissue}}{\text{mM PAH/ml of medium}}$ and T:M ratios of PAH were compared to T/M mannitol.

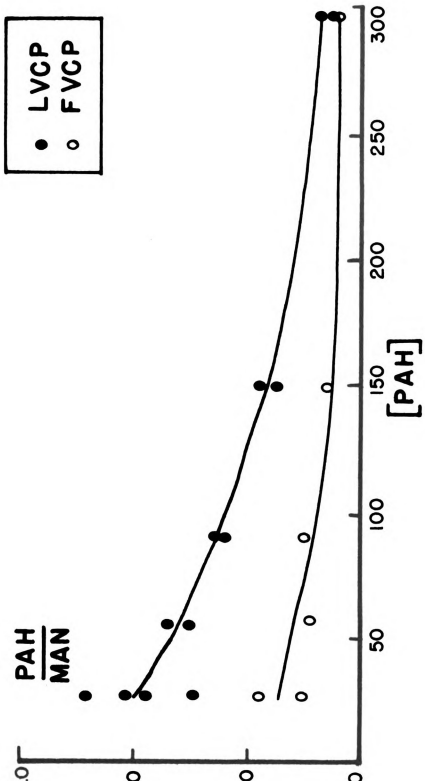


Figure 5

Figure 6.--Relationship between intraventricular pressure and outflow cannula height. Both intraventricular pressure (cm HOH pressure; ordinate) and outflow cannula height (cm; abscissa) measured relative to external auditory meatus. Each point represents 1 observation during 30-90 minutes perfusion time. Equation of the line, determined by the method of linear regression, is: $Y = 0.65 \pm 0.08X + 1.31$ ($n = 27$).

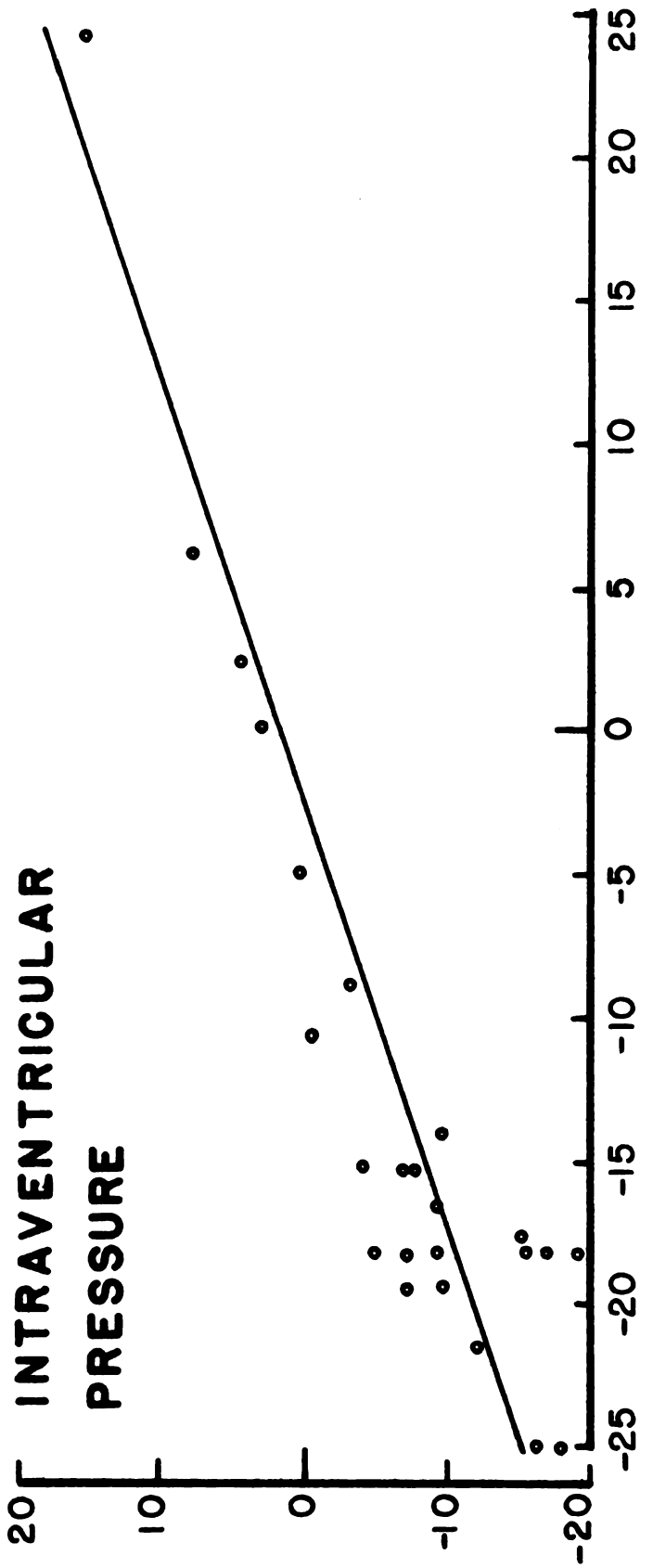

INTRAVENTRICULAR
PRESSURE
20
10
0
-10
-20
-25
-20
-15
-10
-5
0
5
10
15
20
25
OUTFLOW HEIGHT

Figure 6

Figure 7.--Clearance (μl/min; ordinate) of inulin (x——x), mannitol (●—·—●) and PAH (o– – –o) and its relationship to intraventricular pressure (cm HOH; abscissa) measured relative to the external auditory meatus. Clearance of inulin was calculated by Equation 1 (Methods); that of PAH and mannitol, by Equation 2 (Methods) and are shown in Table 7. Perfusion fluid concentrations: inulin, 0.8-1.0 mg/ml; mannitol, 0.25 mg/ml; PAH, < 10 μg/ml. Each point represents 1 observation between 30-90 minutes perfusion time. Equations for lines through data points were determined by the method of linear regression:

inulin: $Y = 3.45 \pm 2.18X + 50.67$ (n = 10)

mannitol: $Y = 3.89 \pm 1.96X + 62.29$ (n = 6)

PAH: $Y = 5.00 \pm 1.26X + 91.71$ (n = 10)

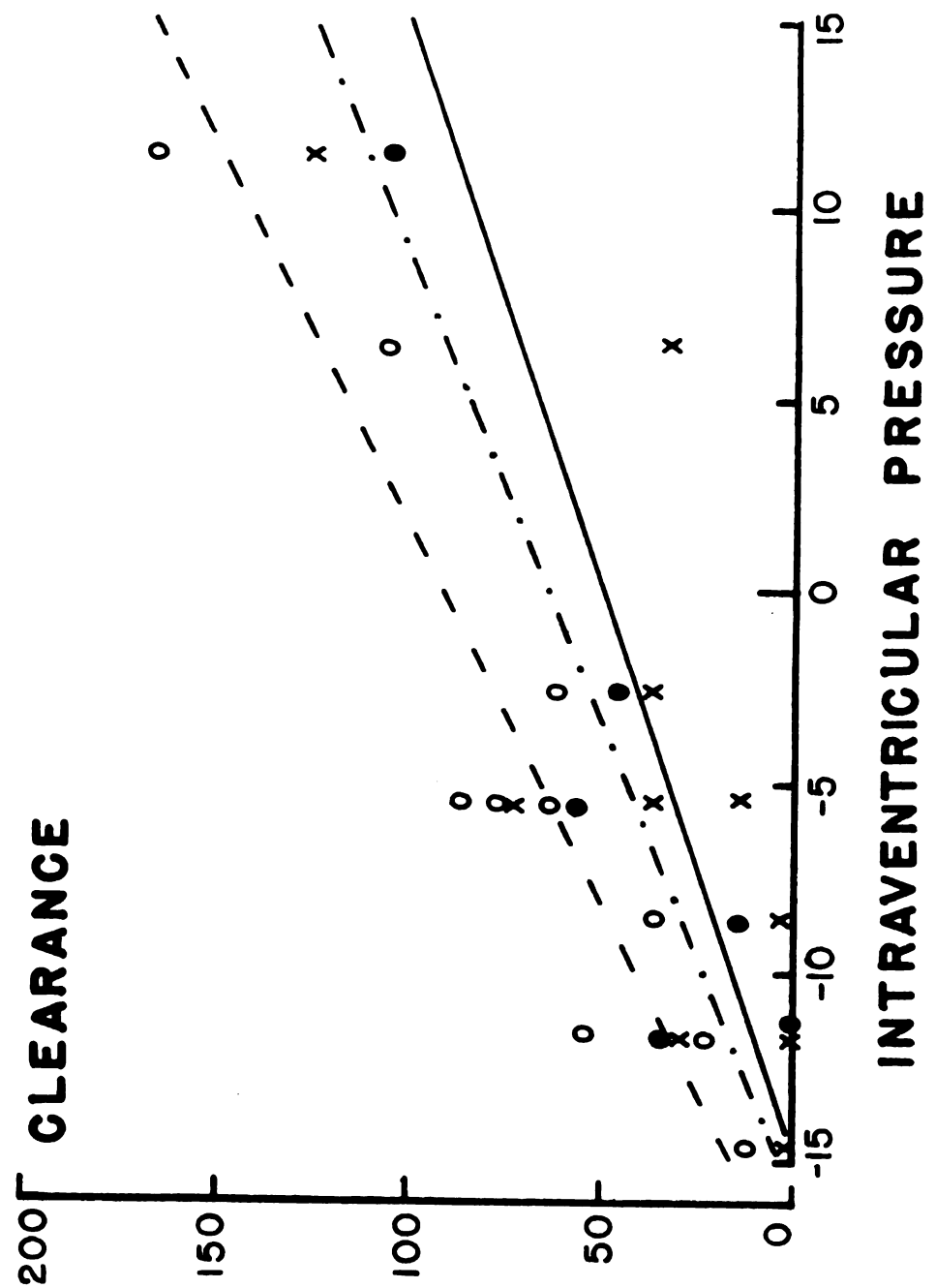

CLEARANCE
200
150
100
50
0
-15
-10
-5
0
5
10
15
INTRAVENTRICULAR PRESSURE

Figure 7

Figure 8.--Efflux coefficients (K_o, μl/min; ordinate) of mannitol (●– · –●) and PAH (o– – –o) and its relationship to intraventricular pressure (cm HOH; abscissa) measured relative to the external auditory meatus. Efflux coefficients were calculated by Equation 3 (Methods) and are shown in Table 7. Perfusion fluid concentrations: mannitol, 0.25 mg/ml; PAH, < 10 μg/ml. Each point represents 1 observation during 30-90 minutes perfusion time. Equations for lines were determined by the method of linear regression:

mannitol: $Y = 0.53 \pm 2.52X + 18.65$ (n = 6)

PAH: $Y = 2.97 \pm 1.01X + 55.75$ (n = 10)

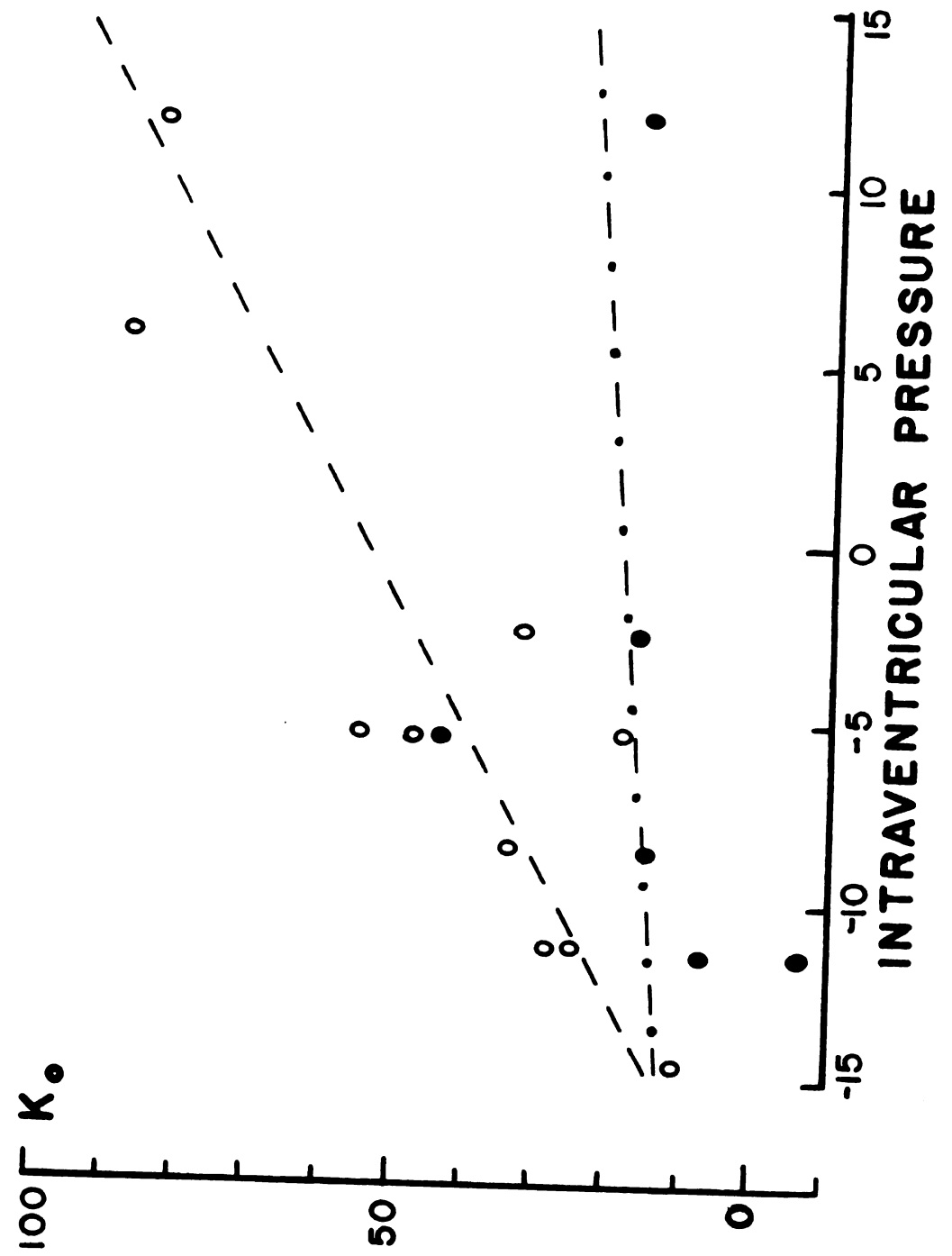


Figure 8

Figure 9. --Efflux coefficients of 3 molecules of similar molecular weight perfused simultaneously in 5 animals (original data in Table 7). Efflux coefficients (K_o, μl/min; ordinate) were calculated by Equation 3 (Methods). Perfusion concentrations: creatinine, 1.0-2.0 mg/ml; PAH, < 10 μg/ml; mannitol, 0.25 mg/ml. Each bar represents mean ± S.E.M.

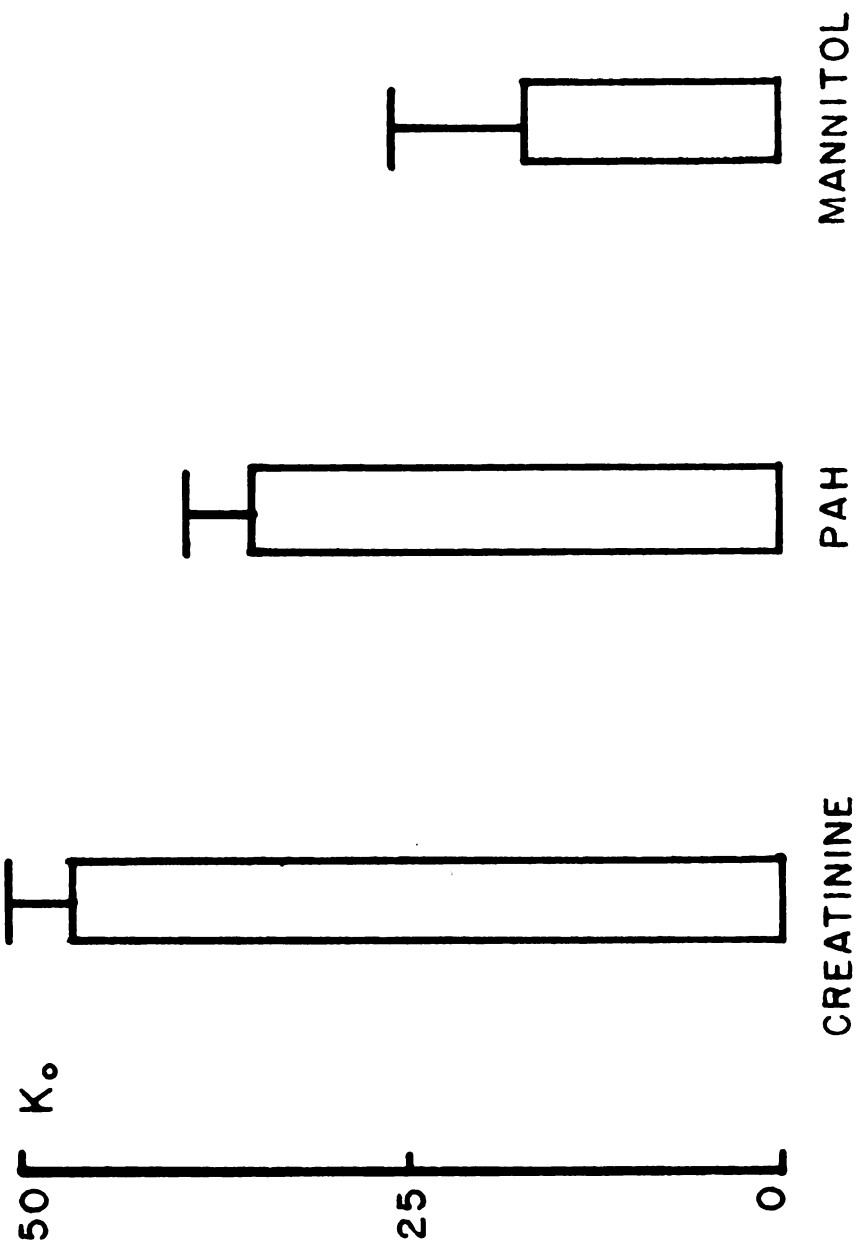


Figure 9

Figure 10. --Inhibition of PAH transport from CSF by the addition of Diodrast to the perfusion fluid. PAH and mannitol efflux coefficients (K_o, μl/min) are shown on the ordinate and perfusion fluid Diodrast concentrations (mg/ml) are shown under each set of bars. Perfusion fluid concentrations: PAH, < 10 μg/ml; mannitol, 0.25 mg/ml. Each bar represents 1 perfusion period; data taken from Table 8 (Dog Dio. I).

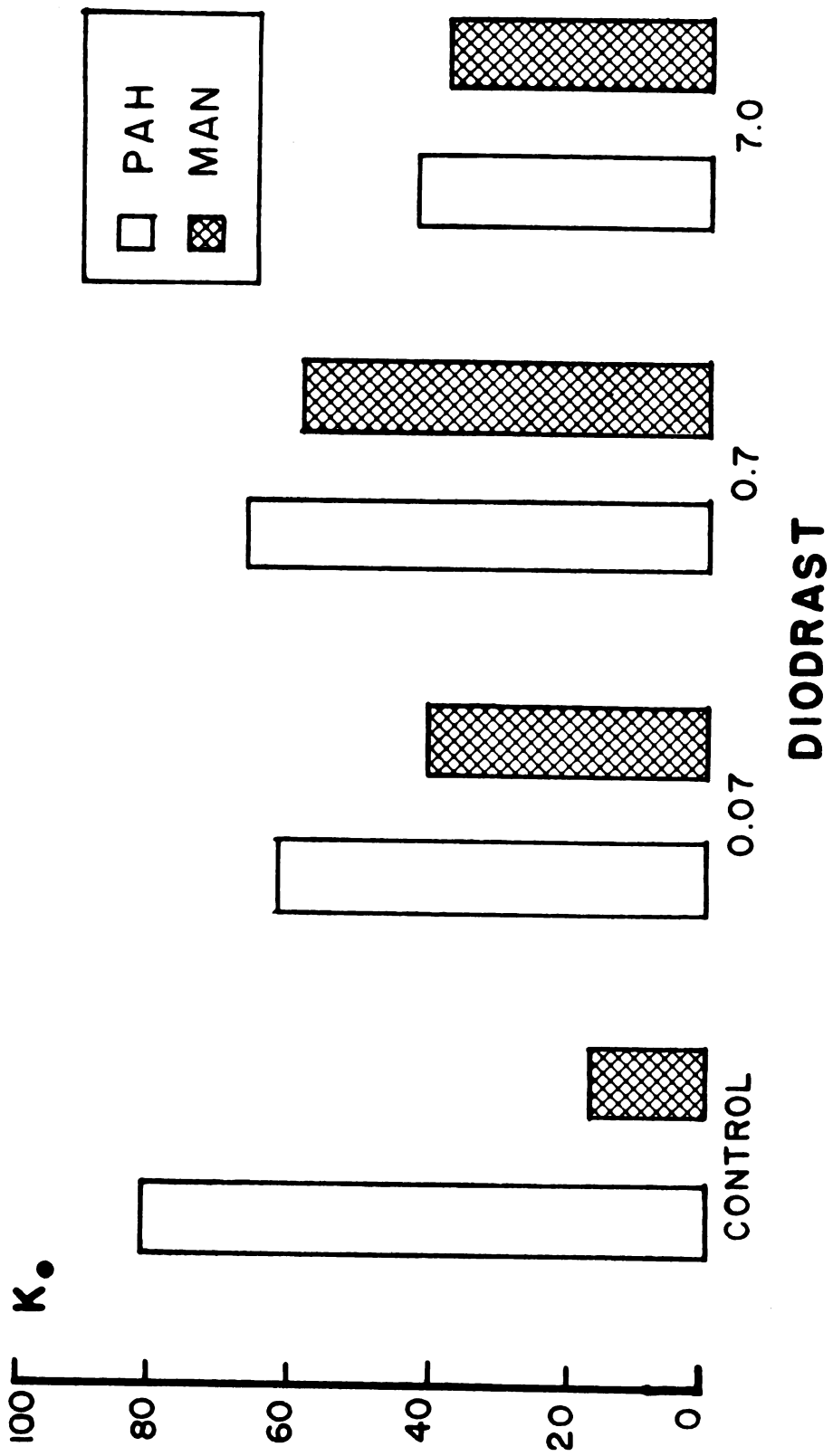


Figure 10

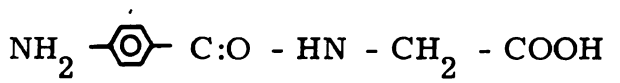
DISCUSSION

Organic anions are products of metabolism which cannot be further metabolized by the cell. Elimination of organic anions from the organism is necessary to prevent slowing of reversible chemical reactions and accumulation of these compounds to toxic levels. In the central nervous system high concentrations of some organic anions cause seizures and convulsions (Pilchner et al., 1947; Walker et al., 1945). Therefore development of an organic anion transport mechanism is of biological importance since the active transport may constitute a barrier which prevents toxic substances in blood from entering cerebrospinal fluid (CSF) and brain, and also may be responsible for excreting into blood waste products generated in the brain.

Organic anions are actively transported from blood into urine by the proximal tubule cells of the kidney. This function in the kidney is thought to involve transport of a molecule having the general formula:

$$R - C{:}O - NX - (CHR')_n - COOH$$

where R or R' may be an aliphatic chain or aromatic ring; X is a hydrogen or methyl group and when R' is hydrogen, n varies from 1 to 5. Organic anions which lack correspondence to the general formula are not actively transported (Despopoulus, 1965). p-Aminohippuric acid (PAH), Diodrast and penicillin share a common transport mechanism and are actively transported by the renal tubule cells (Sperber, 1954). PAH conforms to the general formula (R is an animated benzene ring, X is hydrogen and n equals 1) and its formula is:



Pappenheimer et al. (1961) perfused the brain ventricular system of goats with an artificial CSF containing low concentrations of Diodrast and phenolsulfonphthalein and observed that these organic anions were actively transported from CSF in the region of the fourth ventricle; possibly by the fourth ventricular choroid plexus (FVCP). Becker (1961) reported preliminary evidence that the in vitro lateral ventricular choroid plexus (LVCP) of the rabbit actively accumulated organic anions. Organic anion transport has not been studied in the dog, nor have any studies compared the in vivo and in vitro organic anion transport system. Data presented in this thesis suggest that PAH, an organic anion, is actively

accumulated by both the LVCP and FVCP in vitro and that PAH in low concentrations is actively transported from CSF in vivo.

Perfusion of an artificial CSF solution through the brain ventricular system of the dog permitted the study of the exchange rates of test molecules between CSF, blood and brain. The clearance of a molecule from CSF is defined as the volume of CSF from which a substance is completely removed per unit time; analogous to clearance as used in renal physiology. This volume depends upon the amount entering the brain ventricular system, the amount leaving and the concentration of the substance in the ventricle (Equation 2, Methods; Pappenheimer et al., 1961). In the case of a large molecule such as inulin (M.W. = 5000), Rall and Oppelt (1962) demonstrated that after 5 hours of perfusion of the ventriculocisternal system of the dog, only small amounts of inulin penetrated the ependymal walls of the lateral ventricles and diffused into the brain parenchyma. Heisey et al. (1962) found that the transependymal flux rates of inulin were not detectable and concluded that the loss of inulin from CSF could be used to estimate bulk absorption of fluid distal to the fourth ventricle; possibly through large openings on the arachnoid villi leading from the subarachnoid spaces into the venous sinus (Davson, 1967). The clearance of inulin is a linear function of intraventricular pressure (Figure 7)

which supports observations in the goat (Heisey et al., 1962) and dog (Bering and Sato, 1963; Cserr, 1965).

The clearance of mannitol, a passively diffusing molecule, was always greater than the clearance of inulin (Figure 7), but the slope of mannitol clearance as a function of intraventricular pressure was equal to that of inulin, indicating that the pressure dependence of mannitol clearance can be accounted for by the bulk absorption of fluid from CSF. The difference between the clearance of mannitol and inulin represents the diffusional component of mannitol clearance (Bering and Sato, 1963). Heisey et al. (1962) observed that the passive diffusion of tritiated water (TOH) from the brain ventricular system in goats was dependent upon intraventricular pressure but the increase in TOH clearance with intraventricular pressure could be accounted for by the bulk absorption of CSF.

The outflux coefficient of a molecule represents the clearance of a molecule by means other than bulk absorption (Equation 3, Methods). The slope of the line relating mannitol efflux (K_o mannitol) to intraventricular pressure is zero (Figure 8), indicating that the movement of mannitol, a passively diffusing molecule, out of CSF is independent of intraventricular pressure. When the bulk absorption component was subtracted from the

clearance of tritiated water (TOH), efflux of TOH was also independent of intraventricular pressure (Heisey et al., 1962).

In contrast PAH efflux (K_o PAH) increased with intraventricular pressure over the range -15 to +15 cm HOH pressure. A pressure-dependent efflux of any molecule has not been previously reported. The pressure-dependent PAH efflux might be explained by the course of the perfusion fluid through the ventriculocisternal system and the location of the FVCP. Perfusion fluid flows from the lateral ventricle through the third and fourth ventricles, through the foramina of Luschka, at the lateral recesses of the fourth ventricular cavity, and into the cisterna magna where the outflow needle is placed. At increased intraventricular pressures, fluid is reabsorbed distal to the fourth ventricle, involving a greater surface area of the subarachnoid space. Much of the FVCP projects through the foramina of Luschka into the subarachnoid space. It is possible that at low intraventricular pressures, perfusion fluid takes a more direct route through the foramina of Luschka to the outflow cannula in the cisterna magna and bypasses much of the subarachnoid space and the surface of the FVCP; while at increased intraventricular pressures perfusion fluid passes over more surface of the FVCP en route to more rostral portions of the subarachnoid space and consequent reabsorption into the venous sinuses. Therefore, increased

intraventricular pressures could effectively increase the surface area not only for the bulk absorption of fluid but also increase the perfused surface area of the FVCP for active transport of PAH.

PAH transport exhibits some criteria of active transport, i.e., saturation of the transport mechanism at high PAH perfusion fluid concentrations and inhibition of transport by the addition of competitive inhibitors to the perfusion fluid which are structurally similar, Diodrast and penicillin (Despopoulus, 1965; Sperber, 1954). At PAH perfusion concentrations between 150 and 800 μg/ml PAH, the transport system appeared saturated since the ratio of PAH to mannitol efflux approached 1.0 (Table 8). Pappenheimer et al. (1961) reported that the organic anion transport mechanism in the goat was saturated at 20-40 μg/ml. Comparison of these saturation concentrations would indicate that the dog has a larger capacity for organic anion transport than the goat.

PAH transport was inhibited by the addition of 70-700 μg/ml Diodrast to the perfusion fluid (Table 8), supporting the observation that the organic anion transport mechanism is saturated between 150 and 800 μg/ml PAH. Similarly, Pappenheimer et al. (1961) reported Diodrast transport was inhibited by the addition of 500 μg/ml PAH. The addition of 10,000 IU penicillin/ml to the perfusion fluid produced slight inhibition of PAH transport (Table 8).

Fishman (1966) reported that intracisternal injection of 8×10^{-5} moles PAH in the dog blocked active transport of penicillin from CSF. It appears that PAH, Diodrast and penicillin share a common transport system for the removal of these compounds from the CSF.

Although the molecular weights of mannitol, PAH and creatinine are similar (182, 194 and 113, respectively), efflux coefficients of both creatinine (46 ± 4 μl/min) and PAH (34 ± 4 μl/min) are significantly greater ($p < 0.05$) than mannitol (16 ± 8 μl/min; Figure 9), suggesting that creatinine and PAH leave CSF by a process other than passive diffusion. Others have assumed creatinine leaves CSF by passive transport (Bering and Sato, 1963; Heisey et al., 1962; Cserr, 1965) but none of these investigators have compared efflux coefficients of creatinine to mannitol. Creatinine efflux coefficients of 27 μl/min (Cserr, 1965) and 47 μl/min (Bering and Sato, 1963) were found for dog ventricles; values which are greater than the mannitol efflux coefficient (16 ± 8 μl/min; Figure 9). Heisey et al. (1962) reported that the creatinine efflux coefficient for goat ventricles was 76 ± 15 μl/min; a value greater than those reported for the dog. These differences may be due to species differences in the surface areas of the walls of the ventricles and choroid plexuses having an unknown effect upon the efflux coefficient. My data suggest that creatinine and PAH may be actively transported from CSF.

The tissue:medium concentration ratio (T/M) is a measure of the concentration gradient maintained between the tissue and medium *in vitro*. If a molecule were distributed between the tissue and medium by passive diffusion alone, then the expected T:M ratio would be less than 1.0 due to proteins and salts within the tissue; while T:M ratios greater than 1 indicate active accumulation against a concentration gradient since energy must be supplied to maintain this ratio of concentrations. The T:M ratio is a function of medium concentration and since T:M ratios are not reported at either saturation concentrations or even at the same medium concentration, T:M ratios of different molecules (Table 1) are not comparable.

In diaphragm muscle neither PAH nor mannitol is actively accumulated since T/M PAH and T/M mannitol are not greater than 1.0 (Table 2). Rubin *et al.* (1968) reported that methotrexate, actively transported by the choroid plexus *in vitro*, is not actively accumulated by either diaphragm muscle or cerebral cortical slices; T/M methotrexate ranged from 0.8 to 1.0.

In both the LVCP and FVCP T:M mannitol ratios decreased with increasing age, suggesting that the volume into which mannitol can diffuse decreases. Histological slides of 1-day and adult choroid plexuses (Figure 4) provide anatomical evidence that as the plexus matures the stromal volume of the LVCP and FVCP decreases.

Kappers (1958) observed a similar change in the choroid plexus from 6-week-old human fetuses compared to the fourth month fetus. In the 6-week-old fetus the choroidal epithelium consisted of single-layered cuboidal cells surrounding a stroma composed of gelatinous connective tissue. By the fourth month, the gelatinous connective tissue was replaced by dense fibrous connective tissue and accompanied by a significant increase in the ratio of epithelium to stroma. Hilton (1956) observed that the adult human choroid plexus is extremely lobulated with each lobule composed of finer divisions; a phenomenon not observed in the embryo. An increase in the density of the stromal connective tissue in the developing plexus could decrease the space into which mannitol diffuses. The change in T/M mannitol presumably represents a change in the extracellular fluid (ECF) of the choroid plexus and in order to correct for age differences in the ECF, data is expressed as T/M PAH:T/M mannitol ratios (PAH/MAN). At 1 week of age PAH transport is poorly developed since neither the LVCP nor the FVCP is able to concentrate organic anions as can the adult (Figure 4). T/M for sulphate in cats and rabbits (Robinson et al., 1968) also suggests lower accumulation in the newborn than in the adult. An increased surface area on the adult might account for the greater PAH transport by the adult.

Both the adult LVCP and FVCP actively accumulate PAH and there is no statistical difference between PAH:MAN ratios of the LVCP and FVCP in the adult. Similarly, Tochino and Schanker (1965b) reported that accumulation of serotonin and norepinephrine by the LVCP was not different from the FVCP. In contrast, accumulation of an organic acid, 5-hydroxyindoleacetic acid, was greater in the FVCP of the adult rabbit than in the LVCP (Cserr and Van Dyke, 1971). In vivo results of Pappenheimer et al. (1961) suggest that Diodrast is actively transported from CSF in the region of the fourth ventricle, possibly by the FVCP, and that minimal transport occurs in the region of the lateral ventricles.

Both the LVCP and FVCP exhibit some criteria for the active uptake of PAH: PAH is accumulated against a concentration gradient (T/M > 1.0; Table 2); shows a saturable transport mechanism at high medium concentrations of PAH (> 200 mM/l; Figure 7); is inhibited by other organic anions (Diodrast and penicillin; Table 5) and has a metabolic energy dependent transport mechanism (iodoacetic acid; Table 5).

The LVCP's ability to accumulate organic anions during the 2- and 3- to 4-week age is greater than either the adult or 1-week-old dog (Table 1; Figures 1, 2). Maximum transport at the second week by the LVCP parallels the observations that

sulphate transport is maximal in the 10-day-old rabbit (Robinson et al., 1968) and morphine transport peaks in the 15-day-old rabbit (Asghar and Way, 1970). The FVCP PAH/MAN is greater in the adult than at 1 or 2 weeks of age (Figure 4). The later development of the FVCP transport mechanism contrasts anatomical data which indicate that the myelencephalic choroid plexus (FVCP) develops before the telencephalic plexus (LVCP) in fetal humans (Kappers, 1958) and fetal pigs (Hilton, 1956).

Hirsch and Hook (1969a, 1969b, 1969c, 1970) induced organic anion transport in renal cortical slices from animals previously treated with penicillin. In 2-week-old rabbits, PAH transport by cortical slices was increased 4 times by prior treatment with penicillin, but no increase in transport was noted at 4 weeks of age when the organic anion transport system was well developed. In the LVCP, PAH transport was induced at 1 week postnatal by prior administration of 300,000 IU but not 600,000 IU penicillin G. At 2 weeks no induction was produced in penicillin-treated animals (Table 6). At 1 week PAH transport by the LVCP is not well developed but is highly developed at 2 weeks (Figure 4). Prior administration of 300,000 IU penicillin induced PAH transport at 1 week when the transport mechanism was developing but failure to produce any effect on PAH transport might be that this

dosage exceeded the optimal dosage response for penicillin induction. In the 1-week-old FVCP no induction of PAH transport was produced with either 300,000 or 600,000 IU penicillin, but at 2 weeks PAH transport was induced by treatment with 120,000 IU penicillin. PAH transport by the FVCP is not developed at 1 or 2 weeks but begins to develop at 3-4 weeks (Figure 4). Failure of penicillin to induce PAH transport in the FVCP at 1 week might either be due to the immaturity of the transport mechanism at that age or the low dosage of penicillin administered to the animal. Induction of PAH transport at 2 weeks supports the view that transport induction occurs at a time before transport normally develops. These data support the hypothesis that an increased or prolonged presence of organic anions in the CSF during the period of transport stimulates maturation or activity of the organic anion transport system. Hirsch and Hook (1969a) suggest that the stimulatory effect of penicillin might be caused by enhanced development of existing transport processes or synthesis of new enzyme proteins responsible for organic anion transport.

Asghar and Way (1970) suggest that the increased resistance to drug toxicity in adults could be dependent upon the development of the blood-brain and blood-CSF barriers. They observed that brain concentrations of morphine/gm body weight in

younger animals (< 10 days postnatal) are higher than in adults; a difference which corresponds well to observed toxic effects of morphine in young animals and adults. Furthermore they suggest that high brain morphine levels in the newborn might result from a decreased outflux of morphine from the CNS. Penicillin, an organic anion, is actively transported from CSF into blood and brain (Fishman, 1966) and is a convulsive agent, causing seizures when topically applied to the cerebral cortex of monkeys (Walker et al., 1945) or injected intracisternally (3,000 IU) or intrathecally (7,500 IU) in dogs (Pilchner et al., 1947). Similar to morphine, penicillin transport may be poorly developed in the young since intrathecal injection of 3,000 IU penicillin G causes convulsions in children whereas in adults 10,000 IU penicillin are required to produce the same effects. Challenging the choroid plexus with organic anions during the period of development enhances maturation of the organic anion transport mechanism and provides a mechanism for protecting the CNS of the young from potentially toxic compounds.

Further studies in the area of transport development are suggested since it is not known why young animals transport less organic anions than the adult. Possible explanations could be an increased permeability of the choroid plexus in the young, or a

decreased rate of transport in the young compared to the adult or a combination of both factors. My data indicate that there is an optimal dosage for penicillin induction; all dosages of penicillin were not investigated. Also there may be an optimal time after the last injection of penicillin is administered and PAH accumulation is measured.

SUMMARY

In vitro incubations

1. T/M mannitol in LVCP and FVCP from adult animals were significantly less ($p < 0.05$) than in animals 1 week old; T/M mannitol in diaphragm did not change with age.
2. PAH accumulation by both the LVCP and FVCP was an active process: PAH was accumulated against a concentration gradient, suggesting a metabolic energy dependence, and showed competitive inhibition.
3. PAH accumulation was poorly developed in the LVCP and FVCP from 1-week-old animals (T/M = 2.12; T/M = 1.97, respectively) but well developed in the adult (T/M = 4.04; T/M = 3.84, respectively).
4. Maximum accumulation of PAH occurred in the LVCP from 2-week-old animals and in the FVCP from adult animals.
5. LVCP's from 1-week-old dogs treated with 300,000 IU penicillin showed a significant increase ($p < 0.05$) in PAH accumulation compared to controls. No induction ($p > 0.05$)

of PAH accumulation was produced by treatment with 600,000 IU in 1-week-old dogs or 120,000 IU in 2-week-old animals.

6. FVCP's from 1-week-old dogs treated with 300,000 or 600,000 IU penicillin showed no increase ($p > 0.05$) in PAH uptake but FVCP's from 2-week-old dogs treated with 120,000 IU penicillin produced significantly greater ($p < 0.05$) PAH accumulation than control animals.

In vivo perfusions

1. Inulin is removed from the ventricular system by bulk absorption of fluid at rates which vary linearly with intraventricular pressure.
2. Efflux coefficients of mannitol, a passively diffusing molecule, is independent of intraventricular pressure over the range -15 to +15 cm HOH pressure.
3. PAH efflux from CSF varies directly with intraventricular pressure over the pressure range -15 to +15 cm HOH pressure, indicating that PAH efflux may be a function of the perfused surface area of the FVCP.
4. Efflux coefficients of creatinine and PAH are significantly greater ($p < 0.05$) than mannitol, indicating that creatinine

and PAH may be removed from CSF by a process other than passive diffusion.

5. Active transport of PAH from CSF was indicated by: an efflux coefficient of PAH significantly greater ($p < 0.05$) than mannitol, inhibition of transport by other organic anions and self-saturation.

APPENDICES

APPENDIX 1

PREPARATION OF ARTIFICIAL CEREBROSPINAL FLUID (CSF)

Artificial dog CSF contains:

Cations		Anions	
Na^+	150.0 mEq/l	Cl^-	133.2 mEq/l
K^+	3.0 mEq/l	HCO_3^-	25.0 mEq/l
Ca^{++}	2.3 mEq/l	HPO_4^{-2}	0.5 mEq/l
Mg^{++}	1.6 mEq/l		

Reagents:

1. $Na_2HPO_4 \cdot 7HOH$
2. KCl
3. $NaHCO_3$
4. NaCl
5. $CaCl_2$
6. $MgCl_2 \cdot 6HOH$
7. Dextrose

Solutions:

A. Dissolve 0.00345 g $Na_2HPO_4 \cdot 7HOH$, 0.2237 g KCl, 2.1007 g $NaHCO_3$ and 7.2467 g NaCl in distilled water q.s. 1 liter.

B. $MgCl_2$

Dissolve 0.8133 g $MgCl_2 \cdot 6HOH$ in distilled water; q.s. 100 ml.

C. $CaCl_2$

Dissolve 1.2763 g $CaCl_2$ in distilled water; q.s. 100 ml.

Procedure:

100 ml of solution A is equilibrated with 3-9% CO_2; 0.1 ml of each of solutions B and C and 0.7-1.0 mg/ml dextrose are added.

APPENDIX 2

p-AMINOHIPPURIC ACID ASSAY

Modified from H. W. Smith, 1956, pp. 212-213.

Principle:

A method in which the p-amino group of p-aminohippuric acid (PAH) is diazotized with HNO_2. Excess HNO_2 is destroyed by sulfamate and the diazotized group is coupled with N-(1-napthyl) ethylenediamine to yield a colored complex. The intensity of the color is proportional to the amount of PAH present.

Reagents:

1. HCl
2. Sodium nitrite
3. Ammonium sulfamate (Sigma Chemical Co., St. Louis, Missouri)
4. N-(1-napthyl) ethylenediamine dihydrochloride (Eastman Kodak Co., Rochester, New York)

Solutions:

A. HCl (1.2N)

Add 10.0 ml conc. HCl q.s. 100 ml with distilled water.

B. $NaNO_2$ (1.0 mg/ml)

Dissolve 100 mg $NaNO_2$ q.s. 100 ml with distilled water. Prepare fresh every 3 days.

C. Ammonium sulfamate (5.0 mg/ml)

Dissolve 500 mg ammonium sulfamate q.s. 100 ml with distilled water. Prepare fresh every 2 weeks.

D. N-(1-napthyl) ethylenediamine dihydrochloride (1.0 mg/ml)

Dissolve 100 mg N-(1-napthyl) ethylenediamine dihydrochloride q.s. 100 ml with distilled water. Store in dark glass bottle and refrigerate at 4° C.

PAH standard solutions:

Dissolve 1.0 mg PAH (sodium salt; Sigma Chemical Co., St. Louis, Mo.) in distilled water q.s. 100 ml (10 μg/ml PAH). Dilute 8.0, 6.0, 4.0, 2.0 and 1.0 ml of 10 μg/ml PAH q.s. 10 ml to obtain 8.0, 6.0, 4.0, 2.0 and 1.0 μg/ml PAH standards respectively. Standards are stored at 4° C.

Procedure:

To duplicate 0.50 ml unknown samples, PAH standards and water blank add 0.10 ml of solution A and 0.05 ml of solution B

and mix. Not before 3 and not after 5 minutes later add 0.05 ml of solution C and mix. Three to 5 minutes later add 0.05 ml of solution D and mix. Fifteen minutes after adding reagent D, read all tubes against the water blank at 540 mμ (peak of absorbency).

Calculations:

Absorbency of the standards plotted as a function of PAH concentration (0-10 μg/ml PAH) yields a straight line. Concentration of PAH in unknown samples can be calculated by multiplying the optical density of the unknown by the slope of the standard curve:

$$C_{un} = \left[\frac{C_{std}}{OD_{std}}\right]_{ave} \times OD_{un}$$

where: C = concentration

OD = optical density

std = standard

un = unknown

ave = average

APPENDIX 3

INULIN ASSAY

Direct Resorcinol Method Without Alkali Treatment

Modified from H. W. Smith, 1956, p. 209.

Principle:

A method in which inulin is hydrolyzed to fructose by heating in acid. Fructose molecules combine with resorcinol to yield a colored complex; the intensity of the color is proportional to the amount of fructose present.

Reagents:

1. Resorcinol (Fisher Sci. Co., Fairlawn, New Jersey)
2. Ethanol (95%)
3. HCl

Solutions:

A. Resorcinol (1.0 mg/ml)

Dissolve 100 mg resorcinol q. s. 100 ml with 95% ethanol.

Prepare fresh daily.

B. HCl (approximately 8N)

Add 224 ml of distilled water to 1000 ml conc. HCl.

Inulin standard solutions:

Dissolve 200 mg inulin (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) in distilled water q.s. 100 ml (2.0 mg/ml). Dilute 7.5, 5.0, 4.0, 3.0, 2.0 and 1.0 ml of 2.0 mg/ml inulin solution to 10 ml with distilled water yielding 1.5, 1.0, 0.8, 0.6, 0.5 and 0.2 mg/ml inulin standards. Standards are stored at 4° C.

Procedure:

To duplicate 0.05 ml unknown samples, inulin standards and water blank, 1.0 ml solution A and 2.5 ml solution B are added and mixed under a hood. A glass marble is placed on top of the tubes and tubes are incubated for 25 minutes at 80° C. Tubes are cooled to room temperature and optical density determined within 1 hour at 490 mμ (peak of absorbency) against the water blank in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Calculations:

Optical density at 490 mμ plotted as a function of inulin concentration yields a straight line over the range 0-2.0 mg/ml inulin. Inulin concentration in unknown samples is calculated by

multiplying the optical density of the unknown by the slope of the standard curve:

$$C_{un} = \left[\frac{C_{std}}{OD_{std}}\right]_{ave} \times OD_{un}$$

where: C = concentration

OD = optical density

std = standard

un = unknown

ave = average

APPENDIX 4

CREATININE ASSAY

Modified from S. Natelson, 1961, pp. 197-202.

Principle:

A method in which picric acid forms a colored complex with creatinine in alkaline solution. Maximum absorbency of the complex is 490 mμ. Color intensity is proportional to the concentration of creatinine; color intensity is also dependent on the concentrations of alkali and picric acid and on temperature and time, but these factors are maintained constant in the analysis.

Reagents:

1. Picric acid (J. T. Baker Chemical Co., Phillipsburg, New Jersey)
2. NaOH
3. HCl

Solutions:

A. Picric acid (1.0%)

Dissolve 10.0 g picric acid in distilled water q.s. 1 liter.

B. NaOH (10%)

Dissolve 100.0 g NaOH in distilled water q.s. 1 liter.

C. HCl (0.1 N)

Dilute 8.5 ml conc. HCl in distilled water q.s. 1 liter.

Creatinine standard solutions:

Dissolve 200 mg creatinine (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) in 0.1 N HCl q.s. 100 ml (2.0 mg/ml creatinine). Dilute 8.75, 7.50, 6.25, 5.00, 3.75, 2.50 and 1.25 ml of 2.0 mg/ml creatinine solution to 10 ml with 0.1 N HCl yielding 1.75, 1.50, 1.25, 1.00, 0.75, 0.50 and 0.25 mg/ml creatinine standards respectively. Standards are layered with toluene and stored at 4° C.

Procedure:

Mix 8 parts of solution A with 2 parts of solution B forming alkaline picrate. Allow mixture to stand for 10 minutes before use. To duplicate 0.025 ml unknown samples, standards and water blank add 2.5 ml alkaline picrate and mix. After 10 minutes add 2.5 ml distilled water and mix. Read all tubes against the water blank at

490 mμ (peak of absorbency) before 15 minutes in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Calculations:

Optical density of creatinine standards, read at 490 mμ, plotted as a function of creatinine concentration yields a straight line over the range 0-2.0 mg/ml creatinine. Concentration of creatinine in unknown samples can be calculated by multiplying the measured optical density of the unknown sample by the slope of the standard curve:

$$C_{un} = \left[\frac{C_{std}}{OD_{std}}\right]_{ave} \times OD_{un}$$

where C = concentration

OD = optical density

std = standard

un = unknown

ave = average

APPENDIX 5

DIAL AND URETHANE SOLUTION

Reagents:

1. Diallyl barbituric acid (crystalline; K & K Laboratories, Inc., Plainview, New York).
2. Monoethyl urea (Pfaltz and Bauer, Inc., Flushing, New York).
3. Urethane (Aldrich Chemical Co., Milwaukee, Wisconsin).
4. Disodium calcium ethylene diamine tetra acetate trihydrate (Pfaltz and Bauer, Inc., Flushing, New York).

Procedure:

Dissolve 10.0 g urethane, 40.0 g monoethyl urea and 40.0 g diallyl barbituric acid in 25 ml of distilled water. Heat in a water bath to dissolve chemicals. Cool to room temperature, add 50.0 mg disodium calcium ethylene diamine tetra acetate trihydrate and dilute q.s. 100 ml with distilled water. Store in stoppered dark glass bottle at room temperature.

APPENDIX 6

LIQUID SCINTILLATION COUNTING

Reference: Instruction Manual, Mark I liquid scintillation counter Model 6860, Nuclear Chicago Corp., Des Plaines, Ill.

Principle:

Liquid scintillation counting is a method for detecting the energy of the primary particle emitted by a radioactive molecule. This energy is converted into light energy by a solution of fluors and detected by a photomultiplier tube connected to amplifiers and a scaler circuit. The radioactive substance is placed in close proximity to the scintillation fluor, making the technique well adapted for use with low energy beta emitters such as tritium and carbon-14.

Counting channel selection:

The energy spectra of tritium and carbon-14 overlap; and when both beta emitters are present in the same sample, they are counted simultaneously on 2 separate analyzer channels. Channel A amplifiers are adjusted to give high efficiency of counting tritium

with minimal interference of carbon-14; amplifiers of Channel C were set so that counting efficiency of tritium was insignificant while efficiency for carbon-14 was maximal. Channel B amplifiers were set to maximize energy pulses from an external standard of known disintegration rate (^{133}Ba).

Quench correction curve:

A non-fluorescent solute or solvent will absorb or quency energy emitted from the primary particle and reduce the efficiency of counting the radioactivity. A set of tritium and carbon-14 standards (Nuclear Chicago Corp., Des Plaines, Ill.) containing known amounts of isotope (419,000 dpm and 255,000 dpm, respectively) and varying degrees of quenching for each isotope over the range used in these experiments was used to determine:

1. tritium counting efficiency in tritium channel (A)
2. carbon-14 counting efficiency in tritium channel (A)
3. carbon-14 counting efficiency in carbon-14 channel (C).

The external standard, ^{133}Ba, is used to determine the amount of quenching present in each sample. The channels ratio relates the net barium count rate in the barium channel (B) to the net barium count rate in the tritium channel (A). Since the sample will quench energy emitted by the gamma source, the channels ratio $\frac{B}{A} = \frac{\text{channel B (cpm)}}{\text{channel A (cpm)}}$ increases as the amount of quenching increases.

The quench correction curve (Figure 11) is a plot of the efficiencies of tritium and carbon-14 standards as a function of the standards' channels ratio (B/A). Counting efficiencies of each isotope in an unknown sample can be read directly from the quench correction curve corresponding to the unknown sample's channels ratio (B/A).

The disintegration rates of unknown samples can be calculated from the count rates of tritium and carbon-14 and the efficiencies of counting each isotope in channels A and C.

$$D_H = \frac{N_1 c_2 - N_2 c_1}{h_1 c_2}$$

$$D_C = \frac{N_2}{c_2}$$

where: D_H = disintegration rate (dpm) of tritium

D_C = disintegration rate (dpm) of carbon-14

N_1 = count rate in Channel A (cpm)

N_2 = count rate in Channel C (cpm)

h_1 = counting efficiency for tritium in Channel A (%)

c_1 = counting efficiency for carbon-14 in Channel A (%)

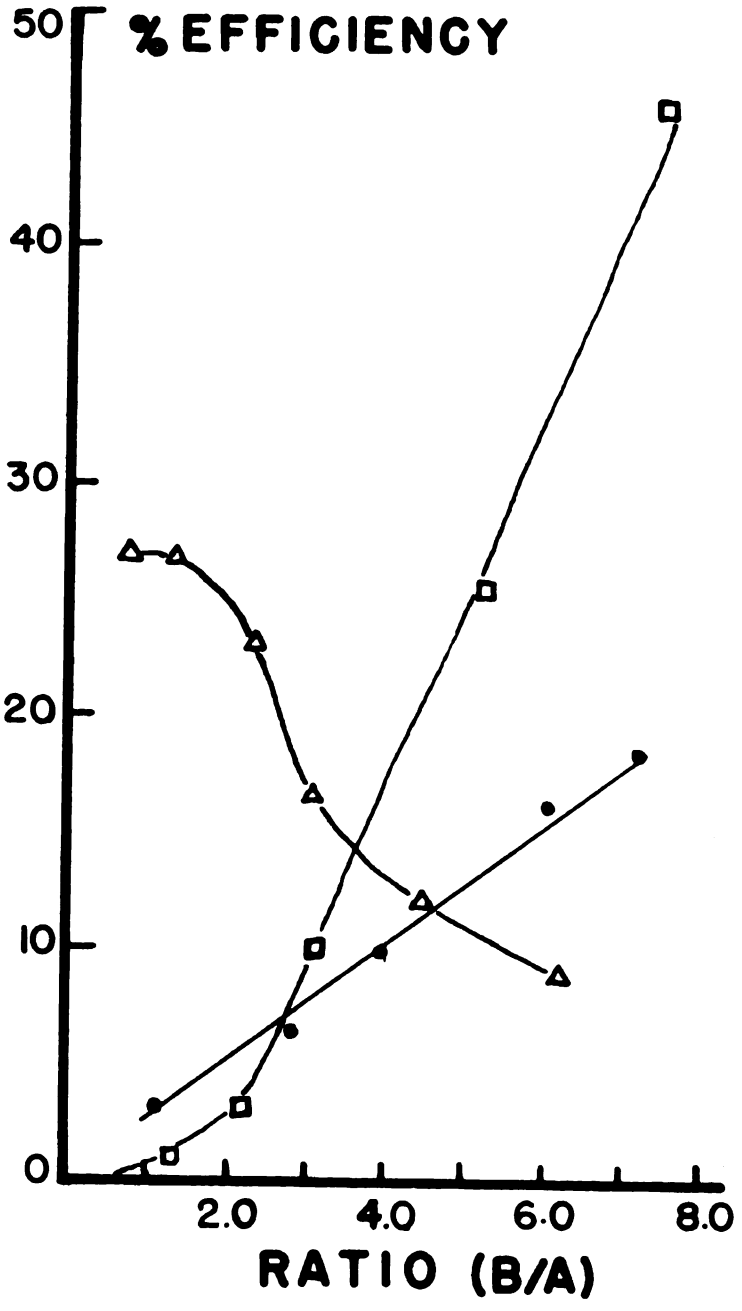
c_2 = counting efficiency for carbon-14 in Channel C (%)

Figure 11. --Barium-133 external standard quench correlation curve for differential counting of tritium and carbon-14 samples. Efficiencies calculated as CPM on scaler A or C/dpm of tritium or carbon-14 standards × 100, respectively. Carbon-14 quench correction curve for carbon-14 efficiency in Channel C (squares); tritium quench correction curve for tritium efficiency in Channel A (circles); and carbon-14 quench correction curve for carbon-14 efficiency in Channel A (triangles) are plotted as a function of Channels ratio $\left[\frac{B}{A} = \frac{\text{Channel B (CPM)}}{\text{Channel A (CPM)}}\right]$.

Discriminator settings:

Channel	Window	Attenuation
A	0.0-2.5	B524
B	0.0-9.9	F670
C	1.5-9.9	E738

Figure 11



APPENDIX 7

PROGRAM RATIO: EXTENDED FORTRAN VERSION FOR THE CALCULATION OF T:M PAH AND MANNITOL RATIOS

```
      PROGRAM RATIO (INPUT,OUTPUT,TAPE60=INPUT,TAPE61=OUTPUT)
      REAL MLHOM,MLASS,MANM1,MANM2,MANCP1,MANCP2,MANM,MANCP,MCP,MMED,MSM
1     READ(60,900)
  900 FORMAT(*                                                        *)
      WRITE(61,900)
      READ(60,901) N
  901 FORMAT (I2)
      READ(60,100) BPAH,BMAN,MLHOM,MLASS
  100 FORMAT (4F10.2)
      KNTR = 0
      DO 800 I=1,N
      READ(60,101) WT,PAHM1,PAHM2,PAHCP1,PAHCP2
  101 FORMAT(1F10.5,4F10.2)
      KNTR = KNTR + 1
C     COMPUTE WEIGHT OF TISSUE IN SAMPLE ASSAYED
      GM = (WT/MLHOM) * MLASS
C     COMPUTE T/M RATIO OF 3H-PAH
      PAHM = ((PAHM1 + PAHM2)/2) - BPAH
      PAHCP = ((PAHCP1 + PAHCP2)/2) - BPAH
      PCP = PAHCP/GM
      PMED = PAHM/MLASS
      PSM = PCP/PMED
      TOTACT = PCP * WT
      READ(60,102) MANM1,MANM2,MANCP1,MANCP2
  102 FORMAT (4F10.2)
C     COMPUTE T/M RATIO OF 14C-MANNITOL
      MANM = ((MANM1 + MANM2)/2) - BMAN
      MANCP = ((MANCP1 + MANCP2)/2) - BMAN
      MCP = MANCP/GM
      MMED = MANM/MLASS
      MSM = MCP/MMED
      WRITE(61,500)  KNTR
  500 FORMAT(*0*,I5)
      WRITE (61,501)  WT
  501 FORMAT(*     WEIGHT OF CHOROID PLEXUS = *,1F11.10,* GMS*)
      WRITE(61,502) GM
  502 FORMAT(*     WEIGHT OF TISSUE IN SAMPLE = *,1F11.10,* GMS*)
      WRITE(61,505) PSM
  505 FORMAT(*     T/M RATIO PAH = *,1F10.2)
      WRITE(61,508) MSM
  508 FORMAT(*     T/M RATIO MANNITOL = *,1F10.2)
      WRITE (61,499)  PCP
  499 FORMAT(*     SPECIFIC ACTIVITY OF PAH = *,1F15.2)
      WRITE(61,509) TOTACT
  509 FORMAT(*0     TOTAL ACTIVITY IN CHOROID PLEXUS = *,1F15.2)
  800 CONTINUE
      GO TO 1
      END
```

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