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Research Article

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Riboflavin Transport in the Central Nervous System

CHARACTERIZATION AND EFFECTS OF DRUGS

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ABSTRACT The relationship of riboflavin transport to the transport of other substances including drugs in rabbit choroid plexus, the anatomical locus of the blood-cerebrospinal fluid barrier, and brain cells were studied in vivo and in vitro. In vitro, the ability of rabbit choroid plexus to transport riboflavin from the medium (cerebrospinal fluid surface) through the choroid plexus epithelial cells into the extracellular and vascular spaces of the choroid plexus was documented using fluorescence microscopy. These studies provided further evidence that riboflavin is transported from cerebrospinal fluid to blood via the choroid plexus. The transport of [^{14}C]riboflavin by the isolated choroid plexus was inhibited by thiol agents, ouabain, theophylline, various flavins (lumiflavin and lumichrome > sugar containing flavins), and cyclic organic acids including penicillin and fluorescein. Riboflavin inhibited [^{14}C]penicillin transport competitively and the inhibition constant (K_I) for riboflavin equaled the concentration of riboflavin at which the saturable transport system for riboflavin is 50% saturated (K_T). These and other data suggest that riboflavin, penicillin, and possibly fluorescein are transported by the same transport system in choroid plexus. In vivo, the intraventricular injection of riboflavin and [^{14}C]penicillin inhibited [^{14}C]penicillin transport from cerebrospinal fluid. In vitro, various flavins (riboflavin > other sugar-containing flavins > lumiflavin > lumichrome) inhibited [^{14}C]riboflavin accumulation by brain slices. These studies support the notions that: (*a*) riboflavin accumulation by choroid plexus (active transport) is quite different from that in brain cells (facilitated diffusion and intracellular trapping), and (*b*) therapeutically important cyclic organic acids (e.g., penicillin) are transported from cerebrospinal fluid by the riboflavin transport system in choroid plexus.

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INTRODUCTION

Many foreign substances including several important drugs are transported from cerebrospinal fluid (CSF)[1] into blood by active transport (efflux) processes[2] located, in part, within the choroid plexus (1, 2). The choroid plexus, the anatomical location of the blood-CSF barrier, contains at least three separate efflux transport systems for anions (the iodide, sulfate, and cyclic organic acid systems) as well as the efflux system(s) for organic bases (1–3). In vitro, the isolated choroid plexus concentrates these substances by separate, active transport mechanisms (1, 2). The cyclic organic acid transport system within the choroid plexus (and possibly other sites in the central nervous system) is the principal mechanism for maintaining extremely low levels of certain drugs in CSF (e.g., penicillin G) (1, 4). The transport of these drugs out of CSF complicates the therapy of several serious disorders of the central nervous system (CNS), e.g., meningitis with certain penicillins and cephalosporins, and meningeal cancer with methotrexate (2, 4, 5). We and others have speculated that these drugs are transported (by chance) from CSF on systems that transport endogenous substances out of CSF.

This view of drug transport from CSF became tenable during our studies of riboflavin homeostasis in the CNS. Total riboflavin levels (riboflavin, flavin

[1] *Abbreviations used in this paper*: CNS, central nervous system; CPDS, 6,6′ dithiodinicotinic acid; CSF, cerebrospinal fluid; T/M, tissue-to-medium ratios; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; I_{c50}, the concentration of inhibitor to depress uptake by 50%; NEM, *N*-ethylmaleimide; K_T, the concentration of ligand at which the saturable transport system is 50% saturated; Y_{max}, the maximal transport rate by the saturable transport system.

[2] In the present studies, the word "transport" refers to the vitamer (riboflavin) or unchanged drug crossing the cell boundary whereas "accumulation" and "uptake" refer to the observed level of intratissue vitamin (riboflavin + FMN + FAD) or drug.

mononucleotide [FMN], and flavin adenine nucleotide [FAD]) in brain, unlike in liver and other organs, are maintained relatively constant even in the face of severe riboflavin deficiency or excess (6). In brain, as in other tissues, riboflavin is enzymatically phosphorylated to FMN, which can then be converted to FAD (6, 7). In vivo, total riboflavin homeostasis in brain depends, in large part, on saturable transport of riboflavin through the blood-brain barrier, saturable accumulation of riboflavin by brain cells via a high affinity (0.1 μM) accumulation process, and rapid, saturable probenecid-sensitive (efflux) transport of riboflavin from CSF (6–8). In these three locations, riboflavin (not FMN or FAD) is the principal moiety (vitamer) transported across cell membranes (8, 9). In vitro, brain slices accumulate riboflavin by facilitated diffusion and intracellular trapping of riboflavin as FMN (and FAD); the isolated choroid plexus accumulates riboflavin by an active transport mechanism with a Michaelis-Menten transport constant (K_T) = 78 μM and a maximal transport rate by the saturable transport system (Y_{max}) = 0.11 mmol/min per kg (7, 8).

The purposes of the present study were to: (*a*) characterize further the riboflavin transport and accumulation systems of the CNS, and (*b*) explore the relationship of the transport of several cyclic anionic drugs (e.g., penicillin) to riboflavin transport in the CNS. Besides extending our knowledge of the mechanisms of riboflavin transport and accumulation in the CNS, the results showed that penicillin and possibly fluorescein and other weak cyclic organic anions are transported from CSF to blood by the riboflavin transport system of choroid plexus.

METHODS

The following radiochemicals were purchased from Amersham Corp., Arlington Heights, Ill. [^{14}C]riboflavin (31 mCi/mmol), [^{14}C]penicillin (51 mCi/mmol), [^{14}C]niacinamide (52 mCi/mmol), [^{3}H]folic acid (56 Ci/mmol) and [^{3}H]sucrose (15.5 Ci/mmol). All experiments were performed on New Zealand white rabbits. Other materials were obtained from sources previously described (6–8) or are noted in Acknowledgments.

Fluorescent microscopy. To study the uptake of riboflavin by the isolated rabbit choroid plexus, advantage was taken of the marked fluorescence of riboflavin and methodology recently described by Bresler et al. (10). Briefly, fluorescence microscopy was performed with a Leitz-Wetzlar Orthoplan Universal Large Field Microscope with a xenon lamp for fluorescence (E. Leitz, Inc., Rockleigh, N. J.). When fluorescence microscopy was used, epi-illumination was employed. Trans-illumination was used for white light photographs. An automatic Orthomat W microscopy camera with Kodak EK-135 Ectachrome film (ASA 400) (Eastman Kodak Co., Rochester, N. Y.) was used. To shorten exposure times, the film was used and developed at an ASA of 800. For the fluorescence pictures, a G (3) filter block was used with an exciting filter (BP) of 350–460 nm and a suppression filter (LP) of 515 nm.

In our fluorescence microscopic studies of choroid plexus accumulation of riboflavin, isolated choroid plexuses (obtained as described below) were incubated in artificial CSF containing 0.1 mM riboflavin at 37°C under 95% O_2 5% CO_2 for various times. At the end of the incubation, the choroid plexus was removed from the incubation medium and placed on a glass slide. A drop of incubation medium was placed on the choroid plexus, which was then gently covered and compressed lightly with a glass cover slip. In some cases, the choroid plexus was washed for 2 s in artificial CSF (37°C) before placing the choroid plexus on the glass slide. In these experiments, a drop of artificial CSF, which contained no riboflavin, was placed on the choroid plexus before the glass cover slip was applied. At various times after placing the choroid plexus on the glass slide, fluorescence, and white light photomicrographs at various magnifications were taken.

Accumulation and release of substances by the isolated choroid plexus. To study the effects of various experimental conditions on the accumulation of [^{14}C]riboflavin (or other radiolabeled substances), isolated intact New Zealand white rabbit choroid plexuses (weighing ~6 mg) were incubated for various times at 37°C under 95% O_2:5% CO_2 in 3 ml artificial CSF containing 5 mM glucose, [^{14}C]riboflavin and/or various other substances in a metabolic shaker by methods previously described in detail (1, 8). At the end of the incubation, the choroid plexuses were weighed, homogenized in either H_2O or 5% TCA, and the content of ^{14}C or ^{3}H in the homogenates and medium determined. Tissue-to-medium ratios (T/M) were determined by dividing the ^{14}C disintegrations per minute per gram of tissue by the ^{14}C disintegrations per minute per milliliter of medium (8).

Although previous studies showed that the release of accumulated [^{14}C]riboflavin by choroid plexus was not saturable or temperature dependent (i.e., simple diffusion) (8), the possibility that theophylline might modify the release of accumulated [^{14}C]riboflavin was checked. Briefly, the release of ^{14}C from choroid plexuses (that had been incubated in artificial CSF containing 0.7 μM [^{14}C]riboflavin for 30 min as above) was measured. At the end of the accumulation incubation in artificial CSF containing [^{14}C]riboflavin, each choroid plexus was washed for 2 s in 10 ml artificial CSF (37°C) and transferred to 3 ml release media (i.e., artificial CSF containing 5 mM glucose, and in some cases, other substances). At the end of the release incubation, the concentration of ^{14}C in the choroid plexus and release medium was determined.

In some experiments, choline chloride was substituted for the sodium chloride in artificial CSF. This reduced the sodium content of the artificial CSF from 150 to 26 mM. The choline-containing artificial CSF is called "choline artificial CSF."

The kinetics of [^{14}C]penicillin accumulation by isolated choroid plexuses were measured during the initial (linear) part of the accumulation process. The kinetic data were fitted to Michaelis-Menten transport kinetics as previously described in detail (1, 8).

Accumulation of [^{14}C]riboflavin by brain slices. To measure the ability of rabbit brain slices to accumulate [^{14}C]-riboflavin under various experimental conditions, brain slices were prepared by methods previously described (7). The brain above the left lateral ventricle (~1,000 mg including cerebral cortex, white matter, and periventricular grey matter) was weighed, sliced in two different directions in a McIlwain tissue chopper at 90° angles at a setting of 0.25 mm, and dispersed in 10 ml of ice-cold artificial CSF (pH = 7.3) containing 1 mg/ml glucose (7). After centrifugation at 400 *g* for 3 min, the tissue was redispersed and washed twice in 10 ml iced artificial CSF. Then, 400 μl of the dispersed brain

slices (~30 mg) were added to 25-ml Erlenmeyer flasks containing 3 ml of artificial CSF with 1 mg/ml glucose, [^{14}C]riboflavin, and various other substances. The incubation was performed under 95% O_2:5% CO_2 at 37°C in a Dubnoff metabolic shaker (80 rpm) for various times (7). At the end of the incubation, the dispersed brain slices were filtered onto Whatman GF/A Glass Microfibre filter paper discs (Whatman, Inc., Clifton, N. J.) on Buchner funnels by vacuum and washed with two 5-ml samples of ice-cold artificial CSF. The ^{14}C activity on the filter paper was determined by adding the filter paper to 3.0 ml of H_2O, allowing the brain slices and filter paper to sit for 1 h, adding 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) and measuring the ^{14}C radioactivity in the gel (7). T/M were obtained by dividing the disintegrations per minute per gram of tissue by the disintegrations per minute per milliliter of medium (7). A 20% weight correction was made for tissue losses during the tissue preparation, incubation, and filtration steps (7).

Clearance of [^{14}C]*penicillin from CSF.* To study whether [^{14}C]penicillin clearance from CSF can be inhibited in vivo by riboflavin, we measured the clearance of intraventricularly injected [^{14}C]penicillin relative to [^{3}H]sucrose[3] with and without the addition of unlabeled FMN to the injectate by methods previously described (4, 6). FMN rather than riboflavin was used because of the limited solubility of riboflavin and the rapid conversion of FMN to riboflavin in CSF (6). Briefly, 0.15 ml of artificial CSF containing 2.3 μCi [^{3}H]sucrose (0.15 nmol), 0.34 μCi [^{14}C]penicillin (6.7 nmol), and, in some cases, 3.5 μmol FMN were injected into the left lateral ventricle of sodium pentothal-anesthetized rabbits by previously described methods (4, 6). After 2 h, the conscious rabbit was reanesthetized and killed with a cardiac puncture through the chest wall. Cisternal CSF was immediately withdrawn and the brain, and choroid plexuses were removed as rapidly as possible. The total ^{14}C and ^{3}H content of the left brain, right brain, CSF, and choroid plexuses were determined (4). The ratios of the ^{14}C to ^{3}H in CSF, choroid plexus, left and right brain homogenates were divided by the comparable ratio in the injectate in all experiments (4).

Analytical methods and assays. ^{3}H and ^{14}C scintillation spectroscopy were performed as previously described (6–8). All samples were made up to 3.3 ml of water and 10 ml of Scintiverse was added (8). The samples were assayed in a Packard 3375 scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) (8).

The concentration of total riboflavin and the various riboflavin vitamers in various solutions or CSF was determined fluorometrically (8).

To determine the purity of the [^{14}C]riboflavin and the integrity of [^{14}C]riboflavin after various experimental manipulations, chromatography was performed in three separate systems; DEAE-Sephadex or Dowex 1 ion exchange chromatography (Dow Corning Corp., Midland, Mich.) (8, 11), thin-layer chromatography in sodium phosphate buffer (6–8, 11), and Sephadex G-15 or G-10 gel exclusion chromatography (8, 11). Riboflavin was separated from FMN and FAD on Sephadex G-15 gels; lumiflavin and lumichrome was separated from riboflavin on Sephadex G-10 gels (11).

The nature of the ^{14}C within choroid plexuses and brain slices (that had been incubated in media containing various concentrations of [^{14}C]riboflavin) was determined by methods previously described in detail that used both the column and thin-layer chromatographic methods described above (8, 11). In all these assays, to assess recovery, duplicate aliquots of homogenates, the various supernates, and appropriate controls were assayed for ^{14}C activity (8).

The purity of [^{14}C]penicillin, [^{3}H]folic acid, [^{14}C]niacinamide, and [^{3}H]sucrose were determined on at least one of the paper or thin-layer chromatographic systems recommended by the supplier (1, 12, 13). [^{3}H]folic acid needed at least bimonthly purification (12), and [^{14}C]penicillin was made up from powder immediately before use in all experiments because of its tendency to decay rapidly in water solutions (1, 4).

Statistical analysis. Student's t test and Scheffe's method for multiple comparisons in the Gaussian analysis of variance were used for comparisons (14). A P value of <0.05 was considered statistically significant.

RESULTS

In previous studies, we have provided indirect evidence that the choroid plexus transports riboflavin from CSF into blood (6, 8). In Figs. 1 and 2, the ability of the choroid plexus' epithelial cells to accumulate riboflavin is pictorially confirmed. Within the choroid plexus, there is marked fluorescence of the cytoplasm but not of the nuclei. Under our conditions, no autofluorescence was detectable. In Figs. 3 and 4, the choroid plexus was allowed to accumulate riboflavin for a longer period of time. Then the choroid plexus was rinsed to remove most of the riboflavin in the adherent medium and the fluorescence measured. As can be seen in Fig. 3, the fluorescence in the choroid plexus epithelial cells is less than in Fig. 1, whereas the fluorescence at the base of the choroid plexus epithelial cells in the extracellular space and, to a certain extent, in the blood vessels, is much more marked. In Figs. 1–4 and many other photomicrographs (not shown), it is visually apparent that riboflavin can first be concentrated from the medium (corresponding to the CSF in vivo) within the choroid plexus epithelial cells, and then transferred through the epithelial cells into the extracellular space of the choroid plexus. Presumably, when blood is coursing through the choroid plexus in vivo, the riboflavin that has been transported through the epithelial cells from the CSF side would be carried away in the blood. There are no tight junctions in the vascular spaces of choroid plexus to inhibit the diffusion of substances from the extracellular space into the vascular space of choroid plexus (15).

The ability of various experimental conditions to alter the accumulation of [^{14}C]riboflavin by choroid plexus is shown in Table I. Lumiflavin and lumichrome, both of which are photodegradation products of riboflavin and do not contain ribose, were more potent inhibitors of [^{14}C]riboflavin transport (concentration of inhibitor to depress uptake by 50% [I_{c50}] $\simeq$ 10 μM) than the sugar-containing flavins isoriboflavin, lyxoflavin, galactoflavin, or riboflavin itself ($I_{c50} \simeq 100$ μM). The cyclic organic acids, fluorescein and penicillin were slightly more potent inhibitors of [^{14}C]ribo-

[3] Sucrose was chosen as an internal standard because it: (*a*) is not metabolized, (*b*) traverses the central nervous system by simple diffusion, and (*c*) is similar in size, shape, and solubility characteristics to penicillin.

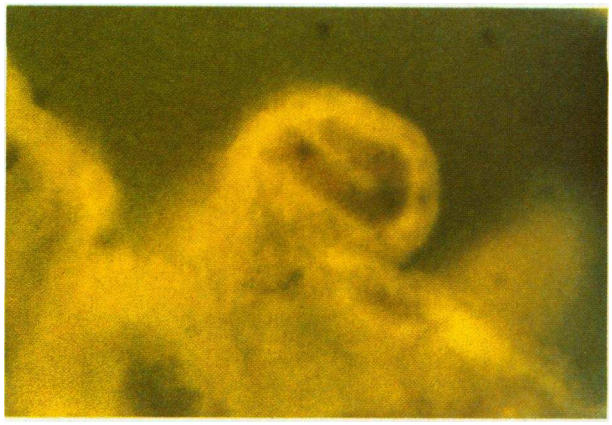

FIGURE 1 Fluorescence micrograph of choroid plexus incubated in medium containing riboflavin. The fourth ventricular choroid plexus was incubated at 37°C under 95% O_2:5% CO_2 in artificial CSF containing 0.1 mM riboflavin for 4 min. Then, the choroid plexus was removed, placed on a glass slide in a drop of riboflavin-containing medium, and covered. The fluorescence micrograph was obtained 5 min after placing the choroid plexus on the slide. In the central frond, the intense yellowish green fluorescence of riboflavin in the cytoplasm but not nuclei of the choroid plexus epithelial cells is apparent. ×339.

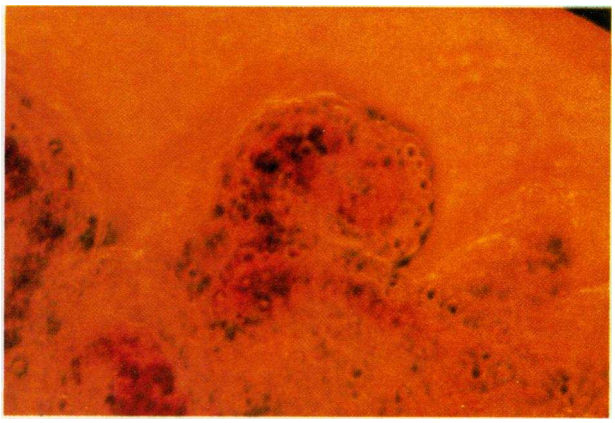

FIGURE 2 Same specimen as in Fig. 1 photographed with white light. The outer portion of the tissue exposed to the medium and, in vivo, to CSF is the single layer of choroid plexus' epithelial cells that are joined by tight junctions (15). Within the choroid plexus is the extracellular space of choroid plexus and large vascular spaces (15). The pink worm-like vascular spaces within the choroid plexus are apparent and due to retained erythrocytes. ×339.

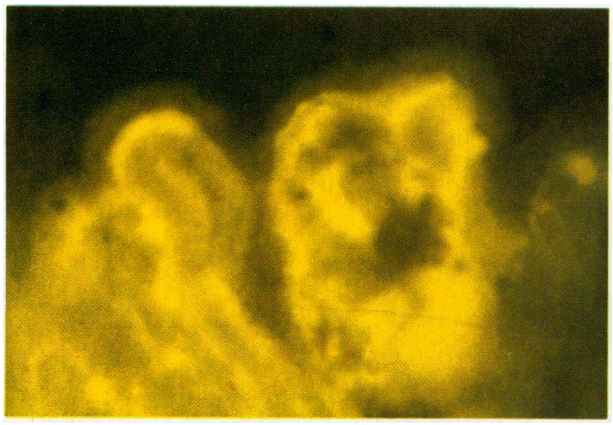

FIGURE 3 Fluorescence micrograph of choroid plexus incubated in riboflavin and then rinsed. The fourth ventricular choroid plexus was incubated in 0.1 mM riboflavin as in Fig. 1 for 20 min. Then, the choroid plexus was removed, rinsed in artificial CSF, placed on a glass slide in a drop of artificial CSF, and covered. The fluorescence micrograph was obtained 3 min after placing the choroid plexus on the slide. The minimal yellowish green fluorescence in the choroid plexus epithelial cells and the bright yellowish green (riboflavin) fluorescence in the extracellular and vascular spaces are apparent. ×339.

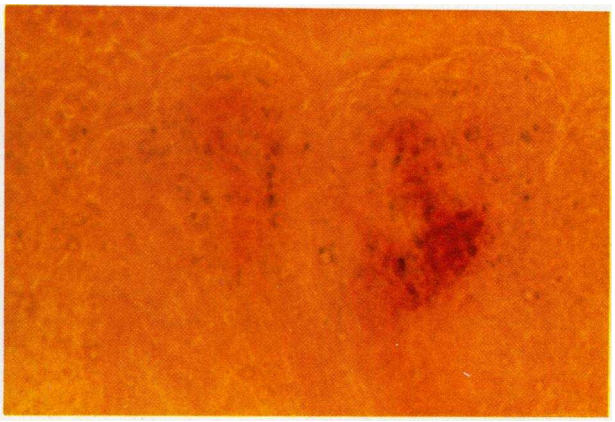

FIGURE 4 Same specimen as in Fig. 3 photographed with white light. The pink wormlike vascular spaces within the choroid plexus are apparent. ×339.

flavin transport than riboflavin itself (Table I). Among the bases tested, both quinidine and its stereoisomer quinine inhibited [^{14}C]riboflavin accumulation (I_{c50} $\simeq$ 100 μM) (Table I). Of the thiol reagents tested, the nonpenetrating thiol reagent 6,6′dithiodinicotinic acid (CPDS) (16), was a much less potent inhibitor of [^{14}C]riboflavin accumulation than the penetrating thiol reagent *N*-ethylmaleimide (NEM) (Table I). Incubation of choroid plexuses in choline artificial CSF, or CSF containing ouabain or theophylline (both 1 mM) also significantly inhibited the choroid plexus' ability to accumulate [^{14}C]riboflavin. Similarly, incubation in chlorpromazine (50 μM) caused significant inhibition of ^{14}C accumulation by the isolated choroid plexus. The addition of imipramine (100 μM), folic acid (50 μM), sulfate (100 μM), and iodide (1.0 mM) did not significantly affect [^{14}C]riboflavin accumulation.

The ability of the substances in Table I to inhibit

TABLE I

*Effect of Various Experimental Conditions on Choroid Plexus Uptake of [^{14}C]Riboflavin**

Inhibitor class	Inhibitor	Concentration	T/M±SEM n‡	Percent control
		μM		
—	Control	0.7	19.51±0.64 (58)	—
Flavins				
	Riboflavin	100	9.58±0.68 (21)	49§
	Galactoflavin	100	12.64±1.71 (6)	65§
	L-lyxoflavin	100	9.90±0.92 (6)	51§
	Isoriboflavin	67	7.32±0.92 (6)	38§
	Lumichrome	1	19.97±0.82 (6)	103
	Lumichrome	10	9.51±1.50 (6)	49§
	Lumichrome	100	4.94±0.28 (6)	25§
	Lumiflavin	110		27§,‖
Acids				
	Sodium fluorescein	10	10.49±1.57 (4)	54§
	Sodium fluorescein	100	6.74±1.11 (3)	34§
	Sodium penicillin G	10	15.59±1.52 (3)	80
	Sodium penicillin G	50	8.71±0.77 (10)	45§
	Na_2SO_4	100	18.54±1.93 (4)	95
Bases				
	Quinidine SO_4	100	7.65±0.74 (4)	39§
	Quinine SO_4	10	16.36±2.60 (4)	83
	Quinine SO_4	100	10.66±1.14 (4)	54§
	Quinine SO_4	10^3	1.68±0.11 (4)	9§
Thiol agents				
	NEM	50	7.28±1.25 (5)	37§
	NEM	10^3	1.41±0.21 (4)	7§
	CPDS	100	10.42±1.03 (6)	43§
	CPDS	10^3	6.46±1.07 (4)	33§
Others				
	Choline artificial CSF		10.46±1.73 (6)	54§
	Ouabain	10^3	6.71±1.20 (4)	34§
	Theophylline	10^3	6.17±0.68 (5)	32§
	Chlorpromazine	10	14.53±1.02 (3)	74
	Chlorpromazine	50	5.06±0.82 (7)	26§
	Imipramine	100	15.55±0.62 (6)	80
	Folic acid	50	17.20±2.18 (6)	88
	Sodium iodide	10^3	26.60±3.43 (5)	136

* Choroid plexuses were incubated at 37°C for 30 min under various conditions in artificial CSF containing 0.7 μM [^{14}C]riboflavin under 95% O_2:5% CO_2 in a metabolic shaker. At the end of the incubation, the T/M were measured. All values are means.

‡ n = number of experiments.

§ $P < 0.05$ by Scheffe's method for multiple comparisons in the Gaussian analysis of variance.

‖ Value from ref. (8).

[^{14}C]riboflavin accumulation was not due to interference with [^{14}C]riboflavin metabolism within the choroid plexus because 96±1 (SEM; n = 5) % of the ^{14}C within the choroid plexus in the controls in Table I was unchanged [^{14}C]riboflavin. We have previously shown that [^{14}C]riboflavin is not bound within the choroid plexus (8). It was also not the result of degradation, reduction or complexing (17) of the [^{14}C]riboflavin by the inhibitors. The addition of the following substances at the concentrations given in Table I did not decrease the fluorescence (excitation 450 nm; emission 530 nm) of artificial CSF containing 0.7 μM [^{14}C]-riboflavin incubated at 37°C for 30 min in 95% O_2:5% CO_2 by >5%: theophylline (2.0 mM), penicillin G, CPDS, NEM (all 1.0 mM), and chlorpromazine, imipramine, quinidine, and quinine (all 100 μM). Also, penicillin, NEM, CPDS (all 1 mM) and chlorpromazine or quinidine (100 μM) did not degrade the [^{14}C]riboflavin to [^{14}C]lumiflavin or [^{14}C]lumichrome after 30-min incubations at 37°C as determined by thin-layer and gel-exclusion chromatography.

As a control, the ability of lumichrome (100 μM) and

quinidine (100 μM) to interfere with the uptake of [^{3}H]folic acid from artificial CSF containing 2 nM [^{3}H]folic acid was measured. Neither quinidine nor lumichrome interfered with the ability of the isolated choroid plexus to concentrate [^{3}H]folic acid (data not shown). However, the ability of the isolated choroid plexus to concentrate [^{3}H]folic acid or [^{14}C]niacinamide after 30-min incubations in medium containing 2 nM [^{3}H]folic acid or 1 μM [^{14}C]niacinamide was markedly reduced when chlorpromazine (100 μM) was placed in the medium. In these cases, chlorpromazine decreased [^{3}H]folic acid uptake and [^{14}C]niacinamide uptake by ~60% ($P < 0.01$ in both cases; data not shown). Thus, chlorpromazine seemed to have the ability to depress the accumulation of [^{14}C]riboflavin, [^{3}H]folic acid, and [^{14}C]niacinamide by choroid plexus.

The addition of 1.0 mM theophylline to the release media did not modify the rate of release of [^{14}C]riboflavin. After a 30-min preincubation in 0.7 μM [^{14}C]-riboflavin, the choroid plexuses were washed and transferred to release medium containing 0.0 or 1.0 mM theophylline. After a 15-min incubation in the release medium, 57.2±3.4% (SEM, $n = 4$) of the [^{14}C]riboflavin in the choroid plexus, at the start of the release incubation, was released into the media that did not contain theophylline vs. 66.4±2.7% (SEM; $n = 4$) in the media that did contain theophylline ($P > 0.05$).

The ability of flavins to protect the choroid plexus against thiol reagent inhibition of [^{14}C]riboflavin accumulation is shown in Table II. Because of the limited solubility of the various flavins and because of the ability of lyxoflavin, isoriboflavin, and riboflavin to inhibit [^{14}C]riboflavin accumulation (Table I), 0.1 mM concentrations of each of these three flavins were added to the preincubation media with the thiol reagent in an attempt to protect the choroid plexus from the damage due to the thiol reagent. No protection was seen with NEM (Table II); however, with CPDS, the addition of the flavins to the preincubation medium appeared to protect the choroid plexus' ability to accumulate [^{14}C]riboflavin slightly (Table II).

The ability of sodium fluorescein and chlorpromazine to inhibit [^{14}C]penicillin accumulation by choroid plexus is shown (Table III). The I_{c50} for fluorescein was slightly greater than 10 μM. FMN is a weaker inhibitor of [^{14}C]penicillin accumulation by the choroid plexus than riboflavin itself (Table III; Fig. 5).

The ability of riboflavin to inhibit competitively the accumulation of [^{14}C]penicillin by the isolated rabbit choroid plexus during the linear part of the penicillin accumulation process (1) is shown in Fig. 5. The K_T for penicillin was determined to be 42 μM and the inhibition constant (K_I) for riboflavin equal to 77 μM. The K_T for riboflavin, when determined directly, was 78 μM (8). As shown in Table I, the concentration of penicillin in the medium necessary to decrease the accumulation of [^{14}C]riboflavin by 50% (I_{c50}) was ~50 μM.

The ability of FMN and/or riboflavin to inhibit the rapid efflux of [^{14}C]penicillin from the CNS is shown in Table IV. The rapid efflux of [^{14}C]penicillin from CSF, choroid plexus, and brain is significantly decreased by the concurrent injection of 3.5 μmol FMN.

The ability of various flavins to inhibit the accumulation of [^{14}C]riboflavin by brain slices is shown in Table V. Unlike choroid plexus, riboflavin was the most potent inhibitor of [^{14}C]riboflavin accumulation by brain slices. Galactoflavin, lyxoflavin, and isoriboflavin were less potent inhibitors of [^{14}C]riboflavin accumulation by brain slices than riboflavin. All the sugar-containing flavins, however, were much more

TABLE II

*Reversibility of Thiol Reagent Inhibition of [^{14}C]Riboflavin Uptake by Choroid Plexus**

Preincubation solution	T/M±SEM *n*	Percent control	Preincubation solution	T/M±SEM *n*	Percent control
Control	17.30±1.10 (6)	—	Control, 0.3 mM	13.89±0.89 (11)	—
NEM, 10 μM	13.67±2.27 (4)	79	—	—	—
NEM, 50 μM	2.90±0.61 (3)	17‡	—	—	—
NEM, 200 μM	1.91±0.14 (4)	11‡	NEM, 200 μM	2.03±0.12 (4)	15‡
CPDS, 100 μM	16.94±1.18 (4)	98	—	—	—
CPDS, 200 μM	9.00±1.33 (4)	52‡	CPDS, 200 μM	9.61±1.74 (4)	69
CPDS, 1.0 mM	6.53±1.14 (4)	38‡	—	—	—
CPDS, 2.0 mM	5.06±0.93 (4)	29‡	CPDS, 2.0 mM	5.82±1.34 (4)	42‡

* Choroid plexus were preincubated at 37°C under 95% O_2:5% CO_2 in a metabolic shaker containing the thiol reagent (left) and the thiol reagent and 0.1 mM riboflavin + 0.1 mM galactoflavin + 0.1 mM L-lyxoflavin (0.3 mM flavins total) (right). Then, the choroid plexuses were rinsed in artificial CSF and transferred to artificial CSF containing 0.7 μM [^{14}C]riboflavin, incubated for 30 min, and the T/M ratios were determined. All values are means.

‡ $P < 0.05$ by Scheffe's method for multiple comparisons in the Gaussian analysis of variance. Each value in the column percent control was compared with the control value in that column.

TABLE III

*Effect of Various Substances on Choroid Plexus Uptake of [^{14}C]Penicillin**

Inhibitor	Concentration	T/M + SEM *n*	Percent control
	μM		
Control	0.1	15.18±1.12 (16)	—
Sodium fluorescein	10	7.94±0.88 (3)	52‡
Sodium fluorescein	100	2.34±0.40 (3)	15‡
FMN	10	11.81±3.07 (3)	78
FMN	100	10.42±0.74 (5)	69
Chlorpromazine	100	6.57±0.78 (6)	43‡

* Choroid plexuses were incubated at 37°C for 5 min in artificial CSF containing 0.1 μM [^{14}C]penicillin and various substances under 95% O_2:5% CO_2 in a metabolic shaker. At the end of the incubation, the T/M were measured. All values are means.

‡ $P < 0.05$ by Scheffe's method for multiple comparisons in the Gaussian analysis of variance.

potent inhibitors of [^{14}C]riboflavin accumulation than lumiflavin and lumichrome (Table V). Lyxoflavin did not interfere with the formation of intracellular FMN and FAD. In the controls (Table V), 41±2% (SEM; $n = 6$) of the ^{14}C within the choroid plexus was [^{14}C]-FMN and [^{14}C]FAD; with lyxoflavin (10 μM) in the media (Table V), 45±3% (SEM: $n = 5$) was [^{14}C]-FMN and [^{14}C]FAD.

A large number of other substances were tested and did not significantly interfere with the ability of the brain slices to accumulate [^{14}C]riboflavin under the same conditions as in Table V. These substances included fluorescein, penicillin G, chlorpromazine, haloperidol, and imipramine (all 100 μM); quinidine, tolazoline, CPDS, and pyridoxine (all 1 mM). These conclusions were based on 8–20 experiments for each of these substances. However, the addition of 1 mM NEM to the medium decreased [^{14}C]riboflavin accumulation to 57% ($n = 16$) of control values ($P < 0.01$).

DISCUSSION

The principal findings reported herein are: (*a*) the accumulation systems for riboflavin in the isolated choroid plexus and brain slices are very different and (*b*) that penicillin and possibly fluorescein and other cyclic organic acids are transported from CSF into blood via the riboflavin transport system within the choroid plexus.

In previous studies, we have shown that the isolated choroid plexus accumulated [^{14}C]riboflavin from artificial CSF by a saturable, energy-dependent mechanism with a $K_T = 78$ μM and a $Y_{max} = 0.11$ mmol/kg per min (8). This process did not depend on intracellular metabolism or binding of the riboflavin when the riboflavin concentration in the medium was 0.7 μM or greater (8). In vivo, [^{14}C]riboflavin is rapidly cleared from the CSF after intraventricular injections by a saturable probenecid-sensitive process (6). The photomicrographs (Figs. 1–4) in this study provide strong, direct, pictorial evidence of the validity of our previous view that riboflavin is picked up from the CSF side of the choroid plexus by the choroid plexus epithelial cells and then transferred through these cells into the extracellular space of the choroid plexus (6, 8). If blood were flowing through the choroid plexus, presumably the riboflavin would be transported out of the choroid plexus by the blood. Bresler et al. (10) have published fluorescence photomicrographs of fluorescein transport by isolated rabbit choroid plexus in vitro. As with riboflavin, fluorescein is transferred from the medium into the extracellular space of the choroid plexus via the choroid plexus epithelial cells (10). As with riboflavin, fluorescein did not enter the erythrocytes within the choroid plexus or the nuclei of the choroid plexus epithelial cells (10).

The riboflavin accumulation system within the choroid plexus appeared to be very sensitive to the penetrating thiol agent NEM (Table I). The nonpenetrating thiol agent CPDS (16) inhibited riboflavin accumulation by the isolated choroid plexus less markedly (Table I). The addition of flavins to the preincubation medium (that contained the thiol reagent) seemed to protect the choroid plexus ability to accumulate [^{14}C]riboflavin when CPDS, but not NEM, was placed in the preincubation medium. These data suggest that: (*a*) intracellular sulfhydryl groups are necessary for riboflavin accumulation by the choroid plexus (Tables I and II); and (*b*) raise the possibility that sulfhydryl groups on the membrane surface are involved in the transport of riboflavin through the choroid plexus (Table II).

Several investigators have suggested that certain

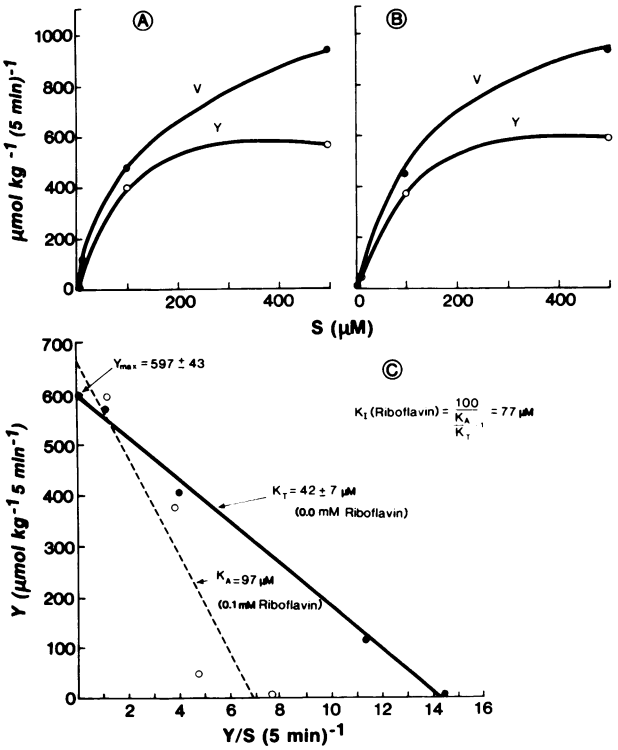

FIGURE 5 Competitive inhibition by riboflavin of penicillin accumulation by choroid plexus. Choroid plexuses were incubated for 5 min at 37°C in artificial CSF containing 0.1 μM [^{14}C]penicillin and various concentrations of unlabeled penicillin (A). In B, the experiments were performed as in A except riboflavin was present at a fixed concentration (0.1 mM). After 5 min, the T/M ratios of [^{14}C]penicillin were determined. The total uptake (V) of penicillin as a function of the penicillin concentration was determined in A and B by multiplying the mean T/M ratio at that concentration by the medium penicillin concentration (1). The saturable uptake (Y) of penicillin was determined by multiplying the mean T/M ratio at that concentration of penicillin minus the T/M ratio with 5 mM penicillin in the medium [T/M = 0.75 in A and 0.74 in B] by the medium concentration of penicillin (1). The total uptake (V) and saturable uptake (Y) as a function of concentration are shown in A and B (1). Each point is the mean of 6 to 13 determinations. In C, Hofstee transformations of the saturable uptake of penicillin (Y) with (B) and without (A) 0.1 mM riboflavin in the medium, and the concentration of penicillin in the medium (S) by the method of least squares are shown (1). The saturable uptake of [^{14}C]penicillin and carrier penicillin by choroid plexus is assumed to follow a Michaelis-Menten transport model (1). The K_T and Y_{max}±the 95% confidence limits for penicillin transport are shown without riboflavin in the medium. Also shown is the apparent K_T (K_A) for penicillin with 0.1 mM riboflavin in the medium. Because the slopes (K_T and K_A) but not Y_{max} values were significantly different, the assumption of competitive inhibition of penicillin transport by riboflavin was made (1). The derived K_T (K_I) for riboflavin is indicated in (C) (1). The T/M ratios with 0.1, 10, 100, or 500 μM [^{14}C]penicillin in the media were 15.17±1.12 (SEM; n = 13), 12.11±0.91 (n = 12), 4.78±0.26 (6), and 1.89±0.15 (6), respectively. With 0.1 mM riboflavin and 0.1, 10, 100, or 500 μM [^{14}C]penicillin in the media, the T/M ratios were 8.42±0.44 (n = 5), 5.45±0.37 (12), 4.53±0.26 (6), and 1.93±0.15 (6), respectively.

TABLE IV
*Clearance of Penicillin from CNS with and without Carrier FMN**

Tissue	Ratios±SEM without FMN (n = 4)	Ratios±SEM with FMN (n = 3)
CSF	0.04±0.01	0.18±0.02‡,§
Choroid plexus	0.28±0.04	0.67±0.01‡
Left brain	0.15±0.01	0.47±0.04‡
Right brain	0.09±0.02	0.35±0.01‡

* Into the left lateral ventricle of anesthetized rabbits, 0.15 ml artificial CSF containing 0.34 μCi [^{14}C]penicillin, 2.3 μCi [^{3}H]-sucrose and, in some cases, 3.5 μmol FMN was injected. After 2 h, the conscious rabbits were killed and the concentrations of ^{14}C and ^{3}H in CSF, choroid plexus and brain were determined. The ratio of ^{14}C to ^{3}H in each tissue was divided by the ratio of ^{14}C to ^{3}H in the injectate. All values are means.
‡ $P < 0.01$ by Student's t test; two-tailed.
§ The concentration of riboflavin plus FMN in the withdrawn CSF was determined fluorometrically and equalled 593±74 μM (SEM). 53±3% of the total was FMN, the remainder was riboflavin.

weakly basic drugs are riboflavin antagonists. Madinaveitia (18) showed that quinine inhibited growth of *Lactobacillus casei*, an effect that was reversed by riboflavin. Recently, several investigators have suggested that chlorpromazine interferes with riboflavin

TABLE V
Effect of Various Flavins on Uptake of [^{14}C]*Riboflavin by Brain Slices**

Flavin	T/M±SEM	n	Percent control
Control, 80 nM	2.42±0.08	(107)	—
Riboflavin, 0.7 μM	1.46±0.10	(10)	60‡
Riboflavin, 100 μM	0.96±0.09	(5)	40‡
Lumiflavin, 10 μM	1.73±0.16	(17)	71
Lumiflavin, 100 μM	0.86±0.08	(5)	36‡
Lumichrome, 10 μM	2.69±0.30	(10)	111
Lumichrome, 100 μM	2.06±0.18	(18)	85
Galactoflavin, 1 μM	2.11±0.09	(10)	87
Galactoflavin, 10 μM	1.40±0.15	(10)	58‡
Galactoflavin, 100 μM	0.94±0.07	(10)	39‡
L-lyxoflavin, 1 μM	1.98±0.13	(10)	81
L-lyxoflavin, 10 μM	1.28±0.08	(10)	53‡
L-lyxoflavin, 100 μM	0.86±0.07	(10)	35‡
Isoriboflavin, 1 μM	1.94±0.09	(11)	80
Isoriboflavin, 10 μM	1.56±0.12	(10)	64‡
Isoriboflavin, 67 μM	1.02±0.11	(10)	42‡

* Brain slices were incubated for 30 min at 37°C in artificial CSF containing 80 nM [^{14}C]riboflavin and various flavins under 95% O_2:5% CO_2. At the end of the incubation, the T/M were measured. All values are means.
‡ $P < 0.05$ by Scheffe's method for multiple comparisons in the Gaussian analysis of variance.

metabolism (19). The choroid plexus transport system for riboflavin (which does not depend on riboflavin binding or metabolism) seemed to be a likely place to test the effect of these drugs on riboflavin transport.

As shown in Table II, quinine, quinidine, and chlorpromazine seemed to inhibit [^{14}C]riboflavin accumulation by the choroid plexus. In previous studies, we have shown that tolazoline (1 mM), which is often used as an inhibitor of the weak basic transport systems in the kidney, had no significant effect on [^{14}C]-riboflavin accumulation by the choroid plexus in vitro (8). In the present study, chlorpromazine not only decreased riboflavin uptake by the isolated choroid plexus (Table I), but decreased folic acid and niacinamide accumulation by the choroid plexus comparably. Folic acid is accumulated by the choroid plexus unchanged (12) whereas niacinamide is accumulated and transformed (13). Thus, chlorpromazine appears to be a nonspecific inhibitor of choroid plexus transport and this may be due to the well-known membrane effect of chlorpromazine (20). A clear explanation of the ability of quinidine and quinine to inhibit [^{14}C]riboflavin accumulation by the choroid plexus is not apparent. Quinidine, however, did not have a nonspecific toxic effect on the choroid plexus in that it did not significantly decrease [^{3}H]folic acid accumulation by the isolated choroid plexus. Also, the inhibitory effects of quinidine and quinine and, for that matter, all the inhibitory substances in Table I on [^{14}C]riboflavin accumulation by the choroid plexus were not due to the formation of intermolecular complexes between the inhibitor and the [^{14}C]riboflavin, or reduction or degradation of the [^{14}C]riboflavin in the medium (17, 21).

The sodium dependence of [^{14}C]riboflavin accumulation by the isolated choroid plexus is also noteworthy. In Table I, the substitution of choline for ~75% of the sodium in artificial CSF significantly inhibited [^{14}C]riboflavin accumulation. Similarly, ouabain inhibited [^{14}C]riboflavin accumulation. These results are consistent with the importance of sodium and/or sodium and potassium transport for riboflavin accumulation.

Theophylline, which is known to increase intracellular cyclic AMP, also inhibited [^{14}C]riboflavin accumulation (22). In the kidney, organic anion transport is also inhibited by theophylline in the medium (22). Theophylline did not increase the efflux of [^{14}C]-riboflavin from the choroid plexus.

In previous studies, we have shown that 1 mM niacinamide, thiamine, pyridoxine, and tolazoline did not inhibit [^{14}C]riboflavin accumulation by the choroid plexus (8) and, in Table I, we have shown that iodide, folic acid, and imipramine also did not inhibit [^{14}C]riboflavin accumulation. Because these studies were performed at concentrations of these substances much higher than their respective K_T values, these results strongly support the notion that riboflavin is not accumulated by any of the separate systems in choroid plexus that accumulate iodide, folic acid, niacinamide, thiamine, weak bases (tolazoline), and pyridoxine (3, 12, 13, 23, 24).

The specificity of the [^{14}C]riboflavin accumulation system in choroid plexus is peculiar, indeed. The photodegradation products of riboflavin (lumiflavin and lumichrome) are more potent inhibitors of [^{14}C]-riboflavin accumulation than the sugar-containing flavins (riboflavin, galactoflavin, lyxoflavin, and isoriboflavin) which are approximately equipotent inhibitors of [^{14}C]riboflavin accumulation (Table I). The ability of fluorescein, penicillin G (Table I) and probenecid (8) to inhibit [^{14}C]riboflavin transport is surprising in view of the differences in the structures of the molecules. In previous studies, we have shown that these weak organic acids are not general inhibitors of choroid plexus transport (1, 4). Moreover, fluorescein, penicillin, and probenecid are all anions at physiological pH whereas riboflavin is predominantly a neutral, uncharged molecule at pH = 7.0–8.0. Although these molecules are structurally dissimilar, riboflavin was a competitive inhibitor of [^{14}C]penicillin uptake by choroid plexus (Fig. 5). The K_I for riboflavin (determined in Fig. 5) is almost identical to the K_T (78 μM determined directly (8). On the other hand, the I_{c50} for the ability of penicillin to inhibit [^{14}C]riboflavin accumulation by the isolated choroid plexus (~40 μM) (Table I) is about the same as the K_T for [^{14}C]-penicillin determined directly (Fig. 5). In a previous study, the K_T for penicillin was 43 μM (1). These results strongly support the notion that pencillin and riboflavin are transported by the same system within the choroid plexus. Consistent with this view was our finding that in vivo, riboflavin inhibited the transport of penicillin from CSF (Table IV). We have previously shown that probenecid parenterally increases both penicillin and riboflavin levels in CSF (1, 6).

Using fluorescence microscopy, Bresler et al. (10) have shown that fluorescein is concentrated by the isolated rabbit choroid plexus by a mechanism very similar to what we have shown for riboflavin as discussed above. The K_T for fluorescein accumulation by the choroid plexus is ~40 μM (10). In Table I, the I_{c50} for fluorescein is between 10 and 100 μM. These results, taken as a whole, are consistent with the view that fluorescein is transported from CSF to blood through the choroid plexus by the riboflavin transport system. It is likely that *p*-aminohippurate and probenecid, which are also transported from CSF to blood (2), and inhibit [^{14}C]riboflavin, penicillin and/or fluorescein accumulation by the isolated choroid plexus (1, 8, 10), travel on the riboflavin transport system.

Previous studies have shown that [^{14}C]riboflavin accumulation by isolated rabbit brain slices was an energy-dependent process that depended, in part, on the formation of [^{14}C]FMN via flavokinase and [^{14}C]-FAD via FAD pyrophosphorylase within the brain slices (7). In the present studies (Table V), we have shown that riboflavin is the most potent inhibitor of [^{14}C]riboflavin accumulation by brain slices of the flavins tested. Galactoflavin, lyxoflavin, and isoriboflavin are less potent inhibitors of [^{14}C]riboflavin accumulation than riboflavin but more potent than lumiflavin and lumichrome (Table V). The addition of lyxoflavin (10 μM) to the medium, although it inhibited the ability of the brain slices to accumulate [^{14}C]riboflavin, did not decrease the percentage of riboflavin phosphorylated within the brain slices. Unlike lumiflavin and lumichrome, galactoflavin, lyxoflavin, and isoriboflavin have no inhibitory activity for flavokinase (25, 26), whereas isoriboflavin does have inhibitory effects on FAD pyrophosphorylase (27). The lack of effect of galactoflavin and lyxoflavin on the riboflavin metabolizing enzymes (flavokinase and FAD pyrophosphorylase) and on intracellular phosphorylation of riboflavin to FMN and FAD in brain slices strongly suggests that these flavins inhibit riboflavin transport into the tissue rather than subsequent phosphorylation and conversion to FAD. These results, taken as a whole, are consistent with our previous view that riboflavin enters the brain slices by facilitated diffusion and is trapped intracellularly as FMN after phosphorylation by flavokinase (7).

Unlike in choroid plexus, fluorescein, penicillin, and chlorpromazine (all 100 μM) as well as quinidine and CPDS (both 1.0 mM) did not inhibit [^{14}C]riboflavin accumulation by isolated brain slices. However, NEM (1 mM) did inhibit the accumulation of [^{14}C]riboflavin. The inhibition by NEM suggests the importance of sulfhydryl groups in the process of ^{14}C accumulation by brain slices.

The results reported herein tend to simplify the numerous efflux transport systems reported within the choroid plexus. The iodide and sulfate transport systems within the choroid plexus are separate from the cyclic organic acid (riboflavin) transport system discussed herein (Table I) (1–3). Our finding that penicillin and probably fluorescein travel on the riboflavin transport system of the choroid plexus of rabbits suggests that many other organic acids that are transported from CSF may also travel on the riboflavin transport system. In man, there is very strong indirect evidence that these systems exist (5, 28). As discussed above, the transport of these substances out of CSF may cause therapeutic problems due to low drug levels in CSF (4, 5).

The reason for the riboflavin carrier in the central nervous system is not completely clear. However, as we previously discussed (6), it is probably there to protect the CNS from excessive riboflavin concentrations and help provide homeostasis of total riboflavin within the brain.

As in the choroid plexus, riboflavin is transported in gut, kidney, and liver by specialized processes (29). There is strong evidence that riboflavin and weak cyclic organic acids (e.g., p-aminohippurate) are secreted into the urine by a single low-affinity, high-capacity, probenecid-sensitive saturable transport mechanism (29, 30). The human gut has a saturable absorptive system for riboflavin (29) and the biliary tract also is able to secrete large amounts of riboflavin from the blood into the bile (29, 31).

In conclusion, the brain appears to be protected from fluctuations in dietary intake of riboflavin by several mechanisms. First, there is the saturable absorptive system in the gut (29); second, there are mechanisms in the biliary tract and kidney either to conserve or excrete excessive amounts of riboflavin (29–31), and finally there are riboflavin transport systems at the blood-brain barrier, in the brain cells themselves, and within the choroid plexus that all tend to maintain constant total riboflavin levels in brain (6–8). By chance, several exogenous substances, including penicillin, appear to travel on the riboflavin transport systems in choroid plexus and, probably, the renal tubules. In some cases, transport of drugs from essential loci (e.g., the CSF) complicates therapy.

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