Transport Characteristics of Folates in Cerebrospinal Fluid; a Study Utilizing Doubly Labeled 5-Methyltetrahydrofolate and 5-Formyltetrahydrofolate

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Abstract Serum disappearance curves in dogs after the intravenous injection of radioactive methotrexate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and folic acid followed first-order kinetics with half-disappearance times ranging from 1.3 to 9 hr respectively. Equilibration of spinal fluid tritium levels with those in serum was rapid (3.0 hr) for both of the reduced folates but was not observed at any time after folic acid and methotrexate. The only radioactive folate identified in the spinal fluid after intravenous injection of either 5-formyltetrahydrofolate or folic acid, as well as 5-methyltetrahydrofolate was 5-methyltetrahydrofolate. These findings indicated that 5-methyltetrahydrofolate was taken up preferentially into the spinal fluid and that the other folate congeners were converted to this compound before uptake. Diphenylhydantoin administration did not alter the uptake of 5-methyltetrahydrofolate into the spinal fluid but was associated with reduced renal excretion of this compound.

INTRODUCTION

5-Methyltetrahydrofolate ($MeFH_4$)[1] is the predominant folate form in serum (1); however, relatively little is known concerning its distribution and utilization in vivo. The observation of higher concentrations of $MeFH_4$ in the cerebrospinal fluid (CSF) than in serum (2–5), the selective conservation of folates in the central nervous system (6), and the association of neuropsychiatric disorders with disturbances of folate metabolism (7–9) suggest that folate coenzymes play an important role in neural metabolism. The association of mental retardation, cerebral calcification, and megaloblastic anemia with combined defects in gastrointestinal and CSF transport of folates has been reported (10). The low concentration of vitamin B_{12} (11) in CSF suggests that the $B_{12}MeFH_4$ transmethylase is not essential to neural folate metabolism. The relatively constant ratio between serum and CSF folate concentrations observed in individual patients (4, 5) suggests the existence of a homeostatic mechanism for the maintenance of CSF folate concentration, perhaps involving a transport function at the "blood-brain barrier."

Past studies of folate transport have been hampered by the limitations of microbiologic assays or have employed tritiated folic acid (FA), which is unphysiologic, rather than $MeFH_4$. In the present study, radiochemically pure, doubly labeled 5-methyl-^{14}C-tetrahydrofolate-9,3′,5′-^{3}H (Me*FH*$_4$) and 5-formyl-^{14}C-tetrahydrofolate-9,3′,5′-^{3}H (Me*FH*$_4$) were utilized to compare the metabolic disposition of these folate coenzyme with that of FA-9,3′,5′-^{3}H (FA*) and methotrexate-9,3′,5′-^{3}H (MTX*).

Diphenylhydantoin (DPH) has been implicated as an etiologic agent in cases of megaloblastic anemia which have responded to folate therapy (12). More recently it has been suggested that some cases of dementia in epileptics may result from a DPH-dependent lowering of folate levels in both serum and CSF (7). Since Woodbury and Kemp (16) demonstrated decreased FA* uptake into the CSF after DPH, it was of interest to determine the effect of DPH treatment upon the transport of Me*FH*$_4$ between the CSF and blood.

This work was presented in part at the American Society of Hematology Meeting in Cleveland, Ohio, 9 December 1969.

Dr. Levitt is a recipient of a Fellowship award of the Leukemia Society of America. Dr. Nixon is the recipient of a Merck, Sharp & Dohme International Fellowship in Clinical Pharmacology. Dr. Bertino is the recipient of a Career Development Award of the National Cancer Institute.

Received for publication 18 December 1970 and in revised form 29 January 1971.

[1] *Abbreviations used in this paper:* CSF, cerebrospinal fluid; DPH, diphenylhydantoin; FA, folic acid; FA*, folic acid-9,3′,5′-^{3}H; f*FH*$_4$, 5-formyl-^{14}C-tetrahydrofolate-9,3′,5′-^{3}H; $MeFH_4$, 5-methyltetrahydrofolate; Me*FH*$_4$, 5-methyl-^{14}C-tetrahydrofolate-9,3′,5′-^{3}H; MTX*, methotrexate-9,3′,5′-^{3}H; p-ABG, *p*-aminobenzoylglutamate.

METHODS

Materials. DPH was obtained from Parke, Davis & Co., Detroit, Mich. MTX* and FA*, each of SA 250 μCi/μmole and 98% radiochemically pure, were obtained from Amersham-Searle Corp., Des Plaines, Ill. Formaldehyde-^{14}C of SA 15–60 μCi/μmole was obtained from New England Nuclear Corp., Boston, Mass. Using these materials, the physiological diasterioisomers of f*FH*$_4$ and Me*FH*$_4$ were synthesized (13). In brief, FA* was chemically reduced to dihydrofolate, then enzymatically reduced to FH$_4$ in the presence of formaldehyde-^{14}C, which resulted in the formation of 5,10-methylene-^{14}C-FH$_4$-^{3}H. This material was either reduced with KBH$_4$ to prepare Me*FH*$_4$, or the enzyme 5,10-methyleneFH$_4$ dehydrogenase was used to produce 5, 10-methenyl-^{14}C-FH$_4$-^{3}H; when autoclaved at neutral pH in the presence of ascorbate, this latter was converted to f*FH*$_4$. In both cases the products were purified by ion-exchange chromatography on DEAE-Sephadex and gel filtration on Sephadex G15. The final product was more than 99% radiochemically pure in the case of f*FH*$_4$ and more than 95% radiochemically pure in the case of Me*-FH*$_4$ as assessed by chromatography in three systems.

General procedures. Mongrel dogs of either sex weighing 10–20 kg were anesthetized with intravenous sodium pentobarbital, 30 mg/kg. Urine and blood sampling were then carried out by Foley and intravenous (i.v.) polyethylene catheters, respectively. Spinal fluid sampling or injection was performed through an indwelling intracisternal needle. All i.v. injections of labeled materials were performed in a limb other than that containing the blood sampling catheter.

No experiment was included in the study if grossly bloody CSF was obtained at any time. Sampling was carried out for periods up to 6 hr after injection. Occasionally a second smaller injection of anesthetic was required. All animals recovered fully from the procedure. Radioactivity of CSF, serum, and urine samples was measured the same day as collection in all cases. Samples for chromatography were frozen at −70°C in the presence of 0.2 M 2-mercaptoethanol to prevent oxidation while pending chromatography which was carried out within 2 wk.

Uptake of folic acid and congeners into spinal fluid. The initial study consisted of a single rapid i.v. injection of 25–50 μg/kg of one of the compounds (Me*FH*$_4$, f*FH*$_4$, FA*, and MTX*) into anesthetized dogs. The specific activity of the compounds was 50 μCi/mg for ^{3}H and 20 μCi/mg for ^{14}C; each animal received 25 μCi of ^{3}H and 10 μCi of ^{14}C (in double-labeled compounds). Blood and CSF samples were then drawn at intervals, and their radioactivity was monitored by liquid scintillation spectrometry as described below. Samples of CSF after i.v. FA*, Me*-FH*$_4$, and f*FH*$_4$ were also analyzed chromatographically as described below. Duplicate experiments utilizing each compound were performed.

Diphenylhydantoin. DPH was administered to four dogs before i.v. injection of Me*FH*$_4$. Daily oral doses of DPH (75 mg/kg) were given to two of these dogs for 30 days to simulate a chronic therapeutic regimen. Two other animals received 25 mg/kg of DPH intraperitoneally 24 hr preceding the experiment and an additional 25 mg/kg i.v. 1 hr preceding the injection of the radioisotope. No ataxia or other manifestations of DPH toxicity were observed in any of the animals.

To measure the efflux kinetics of Me*FH*$_4$ from the CSF in both control and DPH-treated dogs, four animals were utilized. An intracisternal injection of 25 μg/kg of Me*-FH*$_4$ in 0.4 ml 0.9% saline was carried out over a period of 5 min in each of the four animals. Blood samples and in some cases urine samples were obtained at intervals. Two of the dogs received no DPH; the third dog was given 10 mg/kg and the fourth 30 mg/kg of DPH i.v. 1 hr before radioisotope injection.

Scintillation counting. Serum specimens were prepared for counting as follows: 2.0-ml portions of serum were deproteinized by the addition of 0.5 ml 10% perchloric acid. After centrifugation at 3500 rpm for 15 min, 0.5 ml of each supernatant was placed in a glass counting vial with 20 ml of scintillation fluid. The latter consisted of two parts of toluene containing 4 g PPO (2,5-diphenyloxazole) plus 50 mg POPOP (1,4-bis[2-(5-phenyloxazolyl)]-benzene) per liter and one part of 100% ethanol. Portions of 0.5 ml of CSF and urine were counted directly in 20 ml of the same scintillation fluid. When low levels of radioactivity were expected, Aquafluor scintillation mixture (1 ml sample to 10 ml of Aquafluor) was employed.

For counting of ^{3}H and of ^{14}C in samples containing both radioisotopes, four channels of the liquid scintillation spectrometer were utilized. In two channels, external standards were counted and expressed as a channels ratio. One channel was set so as to optimize ^{14}C counting in the quenched samples while excluding ^{3}H. The fourth channel was set to both optimize ^{3}H counting and minimize ^{14}C counting in the quenched samples. The spillover of ^{14}C into the ^{3}H channel and the efficiency of counting of each isotope were determined from a calibration chart constructed with reference to the external standard channels ratio (14).

Spinal fluid chromatography. Identification of the radiolabels in CSF was determined by the method of Nixon and Bertino (15). To 10–20 ml of labeled CSF was added 2 μmoles of *p*-aminobenzoylglutamate (p-ABG) and 2 μmoles of unlabeled MeFH$_4$. The material was then applied to a column of A-25 DEAE-Sephadex equilibrated with 0.1 M potassium phosphate buffer at pH 6 containing 10 mM 2-mercaptoethanol. Elution was carried out by means of a linear concentration gradient constructed by placing 250 ml of 0.1 M phosphate buffer in the mixing chamber and 250 ml of 0.8 M phosphate buffer in the reservoir. Fractions of 2.5 ml each were collected and monitored for radioactivity. Fluorometry or UV-absorbence spectrometry was carried out over the range 240–350 mμ to establish the identity of marker compounds. Radioactivity was determined by counting 1 ml of each fraction in 10 ml of Aquafluor scintillation fluid on a liquid scintillation spectrometer using the double label corrections where appropriate.

RESULTS

With one exception, the results presented are those from a single experiment which is representative of almost identical replicate experiments. In the case of the data shown in Fig. 3, the results of duplicate experiments have been included since they were not entirely consistent.

Uptake of folate congeners into spinal fluid. The serum disappearance and spinal fluid uptake data after intravenous injection of either Me*FH*$_4$, f*FH*$_4$, FA*, or MTX* are shown in Fig. 1 and Table I. With the reduced compounds the serum isotope disappearance curves for all four compounds were parallel and consistent with a biphasic (A and B) first-order disappearance rate plot.

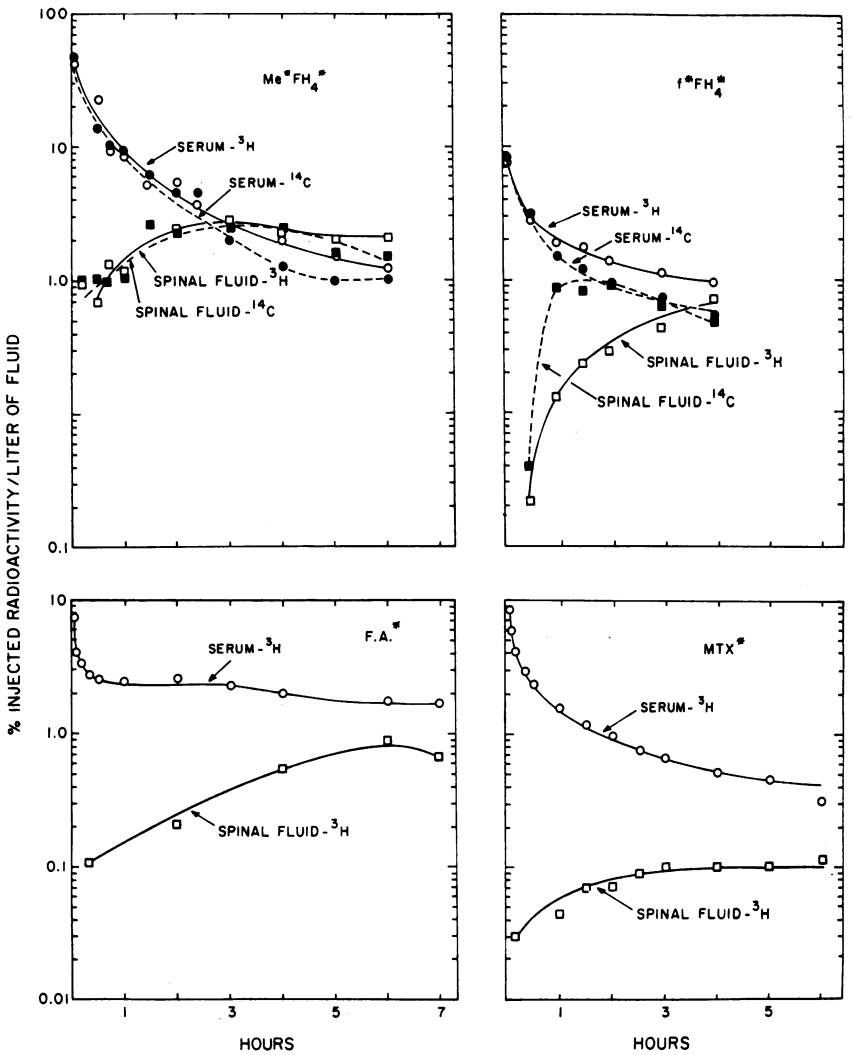

FIGURE 1 Serum clearance and cerebrospinal fluid (CSF) appearance of radioisotope after i.v. injection of radiolabeled folates. Dogs received i.v. injections of either 5-methyl-^{14}C-tetrahydrofolate-9,3',5'-^{3}H (Me*FH*$_4$), 5-formyl-^{14}C-tetrahydrofolate-9,3',5'-^{3}H (f*FH*$_4$), folic acid-9,3',5'-^{3}H (FA*), or methotrexate-9, 3',5'-^{3}H (MTX*). Serum levels are shown as circles and CSF levels as squares. Closed circles and squares and the broken line depict ^{14}C activity, whereas open ones and the solid line depict ^{3}H activity.

Phase A of serum disappearance (Table I) appeared to be complete before the appearance of measurable amounts of radioactivity in CSF (Fig. 1). Equilibration of CSF tritium levels with those in serum was rapid for both Me*FH*$_4$ and f*FH*$_4$ at 3.0 hr, but was not observed at any time after FA* or MTX*. The CSF: serum-^{14}C equilibration rate after f*FH*$_4$ was significantly more rapid than that of tritium. CSF: blood equilibration was observed at the following serum isotope concentrations: Me*FH*$_4$ 2.5% (of injected radioactivity per liter); f*FH*$_4$, 1%.

Diphenylhydantoin. Pretreatment of dogs with DPH (cf. experimental) resulted in serum disappearance and CSF uptake curves after i.v. Me*FH*$_4$ which were indistinguishable from those in untreated animals (Fig. 2, Table I).

The maximum serum levels observed after intrathecal injection of Me*FH*$_4$ were 4.0–5.1% of the injected radioactivity per liter in two control dogs and 6.3–7.8% in two DPH-pretreated dogs (Fig. 3). The data for serum appearance of radiolabels differed within both the control and the pretreated animals pairs (Fig. 3).

TABLE I

Uptake Kinetics of Folic Acid and Congeners into Spinal Fluid

Compound	Serum-^{3}H $t_{1/2}$		Spinal fluid equilibration	
	Phase A	Phase B	^{3}H	^{14}C
	hr		*hr*	
FA*	0.5	9.0	Never	—
f*FH*$_4$	0.3	2.5	3.0	1.7
MTX*	0.6	1.3	Never	—
Me*FH*$_4$	0.3	1.4	3.0	3.0
Me*FH*$_4$, DPH	0.3	1.5	3.0	3.0

The appearance of tritium and carbon-14, however, was parallel in all experiments, suggesting that the compound was chemically unaltered. The mean serum isotope concentrations attained were 4.5 and 7.0% per liter of the injected radioactivity for the control and DPH-treated animals, respectively.

Observations concerning urinary isotope excretion in these animals are presented in Table II. These data were not comparable following the two routes of injection because of the different periods of observation. It did appear, however, that DPH inhibited urinary Me*FH*$_4$ excretion for both routes, particularly the intracisternal. These observations strongly suggest that DPH exerts an inhibitory effect on the renal excretion of Me*FH*$_4$; in addition, an enhancement of CSF: serum efflux is possible.

Spinal fluid chromatography. Chemical identification of radioisotopes appearing in the CSF after injection of Me*FH*$_4$, f*FH*$_4$, and FA* was carried out by chromatography (Fig. 4). Coincident elution of radioisotopes with the unlabeled MeFH$_4$ "marker" was interpreted as indicating chemical coidentity. By this criterion Me*FH*$_4$ was found in the spinal fluid after intravenous injection of all of the three labeled folates. All three of the spinal fluids contained variable amounts of radioactivity other than Me*FH*$_4$. The early eluting material was probably not in the form of folates and may have been amino acids, formaldehyde, methionine, pteridines, and PABG. This is discussed below. These findings indicated that whatever the identity of folates in the blood, their appearance in CSF was dependent upon conversion to Me*FH*$_4$.

DISCUSSION

Several striking characteristics of serum: CSF folate transport have been observed in this study. The pre-

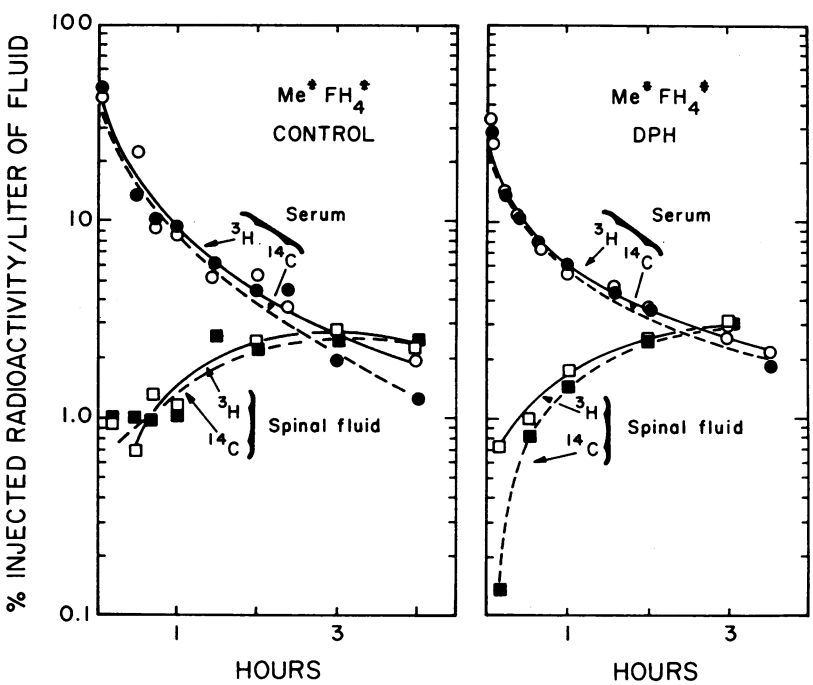

FIGURE 2 The effects of diphenylhydantoin (DPH) on serum clearance and cerebrospinal fluid (CSF) appearance of radioisotope after i.v. injection of 5-methyl-^{14}C-tetrahydrofolate-9,3′,5′-^{3}H (Me*FH*$_4$). Serum levels are shown as circles and CSF levels as squares. Closed circles and squares and the broken line depict ^{14}C activity, whereas open circles and squares and the solid line depict ^{3}H activity.

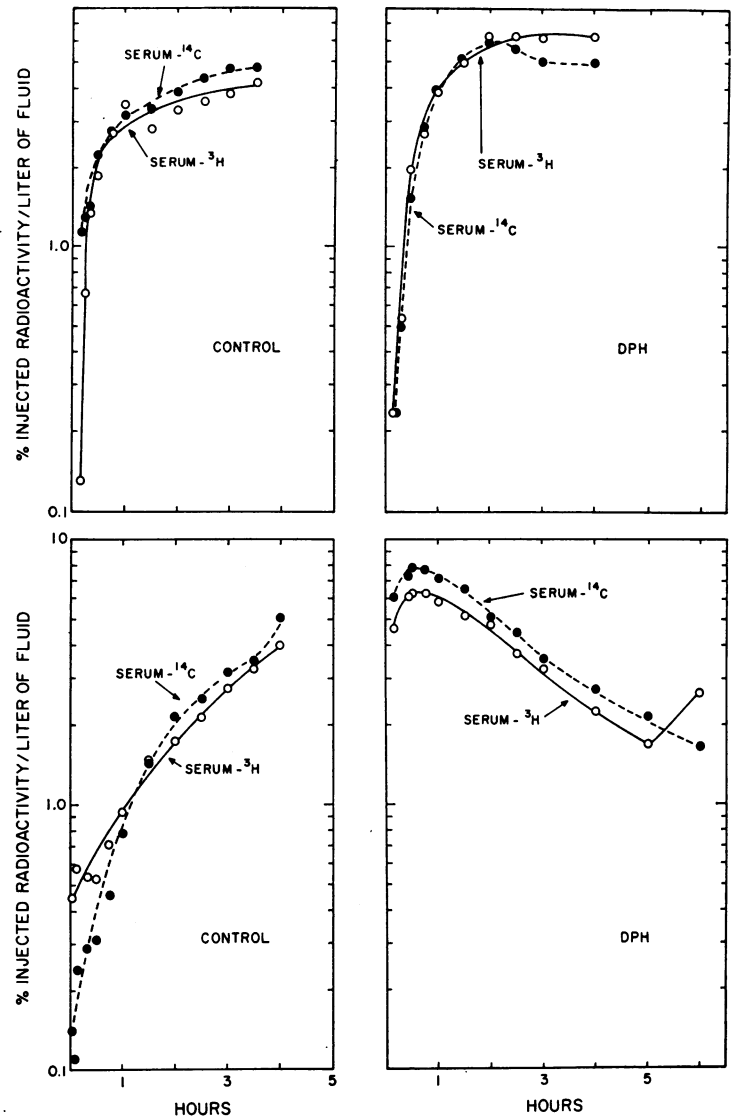

FIGURE 3 The effects of diphenylhydantoin (DPH) on the serum appearance of radioisotope after intracisternal injection of 5-methyl-^{14}C-tetrahydrofolate-9,3′5′-^{3}H (Me*FH*$_4$). Closed circles and broken line depict ^{14}C activity, and open circles with solid line depict ^{3}H activity.

dominant folate form found in the CSF is MeFH$_4$, which is generally the most prevalent form physiologically. The failure to approach a CSF: serum isotope ratio of 3:1 as observed for naturally occurring MeFH$_4$ (4, 5) may be in part due to the short duration of the observation period and/or the kinetics of equilibration of the administered isotope with body pools of intracellular folate. At the 6th hr after i.v. administration of Me*FH*$_4$ (Fig. 1), the CSF: serum isotope concentration ratios were 1.8 and 1.6 for ^{3}H and ^{14}C, respectively, which suggests that a concentrating process was occurring.

The appearance of only MeFH*$_4$ in CSF after the i.v. administration of either FA* or f*FH*$_4$ and the relative delay of appearance of radioactivity in the CSF after these compounds (compared with that of Me*FH*$_4$) suggest that the transport mechanism is specific for MeFH$_4$ and that time is required for the metabolic conversion of these compounds to the biologically prevalent one. The demonstration that acute doses of DPH inhibit the uptake of FA* into the CSF (16), while not affecting the uptake of Me*FH*$_4$ in the current study, suggests that the conversion to MeFH$_4$ is extraneural and sensitive to DPH. The biphasic serum isotope

disappearance curves are consistent with this concept; the phases designated A and B (Table I) may depict equilibration in extracellular fluid followed by cellular uptake and metabolism, respectively. This is suggested by the relative constancy of the $t_{\frac{1}{2}}$ for all four compounds in phase A in contradistinction to the divergent values for phase B; these may reflect differing rates of cellular metabolism and uptake of the various compounds. Definition of the two phases was most distinct after FA*, which is consistent with the extensive metabolism this compound undergoes in its conversion to MeFH*$_4$. That MTX* has the most rapid serum disappearance in phase B can be explained neither by rapid conversion to MeFH$_4$, avid uptake into the CSF, nor by excessively rapid renal excretion (Table II). It is suggested that rapid binding to dihydrofolate reductase, presumably in liver and kidney (17), accounts for this observation.

The much earlier appearance of ^{14}C than tritium in the CSF after f*FH*$_4$ indicates that its 1-carbon moiety is donated before CSF uptake. This early appearing ^{14}C is probably identical with the chromatographically early appearing ^{14}C peak (Fig. 4) and may represent incorporation of the ^{14}C formyl group into serine or methionine. The more extensive metabolic interconversion required after FA* administration may have been too slow to allow achievement of CSF: serum equilibration during the observation period. MTX was least well taken up into the CSF, as expected from previous studies.

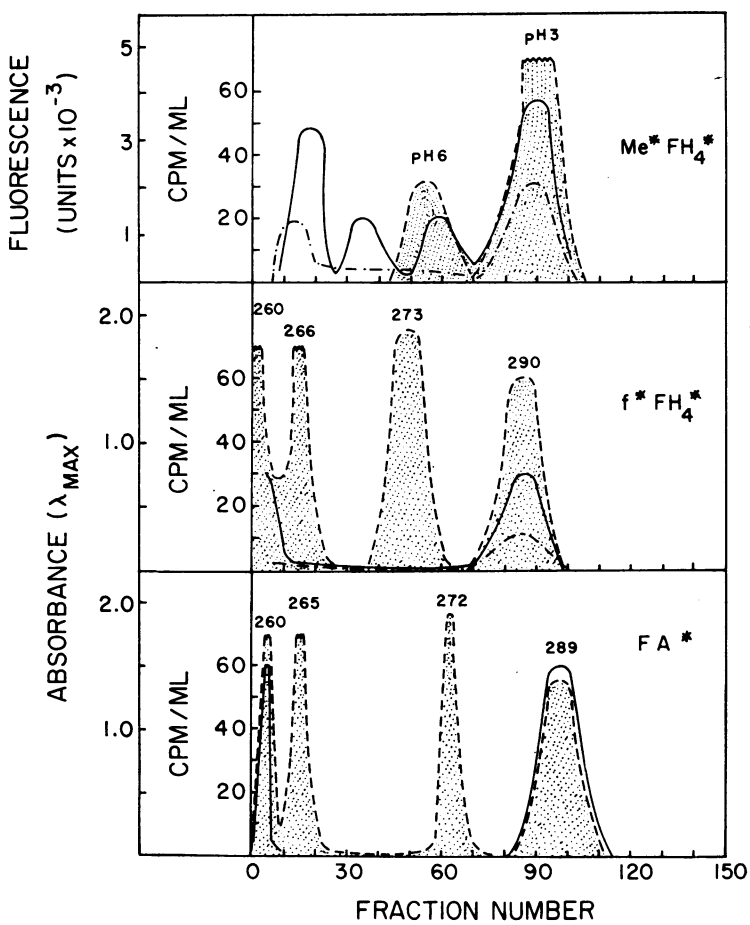

FIGURE 4 The chromatographic identity of cerebrospinal fluid (CSF) radioactivity after i.v. injection of 5-methyl-^{14}C-tetrahydrofolate-9,3',5'-^{3}H (Me*FH*$_4$), 5-formyl-^{14}C-tetrahydrofolate-9,3',5'-^{3}H (f*FH*$_4$), or folic acid-9,3',5'-^{3}H (FA*). The shaded areas enclosed by broken lines represent either fluorescence or absorbence maxima as indicated; in each case the peak between fractions 50 and 60 represents unlabeled marker *p*-aminobenzoylglutamic acid (p-ABG) and the one between fractions 80 and 100, unlabeled marker MeFH$_4$. The solid lines indicate ^{3}H activity and the broken-dotted lines indicate ^{14}C activity.

Some interesting information concerning the interaction of DPH and $MeFH_4$ at the transport level was obtained from this series of studies. The uptake of $Me^*FH^*_4$ into CSF was normal in dogs pretreated with DPH. Combined with the unchanged serum $MeFH_4$ disappearance curves, these observations suggest that the transport of $MeFH_4$ was unaffected, but they are also compatible with a series of mutually compensating and, therefore, unobserved changes. The significance of this finding has been discussed above in relation to the demonstration of inhibition of FA* uptake into the CSF by DPH (16).

However, after intracisternal administration of $Me^*FH^*_4$, the data suggested that higher serum levels were achieved after DPH pretreatment than in control animals (Fig. 3). Inconsistencies in the rate of appearance of radioactivity in the serums of the replicate pairs may have been due to technical difficulties, such as intravascular leakage of the injected material around the intracisternal needle, or to individual differences in the rate of CSF circulation, which is known to be slow and sluggish (18). Urinary excretion of radioactivity was lower in DPH-pretreated animals whether the route of $Me^*FH^*_4$ administration was i.v. or intracisternal. That the difference in urinary excretion between control and DPH-treated animals was more marked when $Me^*FH^*_4$ injection was intracisternal suggests that folate transport may have been inhibited at the CSF : serum interface as well as at the serum : urine interface. However, inhibition at the latter site alone could well account for the increased serum levels in DPH-treated animals. Such a reduction in renal excretion might be a reflection of the cardiodepressive effects of DPH, a direct action on the tubular epithelium affecting folate transport, or a combination of both.

TABLE II
Urine Isotope Excretion

Compound injected and route	Duration	Collection	Per cent of injected ^3H excreted	Per cent of injected ^{14}C excreted
	hr			
$Me^*FH^*_4$ i v.	6	100 ml	34.3	31.0
$Me^*FH^*_4$ i.v. (DPH)	6	44 ml	17.6	10.0
$f^*FH^*_4$ i.v.	6	46 ml	19.6	7.0
FA* i.v.	6	119 ml	7.5	
MTX* i.v.	6	66 ml	17.0	
$Me^*FH^*_4$ Intracisternal	4	51 ml	15.2	8.3
$Me^*FH^*_4$ Intracisternal (DPH)	4	87 ml	2.86	2.63

The nonfolate radioactive peaks in Fig. 4 may be tentatively identified as follows: very early ^{14}C peaks are thought to represent either methionine derived from the transformation of $MeFH_4$ to FH_4 or other amino acids. The unidentified peaks containing either ^3H or both ^3H and ^{14}C are thought to represent pteridines, and finally the ^3H peak which coincides with the p-ABG marker probably represents that compound.

The specific role of folate coenzymes in neural metabolism is unknown. That severe folate deficiency may be the etiologic factor in organic neurologic defects is suggested by several clinical reports (19–21). In all of these, neurologic deficiency was associated with megaloblastic anemia. Luhby, Eagle, Roth, and Cooperman (19, 20) reported the case of a female infant with intestinal folate malabsorption, and megaloblastic anemia which responded to folate therapy and severe mental retardation which, however, did not improve. Strachan and Henderson (21) reported two patients with folate deficiency, megaloblastic anemia, and severe dementia. Both the hematologic and neurologic defects resolved after folic acid therapy.

That specificity is involved in the CSF transport of FA and the congeners studied has been demonstrated. It is tempting to implicate an active cellular process in the performance of this function, particularly in view of the putative relationship between neuropsychiatric function and folate metabolism as well as the folate-concentrating activity of CSF (4) and the folate-retaining capacity of brain tissue (6). A number of selectively concentrative functions including one for MTX (22), have been demonstrated to reside in the choroid plexus (23, 24). On the other hand, the more rapid uptake of intravenously administered D_2O by cerebral tissue than CSF (18) suggests that the selective function may reside in the endothelial cells of the cerebral microcirculation and that events occurring in the CSF may provide only a distant reflection of the vital process. The known sluggish circulation of the CSF (18) detracts from its possible role as a primary mediator in cerebral metabolism; however, the demonstration that numerous substances such as ascorbic acid (18), penetrate cortical slices of cerebral tissue from spinal fluid reinforce the possibility of its importance.

The relative lipid solubility of substances has been suggested as a factor which determines their distribution

in neural tissue (18), and this must be considered either as a primary mechanism or an additional factor to the cellular mechanisms discussed above. In studying a number of intravenously administered drugs, Brodie and Hogben (25), demonstrated an inverse relationship between the time required to achieve a CSF: plasma concentration ratio of unity and the chloroform: water partition coefficient of these drugs. That such a mechanism does not apply to the CSF uptake of folates is implied by the chloroform insolubility of FA, fFH_4, and $MeFH_4$.[2] The present studies which demonstrate that $MeFH_4$ is preferentially taken up into the CSF prompt further studies of folate metabolism in the central nervous system.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Miss Patricia O'Brien and are indebted to Dr. D. G. Johns for critically reviewing the manuscript.

This work was supported in part by U. S. Public Health Service grants 5-PO1-NS-06208, CA 08341, CA 05138, and CA 08010.

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