[\textsuperscript{3}H] Methotrexate Loss from the Rat Brain following Enhanced Uptake by Osmotic Opening of the Blood-Brain Barrier

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ABSTRACT

Right brain regions of anesthetized rats were loaded with [\textsuperscript{3,5,7-\textsuperscript{3}H]methotrexate ([\textsuperscript{3}H]MTX) or with [\textsuperscript{14}C]sucrose by infusing the tracers into the right carotid artery, after the blood-brain barrier had been opened by right carotid infusion of a hypertonic arabinose solution. During the 6 hr following the procedure, the [\textsuperscript{3}H]MTX concentration in 7 right-sided brain regions, when normalized to the plasma concentration integral during tracer infusion, fell, with an average half-time of 4.8 hr as compared to less than 20 min for the initial rate of loss of [\textsuperscript{14}C]sucrose. Right-left brain concentration differences 3 hr after treatment were statistically significant (p < 0.05) for [\textsuperscript{3}H]MTX but not for [\textsuperscript{14}C]sucrose. The results indicate that intracerebral [\textsuperscript{3}H]MTX is lost more slowly than is intracerebral [\textsuperscript{14}C]sucrose, possibly because [\textsuperscript{3}H]MTX enters brain cells, whereas [\textsuperscript{14}C]sucrose remains largely extracellular.

INTRODUCTION

The BBB to water-soluble drugs arises at the continuous layer of cerebrovascular endothelial cells that are connected by tight junctions (zonulae occcludentia) (3, 28, 7, 39, 40). In animals and humans, the barrier can be opened reversibly, presumably because the tight junctions are widened, by intracarotid infusion of a hypertonic solution of urea, lactamide, arabinose, or mannitol (3, 6, 7, 13, 22–30, 36–39). BBB opening, followed by intracarotid infusion of a drug such as MTX, can enhance brain uptake by up to 70-fold, as compared to i.v. infusion of MTX without osmotic treatment (23, 31).

Osmotic BBB opening can be achieved without producing gross functional neurological deficits or evidence of long-term brain edema or of brain pathology (26, 38, 36, 39, 43). However, if the method is to be used as a clinical tool for the chemotherapy of brain tumors (4, 25, 31), it would be useful to know how rapidly drugs are lost from the brain and the factors that determine their rates of loss. In this study, we examine the rate of loss of [\textsuperscript{3}H]MTX, as compared to that of [\textsuperscript{14}C]sucrose, from the rat brain following loading by osmotic opening of the BBB followed by intracarotid tracer infusion. [\textsuperscript{14}C]Sucrose was chosen as a reference tracer because sucrose has a very low permeability at the intact BBB, remains extracellular when within the brain, and is not metabolized by the rat (9–11, 32, 33, 42).

MATERIALS AND METHODS

MTX Metabolism. Adult male Osborne-Mendel rats, 250 to 300 g, were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Catheters filled with 100 IU sodium heparin per ml 0.9% (w/v) NaCl solution (saline) were tied into a femoral artery and vein and into the right external carotid artery in a caudad direction. A filtered solution of 1.8 molal arabinose (Sigma Chemical Co., St. Louis, MO), warmed to 37°, was infused for 30 sec into the right external carotid catheter, at a constant rate of 0.12 ml/sec (31). During infusion, the carotid bifurcation was examined under low-power magnification to ascertain whether the infusion passed cephalad into the internal carotid artery or caudal into the common carotid artery. Experiments were discontinued in the latter case, as preliminary studies showed that the brain then was not perfused.

Five min following the start of arabinose infusion, a rat was given an infusion for 10 min at a rate of 0.12 ml/min through the right external carotid catheter with a solution containing 200 µCi [\textsuperscript{3}H]MTX (specific activity, 0.4 to 0.55 mCi/mg; Amersham Corp., Arlington Heights, IL). Fifteen min or 1, 2, 3, 6, or 12 hr after arabinose treatment, the rat was decapitated, and the right (perfused) cerebral hemisphere was removed. It was frozen at −70°, together with plasma aliquots from centrifuged arterial blood samples.

Brain tissue was sonicated (Sonicator Cell Disruptor, Heat Systems-Ultrasonics, Plainview, NY) with 4 volumes of ethanol and sodium acetate (9:1, v/v). Five µg each of unlabeled 7-OH-MTX (courtesy of D. G. Johns, Sigma) and unlabeled MTX (Sigma) were added as cold carriers. In addition, [\textsuperscript{3}H]MTX, unlabeled MTX, and 7-OH-MTX were added to sonicated brain tissue (spiked brain) from untreated rats. The material was centrifuged in a model B microfuge (Beckman Instruments, Fullerton, CA), and supernatants were concentrated at room temperature under N\textsubscript{2} gas. Plasma samples were similarly treated but were not sonicated, and nonexperimental plasma was spiked with cold MTX, 7-OH-MTX, and [\textsuperscript{3}H]MTX as well. Aliquots were spotted on ITLC-SA plates (Gelman Instruments Co., Ann Arbor, MI) and chromatographed in a system of ethanol and 1 M ammonium acetate (5:2, v/v).\textsuperscript{*} The plates were dried overnight and then cut into 0.5-cm strips, placed in scintillation vials, soaked in 1 ml distilled water, and counted with 6 ml Ready-Solve MP (Beckman) in a liquid scintillation counter.

To determine if a fraction of brain radioactivity following [\textsuperscript{3}H]MTX administration were due to accumulation of [\textsuperscript{3}H]H\textsubscript{2}O, 3 rats were killed by vital injection of [\textsuperscript{3}H]H\textsubscript{2}O at 6 hr after BBB opening followed by carotid infusion of 100 µCi [\textsuperscript{3}H]MTX. Radioactivity (per g wet weight) was determined by scintillation spectroscopy in paired weighed brain samples that were or were not first freeze-dried (Tray Drying Chamber, FTS Systems, Stone Ridge, NY) for 24 hr. Dehydration did not significantly reduce net brain radioactivity (p > 0.05), indicating that [\textsuperscript{3}H]H\textsubscript{2}O did not measurably contribute to net brain radioactivity at 6 hr.

[\textsuperscript{14}C]Sucrose and [\textsuperscript{3}H]MTX Pharmacokinetics. Adult male rats were prepared and catherized as described above. In addition, 2 ml of a solution of 2% (w/v) Evans blue (Chroma-Gesellschaft, Stuttgart, West Germany) per kg in saline were injected i.v. as a visual tracer of BBB integrity. This quantity of Evans blue completely to plasma albumin and normally does not stain the brain (37).

Warmed hypertonic arabinose solution was infused into the right external carotid catheter as described above, followed after 5 min by a 10-min infusion of either [\textsuperscript{3}H]MTX (20 µCi/ml) or of [\textsuperscript{14}C]sucrose (20 µCi/ml; specific activity, 4.9 mCi/mmol; New England Nuclear, Boston MA), at a rate of 0.12 ml/min. The purity of the [\textsuperscript{3}H]MTX was confirmed by
These results indicate that, up to and including 6 hr after [3H]-MTX infusion, all brain and plasma radioactivities were due to [3H]MTX. We therefore limited our studies of [3H]MTX kinetics to 6 hr or less.

We have published (31) that the osmotic BBB treatment, followed by carotid infusion of 200 μCi [3H]MTX, the peak radioactivity at 6 hr or earlier corresponded entirely to the peak in the spiked brain to which [3H]MTX was added, whereas, at 12 hr, 50% of the radioactivity appeared in 2 additional peaks. Similarly, plasma taken at 6 hr or earlier had a radioactive peak which corresponded entirely to [3H]MTX but, at 12 hr, 83% of plasma radioactivity did not chromatograph as [3H]MTX. As noted in "Materials and Methods," no measurable brain radioactivity was due to [3H]H2O. These results indicate that, up to and including 6 hr after [3H]MTX infusion, all brain and plasma radioactivities were due to tracer MTX. We therefore limited our studies of [3H]MTX kinetics to 6 hr or less.

[3H]MTX was infused for 10 min into the right internal carotid artery circulation via the external carotid artery catheter, starting 5 min after a 30-sec infusion of 1.8 molal arabinose solution through the same arterial catheter. The same experiment was performed with [14C]sucrose. Charts 2 and 3 present femoral artery plasma concentrations of [3H]MTX and of [14C]sucrose, respectively, measured in 2 animals that were decapitated after 10 min of infusion. Brain concentrations, dpm/g, were corrected for intravascular radioactivity, equal to whole-blood radioactivity x regional blood volume, from net regional radioactivity. Regional blood volumes have been published (31).

RESULTS

Chart 1 illustrates patterns of radioactivity in various chromatographic samples of brains of rats that were decapitated 6 and 12 hr after osmotic BBB treatment, followed by carotid infusion of 200 μCi [3H]MTX. The peak radioactivity at 6 hr or earlier corresponded entirely to the peak in the spiked brain to which [3H]MTX was added, whereas, at 12 hr, 50% of the radioactivity appeared in 2 additional peaks. Similarly, plasma taken at 6 hr or earlier had a radioactive peak which corresponded entirely to [3H]MTX but, at 12 hr, 83% of plasma radioactivity did not chromatograph as [3H]MTX. As noted in "Materials and Methods," no measurable brain radioactivity was due to [3H]H2O. These results indicate that, up to and including 6 hr after [3H]MTX infusion, all brain and plasma radioactivities were due to tracer MTX. We therefore limited our studies of [3H]MTX kinetics to 6 hr or less.

As shown in Table 1 and Charts 2 and 3, Cbrain at decapitation exceeded the femoral artery plasma concentration by a factor of 2 or more, for both [14C]sucrose and [3H]MTX. Because the carotid artery plasma concentration during infusion of a tracer is about 7 times greater than the femoral artery plasma concentration (31), back diffusion of tracer from brain to plasma can be neglected during radiotracer infusion, and a simplified 2-compartment model represents brain-plasma exchange in this period. According to this model (32, 38), the brain concentration of tracer during infusion, Cbrain dpm/g, is given as follows, where t = time from beginning of infusion, PA sec−1 = cerebrovascular permeability-area product, Ccap dpm/ml = brain capillary plasma con-
METHOTREXATE PHARMACOKINETICS

1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5

Minutes After Hypertonic Arabinose Infusion

[14c]sucrose Infusion
Decapitation

92/300 [Cbrain] = 4.33 x 10^6 dpm x ml^-1 x sec

Table 3. Femoral artery plasma radioactivity, during 10 min of right-sided carotid infusion of [14c]sucrose. Tracer infusion was started 5 min after right-sided carotid infusion of 1.8 molal arabinose. Table 1 (bottom) gives regional brain concentration at decapitation, in relation to the grade of staining by Evans blue (Experiment 345).

Table 1
Regional brain radioactivity in relation to grade of brain staining by Evans blue, in individual rats in which [3H]MTX or [14c]sucrose was infused into the carotid circulation for 9 min, commencing 5 min after hypertonic arabinose was infused into the same artery.

Whole-blood concentration at decapitation equaled 303,100 dpm/ml in Experiment 352 and 671,950 dpm/ml in Experiment 345. [Cbrain] equaled 1.8 x 10^6 dpm/ml/sec in Experiment 352 and 4.33 x 10^6 dpm/ml/sec in Experiment 345. Data correspond to Charts 2 and 3.

Right side

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Grade of staining (dpmlg)</th>
<th>Concentration (dpm/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate nucleus</td>
<td>2+</td>
<td>278,600</td>
</tr>
<tr>
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<td>2+</td>
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<td>125,241</td>
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<tr>
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<td>228,223</td>
</tr>
<tr>
<td>White matter</td>
<td>1+</td>
<td>53,527</td>
</tr>
</tbody>
</table>

[3H]MTX infusion (Experiment 352)

Left side

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[14c]Sucrose infusion (Experiment 345)

Concentration, and r = plasma fraction of tracer not bound to protein (r = 0.5 for [3H]MTX; r = 1 for [14c]Sucrose) (31, 32):

\[ dC_{brain}/dt = PA/(r)C_{brain} \]  

(A)

During carotid infusion of [3H]MTX in the rat, it has been demonstrated that C_{brain} exceeds the measured femoral artery plasma concentration, C_{art} dpm/ml, by a factor s = 7 on the cerebral hemisphere ipsilateral to infusion and by a factor s = 2 on the contralateral hemisphere (31, 32). Thus, during infusion, C_{brain} can be calculated from the femoral artery concentration by the following equation:

\[ C_{brain} = (s)C_{art} \]  

(B)

Substituting Equation B into Equation A gives the following expression for the unidirectional tracer flux from plasma to brain:

\[ dC_{brain}/dt = PA/(r)C_{art} \]  

(C)

Integrating Equation C during the 10 min of tracer infusion (between 300 and 900 sec after hypertonic arabinose treatment), provides a value for PA as follows, where C_{brain} (15 min) is the brain radiotracer concentration 15 min after arabinose treatment.

\[ PA = C_{brain}(15 \text{ min}) \]  

(D)

To calculate the rate of loss of intracerebral tracer after initial loading by 10 min of infusion, we define as follows a normalized brain concentration, C_{brain}, where T is the time of decapitation after right-sided arabinose treatment, and C_{brain}(T) is the measured brain concentration at decapitation:

\[ C_{brain} = C_{brain}(T) \]  

(E)

Comparison of Equations D and E indicates that C_{brain}(T) equals PA if the rat is decapitated immediately after the brain is loaded with tracer, but it is less or equal to PA at later times because of possible back-diffusion from brain to plasma. Note that normalization of brain radioactivity to the plasma concentration integral gives C_{brain}(T) in units of time^-1.

Chart 4 summarizes mean normalized concentrations of [3H]MTX and of [14c]Sucrose in right and left gray matter of the parietal lobe, following right-sided osmotic treatment and radiotracer infusion. Right-sided concentrations were obtained at regions with Grade 2+ and 3+ staining by Evans blue, but not...
with Grade 1+ staining, which is associated only with a slight elevation of PA (32). Left-sided concentrations were calculated at unstained regions (Grade 0). The line in the figure is a least-squares fit (21) of $C_{brain}$ for $[^{3}H]$MTX by the following equation, where $A_{1}$ is a constant concentration and $t_{m}$ is a half-time:

$$C_{brain} = A_{1} \exp(-0.693/t_{m})$$ (F)

The mean right-sided $[^{3}H]$MTX concentration in brain exceeded the left-sided concentration, even 6 hr after treatment. The single exponential expression of Equation F was sufficient to fit the $[^{3}H]$MTX concentration data and provided a value of $t_{m}$ equal to 3.5 hr. Table 2 lists half-times for the parietal cortex as well as for other brain regions, calculated as described for Chart 4. The average $t_{m}$ equaled 4.8 hr.

Unlike the normalized brain concentration for $[^{3}H]$MTX, $C_{brain}$ for $[^{14}C]$sucrose did not decline monoexponentially with time. Chart 4 shows that the value in right-sided parietal lobe gray matter fell between 10 and 55 min after osmotic loading by a factor of 6.5, from a mean of $24 \times 10^{-5}$ sec$^{-1}$ to $3.7 \times 10^{-5}$ sec$^{-1}$. After 55 min, right-sided $C_{brain}$ remained essentially unchanged for up to 24 hr, approximating the 55-min mean value. Furthermore, after 3 hr, right- and left-sided values for $C_{brain}$ did not differ significantly ($p > 0.05$). These observations indicate that the extra quantity of $[^{3}H]$MTX that entered the right brain in relation to perfusion and osmotic BBB opening had disappeared by 3 hr. The remaining radioactivity in both the right and left sides after 3 hr therefore reflected a non-steady-state brain in relation to perfusion and osmotic BBB opening which had disappeared by 3 hr.

The rapid disappearance of loaded $[^{14}C]$sucrose within the first hr suggests that, to obtain an accurate estimate of the $t_{m}$ for initial loss, more time points should be available in the first hr. Without these points, it nevertheless is possible to estimate an upper limit for $t_{m}$ from the available data, by noting that $C_{brain}$ for $[^{14}C]$sucrose never declined by less than a factor of 5 in any of the right-sided, perfused brain regions between 10 and 55 min. Thus, $t_{m} = 20$ min (equal to $-0.693/0.693$ min)/$t_{m}$ (0.2). This value is about one-fifteenth the estimated $t_{m}$ for $[^{3}H]$MTX loss from brain (see above).

**DISCUSSION**

Following loading of either $[^{3}H]$MTX or of $[^{14}C]$sucrose into the rat brain in relation to osmotic opening of the BBB, $[^{3}H]$MTX is lost much more slowly than is $[^{14}C]$sucrose. The initial half-time for $[^{3}H]$MTX efflux from a loaded brain has an average value of 4.8 hr in 7 brain regions, as compared to $<20$ min for $[^{14}C]$sucrose. The $[^{3}H]$MTX half-time approximates a half-time of 1.5 to 3 hr obtained in the dog by a semiquantitative study (27) and of 4 hr obtained in the mouse after intracerebral injection of $[^{3}H]$MTX (45).

The more rapid loss of $[^{14}C]$sucrose than of $[^{3}H]$MTX probably is due to patency of the BBB for up to 1 hr after osmotic treatment and to the distribution of intracerebral $[^{14}C]$sucrose exclusively within the brain extracellular space (9, 10, 36, 37, 47). If $[^{3}H]$MTX also remained only extracellular, its molecular weight similar to that of $[^{14}C]$sucrose (454 daltons for MTX; 360 daltons for sucrose) would have it diffuse from the brain at approximately the same rate as that of $[^{14}C]$sucrose (35, 37, 41). Therefore, the slower loss of $[^{3}H]$MTX may represent cellular uptake or binding. This interpretation is consistent with direct evidence of $[^{3}H]$MTX uptake by mammalian cells, including brain cells (12, 15, 17, 44, 45). Furthermore, the brain distribution space for MTX is 25 to 30% of the wet weight, as compared to spaces of less than 20% for sucrose, inulin, and EDTA:Ca$^{2+}$ (2, 9, 33). In addition, the ionization of MTX but not of sucrose may prolong the half-time of MTX in the brain. This explanation has been suggested to explain the much greater half-time of an ionized radiographic contrast agent (meglumine iohidate) as compared to a nonionized contrast agent (metrizamide) (29).

The experiments with $[^{3}H]$MTX were limited to not more than 6 hr, because metabolites of $[^{3}H]$MTX appeared in blood and brain after 6 hr. Although several studies indicate that MTX is not metabolized by the rat (8, 14), sufficient amounts of aldehyde oxidase exist in the rat liver to promote the slow oxidation of MTX to 7-OH-MTX; metabolism is also quite significant in rabbits, guinea pigs, monkeys, and humans (16, 20, 46). One study reports that MTX metabolites appear after 4 hr in awake rats (19). The appearance of metabolites only after 6 hr in our study may reflect inhibition of liver metabolism by barbiturate anesthesia (34).

For $s = 0.5$ and $r = 7$ and from the data in Chart 2, it can be calculated that intracarotid infusion of 20 $\mu$Ci of $[^{3}H]$MTX, at a rate of 2 $\mu$Ci/min for 10 min, will produce an approximate femoral artery plasma concentration integral equal to 40 $\mu$Ci/ml/sec.

Following these proportionalities, infusion of 0.5 $\mu$mol of MTX should produce a concentration integral equal to 1 $\mu$mol/ml/sec. Chart 4 shows that $C_{brain}$ approximates $25 \times 10^{-5}$ sec$^{-1}$ following carotid infusion of hypertonic arabinose plus 20 $\mu$Ci $[^{3}H]$MTX. Thus, by Equation E, infusion of 0.5 $\mu$mol of MTX should produce an initial ipsilateral brain concentration equal to 0.9 $\mu$mol/kg, or approximately 1 $\mu$M (product of $C_{brain} = 25 \times 10^{-5}$ sec$^{-1}$; $s = 0.5$; $r = 7$; and 1 $\mu$mol/ml/sec). This is the threshold concentration of MTX which has proven effective against many cancers (1, 5, 16). Without the 10-fold BBB effect due to arabinose treatment, 5 $\mu$mol would have to be infused through the carotid artery, whereas 35 $\mu$mol would have to be given i.v. (31).

Malignant gliomas have a net cell cycle time of about 60 hr (15). To treat these neoplasms by osmotic BBB opening to an S-phase-effective agent such as MTX, several approaches are possible. An initial very large dose might be administered after hypertonic arabinose infusion to maintain the brain concentration above 1 $\mu$M for 60 hr, but this approach is impractical, because 60 hr includes 12.5 half-lives within the brain. On the other hand, hypertonic arabinose plus MTX could be administered at repeated intervals during 60 hr to maintain a brain concentration.
at or above 1 μM. On the basis of the above calculation and of a half-time in brain of 4.8 hr, 4 μmol of MTX would have to be given every 14.4 hr (3 half-lives), or 38 μmol once, at 30 hr, during the 60-hr period.

REFERENCES


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