Transport of Methotrexate by the Choroid Plexus

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SUMMARY

Methotrexate-3H was accumulated in vitro against a concentration gradient by the rabbit choroid plexus. This accumulation was energy dependent, inhibited by metabolic inhibitors, and exhibited self-saturation and competitive inhibition. Several folic acid analogs with a 4-amino group were inhibitors of this accumulation, while folic and folinic acid, compounds which do not contain a 4-amino group and are not effective antimetabolites, were ineffective as inhibitors of Methotrexate-3H accumulation.

When Methotrexate-3H clearance exceeded inulin clearance, no accumulation of Methotrexate-3H was demonstrable in vivo. This suggests that in vitro accumulation is equivalent to in vivo transport from cerebrospinal fluid to blood. Such a system would influence the spinal fluid levels of orally or parenterally administered Methotrexate, as well as the systemic toxicity from intrathecally administered drug.

INTRODUCTION

Many chemotherapeutic agents are highly effective against systemic disease but relatively ineffective against central nervous system manifestations of the same disease. Two examples of such agents are the antibiotic penicillin and the antimetabolite Methotrexate. The explanation given for this has been that these substances fail to achieve therapeutic brain and spinal fluid levels because of the blood brain barrier. However, the concentration of a substance in cerebrospinal fluid reflects not only its rate of entry but also its rate of exit from cerebrospinal fluid.

Considerable attention has been given to the entry of compounds into cerebrospinal fluid but less has been focused on the transport of substances out of cerebrospinal fluid. Compounds can be introduced directly into the cerebrospinal fluid by intrathecal injection (22, 23) or intrathecal perfusion (26). This places new emphasis on the exit of these substances from cerebrospinal fluid.

Methotrexate, 4-amino-N10-methylpteroylglutamic acid, is a folic acid antagonist which has been used successfully for the intrathecal treatment of meningeal leukemia (23). It has also been administered by intraventricular perfusion for the treatment of primary intracranial neoplasms (26). Limiting toxicity has been systemic, not local. The increasing intrathecal use of this compound made it of interest to determine if a system exists within the choroid plexus for the transport of Methotrexate and, if such a system exists, to determine its direction and specificity.

MATERIALS AND METHODS

Albino and New Zealand rabbits weighing 1.5-3.0 kg were sacrificed by air embolism. The brains were quickly removed and placed into chilled Krebs-Ringer's phosphate solution (pH 7.4) (5). The choroid plexuses of the lateral and 4th ventricles were then placed in Krebs-Ringer's phosphate containing glucose (1 gm/liter). Varying concentrations of tritium-labeled Methotrexate were then added, in addition to other unlabeled folic acid analogs or other organic compounds. The resulting mixture was shaken in a Dubnoff metabolic shaker at 37°C in an atmosphere of O2. The effects of anaerobic conditions were tested by incubating choroid plexus in a nitrogen atmosphere. Uptake ratios were compared directly with plexuses incubated concurrently in the Dubnoff shaker under an oxygen atmosphere. Effects of low temperature were assessed by maintaining the incubation bath at 4°C. After incubation, the plexus was removed from the medium, blotted, and placed in a preweighed plastic counting vial, reweighed, and then digested in 0.1 ml of 5 N KOH overnight at 40°C. The effects of anaerobic conditions were tested by incubating choroid plexus in a nitrogen atmosphere. Uptake ratios were compared directly with plexuses incubated concurrently in the Dubnoff shaker under an oxygen atmosphere. Effects of low temperature were assessed by maintaining the incubation bath at 4°C. After incubation, the plexus was removed from the medium, blotted, and placed in a preweighed plastic counting vial, reweighed, and then digested in 0.1 ml of 5 N KOH overnight at 40°C in the Dubnoff shaker. Eighteen ml of scintillation fluid (7) were added to each vial; the radioactivity was measured in a liquid scintillation spectrometer. Radioactivity in all samples exceeded background by at least ten-fold.

Methotrexate-3H binding was determined by incubating tissue slices as described above. Fifty to 100 mg of tissue were then homogenized with 2 ml of Krebs-Ringer's buffer and placed in a cellophane dialysis bag and centrifuged overnight at 2,500 X g in accordance with the method of Toribara et al. (28) as modified by Dixon and Adamson (4). Weighed aliquots of tissue homogenate and ultrafiltrate were then digested with 5 N KOH, and Methotrexate-3H activity was counted in the liquid scintillation counter.

Ventriculocisternal perfusions were carried out in 5 dogs as described by Leusen (15). The inflow rate was 0.191 ml/min. The cisternal outflow cannula was maintained at the level of the external auditory meatus. Methotrexate-3H (10^-8 M) or carboxylinulin-14C was added to the inflow, and after an equilibration period, the concentration of Methotrexate-3H or carboxylinulin-14C in the cisternal outflow was measured and expressed as a percent of the inflow. In 2 of these experiments, unlabeled Methotrexate was added to the inflow, and the effect of this on Methotrexate-3H clearance was measured. In experiments utilizing only labeled Methotrexate, the choroid plexuses were removed, and the Methotrexate-3H activity per mg of plexus...
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was determined and compared with the Methotrexate-3H activity in the perfusate (actually an average of inflow and outflow concentration).

The results of tissue uptake experiments were expressed as tissue (T) to medium (M) concentration ratios, T/M. Tissue concentration was expressed as cpm/gm wet weight of tissue; medium concentration was in cpm/gm of medium. The volume of incubation medium was much larger than that of the tissue and did not appreciably change during the incubation.

The percent of bound Methotrexate-3H was calculated from the following equation:

\[
\frac{\text{cpm/mg tissue} - \text{cpm/mg ultrafiltrate}}{\text{cpm/mg tissue}} \times 100
\]

Results, unless otherwise stated, are expressed as the mean ± S.E.

Tritiated Methotrexate was prepared chromatographically pure utilizing the method described by Henderson et al. (10). The specific activity of the Methotrexate-3H employed was 4.2 \( \mu \text{c}/\mu \text{g} \) Methotrexate; the stock solution contained 5 \( \mu \text{g} \) (24 \( \mu \text{c} \))/ml. Additional Methotrexate-3H was prepared and supplied by Dr. V. Oliverio utilizing Methotrexate-3',5'-5 obtained from Nuclear Chicago Corporation. This had a specific activity of 5.55 mc/mg (2.52 c/mmole). Following dilution with unlabeled Methotrexate, the sample was purified by chromatography on a DEAE-cellulose ion-exchange column according to the method of Oliverio (18), but using linear gradient elution with pH 8.3 ammonium bicarbonate buffer (0.1-0.4 M). The buffer solution from the main peak containing the radioactive Methotrexate was removed by lyophilization in the dark at -70°C and 100 microns of pressure. The final specific activity of 124.4 \( \mu \text{c}/\mu \text{mole} \) (0.276 \( \mu \text{c}/\mu \text{g} \)) was determined spectrophotometrically by the percent of its absorption at 302 \( \mu \text{m} \) (O.D. = 0.53) and 370 \( \mu \text{m} \) (O.D. = 0.17). The stock solution containing 308 \( \mu \text{g} \)/ml was added to Krebs-Ringer's phosphate buffer immediately before use, so that 0.1 ml of buffer contained 1000 cpm.

Dichloromethotrexate was supplied by the Cancer Chemotherapy National Service Center and by Lederle Laboratories, Pearl River, New York.

RESULTS

In Vitro

Accumulation of Methotrexate-3H. At a total medium concentration of \( 10^{-8} \) M, Methotrexate-3H was accumulated within the choroid plexuses against a concentration gradient.

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**Table 1**

<table>
<thead>
<tr>
<th>Ions removed from Krebs-Ringer's phosphate</th>
<th>No. of experiments</th>
<th>% depression of 2-hour T/M (S.E.)</th>
<th>% of 2-hour control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca ++</td>
<td>3</td>
<td>60 ± 3</td>
<td>32</td>
</tr>
<tr>
<td>Mg ++</td>
<td>3</td>
<td>68 ± 4</td>
<td>40</td>
</tr>
<tr>
<td>PO₄⁻³</td>
<td>3</td>
<td>71 ± 2</td>
<td>29</td>
</tr>
<tr>
<td>Mg ++ + PO₄⁻³</td>
<td>3</td>
<td>58 ± 4</td>
<td>42</td>
</tr>
</tbody>
</table>

Effect of changes in incubation medium uptake by choroid plexus.

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Chart 1. A typical uptake curve in which uptake \( \text{T/M} \) of Methotrexate-3H by choroid plexus is plotted against time in hours.

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Chart 2. A typical experiment in which the uptake \( \text{T/M} \) of Methotrexate-3H in a nitrogen or oxygen atmosphere is plotted as a function of time.
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Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of experiments</th>
<th>% depression of T/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dinitrophenol</td>
<td>6</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>4</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>4</td>
<td>45 ± 4</td>
</tr>
</tbody>
</table>

Effect of metabolic inhibitors on 2-hour uptake of Methotrexate-3H by choroid plexus. Each compound was used at a concentration of 10^{-4} M.

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of experiments</th>
<th>% depression of 2-hour T/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminohippurate</td>
<td>4</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>Penicillin</td>
<td>4</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Potassium thiocyanate</td>
<td>4</td>
<td>13 ± 6</td>
</tr>
</tbody>
</table>

Effect of weak acids (at 10^{-4} M) on 2-hour Methotrexate-3H uptake by choroid plexus.

(Chart 1). The T/M increased to a maximum of 6-8 at 3 hours and then began to decline. The maximum T/M for any plexus was 8. Slices of cerebral cortex or diaphragm incubated under similar conditions achieved a 2-hour T/M of only 0.8-1.0 and showed no further accumulation. Incubating the plexuses at 4°C for 4 hours resulted in a T/M of only 1.2.

Effect of Anerobic Conditions on Methotrexate-3H Uptake. In N₂ atmosphere, accumulation of Methotrexate-3H consistently lagged behind its accumulation in O₂. A typical experiment (Chart 2) shows a 3-hour T/M in N₂ of 4.5, while the T/M of duplicate slices incubated in O₂ was 8.

Effect of Glucose on Methotrexate-3H Uptake. Plexuses incubated over a 20-hour period and sampled hourly exhibited parallel uptake curves in a medium containing or devoid of glucose. The 3-hour T/M in a medium without glucose was 99 ± 4% of the control.

Effect of Various Inorganic Ions on Methotrexate-3H Uptake (Table 1). Removal of phosphate from the incubation medium (bicarbonate ion added as a buffer) or removal of Mg²⁺ resulted in approximately a 70% reduction of the T/M; removal of Ca²⁺ resulted in a 60% reduction. Doubling the Mg²⁺ concentration resulted in no significant increase in uptake, but the removal of both Mg²⁺ and PO₄⁻³ (bicarbonate added as a buffer) resulted in a 58% reduction.

Effect of Dichloromethotrexate on Methotrexate-3H Uptake. Dichloromethotrexate interfered with the uptake of Methotrexate-3H. The results are plotted according to the method of Lineweaver and Burk (16) (Chart 3) and show a common intercept on the vertical axis, suggesting a competitive nature to the inhibition.

Effect of Metabolic Inhibitors on Methotrexate-3H Uptake (Table 2). The presence of 2,4-dinitrophenol, iodoacetate, or sodium fluoride, each at 10^{-4} M resulted in a decreased T/M. Dinitrophenol and iodoacetate reduced the T/M by 50%, while sodium fluoride resulted in a 45% decrease in T/M.

Effect of Weak Acids on Methotrexate-3H Uptake (Table 3). Methotrexate is a weak acid at pH 7.4 (1) and, as such, may be nonspecifically transported. Other organic acids, such as p-aminohippurate, penicillin, and potassium thiocyanate were individually added to the incubation media, and the pH was readjusted to 7.4. Paraminohippurate 10^{-4} M reduced the T/M by 28%. Penicillin 10^{-4} M and potassium thiocyanate 10^{-4} M produced no significant change.

Effect of Miscellaneous Substances on Methotrexate-3H Uptake. Ouabain (19) and acetazolamide (24, 29) have been reported to depress cerebrospinal fluid production, ouabain by interfering with the NaK-activated ATP enzyme system and acetazolamide by inhibiting carbonic anhydrase (17). The addition of ouabain 10^{-5} M or acetazolamide 10^{-4} M to the incubation medium resulted in no significant change in the T/M.

Effect of Folate Acid and its analogs on 2-hour Methotrexate-3H uptake by choroid plexus (Table 2). Table 5. Each compound was used at a concentration of 10^{-4} M.

Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>% bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate-3H (alone)</td>
<td>25</td>
</tr>
<tr>
<td>Methotrexate-3H + dinitrophenol</td>
<td>23</td>
</tr>
</tbody>
</table>

Effect of 2,4-dinitrophenol on Methotrexate-3H binding.

Table 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of experiments</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic Acid</td>
<td>5</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>Folinic Acid</td>
<td>6</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>3</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>3-Bromomethotrexate</td>
<td>3</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>Dichloromethotrexate</td>
<td>4</td>
<td>50 ± 5</td>
</tr>
</tbody>
</table>

Effect of folate acid and its analogs on 2-hour Methotrexate-3H uptake by choroid plexus. Each compound was used at a concentration of 10^{-4} M.
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Chart 4. Outflow concentration of Methotrexate-3H (MTX-3H) and inulin-14C as a percent of inflow concentration. The effect of the addition of unlabeled Methotrexate $10^{-4}$ M on the outflow concentration of Methotrexate-3H and inulin-14C is shown.

Presence of dinitrophenol, as opposed to 25% in the absence of dinitrophenol. From this it was concluded that, although tissue binding may contribute to Methotrexate uptake, the accumulation of Methotrexate was largely independent of tissue binding. Furthermore, the inhibitory effect of dinitrophenol was not dependent on tissue binding.

**Effect of Some Folic Acid Analogs on Methotrexate-3H Uptake.** Dichloromethotrexate, Methotrexate, and bromomethotrexate are all folic acid analogs containing a 4-amino group (Table 5). Each of these compounds, when added to the incubation medium so that its final concentration was $10^{-4}$ M, resulted in a Methotrexate-3H uptake approximately 50% of control. Folic acid $10^{-4}$ M, which has a hydroxyl group in position 4 rather than an amino group, and folic acid (citrovorum factor), a compound in which the pyrazine ring is saturated, did not significantly affect Methotrexate-3H uptake.

**In Vivo**

*In vivo* studies were undertaken to clarify the direction of transport, i.e., whether from CSF to blood or blood to CSF, and to determine whether T/M ratios represented cellular accumulation or transport through the plexus. Methotrexate-3H or inulin-14C was added to the perfusion fluid and introduced through a ventricular cannula (15, 20). The outflow was collected through a cisternal cannula. When steady-state conditions were established, the outflow concentration of inulin, a non-metabolized molecule, was 70-80% of its inflow concentration, while that of Methotrexate-3H, also a non-metabolized substance (10), was 36% of its inflow. The addition of unlabeled Methotrexate to a final concentration (Chart 4) of $10^{-4}$ M raised the outflow concentration of Methotrexate-3H to 52% of inflow, suggesting a saturable system. The choroidplexuses from these animals were then removed, and Methotrexate-3H concentration was compared with that of the perfusate (inflow concentration and outflow concentration /2).

There was no Methotrexate accumulation in these plexuses, as demonstrated by a T/M of only 0.20. This suggested that either Methotrexate-3H was not accumulated in *vivo* or, more likely, that the *in vitro* accumulation of Methotrexate-3H represented *in vivo* transport from cerebrospinal fluid to blood.

**DISCUSSION**

The accumulation of Methotrexate-3H by choroidal epithelium against a concentration gradient has the characteristics of an active transport system. It is oxygen and temperature dependent and inhibited by metabolic inhibitors. The ions Ca$^{++}$, Mg$^{++}$, and PO$_4^{-3}$ are required, and self-saturation and competitive inhibition are demonstrable. In the rabbit, choroidal epithelium and kidney, but not muscle or cerebral cortex, were able to concentrate Methotrexate-3H (25).

Choroidal epithelium has been shown to transport iodide (31) and thiocyanate (30), as well as organic molecules such as Diodrast and phenolsulphonphthalein (20) and certain quaternary ammonium cations (27). The inability of weak organic acids such as $p$-aminohippurate, penicillin, and thiocyanate to significantly inhibit Methotrexate-3H accumulation suggests that Methotrexate-3H accumulation involves more than a nonspecific carrier transport system for weak organic acids.

The accumulation of Methotrexate-3H may be explained by several alternate hypotheses. Possibly, Methotrexate-3H may be metabolized and the tritium label retained. However, Methotrexate-3H has been shown to be excreted essentially unchanged in mice, rats, dogs, monkeys, and humans (10, 11). With the exception of Methotrexate metabolism reported only in rabbit liver and intestinal mucosa (21), and not in muscle and cerebral cortex (25), significant metabolism of Methotrexate has not been shown. Furthermore, the consistency of data utilizing Methotrexate-3',5'-T and randomly labeled Methotrexate make significant metabolic alteration unlikely.

Binding, either extra- or intracellular, has been suggested as an alternate explanation for tissue accumulation. Ultrafiltration experiments demonstrate that, at a concentration of $10^{-8}$ M, 25% of the Methotrexate-3H is bound to homogenates of choroid plexus. However, dinitrophenol is able to significantly reduce the tissue uptake without modifying the percent of bound Methotrexate-3H. This suggests that tissue binding alone cannot account for the accumulation phenomenon.

It is difficult to distinguish between active transport and intracellular binding. Methotrexate has been shown to bind to
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folic reductase (32, 33). However, in addition to the bound Methotrexate, Fischer (6) and Werkheiser (33, 34), have found free intracellular Methotrexate. The existence of free intracellular Methotrexate in excess of the binding capacity of folic reductase substantiates the view that a transport mechanism exists in addition to intracellular binding.

Johns et al. (13), on the basis of data obtained with Methotrexate-3H in humans, suggested that separate transport mechanisms exist for folic acid and Methotrexate. They suggested that the uptake of folic acid was a specific process dependent both on the pteridine structure and on substituents on the pteridine ring. This supposition is in keeping with the ability of 4-amino folic acid analogs to markedly inhibit Methotrexate-3H uptake while folic acid (a compound in which the pyrazine ring is saturated) was ineffective. Competition for limited intracellular binding sites might also account for the inhibitory effect of 4-amino folic acid compounds on Methotrexate-3H accumulation. This, however, would not explain free intracellular Methotrexate. Interestingly, the 4-amino analogs are effective antimetabolites while folic and folic acid are not.

Some Methotrexate enters the choroid plexus by diffusion. Hakala (8, 9) and Jacquez (12) have suggested that the influx of Methotrexate into cells is a passive process with a low permeability constant. Unfortunately, Hakala does not mention purification of Methotrexate. This makes it difficult to interpret her data, as several active compounds may be involved. Jacquez suggested a low permeability constant but used Methotrexate concentrations of 10^-2 or 10^-3 M. These concentrations would saturate and mask any active transport. Additional data for a specific active transport system which is functional at low concentrations have recently been reported by Kessel and Hall (14). They reported that the accumulation of amethopterin by L1210 and Ehrlich ascites cells was mediated by a slow, temperature-sensitive, and partly saturable process. They further found that the uptake of labeled amethopterin was inhibited by several folate derivatives with an amino group in the 4 position of the pteridine ring. They concluded that a specific mediation process is involved in cellular accumulation of amethopterin by many mammalian cell types.

In vivo, without an efferent vascular supply, the choroid plexus has the ability to accumulate Methotrexate-3H. However, in vitro, with an intact efferent vascular supply and at a time when Methotrexate-3H clearance exceeds inulin clearance, no Methotrexate-3H accumulation is demonstrable. This might be explained by Methotrexate accumulation within the brain. Diffusion studies, however, demonstrate that Methotrexate-3H is not accumulated within the brain but, like inulin, diffuses through brain substance. (R. Rubin and D. Rall, unpublished data). In vitro accumulation may then be equivalent to in vivo transport of Methotrexate-3H from spinal fluid to blood.

The probable transport of Methotrexate from cerebrospinal fluid to blood suggests that this system should be critically evaluated in the planning of central nervous system therapy. Oral and parenteral administration resulting in low cerebrospinal fluid levels are the routes most critically affected. The transport of Methotrexate from cerebrospinal fluid to blood must also be considered in anticipating systemic toxicity from intrathecal perfusion chemotherapy (26). More generally, it must be recognized that low spinal fluid concentrations result not only from the inability of compounds to enter cerebrospinal fluid, but also from their transport out of cerebrospinal fluid. Although it is not meant to imply that the active transport of Methotrexate accounts for either its antifolic activity or its relative ineffectiveness against central nervous system neoplasms, data are rapidly accumulating (6, 14, 25, 33-35) suggesting that transport may account, at least in part, for these observations.

REFERENCES


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