Uptake of Methotrexate-\(^3\text{H}\) by Rabbit Kidney Slices

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SUMMARY

Methotrexate-\(^3\text{H}\) (MTX-\(^3\text{H}\)) was accumulated against a concentration gradient by renal cortical slices. This accumulation was energy dependent, inhibited by metabolic inhibitors, Diamox, and high concentrations of ouabain.

Several folic acid analogs containing the pteridine ring were inhibitors of this accumulation while certain weak organic acids were less striking inhibitors. This suggests that MTX-\(^3\text{H}\) is carrier transported into cells nonspecifically as a weak organic acid and specifically as a folic acid analog.

The accumulation of MTX-\(^3\text{H}\) may represent transport from blood to urine and may be clinically important in predicting drug toxicity.

INTRODUCTION

Methotrexate, 4-amino-\(N^\text{10}\)-methylpteroylglutamic acid, a folic acid antagonist, has been a highly effective antineoplastic agent against a variety of human malignancies. Severe hematologic, gastrointestinal, and hepatic toxicity is, however, often encountered (2). Impaired renal function has been noted to increase the toxicity of a given dose, and renal clearance studies in patients suggest the existence of an active transport system (9). Methotrexate is essentially unmetabolized in man (1, 8, 12) and from 54 to 88\% (8) of an intravenous dose in humans is excreted unchanged in the urine during the 1st 24 hr.

It is the purpose of this report to present data suggesting the existence in vitro of an active transport system for MTX-\(^3\text{H}\) in renal cortical tissue.

MATERIALS AND METHODS

Albino and New Zealand rabbits weighing 1.5-3.0 kg were sacrificed by injecting 20-30 ml of air into an ear vein. The kidneys were quickly removed and placed into chilled Krebs-Ringer’s phosphate solution (pH 7.4) (4). The capsules were stripped and cortical sections approximately 0.5 mm x 0.5 mm were then cut free hand and placed in Krebs-Ringer’s phosphate containing glucose, 1 gm/liter. Varying concentrations of tritium-labeled MTX were then added in addition to other unlabeled folic acid analogs or other organic compounds. The resulting mixture was shaken in a Dubnoff shaker at 37°C in an atmosphere of \(\text{O}_2\). The effects of anaerobic conditions were tested by incubating kidney slices in a nitrogen atmosphere. Uptake ratios from these slices were compared directly with paired slices 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 tissue was removed from the medium, blotted, and placed in a pre-weighed plastic counting vial, reweighed, and the tissue digested in 0.1 ml of 5 N KOH overnight at 40°C in the Dubnoff shaker. Eighteen ml of scintillation fluid (6) were added to each vial and the radioactivity measured in a liquid scintillation spectrometer. Radioactivity in all samples exceeded background by at least tenfold.

MTX-\(^3\text{H}\) binding was determined by incubating tissue slices as described above. The tissue was then homogenized and placed in a cellophane dialysis bag and centrifuged overnight at 2500 \(\times g\) in accordance with the method of Toribara et al. (14) as modified by Dixon and Adamson (3). Weighed aliquots of tissue homogenate and ultrafiltrate were then digested with 5 N KOH and MTX-\(^3\text{H}\) activity counted in the liquid scintillation counter.

Analytic Methods. The results of tissue uptake experiments were expressed as tissue to medium 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 MTX-\(^3\text{H}\) 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 MTX was prepared chromatographically pure uti-

\begin{tabular}{|c|c|c|}
\hline
Constituent & Concentration & Parts \\
\hline
\text{NaCl} & 0.154 & 0.90 \% 100 \\
\text{KCl} & 0.154 & 1.15 \% 4 \\
\text{CaCl}_2 & 0.110 & 1.22 \% 3 \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & 0.154 & 3.82 \% 1 \\
\text{Phosphate buffer} & & 21 \\
\hline
\end{tabular}

The phosphate buffer is prepared by mixing 40 ml of \(\text{m}/4\) \text{NaHPO}_4 and 2 ml of \(\text{m}/1\) \text{HCl} and diluting this mixture to a volume of 100 ml.

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Chart 1. Uptake (T/M) of tritiated methotrexate by rabbit kidney slices plotted against time in hours. Each point represents the average of 4-6 experiments.

Using the method described by Henderson et al. (7). The specific activity of the MTX-3H employed was 4.2 mc/µg MTX; the stock solution contained 5 µg (24 µc)/ml. Additional MTX-3H was supplied and prepared by Dr. V. Oliverio utilizing MTX-3'-5'-T obtained from Nuclear Chicago Corporation. This had a specific activity of 5.55 mc/mg (2.52 c/mmole). Following dilution with unlabeled MTX, the sample was purified by chromatography on a diethylaminoethyl cellulose ion-exchange column according to the method of Oliverio (11), but using linear gradient elution with pH 8.3 ammonium bicarbonate buffer (0.1 M to 0.4 M). The final specific activity of 124.4 mc/µmole (0.276 µc/µg) was determined spectrophotometrically using extinction coefficient values of 302 mµ and 370 mµ of pure unlabeled MTX as a reference. The stock solution containing 308 µM was added to Krebs-Ringer's phosphate buffer immediately before use so that 0.1 ml of buffer contained 1000 cpm.

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

RESULTS

Accumulation of MTX-3H. At a total medium concentration of 10⁻⁴ M, MTX-3H was accumulated within the tissue slices against a concentration gradient (Chart 1). The T/M increased to a maximum of 6-8 at 6 hr and then began to decline. The maximum T/M for any kidney slice was 12. 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.

When kidney and muscle slices were incubated at 4°C, a T/M of 1.5 was reached and maintained after 1 hour and 4 hours respectively for muscle and kidney (Chart 2). Kidney incubated simultaneously at 37°C showed a T/M of 8, while muscle evidenced no additional uptake at 37°C.

Effect of Anaerobic Conditions on MTX-3H Uptake (Chart 3). In an N₂ atmosphere kidney slices obtained a maximum T/M for MTX-3H of 0.8 in 15 min and failed to achieve higher T/M. The two hour T/M of duplicate slices incubated in an atmosphere of O₂ + 5% CO₂ was 6.5.

Effect of Glucose on MTX-3H Uptake. Kidney slices incubated aerobically with or without glucose showed identical uptake curves for MTX-3H over a six hour period.

Effect of Various Inorganic Ions on MTX-3H Uptake (Table 1). Removal of phosphate from the incubation medium (bicarbonate ion added as a buffer) resulted in a 50% reduction of the T/M ratio for MTX-3H, while removing either the Mg²⁺ or Ca²⁺ resulted in a 63% depression of the ratio. Doubling the Mg²⁺ concentration resulted in no significant increase in uptake while removing Mg²⁺ and PO₄³⁻ resulted in a 30% reduction.

Effect of DCM on MTX-3H Uptake. DCM interfered with the renal tissue uptake of MTX-3H. Chart 4 shows the uptake of MTX-3H in the presence and absence of 10⁻³ M DCM. The results are plotted according to the method of Lineweaver and Burk (10) and show a common intercept on the vertical axis suggesting the competitive nature of the inhibition between DCM and MTX.

The Effect of Metabolic Inhibitors on MTX-3H Uptake (Table 2). The presence of DNP, iodosacetate, sodium fluoride, and mercuric chloride in concentrations of 10⁻³ or 10⁻⁴ M resulted in a decreased T/M. DNP 10⁻³ M consistently decreased the T/M of MTX-3H by 50% while mercuric chloride 10⁻⁴ M and iodosacetate 10⁻⁴ M reduced the T/M 16% and 48%, respectively. NaF 10⁻⁴ M resulted in no significant reduction. The T/M was 95% of control.

Effect of Weak Acids on MTX-3H (Table 3). An active transport system for weak acids such as Diodrast, penicillin, and...
Methotrexate-3H Uptake

CHART 2. Uptake (T/M) of tritiated methotrexate (10⁻⁸ M) by kidney and muscle slices at 4°C and 37°C as a function of time.

PAH is well documented (13). MTX is a weak acid at physiologic pH and may be handled in an analogous fashion. Penicillin, PAH, and potassium thiocyanate were individually added to the incubation medium and the pH readjusted to 7.4. Solutions containing 10⁻⁴ M PAH, 10⁻⁴ M penicillin, and 10⁻⁴ M KSCN were found to reduce the T/M by 30%, 20%, and 35%, respectively.

Effect of Miscellaneous Substances on MTX-3H Uptake. Kidney slices were incubated in the presence of varying concentrations of ouabain, acetazolamide 10⁻⁴ M, a methazolamide analog (C1 17,262, American Cyanamid Co. Pearl River, N.Y.) (structurally similar to acetazolamide but inactive as a carbonic anhydrase inhibitor) 10⁻⁴ M (Table 4). All depressed the T/M as follows: ouabain at 10⁻⁵, 10⁻⁴, and 10⁻³ depressed the T/M by 0%, 33%, and 42%, respectively; acetazolamide by 20%; methazolamide analog by 30%.

Tissue Binding of MTX (Table 5). Forty-three percent of MTX-H is bound to nondialyzable tissue components. The possibility that inhibitors of MTX-H uptake function by competitively debinding MTX-H from cells was tested by measuring the effect of a known inhibitor, DNP on MTX-H binding. MTX-H was incubated with and without DNP 10⁻³ M and the % of bound MTX-H determined; 41% of MTX-H was bound in the presence of DNP as opposed to 43% bound in the absence of DNP. From this, it was concluded that the inhibition of MTX uptake by DNP was not dependent on competitive debinding.

TABLE 1

<table>
<thead>
<tr>
<th>Ions removed from Krebs-Ringer’s phosphate</th>
<th>No. of experiments</th>
<th>% Depression of 2-hr T/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>4</td>
<td>63 ± 1.7</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4</td>
<td>63 ± 2.0</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>4</td>
<td>50 ± 2.0</td>
</tr>
<tr>
<td>Mg²⁺ + PO₄³⁻</td>
<td>4</td>
<td>30 ± 4.2</td>
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</tbody>
</table>

Effect of Some Folic Acid Analogs on MTX-3H Uptake. DCM, MTX, and bromomethotrexate are all folic acid antagonists containing a 4-amino group (Table 6). These compounds when added to the incubation medium at a final concentration of 10⁻⁴ M resulted in MTX-H uptake approximately 50% of control. Triamterene (Dyrenium, Smith, Kline, and French Laboratories, Philadelphia, Pa.) a compound containing the pteridine moiety of folic acid and demonstrating in vitro anti-folic acid activity and diuretic properties resulted in uptake 46% of control. Folic acid 10⁻⁴ M with a hydroxyl group in position 4 rather than an amino group was questionably effective as an inhibitor. Folinic acid (citrovorum factor), a compound in which the pyrazine ring is saturated, was essentially ineffective as an inhibitor.
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**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molarity</th>
<th>No. of experiments</th>
<th>% Depression of 2-hr T/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dinitrophenol</td>
<td>$10^{-3}$</td>
<td>8</td>
<td>50 ± 4.6</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$10^{-4}$</td>
<td>5</td>
<td>48 ± 1.0</td>
</tr>
<tr>
<td>Mercureic chloride</td>
<td>$10^{-4}$</td>
<td>3</td>
<td>16 ± 5.0</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>$10^{-4}$</td>
<td>5</td>
<td>5 ± 3.0</td>
</tr>
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</table>

**DISCUSSION**

The uptake of MTX-3H by renal cortical slices against a concentration gradient has the characteristics of an active transport system. It is inhibited by low temperatures and requires an oxygen atmosphere. The system works well in the absence of glucose and may utilize stored metabolites. Calcium and magnesium ions appeared to be essential to MTX-3H accumulation as is adequate phosphate in the medium. The process was inhibited in varying degrees by the metabolic inhibitors tested with the possible exception of NaF. None of these, however, were able to completely inhibit the uptake of MTX-3H. Ouabain $10^{-6}$ M was surprisingly ineffective as an inhibitor.

Weak organic acids such as penicillin, Diodrast, and PAH have been shown to be transported by kidney cortical slices (13). The ability of these compounds to inhibit MTX-3H accumulation suggests that at least some MTX-3H is accumulated by a nonspecific carrier transport for weak organic acids. The inhibition of MTX-3H uptake by DCM in accordance with Lineweaver-Burk kinetics for competitive inhibition (10) and the strong inhibitory effect of other 4-amino pteridines suggest that a further transport mechanism may exist based on the pteridine ring. This is supported by the ineffectiveness of folic acid as an inhibitor. The minimal inhibitory effect of folic acid, a compound without a 4-amino group, suggests that the transport mechanism may be specific for 4-amino pteridines.

Binding, either extra- or intracellular, has been suggested as...
an alternate explanation. Ultrafiltration experiments demonstrate that 40-45% of MTX-3H is bound to homogenates of renal cortical slices. However, 2,4-DNP is able to significantly reduce the tissue uptake without modifying the percent of bound MTX-3H. This strongly suggests that tissue binding alone cannot account for the accumulation phenomenon.

MTX has been shown to bind to folic reductase (15, 16, 19). In addition, however, to the bound MTX, Fischer (5) and Werkheiser (16, 17) have found free intracellular MTX. Fischer (5) found that a clone of murine leukemic cells (L5178Y) sensitive to MTX was capable of taking up 10 times the amount of MTX required to titrate the total folic reductase activity within the cell. He suggested that MTX-3H is carrier transported into cells. Furthermore, we feel that this transport may be oriented both specifically to 4-amino pteridines and nonspecifically to weak organic acids. This transport may also be coupled to the intracellular binding of folic acid analogs to folic reductase.

The existence of a system capable of transporting MTX-3H from blood to urine is suggested by renal clearance studies (9). In vitro accumulation of MTX may represent, in part, in vivo transport from blood to urine. Although it is not meant to imply that the active transport of MTX accounts for either its antifolic activity (16-18) or its rapid excretion, this transport may account at least in part for these observations.

REFERENCES
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