Distribution and Degradation of $[^3\text{H}]$Methotrexate after Intravenous and Cerebral Intraventricular Injection in Primates

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SUMMARY

Four hr after either a single injection or continuous infusion of methotrexate (MTX) plus purified $[3',5',9(n)-^3\text{H}]$MTX in cynomolgus or rhesus monkeys, 80 to 99% of the $^3\text{H}$ radioactivity present in the plasma was found not to represent intact MTX. The percentage of $^3\text{H}$-containing MTX products in the urine after 4 hr was considerably less, although more variable. This variability seemed to be related to variability in the amount of the total dose excreted. Non-MTX products were also found in selected tissues and the percentage of intact MTX found 4 hr after i.v. injection varied from 2 to 26%. The percentage of intact MTX was routinely measured by comparing the values obtained using the dihydrofolate reductase assay with values based on the specific activity of $[3',5',9(n)-^3\text{H}]$MTX. Results obtained by diethylaminoethyl column chromatography on a few samples, however, showed good agreement with results from the reductase assay. $[3',5',9(n)-^3\text{H}]$MTX products appeared in peaks eluting from the diethylaminoethyl column both earlier and later than the MTX peak, with the earlier peaks being present in only small amounts in the urine.

After continuous i.v. infusion, only 2% or less of the radioactivity found in the cerebrospinal fluid after 4 hr represented intact MTX, with the remaining radioactivity eluting much earlier than MTX. In contrast, after direct injection into the left lateral ventricle, all the $^3\text{H}$ radioactivity in both cerebrospinal fluid and brain tissue represented intact MTX for up to 4 hr after injection.

The appearance of MTX products in the plasma and selected tissues of these primates a short time after i.v. injection is compared to other work in experimental animals and man and suggests a greater metabolism of MTX than was previously suspected.

INTRODUCTION

MTX$^2$ has been used extensively in the treatment of a variety of malignant conditions. It has been administered both i.v. and i.t. for clinical use (2, 5) and, in addition, i.p. for experimental studies (8). MTX is, however, rapidly excreted in the urine, resulting in very low plasma levels (7). Earlier studies had found that MTX in the urine is chemically intact for a considerable period after injection of relatively low doses, suggesting that the plasma MTX also remained essentially unchanged (8). In this study we have found, however, the presence of a considerable amount of non-MTX products in the plasma 4 hr after i.v. injection in both cynomolgus (Macaca fascicularis) and rhesus (Macaca mulatta) monkeys. This was associated with lesser proportions of non-MTX products in the urine over a comparable time period, suggesting some selective excretion of intact MTX by the kidney. We have also compared the integrity of MTX in brain and CSF after i.v. injection and after injection directly into the lateral ventricle.

MATERIALS AND METHODS

Purification of MTX. MTX was purified essentially according to the method of Henderson et al. (8) and Oliverio (18). The sodium salt of $[^3\text{H}]$MTX was purified on a column of Whatman microgranulated (preswollen) anion exchanger DE52, equilibrated with 0.1 m NH$_4$HCO$_3$ buffer (pH 8.3), and eluted with a 1000-mI continuous gradient of 0.1 to 0.4 M concentrations of the same buffer. The column was eluted at a rate of 30 ml/hr and approximately 10-mI fractions were collected and monitored with a UV recorder at 254 nm. The MTX fractions were combined and lyophilized, reconstituted with a small amount of water, divided into vials of 100 µCi each, and lyophilized again. These vials were stored at −70° until use.

Lederle MTX was used as the cold carrier and was not purified before use. It was stated to contain the specified amount of MTX (25 mg/ml) plus 5 to 6% impurity by weight. We analyzed the Lederle MTX by enzyme assay, UV absorbance, and DEAE column chromatography and calculated the average concentration of pure MTX as 30 mg/ml (range, 28 to 32 mg/ml).

Column Chromatography of Urine, Plasma, and CSF. Urine was applied directly to the DE52 column without prior treatment and run according to the purification procedure for tritiated MTX described above. Plasma and CSF were heated at 70–80° for 30 min and centrifuged to remove the protein precipitate. The protein pellet was washed to remove additional MTX, and the supernatant fractions were combined and then applied to a DE52 column. Eighty to
95% of the radioactivity was recovered in the combined supernatant fractions. From 2 to 4 ml of sample were applied and allowed to enter the column. The eluted volume was collected and with the wash solution represented Fraction 0 which usually contained 4 to 7 ml. The gradient pump was then started and 10-ml fractions were collected beginning with Fraction 1. Cold MTX was added to identify the position of the MTX peak. Recovery from the column was 93 to 100%.

**Measurement of MTX Concentration by Dihydrofolate Reductase Assay.** This determination was performed essentially according to the procedure of Sirotnak and Donsbach (21) in citrate buffer, pH 5.9, using crude dihydrofolate reductase from a guinea pig liver supernatant fraction (1). The assay mixture contained 0.5 ml of a solution of 0.2 mM NADPH in a buffer containing 0.2 mM citrate, 0.3 mM KCl, and 0.02 mM β-mercaptoethanol plus 100 to 250 μl dihydrofolate reductase and 50 to 150 μl sample. Water was added to make a final volume of 1.0 ml. Five-tenths ml of 0.2 mM dihydrofolate was added to initiate the reaction which was run for 20 to 30 min at 30°C (21). From 100 to 200 ng of MTX in 1 ml were then added to stop the reaction, and the absorbance was measured at 340 nm. The amount of MTX was then read off a standard curve run at the same time. The assay was found to be accurate between 0.5 and 5.0 ng of MTX. Plasma and CSF samples were diluted 3 to 5 times and heated for 30 min at 70-80°C; then an appropriate dilution was made for the assay. Urine was diluted and used directly in the assay. Tissues were homogenized, heated in a boiling water bath for 10 min, and centrifuged; the supernatant was used for the assay after an appropriate dilution. The various preparative treatments used were found not to affect the assay of added amounts of purified MTX.

**Procedures for Injection and Sampling.** These were performed essentially as previously described (3, 4, 14). All animals were cannulated via the right femoral vein with 45-cm segments of PE160 polyethylene tubing (Clay-Adams, Parsippany, N. J.), passed to a distance of about 7 cm. The left femoral artery was cannulated similarly with a 30-cm segment of tubing. A size 5 French pediatric feeding tube was then inserted transunethrally into the bladder to provide constant urine collection. Arterial blood pressure was recorded throughout the entire period, except during arterial cannulation procedures were performed following i.m. administration of phencyclidine hydrochloride, 2.0 mg/kg (Sernylan; Bio-Ceutic Laboratories), with appropriate supplemental doses given as needed for monkeys. Anesthetic use of Sernylan was accompanied by 0.1 mg of atropine sulfate. Cats were anesthetized by a total i.p. injection of 60 mg sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, Ill.) per kg over 4 hr.

**RESULTS**

**3H Radioactivity and MTX Levels in the Plasma after a single i.v. Injection of [3H]MTX.** The level of 3H radioactivity

\[
\text{Surface area (sq m)} = \frac{(K \cdot W^2)}{10^4}
\]

where \( W \) is the body weight in g and \( K \) is a conversion factor (= 11.8).

Intraventricular injections into the left lateral ventricle were performed essentially according to the method of Feinsteincher (6). The dose of MTX (7.2 mg/sq m or 0.59 mg/kg for a 3-kg monkey) was made up in 0.3 ml of bicarbonate-buffered “artificial CSF” (4) plus approximately 100 μCi of [3H]MTX. It was injected manually over ~1 min and flushed in with a further 0.1 ml of artificial CSF. Samples of CSF were removed by means of a hypodermic needle inserted into the cisterna magna (4, 6). Aliquots were either removed at intervals during the course of the experiment (0.05 ml), and/or a sample was removed immediately prior to sacrifice (1 to 2.0 ml). The animals were sacrificed by a crushing cervical clamp.

When the animals were treated with probenecid, a 25-mg/ml solution (pH 7.4) at a dose of 300 mg/sq m was injected i.v. 30 min before injecting MTX.

Sampling of brain tissue and systemic organ samples was as previously described (14). Blood volume and blood content of tissues were determined using 125I-labeled albumin (14). Plasma, urine, CSF, and tissue samples were prepared for scintillation counting using protosol and hydrogen peroxide for decolorization where necessary, as previously described (14). Quench correction was determined with standard curves prepared with known standards quenched with blood or chloroform, using either a channels ratio or external standard method in a Packard Model 3330 liquid scintillation counter.

**Animals and Materials.** Young male or female adult cynomolgus monkeys (Macaca fascicularis, 2.6 to 4.2 kg), rhesus monkeys (Macaca mulatta, 2.3 to 3.0 kg), and cats (2.1 kg) as obtained from commercial suppliers were used after a minimum of 1 week of stabilization and quarantine. Cannulation procedures were performed following i.m. administration of phencyclidine hydrochloride, 2.0 mg/kg (Sernylan; Bio-Ceutic Laboratories), with appropriate supplemental doses given as needed for monkeys. Anesthetic use of Sernylan was accompanied by 0.1 mg of atropine sulfate. Cats were anesthetized by a total i.p. injection of 60 mg sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, Ill.) per kg over 4 hr.

[3H]MTX with a specific activity of 9.3 Ci/m mole or 20.5 mCi/mg was generously supplied by the Chemical and Drug Procurement Section of the National Cancer Institute through Monsanto Research Corporation, Dayton, Ohio. Probenecid was a generous gift of Merck, Sharp and Dohme Research Laboratories, West Point, Pa. Sodium MTX as supplied for parenteral injection was from Lederle Laboratories Division, Pearl River, N. Y. DES2 was from Whatman Biochemicals Ltd., Kent, England. NADPH, β-mercaptoethanol, and dihydrofolate were obtained from Sigma Chemical Co., Saint Louis, Mo. All other chemicals were of at least reagent grade quality. Deionized, distilled water was used.

**RESULTS**
in the plasma after a single i.v. injection in a cynomolgus monkey is shown in Chart 1 for 2 different concentrations (210 and 420 mg/sq m) of injected MTX. The results are expressed as percentage of dose in the entire plasma compartment, the value for which (42.7 mI/kg) was the average determination in representative animals using 125l-labeled albumin. It is clear that there is rapid removal of [3H]MTX, since only peak levels of 26 to 27% of the total dose are seen and the 3H level rapidly falls upon completion of the i.v. injection at 3.5 min. From 3 to 1% of the total dose of 3H is found between 40 and 240 min. A similar behavior was found after a dose of 80 mg/sq m (not shown), with 0.9% dose in terms of radioactivity being present at 240 min.

When the amount of intact MTX was determined by the dihydrofolic reductase assay and compared to the MTX computed from the 3H levels, a very small proportion of the radioactivity present in the plasma was found to represent intact MTX. The difference between total 3H radioactivity and intact MTX is shown by the broken line in Chart 1. These values, representing 3H-containing MTX products, increased over the time period of the experiment and after about 80 min closely followed the time course for total 3H levels. At 4 hr only 2 to 5% of the 3H present represented intact MTX. A similar result was found after 80 mg/sq m was injected.

Previous work had studied the integrity and distribution of MTX in rhesus monkeys and other experimental animals (8). We therefore also studied the integrity of MTX after a single i.v. injection in a rhesus monkey (Chart 2). Within experimental error we detected a degree of breakdown of MTX in this experiment comparable to that found in the cynomolgus monkeys.

3H Radioactivity and MTX Levels in the Plasma after Continuous i.v. Infusion of [3H]MTX. Since clinically (7) and in previous studies (3) continuous i.v. infusion of [3H]MTX was used, we performed studies using this mode of injection where one-half of the dose was given in the 1st 5 min and the remaining half was given over the rest of the experimental time period. These results are shown in Chart 3 and are expressed as a percentage of the total dose administered in the total plasma compartment. Under these conditions considerable non-MTX radioactivity in the plasma was also found although, as might be expected, it was somewhat less than in the single-injection experiments. The difference in MTX levels calculated from 3H radioactivity, assuming no breakdown, and the amount of intact MTX found from the enzymic assay is shown as before as a dashed line. This represents products of [3H]MTX containing 3H, based on the assumption that they show no or negligible inhibition of dihydrofolate reductase relative to intact MTX. This assumption is supported by the good agreement found between determinations of intact MTX based on the enzyme assay and DEAE column chromatography (see Chart 3 (inset), Chart 5, and Table 1) and suggests that MTX metabolites that have been reported to be effective inhibitors of dihydrofolate reductase (10) are not present in significant amounts. The large graph in Chart 3 is for a single experiment, and the inset gives the average values ± S.E. for 4 separate experiments.

In 2 experiments performed in cats, we found no breakdown at 240 min after continuous i.v. infusion at a dose of 210 mg/sq m.
In order to confirm our results using the dihydrofolate reductase assay, we also examined samples of the plasma at increasing times by the column chromatography method, described in "Materials and Methods." The results of this are shown in Chart 4. The percentages refer to fractions of the total radioactivity recovered, which was 96 to 100% of the applied radioactivity. As can be seen there is a progressive increase of 1 or perhaps several components eluting at the front, associated with a marked decline in the MTX peak. There is also an increase in a later eluting component the level of which appears to decline at later times. Since these experiments were, however, done on separate animals as described in the chart legend, it need not necessarily reflect a trend.

The percentages of intact MTX determined by both the column chromatography method and the dihydrofolate reductase assay after continuous i.v. infusion of [3H]MTX are compared in Chart 5. The results from the 2 methods are from 3 separate experiments, except for a 240-min sample that was assayed by both methods and that within experimental error gave the same result of 20% intact MTX. These results show that there is very close agreement between the 2 methods and, as mentioned previously, suggest that the MTX products show negligible inhibition of the reductase. This chart also shows the corresponding percentages of intact MTX in the urine for, in some cases, the same experiments. It can be seen that MTX in the urine is 80 to 90% intact, which is considerably higher than the comparable plasma values. This result will be referred to in a later section on integrity of MTX in urine.

Effect of Probenecid. Probenecid had previously been reported to increase the levels of [H] from [3H]MTX in the plasma by around 2-fold during continuous i.v. infusion in a rhesus monkey (3). After pretreatment with probenecid, 350 mg/kg i.v., we found in 1 experiment that probenecid increased the total [H] in the plasma of a cynomolgus monkey by about 50% after continuous infusion, in agreement with previous observations on rhesus monkeys (3). The effect on intact MTX levels, however, measured by the dihydrofolate reductase assay was much greater. At 240 min the radioactivity represented 65% intact MTX, which was ~10-fold greater than the average level found in the absence of probenecid.

Integrity of MTX in Urine. Earlier studies had found no metabolites of MTX in the urine after i.v. injection in a number of species, including rhesus monkeys, at relatively low doses (8). More recent studies have detected evidence for metabolites of MTX appearing in the urine of patients after prolonged periods and higher doses (9, 11, 15). In view of these results and our finding of the presence of considerable amounts of MTX products in the plasma, especially at later time periods, we also examined the integrity of MTX in the urine. Table 1 and Chart 5, where it is directly compared to the integrity of MTX in plasma, summarizes the results for a number of different dose levels and modes of injection. These results were mainly obtained by the dihydrofolate reductase assay, but a few animals were studied using column chromatography, as indicated in Table 1 and Chart 5.

It can be seen from Table 1 that a marked variability in the proportion of non-MTX radioactivity in the urine was obtained. Generally, a high percentage of MTX products in the urine in both cynomolgus and rhesus monkeys, especially at later sampling periods after a single injection, occurred when relatively high percentages of the total dose were excreted. The animals given a continuous infusion of MTX generally showed 90 to 100% intact MTX at 240 min except for 1 cynomolgus monkey which excreted 69% of the dose given and had 76% intact MTX at 240 min (see Table 1). The urine of this animal was monitored by column chromatography (see Chart 6), and 3 and 19% of the MTX metabolites
Table 1
Percentage of intact MTX in urine after a single i.v. injection or continuous i.v. infusion

Continuous i.v. infusion, one-half dose in 1st 5 min, remaining one-half over next 235 min. Single injection 5 to 10 ml, injected in ~4 min. Percentage intact MTX determined by enzyme assay (see "Materials and Methods"), except as otherwise noted. The values determined by the enzyme assay represent the results from a single animal at the different dose levels indicated and are the average of 3 separate determinations on the same sample. These values did not differ by more than 10%. Data are for cynomolgus monkeys except where specified otherwise at the top of the columns.

<table>
<thead>
<tr>
<th>Time period over which sample was taken (min)</th>
<th>Single injection</th>
<th>Continuous infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80 mg/sq m</td>
<td>210 mg/sq m (rhesus)</td>
</tr>
<tr>
<td>10-20</td>
<td>114</td>
<td>90*</td>
</tr>
<tr>
<td>20-30</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>10-30</td>
<td>125</td>
<td>90*</td>
</tr>
<tr>
<td>30-60</td>
<td>115</td>
<td>91*</td>
</tr>
<tr>
<td>50-60</td>
<td>103</td>
<td>92*</td>
</tr>
<tr>
<td>70-100</td>
<td>52</td>
<td>93*</td>
</tr>
<tr>
<td>100-120</td>
<td>92</td>
<td>94*</td>
</tr>
<tr>
<td>120-150</td>
<td>40</td>
<td>31</td>
</tr>
<tr>
<td>150-180</td>
<td>91</td>
<td>64</td>
</tr>
<tr>
<td>180-210</td>
<td>36</td>
<td>63</td>
</tr>
<tr>
<td>210-240</td>
<td>54</td>
<td>35*</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

| % intact MTX                              |                  |
| % total dose excreted in 4 hr             |                  |
| volume of urine in 4 hr (ml)              |                  |

* Column chromatography.

were found in a late peak for the 10- to 30- and 210- to 240-min samples, respectively. This peak occurred at a position similar to that of the late peak found in the plasma (see Chart 4 and recent work in Ref. 11). The remaining non-MTX radioactivity was found in several very small early peaks.

Table 2 shows representative data from Table 1 in terms of percentage of dose excreted during the various collection periods. It can be seen that MTX products represent an average of 3.6 to 8.5% of the 3H radioactivity excreted in the urine during the selected time periods measured between 10 and 240 min.

The urine of the rhesus monkey given the single injection, in which 80% of the radioactivity at 240 min was not associated with intact MTX, as measured by the dihydrofolate reductase assay, was also chromatographed. Twenty-seven % of the radioactivity was found to be present in the MTX peak, in reasonable agreement with the 20% determined by dihydrofolate reductase assay, and 66% was found in a later eluting peak. This is similar to the recent results of Jacobs et al. (11). All the radioactivity in the cat urine was associated with intact MTX as indicated by the dihydrofolate reductase assay, which might be expected from the lack of breakdown found in the plasma.

Integrity of MTX in CSF. Significant 3H radioactivity can be detected in CSF after i.v. injection, which after 4 hr approximates the concentration of non-protein-bound MTX (~50% total [3]) in plasma after both single and continuous i.v. infusion of [3H]MTX. Because of the considerable breakdown found in plasma, however, we considered that this might well not represent intact MTX. As shown in Table 3, only 2% or less of the 3H radioactivity found in the CSF after continuous i.v. infusion in fact represents intact MTX, and this was not significantly affected by pretreatment with probenecid. As shown in Chart 6 (top), DEAE column chromatography performed on these samples showed that 14% of the non-MTX radioactivity eluted in the 1st fraction when the gradient was started (see "Materials and Methods") and 85% eluted in the 2nd fraction. A small percentage, estimated at ~3% of the radioactivity, was found in the usual position of the MTX peak in agreement with the results from the reductase assay shown in Table 3.

In contrast, as shown in Table 3, if MTX is injected directly into the CSF via the left lateral ventricle (see "Materials and Methods"), no breakdown of the MTX was detected by the
MTX and MTX products in urine as a percentage of total dose injected at selected times

Data from selected experiments shown in Table 1. Column 1 for each dose refers to the percentage of the total dose in terms of \(^3\)H radioactivity excreted for the time period specified. Column 2 refers to the percentage of the dose excreted in the corresponding time period, which represents non-MTX.

<table>
<thead>
<tr>
<th>Time sample taken (min)</th>
<th>(^3)H-MTX</th>
<th>% as non-MTX</th>
<th>% as non-MTX</th>
<th>% as non-MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^3)H product</td>
<td>% as non-MTX product</td>
<td>% as non-MTX product</td>
<td>% as non-MTX product</td>
</tr>
<tr>
<td>0-10</td>
<td>3.1</td>
<td>10.9</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>10-20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-30</td>
<td>0.7</td>
<td>8.4</td>
<td>7</td>
<td>0.7</td>
</tr>
<tr>
<td>30-60</td>
<td>33.7</td>
<td>10.7</td>
<td>28</td>
<td>14.0</td>
</tr>
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<td>60-90</td>
<td>9.7</td>
<td>4.9</td>
<td></td>
<td>8.2</td>
</tr>
<tr>
<td>90-120</td>
<td>3.5</td>
<td>4.2</td>
<td>57</td>
<td>1.9</td>
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<td>120-150</td>
<td>1.8</td>
<td>1.8</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>150-180</td>
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</tr>
<tr>
<td>180-210</td>
<td>1.1</td>
<td>2.5</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>210-240</td>
<td>1.9</td>
<td>1.0</td>
<td>65</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Total \(^3\)HMTX excreted as % injected dose

% total injected dose excreted for sample times when MTX was also assayed for chemical integrity

Table 3

**Integrity of MTX in CSF after i.v. and intraventricular injection**

Percentage of counts corresponding to intact MTX represent the average of not less than 3 determinations by the enzyme assay, and each value did not differ by more than 10%. The DEAE column result was a single determination. All i.v. injections were continuous infusions. Intraventricular injection was into the left lateral ventricle as described in "Materials and Methods." Results are from a single animal in which a cisternal needle was present throughout the experiment except for the 2nd values at 70 and 250 min, when the sample was taken only at the end of the experiment.

<table>
<thead>
<tr>
<th>Mode of injection</th>
<th>Dose (mg/sq m)</th>
<th>Assay method</th>
<th>Time (min)</th>
<th>ml based on cpm</th>
<th>(^\mu)g/</th>
<th>% as intact MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>210</td>
<td>DEAE column</td>
<td>240</td>
<td>2.82</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>210</td>
<td>Enzyme assay</td>
<td>240</td>
<td>3.88</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>210(^a)</td>
<td>Enzyme assay</td>
<td>240</td>
<td>3.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Intraventricular</td>
<td>7.2</td>
<td>Enzyme assay</td>
<td>30</td>
<td>590</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Intraventricular</td>
<td>7.2</td>
<td>Enzyme assay</td>
<td>70</td>
<td>174</td>
<td>105</td>
<td></td>
</tr>
<tr>
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<td>7.2</td>
<td>Enzyme assay</td>
<td>250</td>
<td>10.3</td>
<td>77</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Plus probenecid.

\(^b\) Second values; sample taken at end of experiment.

Integrity of MTX in Tissues. It was of interest to see whether the MTX taken up by tissues reflected the same breakdown found for MTX in the plasma at 4 hr, or whether a greater or lesser proportion of breakdown products was taken up. Table 4 shows the percentage uptake of \(^3\)HMTX by a number of tissues based on radioactivity assuming no breakdown, and the amount of recovered \(^3\)HMTX (51 to 97%) that represented intact MTX, after a single i.v. injection. It is clear that there is also considerable breakdown of the MTX taken up by the tissues, but this is variable. Both liver and spleen show considerable breakdown, but bone marrow and especially kidney show less. In the latter case this may partially represent intact MTX present in the tubular lumen. The total liver and spleen weight has been found...
to be 83.5 and 12.3 g, respectively (14). On this basis the ³H radioactivity in liver and spleen represent 2.33 and 0.06% of the total injected dose, respectively, after a dose of 210 mg/sq m. The 26% intact MTX that we obtained for the kidney after 4 hr is very close to the 23% intact MTX also recently reported for the kidney after 24 hr (11).

In sharp contrast the 70% of the MTX recovered from the brain after intraventricular injection shows essentially no breakdown, consistent with the lack of significant breakdown found in the CSF after intraventricular injection (see Table 3).

**DISCUSSION**

The appearance of non-MTX products in the plasma after i.v. injection of [³H]MTX in both cynomolgus (M. fascicularis) and rhesus (M. mulatta) monkeys was unexpected in view of previous reports that only intact MTX was present in the urine of a number of animals, including rhesus monkeys, after i.v. injection (8). Our studies also showed the presence of non-MTX products in the urine. The percentage of MTX products in the urine, however, was very variable but always lower than the proportion of MTX products seen in the plasma for corresponding time periods. Over the 4-hr period studied, an average of 4 to 9% of the excreted dose in the urine were non-MTX products (Table 2) after single injections. During the last 210- to 240-min time period, this proportion was higher (37 to 80%) (Table 1). In the plasma and tissues we studied (see Table 4), an average of 8.9% of the total radioactivity derived from the [³H]MTX left in the body represented intact MTX 4 hr after a single i.v. injection.

The amount of MTX breakdown products in the urine seemed to be proportional to the amount of MTX excreted as indicated by the data in Table 1. Excretion of non-MTX products is also suggested by the data in Chart 1 in which the plasma level of non-MTX products begins to fall after 60 min, reflecting the dominance of removal processes over the production of breakdown products from the declining level of intact MTX. The fact that previous workers did not detect any MTX products in the urine of rhesus monkeys is possibly due to the very low doses of MTX (0.3 mg/kg i.v.) used in previous studies (8). We have used doses in the range of 80 to 410 mg/sq m, which translates to 6.5 to 39.2 mg/kg for a 3-kg monkey. Moderate MTX doses of 30 mg/sq m and 80 mg/sq m (9, 24), and higher doses of > 240 mg/sq m (15), 50 to 200 mg/kg (22), and up to 600 mg/kg with citrovorum rescue (5) are now being used clinically. If it is assumed that MTX is excreted in the urine by a transport mechanism, for which there is some evidence in the rhesus monkey (3), perhaps involving an organic acid transport system (16), then it is quite possible that this system has a higher affinity for MTX than at least some of its metabolic products. This would result in some degree of selective excretion of MTX, which would fit with our finding of consistently more breakdown in plasma than in urine and greater appearance of MTX products when larger percentages of the initial dose are excreted. The very low proportion of MTX products seen in the urine after 4 hr with continuous i.v. infusion of MTX in the face of a considerable proportion of MTX products in the plasma (80 to 90%) at the same time period (see Chart 5), could also be explained by a renal transport system with a higher affinity for intact MTX. By comparing Charts 4 and 6, it can be seen that it is the earlier eluting products that are absent from the urine.

A report in partial agreement with our results on urine appeared while this manuscript was in preparation. These studies showed, using DEAE-cellulose column chromatography, the appearance of a considerable amount of a later and also an earlier peak in the urine of both rhesus monkeys and human patients after i.v. doses of MTX, 200 mg/kg (11). These authors identified the slow-moving component as 7-hydroxymethotrexate, which is known to be formed from the action of hepatic metalloflavoprotein aldehyde oxidase in the rabbit. It is possible that the slower moving component in our studies, found in both plasma and urine in Fractions 60 to 75, is also the same compound, since it appeared at approximately the same point. In the absence of corroborative chemical analysis, however, we are unable to definitely identify it. Assuming that the major route of MTX metabolism was through the hepatic aldehyde oxidase, it was suggested (11) that high-dose MTX (200 mg/kg) would exceed the $K_m$ for the enzyme, while the lower doses
(0.3 mg/kg) previously used might not. Since we found the same proportion of MTX products at 80 mg/sq m (6.5 mg/kg, as at higher doses, it appears to be saturated at doses at least as low as 6.5 mg/kg. Therefore elucidation of the concentration dependence of MTX metabolism would require further studies of the dose dependency at MTX doses <6 mg/kg in the cynomolgus monkey.

Apart from the recent study mentioned above showing the appearance of metabolites in human urine after high doses of MTX (11), there have been a few earlier studies indicating the appearance of breakdown products in patients, although detailed clinical use of [3H]MTX was clearly more limited. An earlier eluting component had been noted after DEAE chromatography of the urine of a few patients after i.v. injection of [3H]MTX at 30 mg/sq m (9, 24). Pharmacokinetic studies on the plasma and urine of several patients after i.v. injection of 80 and, in some cases, 1800 mg/sq m suggested the existence of a plasma component that did not behave kinetically as a source of the [3H]MTX in the urine, and it was suggested that this component might be a byproduct (15). Thus, there is a small body of evidence suggesting the existence of what might be considerable metabolism of MTX in man.

In experimental animals other than the rhesus monkey (11), there has been 1 report of 50% of the [3H]MTX present in rat plasma 6 hr after i.v. injection, not eluting with intact MTX by a paper chromatography method (13). Considerable breakdown of MTX has also been reported, especially in the rabbit, due to hepatic aldehyde oxidase activity as mentioned above (12). A number of other reports have shown breakdown products in the urine of several species 3 to 12 hr after i.p. injection, but these were considered to be principally due to metabolism by intestinal bacteria (19, 23). The relatively rapid breakdown of MTX found in the present study after i.v. injection suggests that this bacterial pathway should be minimal during the time period of the experiments.

This study shows that the proportion of non-MTX products found in the plasma is considerably greater than that found in the urine during the latter part of the experimental time period and indeed can occur when only a minimal amount of MTX products are detected in the urine. This suggests a certain degree of selective excretion of intact MTX as discussed above. It is not clear whether these breakdown products have any significant toxicity or whether they result from detoxification mechanisms, as might be inferred if they are largely a result of hydroxylation by liver enzymes. It has been suggested, however, that accumulation of the relatively insoluble 7-hydroxy derivative and MTX itself might cause renal toxicity (11, 22). The accumulation of exclusively MTX breakdown products in the CNS after i.v. injection in a normal animal, as well as the fact that they seem rapidly to form such a large proportion of the residual dose in the plasma, suggests a need for examining the possibility of their toxicity. The non-MTX radioactivity eluting in the 1st few fractions could have been titrated water (H2O). The fact that the radioactivity in the early peaks did not appear in Fraction 0 but mainly in the 2nd and 3rd fraction after the gradient was started in Fraction 1 for either CSF or plasma suggests that it was not H2O. Free diffusion of H2O might be expected to result in its appearance in early fractions after the sample entered the column, starting with Fraction 0. Also H2O should equilibrate throughout the body water, and no significant amount of the early peak was found in the urine (see Chart 6). Finally, [3H] exchange alone would produce nonradioactive MTX which, if present in sufficient amounts, would lead to a discrepancy between the values for intact MTX obtained from the dihydrofolate reductase assay and DEAE column chromatography.

The absence of MTX products in the CNS after intraventricular injection as distinct from the 95% non-MTX products found in CSF after i.v. injection reinforces the necessity of this mode of treatment for CNS cancer. However, it also emphasizes the necessity of further refining the lumbar i.t. and intraventricular modes of injection presently in use (2, 20), since they sometimes result in severe neurological deficits (2, 17). If general toxicity after i.v. injection is not due simply to MTX but to some combination of MTX and its metabolic products, then the elevation of intact MTX levels by probenecid may have some therapeutic use. In addition, we have recently shown that microencapsulation of MTX inside liposomes (phospholipid vesicles) markedly increases the plasma levels of the drug, possibly by inhibiting its renal excretion, and completely prevents the metabolic breakdown of encapsulated MTX while in the plasma (14).

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Distribution and Degradation of $[\text{H}]$Methotrexate after Intravenous and Cerebral Intraventricular Injection in Primates

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