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DIURNAL VARIATION OF METHOTREXATE TRANSPORT AND ACCUMULATION IN HEPATOCYTES—A CONSEQUENCE OF VARIATIONS IN CELLULAR GLUTATHIONE*

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Abstract—Diurnal variation of the methotrexate (MTX) initial influx and net uptake in isolated rat liver cells was studied in dependence on diurnal variation of cellular glutathione. It was found that the most significant differences concerning the MTX initial transport and accumulation were observed between hepatocytes prepared at 1200 hr when cellular glutathione reached its maximum, and those isolated at 0000 hr when liver glutathione had its minimal concentration. The initial influx of MTX was the biggest in cells isolated at 0000 hr and the smallest in cells prepared at 1200 hr. The K_m values in cells with low cellular glutathione (at 1800 and 0000 hr) were about three times smaller than in cells with high glutathione (at 0700 and at 1200 hr), whereas the V_{max} value remained unchanged. Titration of external membrane SH groups by [^{203}Hg]*p*-CMBS revealed a much larger amount of free SH groups on cells having low glutathione level than on those with high glutathione. Despite big initial influx of MTX found in cells with low cellular glutathione, net accumulation of MTX was significantly smaller in these cells as compared with hepatocytes having high glutathione level. In conclusion, the present studies confirmed a statement of the preceding paper on the important role of cellular glutathione played in methotrexate transport and accumulation in rat liver cells.

The preceding paper [1] described the role of cellular glutathione on influx and accumulation of methotrexate (MTX)‡ in isolated liver cells. Since it has been reported [2, 3] that liver glutathione is subject to diurnal variation, it seemed very likely that MTX uptake by hepatocytes should also vary with day time, depending on the actual level of glutathione in the liver. On the other hand, the diurnal variability of the cure rate and toxicity of some cytotoxic drugs have already been observed by clinicists and in animal experiments [4–10]. English *et al.* [10] reported a diurnal rhythmicity in the toxicity of methotrexate in rats, but the reason for these variations could not be explained.

In view of the above, we found it interesting and of practical importance to examine diurnal variation in methotrexate transport and accumulation in isolated hepatocytes.

MATERIALS AND METHODS

For experiments, female Wistar rats of 150–180 g body wt were used. The animals were housed under standard laboratory conditions, and a 12-hr light (0600–1800 hr) and 12-hr darkness (1800–0600 hr) constant cycle was maintained. Standard laboratory diet and water was given *ad libitum*.

The preparations of liver cells were carried out at different times of day and night, namely: 0700, 1200, 1800, and 0000 hr. The investigations of initial influx and net accumulation of [^{3}H]MTX in isolated hepatocytes, and determination of free membrane SH groups titrable by [^{203}Hg]*p*-CMBS were performed. Additionally, the intracellular glutathione as well as cellular Na^+ and K^+ were examined in cells from each preparation. All methods used were identical to those in a preceding paper [1]. [^{3}H]MTX was purified as described previously [1].

RESULTS

Table 1 demonstrates levels of cellular glutathione and concentrations of K^+ and Na^+ found in cells prepared at different times of day and night. As is shown, cellular glutathione was found to have the highest level at 1200 hr and the lowest one at 0000 hr. Hepatocytes from the preparation at 0000 hr had also the lowest cellular Na^+/K^+ ratio, contrary to cells prepared at 1200 hr, which showed the highest Na^+/K^+ ratio.

The initial influx of MTX (measured during the first min of cell incubation with [^{3}H]MTX) was the biggest in hepatocytes isolated at 0000 hr and the smallest in cells prepared at 1200 hr (Fig. 1). The double-reciprocal Lineweaver–Burk plots of MTX influx (Fig. 2) indicate that the values of K_m varied

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‡ Abbreviations used: GSH, (reduced) glutathione; MTX, methotrexate (amethopterin); *p*-CMBS, *p*-chloromercuribenzene sulfonate.

Table 1. Diurnal variation of cellular glutathione, Na^+ and K^+ in isolated hepatocytes of fed rats

Time of cell preparation	Cellular glutathione (mM)	Cellular K^+ (mM)	Cellular Na^+ (mM)	Na^+/K^+	Number of experiments
0700 hr	3.8 ± 0.55*	85.6 ± 8.2	45.2 ± 5.6	0.53	8
1200 hr	4.5 ± 0.68	82.2 ± 7.8	40.6 ± 5.3	0.49	4
1800 hr	2.5 ± 0.75	90.5 ± 7.5	35.5 ± 4.2	0.39	3
0000 hr	2.0 ± 0.70	100.6 ± 8.2	35.3 ± 3.6	0.35	4

* ± S.E.

Statistical significances: Cellular glutathione: $P_{1200/0700hr} > 0.05$, $P_{1800/1200hr} < 0.05$, $P_{0000/1800hr} > 0.05$, $P_{0000/1200hr} < 0.02$ cellular K^+ and cellular Na^+: $P > 0.05$ (in all cases).

in hepatocytes depending on the time of cell preparations from 4.7 μM at 0700 hr to 1.7 μM at 0000 hr. On the contrary, the V_{max} value remained unchanged and was 5.11 pmoles/min/mg protein in all cells studied.

The investigation of net accumulation of MTX within cells revealed that the amount of drug found in cells during 10 min incubations with 1 μM and with 10 μM MTX also differed in dependence on time of cell preparation. However, contrary to the initial influx, the biggest amount of methotrexate accumulated in cells was found in hepatocytes prepared at 1200 hr and the smallest one after preparation at 0000 hr (Fig. 3). Addition of 5 mM GSH to cell incubate caused a nearly two-fold increase of MTX accumulation within the cells prepared at 0700 and 1200 hr and only relatively small increases in MTX accumulated in hepatocytes prepared at 1800 and 0000 hr (about 30%, respectively) (Fig. 3). The experiments on titration of membrane free SH groups by [^{203}Hg]*p*-CMBS brought out significant variations in the amount of titrable SH groups depending on the time of cell preparation (Fig. 4). Namely, cells prepared at midnight, as well as those prepared at 1800 hr exposed about 3 times more membrane SH groups than cells isolated at 1200 or 0700 hr. It should be pointed out that increase in titrable membrane SH groups and increase in cellular K^+ (Table 1) are in parallel. The pretreatment of cells with 5 mM GSH caused very strong increases in the amount of titrable membrane SH groups in hepatocytes prepared at 0700 and 1200 hr, whereas in cells isolated at 1800 and 0000 hr a significant effect of exogenous GSH could not be shown (Fig. 4).

DISCUSSION

The results of the present paper are consistent with reports of other authors, that liver glutathione is subject to diurnal variation [2, 3]. In our experiments, the highest level of cellular glutathione was

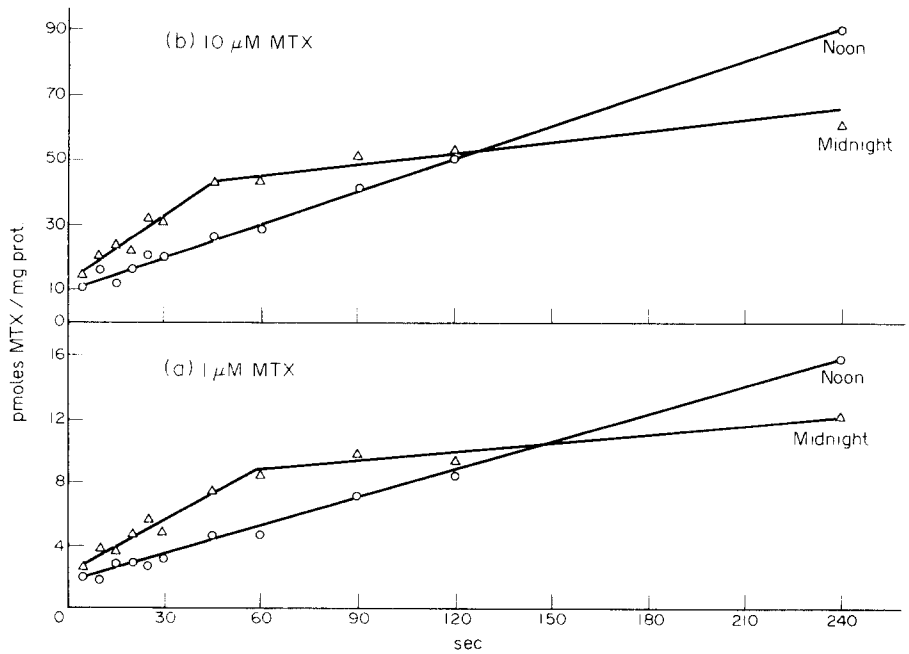

Fig. 1. Time course of initial uptake of [^{3}H]MTX in hepatocytes prepared at 1200 hr (○—○) and at 0000 hr (△—△) over the first 240 sec after addition of either 1 μM MTX, or 10 μM MTX. The incubations were carried out always at 37°. Before MTX addition, cells were preincubated for 10 min.

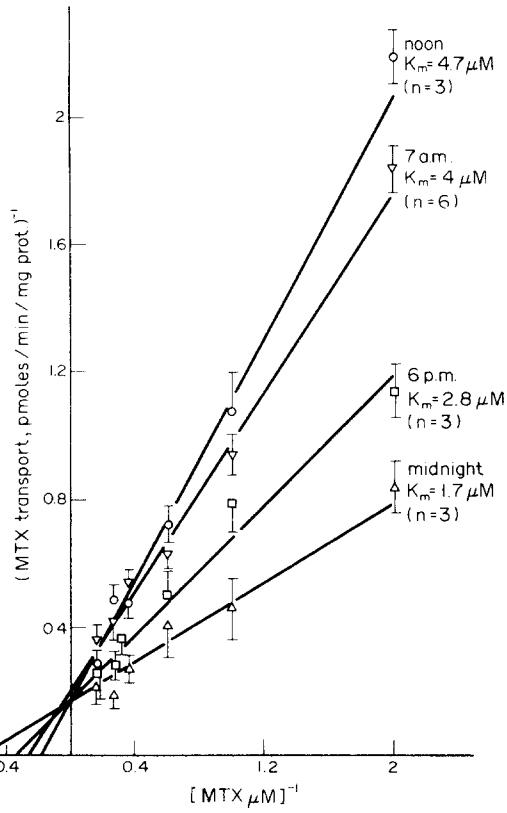

Fig. 2. Double-reciprocal plots of the MTX initial transport rate as a function of MTX concentration in hepatocytes prepared at various times: at 0700 hr (▽—▽), at 1200 hr (○—○), at 1800 hr (□—□), and at 0000 hr (△—△). Mean ± S.E., N = number of experiments.

observed between 0700 and 1200 hr, with the maximum reached at 1200 hr. Subsequently, the liver glutathione level fell down to reach the minimum at 0000 hr. It has been clearly demonstrated that methotrexate initial influx and net accumulation were also subject to diurnal variation and were closely dependent on the level of cellular glutathione. Thus, the most significant differences concerning the MTX initial influx and accumulation were observed between cells prepared at 1200 hr when cellular glutathione reached its maximum, and hepatocytes isolated at 0000 hr, when liver glutathione had its minimal concentration. The initial transport of MTX to hepatocytes was much faster in cells having low glutathione level than in cells with high concentrations of endogenous glutathione. The K_m value of MTX transport in hepatocytes with maximum glutathione level (at 1200 hr) was about 3 times larger than in cells having minimal level of cellular glutathione (at 0000 hr). This finding indicates that in the latter cells the efficiency of methotrexate transport was increased. On the other hand, the cells with low level of cellular glutathione exposed much more free membrane SH groups than cells with high cellular glutathione level (Fig. 4). As in the preceding paper, increase in titrable membrane SH groups and increase in cellular K^+ (Table 1) are in parallel. The present results are also very similar to those described in the preceding paper concerning the differences between initial influx of MTX in cells from fed rats and cells with experimentally diminished cellular glutathione (by starvation and by treatment of rats with phorone). Likewise, the changes in net accumulation of MTX in cells with high and low glutathione level are identical to those found in the preceding studies between cells of fed

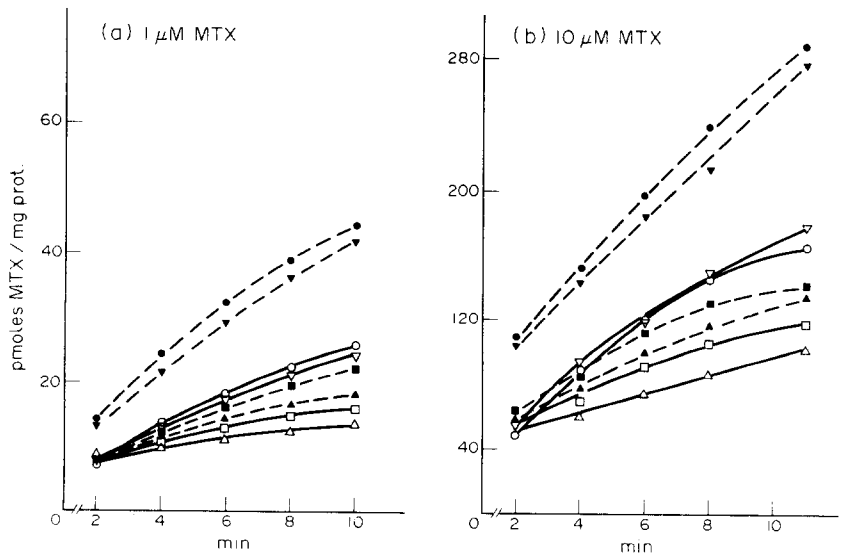

Fig. 3. Diurnal variation of methotrexate accumulation in hepatocytes over 10 min of exposure of cells to either 1 μM MTX (a) or to 10 μM MTX (b). Cells were preincubated for 10 min prior to MTX addition either without any further additions (——) or with 5 mM GSH (– –). (▽), hepatocytes prepared at 0700 hr; (○), hepatocytes prepared at 1200 hr; (□), hepatocytes prepared at 1800 hr; (△), hepatocytes prepared at 0000 hr.

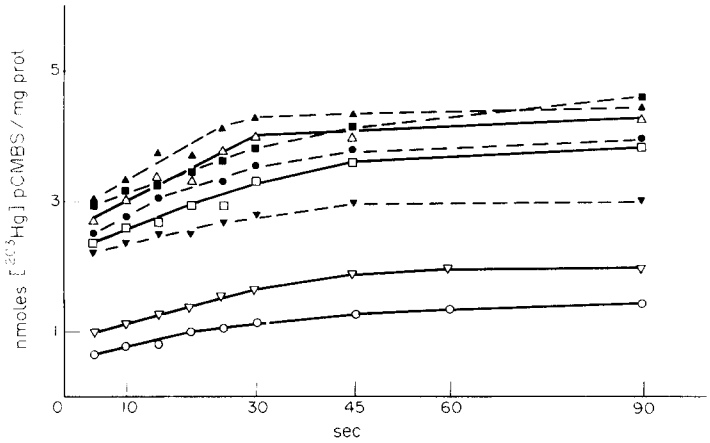

Fig. 4. Time course of titration by [^{203}Hg]*p*-CMBS (0.1 mM) of membrane SH groups in liver cells prepared at various times: at 0700 hr (▽), at 1200 (○), at 1800 hr (□) and at 0000 hr (△). ——, cells preincubated for 10 min without any additions, then titration started, – – –, cells preincubated with 5 mM GSH, then washed, resuspended in fresh medium and *p*-CMBS was added.

rats and cells with experimentally diminished cellular glutathione. Namely, much bigger amounts of MTX were accumulated in hepatocytes with high cellular glutathione than in cells having low glutathione level.

In view of the above, we can conclude that the present studies confirmed a statement of the preceding paper [1] on the important role of cellular glutathione played in methotrexate transport and accumulation in rat liver cells. On the other hand, the diurnal variation of methotrexate accumulation in liver cells, demonstrated in the present paper can have important practical implications concerning the cancer therapy with methotrexate. It has already been reported [10] that MTX toxicity is subjected to diurnal variation and maximum toxicity occurred after MTX administration at 0600 hr, whereas the minimum toxicity was found at 0000 hr. Our results showed that at 0000 hr MTX accumulation in the liver cells reached its minimum whereas in the morning the amount of MTX accumulated in cells is significantly increased. Thus, the diurnal variation of MTX accumulation in the liver cells, being a consequence of variations of cellular glutathione level, can be a reason of diurnal variation of anticancer efficiency of methotrexate and its toxicity.

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