Biochemical Pharmacology, Vol. 34, No. 10, pp. 1627–1634, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

EFFECT OF DEPLETION OF CELLULAR GLUTATHIONE ON METHOTREXATE INFLUX, EFFLUX AND RETENTION IN HEPATOCYTES*

ANNA LESZCZYŃSKA-BISSWANGER and ERICH PFAFF†
Institut für Toxikologie der Universität Tübingen, Wilhelmstr. 56, D-7400 Tübingen, Federal Republic of Germany

(*Received* 16 *July* 1984; *accepted* 15 *October* 1984)

Abstract—In isolated hepatocytes the influence of cellular glutathione (GSH) on initial influx, net uptake and efflux of methotrexate (MTX) was determined. Endogenous glutathione in rat liver cells was depleted by either fasting of rats or by *in vivo* administration of phorone prior to cell preparation.

The initial rate of influx of MTX was found to be higher in hepatocytes of fasted and phorone-treated rats than in those of untreated, fed control rats. The K_m values for the methotrexate influx in GSH-deficient hepatocytes were up to 3 times lower than in normal cells, whereas V_{max} remained unchanged. These results disclose an increased efficiency of the MTX transport system in cells with diminished cellular GSH levels. On the other hand, titration of external membrane SH groups by ^{203}Hg *p*-CMBS revealed up to three times higher amounts of free SH groups on cells from starved and phorone-treated rats than on hepatocytes of fed rats. Increased efficiency of the MTX transport system in GSH-deficient cells may, therefore, be interpreted as increased capacity of the MTX transport carrier for which free membrane SH groups are known to be essential.

Despite activation of initial transport of MTX here, later net accumulation of MTX became smaller than in cells with normal GSH levels. Efflux of MTX from liver cells was not influenced by fasting or phorone treatment of rats, however, the "nonexchangeable" pool of MTX was found to be decreased, which indicates inhibition of formation of MTX polyglutamates here. This inhibition was most likely responsible for the decreased amounts of MTX finally accumulated in GSH-deficient hepatocytes.

In previous studies [1] it has been demonstrated that methotrexate (MTX)‡ uptake in rat hepatocytes can be activated by addition of exogenous glutathione. This effect was interpreted as an alteration by GSH of the membrane redox state, e.g. by reaction with protein disulfide groups of the cell membrane releasing free SH groups, obligatory for the functioning of MTX carrier system [2–5].

It has been reported [6, 7] that endogenous glutathione plays an important role in the regulation of the redox state of membrane proteins. On this account, it might be expected that changes in intracellular glutathione can also influence the methotrexate transport across the cell membrane. It has already been demonstrated [8–11] that depletion of cellular GSH causes inhibition of transport of some amino acids and α-amino-D-glucoside in rat kidney. However, nothing is known about the role of endogenous GSH in transport processes in liver cells.

The simplest, physiological way to modify GSH levels in liver is starvation. Thus hepatic GSH is significantly diminished during starvation, depending on its duration [12, 13]. On the other hand, GSH in liver can be more efficiently depleted chemically by the use of agents such as phorone, which conjugates with GSH via the glutathione transferases reaction [14]. Using adequate dosing of phorone *in vivo* an 80–90% decrease in liver GSH may be achieved.

In the present paper we used both methods for GSH depletion to investigate methotrexate transport and accumulation in isolated liver cells under the condition of cellular GSH deficiency.

MATERIALS AND METHODS

Animals. Female rats of 150–180 g body wt were used. The animals were divided into three groups which were treated differently: Prior to preparation of liver cells, the first group was fed with standard laboratory diet *ad libitum*, whereas the second group had been fasted for 24–28 hr. The third group had also been kept on normal diet *ad libitum*, but the animals were treated 2 hr before cell preparation either with 250 mg/kg phorone in 20% ethanol i.p. or, for control, with 20% ethanol i.p. only. All animals had free access to tap water. The cell preparation was performed always at the same time, between 0700 and 0800 hr.

Materials. The following substances were purchased: (a) Radioactive compounds: [3′,5′,7′-^{3}H]methotrexate, sodium salt (MTX; 39.6 mCi/g) and *p*-chloro[^{203}Hg] mercuribenzene sulfonate from Amersham Buchler (Braunschweig, West Germany); ^3H_2O (1 mCi/ml) and [carboxyl-^{14}C] dextran (M_T 70,000; 1.4 mCi/g) from NEN Chemicals GmbH (Dreieich, West Germany); (b) unlabelled

* Supported by the Deutsche Forschungsgemeinschaft.
† To whom reprint requests should be addressed.
‡ Abbreviations used: GSH, (reduced) glutathione; MTX, methotrexate (amethopterin); *p*-CMBS, *p*-chloromercuribenzene sulfonate.

compounds: reduced glutathione (GSH) from Sigma Chemicals GmbH (München, West Germany), NADPH, 5,5′-dithiobis-(2-nitrobenzoate), and collagenase and other biochemicals from E. Boehringer (Mannheim, West Germany), silicone oils from Wacker Chemie (München, West Germany). Amethopterin (MTX), was a generous gift from Bristol Arzneimittel (Bergisch-Gladbach, West Germany) and phorone (2,6-dimethyl-2,5-heptadien-4-one) from Prof. A. Wendel (Institute of Physiological Chemistry, University of Tübingen, West Germany).

Methods. Isolation of liver cells, their incubation and separation from incubation medium by centrifugal filtration as well as determination of intracellular water and dextran space were carried out as described previously [1]. The incubation medium consisted of 137 mM NaCl, 5 mM KCl, 0.9 mM $MgSO_4$, 1 mM $CaCl_2$ buffered with 3 mM phosphate (made up of 30 parts KH_2PO_4 and 92 parts Na_2HPO_4) and 20 mM morpholino-3-propanesulfonic acid adjusted with Tris base to pH 7.3. Throughout, incubates contained 1.2–1.4 mg cellular protein/ml (corresponding to about 2×10^6 cells/ml). The following types of experiments were performed:

(1) Measurement of net accumulation, influx, efflux, and exchangeable and nonexchangeable pools of MTX.

(a) After MTX or MTX + GSH additions to the incubate, initial uptake of MTX into the cells was measured over the time span 5–120 sec by withdrawing aliquots, initially at 5-sec intervals. Subsequent accumulation of MTX within the cells was followed by sampling every 2 min over the next 8 min of incubation. Analysis of MTX transport was performed for the concentration range 0.5–100 μM. A preincubation of 10 min always preceded addition of [^{3}H]-MTX (3–400,000 dpm/ml) to the incubates.

(b) For efflux studies, cells were incubated with 1 μM [^{3}H]MTX for 30 min, before they were washed and resuspended in MTX-free medium to the original volume [15]. Thereafter, samples were taken from the cell suspension for analysis of the efflux process over the time span 1–60 min. The influence of exogenous GSH on MTX efflux from hepatocytes was examined under two experimental conditions: Either GSH was added and removed together with MTX or GSH was added after MTX had been washed out.

(c) Exchangeable and nonexchangeable components of intracellular [^{3}H]MTX were experimentally determined by diluting cells 20-fold after incubation with 1 μM [^{3}H]MTX between 1 and 20 min with MTX-free incubation medium as described by Gewirtz *et al.* [5]. In the first minutes intracellular MTX declined to a constant level. The amount of [^{3}H]MTX that left the cells was defined as "exchangeable fraction". The portion of MTX which remained within the hepatocytes was referred to as "nonexchangeable". Extrapolation of the nonexchangeable component (polyglutamate derivatives) to the time of dilution permits quantification of the exchangeable and nonexchangeable components at that time. This extrapolation also gives hints to the time course of MTX polyglutamate formation within the cells.

(2) Binding of [^{3}H]MTX to the external surface of liver cells was determined at 4° according to Henderson *et al.* [16]. Cell samples were taken for radioactivity detection over the time span 5–120 sec, initially every 5 sec.

(3) Titration of external membrane SH groups by *p*-CMBS. [^{203}Hg]*p*-CMBS (475–750,000 dpm/ml and about 0.16 mM) was added to incubates of liver cells applying two different procedures:

(a) after a 10 min preincubation of cells *p*-CMBS was added: no further additions.

(b) after a 5 min preincubation, GSH (5 mM) was added and incubation continued for 5 more min; thereafter, cells were washed, resuspended in fresh medium and *p*-CMBS was added. Samples were then taken over the time span from 5 to 120 sec, initially at 5-sec intervals between samples.

Intracellular Na^+ and K^+ concentrations were determined using flame photometry, as described previously [1]. The level of intracellular glutathione (GSH + GSSG) was measured by the catalytic assay with 5,5′-dithiobis-(2-nitrobenzoate) [17, 18].

MTX purification technique: [3′,5′,9-^{3}H]MTX was purified by thin layer SIL gel chromatography using as eluent system: *n*-butanol:methanol:triethylamin:water (in the ratio 7.5:6:1.5:1.5). This method was elaborated by Prof. Ursula Breyer-Pfaff from the Institute for Toxicology at the University of Tübingen.

RESULTS

Levels of endogenous total glutathione found in the various preparations of hepatocytes studied are listed in Table 1. It can be seen that fasting of rats for 24 hr, or treatment with phorone reduced intracellular glutathione by 50 and 70%, respect-

Table 1. Levels of cellular glutathione, K^+ and Na^+ in hepatocytes of fed, fasted and phorone-treated rats

Source of hepatocytes	Cellular glutathione [mM]	Cellular K^+ [mM]	Cellular Na^+ [mM]	Na^+/K^+	Number of experiments
Fed rats (control) (C)	3.8 ± 0.55*	85.6 ± 8.2	45.1 ± 5.6	0.53	8
Fasted rats (F)	1.6 ± 0.48	98.5 ± 8.3	32.6 ± 6.2	0.33	6
Phorone-treated rats (Ph)	0.9 ± 0.23	118.6 ± 9.5	32.3 ± 5.8	0.27	6

* Mean ± S.E.

Statistical significances: cellular glutathione: $P_{F,C} < 0.02$; $P_{Ph,C} < 0.005$; cellular K^+: $P_{F,C} = 0.05$, $P_{Ph,C} < 0.05$ $P_{F,Ph} > 0.05$; cellular Na^+: $P_{Ph,F,C} = 0.05$.

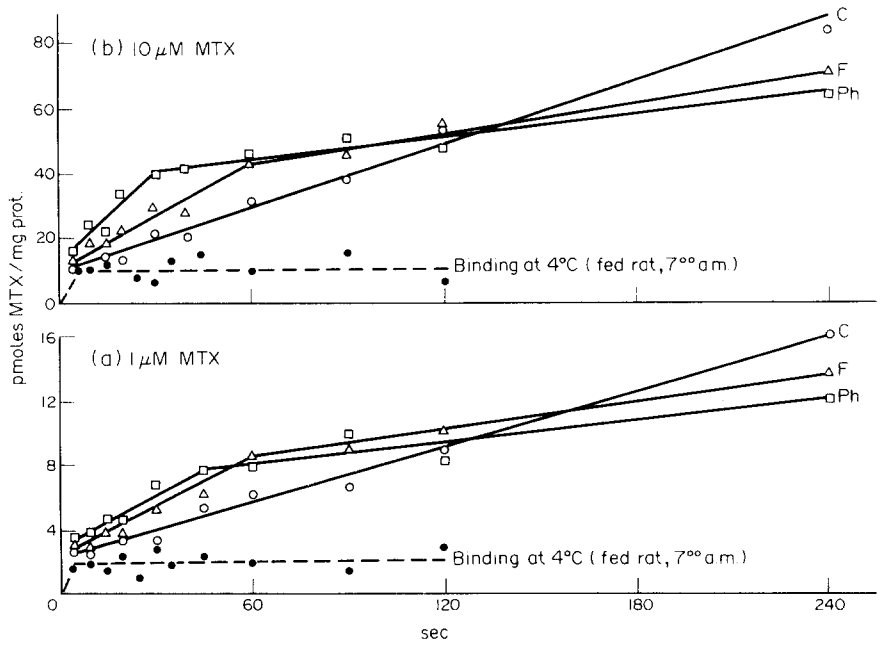

Fig. 1. Time course of initial uptake of [^{3}H]MTX over the first 240 sec after exposure of cells to either 1 μM MTX (a) or 10 μM MTX (b); hepatocytes of fed control (C), fasted (F) or phorone-treated (Ph) rats. The dashed-lines represent "nonspecific" binding (adsorption to the cell surface) of MTX, measured in hepatocytes of fed rats at 4°, instead of the 37° of the other incubations. Cells always were preincubated for 10 min before addition of [^{3}H]MTX.

ively, as compared to a control group of fed rats. The liver cells from fasted and from phorone-treated rats also showed markedly higher intracellular K^+ concentrations and, in consequence, much smaller Na^+/K^+ ratios (Table 1). The results with cells of animals having received an i.p. dose of 20% ethanol solution simultaneously with the phorone-treated rats did not differ from those of the control group of fed rats (not shown).

1. Investigation of initial and net fluxes of [^{3}H]MTX into the isolated liver cells

Analysis of MTX uptake by hepatocytes performed during the first 4 min of cell incubation with [^{3}H]MTX brought out distinct differences between cells of fed, fasted, and phorone-treated rats (Fig. 1). The initial influx of MTX (defined as uptake within the first 60 sec of incubation) into the cells of fasted and phorone-treated rats was found to be

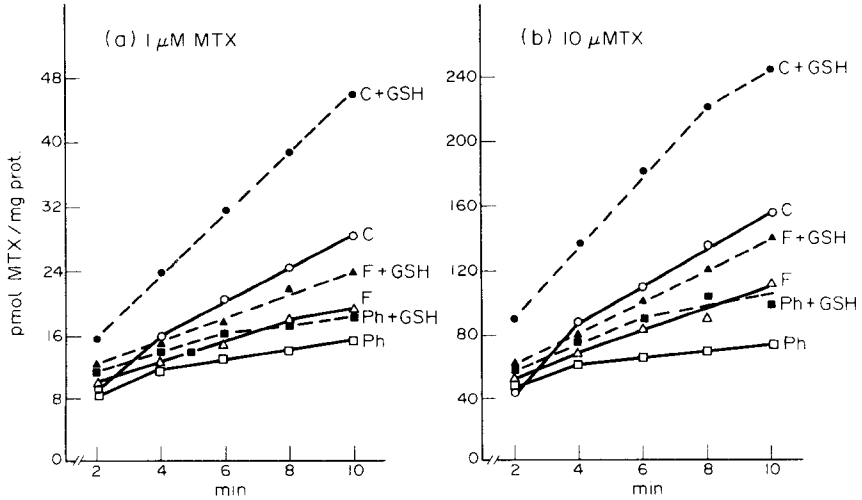

Fig. 2. Time course of [^{3}H]MTX accumulation over 10 min of exposure of cells to either 1 μM MTX (a) or 10 μM MTX (b) in hepatocytes of fed control (C), fasted (F) and phorone-treated (Ph) rats. MTX accumulation, no further addition = solid lines, and after preincubation of cells with 5 mM GSH (dashed lines).

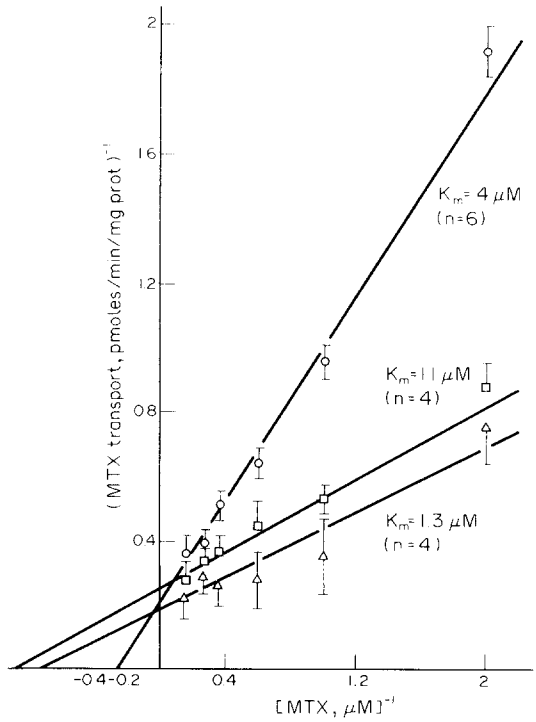

Fig. 3. Double-reciprocal plot of the MTX initial transport rate as a function of MTX concentration in hepatocytes of fed control, (○—○), fasted (△—△), or phorone-treated (□—□) rats. Mean ± S.E., N = number of experiments.

much bigger than in cells of fed rats. After this very rapid initial phase of MTX transport, however, a significant change of the kinetics of MTX uptake was observed in cells of fasted and of phorone-treated rats, namely an expressed deceleration of uptake. The lesser increase in total MTX in these cells resulted in a crossover of the traces of cellular MTX uptake at 2 min incubation time when the intracellular level of MTX became higher in cells of fed control rats. By 10 min of cell incubation this difference was very prominent: the total amount of MTX accumulated in hepatocytes was much higher in the cells of fed rats than in those of fasted and of phorone-treated rats (Fig. 2), in spite of the much faster initial influx of MTX into the latter cells. Addition of GSH (5 mM) activated MTX net uptake in all hepatocyte preparations from the differently treated groups of rats but this activation was more strongly expressed with those of fed rats (60% activation) than of fasted (25% activation) or of phorone-treated rats (20% activation), as shown in Fig. 2. Kinetic analysis of the parameters of initial transport of MTX into the cells revealed significant differences in K_m values between the hepatocytes of the fed and the two other groups studied. In fed rat hepatocytes K_m was 4.0 μM, whereas in cells of fasted and phorone-treated rats this value was 2.9–3.6 times lower (Fig. 3 and Table 2). On the other hand, the V_{max} value remained unchanged after fasting of rats and was only slightly lower (16%) after treatment of rats with phorone. Under the influence of exogenous GSH added to the incubation medium, strong reduction of the K_m to one fourth the original value was observed in fed rat hepatocytes, whereas in cells of fasted and of phorone-treated rats GSH caused only a small (18–20%) reduction in K_m values (Table 2).

Table 2. Changes in K_m [μM] values of MTX initial transport in hepatocytes of rats after various treatments; influence of glutathione addition

Rat treatment	GSH addition to the cell incubate		Number of experiments
	−	+	
Fed rats, no treatment	4.0	0.9	8
Rats fasted 24 hr	1.3	0.9	6
Fed rats, phorone administration	1.1	0.9	6

2. Examination of "nonspecific" binding of MTX

"Nonspecific" binding mainly represents adsorption to the cell surface and only to a small extent the possible specific binding to the transport protein for reduced folates. Binding studies were performed at 4°. As has been reported [16], at this low temperature transport processes are hardly detectable; thus the radioactivity found in the cell sediment under these conditions can be taken to represent [^{3}H]MTX nonspecifically bound to the cell membrane. As illustrated in Fig. 1, this binding was too fast to be kinetically resolved. This initial, rapid binding is part of the total MTX found in cell sediments centrifuged after the first 5 sec of incubation at 37°. It can be visualized here only graphically by the positive intercept on the y-axis, if the uptake slope is extrapolated back to the onset of incubation at zero time. Thus MTX adsorption to the cell surface always has to be taken into account as an essential part of the initial uptake phase.

3. MTX efflux and discrimination of "exchangeable" and "nonexchangeable" ^{3}H MTX pools

(a) *MTX efflux.* As is shown in Fig. 4(a), the kinetics of MTX efflux from liver cells of fed rats were in parallel to efflux kinetics from hepatocytes of fasted and of phorone-treated rats independent of differences in the absolute values for the total initial intracellular MTX or the asymptotically approached residual MTX content in cells. Thus it can be concluded that the efflux processes from liver cells were not influenced by neither fasting nor by treatment of rats with phorone. Likewise, no changes in MTX efflux kinetics from fed rat hepatocytes were found after GSH addition to the incubation medium, no matter when it had been added (Fig. 4(b)).

(b) *Detection of "exchangeable" and "nonexchangeable" fractions of [^{3}H]MTX in cells—influence of GSH addition.* The lower part of Fig. 5 illustrates the time course of MTX uptake and the appearance of "exchangeable" and "nonexchangeable" intracellular ^{3}H over 20 min after incubation of cells of fed rats with 1 μM [^{3}H]MTX. After 4 min of cell incubation with extracellular MTX, exchangeable MTX was detectable, and both, exchangeable and nonexchangeable intracellular ^{3}H components,

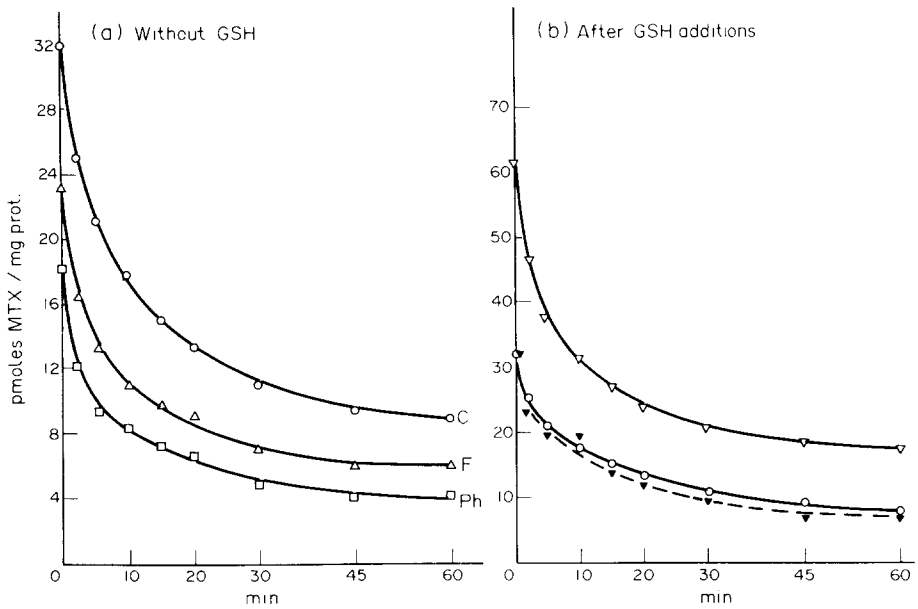

Fig. 4. (a) Time course of MTX efflux from hepatocytes of fed control (C), fasted (F) and phorone-treated (Ph) rats. Before measurement of efflux kinetics, cells were preincubated with 1 μM [^{3}H]MTX for 30 min, then washed and resuspended in MTX-free medium. (b) Influence of exogenous GSH on MTX efflux from hepatocytes of fed rats. Symbols used: in (a) as in fig. 3; in (b): ○—○, cells preincubated with MTX only, then washed and resuspended in MTX-free medium (identical to control showed in part a); ▽—▽, cells preincubated with MTX together with 5 mM GSH, then washed and resuspended in MTX-free medium; ▼—▼, cells preincubated with MTX alone, then washed and resuspended in MTX-free medium containing 5 mM GSH.

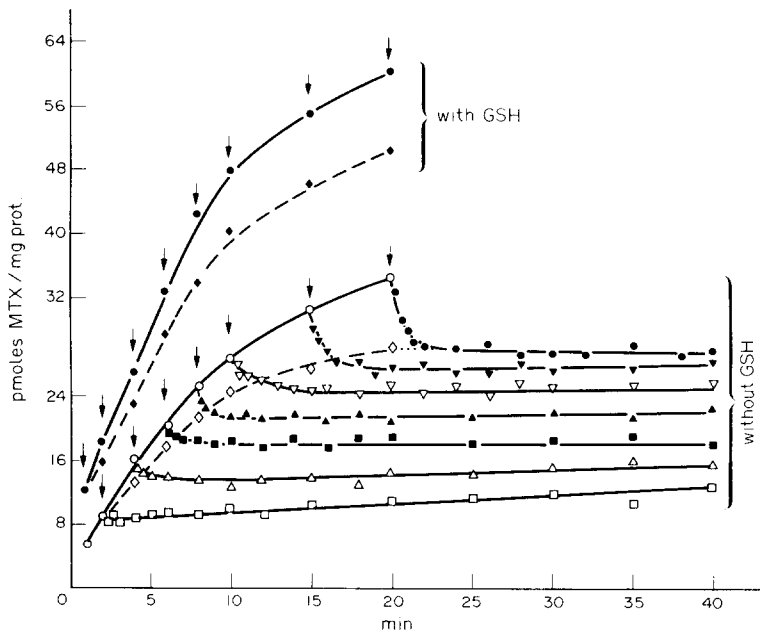

Fig. 5. Time course of uptake and determination of exchangeable and nonexchangeable [^{3}H]MTX in hepatocytes of fed rats over 20 min of cell incubation with 1 μM MTX either alone (lower part of the figure) or together with 5 mM GSH (upper part of the figure). Large symbols: ○—○, ●—●, time course of total uptake of MTX. At the arrows, portions of the cell suspension were transferred to separate flasks where extracellular MTX was diluted 20-fold with MTX-free medium and the time course of subsequent efflux of ^{3}H from cells was monitored. ····· Extrapolation of the nonexchangeable component to the time of dilution (◇) that permits quantification of the exchangeable and nonexchangeable components at that time. ◇--◇, ◆--◆, time course of accumulation of nonexchangeable [^{3}H]MTX (open symbols: cells incubated with MTX alone; closed symbols: cells incubated with MTX together with 5 mM GSH). In the case of MTX + GSH, only the development of the nonexchangeable component by time is plotted as obtained by extrapolation of individual efflux kinetics obtained after interruption of MTX incubation by dilution at various times (not shown).

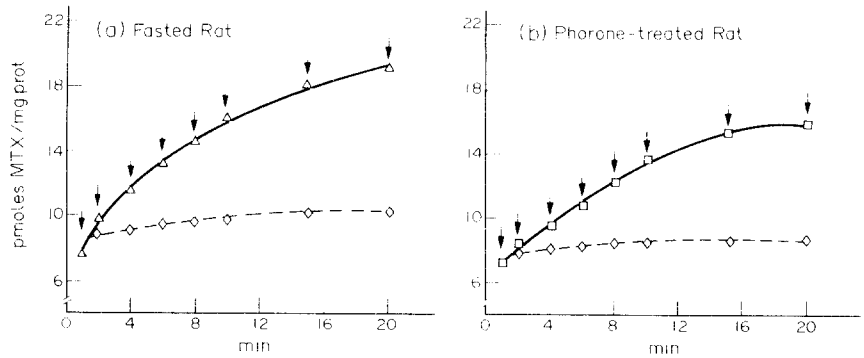

Fig. 6. Time course of MTX uptake and accumulation of nonexchangeable MTX over 20 min of incubation of hepatocytes of fasted (a) and phorone-treated (b) rats with 1 μM MTX. Experimental procedure was identical to that described in Fig. 5. For better readability, only MTX uptake (solid lines) and the extrapolated polyglutamate formation are plotted; MTX uptake: solid lines (as described in Fig. 5), polyglutamate formation: dashed-lines.

increased very fast with time. Within 10 min, the rate of accumulation of the nonexchangeable fraction of MTX, which represents the polyglutamate derivatives [5], decreased. For the sake of better readability of the results on the influence of exogenous GSH on the quantitative relationship between the levels of exchangeable and nonexchangeable intracellular MTX, individual efflux kinetics at various times of incubation were omitted and only MTX influx and the extrapolated polyglutamate formation were illustrated in the upper part of Fig. 5. The size of the exchangeable pool was similar under both conditions as were individual efflux kinetics. On the other hand, the level of nonexchangeable MTX was increased under the influence of GSH which is in line with previously demonstrated increased uptake of total (exchangeable and nonexchangeable) MTX by cells of fed rats after GSH addition [1]. Entirely different results have been found in cells from fasted and phorone-treated rats (Fig. 6(a) and (b)). The experimental procedure for determination of exchangeable and nonexchangeable components of intracellular MTX was identical to that described for cells of fed rats. It can be seen that it was the polyglutamate formation found by the extrapolation method, that was very small here and increased but little by time in contrast to total MTX uptake. In consequence, the non-exchangeable component of MTX found in hepatocytes of fasted and phorone-treated rats represented only 53–55% of total intracellular MTX whereas in cells of fed rats 80% of total cellular MTX was established by the nonexchangeable fraction.

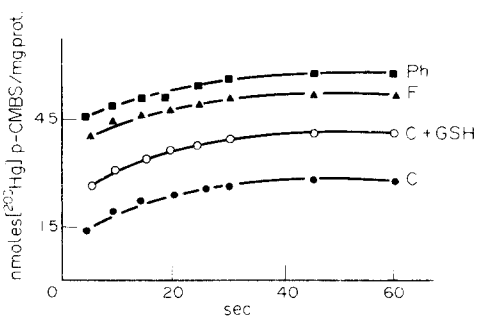

Fig. 7. Time course of titration by [^{203}Hg]p-CMBS (0.16 mM) of membrane SH groups in hepatocytes of fed control (C), fasted (F) and phorone-treated (Ph) rats. Titration of SH groups was started after a 10 min cell preincubation. +GSH: cells were preincubated with 5 mM GSH, then washed, resuspended in fresh medium and titration of SH groups started by addition of [^{203}Hg]p-CMBS.

4. *Study of* [^{203}Hg]*p-CMBS binding to membrane SH groups of liver cells*

In Fig. 7 it has been demonstrated that the amount of free SH groups of cell membrane of fed rat hepatocytes titrable by p-CMBS was about 3–4 times lower than in the other hepatocytes tested. Pretreatment of cells with GSH caused increase in the amount of SH groups titrated in liver cells of fed rat (Fig. 7), but had no significant effect on hepatocytes of fasted and phorone-treated rats (result not shown).

DISCUSSION

These studies revealed some new aspects concerning the MTX transport and accumulation in isolated liver cells. As has been demonstrated, hepatocytes with diminished cellular glutathione accumulated MTX to a lesser extent than those with normal GSH level. However, analysis of initial influx of MTX into hepatocytes brought out opposite results. Namely, the very initial uptake (measured during the first min) of methotrexate by hepatocytes of starved and phorone-treated rats was bigger in comparison with fed rat hepatocytes. While the K_m value in fed rat hepatocytes was 4 μM, about 3 times lower values of K_m were obtained in hepatocytes of rats with diminished cellular GSH. In contrast, the V_{max} value was about the same in any hepatocyte preparation studied. These results indicate an increased efficiency of the MTX transport in GSH-deficient cells.

The results of experiments on titration of exterior membrane SH groups revealed that cells from starved and phorone-treated rats had up to three times higher amounts of free SH groups than hepatocytes of fed rats. On account of this finding, the increased efficiency of MTX transport system in the

latter cells can be interpreted as increased capacity of the MTX transport carrier since it is generally accepted that this carrier requires intact SH groups [2–5].

Increased amounts of free membrane SH groups observed in hepatocytes of starved and phorone-treated rats can also be an explanation for the smaller effect of exogenous GSH on MTX uptake by the latter cells, in comparison with its strong effect demonstrated in fed rat hepatocytes. The effect of GSH addition on MTX transport in liver cells of fed rats was first described in our previous paper [1], and the hypothesis was stated that this effect might result from GSH binding to the cell membrane releasing free SH groups. In the present paper this hypothesis was experimentally verified and confirmed by the finding that after treatment of hepatocytes with GSH, the membrane SH groups titrable by *p*-CMBS were increased (Fig. 7). In liver cells of starved and phorone-treated rats this effect may be expected to be smaller on account of the much higher amount of membrane SH groups already exposed by these cells.

The question arises, why the methotrexate accumulation in hepatocytes with diminished cellular glutathione was decreased, in spite of its increased initial uptake by these cells. Theoretically, two possible explanations should be taken into consideration namely, either efflux processes of MTX from the cells were also increased, or the MTX metabolism was influenced. The first explanation can be ruled out since it has clearly been demonstrated that efflux processes in starved and phorone-treated rats were unchanged (Figs. 4–6). This finding is consistent with a very recent report of Dembo *et al.* that the systems of MTX influx and efflux are separated, and have different requirements [19].

The determination of "exchangeable" and "nonexchangeable" components of intracellular MTX revealed the inhibition of formation of a "nonexchangeable" fraction in cells with cellular GSH deficiency, in comparison to hepatocytes having normal glutathione level. It is generally accepted that this non-exchangeable fraction of cellular MTX is established mainly by MTX γ-polyglutamate derivatives [5, 20–22].

Recent studies by a number of investigators have indicated that formation of methotrexate polyglutamates is widespread and probably plays an important role in the antitumor activity of this compound [21–26]. However, the investigations into the nature of the γ-glutamylation reaction and its metabolic regulators are still in their initial phases. There is some evidence indicating that these processes may be self-regulating. It seems also fairly certain from inhibition studies with partially purified enzyme preparations [27] and cultured cell systems [28, 29] and from genetic and biochemical analyses [30, 31] that the same enzyme in mammalian cells that catalyzes the γ-glutamylation of the folate coenzymes also catalyzes the γ-glutamylation of analogs, such as methotrexate. Some authors reported that insulin can modify the methotrexate polyglutamation *in vitro* in an isolated human breast cancer cell line [32] and in hepatoma cells [33].

Our studies describe the modification by endogenous glutathione of processes leading to formation of MTX polyglutamates in isolated liver cells. The mechanism of this modification remained unclear up to now and to explain it, further biochemical studies are needed.

In conclusion, it is apparent that depletion of cellular glutathione has a double effect on MTX accumulation in isolated hepatocytes. The first one is an influence on initial influx of MTX into the cells changing the efficiency of the MTX carrier system, and the second one is regulation of MTX retention within cells, probably due to the modification of formation processes of MTX polyglutamates. The latter effect is more strongly expressed, which lessens the practical implications of the first one. On this account, further studies on the nature of the moderator role of endogenous glutathione on the processes of MTX polyglutamation seem to be of special interest.

Acknowledgements—The authors wish to thank Mrs. Sylvia Kasperek and Mr. Günther Joachim for skilful technical assistance.

REFERENCES

1. A. Leszczyńska and E. Pfaff, *Biochem. Pharmac.* **31**, 1911 (1982).
2. I. D. Goldman, *Biochim. biophys. Acta* **233**, 624 (1971).
3. D. W. Horne, W. T. Briggs and C. Wagner, *Biochem. biophys. Res. Commun.* **68**, 70 (1976).
4. D. W. Horne, W. T. Briggs and C. Wagner, *J. biol. Chem.* **253**, 3529 (1978).
5. D. A. Gewirtz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.* **40**, 1573 (1980).
6. N. S. Kosower and E. M. Kosower, *Intern. Rev. Cytol.* **54**, 109 (1978).
7. J. Isaacs and F. Binkley, *Biochim. biophys. Acta* **197**, 192 (1977).
8. J. Hewitt, D. J. Pillion and F. H. Leibach, *Biochim. biophys. Acta* **363**, 267 (1974).
9. D. J. Pillion and F. H. Leibach, *Biochim. biophys. Acta* **382**, 246 (1975).
10. D. J. Pillion, F. H. Leibach, J. von Tersch and J. Mendicino, *Biochim. biophys. Acta* **419**, 104 (1976).
11. F. H. Leibach, D. J. Pillion, J. Mendicino and P. Pashley, in *Functions of Glutathione in Liver and Kidney* (Eds. H. Sies and A. Wendel), p. 84. Springer, Berlin (1978).
12. N. Tateishi, T. Higashi, S. Shinya, A. Naruse and Y. Sakamoto, *J. Biochem.* **75**, 93 (1974).
13. R. J. Richardson and S. D. Murphy, *Toxicol. appl. Pharmac.* **31**, 505 (1975).
14. R. Van Doorn, C. M. Leijdekkers and P. T. Henderson, *Toxicology* **11**, 225 (1978).
15. G. B. Henderson and E. M. Zevely, *Archs Biochem. Biophys.* **200**, 149 (1980).
16. G. B. Henderson, B. Grzelakowska-Sztabert, E. M. Zevely and F. M. Huennekens, *Archs Biochem. Biophys.* **202**, 144 (1980).
17. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
18. H. Sies and K. H. Summer, *Eur. J. Biochem.* **57**, 503 (1975).
19. H. Dembo, F. M. Sirotnok and D. M. Moccio, *J. membrane Biol.* **78**, 9 (1984).
20. D. A. Gewirtz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.* **39**, 2914 (1979).

21. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
22. M. Balinska, J. Galivan and J. K. Coward, *Cancer Res.* **41**, 2751 (1981).
23. D. S. Rosenblatt, V. M. Whitehead, N. Vera, A. Pottier, M. Dupont and M. J. Vuchich, *Molec. Pharmac.* **14**, 1143 (1978).
24. J. Galivan, *Cancer Res.* **39**, 735 (1979).
25. D. W. Fry, J. C. Yalowich and I. D. Goldman, *J. biol. Chem.* **257**, 1890 (1982).
26. J. Jolivel, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
27. D. S. Rosenblatt, V. M. Whitehead, M. J. Vuchich, A. Pottier, N. V. Matiaszuk and D. Beaulieu, *Molec. Pharmac.* **19**, 87 (1981).
28. J. J. McGuire, P. Hsieh, J. K. Coward and J. R. Bertino, *J. biol. Chem.* **225**, 5776 (1980).
29. M. W. McBurney and G. F. Whitmore, *Cell.* **2**, 173 (1974).
30. M. Balinska, Z. Nimec and J. Galivan, *Archs Biochem. Biophys.* **216**, 466 (1982).
31. R. T. Taylor and M. L. Hanna, *Archs Biochem. Biophys.* **181**, 331 (1977).
32. D. G. Kennedy, R. Clarke, H. W. van den Berg and R. F. Murphy, *Biochem. Pharmac.* **32**, 41 (1983).
33. J. Galivan, Z. Nimec and M. Balinska, *Biochem. Pharmac.* **32**, 3244 (1983).