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ACTIVATION BY REDUCED GLUTATHIONE OF METHOTREXATE TRANSPORT INTO ISOLATED RAT LIVER CELLS

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Abstract—The uptake of methotrexate (MTX) by isolated rat hepatocytes and its changes under the influence of exogenous GSH have been studied under various conditions: GSH concentration, pH of incubation medium, preincubation of cells prior to MTX and GSH addition, ionic composition of the incubation medium (standard saline, Na^+-free, Na^+ and K^+-free, or ion-deficient), after prior treatment of cells by membrane -SH blockers (*p*-CMBS, 4-CMB and DIP^{2+}) and ATP.

It was found that GSH strongly accelerated MTX uptake. This effect depended on GSH concentration and on preincubation of cells. The GSH effect was not dependent on medium pH in spite of an observed close relationship between pH of incubate and MTX transport itself. Activation by GSH of MTX transport was connected to an increase in intracellular K^+. It was also noted that while blockers of membrane -SH groups like *p*-CMBS and 4-CMB inhibited MTX uptake and increased the intracellular Na^+/K^+ ratio, both effects were partially overcome by GSH. After treatment by DIP^{2+}, Na^+/K^+ ratio was unaffected, but MTX uptake inhibited. Still GSH abolished inhibition. Added ATP also inhibited MTX uptake and caused loss of cellular K^+ and accumulation of Na^+. Here neither effect could be reversed by GSH; consequently, high cellular amounts of K^+ and MTX accumulated by previous action of GSH were depleted on subsequent ATP addition. MTX uptake was low in sucrose medium. But in this ion-deficient medium, GSH had the greatest stimulatory effect on MTX uptake.

It is concluded that binding GSH can affect the redox state of the -S-S-/-SH groups of the cellular plasma membrane and that this effect of GSH might demonstrate involvement of the redox state in the control of MTX permeability.

In previous studies [1] it has been demonstrated that using exogenous glutathione it is possible to increase the penetration of some drugs (e.g. rifamycin SV) from blood to particular tissues. The hypothesis was stated that GSH‡ can change the permeability of cell membranes to some drugs and that this effect is determined by the free SH-groups of GSH. As the mechanism of this effect could not be explained applying *in vivo* methods, in the present study we decided to use isolated rat liver cells to investigate the effect of GSH on drug transport across the cell membrane, since hepatocytes are a widely used model for membrane permeability and drug transport studies. The drug of our choice was methotrexate (MTX) due to its common use in the therapy of hepatoma and other cancers, and because its uptake into liver and other cells has been intensively studied [2–7], though with contradictory results especially for hepatocytes [2, 3]. On the other hand, anticancer pharmacotherapy is especially limited by the toxic effect of cytostatics. In view of the above, the possible increase of the cytostatic concentration in cancer cells without increasing the dose seemed of special interest. First of all, however, investigations on normal cells should be performed to find out whether the activation of MTX transport is possible and in case of positive results to examine the membrane mechanism of this effect.

MATERIALS AND METHODS

Animals

Female Wistar rats of 200–250 g body wt were used. Standard laboratory diet (Altromin) and water were given *ad libitum*.

Materials

The following substances were purchased. (a) Radioactive compounds: [3′, 5′, 7′-^{3}H]methotrexate sodium salt (MTX; 39.6 mCi/mg) from Amersham–Buchler (Braunschweig, West Germany), ^3H_2O (250 ±Ci/ml) and [carboxyl-^{14}C]dextran (M_T 70,000; 1 mCi/g) from NEN Chemicals GmbH (Dreieich, West Germany); (b) unlabelled compounds: amethopterin (MTX), reduced glutathione (GSH) and *p*-chloromercuribenzene sulfonic acid (*p*-CMBS) from Sigma Chemie GmbH (München, West Germany), 4-chloromercuribenzoic acid sodium salt (4-CMB) from Fluka AG (Buchs SG, Switzerland), ATP, NADPH, 5,5-dithiobis(nitrobenzoate), collagenase and other biochemicals from E. Boehringer (Mannheim, West Germany), sucrose and choline chloride from E. Merck (Darmstadt, West Germany) and silicone oils

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‡ Abbreviations used: GSH, reduced glutathione; MTX, methotrexate (amethopterin); 4-CMB, 4-chloromercuribenzoate; *p*-CMBS, *p*-chloromercuribenzene sulfonate; DIP^{2+}, bis-*N*-methyl iodide salt of diazene dicarboxylic acid-bis(*N*-methylpiperazide).

from Wacker Chemie (München, West Germany). DIP^{2+} [bis-*N*-methyl iodide salt of diazene dicarboxylic acid-bis(*N*-methylpiperazide)] was a generous gift from Professor Ammon (Pharmaceutical Institute, University of Tübingen, West Germany).

Methods

Isolation of liver cells. Liver cells were isolated according to the procedure of Berry and Friend [8] with modifications by Seglen [9] and Baur *et al.* [10]. During preparation the liver was perfused by Ca^{2+}-free Hank's medium at 37° via the vena portae. After preparation of the liver, perfusion was carried out with a recirculating medium containing 1.25 mM Ca^{2+}, 2 mM pyruvate and 0.05% collagenase. After a perfusion of 10–15 min, it was continued via the vena cava. After 30–40 min the liver disintegrated. Subsequently, the tissue was transferred to a round-bottomed flask and enzymic treatment was continued for another 15 min under slow rotation. After washing, the cells were stored in suspension at 0° in the standard medium containing 137 mM NaCl, 5 mM KCl, 0.9 mM $MgSO_4$, 0.12 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

Incubation of liver cells. Incubation of cells was carried out at 37°. Various media were used:

(a) *Normal incubation medium.* Normal incubation medium was essentially standard medium, except for $CaCl_2$ being 1 mM. The medium was used at the following pH values: 6.2, 6.5, 6.8, 7.1, 7.3 and 7.8. Two different types of experiments were performed:

(1) *Investigation of MTX uptake into the isolated cells.* In these experiments [^{3}H]MTX (10 μM) was added to the incubation medium alone or together with GSH (0.1, 1, 2 and 5 mM) in two different variants: MTX was present in medium prior to addition of cells which started incubation (variant 1), or after a 10 min preincubation of cells at 37°, MTX was added starting incubation (variant 2).

(2) *Examination of intracellular* Na^+ *and* K^+*-concentrations.* Unlabelled MTX (10 μM) was added to the incubation medium (containing ^3H_2O and [carboxyl-^{14}C]dextran) alone or together with GSH (5 mM); apart from that, the same procedure as in MTX uptake studies was used.

In some series, one of the following substances was applied in both types of experiments: ATP, 4-CMB, *p*-CMBS, or DIP^{2+}. ATP (1 mM) was given to the medium, pH 7.3, in three variants: either used alone after a 10 min preincubation of cells or at the same time together with MTX, or in the third variant after a 10 min preincubation, MTX was added first together with 5 mM GSH and after a further 10 min incubation, ATP was also given and the experiment was carried on for a further 20 min. DIP^{2+}, 4-CMB, or *p*-CMBS, respectively, were added at 0.1 mM to the medium after a 10 min preincubation. Then incubation continued for 2 min. Subsequently, cells were washed by short centrifugation (½ min) and resuspended in new medium to the original volume. After 2 more min, MTX or MTX + GSH was added and incubation was started. To make sure that the washing procedure itself did not influence MTX uptake and intracellular Na^+ and K^+-concentration, in control experiments cells were also washed prior to MTX incubation. Samples of the incubation suspension for measurement of MTX uptake and intracellular Na^+ and K^+-concentrations were taken over a time span from 15 sec to 40 min after MTX addition.

(b) *Choline medium without* Na^+. This medium contained all components of normal incubation medium except for NaCl being replaced by choline chloride (pH 7.3).

(c) *Choline medium without* Na^+ *and* K^+. Here, besides replacement of NaCl by choline chloride, KCl was omitted from normal incubation medium (pH 7.3).

(d) *Sucrose medium.* This medium contained nothing but 250 mM sucrose and 10 mM Tris (pH 7.3). MTX uptake was studied in each of these three media. To this end, after a 10 min preincubation of cells, MTX (10 μM) or MTX with 5 mM GSH was added.

Throughout, 1.4–2.0 mg cellular protein/ml incubate was applied. Cells were separated from incubation medium by centrifugal filtration [11]. Radioactivity in the cell precipitate was analysed in Unisolve solution in a Berthold liquid scintillation counter. MTX was computed as nmole/mg protein.

Special analytical methods. Cellular protein was determined by a modified biuret method [12]. For a quick check of cell viability in the preparation, the trypan blue staining test was used. The cells were taken for use in experiments if the susceptibility of the preparation to staining was less than 6%. The aqueous cell volume was measured using ^3H_2O and [carboxyl-^{14}C]dextran as described by Baur *et al.* [10]. The concentrations of intracellular Na^+ and K^+ were determined by flame photometry [10]. Oxidized glutathione was determined by a decrease in NADPH absorption in a glutathione reductase-catalysed reaction [13]. Total glutathione was measured by the catalytic assay with 5,5-dithiobis-(nitrobenzoate) [14].

RESULTS

Experiments with normal medium

The influence of GSH on MTX uptake from standard medium, pH 7.3. It was found that GSH accelerated the uptake of MTX by hepatocytes (Fig. 1a and b). This effect was stronger when cells were preincubated for 10 min prior to GSH and MTX addition (Fig. 1a), as compared to the effect observed in experiments without preincubation of cells (Fig. 1b). In both cases, however, the glutathione effect was concentration-dependent. The strongest activation of MTX transport was obtained after using 5 mM GSH.

Effect of pH variations of incubation media on MTX transport in the presence and absence of GSH. As shown in Fig. 2 the highest uptake of MTX was found in incubation medium, pH 6.2. Increasing pH to 6.5, 6.8, 7.1 or 7.8, a progressive decrease of MTX uptake was observed. However, it was repeatedly found that MTX transport at pH 7.3 (standard medium) was larger than at pH 7.1. This phenomenon occurred independently of whether MTX was

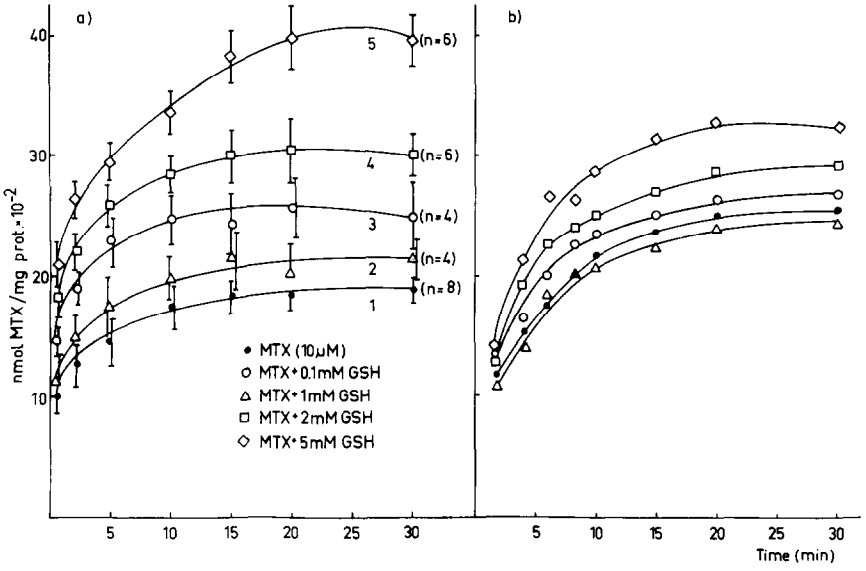

Fig. 1. The influence of GSH on MTX uptake by hepatocytes; dependence on GSH concentration. (a) Left panel: MTX (alone or together with GSH) added after 10 min preincubation of cells. The results of these experiments were statistically evaluated with the use of Student's *t*-test for the difference between compared groups (n = number of experiments). Statistical significances: $P_{1,5}$ and $P_{1,4} = 0.005$; $P_{1,3} = 0.02$; $P_{1,2} = 0.05$; $P_{5,4} = 0.02$; $P_{4,3}$ and $P_{3,2} = 0.05$. (b) Right panel: MTX (alone or together with GSH) added prior to addition of cells.

added to the medium after a 10 min preincubation or together with cells.

The activatory effect of GSH on MTX transport found in standard medium at pH 7.3 also occurred in media of various pH, except for pH 7.8 (Fig. 2). At this pH no GSH effect was found. However, measurement of total and oxidized glutathione in medium of pH 7.8 containing no cells, revealed the loss of 60–70% of GSH. It means that at such a high pH, GSH is not stable. Hence, an effect cannot be expected. In the experiment to Fig. 2, the strongest effect of GSH was observed at pH 7.3.

Changes in intracellular Na^+ *and* K^+*-concentrations in incubation media of various pH.*

(a) *After addition of MTX only.* It was found that intracellular K^+ increased with decrease of medium pH, progressively from 7.8 to 6.5 (here, pH 7.3 was

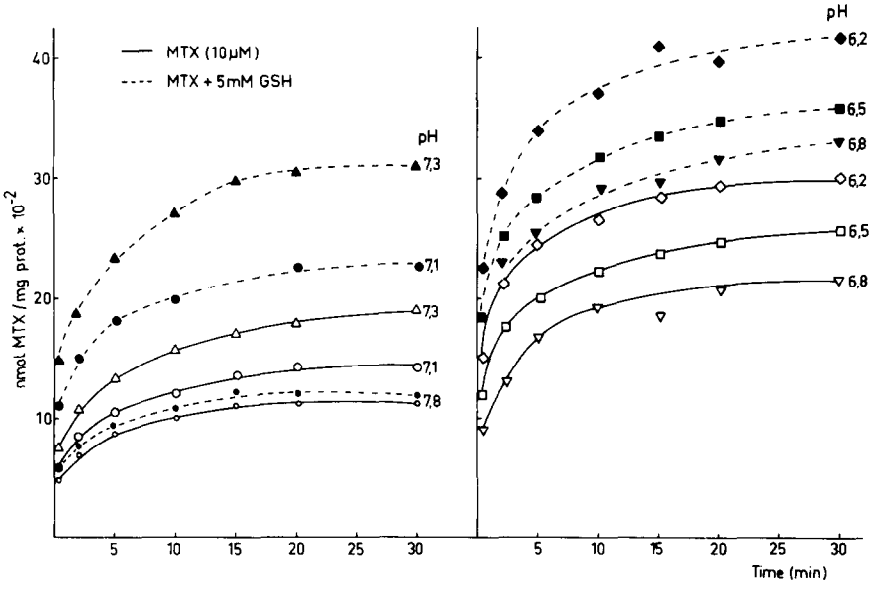

Fig. 2. MTX uptake by hepatocytes after its addition alone or together with GSH (5 mM) at various pH of incubation media, 10 min after preincubation of cells. Symbols (open or closed) pertain to the various values of medium pH. Open symbols are used, if MTX only was added, closed symbols if MTX was given together with GSH.

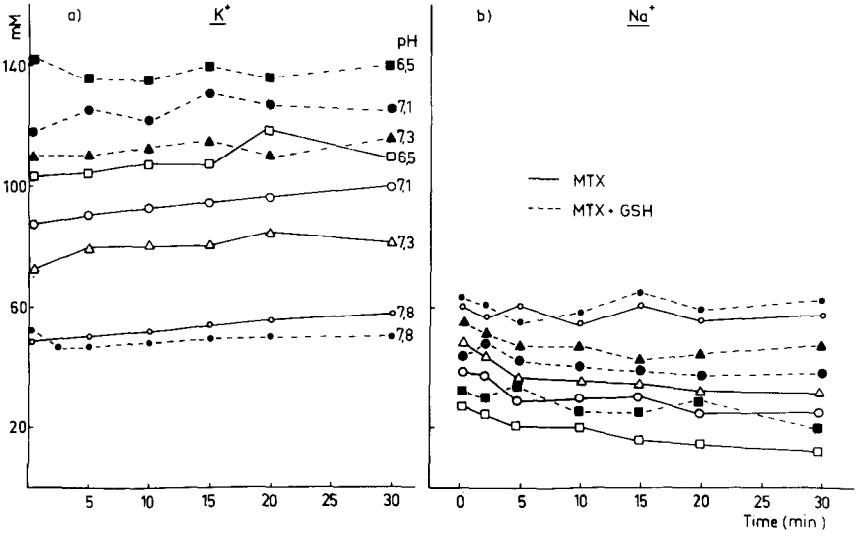

Fig. 3. Concentrations of intracellular Na^+ and K^+ after addition of MTX alone or together with GSH (5 mM) at various pH of media, 10 min after preincubation of cells. Indications as in Fig. 2.

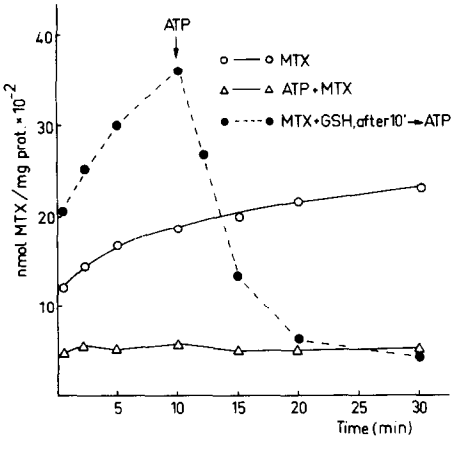

Fig. 4. Influence of ATP (1 mM) on the GSH effect on MTX uptake by hepatocytes. 5 mM GSH and 10 μM MTX were added 10 min after preincubation of cells.

in sequence), (Fig. 3a). Simultaneously, a small decrease in intracellular Na^+ was found with lower pH values (Fig. 3b).

(b) *After addition of GSH.* GSH caused a very strong increase in intracellular K^+ at any medium pH except for pH 7.8 (Fig. 3a). Small increases in intracellular Na^+ were also observed (Fig. 3b).

Effect of ATP on MTX uptake and on intracellular Na^+ *and* K^+*-concentrations.* It was found that ATP caused a pronounced inhibition of MTX transport and on the other hand, rapidly abolished the activatory effect of GSH (Fig. 4). Simultaneously, ATP generated strong disturbance in the intracellular Na^+/K^+ ratio indicated by a sharp loss of intracellular K^+ and accumulation of intracellular Na^+ (Fig. 5). When ATP was added after commencement of GSH action, instantaneous and very drastic decrease of intracellular K^+ and increase of Na^+ can be noted, in spite of the previous strong increase of K^+ level brought about by GSH (Fig. 5).

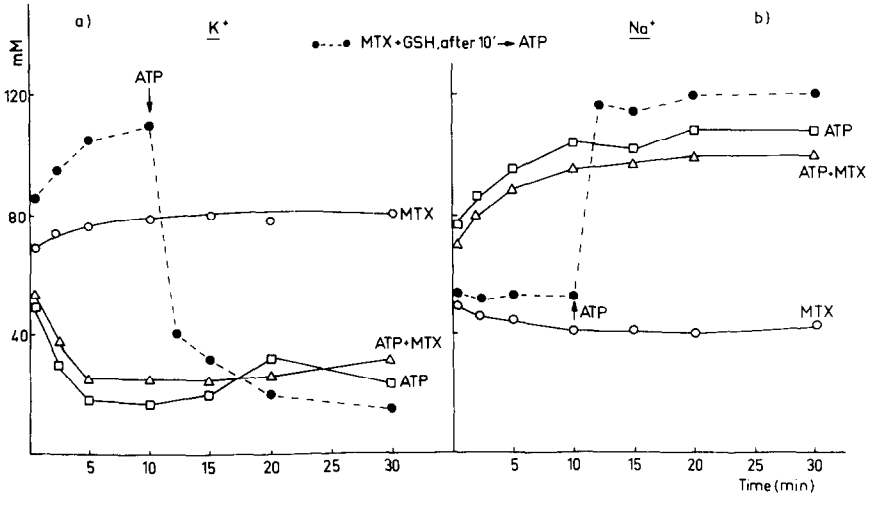

Fig. 5. Changes in intracellular concentrations of Na^+ and K^+ after addition of ATP alone, together with MTX, and 10 min after MTX + GSH addition. Conditions as in Fig. 4.

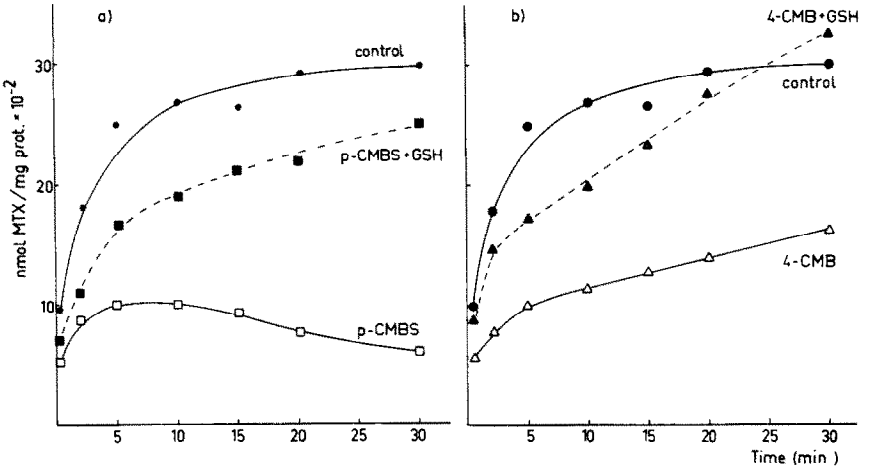

Fig. 6. Effect on MTX uptake of (a) *p*-CMBS and (b) 4-CMB. Key: control, addition of MTX to a suspension of preincubated, but otherwise untreated cells; p-CMBS, addition of MTX after prior treatment of cells with *p*-CMBS (0.1 mM); *p*-CMBS + GSH, the same but addition of MTX together with GSH (5 mM); 4-CMB, MTX addition after prior treatment of cells with 4-CMB (0.1 mM); 4-CMB + GSH, the same but simultaneous addition of MTX and GSH.

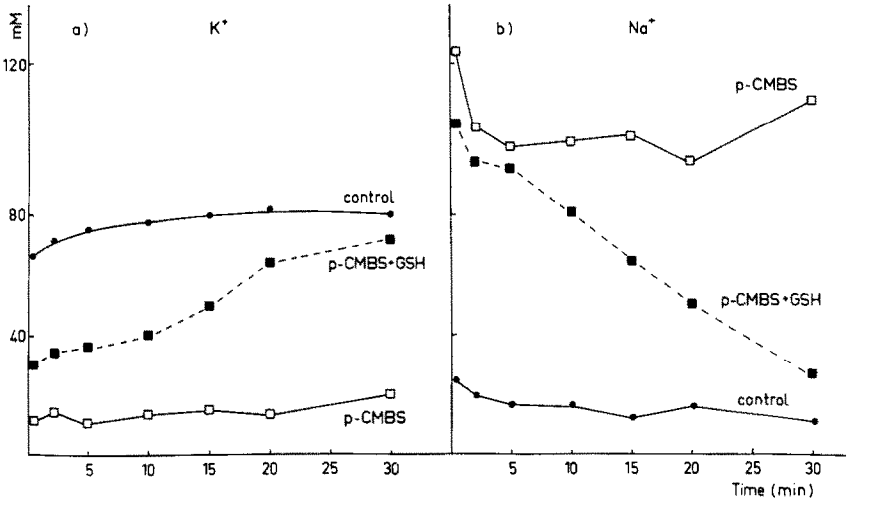

Fig. 7. Effect on intracellular Na^+ and K^+ concentrations of *p*-CMBS pretreatment of cells. Symbols and conditions as in Fig. 6a.

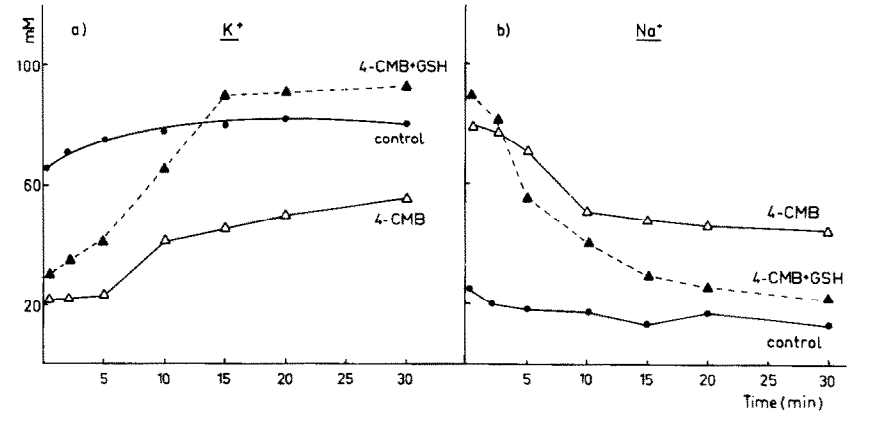

Fig. 8. Effect on intracellular Na^+ and K^+ concentrations of 4-CMB pretreatment of cells. Symbols and conditions as in Fig. 6b.

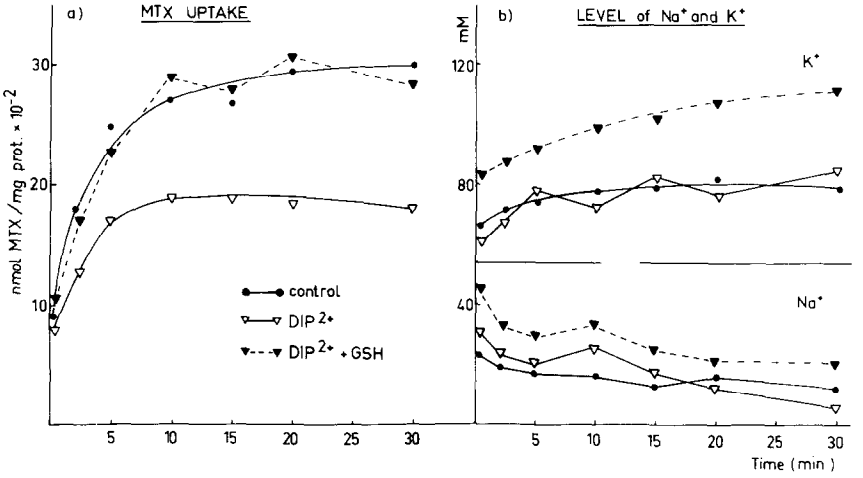

Fig. 9. Effect of pretreatment of cells with DIP^{2+} (a) on MTX uptake by hepatocytes and (b) on intracellular concentrations of K^+ (upper half of figure) and Na^+ (lower half of figure). Key: control, MTX added to a suspension of preincubated, but otherwise untreated cells; DIP^{2+}, MTX addition after pretreatment of cells with DIP^{2+} (0.1 mM); DIP^{2+} + GSH, the same but MTX added together with GSH (5 mM).

Effect of blockers of membrane SH-groups on MTX transport and on intracellular Na^+ *and* K^+ *concentrations.* *p*-CMBS and 4-CMB brought on a strong decrease of MTX uptake by hepatocytes (Fig. 6a and b). A very pronounced inhibition of MTX transport was observed after using *p*-CMBS (Fig. 6a). Treatment of cells with *p*-CMBS and 4-CMB also resulted in loss of intracellular K^+ and accumulation of intracellular Na^+ (Figs. 7 and 8).

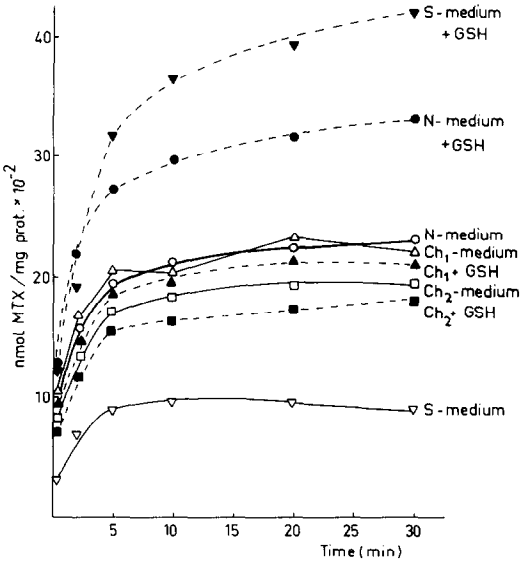

Fig. 10. MTX uptake into hepatocytes in various media as affected by GSH. Key: N-medium, MTX added in normal incubation medium; N-medium + GSH, the same but MTX added together with GSH (5 mM); Ch_1-medium, MTX added to a normal medium in which NaCl is replaced by choline chloride; Ch_1-medium + GSH, the same but MTX and GSH added together; Ch_2-medium, MTX added to a medium, in which, besides replacement of NaCl by choline chloride, KCl is omitted; Ch_2-medium + GSH, the same but GSH added together with MTX; S-medium, MTX added to a medium containing only sucrose and Tris; S-sucrose + GSH, the same but MTX added together with GSH.

DIP^{2+}, an agent not penetrating the cell membrane [15] also decreased MTX uptake by hepatocytes (Fig. 9a); it did not cause, however, any significant changes in intracellular Na^+ and K^+ (Fig. 9b).

Effect of GSH on disturbances of MTX uptake and intracellular Na^+ *and* K^+ *caused by blockers of membrane SH-groups.* GSH greatly diminished the inhibitory effect of *p*-CMBS and 4-CMB on MTX transport (Fig. 6a and b) and completely abolished the effect of DIP^{2+} with regard to the control (MTX alone), (Fig. 9a). Simultaneously, time-dependent compensation of the disturbance in intracellular Na^+/K^+ distribution caused by *p*-CMBS and 4-CMB took place under the influence of GSH (Figs. 7 and 8).

Experiments with choline chloride medium

Without NaCl *(Ch_1).* MTX transport was found not to be changed in normal medium containing choline chloride instead of NaCl (Fig. 10). However, no GSH effect was observed in NaCl-free choline medium.

Without NaCl *and* KCl *(Ch_2).* MTX uptake in this medium deprived of Na^+ and K^+ ions was smaller than in normal medium, though these differences were not very expressed (Fig. 10). GSH in this medium caused no activation, but rather a small decrease of MTX uptake.

Experiments with sucrose medium

In medium containing only sucrose and, except for Tris, no ions, a strong inhibition of MTX uptake into isolated hepatocytes was noted (Fig. 10). On the other hand, GSH added to this medium caused a large and very fast activation of MTX uptake. This effect was much stronger than in normal medium.

DISCUSSION

The results of the present studies disclose a strong activation by GSH of the transport of MTX into isolated rat liver cells. This effect is not dependent on pH of incubation medium irrespective of a close relationship between medium pH and extent of MTX uptake itself, the latter being higher at lower pH values. However, stimulation of MTX transport by both, GSH and low pH, is associated with simultaneously occurring changes in Na^+/K^+ distribution (intracellular K^+ being increased). The fact that the GSH effect persists also at low pH when cellular K^+ is already high, indicates that the increase of K^+ caused by GSH adds to that by low pH.

The smaller effect of GSH on MTX transport found in experiments with cells not preincubated shows that for a maximal GSH effect the cellular Na^+/K^+ ratio should be stabilized. It is known that cells stored at 0° have a very high Na^+/K^+ ratio due to a decrease in Na^+/K^+ ATPase activity [16, 17]. In our experiments after a 10 min preincubation at 37°, the Na^+/K^+ ratio was found to be stabilized at a lower level.

As to the mechanism of GSH action, an answer is not yet possible, but let us collect the facts pertaining to this question. It can be safely excluded that GSH needs to permeate to exert its action since liver is essentially impermeable to exogenous GSH [18]. As it is known that endogenous GSH regulates the -S-S-/-SH ratio of membrane proteins [19], it is very likely that exogenous GSH also reacts with the disulfide bridges of proteins on the outer surface of the plasma membrane. This action of GSH on the redox state of the membrane were highly relevant for MTX uptake itself, since it was reported that the carrier responsible for transport requires intact membrane-SH groups [3]. Very recent data with *p*-CMBS of Henderson and Zevely [20] and our results with *p*-CMBS and 4-CMB (commonly used as blockers of membrane -SH groups [21, 22]) are in agreement with this concept. The interesting new finding of the present paper is that exogenous GSH simultaneously abolishes the inhibitory effect of *p*-CMBS and 4-CMB on MTX uptake and overcomes the disturbances in Na^+/K^+ distribution caused by these blockers. It is known that the extent of -SH group blockade of the cell membrane is related to the extent of K^+ loss and accumulation of Na^+ [22]. Hence restoration of the Na^+/K^+ ratio by GSH can be assumed to be brought about by liberation of blocked -SH groups and by breaking up disulfide bridges on binding to the membrane. The activatory effect on MTX uptake also appears to be linked to this binding.

Membrane binding of GSH would also alter the surface charge density of the membrane by virtue of the negative surplus charge of the glutathione moiety. A regulatory role of surface potential in ion transport is generally accepted; thus changes in permeability parameters of hepatocytes have been reported by amphiphilic ions as effectors of surface potential [23]. MTX uptake can be also expected to be affected by increase in surface charge density through GSH, since MTX at neutral pH carries positive and negative charges.

ATP addition shifts the Na^+/K^+ gradient to a high level resulting in very little uptake of simultaneously added MTX. Even if MTX together with GSH were added prior to ATP, ATP addition reduced the cellular content of MTX and simultaneously increased the cellular Na^+/K^+ to the same high ratio observed when ATP was present from the start of the incubation. This parallelism of Na^+/K^+ ratio and MTX distribution across the cellular membrane makes it difficult to describe MTX uptake as energetically dependent on the Na^+ gradient, but rather that a given membrane state similarly affects Na^+, K^+ and MTX distribution. This also would solve controversies arising from reports on the role in MTX uptake of extracellular Na^+ [2, 3]. Also, our data with Na^+-free choline clearly support the finding of Horne *et al.* [2] that MTX uptake is not dependent on extracellular Na^+ and not the opposite data of Gewirtz *et al.* [3]. On the other hand, our observations that GSH has no effect on MTX uptake in choline medium free of Na^+ or Na^+ and K^+, but a strong activatory effect in ion-deficient sucrose medium, cannot be explained as yet and requires further studies.

As to the mechanism of ATP action, especially why ATP completely abolishes the GSH effects, an answer is not yet possible. Likewise, as yet an inter-dependent action of both, a high intracellular K^+ and a suitable redox state of the membrane, in MTX uptake is not so clear as might be expected, e.g. from the results of experiments with *p*-CMBS and 4-CMB. Thus, using the non-permeant DIP^{2+} which is known to strongly react with membrane-SH groups [15], the decrease in MTX uptake was not connected with a decrease in intracellular K^+ (the Na^+/K^+ ratio being unchanged). However, GSH also abolished its inhibitory effect, even more pronounced than in the case of the *p*-CMBS and 4-CMB experiments.

As yet, the effect of GSH is consistent with the assumption that the redox state of -S-S-/-SH groups of the cellular plasma membrane might be involved in the control of MTX permeability. The inherent therapeutic potency of simultaneous administration of GSH and MTX could be especially advantageous against cancer cells, if they differed in the redox state of their plasma membrane. This will be the subject of further experiments with cancer cells.

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