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EFFECT OF MODULATORS OF GLUTATHIONE SYNTHESIS ON THE HEPATOTOXICITY OF 2-METHYLFURAN

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Abstract—Treatment of male Sprague–Dawley rats with buthionine sulfoximine (BSO), prior to administration of carbon-14(^{14}C)-labelled 2-methylfuran (2MF) caused a marked decrease in the covalent binding of ^{14}C-labelled 2MF metabolites to both DNA and protein, although there was no apparent change in the distribution of the labelled parent 2MF. BSO pretreatment also protected against hepatotoxicity of 2MF, as indicated by lower serum glutamic pyruvic transaminase (GPT) levels. Pretreatment with BSO offered protection only if administered 1.5 hr before 2MF dosage. Administration of 2MF, 4 and 6 hr after BSO resulted in manifestation of the hepatotoxicity of 2MF. Prior treatment with diethylmaleate (DEM), increased covalent binding of [^{14}C]2MF to liver proteins and also elevated serum GPT levels. Thus, depletion of tissue glutathione (GSH) by two different chemicals acting by different mechanisms produced opposite effects on the covalent binding and toxicity of 2MF. Pretreatment with L-2-oxothiazolidine-4-carboxylate (OTZ), a promoter of GSH biosynthesis, increased the hepatic covalent binding of [^{14}C]2MF and potentiated hepatotoxicity. However, administration of OTZ and BSO prior to an i.p. dose of 100 mg/kg of 2MF, decreased the hepatic covalent binding of [^{14}C]2MF and decreased the hepatotoxicity. The marked instability of the GSH conjugate of the reactive metabolite of 2MF may account for the potentiation of hepatotoxicity of 2MF by OTZ. A single s.c. dose of BSO, caused a transient increase in plasma cystine levels concurrent with the depletion of liver GSH. Administration of 2MF, 1.5 hr after BSO, significantly decreased plasma cystine levels as compared to control animals that received vehicle alone. Pretreatment with BSO also resulted in increased excretion of urinary metabolites in 2MF treated animals as compared to animals receiving 2MF alone. Thus, BSO probably protects against hepatoxicity of 2MF by indirectly causing more detoxification of the reactive metabolite of 2MF, as it does not alter the distribution of unmetabolized 2MF and does not have any apparent effect on the microsomal mixed-function oxidase which mediates the activation of 2MF. The enhanced detoxification of 2MF in BSO treated animals appears independent of the depleted GSH levels; it may result from increased availability of a better alternative nucleophile (i.e. cysteine), capable of conjugating with acetyl acrolein. Acetyl acrolein (AA) appears to be the principal reactive metabolite of 2MF which binds covalently to tissues. Previous *in vitro* studies have shown that cysteine is a better trapping agent of AA than GSH or *N*-acetyl cysteine.

2-Methylfuran (2MF), a commonly occurring furan, causes centrilobular hepatic necrosis when administered to rats. 2MF is metabolically activated by hepatic mixed-function oxidase to a reactive metabolite that binds covalently to tissue macromolecules [1]. The covalent binding of the reactive metabolite(s) to hepatic macromolecules is correlated with the extent of liver damage and a corresponding rise in serum GPT levels [1].

Oxygen- and NADPH-dependent metabolism of 2MF by rat hepatic microsomes leads to the formation of reactive intermediates that bind covalently to microsomal proteins, *in vitro* [2]. The major alkylating metabolite of 2MF in rat hepatic microsomal systems has been identified as AA [3]. Unsaturated aldehydes, like AA are very reactive and can bind covalently with tissue macromolecules either via Michael addition of sulfhydryls across the activated double bond or by nucleophilic addition to the aldehyde. *In vitro* studies using liver microsomes have demonstrated that cysteine is a better nucleophilic trap for AA than GSH or *N*-acetyl cysteine [2]. Earlier attempts to isolate the reactive metabolite of methylfurans as GSH conjugates have failed, as the GSH conjugates of the unsaturated aldehydes are extremely unstable [3].

The endogenous thiol nucleophile GSH, generally acts as an efficient trapping agent for reactive electrophilic metabolites formed *in vivo* by forming stable GSH adducts. Depletion of intracellular GSH, results in potentiation of toxicity of the reactive metabolite [4, 5], while increasing intracellular GSH, results in protection from the toxicity caused by the reactive electrophilic metabolite [6]. However, in view of the instability of the GSH conjugate of AA and the stability of cysteine adduct of AA, the major alkylating reactive metabolite of 2MF, the effect of modulation of intracellular GSH and cysteine on 2MF induced hepatotoxicity might be important.

A study of the effect of various inhibitors and inducers of endogenous GSH on the tissue

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distribution, metabolic activation and toxicity of 2MF was therefore undertaken to determine the role of GSH in the hepatotoxicity of 2MF.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 150–200 g were obtained from Taconic farms (Germantown, NY). Animals were given laboratory diet and water *ad lib.* and were not fasted prior to use.

Radiochemical. 2-([^{14}C]Methyl) furan ([^{14}C]2MF) was synthesized and purified as previously described [7]. The specific activity of [^{14}C]2MF was 0.1 mCi/mmol and the radiochemical purity was greater than 98%. 2MF dose solutions were prepared in sesame oil such that 1 mL per 100 g body weight was administered i.p. to yield the desired dose. Due to the high volatility of 2MF, dose solutions were prepared just prior to injection. Unlabelled 2MF was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was distilled before use. All other chemicals were of reagent grade. DL-Buthionine sulfoximine (BSO) and L-2-oxothiazolidine-4-carboxylate (OTZ) were purchased from Chemical Dynamics Corporation (South Plainfield, NJ) and diethylmaleate (DEM) from Eastman Kodak Co. (Rochester, NY).

Pretreatments. BSO was administered s.c. as a solution in phosphate-buffered saline at a dose of 900 mg/kg body weight, 1.5 hr before administration of 2MF unless otherwise indicated. DEM was administered s.c. as a solution in sesame oil at a dose of 0.4 mL/kg body weight, 0.5 hr before dosing with 2MF. The pretreatment time was based on earlier experiments [8] with DEM, which indicated that 0.5 hr pretreatment time was most suitable, since hepatic GSH levels recovered to normal levels 4 hr after DEM administration. OTZ was given s.c., 5 min before administration of 2MF, at a dose of 1000 mg/kg body weight. Dose solutions of OTZ were prepared in distilled water and adjusted to pH 7.4 with sodium hydroxide.

Covalent binding studies. Rats were sacrificed by an overdose of pentobarbital and a sample of blood was removed by cardiac puncture, serum separated and measured for GPT activity [9]. A small portion of the liver was removed and immediately frozen in liquid nitrogen. Radioactivity covalently bound to proteins and DNA was assayed as described elsewhere [1]. There was no significant difference in the serum GPT levels in control animals treated different vehicles.

Measurement of plasma cystine. For the study of the effect of BSO on plasma cystine levels, rats were killed at specified times after treatment with BSO, by an overdose of pentobarbital. Blood (2 mL) was removed by cardiac puncture and transferred to a test tube containing 50 μL of 0.5 M ethylenediamine tetracetic acid disodium salt. Plasma was separated by centrifugation and analysed for cystine on a Beckman System 6300 high performance amino acid analyser. The instrument was operated with a cation exchange column and eluted with citrate buffers using elution sequences recommended by the manufacturer.

Estimation of liver GSH. Rats dosed with DEM, BSO or OTZ were killed at specified time intervals by an overdose of pentobarbital. A small portion of the liver was removed and frozen immediately in liquid nitrogen. Liver GSH levels were measured as described elsewhere [10].

RESULTS

Total radioactivity present in livers of control, BSO pretreated and DEM pretreated rats was monitored up to 12 hr after a single i.p. dose of 100 mg/kg of [^{14}C]2MF (Fig. 1a). In animals that received [^{14}C]2MF alone, the maximal level of radioactivity in liver was observed 4 hr after dosage. The total amount of label, however, was decreased at the end of 12 hr. Livers of animals pretreated with BSO had consistently lower levels of radioactivity at all time points. Livers of rats pretreated with DEM had lower amounts of total radioactivity 1 hr after dosage, compared to unpretreated rats. However, at the end of 4 hr, DEM pretreated and control rats had the same amount of label in the liver (Fig. 1a). By the end of 8 and 12 hr after a single dose of [^{14}C]2MF, livers from DEM-pretreated rats had significantly higher amounts of label than those from the group of that received [^{14}C]2MF alone.

Total methanol-extractable radioactivity in liver was also measured over a period of 12 hr after administration of [^{14}C]2MF in control, DEM or BSO pretreated rats (Fig. 1b). DEM pretreated rats had lower levels of methanol extractable radioactivity 1 hr after dosage, but at 4, 8 and 12 hr after dosage, there was no significant difference as compared with the group that received [^{14}C]2MF alone. Prior administration of BSO had no effect on the distribution of methanol-extractable label in the liver; there was no difference between BSO-pretreated groups and the control animals that received only [^{14}C]2MF.

In contrast to the above results, the covalent binding of the label derived from [^{14}C]2MF to liver protein was markedly affected by the pretreatments with DEM or BSO (Fig. 2). Prior administration of BSO caused a striking decrease in the covalent binding of the label to liver protein measurable 1 hr after administration of [^{14}C]2MF; the decrease was even more prominent at 4, 8 and 12 hr. Prior treatment with DEM, which strikingly depleted hepatic GSH (data not shown) to a degree consistent with previous studied [4], did not cause a marked change in the covalent binding to liver proteins up to 4 hr after dosage. Thereafter, at the end of 8 and 12 hr, covalent binding to liver proteins was significantly higher in DEM pretreated groups as compared to rats that received [^{14}C]2MF alone.

Covalent binding of the reactive metabolite to DNA was also significantly lower in the livers of BSO treated rats at all time points observed (Fig. 3a and 3b). In rats that received only [^{14}C]2MF, maximal hepatic covalent binding per mg DNA was observed 4 hr after dosage and decreased thereafter. Prior administration of DEM did not seem to cause any change in the amount of label bound per mg DNA at 4, 8 and 12 hr compared to the rats that received [^{14}C]2MF alone. However, there was an

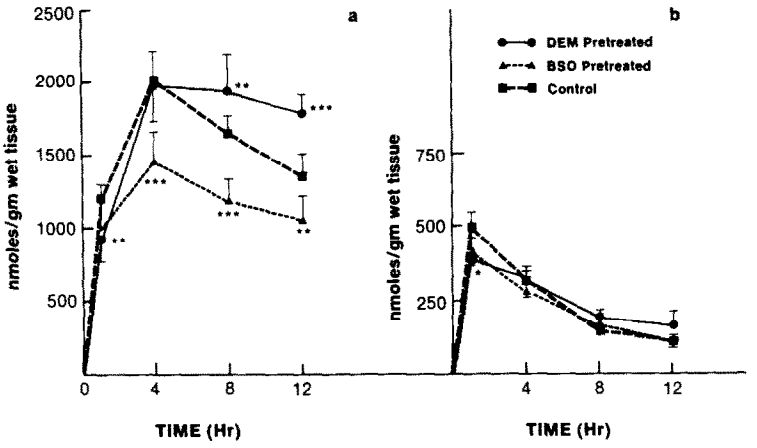

Fig. 1. Distribution of (a) total radioactivity and (b) methanol extractable radioactivity in the liver following a single administration of 100 mg/kg of [^{14}C]2MF. Rats were pretreated with BSO, DEM or vehicle as described in Methods. Rats were killed 1, 4, 8 and 12 hr after administration of [^{14}C]2MF. Values are expressed as mean ± SD (N = 4). Mean values indicated were significantly different from control (**P < 0.01 and ***P < 0.001).

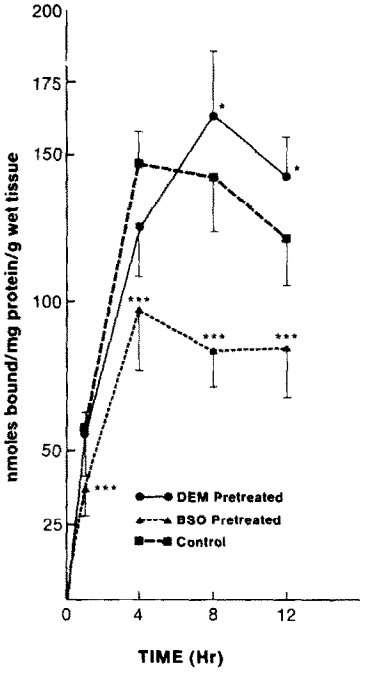

Fig. 2. Effect of pretreatment of BSO or DEM on the covalent binding of [^{14}C]2MF to liver proteins. After pretreatment with BSO or DEM as described in Methods, rats were administered 100 mg/kg of [^{14}C]2MF and killed 1, 4, 8 and 12 hr after dosage. Values are represented as mean ± SD (N = 4). Mean values indicated were significantly different from control (*P < 0.05 and ***P < 0.001).

increase in DNA content in the liver of rats (~130%) that received both DEM and [^{14}C]2MF, and the covalent binding to DNA per gram of liver was higher in DEM-pretreated groups as compared to controls (Fig. 3a), at 8 and 12 hr after dosage.

Administration of OTZ, an inducer of liver GSH, prior to a single dose of [^{14}C]2MF caused increased levels of total radioactivity as compared to control (Fig. 4a). However, s.c. administration of both OTZ and BSO prior to [^{14}C]2MF resulted in a significantly lower amount of total radioactivity in the liver. The pretreatments, however, had no effect on the amount of methanol extractable radioactivity; all three groups, the control, OTZ-pretreated and OTZ- and BSO-pretreated contained comparable amounts of the label (Fig. 4b).

Prior treatment with OTZ caused a large increase in the amount of label that was bound covalently to liver proteins, while administration of OTZ and BSO prior to [^{14}C]2MF caused a marked decrease in the covalent binding to liver proteins as compared to control animals (Fig. 5a). However, the covalent binding to DNA was decreased by both of the pretreatments (Fig. 5b). Although pretreatment with OTZ increased covalent binding to proteins, it decreased the covalent binding to DNA. Administration of BSO before dosage with [^{14}C]2MF resulted in a decrease in the covalent binding to both protein and DNA.

Serum GPT levels were estimated 24 hr after a single i.p. dose of 100 mg/kg body weight of 2MF. Administration of 2MF caused an increase in serum GPT levels as compared to rats that received vehicle alone. Prior administration of DEM or OTZ potentiated the hepatotoxicity of 2MF; the serum GPT levels were markedly elevated compared to the rats that received only 2MF (Fig. 6). Prior administration of the GSH synthesis inhibitor, BSO, resulted in markedly reduced serum GPT levels. A similar effect was noticed when both OTZ and BSO were given s.c. before a single i.p. dose of 100 mg/kg of 2MF. Administration of OTZ, BSO or DEM alone had no effect on the serum GPT levels (data not shown).

Liver GSH levels were monitored over a period of 4 hr following a single s.c. dose of 1000 mg/kg of

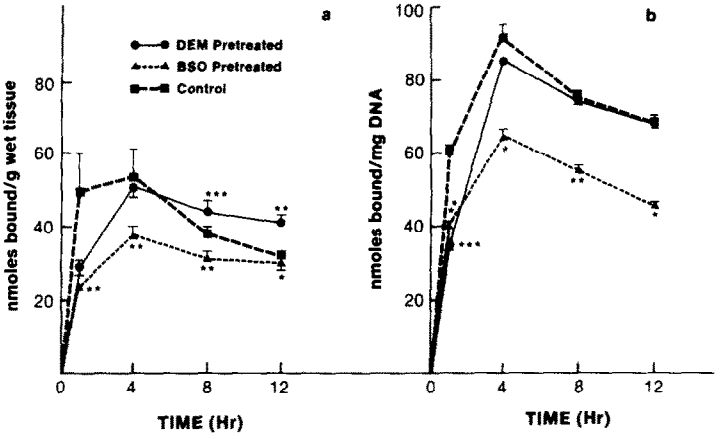

Fig. 3. Covalent binding of [^{14}C]2MF to liver DNA in control, BSO or DEM pretreated rats, expressed as (a) per g liver and (b) per mg DNA. After pretreatment with DEM or BSO (see Methods) rats were administered 100 mg/kg of [^{14}C]2MF i.p. and killed at 1, 4, 8 and 12 hr. Values are mean ± SD of four rats. Mean values indicated were significantly different from control (*$P < 0.05$ and ***$P < 0.001$).

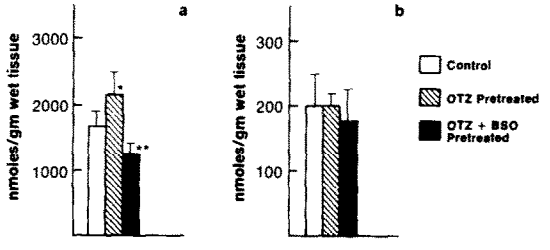

Fig. 4. Distribution of (a) total radioactivity and (b) methanol extractable radioactivity in liver following a single administration of 100 mg/kg of [^{14}C]2MF. Rats were pretreated with OTZ or OTZ and BSO as described in Methods. Rats were killed 8 hr after the administration of [^{14}C]2MF. Values are expressed as mean ± SD ($N = 4$). Mean values indicated were significantly different from control (*$P < 0.05$, **$P < 0.01$ and $P < 0.001$).

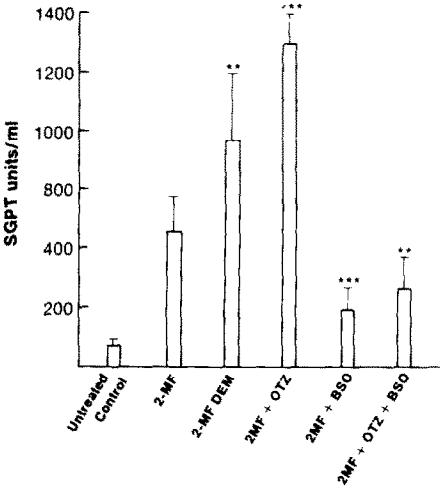

Fig. 6. Serum GPT levels after a single dose of 100 mg/kg of 2MF with and without pretreatments as described in Methods. Rats were killed 24 hr after administration of 2MF. Values are expressed as mean ± SD ($N = 4$). Mean values indicated were significantly different from control (**$P < 0.01$ and ***$P < 0.001$).

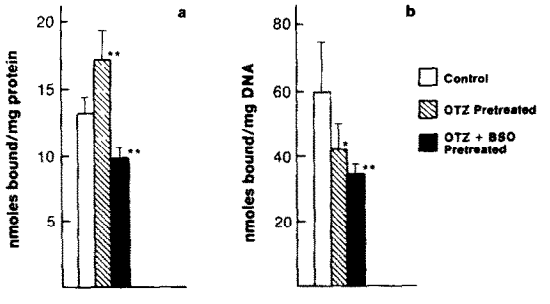

Fig. 5. Effect of pretreatment with OTZ and OTZ and BSO on the covalent binding of [^{14}C]2MF to liver (a) proteins and (b) DNA. Pretreatment was given as described in Methods, followed by a single i.p. dose of 100 mg/kg body weight of [^{14}C]2MF. Rats were killed 8 hr after administration of [^{14}C]2MF. Values are represented as mean ± SD ($N = 4$) (*$P < 0.05$ and **$P < 0.01$).

OTZ (Fig. 7). Within 15 min after dosage, there was a significant increase ($P < 0.01$) in the liver GSH levels. It was for this reason that a very short pretreatment time (5 min) was used in experiments, where both OTZ and 2MF were administered. Maximal levels of GSH in the liver were reached 2 hr after dosage (158% of control); liver GSH was significantly higher than control levels for up to 4 hr after dosage.

Liver GSH and plasma cystine were measured at various time intervals following a single s.c. dose of 900 mg/kg body weight of BSO (Fig. 8). Plasma cystine levels were measured to provide an indirect

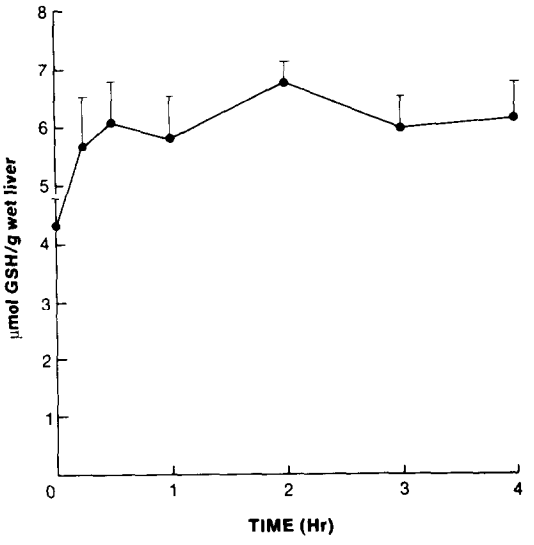

Fig. 7. Liver GSH levels after a single s.c. dose of 1000 mg/kg of OTZ. Groups of four rats were killed at various time intervals after dosage. Values are expressed as mean ± SD (N = 4).

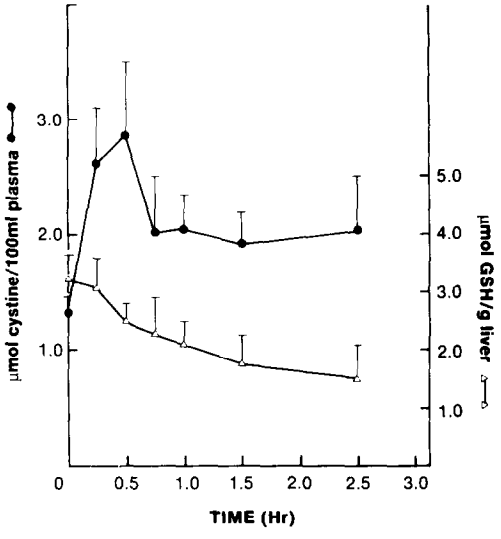

Fig. 8. Liver GSH (△—△) and plasma cystine levels (●—●) after a single s.c. dose of 900 mg/kg of BSO. Groups of four rats were killed at various intervals after dosage. Values are expressed as mean ± SD (N = 4).

indication of changes in cysteine levels in the liver. Direct measurement of liver cystine or cysteine levels proved impractical. Plasma cystine levels at 15 and 30 min after dosage with BSO were nearly two times control levels, and remained significantly higher than control for up to 2.5 hr after administration of BSO. There was concurrent depletion of liver GSH, at the end of 2.5 hr, liver GSH levels were only 50% of control (Fig. 8).

Administration of a single dose of BSO caused a

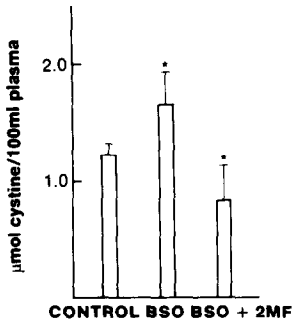

Fig. 9. Plasma cystine levels after a single dose of BSO or BSO and 2MF. An i.p. dose of 100 mg/kg of [^{14}C]2MF was administered 1.5 hr after a s.c. dose of 900 mg/kg of BSO. All animals were killed 2 hr after the administration of [^{14}C]2MF. Values are expressed as mean ± SD (N = 4). Mean values indicated were significantly different from control (*$P < 0.05$).

significant increase (137% over control) in plasma cystine levels over that of control animals that received vehicle alone. Administration of a single dose of 100 mg/kg of 2MF, 1.5 hr after BSO, lowered the plasma cystine levels; they were only 66% of control (Fig. 9). Thus, administration of 2MF not only counteracted the increase in plasma cystine caused by BSO, but also significantly lowered plasma cystine as compared to control. BSO-mediated increase in cysteine levels are known to be transient [11] and the cysteine levels return to control levels by 4 hr after dosage. Hence, rats were killed 2 hr after 2MF administration, that is 3.5 hr after BSO dose. The time point chosen was such that it permitted adequate time for 2MF metabolism to AA by hepatic cytochrome P450 and plasma cysteine levels also remained high in animals following BSO dose.

[^{14}C]2MF was administered 4 and 6 hr after s.c. dose of BSO, instead of the 1.5 hr pretreatment time used in earlier experiments. The rats were killed 2 hr after administration of 2MF and the covalent binding to liver proteins was measured. The hepatic covalent binding was not affected by pretreatment with BSO (Fig. 10). The protective effect observed when 2MF was administered 1.5 hr after BSO was not evident if the pretreatment time was increased to 4 or 6 hr.

DISCUSSION

The protective role of endogenous GSH against damage by reactive metabolites has been well established in hepatic [12], as well as extrahepatic tissues [4]. Depletion of GSH by direct conjugation with diethyl maleate typically increases the covalent binding of a reactive metabolite to tissue macromolecules and thereby enhances the toxicity caused by the parent compound [12]. Such an effect was observed with 2MF. Prior administration of DEM increased the covalent binding of the label to liver proteins and caused a marked enhancement in hepatotoxicity (as indicated by the rise in serum

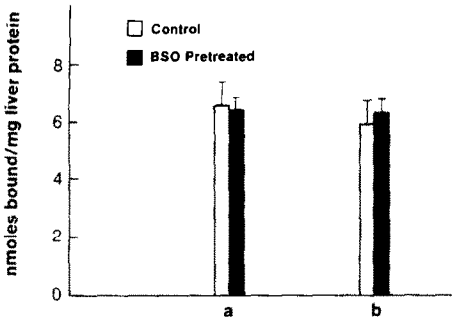

Fig. 10. Effect of BSO on the covalent binding of [^{14}C]2MF to rat liver protein. (a) [^{14}C]2MF was administered 4 hr after a single s.c. dose of 900 mg/kg of BSO. (b) [^{14}C]2MF was administered 6 hr after a single s.c. dose of 900 mg/kg of BSO. Rats were killed 2 hr after administration of [^{14}C]2MF. Values are mean ± SD (N = 4).

GPT levels). Pretreatment with DEM seemed to alter the distribution of 2MF at earlier time intervals after dosage; there was a significantly lower amount of label 1 and 4 hr after dosage, but at the end of 8 and 12 hr, there was no difference in the amount of extractable label between DEM-pretreated and control livers. Thus, the enhanced toxicity and covalent binding of [^{14}C]MF caused by DEM does not appear to be due to an alteration in the tissue distribution of the parent [^{14}C]2MF.

Varying pretreatment time has been used for the different modulators of hepatic GSH. This is based on the pharmacokinetics of the compounds used. A short pretreatment time of 5 min was used for OTZ, since OTZ induces liver GSH levels within 15 min. This induction is sustained for 4 hr and by 8 hr, GSH levels return to normal [13]. Similarly, administration of DEM causes a rapid fall in liver GSH, within 0.5 hr, and by 6 hr, the GSH levels return to control levels (data not shown). Hence, the pretreatment time used for studies with DEM was 0.5 hr. In contrast, dosage with BSO results in depletion of hepatic GSH by 50%; 1.5–2 hr after treatment. The GSH levels are restored 12 hr after administration of BSO (data not shown). Hence, pretreatment time of 1.5 hr was used for studies wherein the effect of BSO on 2MF toxicity was examined. Further, the maximal covalent binding of the radiolabelled 2MF administered alone, was observed 4–8 hr after dosage (Fig. 2). In view of this fact, animals were killed 8 hr after 2MF administration, in experiments wherein the combined effect of BSO and OTZ on 2MF toxicity was examined.

Paradoxical effects were, however, observed when BSO was administered before 2MF. BSO markedly decreased both the hepatic covalent binding of [^{14}C]MF and the hepatotoxicity caused by administration of 2MF. BSO is an inhibitor of γ-glutamyl cysteine synthetase, a key enzyme in GSH biosynthesis, and hence lowers liver GSH *in vivo* [14]. BSO does not have any effect on the conjugating enzymes or mixed-function oxidase activity in 105,000 *g* supernatants or microsomes prepared from liver of mice administered 1600 mg/kg of BSO [15]. Thus, it seems unlikely that BSO significantly inhibits the bioactivation of 2MF by the hepatic mixed-function oxidase system. Further, BSO does not appear to alter the distribution of parent [^{14}C]2MF in the rat. The amount of methanol-extractable radioactivity in the liver was the same in control and BSO-pretreated rats. Thus, the protection against hepatotoxicity afforded by BSO is not likely due to inhibition of metabolism of 2MF or alteration in the distribution of the unmetabolized compound. Prior administration of BSO caused a significant increase in urinary excretion of labelled metabolites (20% over control) as compared to rats that received [^{14}C]2MF alone (data not shown). These results seem to suggest that pretreatment with BSO affords protection against the damage caused by reactive metabolites of 2MF, although the levels of the endogenous protective nucleophile, GSH, are lowered. All these findings appear consistent with an enhanced detoxification of 2MF in BSO treated rats, probably through conjugation resulting in increased urinary excretion of the label. Unchanged 2MF was not detected in the urine, which was expected considering the extremely volatile nature of the parent compound.

The effect of various nucleophiles has previously been examined on the *in vitro* covalent binding of 2MF in rat liver microsomal systems in the presence of oxygen and NADPH [2]. Cysteine was found to be a better trapping agent of acetyl acrolein, the reactive metabolite of 2MF, than GSH. Cysteine could completely prevent the covalent binding of the label from [^{14}C]2MF to microsomal protein. Cysteine forms a stable cyclic conjugate with α,β unsaturated aldehydes [16] which would explain the efficient trapping ability of cysteine over other nucleophiles such as GSH and *N*-acetyl cysteine.

In light of the above, an increased tissue content of cysteine, which could be expected to occur in concert with inhibition of GSH biosynthesis by BSO, could conceivably account for the enhanced detoxification of 2MF *in vivo*. Because of its facile oxidation to cystine, we were not able to measure tissue cysteine directly. We therefore assayed plasma cystine as an indirect measure of tissue cysteine status. BSO had been previously shown to produce an increase in plasma cysteine in mice [11]. A similar result was obtained here in rats (Fig. 8); plasma cysteine remained significantly elevated up to 2.5 hr after dosage with BSO. Administration of 2MF after a s.c. dose of BSO resulted in substantially lower plasma cysteine levels than control values from animals receiving BSO alone. Further pretreatment with BSO protected against hepatotoxicity of 2MF, only if administered 1.5 hr before 2MF. BSO did not protect against 2MF induced hepatotoxicity if 2MF was administered 4 or 6 hr after BSO treatment. The increase in plasma cysteine following BSO treatment is known to be transient [11].

Prior administration of BSO potentiates the hepatotoxicity of acetaminophen [16]. It has also been observed that the anticancer drug, 1-(2-chlorethyl)-3-(*trans*-4-methyl-cyclohexyl)-1-nitrosourea, methyl-CCNU, produced hepatotoxicity in rats pretreated with BSO; liver toxicity is not observed when methyl-CCNU is given alone [6]. Thus, in

comparison to these precedents, the observed protection by BSO against the hepatotoxicity of 2MF is paradoxical, i.e. opposite to that which might have been expected, and the mechanism of the effect is as yet unclear. Isolation and detailed characterization of urinary conjugates of 2MF after the BSO pretreatment would help provide an answer. Increased urinary excretion of the label is observed and preliminary evidence shows that the urinary metabolite profile of BSO pretreated rats is markedly different from the control rats, as might be expected if BSO caused a shift towards more metabolism via conjugation with cysteine. Characterization of the urinary metabolites of 2MF was attempted, but the definitive identification of metabolites has thus far been precluded due to difficulty in separation and sufficient purification of the many different urinary metabolites formed.

Although there was a decrease in the covalent binding to liver protein following pretreatment with BSO, the covalent binding to lung and kidney proteins was significantly increased (data not shown). Thus, the paradoxical effect of BSO pretreatment was observed only in the liver and not in other tissues.

5-Oxo-L-prolinase is a key enzyme in GSH biosynthesis and converts 5-oxoproline to L-glutamate that is required for GSH synthesis. OTZ is a substrate for 5-oxo-L-prolinase, resulting in the formation of cysteine, which in turn is used for the biosynthesis of GSH. The administration of OTZ, thus, stimulates the formation of hepatic GSH [13]. Administration of OTZ protects against acetaminophen hepatotoxicity; OTZ is converted to L-cysteine thereby promoting the synthesis of GSH which is the actual protectant [17]. It was also observed that the effect of OTZ in increasing hepatic GSH was prevented by coadministration of BSO. Although we found increased cystine levels after BSO pretreatment, administration of OTZ did not further increase cystine levels (data not shown). Moreover, OTZ has not been shown to increase plasma cystine levels as is observed with BSO. Administration of OTZ results in selective increase of hepatic GSH concentration, while cysteine levels are not changed [18]. OTZ administration increases cysteine levels in brain probably due to the low turnover of GSH in this tissue [19].

Although administration of OTZ increased covalent binding to liver protein and enhanced the hepatotoxicity of 2MF, administration of OTZ and BSO decreased covalent binding to liver protein and protected against hepatotoxicity of 2MF (Fig. 5a).

The GSH conjugates of α,β-unsaturated carbonyl compounds vary in their stability [20]. The GSH conjugate of acetyl acrolein, the *in vitro* microsomal metabolite of 2MF, has been reported to be unstable and not readily isolatable [21]. Although GSH initially forms a conjugate with the reactive metabolite of 2MF, the relative instability of this conjugate probably precludes any protection that might otherwise have been afforded by conjugation with GSH. It is also interesting to note that although OTZ treatment increases covalent binding to liver protein, it decreases covalent binding to DNA. Further, pretreatment with OTZ had no effect on the covalent binding to lung, kidney, blood or heart protein (data not shown). Depletion of liver GSH by DEM or promotion of GSH synthesis by OTZ both cause similar effects on 2MF toxicity, which seems to indicate that a GSH threshold exists, above or below which there is a stimulation of the toxic effects caused by the reactive intermediates of 2MF. Since cysteine acts as a better nucleophilic trapping agent for the reactive metabolite of 2MF, cysteine levels within the cell are also likely to play an important role in 2MF toxicity.

The toxicity exhibited by 2MF did not seem to necessarily require extensive prior depletion of GSH. At a dose of 100 mg/kg, 2MF caused hepatic damage although the GSH levels were reduced by only 20% at this dose [1]. In addition to the depletion of tissue GSH, DEM pretreatment can lead to inhibition or induction of hepatic mixed function oxidases and changes in the tissue distribution of xenobiotics [8]. This may explain the lowered levels of radioactivity seen in the liver at early time points following treatment of animals with 2MF and DEM.

Development of resistance of tumors to alkylating agents and irradiation has been suggested to be related to increased intracellular GSH levels [22]. Depletion of intracellular GSH of tumor cell lines by BSO has been shown to sensitize the cells to irradiation [23] and certain anticancer drugs [24]. Such studies have led to a growing interest in the use of BSO as a chemosensitizing agent in cancer chemotherapy. The use of OTZ as an intracellular cysteine delivery system that would protect against toxicity by promoting GSH synthesis has also been suggested [25]. The studies described in this paper, however, present instances where BSO or OTZ may have an effect that is opposite of the predicted one. The precise molecular mechanisms underlying these paradoxical observations are as yet unclear. However, it demonstrates that it is necessary to have an understanding of the mechanism of action of the anticancer drugs and the specific effect of the modulators of intracellular GSH levels in target organ toxicity before the use of combination therapy can be rationally made in an actual clinical situation.

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