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BIOACTIVATION OF *S*-METHYL *N,N*-DIETHYLTHIOLCARBAMATE TO *S*-METHYL *N,N*-DIETHYLTHIOLCARBAMATE SULFOXIDE

IMPLICATIONS FOR THE ROLE OF CYTOCHROME P450

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Abstract—Diethyldithiocarbamate (DDTC), diethyldithiocarbamate methyl ester (DDTC-Me), *S*-methyl *N,N*-diethylthiolcarbamate (DETC-Me) and *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide (DETC-MeSO) are all metabolites of disulfiram. All inhibit rat liver low K_m aldehyde dehydrogenase (ALDH) *in vivo*, with the order of potency being DETC-MeSO > DETC-Me > DDTC-Me > DDTC. Studies were carried out both *in vivo* and *in vitro* to further investigate the role of bioactivation as a requirement for the action of disulfiram as a liver ALDH inhibitor. The cytochrome P450 inhibitor 1-benzylimidazole (NBI) was employed as a pharmacological tool to study the metabolism of DETC-Me to DETC-MeSO. Administration of NBI to rats prior to DETC-Me treatment blocked the inhibition of liver mitochondrial low K_m ALDH by DETC-Me. This was accompanied by an increase in plasma DETC-ME and a decrease in plasma DETC-MeSO. Pretreatment of rats with NBI prior to DETC-MeSO administration did not block the inhibition of liver mitochondrial low K_m ALDH by DETC-MeSO. In *in vitro* studies, the inclusion of NBI in an incubation containing rat liver microsomes, mitochondria and an NADPH-generating system blocked the formation of DETC-MeSO and inhibition of liver mitochondrial low K_m ALDH by DETC-Me. DETC-MeSO was found to be a potent inhibitor of rat liver mitochondrial low K_m ALDH both *in vivo* and *in vitro*. The data suggest that the metabolism of DETC-Me to DETC-MeSO is mediated by cytochrome P450, and that inhibition of cytochrome P450 by inhibitors such as NBI block the inhibition of low K_m ALDH by DETC-Me.

The pharmacological basis for the clinical use of disulfiram is its inhibition of liver aldehyde dehydrogenase (ALDH)‡ and the subsequent onset of a disulfiram–ethanol reaction (DER) after ethanol ingestion. Disulfiram metabolism has been studied extensively by many investigators. Disulfiram is reduced rapidly *in vivo* to diethyldithiocarbamate (DDTC) by plasma glutathione reductase [1] and albumin [2]. DDTC is glucuronidated to its *S*-glucuronide [3–6], and *S*-methylated to form diethyldithiocarbamate-methyl ester (DDTC-Me) in rats [7], mice and dogs [5], and humans [6, 8]. Other metabolites of disulfiram also have been reported, and a metabolic scheme has been outlined in detail [9]. The disulfiram metabolites reported to inhibit rat liver mitochondrial low K_m ALDH *in vivo* are given in Fig. 1.

The chemical species responsible for liver mitochondrial low K_m ALDH inhibition after the administration of disulfiram is controversial [10]. The seminal finding that DDTC-Me inhibits rat liver mitochondrial low K_m ALDH *in vivo* [11] suggested that disulfiram requires bioactivation in order to inhibit the low K_m ALDH present in liver mitochondria. *S*-Methyl *N,N*-diethylthiolcarbamate (DETC-Me) was identified subsequently as a disulfiram metabolite [12], and its low K_m ALDH inhibitory profile was described by Hart *et al.* [13]. The identification of DETC-Me in both rats and humans as a disulfiram metabolite also was reported independently by Johansson *et al.* [14]. However, DETC-Me is relatively ineffective as an ALDH inhibitor *in vitro*, and furthermore, the cytochrome P450 inhibitor 1-octylimidazole (NOI) blocks the inhibition of rat liver mitochondrial low K_m ALDH by disulfiram, DDTC, DDTC-Me, and DETC-Me [15]. Those studies suggested that another metabolite was responsible for the action of disulfiram as an ALDH inhibitor *in vivo*. This led to the discovery by Hart and Faiman [16] that *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide (DETC-MeSO) was a natural metabolite of disulfiram and a very potent inhibitor of rat liver mitochondrial low K_m ALDH both *in vivo* and *in vitro*. In rats, 21.5 μmol/kg (3.5 mg/kg, i.p.) DETC-MeSO inhibited liver mitochondrial low K_m ALDH by 50% (ID_{50}). *In vitro*, inhibition of the enzyme was rapid, and 750 nM DETC-MeSO inhibited the low K_m ALDH by 50% (IC_{50}). It was therefore proposed that DETC-MeSO is the disulfiram metabolite responsible for *in vivo*

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‡ Abbreviations: ALDH, aldehyde dehydrogenase; DDTC, diethyldithiocarbamate; DDTC-Me, diethyldithiocarbamate-methyl ester; DETC-Me, *S*-methyl *N,N*-diethylthiolcarbamate; DETC-MeSO, *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide; NBI, 1-benzylimidazole; and DER, disulfiram–ethanol reaction.

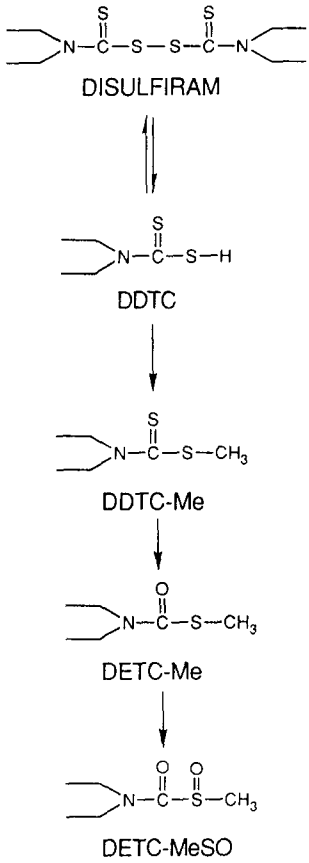

Fig. 1. Proposed scheme for disulfiram metabolites inhibiting rat liver mitochondrial low K_m ALDH *in vivo*.

inhibition of rat liver mitochondrial low K_m ALDH [16]. The present studies, in conjunction with the studies by Madan *et al.* [17], provide additional support for the hypothesis that DETC-MeSO is the disulfiram metabolite responsible for rat liver mitochondrial low K_m ALDH inhibition, and that cytochrome P450 plays an important role in this bioactivation.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (250–400 g), bred from a resident colony maintained in the Animal Care Unit at The University of Kansas, were used throughout the studies. The rats were maintained on a 12-hr light–dark cycle with access to lab chow and water *ad lib.* until the night before an experiment, at which time food was removed. Animals were fasted for 12 hr prior to drug administration.

Drug administration. 1-Benzylimidazole (*N*-benzylimidazole; NBI) was used because of its commercial availability rather than 1-octylimidazole (*N*-octylimidazole; NOI), which had been used previously [15]. The dissolution of NBI (Aldrich Chemical Co., St. Louis, MO) in saline was aided by the addition of a few drops of 4 N HCl. Rats were dosed with NBI (20 mg/kg; 3 mL/kg, i.p.) or 3 mL/kg saline i.p. 30 min prior to the administration of DETC-Me or DETC-MeSO. DETC-Me was synthesized as described by Hart *et al.* [13] and was administered at a dose of 126 μmol/kg (18.6 mg/kg) i.p. in corn oil (1 mL/kg). Control rats received 1 mL/kg corn oil i.p. DETC-MeSO was synthesized as described by Hart and Faiman [16], and was administered at a dose of 32 μmol/kg (5.2 mg/kg) i.p. in polyethylene glycol 200 (PEG 200, 1 mL/kg). Different vehicles were used for DETC-Me and DETC-MeSO, because DETC-MeSO is insoluble in corn oil and DETC-Me is insoluble in PEG 200. Control rats received 1 mL/kg PEG 200.

Aldehyde dehydrogenase assay. Drug-treated and control rats were anesthetized with CO_2 and decapitated. A portion of liver was removed and homogenized in 0.25 M sucrose, and the mitochondria were isolated by differential centrifugation. Then the mitochondria were solubilized with sodium deoxycholate, and low K_m ALDH activity was determined by the method of Tottmar *et al.* [18].

Microsomal activation of DETC-Me in vitro. Mitochondria were isolated from the liver of an untreated rat as described above. Rat liver microsomes were isolated and incubated with mitochondria in the presence of an NADPH-generating system as described by Yourick and Faiman [15]. DETC-Me was added to the incubation in 100 μL ethanol to give a final concentration of 200 μM. NBI, when present, was added in 10 μL acetonitrile to give a final concentration of 1 mM. Control incubations contained 100 μL ethanol and 10 μL acetonitrile.

Determination of DETC-Me and DETC-MeSO in vitro *by HPLC.* The incubation mixture contained 200 μM DETC-Me, microsomes and an NADPH-generating system. After incubation at 37° for 60 min, the reaction was terminated by the addition of an equal volume of acetonitrile containing 1 μg/mL ethiolate sulfoxide as the internal standard. The precipitated proteins were removed by centrifugation, and 0.5 mL of the supernatant and 1 mL methylene chloride were mixed vigorously for 60 sec. The organic phase was isolated and removed under a stream of nitrogen, and the residue was dissolved in the mobile phase. DETC-Me and DETC-MeSO concentrations were determined by HPLC on a Beckman/Altex 2 mm × 25 cm C18 reversed-phase column. The mobile phase was 20:80 acetonitrile:water, and a flow rate of 0.2 mL/min was employed. Detection was carried out at 215 nm.

Determination of plasma DETC-Me and DETC-MeSO by HPLC. Drug-treated rats were anaesthetized with CO_2 and decapitated, and trunk blood was collected. The plasma was isolated and 1 mL extracted (Labquake shaker) for 15 min with 5 mL methylene chloride which contained 200 ng/mL ethiolate sulfoxide as the internal standard. The organic phase was isolated and removed under a stream of nitrogen. The residue was dissolved in 20:80 acetonitrile:water, and DETC-Me and DETC-MeSO concentrations were determined by HPLC as described above.

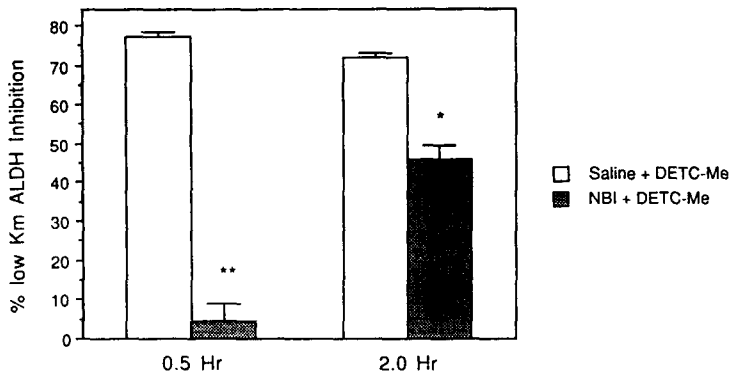

Fig. 2. Effect of 1-benzylimidazole (NBI) on *in vivo* rat low K_m ALDH inhibition by DETC-Me. Saline (3 mL/kg, i.p.) or NBI (20 mg/kg, i.p.) was administered 30 min prior to DETC-Me (126 μmol/kg; 18.6 mg/kg, i.p.). The rats were killed either 0.5 or 2 hr later, and low K_m ALDH activity was determined. The data for each group represent the means ± SEM for four rats. The control value for low K_m ALDH activity was 25.8 ± 1.46 nmol NADH/min/mg mitochondrial protein. Key: (*) $P < 0.001$, and (**) $P < 0.0001$ when compared with the saline control.

Statistical analyses. Differences between group means were determined using a Student's two-tailed *t*-test.

RESULTS

In vivo *studies.* Inhibition of rat liver mitochondrial low K_m ALDH by DETC-Me was blocked almost completely in rats treated with NBI and killed 30 min after DETC-Me, whereas ALDH inhibition was only partially blocked in rats treated with NBI and killed 2 hr after DETC-Me administration (Fig. 2). Since 1-arylimidazoles such as NBI are non-specific competitive reversible inhibitors of cytochrome P450 [19], inhibition of cytochrome P450 by NBI would be expected to be partially reversed during the 2-hr interval. Thus, more DETC-MeSO would be formed from DETC-Me producing a greater degree of mitochondrial low K_m ALDH inhibition. The importance of cytochrome P450 in the oxidation of DETC-Me to DETC-MeSO is illustrated by the data shown in Fig. 3. Rats treated with NBI 30 min prior to DETC-Me administration exhibited almost a 4-fold increase in plasma DETC-Me compared with rats not receiving NBI (Fig. 3A). This is consistent with the finding that after DETC-Me administration, a large concentration of DETC-MeSO was found in plasma, whereas if NBI was given 30 min before DETC-Me administration, only a trace amount of DETC-MeSO was detected (Fig. 3B). These observations are in agreement with the data that liver mitochondrial low K_m ALDH was inhibited by DETC but not inhibited in rats first treated with NBI, since formation of DETC-MeSO was blocked (Fig. 2). Cytochrome P450 does not play a role in the further bioactivation of DETC-MeSO since liver mitochondrial low K_m ALDH was inhibited to the same degree whether rats were or were not pretreated with NBI prior to DETC-MeSO administration (Fig. 4).

In vitro *studies.* Studies also were carried out *in vitro* to verify the *in vivo* findings. Incubation of DETC-Me with rat liver mitochondria, microsomes, and an NADPH-generating system for 60 min resulted in 75% inhibition of liver mitochondrial low K_m ALDH, whereas the liver mitochondrial low K_m ALDH was inhibited only 15% when NBI was added to the incubation (Fig. 5). Furthermore, the rate of metabolism of DETC-Me and the corresponding rate of DETC-MeSO formation were not only similar, but when NBI was added to the incubation, the rate of DETC-Me metabolism and the rate of DETC-MeSO formation both decreased in a proportional manner (Fig. 6).

The data from these *in vivo* and *in vitro* studies are consistent with the concept that formation of DETC-MeSO was required for the inhibition of rat liver mitochondrial low K_m ALDH, and that DETC-MeSO is a potent inhibitor of rat liver mitochondrial low K_m ALDH both *in vivo* and *in vitro*, confirming the previous findings of Hart and Faiman [16]. Inhibition of cytochrome P450 blocked the formation of DETC-MeSO from DETC-Me and prevented the inhibition of rat liver mitochondrial low K_m ALDH. This suggested that this oxidative mechanism plays an important role in the formation of DETC-MeSO and its action as a low K_m mitochondrial ALDH inhibitor.

DISCUSSION

DETC-MeSO was found to be a potent inhibitor of rat liver mitochondrial low K_m ALDH both *in vivo* and *in vitro*; this finding is consistent with the proposal that DETC-MeSO is the disulfiram metabolite responsible for the inhibition of this enzyme [16]. The observation that plasma DETC-Me was increased markedly while only trace amounts of DETC-MeSO were found in rats treated with NBI prior to DETC-Me administration (Fig. 3) is in keeping with the concept that DETC-MeSO must be formed in order for rat liver mitochondrial low K_m ALDH to be inhibited either *in vivo* or *in vitro* (Figs. 2, 3, and 5). Pretreatment of rats with NBI

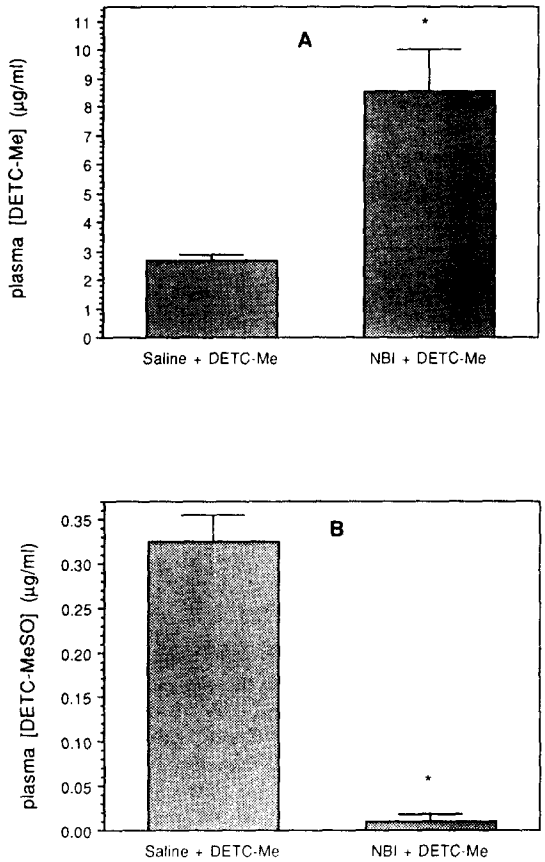

Fig. 3. (A) Effect of 1-benzylimidazole (NBI) on plasma DETC-Me after DETC-Me administration. Saline (3 mL/kg, i.p.) or NBI (20 mg/kg, i.p.) was administered 30 min prior to DETC-Me (126 μmol/kg; 18.6 mg/kg, i.p.). The rats were killed 0.5 hr later, and plasma DETC-Me was determined. The data for each group are the means ± SEM for four rats. Key: (*) $P < 0.001$ when compared with the saline control. (B) Effect of 1-benzylimidazole (NBI) on plasma DETC-MeSO after DETC-Me administration. Saline (3 mL/kg, i.p.) or NBI (20 mg/kg, i.p.) was administered 30 min prior to DETC-Me (126 μmol/kg; 18.6 mg/kg, i.p.). The rats were killed 0.5 hr later, and plasma DETC-MeSO was determined. The data for each group are the means ± SEM for four rats. Key: (*) $P < 0.001$ when compared with the saline control.

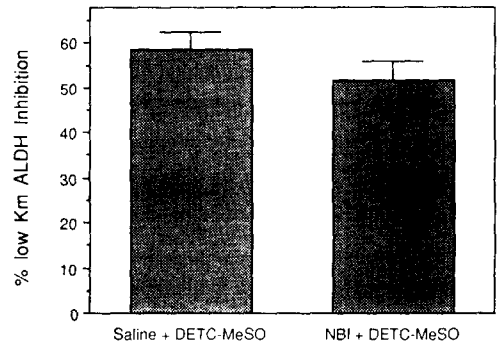

Fig. 4. Effect of 1-benzylimidazole (NBI) on *in vivo* rat low K_m ALDH inhibition by DETC-MeSO. Saline (3 mL/kg, i.p.) or NBI (20 mg/kg, i.p.) was administered 30 min prior to DETC-MeSO (32 μmol/kg; 5.2 mg/kg, i.p.). The rats were killed 0.5 hr later, and low K_m ALDH activity was determined. The data for each group represent the means ± SEM for four rats. The control value for low K_m ALDH activity was 27.2 ± 0.44 nmol NADH/min/mg mitochondrial protein.

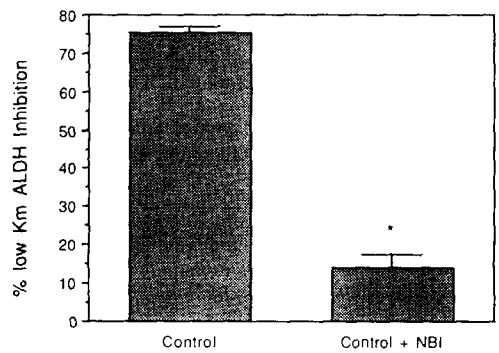

Fig. 5. Effect of 1-benzylimidazole (NBI) on the microsomal activation of DETC-Me and *in vitro* rat mitochondrial low K_m ALDH inhibition. Rat liver mitochondria were incubated with 200 μM DETC-Me, microsomes and an NADPH-generating system in the absence (control) or presence of 1 mM NBI. After 60 min, the mitochondria were isolated, and low K_m ALDH activity was determined. The data for each group represent the means ± SEM for four incubations. The control value for low K_m ALDH activity was 12.2 ± 0.49 nmol NADH/min/mg mitochondrial protein. Key: (*) $P < 0.0001$ when compared with the control.

prior to DETC-MeSO administration had no effect on the inhibition of liver mitochondrial low K_m ALDH inhibition by DETC-MeSO (Fig. 4). Metabolism of DETC-MeSO to a metabolite that also inhibited mitochondrial low K_m ALDH by a mechanism not requiring cytochrome P450 is unlikely. Thiocarbamate sulfoxides are known to be conjugated with reduced glutathione to form *S*-diethylcarbamoyl glutathione and rapidly excreted as the corresponding mercapturic acid [20]. Furthermore, glutathione conjugation reflects an excretory pathway whose products generally lack pharmacological activity. Disulfiram, DDTC, DDTC-Me, DETC-Me and DETC-MeSO have all been identified in rat plasma, all inhibit rat liver mitochondrial low K_m ALDH, and all produce similar dose–response curves [13, 16]. In addition, disulfiram, DDTC, DDTC-Me and DETC-Me treatments all produce a DER (hypotension) in rats after an ethanol challenge [13, 21]. Although DETC-MeSO treatment in rats has only been investigated in a preliminary manner, it too produced a DER in ethanol-challenged rats (unpublished results). The 8-hr ID_{50} in rats for disulfiram, DDTC, DDTC-Me, DETC-Me, and DETC-MeSO is 190.0, 90.0, 95.1, 44.2, and 21.5 μmol/kg, i.p. [13, 16]. Thus, as the

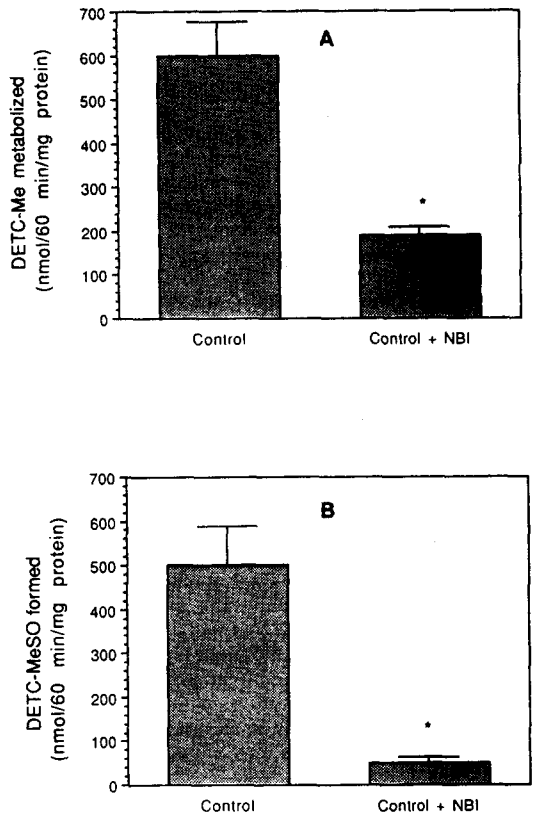

Fig. 6. (A) Effect of 1-benzylimidazole (NBI) on the microsomal metabolism of DETC-Me. DETC-Me (2 mM) was incubated with microsomes and an NADPH-generating system in the absence (control) or presence of 1 mM NBI. One hour later, the DETC-Me concentration in the incubations was determined. The data for each group represent the means ± SEM for four incubations. Key: (*) $P < 0.01$ when compared with the control. (B) Effect of 1-benzylimidazole (NBI) on the microsomal metabolism of DETC-Me to DETC-MeSO. DETC-Me (2 mM) was incubated with microsomes and an NADPH-generating system in the absence (control) or presence of 1 mM NBI. One hour later, the DETC-MeSO concentration in the incubations was determined. The data for each group represent the means ± SEM for four incubations. Key: (*) $P < 0.01$ when compared with the control.

active metabolite is approached (Fig. 1), the respective metabolites become more potent which is to be expected. These data thus support earlier studies [16] that DETC-MeSO is the metabolite proposed to be responsible for the *in vivo* inhibition of rat liver mitochondrial low K_m ALDH by disulfiram.

The suggestion that DETC-MeSO is the active metabolite of disulfiram is inconsistent with studies by Johansson [22]. DETC-Me is a metabolite of disulfiram reported independently by both Johansson *et al.* [14] and Hart *et al.* [12, 13]. Johansson [22] proposed that DETC-Me is the active metabolite of disulfiram, with DETC-Me interacting with ALDH by a mechanism similar to that of suicide inhibitors. Although this suggestion cannot be ruled out completely, this interpretation may not be correct. In those *in vitro* studies employing a partially purified bovine liver low K_m ALDH, Johansson [22] found ALDH to be inhibited only 25% after 24 hr of incubation with 2.76 mM DETC-Me. This low potency does not suggest that DETC-Me is the active metabolite of disulfiram. The active metabolite should be potent *in vitro*, and inhibit the enzyme rapidly, a view also supported by others [10]. Kitson [10] proposed that DETC-Me may be metabolized to bis (diethylcarbamoyl) disulfide (dioxiram). However, this postulated mechanism requires demethylation of DETC-Me to the diethyl-monothiocarbamate ion and subsequent oxidation to dioxiram. Other metabolites also have been proposed as the active metabolite of disulfiram. For example, MacKerell *et al.* [23] suggest that methyldiethylthiocarbamyl disulfide could be formed from disulfiram or DDTC *in vivo* and be responsible for the inactivation of cytoplasmic and mitochondrial ALDH. Kitson [10] alternatively suggested that the diethylmonothiocarbamate ion could be co-oxidized with methanethiol giving the mixed disulfide as proposed by MacKerell *et al.* [23]. Although these are all plausible suggestions, or another natural metabolite of disulfiram responsible for the liver ALDH inhibition may exist, to date none has been identified.

The *in vitro* studies with a microsomal activating system and the cytochrome P450 inhibitor NBI confirmed the findings from the *in vivo* studies, and illustrated the importance of cytochrome P450 in the formation of DETC-MeSO from DETC-Me (Figs. 5 and 6). This is consistent with earlier studies *in vitro* in which DETC-Me inhibited rat liver mitochondrial low K_m ALDH, but only if microsomes were included in the incubation [15]. Addition of NBI to the complete incubation containing both mitochondrial and rat liver microsomes blocked the inhibition of rat liver mitochondrial low K_m ALDH by DETC-Me (Fig. 5). The rate of DETC-Me metabolized and the rate of DETC-MeSO formation was approximately 600 and 500 nmol/60 min/mg protein, respectively (Fig. 6). Although this study was carried out for only one time period (60 min), in other studies carried out from 0 to 30 min a mass balance between the rates of DETC-Me metabolism and DETC-MeSO formation was observed [17]. DETC-MeSO also has been found to be a potent inhibitor of purified beef mitochondrial low K_m ALDH (unpublished results).

In conclusion, these studies provide supporting evidence that disulfiram must be bioactivated in order to inhibit rat liver mitochondrial low K_m ALDH. It is true that disulfiram is a potent inhibitor of mitochondrial low K_m ALDH both *in vitro* and *in vivo*, and reacts rapidly with ALDH [10]. However, the concept first proposed by Hart and Faiman [16] that DETC-MeSO is the active metabolite of disulfiram responsible for the inhibition of rat liver mitochondrial low K_m ALDH seems to be most likely. Cytochrome P450 is important in the metabolism of DETC-Me to DETC-MeSO, although the particular enzyme has not yet been identified. These studies are presently in progress.

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