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# ABILITY OF 1-METHYLTETRAZOLE-5-THIOL WITH MICROSOMAL ACTIVATION TO INHIBIT ALDEHYDE DEHYDROGENASE

JAMES J. LIPSKY*

Division of Clinical Pharmacology, Departments of Medicine and Pharmacology and Molecular Sciences, The Johns Hopkins University, School of Medicine, Baltimore, MD 21205, U.S.A.

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**Abstract**—Antibiotics that contain the 1-methyltetrazole-5-thiol (MTT) leaving group are associated with an adverse effect when alcohol is ingested after their administration. Therefore, the ability of MTT to inhibit an enzyme in alcohol metabolism, aldehyde dehydrogenase (ALDH), was examined. In the absence of microsomes, MTT did not inhibit ALDH obtained from either yeast or rat liver. In the presence of rat hepatic microsomes, MTT was able to inhibit the enzyme from both sources. The characteristics of the inhibition were studied, using the yeast enzyme, and found to be dependent upon the length of incubation with the hepatic microsomes and upon the concentration of MTT. Inhibition required the presence of NADH and was not detected if the microsomes were heat treated. Dilution did not reverse the inhibition. Intact antibiotics which contain the MTT moiety did not cause an inhibition of yeast ALDH unless the antibiotics were first treated with potassium hydroxide and then incubated with microsomes. Inhibition of ALDH activity measured in the mitochondrial plus microsomal fractions of rat liver also required NADH and was prevented by glutathione and heat treatment of the microsomes. These results indicate that microsomal activation of MTT is necessary for inhibition of aldehyde dehydrogenase. The behavior of MTT described here may explain the adverse effect observed if alcohol is ingested following administration of MTT-containing antibiotics.

Several cephalosporin and related antibiotics including cefamandole [1, 2], moxalactam [3, 4] and cefoperazone [5, 6] are associated with a toxic reaction when alcohol is ingested after their administration. This reaction is similar to that seen with disulfiram, which is used for aversion therapy in the treatment of alcoholism. The mechanism of action of disulfiram is believed to be related to its ability to inhibit aldehyde dehydrogenase (ALDH), a key enzyme in alcohol metabolism [7]. Inhibition of ALDH results in an accumulation of acetaldehyde and a consequent systemic toxic reaction.

The antibiotics associated with a "disulfiram-like reaction" all contain a methyltetrazole-thiol (MTT) leaving group (Fig. 1). In animals, MTT-containing antibiotics, as well as the MTT group itself, have been shown to alter alcohol metabolism in a manner consistent with the inhibition of ALDH [8–11]. Aldehyde dehydrogenase activity has also been found to be decreased in the livers of rats that have received MTT [9, 11, 12]. However, *in vitro*, under conditions used by other laboratories, MTT has not been found to be a potent inhibitor of ALDH [9, 13–15]. This result suggests that a metabolite of MTT may be responsible for the inhibition of ALDH. Indeed, a potential metabolite, the disulfide dimer of MTT (Fig. 1), has been found to inhibit this enzyme *in vitro* [14]. Interestingly, it has been suggested that disulfiram, which is a disulfide dimer, may exert its inhibitory effect via a mechanism involving the disulfide bond [7]. To determine whether metabolic activation of MTT is required for the inhibition of ALDH, we employed a microsomal system which is known to metabolize MTT to forms capable of inhibiting the gamma-carboxylation of glutamic acid [16]. This inhibition by MTT, or the metabolites produced in this system, has been shown to play a role in the hypoprothrombinemic effect of MTT-containing antibiotics.

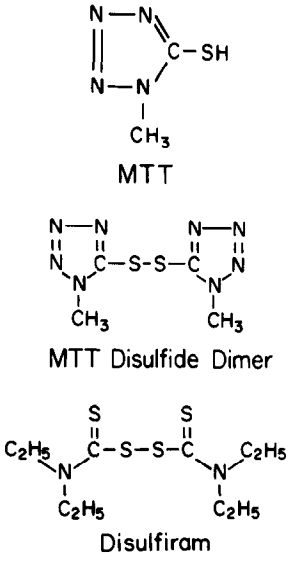


Fig. 1. Structures of MTT, the disulfide dimer of MTT, and disulfiram.

* Address correspondence to: James J. Lipsky, M.D., Osler 527, The Johns Hopkins Hospital, Baltimore, MD 21205, U.S.A.

## METHODS

The activity of purified yeast ALDH, or ALDH activity within microsomal or mitochondrial fractions of rat liver, was assayed spectrophotometrically [17] at 25° as described in the text. Solubilized microsomes were prepared from rat livers obtained from male, Sprague–Dawley (Harlan) rats weighing 200–250 g [16]. Livers were homogenized, 33% (w/v), in 250 mM sucrose/80 mM KCl/25 mM imidazole, pH 7.4, at 2°. The homogenate was centrifuged at 10,000 *g* for 10 min, and the resulting supernatant fraction was centrifuged at 100,000 *g* for 60 min. The pellet from this centrifugation was resuspended in the imidazole buffer which also contained a final concentration of 1.5% (v/v) Triton X-100. The final volume of this suspension was equal to the volume of the supernatant fraction of the initial centrifugation. It was then centrifuged at 100,000 *g* for 60 min, and the supernatant fraction was taken as the microsomal preparation. The protein concentration of the microsomal preparation was adjusted to 10 mg/ml [18]. In the experiments described, the microsomal activating system contained 0.5 mg of rat liver microsomal protein in 250 mM sucrose/80 mM KCl/25 mM imidazole, pH 7.4, 1.5% Triton X-100 and 2 mM NADH.

A mitochondrial fraction with ALDH activity was obtained from rat liver by the method of Kamei *et al.* [15]. A homogenate of 10 g rat liver in 50 ml of 0.25 M sucrose in 5 mM Tris–HCl buffer (pH 7.2) was centrifuged for 10 min at 700 *g*. The supernatant fraction was centrifuged for 15 min at 10,000 *g* and the pellet was resuspended at a concentration of 5 mg protein/ml in the Tris/sucrose buffer containing 0.25 mg sodium deoxycholate/mg mitochondrial protein.

1-Methyltetrazole-5-thiol, obtained from ICN Biomedical, Plainview, NY, was dissolved in the imidazole buffer and used at the concentrations indicated in the text. NAD, NADH, and glutathione were obtained from the Sigma Chemical Co., St. Louis, MO. Yeast ALDH was obtained from Boehringer Mannheim, Indianapolis, IN. Results are expressed as the average of two to four determinations. Replication of results was usually within 10%.

Statistical analysis was performed using the Wilcoxon Rank Sum Test (Epistat Statistical Package).

Table 1. Effect of MTT on yeast aldehyde dehydrogenase in the absence of microsomes

| Time (min) | % ALDH activity |
|---|---|
| 0 | 124 |
| 20 | 121 |
| 30 | 105 |

MTT (1 mM) and 1.0 unit of yeast ALDH were incubated for the indicated times in a total volume of 0.4 ml, in a buffer which contained 250 mM sucrose/80 mM KCl/25 mM imidazole, pH 7.4, and 1.5% Triton X-100. An 80-μl aliquot was then assayed for ALDH activity in 0.1 M Tris buffer, pH 8.0, containing 0.2 M KCl, 1.5 mM NAD and 2.4 mM acetaldehyde. ALDH activity is expressed as percent activity in the absence of MTT.

## RESULTS

If MTT was incubated with yeast ALDH in the absence of microsomes, there was no inhibition of the enzyme, but rather a slight enhancement of activity (Table 1). However, if MTT was incubated with yeast ALDH in the presence of microsomes and NADH, inhibition of the enzyme was observed. The endogenous specific activity of ALDH in the microsomal preparation was about 0.004 units/mg protein/

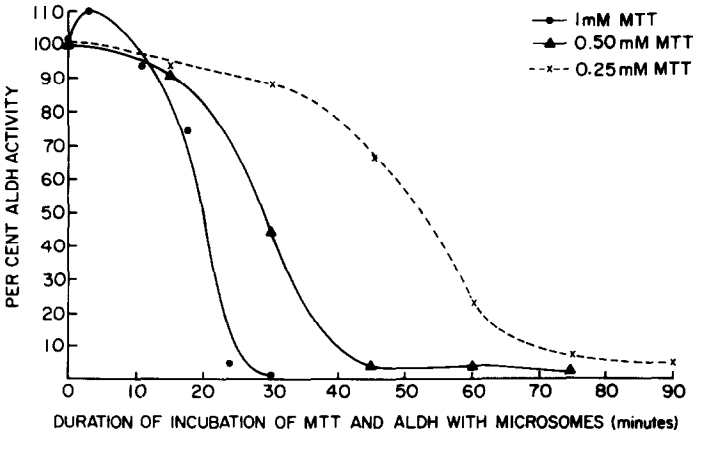


Fig. 2. Inhibition of yeast ALDH by MTT in the presence of microsomes. Yeast ALDH (0.4 units) was incubated in a total volume of 0.1 ml with MTT in the presence of 0.5 mg of rat liver microsomal protein in 250 mM sucrose/80 mM KCl/25 mM imidazole, pH 7.4, 1.5% Triton X-100 and 2 mM NADH. The duration of incubation was for the time indicated on the abscissa. A 20-μl aliquot of the incubation mixture was assayed for ALDH activity in a total volume of 1 ml. The assay mixture contained 0.1 M Tris buffer, pH 8.0, 0.2 M KCl, 1.5 mM NAD and 2.4 mM acetaldehyde. On the ordinate ALDH activity is expressed as the percent activity of ALDH when incubated with microsomes in the absence of MTT.

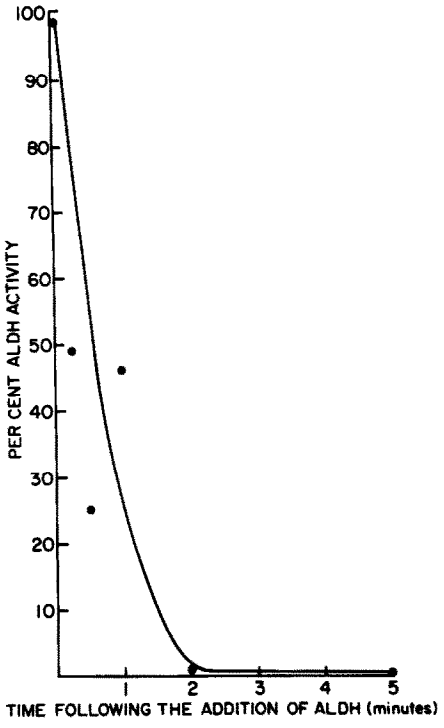


Fig. 3. Ability of MTT to inhibit yeast ALDH following a 30-min preincubation of MTT with microsomes. MTT in the absence of ALDH was incubated with microsomes under the conditions described in the legend of Fig. 2. ALDH (0.4 units) was then added to the incubation mixture and, at the times indicated on the abscissa, a 20-$\mu$l aliquot was assayed for activity of ALDH using the conditions described in Fig. 2. On the ordinate ALDH activity is expressed as the percent of activity of ALDH when incubated with microsomes in the absence of MTT.

min (one unit of activity: 1 $\mu$mol NADH formed/min/mg protein) which was much less than the 0.4 units of specific activity of the yeast ALDH which was added to the microsomes. Figure 2 shows that inhibition by MTT was time and concentration dependent, with inhibition not detected during the first 10 min of incubation. The greater the duration of the incubation as well as the greater the concentration of MTT, the greater the degree of inhibition.

The time dependence of the inhibition by MTT may have been due to the rate of accumulation of a metabolite or metabolites of MTT which had the ability to inhibit ALDH. If this were the case, then incubation of the microsomes with MTT prior to the addition of ALDH should lead to rapid inactivation of ALDH. Therefore, this possibility was examined. MTT at a concentration of 1 mM was incubated with microsomes for 30 min, and then yeast ALDH was added. The results shown in Fig. 3 indicate that 2 min after the addition of ALDH there was nearly complete inhibition of this enzyme.

The question of the reversibility of inhibition was examined by testing the effect of dilution upon the system. MTT at a concentration of 1 mM, microsomes, and ALDH were incubated for 30 min after which time the incubation mixture was diluted 10-fold with buffer. The activity of ALDH was assayed immediately after dilution and at various times for the next 90 min. The results in Fig. 4 demonstrate that the activity of ALDH could not be restored by dilution. Control incubation mixtures, which had no MTT added, lost approximately 50% of their activity over the 90-min observation period. However, even after 90 min, the activity of ALDH would have been detectable if complete reversal of the inhibition had occurred following dilution.

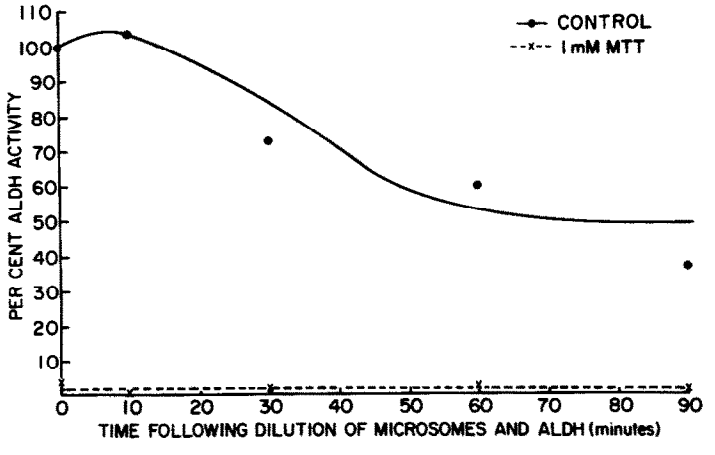


Fig. 4. Effect of dilution upon the inhibition of yeast ALDH by MTT. ALDH was incubated with MTT, NADH, and the microsomes as described in the legend of Fig. 2. After 30 min, the incubation mixture was diluted 1 to 10 with 0.1 M Tris buffer, pH 8. A 20-$\mu$l aliquot of the diluted incubation mixture was then assayed for ALDH activity as described in Fig. 2 at the times indicated on the abscissa. On the ordinate ALDH activity is expressed as the percent of activity of ALDH when incubated with microsomes in the absence of MTT.

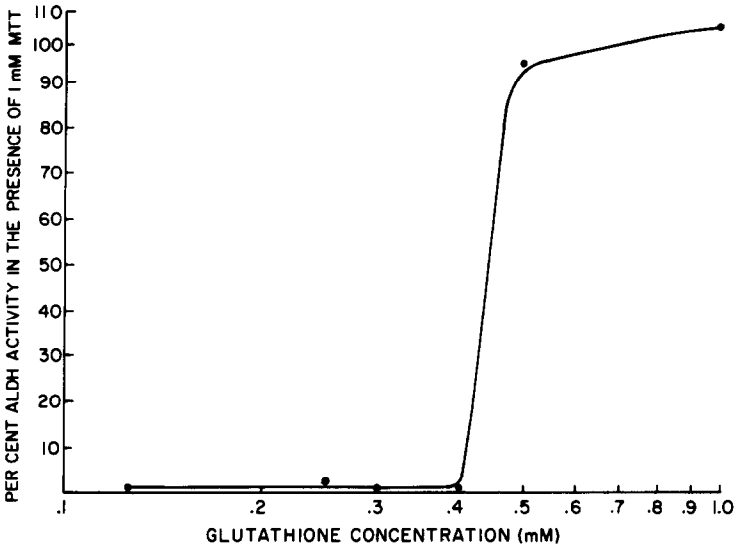


Fig. 5. Effect of glutathione on the ability of 1 mM MTT to inhibit yeast ALDH. Glutathione was incubated with the microsomes, MTT and NADH as described in the legend of Fig. 2. On the ordinate ALDH activity in the presence of 1 mM MTT is expressed as the percent of activity of ALDH incubated with microsomes in the absence of MTT.

Since glutathione has been shown to prevent MTT from inhibiting the gamma-carboxylation of glutamic acid [16] and also to protect ALDH from inhibitors, the effect of glutathione upon the inhibition of yeast ALDH by MTT was examined. MTT (1 mM), microsomes, and ALDH were incubated for 30 min in the presence of various concentrations of glutathione. Figure 5 shows that ALDH activity was protected by concentrations of glutathione greater than 0.5 mM.

The results obtained here indicate that the presence of microsomes enabled MTT to inhibit aldehyde dehydrogenase. This suggests that MTT underwent metabolic activation. One possible microsomal enzyme which may participate in such an activation of MTT is a flavin monooxygenase, which has the potential to metabolize thiol compounds similar in structure to MTT to sulfenic acids (Fig. 6) [19]. This enzyme requires either NADH or NADPH and is heat sensitive [20]. Therefore, the possible influence of these two characteristics of the flavin enzyme on the activation of MTT were examined. Table 2 indicates that NADH was necessary for the activation of MTT. In the absence of NADH, aldehyde dehydrogenase activity was close to the activity measured in the absence of MTT, whereas addition of 2 mM NADH led to almost complete inhibition. Furthermore, preliminary heat treatment of the microsomes at 37° for 30 min destroyed the ability of the microsomes to cause MTT to inhibit aldehyde dehydrogenase.

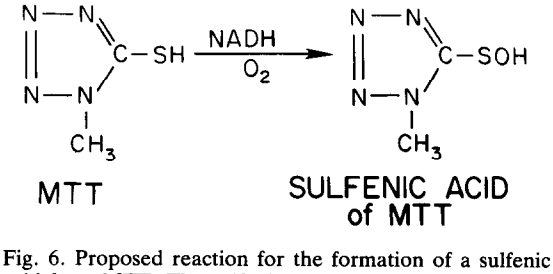


Fig. 6. Proposed reaction for the formation of a sulfenic acid from MTT. The sulfenic acid has been hypothesized to be the active metabolite of MTT.

Since it has been questioned previously [21] whether or not intact, MTT-containing antibiotics have the ability to inhibit ALDH, these as well as one non-MTT-containing antibiotic, cefotaxime, were examined in the microsomal system. As shown in Table 3, none of the antibiotics tested produced significant inhibition of ALDH activity. Since MTT is released from MTT-containing antibiotics upon cleavage of the beta-lactam bond [22], the effect of cleavage of the bond upon the abilities of the antibiotics to inhibit ALDH was also examined. Cleavage of the bond was accomplished by exposing the antibiotics to 0.83 M potassium hydroxide. The data in Table 3 indicate that hydroxide treatment of

Table 2. Effects of 2 mM NADH and heat treatment of the microsomes on yeast aldehyde dehydrogenase inhibition by 1 mM MTT

| Condition | % ALDH activity |
|---|---|
| − NADH | 95 |
| + NADH | 1* |
| 2° | 2* |
| 37° | 100 |

Incubation of the microsomes and the assay of ALDH activity were as described in the legend of Fig. 2. For the heat treatment experiments, preincubations of the microsomes at either 2° or 37° were performed for 30 min. Following this incubation, MTT and ALDH were added and the incubation was continued for an additional 30 min at 25°.

* Significantly different from control, $P < 0.05$ (Wilcoxon Rank Sum Test).

Table 3. Effect of intact or KOH-treated antibiotics on yeast aldehyde dehydrogenase activity

| Drug | % ALDH activity | |
|---|---|---|
| | Intact | KOH treatment |
| Moxalactam | 99 | 0* |
| Cefoperazone | 89 | 0* |
| Cefamandole | 97 | 0* |
| Cefotaxime | 100 | 100 |

Potassium hydroxide treatment of the antibiotic was done in the following manner: 0.1 ml of 7.5 M KOH was added to 0.8 mM of a 12.5 mM solution of the intact antibiotic in water; after 5 min, 0.1 mM of 7.5 M HCl was added. An aliquot of the treated mixture to yield a final concentration of 1 mM of antibiotic was added to the microsomal preparation. Microsomal incubation and assay of ALDH activity were performed as described in the legend of Fig. 2.

MTT-containing antibiotics resulted in inhibition of ALDH. However, hydroxide treatment of the non-MTT-containing antibiotic, cefotaxime, did not result in inhibition.

The effect of MTT on the activity of ALDH from a mammalian source was examined next. The microsomal preparation used in the previous experiments as well as a mitochondrial fraction of a rat liver homogenate were used as sources of ALDH. When 1 mM MTT was added to the mitochondrial fraction which contains about 0.006 units ALDH/mg protein/min, only a 5% reduction in activity of ALDH was detected. However, if the mitochondrial fraction was added to a microsomal fraction that had been preincubated with MTT and NADH for 30 min, over 75% inhibition of activity was detected. As also shown in Table 4, the activity of ALDH in the microsomal fraction itself was inhibited by MTT if NADH was present. Glutathione prevented the inhibition by MTT in both the microsomal and the microsomal plus mitochondrial system. Pretreatment of the microsomal fraction with heat at 37° for 30 min prevented inhibition.

## DISCUSSION

The results presented in this paper indicate that the inhibition of aldehyde dehydrogenase by MTT is made possible by microsomal activation of MTT. The inhibitory effect was observed with yeast as well as mammalian ALDH. This result is consistent with the fact that enzymes from both sources have been shown to have similar mechanisms and similar behavior with respect to the same active-site-directed inhibitor, cyclopropanone hydrate [23].

The results obtained in this *in vitro* study support the hypothesis that it is a metabolite of MTT that is responsible for the inhibition of ALDH. Several lines of evidence lead to this conclusion. First, it was found that MTT must be incubated with microsomes in order for inhibition of both yeast and mammalian ALDH to occur. Without incubation with the microsomes, 1 mM MTT did not inhibit either aldehyde dehydrogenase. Other investigators, under conditions lacking microsomes, found only 13% inhibition of a rat mitochondrial aldehyde dehydrogenase by 1 mM MTT [15]. In our studies, when MTT was preincubated with the microsomes for 30 min prior to the addition of ALDH, very rapid inhibition of ALDH was observed. This result is consistent with the formation of an active metabolite of MTT which increases in amount during the incubation period and then can directly inhibit aldehyde dehydrogenase. Furthermore, the stoichiometry of the reaction between GSH and MTT suggests the formation of a metabolite. Since 0.5 mM glutathione prevents inhibition of yeast aldehyde dehydrogenase by 1 mM MTT, this protective effects is unlikely to be due to a direct reaction between MTT and glutathione. A possible interpretation of this finding is that a small amount of an active metabolite of MTT is formed which is inactivated by glutathione.

The process by which MTT forms an active metabolite may be via a flavin monooxygenase (Fig. 6)

Table 4. Effect of 1 mM MTT on mammalian aldehyde dehydrogenase

| Condition | % ALDH activity | |
|---|---|---|
| | Microsomal | Microsomal + Mitochondrial |
| + NADH | 0* | 23* |
| − NADH | 90 | 92 |
| + GSH + MTT + NADH | 85 | 100 |
| 30-min Preincubation of microsomes at 37° | 96 | 98 |

The microsomal suspension contained 2 mg of protein in a volume of 0.2 ml, and the mitochondrial fraction, 0.5 mg in a volume of 0.1 ml. In the microsomal + mitochondrial experiments, MTT under the various conditions was incubated for 30 min with the microsomes in a total volume of 0.22 ml prior to the addition of 0.1 ml of the mitochondrial fraction. After an additional 5-min incubation, the mixture was assayed in a total volume of 3 ml for ALDH activity as described in the legend of Fig. 2 except that the concentration of acetaldehyde was 0.15 mM. In the experiments with the microsomal fraction alone, this fraction was assayed in a total volume of 3 ml for ALDH activity following the 30-min incubation with MTT under the various conditions.

* Significantly different from control, $P < 0.05$.

[19]. The results in this study indicate that inhibition by MTT requires NADH and that the activation process in the microsomes is sensitive to heat. This is in keeping with the properties of flavin monooxygenase described by Poulsen *et al.* [19]. Evidence has been presented that a sulfenate may be formed as a consequence of oxidation of thiols by the monooxygenase [20]. Sulfenates are very reactive molecules, and such a species is an attractive candidate for an active metabolite of MTT. In addition, sulfenates may serve as precursors to other, more complex, sulfur-containing compounds. One such potential active metabolite of MTT, a disulfide dimer, has been proposed by two groups. This compound has been shown to be a potent inhibitor of the gamma carboxylation of glutamic acid [16], as well as an inhibitor of ALDH obtained from sheep liver [14]. The dimer is very reactive but is very unstable in aqueous solutions [24] and, if it were formed by the oxidation of MTT *in vivo*, its isolation would be difficult. Therefore, failure to isolate this compound would not rule out its transient existence *in vivo*. It is also possible that more than one metabolite contributes to the inhibition by MTT. The nature of the active metabolite(s) of MTT remains to be elucidated.

Our *in vitro* results from the dilution experiments with the yeast ALDH provide no indication that the inhibition of ALDH by MTT is reversible. This finding is consistent with the clinical observation that adverse reactions to alcohol are observed in patients hours [2, 3] to days [5, 6] after the administration of the MTT-containing drugs. Although the concentration of MTT in the blood has not been measured in the patients experiencing the alcohol reactions, MTT is known to be eliminated rapidly in humans [25]. Therefore, irreversible inhibition of aldehyde dehydrogenase by MTT would explain a reaction to alcohol long after MTT had been eliminated.

There has been some controversy as to whether it is the intact MTT-containing antibiotics or MTT itself which is responsible for the disulfiram effect [21]. It has been demonstrated that free MTT has the ability *in vivo* in rats to produce an alteration in alcohol metabolism similar to that of disulfiram [8, 10, 11]. Other researchers have shown that, at high concentrations (greater than 5 mM) of the intact, MTT-containing antibiotics, there is a slight inhibition of a rat liver mitochondrial ALDH [7, 9, 15]. However, the significance of these findings has been questioned because of the high levels of antibiotic used, as well as the low extent of inhibition obtained [21]. Additionally, the results of one study indicated that there is no correlation in the potency of the *in vitro* inhibition of ALDH by intact antibiotics with the *in vivo* alteration in ethanol metabolism produced by these drugs [9]. Our findings that potassium hydroxide treatment was necessary for the inhibition (Table 3), and the observation that cephalosporins which do not contain the MTT or related groups do not alter alcohol metabolism *in vivo* [12] or *in vitro* (Table 3), strongly suggest that it is the presence of the MTT group which confers inhibitory potential, and not the cephalosporin nucleus.

Studies in animals that have undergone biliary diversion provide further evidence supporting the lack of a direct effect of intact, MTT-containing antibiotics upon ALDH. It has been hypothesized that MTT is released from MTT-containing antibiotics in the intestine following biliary secretion. The MTT group would then be absorbed from the intestine prior to its producing inhibition of ALDH. In animals in which the bile duct was cannulated and the bile diverted from the intestine, moxalactam appeared to have no effect on altering alcohol metabolism [10]. Therefore, the exposure of the animal to moxalactam in itself was not sufficient to produce an effect. The investigations concluded that the MTT group must be released from the antibiotic in order for inhibition to occur. This is consistent with the *in vitro* finding presented in this paper (Table 3).

In conclusion, the studies presented here provide evidence for the hypothesis that the disulfiram-like effect associated with MTT-containing antibiotics is attributable to the presence of the MTT group. In order for inhibition of ALDH to occur, the following sequence of events is postulated: (1) release of MTT from the parent antibiotic, (2) the transformation of MTT in a reaction that is catalyzed by microsomes in the presence of NADH, and (3) irreversible inhibition of aldehyde dehydrogenase by a metabolite of MTT.

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