EFFECTS OF BUTHIOBATE, A FUNGICIDE, ON CYTOCHROME P-450 OF RAT LIVER MICROSONES

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Effects of buthiobate (S-butyl S'-p-tert-butylbenzyl N-3-pyridylidithiocarbonimidate), a fungicide which inhibits lanosterol 14α-demethylation catalyzed by a cytochrome P-450 (P-45014DM) of yeast, on cytochrome P-450 of rat liver microsomes were studied. Buthiobate bound to limited forms of cytochrome P-450 in the microsomes with three different Kd values, 0.38, 1.56 and 13.6 μM. Buthiobate inhibited lanosterol 14α-demethylase activity of the microsomes with a 50%-inhibition concentration of 0.4 μM. This concentration was comparable to the lowest Kd of buthiobate for the microsomal cytochrome P-450 and also to the 50%-inhibition concentration of the inhibitor for lanosterol 14α-demethylation by yeast P-45014DM. Buthiobate partially inhibited 7-ethoxycoumalin O-deethylase activity of the microsomes but inhibited neither benzphetamine N-demethylation nor p-nitroanisole O-demethylation. These observations suggest that cytochrome P-450s catalyzing drug oxidations are rather insensitive to buthiobate. These observations indicate that buthiobate is an unique inhibitor for hepatic microsomal cytochrome P-450 system, which inhibits cholesterol biosynthesis effectively but causes a little effect on drug oxidations.

Keywords—buthiobate; fungicide; lanosterol demethylation; drug oxidation; cholesterol biosynthesis; cytochrome P-450

Buthiobate (S-n-butyl S'-p-tert-butylbenzyl N-3-pyridylidithiocarbonimidate, Fig. 1) is a fungitoxic agent which affects sterol biosynthesis in yeasts and fungi and causes large accumulation of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) in their cells. Recently, Aoyama et al. provided lines of evidence indicating that this compound inhibits the 14α-demethylation of lanosterol catalyzed by a cytochrome P-450 (P-45014DM) of yeast microsomes. It was also found that this compound fails to inhibit the 22-desaturation of ergosta-5,7-dien-3β-ol catalyzed by another species of cytochrome P-450 (P-45022DM) in microsomes of the same yeast. These findings suggest that buthiobate selectively inhibits P-45014DM in yeast. Lanosterol 14α-demethylation is involved also in the cholesterol biosynthesis in mammals. Contribution of a type of cytochrome P-450 to this activity has been suggested based on the inhibitory effect of CO to this activity. In the recent years, inhibitory effects of some imidazole- and triazole-fungicides to the lanosterol demethylase activity in rat liver have been reported. It was also reported that buthiobate underwent some oxidative metabolism in rat liver microsomes.

In hepatic microsomes, there are numbers of cytochrome P-450 species catalyzing monoxygenation of various small lipophilic compounds. This communication provides evidence indicating that buthiobate interacts with a few species of cytochrome P-450 in rat liver microsomes. This

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FIG. 1. Structural Formula of Buthiobate
paper also describes that buthiobate inhibits lanosterol 14α-demethylase activity of liver microsomes at low concentration but its inhibitory effect on drug oxidation activity is not so strong.

MATERIAL AND METHODS

Chemicals and Biochemicals — Buthiobate was supplied by Dr. Toshiro Kato of Sumitomo Chemical Co. Benzphetamine and 7-ethoxycoumalin were gifts from Dr. Yoshio Imai of Osaka University. P-45014DM was purified by the method of Yoshida and Aoyama.39 Nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), glucose 6-phosphate and glucose-6-phosphate dehydrogenase were the products of Oriental Yeast Co. Other chemicals used were the commercially available purest reagents.

Preparation of Microsomes — Male Sprague-Dawley rats (5–6 weeks old) were used in most of the experiments. Rats were fasted for 20 h and sacrificed by decapitation. Livers were washed well with cold 0.15 M KCl and homogenized with 5 volumes of the same medium. Microsomes were collected by differential centrifugation between 9000 × g for 10 min and 105000 × g for 60 min. The microsomes were washed with 0.1 M potassium phosphate buffer, pH 7.5, suspended in the same buffer and stored at −70°C under nitrogen atmosphere. Microsomes used in lanosterol 14α-demethylase assay were prepared from the 8–9 week-old male rats of the same strain without fasting prior to sacrifice. The KCl-washed livers were homogenized with 2.5 volumes of 0.1 M potassium phosphate buffer, pH 7.5, and microsomes were prepared and stored as above. Under these conditions, microsomes could be stored for more than 6 months without significant change. For the demethylase assay, the postmicrosomal supernatant was also stored under the same conditions.

Determination of Lanosterol 14α-Demethylase Activity — A lanosterol preparation consisting of 60% lanosterol and 40% dihydrolanosterol (4,4,14α-trimethyl-5α-cholest-8-en-3β-ol) was emulsified with Tween 80. The emulsion containing 100 nmol sterols and 4.0 μg Tween 80 was mixed with an enzyme solution consisting of 6–10 mg protein of the microsomes, 20–40 mg protein of the post-microsomal supernatant, 0.5 mM ethylenediaminetetra acetic acid (EDTA), 1 mM KCN, and 0.1 M potassium phosphate buffer, pH 7.5. Then, 1.5 mM NADH and an NADPH-generating system consisting of 0.15 mM NADPH, 10 mM glucose 6-phosphate and 0.29 unit glucose-6-phosphate dehydrogenase was added to the mixture and the volume of the mixture was made up to 2.0 ml. Reaction was carried out at 37°C for 30 min under constant shaking. Buthiobate was added as 10 μl dimethylsulfoxide solution prior to the addition of the electron donors. The same volume of the solvent was added to the control. The reaction was stopped by the addition of 5 ml of 10% KOH/methanol and the mixture was saponified at 80°C for 60 min. Sterols were extracted from the saponified solution and analyzed with thin-layer and gas-liquid chromatography.40

Assay of Drug Oxidative Activities — Reaction mixture for benzphetamine N-demethylase contained 0.15 mM NADPH, 1.25 mM benzphetamine, 0.5 mM EDTA, and 5.3 mg protein of microsomes in 20 ml of 0.1 M potassium phosphate buffer, pH 7.2. The reaction was started by the addition of NADPH, run at 37°C for 10 min under constant shaking and stopped with 5% trichloroacetic acid. After deproteinization, formaldehyde was determined by the method of Nash.13 7-Ethoxycoumalin O-deethylase activity was assayed in a 2 ml reaction mixture consisting of 0.15 mM NADPH, 0.25 mM 7-ethoxycoumalin, 0.5 mg protein of microsomes and 0.1 M potassium phosphate buffer, pH 7.2. The reaction was started by the addition of NADPH and run at 25°C for a few min in a fluorometer cuvette. Formation of 7-hydroxyneupylal was followed by increasing fluorescence at 455 nm. O-Demethylation of p-nitroanisole was assayed in a reaction mixture consisting of 0.1 mM each of NADPH and NADH, 2 mM potassium phosphate buffer, pH
7.5. Reaction was carried out at 37°C for 10 min under constant shaking. After deproteinization with 5% trichloroacetic acid, p-nitrophenol formed was determined by the method of Shigematsu et al.14) In these three enzyme assays, buthiobate was added as dimethylsulfoxide solution, and the corresponding volume of the solvent was added to the control.

Spectrophotometric Determination of the Binding of Buthiobate to Cytochrome P-450 —— Microsomes were suspended in 0.1M postassium phosphate buffer, pH 7.5 and divided into sample and reference cuvettes. After recording the baseline, the sample was titrated with dimethylsulfoxide solution of buthiobate and the corresponding volume of the solvent was added to the reference. The resulting difference spectrum was recorded and the extent of buthiobate binding was determined from the absorbance difference between 428 and 410 nm. Further details are described in figure captions.

**Analytical Methods** —— Protein was determined by the method of Lowry et al.15) using bovine serum albumin as the standard. Cytochrome P-450 was determined according to the method of Omura and Sato.16)

RESULTS AND DISCUSSION

Spectrophotometric Evidence for the Binding of Buthiobate to Cytochrome P-450

Fig. 2 shows difference spectra induced by the addition of buthiobate or pyridine to rat liver microsomes. The buthiobate-induced difference spectrum (Fig. 2A) indicates that buthiobate bound to cytochrome P-450 in the microsomes with a Type II spectral change. The shape of this difference spectrum was essentially identical with that of yeast P-45014DM (Fig. 3 and ref. 2). The buthiobate-induced difference spectrum of P-45014DM was superimposable on the pyridine-induced one of the same P-450 (Fig. 3). It is now known that the shape of absorption spectrum of

![Figure 2: Spectral Changes of Rat Liver Microsomes Caused by the Addition of Buthiobate and Pyridine](image-url)

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
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an exogenous ligand complex of cytochrome P-450 is determined by the binding functional

group to the heme iron. According to the observation, the above observation indicates that buthiobate binds to the heme iron of P-450_{14DM} by its pyridine moiety. In contrast, the shape and the intensity of the buthiobate-induced difference spectrum of hepatic microsomes (Fig. 2A) was obviously different from the pyridine-induced one (Fig. 2B). Shape of Type II difference spectrum of cytochrome P-450 is known to be affected by its spin state. When a Type II ligand binds to low-spin cytochrome P-450, trough of the difference spectrum is observed around 410 nm. On the other hand, the trough is observed around 390 nm if the ligand interacts with high-spin form of the cytochrome. Judging from the above cri-

FIG. 3. Buthiobate- and Pyridine-Induced Difference Spectra of P-450_{14DM}

Purified P-450_{14DM} was dissolved in 0.1 M potassium phosphate buffer, pH 7.0 containing 20% glycerol and 0.005% Emulgen 913 and the buthiobate-induced difference spectrum was recorded (—). P-450_{14DM} and buthiobate concentrations were 0.62 μM and 3.3 μM, respectively. The pyridine-induced difference spectrum of the same sample was recorded with 52 mM pyridine (— — —).

FIG. 4. Titration of Cytochrome P-450 in Rat Liver Microsomes with Buthiobate

Difference spectra of the rat liver microsomes induced by various concentrations of buthiobate were recorded as Fig. 2A. Binding of buthiobate to cytochrome P-450 was estimated from the absorbance difference between 428 and 410 nm (ΔA_{428-410}) and result was expressed with a Hofsteil-type plot. The concentration of cytochrome P-450 was 3.4 μM. The microsomes used were those prepared for lanosterol 14α-demethylase assay.

FIG. 5. Gas Chromatograms of Sterols Extracted from the Reaction Mixtures of Lanosterol 14α-Demethylase Assay

The reaction was run as described in Materials and Methods in the absence (A) or presence (B) of 10 μM buthiobate. Sterols extracted from the reaction mixture were separated by thin-layer chromatography. Lanosterol fraction on the silica gel plate was extracted, trimethylsilylated, and subjected to gas-liquid chromatography through a glass capillary column of OV-17. Peaks 1 and 2 represent dihydrolanosterol and lanosterol, respectively. Peaks 3 and 4 were identified as the 14-demethylated derivatives of dihydrolanosterol and lanosterol, respectively, from their mass spectra (data not shown; see ref. 4).
teria and the shape of the difference spectrum (Fig. 2B), buthiobate bound to low-spin cytochrome P-450 while pyridine bound to high-spin species of the cytochrome. The pyridine-induced difference spectrum (Fig. 2) had a negative shoulder at 410 nm indicating the interaction of pyridine also with cytochrome P-450 in a low-spin state. Furthermore, no spectral change was induced by the addition of buthiobate to the pyridine-saturated microsomes. As shown in Fig. 2B, the negative shoulder around 410 nm in the pyridine-induced difference spectrum was diminished by subtracting the buthiobate-induced difference spectrum from the pyridine-induced one. These observations indicate that buthiobate could bind only to limited form(s) of cytochrome P-450 in hepatic microsomes. The result shown in Fig. 2 also indicates that buthiobate bound almost selectively to cytochrome P-450 in a low-spin state in the microsomes.

To assume affinity of buthiobate to cytochrome P-450, the microsomes were titrated with various concentrations of the compound. Fig. 4 represents a typical result of the titration of the microsomes prepared for the lanosterol 14α-demethylase assay. The result suggested that buthiobate bound to seemingly three cytochrome P-450 species with observed \( K_d \) values of 0.38, 1.56 and 13.2 \( \mu M \). The lowest \( K_d \) value was comparable to the buthiobate concentration necessary for the half maximal inhibition of lanosterol 14α-demethylation by hepatic microsomes (see below). This fact suggested that this species is likely to be the cytochrome P-450 catalyzing lanosterol 14α-demethylation.

**Inhibition of Lanosterol 14α-Demethylation by Buthiobate**

When a lanosterol–dihydrolanosterol mixture was incubated with the hepatic microsomes in the presence of NADH, NADPH, molecular oxygen and cytosolic protein, both of the sterols were converted to their 14-demethylated derivatives (Fig. 5A). This activity was almost completely inhibited by 10 \( \mu M \) buthiobate (Fig. 5B). Then,

![Image](image_url)

**FIG. 6. Inhibition of Lanosterol 14α-Demethylase Activity of the Rat Liver Microsomes by Buthiobate**

Lanosterol 14α-demethylase activity was assayed as described in Materials and Methods under the presence of the indicated concentrations of buthiobate. Conversion ratio observed in the presence of dimethylsulfoxide (100% activity) was 17%.

![Image](image_url)

**FIG. 7. Effects of Buthiobate on Some Drug-oxidizing Activities of Rat Liver Microsomes**

Benzphetamine \( N \)-demethylation (○), 7-ethoxyccoumalin \( O \)-deethylation (●) and \( p \)-nitroanisole \( O \)-demethylation (▲) activities of the rat liver microsomes were assayed as described in Materials and Methods in the presence of indicated concentrations of buthiobate. Activities observed in the presence of dimethylsulfoxide (100% activities) were 1.18 nmol HCHO formed/min/mg protein for benzphetamine \( N \)-demethylation and 2.78 nmol \( p \)-nitrophenol formed/min/mg protein for \( p \)-nitroanisole \( O \)-demethylation. 7-Ethoxyccoumalin \( O \)-deethylation activity was expressed by an arbitrary unit based on fluorescence intensity.
the effect of buthiobate concentration to this activity was studied. As shown in Fig. 5A, lanosterol and dihydrolanosterol were demethylated with nearly the same rate and lanosterol is known to be converted to dihydrolanosterol in hepatic microsomes. So, in this experiment, apparent 14α-demethylase activity was expressed by the conversion ratio of lanosterol plus dihydrolanosterol to their 14-demethylated derivatives. The conversion ratio thus calculated was linearly dependent on the incubation time of more than 30 min. As shown in Fig. 6, buthiobate caused a dose-dependent inhibition of the demethylase activity and a 50%-inhibition concentration was determined to be 0.4 μM. This concentration was close to the 50%-inhibition concentration of buthiobate for the lanosterol demethylation by yeast P-450\textsubscript{14DM}\textsuperscript{2} and also to the lowest \( K_d \) of buthiobate to the microsomal cytochrome P-450 (Fig. 4). These findings suggest that the hepatic cytochrome P-450 with the highest affinity for buthiobate contributed to lanosterol 14α-demethylation. It has been reported by Bossche \textit{et al.}\textsuperscript{11} that cholesterol synthesis in mammals is less sensitive to imidazole- and triazole-fungicides than the ergosterol synthesis in yeast. Since these fungicides have been reported to inhibit lanosterol 14α-demethylase,\textsuperscript{11} the above fact seems to suggest relatively low sensitivity of the mammalian enzyme to these fungicides. In contrast, 50%-inhibition concentration of buthiobate to the hepatic microsomal enzyme (Fig. 6) was considerably low and was essentially the same as that to the yeast enzyme.\textsuperscript{29}

Contribution of cytochrome P-450 to lanosterol 14α-demethylation in hepatic microsomes has been suggested based on the inhibitory effect of CO\textsubscript{2}.\textsuperscript{6-8} The present comparative study of buthiobate inhibition on lanosterol 14α-demethylase in rat liver and yeast microsomes provides additional evidence for the contribution of cytochrome P-450 to the lanosterol demethylation. Lanosterol 14α-demethylation by the hepatic microsomes was also inhibited by 1 mM metyrapon, a known cytochrome P-450 inhibitor (data not shown). It can thus be concluded that hepatic microsomes surely contain cytochrome P-450 functioning in lanosterol 14α-demethylation.

14α-Demethylation of lanosterol is considered to consist of three oxygenations.\textsuperscript{20,21} It was reported for the hepatic microsomal system that cytochrome P-450 mediated only the first step forming the 32-hydroxylated intermediate and the following two steps were catalyzed by some CO-insensitive enzyme(s).\textsuperscript{9} In contrast, yeast P-450\textsubscript{14DM} is known to catalyze the entire process of the 14α-demethylation.\textsuperscript{4} According to these reports, it can be supposed that the cytochrome P-450s of yeast and mammalia catalyzing lanosterol 14α-demethylation are different from each other in their reaction mechanisms. However, as described in this paper, reactivity of buthiobate to these cytochrome P-450s are essentially the same. Accordingly, it may be worth doing to compare the mammalian and the yeast systems with respect to the sensitivities of the second and the third steps of lanosterol 14α-demethylation\textsuperscript{9} to buthiobate.

\textbf{Effects of Buthiobate to Some Drug Metabolizing Activities}

Fig. 7 represents the effects of buthiobate to some drug oxidizing activities of rat liver microsomes. Buthiobate inhibited about one third of 7-ethoxycoumalin O-deethylase activity of the liver microsomes at the same concentration as that necessary for the 90%-inhibition of lanosterol 14α-demethylation. However, the residual activity was resistant to the inhibitor and more than half of the activity remained even in the presence of 10 μM buthiobate. Other drug-oxidizing activities studied, \textit{i.e.}, N-demethylation of benzphetamine and O-demethylation of p-nitroanisole, were not affected by buthiobate under the same inhibitor concentrations. Similarity between the buthiobate concentrations necessary for the inhibition of lanosterol 14α-demethylation and the partial inhibition of 7-ethoxycoumalin O-deethylase suggests that a part of the deethylase activity was dependent on the lanosterol demethylase. However, the results shown in Fig. 7 seem to indicate that most of
cytochrome P-450s catalyzing drug oxidations were rather insensitive to buthiobate. As shown in Fig. 2, the hepatic microsomes contained some species of cytochrome P-450 which could not react with buthiobate. It is, therefore, likely that most of drug oxidations occurring in hepatic microsomes are catalyzed by these buthiobate-insensitive cytochrome P-450 species.

Taken all together, it is concluded that buthiobate is a unique cytochrome P-450 inhibitor which strongly inhibit lanosterol 14α-demethylation, an intermediate reaction of cholesterol biosynthesis, but shows little inhibition on drug oxidations. As shown in Fig. 4, the hepatic microsomes seem to contain three species of cytochrome P-450 which react with buthiobate. One of them showing the highest affinity for buthiobate is likely to be the lanosterol 14α-demethylase.

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