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MICROSOMAL OXIDATION OF THIOBENZAMIDE. A PHOTOMETRIC ASSAY FOR THE FLAVIN-CONTAINING MONOOXYGENASE.

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SUMMARY

The hepatotoxin thiobenzamide is S-oxidized by the microsomal flavin-containing monooxygenase (MFMO)[1] in liver, lung, and kidney of rabbit, mouse and rat. Its oxidation is accompanied by a large spectral shift which can be used as the basis of a simple convenient photometric assay for the MFMO system.

Introduction

Thiobenzamide (TB) is a hepatotoxin in the rat which, like other toxic thiocarbonyl compounds, appears to require metabolic activation for expression of its toxic effects (1-4). We have now investigated the metabolism of thiobenzamide *in vitro*, and in this communication report that TB is very efficiently S-oxidized by rat liver microsomes, that this reaction is catalyzed by the microsomal flavin containing monooxygenase (MFMO) and not by cytochromes P-450, and that this oxidation can serve as the basis of a simple sensitive photometric assay for MFMO activity in a variety of tissues.

Materials and Methods

Thiobenzamide was obtained from Aldrich Chemical Co.; its S-oxide (TBSO), as well as p-nitrothiobenzamide (PNTB) and its S-oxide (PNTBSO) were prepared according to Walter and Curts (5). [^{3}H]-TB (1.56 Ci/mole) was prepared from ^{3}H-benzonitrile (6, 7). Liver microsomes were isolated from male Sprague-Dawley rats (200-250 g), and were washed and resedimented at 105,000 xg using 0.1 M

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[1]Abbreviations used: EDTA, ethylenediamminetetraacetic acid; GS, generating system; MeCN, acetonitrile; MFMO, microsomal flavin-containing monooxygenase; NOA, n-octylamine; NOI, N-octylimidazole; PNTB, p-nitrothiobenzamide; PNTBSO, p-nitrothiobenzamide-S-oxide; TB, thiobenzamide; TBSO, thiobenzamide-S-oxide.

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Table 1. Physical properties of thiobenzamide derivatives.

Compound	mp, °C.	R_f	$\lambda(\epsilon)$, nm ($M^{-1}cm^{-1}$)
TB	116-118	0.60	248(8280), 286(8180)
TBSO	127-128	0.07	328(8300)
PNTB	160	0.55	255(9600)
PNTBSO	170	0.09	264(14500), 371(6160)

Substrate	$\Delta\epsilon_{max}$ (λ)	λ_{obs} ($\Delta\epsilon$)
TB	7220(335)	370(2930)
PNTB	4840(372)	420(3020)

Spectroscopic data are reported for solutions in buffer; the values change slightly in the presence of microsomes and/or cosolvents. The R_f values are for silica tlc plates eluted with ethylacetate/hexane (60:40).

potassium phosphate buffer (pH 7.6) containing 1 mM EDTA. Washed microsomes were resuspended in buffer (10 mg protein/ml) and stored at -80°C in small vials. For incubations one vial of stock microsome suspension was thawed and diluted with buffer to a protein concentration of 0.5-0.75 mg/ml as determined according to Bradford (8).

Incubations were monitored at 35°C in a Cary 118 spectrophotometer, at 370 nm for TB or 420 nm for PNTB. Stocks of diluted microsomes (0.75 mg protein/ml) and NADPH generating system were maintained on ice. For an assay run 2.0 ml microsomes, 50 ul NADPH generating system, and 25 ul of either buffer or inhibitor were added to both sample and reference cuvettes and warmed to 35°. Reactions were initiated by adding 25 ul of MeCN to the reference cuvette and 25 ul of a solution of TB in MeCN to the sample cuvette. Each cuvette was inverted gently several times to achieve mixing without bubble formation. Absorbance changes were then recorded for 5-7 min to determine initial reaction rates.

Results

The physical properties of TB and related compounds are reported in Table 1. Upon S-oxidation, the absorption spectrum of TB shifts to much longer wavelengths so that TBSO formation can be monitored photometrically with negligible interference from endogenous substances (unless stoichiometric amounts of NADPH are used instead of a regenerating system). The absorption difference between TB and TBSO is greatest at 335 nm, but is large enough even at 370 nm, where the absorption of TB (and benzamide) is essentially zero, to allow monitoring of its formation in microsomal suspension with good sensitivity. With PNTB, S-oxide formation can be monitored with reasonable sensitivity at wavelengths as long as 420 nm, although there is no intrinsic gain in sensitivity with this compound.

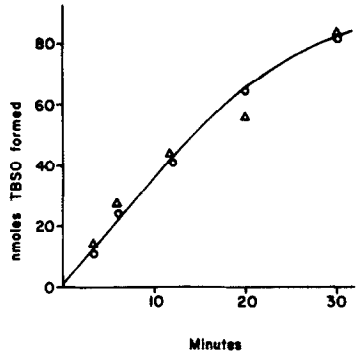

Figure 1. Time course of TBSO formation in a single cuvette as determined photometrically (circles) and radiometrically (triangles).

Repetitive scanning after addition of TB to a sample cuvette containing active microsomes plus an NADPH-generating system showed the pattern of spectral changes expected for the conversion of TB to TBSO. As shown in Figure 1, absorbance at 370 nm increased steadily with time, and the reaction rate remained linear for at least 10 min. In addition, the identity and quantitation of the reaction product as TBSO were confirmed by tlc analysis of ethanol-quenched aliquots taken from a photometric run employing [^{3}H]-TB in the cuvette.

The rate of TBSO formation was proportional to protein concentration in the range 0.5-2.0 mg/ml, and even at the highest concentrations turbidity was not a significant problem. However, with microsomes diluted below 0.2 mg protein/ml, TB would sometimes precipitate briefly as it was added, causing a delay in obtaining a stable initial rate measurement. Data given in Table 2 indicate that TBSO formation requires active microsomes, O_2 and NADPH, and that it is not inhibited

Table 2. Effect of inhibitors on microsomal oxidation of thiobenzamide.

Incubation Conditions	TBSO Formation (nmole/min/mg protein)
Complete system	4.78 ± 0.21
Omit NADPH-GS	0
Omit oxygen (N_2)	0
+SKF-525A (1 mM)	4.38 ± 0.11
+N-Octylimidazole (1 mM)	4.64 ± 0.23
+Thiourea (1 mM)	4.36 ± 0.44

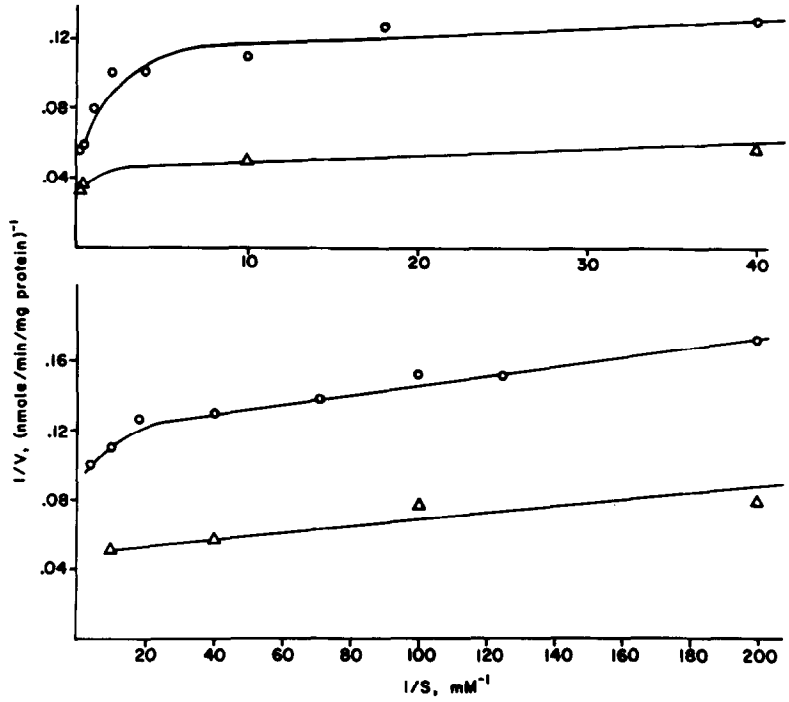

Figure 2. Double reciprocal plot for thiobenzamide oxidation by rat liver microsomes in the absence (circles) and presence (triangles) of 5 mM n-octylamine.

by classical inhibitors of cytochrome P-450, namely SKF-525A and N-octylimidazole (NOI). In fact, n-octylamine (NOA), another good inhibitor of cytochromes P-450, actually enhances TB oxidation.

At low concentrations TB oxidation follows Michaelis-Menten kinetics, as indicated in Figure 2. However, above 25 uM the plot curves downward rapidly, indicating that TB enhances its own oxidation. PNTB shows similar behavior (data not shown). In the presence of 5 mM NOA the rate of TB oxidation is increased ca. 2.5-fold at low concentrations of TB, and at higher concentrations the downward curvature in the double-reciprocal plot is much less pronounced. Extrapolation of the linear portions of these plots leads to apparent K_m and V_{max} values of 2 uM and 8.5 nmol/min/mg protein in the absence of NOA, and 4 uM and 21 nmol/min/mg protein in its presence. In the region above 25 uM substrate, both plots converge toward a common Y-intercept, suggesting a limiting maximum velocity of about 25-30 nmole/min/mg protein.

Table 3. Thiobenzamide oxidation with liver, lung, and kidney microsomes from rats, rabbit, and mice.

Species, Incubation Conditions	TBSO formation (nmol/min/mg protein) Source Tissue		
	Lung	Liver	Kidney
Rat			
Standard	17.7 ± 1.1	10.7 ± 0.4	15.3 ± 0.9
+NOA	17.2 ± 1.8	23.8 ± 2.6	26.2 ± 1.8
+NOI	18.1 ± 1.4	13.6 ± 1.2	16.7 ± 1.2
Rabbit			
Standard	16.3 ± 1.4	23.0 ± 0.9	18.7 ± 1.3
+NOA	37.2 ± 1.7	31.0 ± 0.1	24.3 ± 1.5
+NOI	19.2 ± 0.3	20.0 ± 1.0	12.8 ± 1.1
Mouse			
Standard	14.2 ± 0.9	19.1 ± 0.5	12.1 ± 0.2
+NOA	29.7 ± 0.5	17.0 ± 1.2	20.1 ± 1.0
+NOI	18.9 ± 0.5	13.3 ± 0.4	10.9 ± 0.7

Results given are the mean ± SE for 3-5 determinations with microsomes from pooled tissues of 8 rats (male, 200 g), one rabbit (male, 2.4 kg), or 14 mice (male, 25 g). Standard incubations were conducted as described under Methods; +NOA means n-octylamine (5 mM) was also added; +NOI means N-octylamidazole (1 mM) was also added. In all cases the initial concentration of TB was 1 mM.

As shown by Table 3, liver, lung, and kidney microsomes from rats, rabbits and mice also oxidize TB to TBSO at substantial rates. Except with rabbit kidney and mouse liver microsomes, TB oxidation was not inhibited by NOI, while NOA significantly enhanced TB oxidation in all cases except with mouse liver and rat lung microsomes.

Preliminary studies have shown that over the time course of these experiments, TBSO is quite stable in suspensions of boiled microsomes, with or without added glutathione. However, in the presence of active microsomes, NADPH and oxygen, it is further metabolized. The products of this reaction have not been identified, but their formation is very slow, and they do not absorb in the region of the S-oxide. In contrast, PNTBSO is very stable even in the presence of active microsomes; i.e. it is not detectably oxidized further.

Discussion

In the rat thiobenzamide-S-oxide is significantly more hepatotoxic than thiobenzamide, and the hepatotoxicity of p-substituted thiobenzamides is strongly

dependent on the electron-donating ability of the para substituent. These observations have been interpreted to suggest that S-oxidation is an obligatory step in the expression of toxicity from thiobenzamide (1). In agreement with this hypothesis we have now found that TB is indeed oxidized efficiently by rat liver microsomes in vitro. TBSO formation can easily be monitored spectrophotometrically, and its identification and quantitation by this method have been verified by an independent tlc/radiometric assay. TBSO formation is insensitive to inhibition by SKF-525A and NOI while it is stimulated ca. 2.5-fold by NOA; Table 3 shows that with few exceptions this pattern also holds for S-oxidation of TB with microsomes from a variety of sources other than rat liver. Thus it appears that TB is oxidized largely, if not exclusively, by the MFMO rather than cytochromes P-450.

The kinetics of TB oxidation by rat liver microsomes shows the same complication as observed for the oxidation of amine substrates by the purified MFMO, i.e., downward curvature of double reciprocal plots at high substrate concentrations (Figure 2). This is attributed to substrate binding to an allosteric site which increases the activity of the enzyme (9). Primary amines, although not substrates for the MFMO, still act as positive effectors of enzyme activity. Since the downward curvature of the double reciprocal plot for TB is considerably diminished in the presence of NOA, it seems that TB can also bind to the allosteric site on MFMO and thereby enhance its own oxidation.

The purified MFMO from hog liver is known to oxidize a wide variety of amine and sulfur-containing xenobiotic compounds, including thiobenzamide, and is thought to serve an important physiological role through the oxidation of cysteamine to cystamine (10,11). The activity of the purified MFMO is commonly measured by observing substrate-dependent stimulation of oxygen consumption or NADPH oxidation, while microsomal MFMO may be assayed by colorimetric determination of N-oxides of tertiary amine substrates such as N,N-dimethyloctylamine (9). Based on the present results, it appears that TB may provide a useful alternative method for measuring MFMO activity spectrophotometrically, even in relatively crude preparations

where measurements of oxygen consumption or NADPH oxidation may be ambiguous. The fact that thiobenzamide is a positive effector of its own oxidation could potentially complicate its use as an assay substrate. However, this problem can easily be avoided by using high enough concentrations of TB, and/or by running the assays in the presence of at least 5 mM NOA.

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