

TRICLOSAN AS A SUBSTRATE AND INHIBITOR OF 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE-SULFOTRANSFERASE AND UDP-GLUCURONOSYL TRANSFERASE IN HUMAN LIVER FRACTIONS

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ABSTRACT:

Triclosan is a broad spectrum antibacterial agent used in many household products. Due to its structural similarity to polychlorobiphenyls, which are potent inhibitors of the sulfonation and glucuronidation of 3-hydroxy-benzo[a]pyrene, it was hypothesized that triclosan would inhibit these phase II enzymes. This study was designed to assess the interactions of triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferases and UDP-glucuronosyltransferases in human liver cytosol and microsomes. Triclosan was sulfonated and glucuronidated in human liver. The apparent K_m and V_{max} values for triclosan sulfonation were 8.5 μM and 0.096 nmol/min/mg protein, whereas K_m and V_{max} values for glucuronidation were 107 μM and 0.739 nmol/min/mg protein. Triclosan inhibited the hepatic cytosolic sulfonation of 3-hydroxybenzo(a)pyrene (3-OH-BaP), bisphenol A, *p*-nitrophenol, and acetaminophen with IC_{50} concentrations of 2.87,

2.96, 6.45, and 17.8 μM , respectively. Studies of 3-OH-BaP sulfonation by expressed human SULT1A1*1, SULT1A1*2, SULT1B1, and SULT1E1 showed that triclosan inhibited the activities of each of these purified enzymes with IC_{50} concentrations between 2.09 and 7.5 μM . Triclosan was generally a less potent inhibitor of microsomal glucuronidation. IC_{50} concentrations for triclosan with 3-OH-BaP, acetaminophen, and bisphenol A as substrates were 4.55, 297, and >200 μM , respectively. Morphine glucuronidation was not inhibited by 50 μM triclosan. The kinetics of 3-OH-BaP sulfonation and glucuronidation were examined in the presence of varying concentrations of triclosan: the inhibition of sulfonation was non-competitive, whereas that of glucuronidation was competitive. These findings reveal that the commonly used bactericide triclosan is a selective inhibitor of the glucuronidation and sulfonation of phenolic xenobiotics.

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether; synonym, Irgan DP 300; see Fig. 1) is an antimicrobial agent that has found widespread use in many products, including antiseptic soaps, toothpastes, mouthwashes, cosmetics, dermatological agents, fabrics, and plastics. Triclosan is active against both Gram-positive and Gram-negative bacteria, and has some antifungal and antiviral properties (Jones et al., 2000). Triclosan's effectiveness as an antimicrobial agent is thought to be due to its ability to inhibit the enzyme enoyl-acyl carrier protein reductase, which is involved in bacterial lipid biosynthesis (McMurry et al., 1998; Levy et al., 1999; Heath and Rock, 2000).

Triclosan was first introduced in the health care industry in 1972 and in toothpaste in Europe in 1985 (Jones et al., 2000). In the United States, triclosan has been used for over 40 years and is recognized by

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the Food and Drug Administration as either an over-the-counter or a prescription drug (Jones et al., 2000). A number of consumer products with antibacterial properties, the vast majority containing triclosan, have entered the market. Triclosan is a stable, lipophilic compound ($\log K_{ow} = 4.8$), which can be heated up to 200°C for up to 2 h without significant degradation (Bhargava and Leonard, 1996). Due to its widespread use and stability, triclosan and some of its derivatives can now be readily detected in the environment and in some food sources (Lopez-Avila and Hites, 1980; Miyazaki et al., 1984; Okumura and Nishikawa, 1996). Triclosan has been reported in bile from fish caged close to wastewater treatment plants and in samples of human breast milk, with one milk sample containing 300 $\mu\text{g}/\text{kg}$ lipid weight (Adolfsson-Erici et al., 2002). Likewise, the presence of triclosan in human plasma was reported (DeSalva et al., 1989; Hovander et al., 2002). Triclosan itself has low acute toxicity, with reported LD_{50} values in mice of >1 g/kg (Kanetoshi et al., 1992). Its more highly chlorinated derivatives, some of which may be formed by bleaching textile products containing triclosan as a bactericide, had higher acute toxicity to mice (Kanetoshi et al., 1992). It was shown that triclosan could inhibit several cytochrome P450-dependent activities in rat liver microsomes. Triclosan was a potent inhibitor of pentyxyresorufin *O*-depentylase activity (K_i 1.48 μM) and

ABBREVIATIONS: 3-OH-BaP, 3-hydroxy-benzo(a)pyrene; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SULT, PAPS-sulfotransferase; TCA, trichloroacetic acid; TLC, thin-layer chromatography; UGT, UDP-glucuronosyltransferase; UDPGA, uridine diphosphoglucuronic acid.

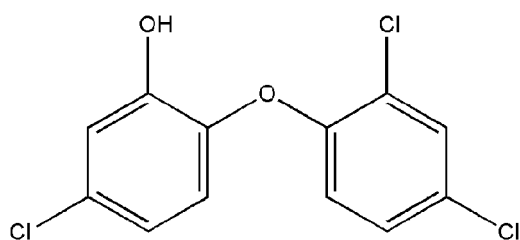


FIG. 1. The structure of triclosan.

ethoxyresorufin *O*-deethylase activity (K_i 0.24 μM) in liver microsomes from phenobarbital-treated and 3-methylcholanthrene-treated rats, respectively, and was a weaker inhibitor (K_i 31 μM) of testosterone 6 β -hydroxylase in liver microsomes from dexamethasone-treated rats (Hanioka et al., 1996). This finding suggested that exposure to triclosan could result in interactions with cytochrome P450 in the CYP1A, 2B, and 3A subfamilies.

There have been limited studies of triclosan metabolism. As a hydroxylated compound, it should be expected that glucuronidation and sulfonation would be the main pathways of triclosan metabolism. One study showed that triclosan was excreted primarily as the glucuronide following application of a 1% formulation to guinea pig or rat skin (Black et al., 1975). Another study showed that topically applied triclosan formed sulfate and glucuronide conjugates in human as well as rat skin (Moss et al., 2000). The human hepatic biotransformation of triclosan has not been reported in the open literature. There have been no studies of the possible interaction of triclosan with phase II enzymes.

Due to the structural similarity of triclosan to hydroxylated polychlorinated biphenyls, which have been shown to inhibit the sulfonation and glucuronidation of 3-hydroxy-benzo[*a*]pyrene (3-OH-BaP) in channel catfish intestine (van den Hurk et al., 2002), the sulfonation of 3-OH-BaP in human liver (Wang et al., 2002), and the sulfonation of estradiol by purified human sulfotransferases (Kester et al., 2000), it was of interest to determine whether triclosan would inhibit sulfonation and glucuronidation in human liver. The substrates selected for study were 3-OH-BaP, a major metabolite of the common environmental contaminant, benzo(*a*)pyrene, in humans and animals (Astrup et al., 1982), bisphenol A, another common environmental pollutant (Brotons et al., 1995), acetaminophen and morphine, widely used analgesics, and *p*-nitrophenol, a model substrate for SULT1A1, the major form of phenol sulfotransferase in human liver (Weinshilboun, 1990).

In the present study, human liver microsomes and cytosol were used to examine the conjugation of triclosan, and the effect of triclosan on the glucuronidation and sulfonation of representative hydroxylated xenobiotics. Some additional studies of the inhibition of sulfotransferase were conducted using expressed purified human sulfotransferases.

Materials and Methods

Materials. Triclosan was purchased from Sigma-Aldrich (St. Louis, MO). 3-OH-BaP, and BaP-3-sulfate and BaP-3-glucuronide were purchased from Midwest Research Institute (Kansas City, MO), through the National Cancer Institute Chemical Carcinogen Reference Standard Repository. ^{35}S -PAPS (3 $\mu\text{Ci/nmol}$, 99.1% pure) and ^{14}C -UDPGA (0.3 $\mu\text{Ci/nmol}$, >99% pure) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). ^{14}C -Acetaminophen (7.5 mCi/mmol, >98% pure) was purchased from Sigma-Aldrich. For use in assays, the ^{14}C -acetaminophen was diluted with unlabeled acetaminophen (>99% pure) to a specific radioactivity of 2.18 mCi/mmol. ^{14}C -Bisphenol A (200 mCi/mmol, >97% pure) was purchased from Moravak Biochemicals (Brea, CA) and diluted with unlabeled bisphenol A (>99% pure)

to 3.4 mCi/mmol for use in assays. 4-Nitro[^{14}C]phenol (118 mCi/mmol, 98.4% pure) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). The ^{14}C -*p*-nitrophenol was diluted with unlabeled *p*-nitrophenol (spectrophotometric grade) to a specific radioactivity of 20.1 mCi/mmol for use in assays. Bisphenol A, acetaminophen, morphine, morphine 3-glucuronide, *p*-nitrophenol, *p*-nitrophenylsulfate, Brij 58, arylsulfatase, and β -glucuronidase were purchased from Sigma-Aldrich. All other chemicals were the highest grade available from Fisher Scientific Co. (Pittsburgh, PA) or Sigma-Aldrich.

Tissue Preparation. Three human liver samples, which were kindly donated by Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN) were used in these studies. Liver cytosolic and microsomal preparations were made by standard differential centrifugation methods. In short, the liver was homogenized in 4 volumes of ice-cold 1.15% KCl dissolved in 50 mM potassium phosphate buffer (pH 7.4) using a glass-Teflon homogenizer. The homogenates were centrifuged at 13,300g for 20 min and the resulting supernatants were centrifuged again at 155,000g for 45 min. The high speed cytosolic fraction was divided into aliquots and stored at -80°C until use. The microsomal pellet was resuspended in the homogenizing buffer, resedimented at 155,000g for 40 min, and then resuspended in buffer containing 0.25 M sucrose, 0.01 M HEPES, pH 7.4, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Aliquots were stored at -80°C until use. The protein concentrations were measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

Purified Enzymes. Expression in *Escherichia coli* of human SULT1A1*1, SULT1B1, and SULT1E1 isoforms has been described previously (Falany et al., 1990; Hagen et al., 1998; Wang et al., 1998). *E. coli* containing the genes for these enzymes were grown as described previously, and harvested cells were used for SULT isolation (Falany et al., 1990, 1994). Expressed human SULT1A1*2 cytosolic extract was purchased from PanVera Corp. (Madison, WI).

Sulfotransferase Assays. Assay conditions with all substrates were optimized such that the rate of reaction was linear with protein and time, and was saturating for PAPS. The concentration of each xenobiotic substrate was selected so that no substrate inhibition was observed. All studies were conducted with hepatic cytosol or microsomes from three different individuals, with duplicate tubes for each assay condition.

Triclosan sulfonation was assessed with a radiochemical assay using ^{35}S -labeled PAPS. A concentration series of 0.5 to 20 μM triclosan was incubated with human liver cytosol (0.3 mg protein), 20 μM ^{35}S -PAPS (diluted with unlabeled PAPS to a specific activity of 750 mCi/mmol), 0.05 M Tris-HCl (pH 7.0) and 0.4% BSA. The reaction mixture was preincubated at 35°C for 1 min; then, the reaction was started by adding the PAPS. After incubation for 30 min at 35°C with gentle shaking, the reaction was terminated by adding 0.10 ml of a 1:1 mixture of acetic acid (2.5%) and tetrabutylammonium dihydrogen phosphate (0.2 mM), and the sulfated product was extracted with 2.0 ml of water-saturated ethyl acetate, as described previously for other xenobiotic substrates (Tong and James, 2000). Blank tubes were stopped immediately after addition of the PAPS mixture. The phases were separated by centrifugation, and a portion (1.8 ml) of the ethyl acetate phase was transferred to clean tubes. An additional 1.0 ml of water-saturated ethyl acetate was added to the aqueous phase. After vortex-mixing and centrifugation, another 1.1 ml of the ethyl acetate phase was combined with the first. The ethyl acetate phase, 0.5 ml, was counted for quantitation of sulfate conjugate. The enzyme activity was determined using the specific radioactivity of PAPS after correction for blanks.

For validation of the triclosan sulfotransferase assay, samples of the ethyl acetate phases and aqueous phases from incubations with triclosan were subjected to thin-layer chromatography (TLC) on microcrystalline cellulose-coated plates (Avicel 250- μm plates from Analtech, Newark, DE). The plates were developed in *n*-butanol/acetic acid/water, 5:0.5:1, by volume, and radioactive spots were visualized by the InstantImager (PerkinElmer Life and Analytical Sciences). The identity of the conjugate of triclosan as a sulfate ester was verified by examining its sensitivity to arylsulfatase. Samples of the dried ethyl acetate extracts were dissolved in 0.5 ml of Tris-Cl buffer, pH 7.0, containing 0 or 1.9 units of arylsulfatase. Tubes were incubated at 37°C for 5 h, and then aliquots were reanalyzed by TLC as above.

3-OH-BaP sulfotransferase activity was determined by a method described previously (James et al., 1997), and optimized for human liver cytosol and

expressed enzymes. In short, the reaction mixture consisted of 0.4% BSA, 50 mM Tris-HCl (pH 7.0), 20 μ M PAPS, 250 μ g of cytosolic protein, and 0.1 or 1 μ M 3-OH-BaP in a total reaction volume of 500 μ l. For studies of inhibition, stock solutions of triclosan were prepared in DMSO, and final concentrations of 0.1 to 500 μ M were added to the incubation mixture, such that DMSO concentration did not exceed 0.5% (v/v). For studies with expressed human SULT, 0.02 to 5.2 μ g of protein was used, and the PAPS concentration was 2 μ M (SULT1A1 and 1E1) or 20 μ M (SULT1B1). Incubation was carried out for 10 min at 35°C. The reaction was stopped with 2 ml of ice-cold methanol. Precipitated protein was pelleted by centrifugation for 10 min at 2000 rpm. Two milliliters of the supernatant was then mixed with 0.5 ml of 1 M NaOH, and the fluorescence of BaP-3-SO₄ was measured at the optimal excitation/emission wavelengths of 294/415 nm and a slit width of 10 nm for excitation and emission. The fluorescence was calibrated from a standard curve of authentic BaP-3-SO₄ prepared with a range of concentrations that covered the range of product formation. The sensitivity of the fluorescence spectrophotometer was adjusted to provide appropriate sensitivity.

Sulfotransferase activity with acetaminophen was conducted as follows. Briefly, the enzyme assay mixture contained 0.5 mg of hepatic cytosolic protein with 0.4% BSA, 50 mM Tris-Cl (pH 7.0), 100 μ M PAPS, 1.5 mM ¹⁴C-acetaminophen, and varying concentrations (0–100 μ M) of triclosan in a total volume of 250 μ l. Samples were incubated at 37°C for 1 h. The reaction was stopped by addition of 1.5 ml of ice-cold methanol. After centrifugation for 10 min at 2000 rpm, 1.6 ml of the supernatant was transferred to a clean tube, and the solvent was removed under vacuum. The residue was redissolved in 200 μ l of 50% aqueous methanol. Then, 20 μ l of this solution was applied to microcrystalline cellulose-coated plates (Avicel 250- μ m plates from Analtech). The plates were developed in *n*-butanol/acetic acid/water, 3:1:1 by volume, and radioactive spots were visualized by the InstantImager (PerkinElmer Life and Analytical Sciences). The identity of the acetaminophen sulfate spot was verified by demonstrating that this spot was sensitive to sulfatase hydrolysis.

The sulfonation of bisphenol A was assessed by a radiochemical extraction method. The assay mixture contained 0.04 mg of hepatic cytosolic protein, 0.4% BSA, 50 mM Tris-Cl (pH 7.0), 75 μ M ¹⁴C-bisphenol A, varying concentrations (0–100 μ M) of triclosan, and 20 μ M PAPS (added last to start the reaction), in a total volume of 100 μ l. Samples were incubated at 37°C for 20 min. The reaction was stopped by addition of 200 μ l of cold 3% trichloroacetic acid (TCA; w/v). Methylene chloride (1 ml) was added and the mixture was extracted to remove unreacted bisphenol A substrate. The mixture was centrifuged for 10 min at 2000 rpm. The aqueous phase was extracted twice more to ensure removal of the substrate. The aqueous phase, 150 μ l, containing the bisphenol A sulfate, was taken for liquid scintillation counting (Beckman LS 6000; Beckman Coulter, Fullerton, CA). The efficiency of separation of bisphenol A from the sulfate conjugate was demonstrated by analyzing each phase by TLC as described above for acetaminophen. The spot corresponding to bisphenol A sulfate was shown to be sensitive to hydrolysis with sulfatase.

p-Nitrophenol sulfotransferase activity was measured with 4 μ M 4-nitro[¹⁴C]phenol, 20 μ M PAPS, 50 mM Tris-HCl (pH 7.0), 0.4% BSA, and 30 to 80 μ g of cytosolic protein. Varying concentrations of triclosan dissolved in DMSO (1.25 μ l) were added to the incubation mixture for a final volume of 250 μ l. The reaction was started by addition of PAPS and stopped with 200 μ l of cold 3% TCA (w/v) after a 10-min incubation at 37°C. Methylene chloride, 1 ml, was added, and the mixture was extracted to remove unreacted *p*-nitrophenol. The mixture was centrifuged for 10 min at 2000 rpm to separate phases. The aqueous phase was extracted twice more with methylene chloride. The final aqueous phase, 200 μ l, containing *p*-nitrophenylsulfate, was used for liquid scintillation counting (Beckman Coulter LS 6000). The efficiency of separation of *p*-nitrophenol from the sulfate conjugate was demonstrated by analyzing each phase by TLC as described above for acetaminophen.

Glucuronidation Assays. Assay conditions with all substrates were optimized such that the rate of reaction was linear with protein and time, and was saturating for UDPGA. A radiochemical method was used to study triclosan glucuronidation. A concentration series of 5 to 500 μ M triclosan was incubated with human liver microsomes (0.3 mg protein) that had been pretreated with Brij 58 at a ratio of 0.25 mg of Brij 58 per mg of microsomal protein, 200 μ M ¹⁴C-UDPGA (diluted with unlabeled UDPGA to a specific activity of 9.9

mCi/mmol), 0.1 M Tris-HCl (pH 7.6), and 5 mM MgCl₂. The concentration of Brij 58 needed to optimally activate the microsomes was determined experimentally. The reaction was started by adding UDPGA, and the extraction procedure was similar to that described above for studies of triclosan sulfonation. The difference was that the glucuronidation assay included a set of blank tubes without triclosan, but with microsomes, which were incubated for 30 min. This was necessary because endogenous compounds or drugs were present in microsomes, which were substrates for glucuronidation. The separation of triclosan-¹⁴C-glucuronide from ¹⁴C-UDPGA by the extraction assay was verified. Samples of the ethyl acetate phases and aqueous phases from incubations with triclosan were subjected to TLC on microcrystalline cellulose-coated plates (Avicel 250- μ m plates from Analtech). The plates were developed in *n*-butanol/acetic acid/water, 5:0.5:1, by volume, and radioactive spots were visualized by the InstantImager (PerkinElmer Life and Analytical Sciences). To confirm the identity of the radioactivity in the ethyl acetate fraction as triclosan glucuronide, samples of the dried ethyl acetate extracts were dissolved in 0.4 ml of 0.5 M Tris-Cl buffer, pH 5.0, containing 3000 units of β -glucuronidase. Tubes were incubated at 35°C for 16 h, and then aliquots were reanalyzed by TLC as above.

The reaction mixture for measuring the glucuronidation of 3-OH-BaP consisted of 0.1 M Tris-HCl buffer (pH 7.6), 5 mM MgCl₂, 200 μ M UDPGA, 104 to 187 μ g of microsomal protein, 1 μ M 3-OH-BaP, and Brij 58 (0.25 mg/mg protein). The assay procedure was identical to the sulfonation assay described above, except the final incubation time was 40 min and the fluorescence of BaP-3-glucuronide was measured at excitation/emission wavelengths of 300/421 nm, with slit widths for excitation and emission of 10 nm (Singh and Wiebel, 1979). The fluorescence was compared to a standard curve prepared with authentic BaP-3-glucuronide in the range of glucuronide produced.

The incubation mixture for acetaminophen glucuronidation contained 0.5 to 0.7 mg of hepatic microsomal protein with 0.25 mg of Brij 58 per mg of protein, 0.1 M Tris-Cl (pH 7.6), 5 mM MgCl₂, 1 mM UDPGA, 1.5 mM acetaminophen, and varying concentrations (25–500 μ M) of triclosan in a total volume of 250 μ l. The subsequent procedures for quantitation of formation of acetaminophen glucuronide were identical to those used to study sulfonation of acetaminophen, except that the identity of the glucuronide conjugate was verified by incubation with β -glucuronidase.

The incubation mixture for bisphenol A glucuronidation contained 0.3 mg of hepatic microsomal protein with 0.25 mg of Brij 58 per mg of protein, 0.1 M Tris-Cl (pH 7.6), 5 mM MgCl₂, 1 mM UDPGA, 50 μ M bisphenol A, and varying concentrations (10–200 μ M) of triclosan in a total volume of 100 μ l. The reaction was stopped with TCA, and unreacted substrate was removed by dichloromethane extraction, as described above in the assay for sulfonation of bisphenol A.

Glucuronidation of morphine was examined by using a Zorbax RX-C8 high performance liquid chromatography column and a SecurityGuard column. The mobile phase consisted 74% (v/v) 10 mM sodium dihydrogen phosphate and 1 mM sodium dodecyl sulfate (pH 2.1) in acetonitrile. Metabolites were detected at excitation/emission wavelengths of 210/340 nm (Glare et al., 1991). The band width was 20 nm and the gain setting was 2. Addition of triclosan at a concentration of 50 μ M did not alter the amount of morphine 3-glucuronide formed; therefore, no further studies were conducted with this substrate.

Kinetic Analysis. The kinetics of sulfonation of 3-OH-BaP were studied in three liver samples using the assay described above, with the exception that the reaction volume was increased to 1 ml, and the reaction was stopped with 1.5 ml of ice-cold methanol. These modifications were necessary to improve the sensitivity of the assay at substrate concentrations below 100 nM. Human liver cytosol (0.2 mg protein), was incubated with 0.035 to 0.25 μ M 3-OH-BaP in the absence or the presence of 0.5, 1.0, and 2.0 μ M triclosan. Similarly, the kinetics of glucuronidation were studied using the glucuronidation assay described above except the substrate 3-OH-BaP concentrations were 2.5, 5, 10, 20, and 40.0 μ M, while UDPGA was kept at 0.4 mM. To study the type of inhibition of triclosan with glucuronosyltransferase, four sets of assays with triclosan (control, 5.0, 10.0, and 20.0 μ M) were used.

Data Analysis. Inhibition data are presented as percentage of activity compared with the controls without inhibitor. The IC₅₀ values were obtained from regression analysis of the relationship between percentage of control activity and log triclosan concentration over the log-linear range using Excel

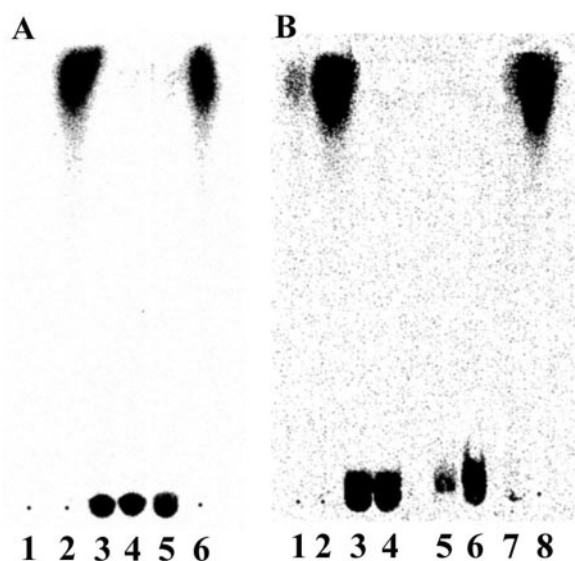


FIG. 2. Thin-layer chromatograms of sulfonation and glucuronidation of triclosan on Avicel microcrystalline cellulose plates. A, lanes 1–2: ethyl acetate extracts of incubations of human hepatic cytosol with ^{35}S -PAPS and triclosan (0, 10 μM) showing the conjugate in lane 2; lanes 3–4: aqueous phases from incubations with triclosan (0, 10 μM) showing ^{35}S -PAPS at the origin; lane 5: the ethyl acetate extract from an incubation with triclosan, 10 μM , was dried and incubated with sulfatase in 0.5 M Tris-HCl buffer, pH 7.0, for 10 h at 37°C, and aliquots were applied to the TLC plate; lane 6: the ethyl acetate extract from an incubation with triclosan, 10 μM , was dried and incubated with 0.5 M Tris-HCl buffer, pH 7.0, for 10 h at 37°C, and aliquots were applied to the TLC plates. B, lanes 1–2: ethyl acetate extracts of incubations of human hepatic microsomes with ^{14}C -UDPGA and triclosan (0, 100 μM) showing the conjugate in lane 1 and small amounts of an unknown conjugate in lane 2; lanes 3–4: aqueous phases from incubations with triclosan (0, 100 μM) showing ^{14}C -UDPGA at the origin; lanes 5–6: the ethyl acetate extracts from incubations with triclosan (0, 100 μM) were dried and then incubated with glucuronidase in 0.5 M Tris-HCl buffer, pH 5.0, for 16 h at 37°C, and aliquots were applied to the TLC plate, showing ^{14}C -UDPGA at the origin; lanes 7–8: the ethyl acetate extract from incubations with triclosan (0, 100 μM) were dried and then incubated with 0.5 M Tris-HCl buffer, pH 5.0, for 16 h at 37°C, and aliquots were applied to the TLC plates.

software. Results are presented as mean values with standard deviation for results from three different individuals. Enzyme kinetic parameters (K_m , V_{\max}) were calculated from the Michaelis-Menten equation with the software package Prism (versions 3.0 and 4.0; GraphPad Software, Inc., San Diego, CA). The effects of triclosan on UGT and SULT activities were analyzed by Dixon plots for each individual liver, as well as by plotting apparent K_m/V_{\max} data for each individual against the concentration of triclosan, and apparent $1/V_{\max}$ data for each individual liver against the concentration of triclosan (Cornish-Bowden, 1995).

Results

Sulfonation and Glucuronidation of Triclosan. Triclosan was shown to be sulfonated and glucuronidated in human liver cytosol and microsomes, respectively. The ion-pair extraction assays were validated by demonstrating that ethyl acetate extraction separated the radiolabeled cosubstrate (^{35}S -PAPS or ^{14}C -UDPGA) from the radiolabeled conjugate. Representative TLC autoradiograms showing that the ethyl acetate extracts contained only the respective conjugates, whereas the aqueous phases contained only the respective cosubstrates, are shown in Fig. 2. Arylsulfatase or β -glucuronidase treatment of the ethyl acetate fractions from assays of sulfonation or glucuronidation, respectively, resulted in loss of the spots corresponding to triclosan sulfate or glucuronide (Fig. 2). Ethyl acetate extracts of human liver microsomes incubated for 30 min with ^{14}C -UDPGA in the absence of added triclosan showed very small amounts of glucuronide conjugate that was presumably formed from a substance

TABLE 1
Apparent kinetic constants for triclosan sulfotransferase and glucuronosyltransferase activity in human liver

Values are mean \pm S.D. ($n = 3$).

	K_m	V_{\max}
	μM	$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$
Sulfonation	8.5 ± 3.2	95.9 ± 28.4
Glucuronidation	107 ± 22.2	739 ± 163

present in human liver microsomes (Fig. 2B, lane 1). This product was minor, compared with the amount of triclosan glucuronide formed in the incubation with added triclosan (Fig. 2B, lane 2).

Both sulfonation and glucuronidation followed Michaelis-Menten kinetics in the substrate concentration range studied. We did not investigate triclosan concentrations higher than 20 μM for sulfonation. The apparent K_m and V_{\max} values for sulfonation and glucuronidation of triclosan are given in Table 1. Graphs showing the data are presented in Fig. 3. Both K_m and V_{\max} for glucuronidation were approximately an order of magnitude higher than for sulfonation.

Inhibition of 3-OH-BaP Sulfonation and Glucuronidation by Triclosan. Addition of triclosan to assay tubes resulted in a concentration-dependent inhibition of 3-OH-BaP sulfonation and glucuronidation using human hepatic cytosol or microsomes (Fig. 4). At a concentration of 0.1 μM , triclosan inhibited both sulfonation and glucuronidation of 3-OH-BaP by about 10%. IC_{50} values for inhibition of 3-OH-BaP sulfotransferase and glucuronosyltransferase activity were, respectively, 2.77 ± 0.94 and 4.55 ± 1.65 μM (mean \pm S.D., $n = 3$ individuals).

The effect of triclosan on the kinetics of 3-OH-BaP sulfotransferase was examined, and Fig. 5 shows mean results for the three livers. Error bars have been omitted for clarity, since there was a large spread of activity between the livers. The transformation of the enzyme activity (Fig. 5A) for substrate concentrations between 25 and 125 nM 3-OH-BaP into Lineweaver-Burk plots (Fig. 5B) generated straight lines, and in this substrate range the data fit the Michaelis-Menten model. The highest concentration of 3-OH-BaP used, 250 nM, showed substrate inhibition and was not used in the kinetic analysis. Double-reciprocal plots of the rate of 3-OH-BaP sulfonation versus the 3-OH-BaP concentration in the absence or presence of different concentrations of triclosan converged at approximately the same point on the x -axis. There was no change in K_m with added triclosan, but there was a reduction in V_{\max} , indicating noncompetitive inhibition. Table 2 summarizes the kinetic parameters K_m and V_{\max} . The K_i value was calculated from Dixon plots for each individual liver as 1.52 ± 0.13 μM (mean \pm S.D., $n = 3$).

Figure 6 shows results for the kinetics of 3-OH-BaP-glucuronosyltransferase in human liver microsomes. In the absence of triclosan, the maximum rate for the formation of 3-OH-BaP glucuronide using human hepatic microsomes was 519 ± 137 pmol/min/mg protein, and the apparent K_m value for 3-OH-BaP was 15.1 ± 3.6 μM (mean \pm S.D., $n = 3$). UGT activity with 3-OH-BaP in human liver was not sensitive to the substrate inhibition, even at a substrate concentration of 40 μM (Fig. 6). Addition of 5 or 10 μM triclosan increased the apparent K_m value but did not affect the apparent V_{\max} , whereas addition of 20 μM triclosan increased K_m and reduced apparent V_{\max} , relative to the uninhibited reaction. This was true for each individual liver, although because of high individual variability, the mean apparent K_m and V_{\max} values, summarized in Table 2, did not show statistically significant differences in the V_{\max} values. Data for each individual liver were analyzed by Dixon plots, plots of K_m/V_{\max} versus triclosan concentration, and plots of $1/V_{\max}$ against triclosan

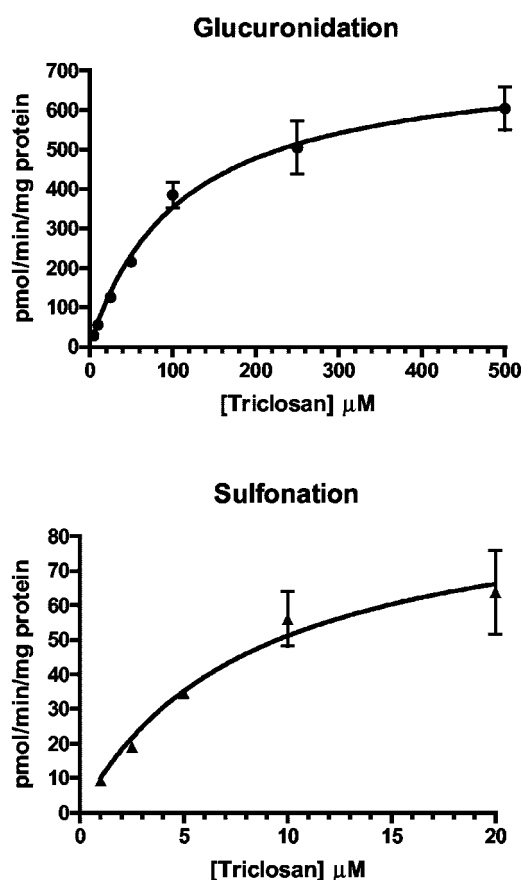


FIG. 3. Results for rates of glucuronidation (top) and sulfonation (bottom) of triclosan in human liver. Data are given as the mean of three experiments \pm S.D. The kinetic parameters are summarized in Table 1.

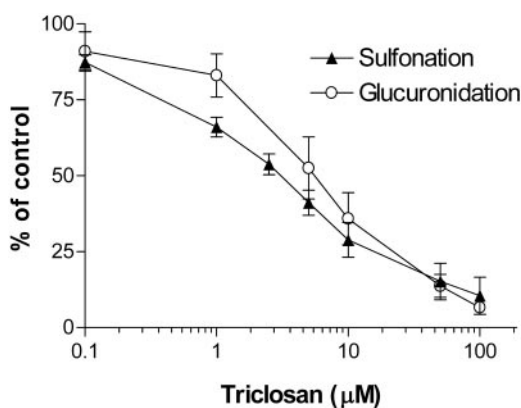


FIG. 4. Inhibition of sulfotransferase and glucuronosyltransferase activities of 3-OH-BaP in human liver by increasing concentrations of triclosan. The 3-OH-BaP concentration was 100 nM for sulfonation and 1 μ M for glucuronidation. Activities are given as percentage of control rates, which were 50.5 ± 10.9 pmol/min/mg protein for 3-OH-BaP sulfonation and 44.7 ± 6.2 pmol/min/mg protein for 3-OH-BaP glucuronidation. Data are given as the mean of three experiments \pm S.D.

concentration. The best fit was for plots of K_m/V_{max} versus triclosan concentration, indicating competitive inhibition with K_i of 8.43 ± 1.94 μ M (mean \pm S.D., $n = 3$).

The susceptibilities of several phenol sulfotransferase isoforms to triclosan were examined with 3-OH-BaP as substrate. Studies showed that 3-OH-BaP was a substrate for SULT1A1*1, SULT1A1*2, SULT1B1, and SULT1E1. SULT1A1 and 1E1 showed substrate inhibition at 3-OH-BaP concentrations >200 nM (manuscript in prep-

aration); therefore, the concentration of 3-OH-BaP used with these isoforms was 100 nM. No substrate inhibition was found with SULT1B1, and the effects of triclosan were assessed with a 3-OH-BaP concentration of 1 μ M. The IC_{50} values for SULT1A1*1, SULT1A1*2, SULT1B1, and SULT1E1 were 2.3 μ M, 2.4 μ M, 2.1 μ M, and 7.5 μ M, respectively (Fig. 7).

Inhibition of the Sulfonation and Glucuronidation of Other Xenobiotics. Triclosan inhibited the sulfonation of bisphenol A, *p*-nitrophenol, and acetaminophen with IC_{50} values of 3.68 ± 0.64 , 6.45 ± 0.31 , and 17.8 ± 2.3 μ M, respectively (mean \pm S.D., $n = 3$). Triclosan was a weak inhibitor of acetaminophen and bisphenol A glucuronidation. The IC_{50} value for inhibition of acetaminophen glucuronidation was 297 ± 5.1 μ M (mean \pm S.D., $n = 3$), whereas that for bisphenol A glucuronidation was >200 μ M (the highest concentration studied). Figure 8 shows the effects of triclosan on the conjugation of these substrates.

Discussion

Due to the widespread use and the stability of triclosan, it can be anticipated that humans may be exposed to triclosan. The present study showed that triclosan was metabolized to glucuronide or sulfate conjugates in the presence of human liver microsomes or cytosol, respectively. As a substrate for phase II enzymes, triclosan behaved similarly to several other hydroxylated xenobiotics, in that both the K_m and V_{max} values for glucuronidation were higher than for sulfonation (Mulder et al., 1990). At low concentrations most likely to be relevant to environmental exposures, such as 1 to 5 μ M, our data showed that triclosan sulfate and glucuronide were formed in the liver at approximately equal rates (Fig. 3). At concentrations below 1 μ M, sulfonation would be expected to predominate. Exposure to concentrations of triclosan higher than 20 μ M should result in glucuronidation being the predominant pathway in the liver (Table 1; Fig. 3). There are no data on the expected hepatic concentrations of triclosan following dermal application or exposure of people to triclosan; however, reports of triclosan residues in fish bile and human breast milk suggest that systemic absorption occurs (Adolfsson-Erici et al., 2002). Although the conjugation of triclosan has not been previously reported in human liver, an in vitro study of the percutaneous penetration and dermal metabolism of triclosan in diffusion cells fitted with human skin following application of 64.5 mM [3 H]triclosan showed that triclosan sulfate was the only metabolite in the skin at 4 h after application, whereas both the sulfate and glucuronide conjugates were present in skin at 8 and 24 h after application (Moss et al., 2000). At all times, there was more unchanged triclosan than either of the metabolites in skin. At the 4-h time point, only a low concentration of triclosan had penetrated the skin (Moss et al., 2000); thus the finding that only triclosan sulfate was present may be due to the lower K_m for sulfonation than for glucuronidation. Similar results were found in diffusion cells with rat skin. In intact rats given a single topical dose of 1.84 mg [3 H]triclosan, part of the dose was absorbed and eliminated in feces and urine. Unchanged triclosan and triclosan glucuronide were identified in urine and feces, with small amounts of triclosan sulfate in urine (Moss et al., 2000). In an earlier study, rats and guinea pigs treated with 1% triclosan in a soap formulation to the skin excreted triclosan glucuronide as the major urinary metabolite (Black et al., 1975).

As well as being a substrate for glucuronidation and sulfonation, this study showed that triclosan inhibited these pathways. Cytosolic SULT enzymes catalyzing the conjugation of 3-OH-BaP, *p*-nitrophenol, bisphenol A, and acetaminophen were inhibited by low micromolar concentrations of triclosan, such as may be attained from exposure to triclosan-treated products. Since 3-OH-BaP was most

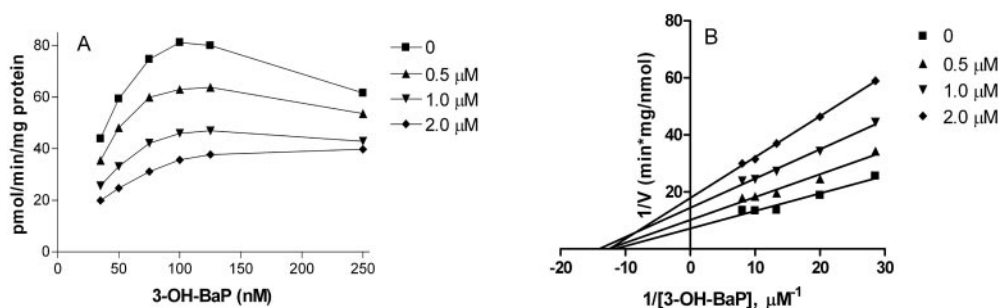


FIG. 5. Representative results of the kinetics of sulfotransferase activity with 3-OH-BaP in human liver cytosol in the presence of triclosan. A shows saturation curves and B shows transformation of the enzyme activities into Lineweaver-Burk plots. The kinetic parameters are summarized in Table 2.

TABLE 2

Apparent kinetic constants for 3-OH-BaP sulfotransferase and glucuronosyltransferase activity in human liver in the presence and absence of triclosan

Values are mean \pm S.D. ($n = 3$).

Triclosan μM	Sulfonation		Triclosan μM	Glucuronidation	
	K_m μM	V_{max} $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$		K_m μM	V_{max} $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$
0	0.057 ± 0.014	121 ± 36.7	0	15.1 ± 3.6	519 ± 137
0.5	0.054 ± 0.015	93.5 ± 39.9	5	24.1 ± 5.4	510 ± 171
1.0	0.060 ± 0.018	71.2 ± 29.2	10	31.3 ± 3.9	481 ± 149
2.0	0.074 ± 0.020	59.7 ± 22.6	20	27.9 ± 7.3	291 ± 138

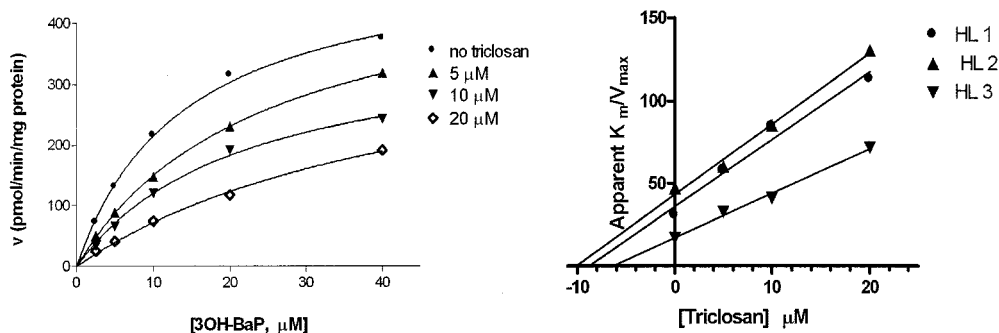


FIG. 6. Effect of triclosan on the kinetics of glucuronosyltransferase with 3-OH-BaP in human liver microsomes. The left graph shows saturation curves, with each point representing the mean of data from three livers. Standard deviations were not shown on the graph, for clarity. The kinetic parameters are summarized in Table 2. The right graph shows plots of apparent K_m/V_{max} versus the concentration of triclosan for each individual human liver, for calculation of K_i values.

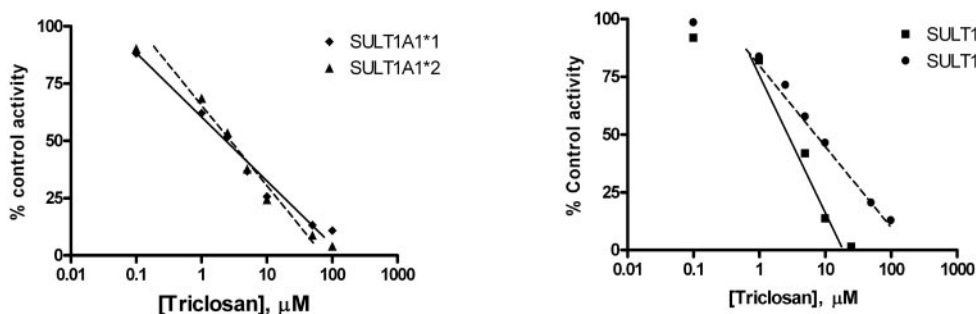


FIG. 7. Effect of triclosan on 3-OH-BaP sulfonation catalyzed by expressed purified human SULT isoforms. Graphs show data as percentage of control activity in the absence of triclosan. IC_{50} values were calculated from regression lines for the log-linear portions of the inhibition curves, as shown.

sensitive to inhibition by triclosan, studies of its conjugation by individual SULT isozymes expected to contribute to its hepatic biotransformation were examined. Phenolic xenobiotics such as 3-OH-BaP are most likely to be substrates for phenol sulfotransferases. SULT1A1 (also known as ST1A3) is the predominant phenol sulfotransferase expressed in human liver, and the other major hepatic phenol sulfotransferases are SULT1E1 (ST1E4) and SULT1B1

(ST1B2) (Honma et al., 2002). SULT1A1 has two common polymorphic variants, SULT1A1*1 and SULT1A1*2 (Bamber et al., 2001). Both SULT1A1 variants readily metabolized 3-OH-BaP and were similarly sensitive to triclosan, with IC_{50} values of 2.3 and 2.5 μM , respectively. These IC_{50} values were similar to the IC_{50} value of 2.8 μM for hepatic cytosol. SULT1B1 and 1E1 also catalyzed the sulfonation of 3-OH-BaP and were susceptible to inhibition by triclosan

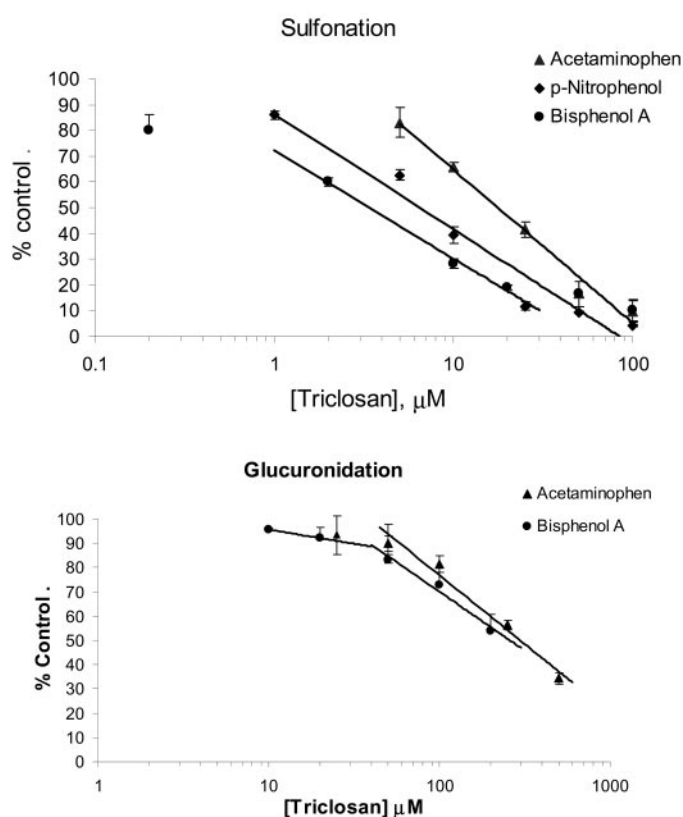


FIG. 8. Inhibition of sulfonation (top) and glucuronidation (bottom) of acetaminophen, bisphenol A, and *p*-nitrophenol (sulfonation only) by triclosan. IC_{50} values were calculated from regression lines for the log-linear portions of the inhibition curves, as shown. Uninhibited activities were as follows: acetaminophen sulfonation 37.9 ± 28.3 pmol/min/mg protein; acetaminophen glucuronidation 35.9 ± 15.8 pmol/min/mg protein; *p*-nitrophenol sulfonation, 798 ± 260 pmol/min/mg protein; bisphenol A sulfonation, 80.4 ± 11.3 pmol/min/mg protein; bisphenol A glucuronidation, 637.8 ± 106.5 pmol/min/mg protein.

at low micromolar concentrations. These results suggested that exposure to triclosan may impair the sulfonation of several phenolic xenobiotics.

The effects of triclosan on glucuronidation varied with substrate. Triclosan inhibited 3-OH-BaP glucuronidation at low micromolar concentrations, whereas it was a much weaker inhibitor of the glucuronidation of acetaminophen and bisphenol A. The glucuronidation of 3-OH-BaP in human liver is thought to be catalyzed by several UGT isozymes including UGT1A6 and UGT1A9 (reviewed by Radominska-Pandya et al., 1999). The conjugation of acetaminophen by several expressed human UGTs has been examined: UGT1A1 and UGT1A9 were shown to have good activity, and UGT1A6 and UGT1A10 had some activity (Court et al., 2001). Of these forms, only UGT1A10 is not expressed in human liver (Tukey and Strassburg, 2000). It was not known whether the difference between 3-OH-BaP and acetaminophen in sensitivity to inhibition by triclosan was due to different isozymes being the major catalysts for glucuronidation of these two substrates in human liver microsomes, or whether there were differences in the way that triclosan bound to UGT in the presence of either 3-OH-BaP or acetaminophen. Morphine is reported to be a substrate of UGT2B7, but not UGT1A isoforms (Radominska-Pandya et al., 1999). Since morphine glucuronidation was not inhibited by triclosan, this suggested the inhibition was isoform-specific, and not a general phenomenon.

To gain more insight into the mechanism of inhibition by triclosan, the kinetics of inhibition of cytosolic SULT were studied with 3-OH-BaP as

substrate in concentrations between 35 and 250 nM. The maximum rate of sulfonation in human liver cytosol was observed at 100 nM 3-OH-BaP, with marked substrate inhibition at higher concentrations (Fig. 5). The results for the kinetics of inhibition in the substrate range 35 to 100 nM suggested a noncompetitive mode of inhibition. Our studies with expressed human SULT enzymes showed that several forms of phenol sulfotransferase can metabolize 3-OH-BaP. Interpretation of the kinetic studies in cytosol is complicated by the properties of SULT1A1 and SULT1E1, two important forms of SULT found in hepatic cytosol, of exhibiting substrate inhibition that is apparently related to two substrate binding sites. Substrate inhibition was shown for the sulfonation of *p*-nitrophenol by SULT1A1*2, and studies of the crystal structure of SULT1A1*2 bound to *p*-nitrophenol provided evidence that this was because there were two substrate-binding sites with differing binding constants and rates of sulfonation (Gamage et al., 2003; Barnett et al., 2004). Assuming that SULT1A1 was the major catalyst for 3-OH-BaP sulfonation in human liver, it was possible that triclosan affected the interaction of 3-OH-BaP with the two substrate binding sites and, therefore, the rates of sulfonation. Studies of the catalytic mechanism of SULT1E1 suggested two binding sites for estradiol, one active site and one allosteric site associated with slower sulfonation (Zhang et al., 1998). Crystallographic studies of the interaction of 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl with human SULT1E1 suggested that this compound binds at the estrogen substrate binding site (Shevtsov et al., 2003). However, Kester et al. (2000) showed noncompetitive inhibition of SULT1E1 by the same compound. It is possible that when 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl occupies the substrate binding site, estradiol binds to the second site, where conjugation with sulfate occurs more slowly (Zhang et al., 1998). A similar mechanism may occur for triclosan.

Kinetic studies of the effect of triclosan on 3-OH-BaP glucuronidation in human liver microsomes most closely fit a competitive mechanism of inhibition (Table 2), suggesting that triclosan and 3-OH-BaP compete for the substrate-binding site. As discussed above, several forms of UGT1A are likely to contribute to 3-OH-BaP glucuronidation, and it is likely that the same forms also conjugate triclosan. Further studies are needed to determine whether the different UGT1A isoforms exhibit different kinetics and interactions with triclosan.

We did not study the effect of triclosan on the *in vivo* fate of coadministered 3-OH-BaP. Our findings suggest that if tissue concentrations of triclosan reached the inhibitory range demonstrated in this study, the phase II conjugation of 3-OH-BaP would be inhibited. This could lead to retention of 3-OH-BaP and perhaps biotransformation by other pathways, such as activation to the mutagenic metabolite, 3-OH-BaP-7,8-dihydrodiol-9,10-epoxide (Glatt et al., 1987).

In summary, the widely used antibacterial agent triclosan was found to inhibit the sulfonation and glucuronidation of 3-OH-BaP and other phenolic xenobiotics in human liver. Triclosan itself was a substrate for human SULT and UGT enzymes. To our knowledge, this is the first *in vitro* study of the conjugation of triclosan using human liver fractions.

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