Interaction of Salicylate and Ibuprofen with the Carboxylic Acid: CoA Ligases from Bovine Liver Mitochondria

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ABSTRACT: Neither salicylate nor ibuprofen was a substrate or inhibitor of the long-chain fatty acid:CoA ligase. In contrast, all three xenobiotic-metabolizing medium-chain fatty acid:CoA ligases (XL-I, XL-II, and XL-III) had activity toward salicylate. The K_m value for salicylate was similar for all three forms (2 to 3 μM), but XL-II and XL-III had higher activity at V_{max}. For ibuprofen, only XL-III catalyzed its activation, and it had a K_m for ibuprofen of 36 μM. Studies of salicylate inhibition of XL-I, XL-II, and XL-III revealed that it inhibited the benzoate activity of all three forms with K_I values of ca. 2 μM, which is in agreement with the K_m values obtained with salicylate as substrate. Kinetic analysis revealed that salicylate conjugation by all three forms is characterized by substrate inhibition when salicylate exceeds ca. 20 μM. Substrate inhibition was more extensive with XL-I and XL-III. Previous work on the ligases employed assay concentrations of salicylate in the range of 0.1 to 1.0 mM, which are clearly inhibitory, particularly toward XL-I and XL-III. Thus, activity was not properly measured in previous studies, which accounts for the fact that salicylate conjugation was only found with one form, which is most likely XL-II since it has the highest V_{max} activity and shows the least amount of substrate inhibition. Studies with ibuprofen indicated that it inhibited XL-I, XL-II, and XL-III, with K_I values being in the range of 75–125 μM. The short-chain ligase was inhibited by both salicylate and ibuprofen with K_I values of 93 and 84 μM, respectively. It was concluded that pharmacological doses of salicylate, but not ibuprofen, will affect the metabolism of medium-chain fatty acids and carboxylic acid xenobiotics and that the previously described mitochondrial ibuprofen:CoA ligase activity is attributable to XL-III. © 1996 John Wiley & Sons, Inc.

KEYWORDS: **Amino acid conjugation, Coenzyme A, Fatty acid:CoA ligase, Ibuprofen:CoA ligase, Ibuprofen, Salicylate:CoA ligase, Salicylate, Xenobiotic/Medium chain ligases.**

INTRODUCTION

A number of xenobiotic carboxylic acids are conjugated with an amino acid prior to excretion (1,2). This conjugation pathway is found in the liver and kidney and occurs in the matrix space of mitochondria. The pathway involves two sequential reactions (3–6). The first step is the activation of the carboxylic acid to the CoA thioester in an ATP-dependent reaction catalyzed by a carboxylic acid:CoA ligase [Reaction (1)]. In a subsequent reaction, the acyl

$$\begin{array}{l} \text{O} \\ \| \\ \text{R-COH} + \text{CoASH} + \text{ATP/Mg}^{2+} \\ \qquad\qquad\qquad\qquad \text{O} \\ \qquad\qquad\qquad\qquad \| \\ \qquad\qquad\qquad\qquad \rightarrow \text{R-C-SCoA} + \text{AMP} + \text{PP}_i \end{array} \qquad (1)$$

group is then transferred from CoA to the amino group of an amino acid in a reaction catalyzed by acyl-CoA:amino acid N-acyltransferases. The N-acyltransferases have been studied extensively (7–12), but the numerous forms of the ligases have only recently been fully identified. Carboxylic acid:CoA ligase activity has also been found to be involved in the pathway by which R(−) ibuprofen and its analogs undergo in vivo chiral inversion (13,14).

Bovine liver mitochondria contain numerous fatty acid activating ligases—a long-chain fatty acid ligase (LC-ligase), a short-chain fatty acid ligase (SC-ligase), and three medium-chain fatty acid ligases (15,16). The three medium-chain ligases also activate a variety of xenobiotics and thus are referred to as X-ligases (16). The nonsteroidal anti-inflammatory drugs (NSAIDS) salicylate (16–18) and ibuprofen (13,14) are among the xenobiotics activated by mitochondrial ligases, but the

Received January 22, 1996.

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0887-2082/96/020073-06

specific enzymes responsible for activating these NSAIDS have not been clearly identified. In view of the extensive use of these two anti-inflammatory drugs, it is important to identify the forms that catalyze their activation. Further, it is important to characterize any inhibitory interactions between these NSAIDS and the different forms of carboxylic acid:CoA ligase. Thus, we report on the interaction of the two NSAIDS, salicylate and ibuprofen, with the three X-ligases, the LC-ligase, and the SC-ligase, all from bovine liver mitochondria.

MATERIALS AND METHODS

[14C]-Benzoic acid was obtained from ICN Radiochemicals (Irvine, CA). [14C]-Butyric acid, [14C]-decanoic acid, [14C]-hexanoic acid, [3H]-ibuprofen, [14C]-palmitic acid, and [14C]-salicylic acid were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [14C]-Propionic acid was from Moravek Biochemicals, Inc. (Brea, CA). ATP and CoA were obtained from Sigma (St. Louis, MO). 2,4,6,8-Decatetraenoic acid (DTA) was obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). DE-52 DEAE cellulose was purchased from Whatman Labsales, Inc. (Hillsboro, OR). Sephacryl S-200 gel was obtained from Pharmacia Biotech, Inc. (Piscataway, NJ). Scintisafe-30% (TM) scintillation cocktail was obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of Carboxylic Acid:CoA Ligases.

Bovine liver was obtained fresh from a local slaughterhouse. The liver was quartered and transported to the laboratory in plastic bags in an ice-water slurry. All subsequent operations were done at 4°C. The liver was ground with a hand meat grinder and homogenized in a blender at low speed in isolation buffer (0.13 M KCl, 20 mM HEPES, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, and 250 μg/L each of the protease inhibitors chymostatin, leupeptin and pepstatin A, at pH 7.5) and mitochondria isolated as described previously (16). The final mitochondrial pellet was resuspended at a concentration of 75 mg protein/mL by homogenization in a solution of 80% isolation buffer and 20% glycerol and then frozen at -80°C. The following day the frozen mitochondria were thawed, and the suspension was centrifuged at 100,000 $\times$ g for 40 minutes. The supernatant was filtered through two layers of cheesecloth, extensively dialyzed, and then loaded onto a column of DEAE-cellulose (DE52). The column was eluted with a 0 to 0.2 M gradient of KCl (16). The protein elution profile was monitored at 280 nm, and the carboxylic acid:CoA ligases activities were detected by [14C]-benzoate, [14C]-propionate, and DTA assays. The fractions containing the SC-ligase and forms XL-I, XL-II, and XL-III were separated. Each enzyme form from the DE52 column was further purified by chromatography on a Sephacryl S-200 gel-filtration column (16).

The long-chain fatty acid ligase (LC-ligase) was solubilized from the mitoplast fraction in 0.1% Triton X-100. A particle-free supernatant containing >50% of the LC-ligase activity was obtained after high-speed centrifugation. The LC-ligase was further purified by gel filtration on Sephacryl S-200 in 0.05% Triton X-100, which removed all activity toward benzoate. Activity was followed using [14C]palmitate as the substrate.

DTA Assay for Ligase Activity.

The conversion of DTA to DTA-CoA was followed spectrophotometrically as described by Garland *et al.* (19). The reaction mixture contained 100 mM Trizma pH 8.1, 250 μM CoA, 3 mM $MnCl_2$, 25 μM DTA, 25 μL of enzyme, and 3 mM ATP in a 1 mL volume at 30°C. The appearance of DTA-CoA was followed at 375 nm.

Radioactive Assays for Ligase Activity.

[14C]- or [3H]-labeled carboxylic acids were used, and the formation of the radioactive CoA-adduct was monitored. Unless otherwise stated, the reaction mixtures contained 100 mM Trizma pH 8.1, 100 μM radioactively labeled carboxylic acid, 3 mM ATP, 3 mM MnCl2, and enzyme solution, in a total volume of 450 μL. The reaction was then initiated by the addition of CoA to 250 μM, and the mixture was incubated in a water bath at 30°C. A 0.1 mL aliquot of the reaction mixture was removed at timed intervals (usually 0, 1, 2, and 3 minutes) to 200 μL of 25 mM EDTA to terminate the reaction. This was followed by the addition of 200 μL of 60 mM succinic acid (or for the [14C]salicylate assays, 400 mM succinic acid, and for the [14C]palmitate assay, just H_2O). The mixture was then extracted twice with 1 mL of water-saturated butanol (or once for [14C]-palmitate). The radioactivity in the final aqueous layer was determined using a Beckman LS1801 Liquid Scintillation counter. Initial rates of reaction were determined from the time course of formation of product. The extraction efficiency for each CoA-adduct was determined spectrophotometrically and used to correct the rates for extraction losses.

Protein Concentration.

The protein concentration was determined with the Coomassie brilliant blue G-250 dye-binding assay

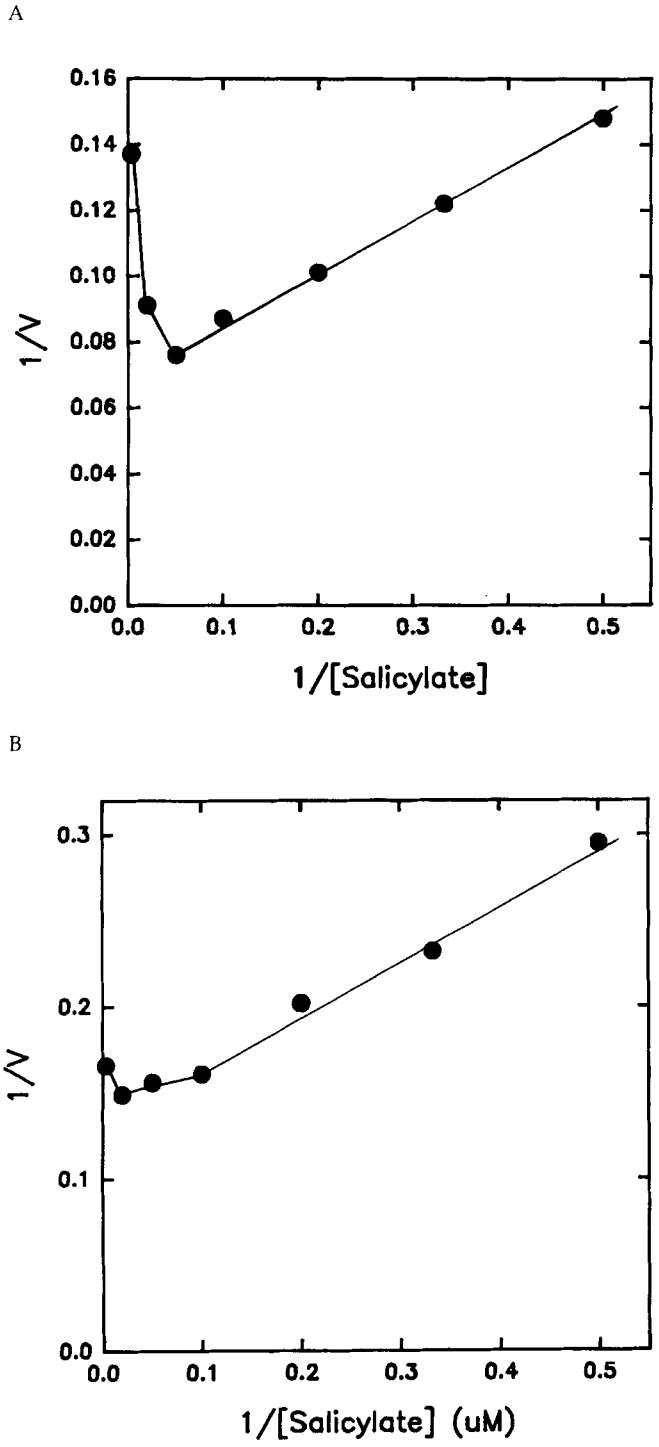

FIGURE 1. Substrate inhibition of forms XL-I (A) and XL-II (B) by salicylate. Ligase activity of XL-I (A) and XL-II (B) was measured with salicylate as the substrate over a range of concentrations. The data are plotted in double reciprocal form, and the reaction velocities are expressed as nmols of product formed per min per mL of enzyme solution.

from Bio-Rad laboratories (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard.

RESULTS

Over 95% of the xenobiotic carboxylic acid:CoA ligase activity in bovine liver mitochondria is found in the high-speed supernatant fraction derived from a freeze–thaw lysis of the mitochondria. Fractionation of this soluble lysate on a DEAE-cellulose column with a gradient of KCl (16) resolves activity into four distinct ligases, a short-chain (SC) ligase, and three xenobiotic-metabolizing medium-chain ligases (X-ligases), which have been denoted XL-I, XL-II, and XL-III. For the current study, the SC-ligase and the three X-ligases were separated on the DEAE-cellulose column and then purified further by gel filtration chromatography on Sephacryl S-200. The long-chain (LC) ligase activity was obtained by detergent extraction of the mitoplast fraction following two freeze–thaw cycles.

Each of the five forms was tested for activity toward salicylate and ibuprofen. Neither the LC-ligase nor the SC-ligase had activity toward either salicylate or ibuprofen. All three X-ligases (XL-I, XL-II, and XL-III) had activity toward salicylate. XL-II showed the greatest salicylate activity. Activity toward ibuprofen was only found with form XL-III.

Kinetic analysis was conducted on the Sephacryl S-200 eluates of the three X-ligases using salicylate as the carboxylic acid substrate. The concentrations of CoA and ATP/Mn^{2+} were fixed at their optimum concentrations, while the concentration of salicylate was varied to determine an apparent K_m (K_m^{App}) and an apparent V_{max} (V_{max}^{App}). Analysis of form XL-I (Figure 1A) revealed substrate inhibition at high concentrations of salicylate. Thus, the maximum activity obtainable is at 20 μM salicylate, and this is only ca. 50% of the theoretical maximum rate. Beyond 20 μM, the rate begins to decrease as the concentration of salicylate is increased. Similarly, for XL-III (data not shown), the double reciprocal plot began to turn up at high salicylate concentrations (in this case, beyond 50 μM); by 300 μM salicylate, the rate was ca. 50% of the theoretical maximum rate. For the XL-II form, substrate inhibition was less apparent, but at 300 μM salicylate, the activity was reduced from the theoretical maximum by ca. 15% (Figure 1B). The results of the kinetic analysis of the linear portion of the plots are shown in Table 1. The K_m^{App} for salicylate as extrapolated from the linear portion of the plots was nearly the same for each of the three forms, approximately 2–3 μM. Form XL-III appeared to have a lower activity at V_{max} than the other forms.

TABLE 1. KInetic Analysis of XL-I, XL-II, and XL-III with Salicylate as Substrate

	K_m^{APP} (μM)	V_{max}^{APP}
XL-I	2.4 ± .1	1.2 ± .1
XL-II	2.2 ± .5	1.4 ± .1
XL-III	2.5 ± .6	0.24 ± .02

The concentration of salicylic acid was varied at fixed concentrations of CoA (0.5 mM), ATP (3 mM), and Mn^{2+} (5 mM). The apparent K_m (K_m^{APP}) and apparent V_{max} (V_{max}^{APP}) were determined by extrapolating the linear portion of the kinetic plots to intersection with the axes. V_{max}^{APP} is expressed as nmoles of salicyl-CoA formed per min per mg of protein. Experimental values are expressed as mean ± standard deviation.

TABLE 2. Inhibition Constants for Salicylate and Ibuprofen Inhibition of Benzoate Activity with the Different X-Ligases

	XL-I	*XL-II*	*XL-III*	*SC-ligase*
Ibuprofen	115 ± 5	67 ± 12	125 ± 25	93 ± 7
Salicylate	2.1 ± .5	2.7 ± .4	6.5 ± 2.0	84 ± 8

The inhibition of benzoate (XL-I, XL-II, and XL-III) or butyrate (SC-ligase) activity by either ibuprofen or salicylate was measured in standard assays. The concentration of salicylate was varied over the range of 0–20 μM, and the concentration of ibuprofen was varied over the range of 0–200 μM, at each of two different concentrations of substrate. The K_I values were determined from the data by the method of Dixon (18) and are expressed as micromolar. Experimental values are expressed as mean ± standard deviation.

Kinetic analysis was conducted on XL-III with ibuprofen as substrate. The K_m^{APP} for ibuprofen was 36 μM. The rate of activation of ibuprofen was relatively low, being only 2% of the rate obtained with benzoate as substrate.

The SC-ligase had activity toward butyrate and propionate, less toward hexanoate, and even less toward decanoate. However, it did not have activity toward any xenobiotic including salicylate and ibuprofen. Using butyrate as substrate, the SC-ligase was assayed for inhibition by ibuprofen and by salicylate. It was found that both salicylate and ibuprofen were inhibitory. The K_I values determined from a Dixon plot (20) were 93 μM for salicylate and 84 μM for ibuprofen.

Benzoate activation by the three X-ligases was found to be inhibited by salicylate. The K_I values for inhibition of benzoate activity were determined by the method of Dixon (20) and are shown in Table 2. Dixon plots of salicylate inhibition were linear over the range of 0–20 μM salicylate and yielded K_I values for salicylate in the micromolar range for all three X-ligases. The inhibition of XL-III by salicylate had a higher K_I (6.5 μM) than for the other two forms. Salicylate inhibition of XL-III was also analyzed using the DTA spectrophotometric assay; this yielded a K_I of 3 μM. A kinetic analysis of the salicylate inhibition of benzoate activation by XL-III (Figure 2) revealed that at micromolar concentrations, salicylate was a competitive inhibitor versus benzoate. Salicylate was also a competitive inhibitor versus benzoate with forms XL-II and XL-I (data not shown). At concentrations of salicylate in excess of 40 μM, the inhibition kinetics became nonlinear, which probably reflects the same event as that causing substrate inhibition by salicylate.

The reverse experiment, testing benzoate as an inhibitor of the activation of salicylate, was also measured. The K_I value for benzoate inhibition of XL-I was 12 μM; for XL-II, it was 2.7 μM; and for XL-III, it was 25 μM. The inhibition pattern in each case was competitive versus salicylate.

All three X-ligases were also inhibited by ibuprofen. Dixon plots were used to determine K_I values for ibuprofen inhibition. These data are shown in Table 2. The K_I values for ibuprofen inhibition are in the range of 67–125 μM. For all three inhibitions, ibuprofen was competitive versus the carboxylic acid substrate.

The LC-ligase was tested for inhibition by salicylate and ibuprofen. Palmitate was used as the substrate at a concentration of 10 μM. Ibuprofen at a concentration of 300 μM did not inhibit and, in fact, showed a slight stimulatory effect. Salicylate at 300 μM did not have a significant effect on the LC-ligase.

DISCUSSION

We have isolated the membrane-associated LC-ligase and the four different soluble forms of carboxylic acid:CoA ligase from bovine liver mitochondria and have characterized their interaction with the two nonsteroidal anti-inflammatory drugs, salicylate and ibuprofen. Neither the specific SC-ligase nor the LC-ligase had activity toward either salicylate or ibuprofen. However, salicylate activity was found with all three X-ligases, which is contrary to previous reports (16–18). As noted in Figure 1, salicylate is capable of substrate inhibition at concentrations exceeding ca. 20 μM. This is very important because all previous work with this enzyme employed concentrations of salicylate in the assay that were never lower than 100 μM, and generally 0.5 or 1 mM. At 100 μM salicylate, forms XL-I and XL-III have reduced rates. This accounts for the past difficulties in accurately measuring salicylate activity using assays containing 0.1 mM salicylate (16). At 500 μM salicylate, the rates are extremely low, which accounts for the inability to detect activity in assays with 0.5 and 1 mM salicylate (17, 18). This clearly shows why salicylate conjugation has not been detectable with all forms. In early studies, only one form was reported to have salicylate activity (17, 18), this form most likely being XL-II (16), which shows the least amount of substrate inhibition.

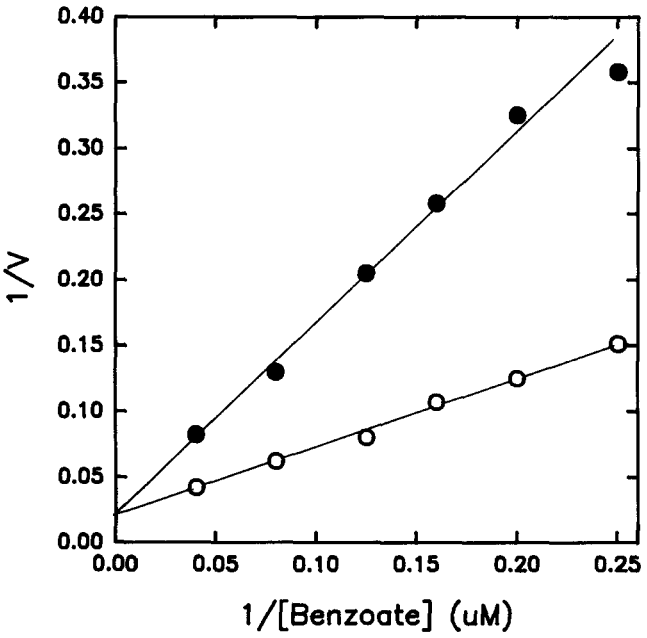

FIGURE 2. Inhibition by salicylate of benzoate activation by form XL-III. Ligase activity of XL-III was measured with benzoate as the substrate at variable concentrations, in the absence (○) and presence (●) of 20 μM salicylate. The data are plotted in double reciprocal form, and the reaction velocities are expressed as nmols of product formed per min per mL of enzyme solution.

Kinetic analysis of all three X-ligases with salicylate as substrate revealed that they have K_m values for salicylate in the range of 2–3 μM. However, the XL-I and XL-II forms have a much higher V_{max}^{App} with salicylate than does XL-III, and thus at low concentrations of salicylate they would be the predominant salicylate-metabolizing forms. As discussed earlier, at high concentrations of salicylate, the role of XL-I becomes diminished due to substrate inhibition.

The XL-III ligase is the only form with activity toward DTA, and, in fact, DTA was the preferred substrate (16). The substrate specificity study suggests that XL-III is more effective at the activation of medium-chain fatty acids, arylacetic acids, and valproate than are either XL-I or XL-II (16). In the present study, we have found that XL-III is also the only form with activity toward ibuprofen. Ibuprofen:CoA ligase activity has previously been demonstrated in mitochondria, where it has been shown to be part of the pathway for the chiral inversion of R(-)ibuprofen and its analogs (13, 14). It is now clear that this mitochondrial activity resides with the XL-III form. XL-III thus appears to have the broadest specificity toward xenobiotics of the three X-ligases.

As expected, salicylate proved to be an effective alternate substrate inhibitor of all three forms of X-ligase. At concentrations of salicylate in excess of 20 μM, the inhibition kinetics became nonlinear, perhaps as a result of the same event as that causing substrate inhibition by salicylate. However, at concentrations of 20 μM or less, linear inhibition kinetics were obtained that allowed us to determine K_I values. The K_I values obtained for salicylate inhibition of benzoate activation by XL-I and XL-II were 2.1 and 2.7 μM, respectively. These are similar to the K_m values for salicylate binding as a substrate to these two forms. This suggests that the salicylate inhibits by binding at the active site. This was confirmed by the finding that salicylate was a competitive inhibitor versus benzoate. With form XL-III, the K_I value for salicylate is slightly higher than the K_m, which may mean that there is an undetected contribution from the same type of inhibition that characterizes salicylate substrate inhibition. At concentrations of salicylate in excess of 20 μM, the inhibition kinetics became nonlinear, probably reflecting the same event as that causing substrate inhibition by salicylate.

Inhibition of salicylate activity by benzoate was also measured. The K_I value for benzoate inhibition of XL-I was 12 ± 2 μM; for XL-II, it was 2.7 μM; and for XL-III, it was 25 μM. The apparent K_m values for benzoate binding to XL-I, XL-II, and XL-III (as determined at fixed saturating concentrations of ATP/Mn^{2+} and CoA) were 1.1, 1.5, and 7.4 μM, respectively (16). Thus, the K_I values are considerably higher than the values for the apparent K_m. This may again be related in some way to the salicylate substrate inhibition effect, or it may be a reflection of the limitedness of the apparent K_m values for interpreting true binding constants.

Both salicylate and ibuprofen were inhibitory to the SC-ligase, having K_I values of 84 μM for salicylate and 93 μM for ibuprofen. This means that large doses of either ibuprofen or salicylate can inhibit all forms of ligase with short-chain fatty acid activity (the SC-ligase and three X-ligases) and, thus, presumably alter short-chain fatty acid metabolism. The three forms with medium-chain fatty acid activity (Xl-I, XL-II, and XL-III) are all inhibited by micromolar concentrations of salicylate. Thus, it is expected that even moderate doses of salicylate will interfere with the metabolism of medium-chain fatty acids and also with the conjugation of carboxylic acid xenobiotics.

In vivo studies of rates of excretion of salicylurate (21) have shown that benzoate inhibits salicylate conjugation. Because the activation step is rate limiting for salicylate (4,16), and because benzoate is a cosubstrate, it can be assumed that benzoate inhibits the activation of salicylate, and this is then the basis for the competition. However, salicylate administration to humans has not been observed to inhibit hippurate excretion

from benzoate (21). Based on the kinetic analysis in this article, salicylate should inhibit benzoate conjugation in vivo. This may be an indication that the ligases in human liver mitochondria are quite different from those in bovine liver.

It has been shown that the ligase reaction is rate limiting for conjugation in the case of 3-phenoxybenzoate, salicylate, benzoate, and phenylacetate (4,16,21,22). Thus, the inhibition of these ligases by salicylate and ibuprofen is expected to have impact on the metabolism of xenobiotics by these enzymes. Clearly, ibuprofen is less inhibitory than salicylate.

ACKNOWLEDGMENT

This work was supported by the Medical Service of the Department of Veterans' Affairs.

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