Biochemical Pharmacology, Vol. 35, No. 2, pp. 289–295, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

INTERACTION OF 2,4-DICHLOROPHENOXYACETATE (2,4-D) AND 2,4,5-TRICHLOROPHENOXYACETATE (2,4,5-T) WITH THE ACYL-CoA:AMINO ACID *N*-ACYLTRANSFERASE ENZYMES OF BOVINE LIVER MITOCHONDRIA

MICHAEL KELLEY* and DONALD A. VESSEY†‡

*† Liver Study Unit, Veterans Administration Medical Center, San Francisco, CA 94121; and the Departments of † Medicine and *† Pharmacology, University of California, San Francisco, CA 94143, U.S.A.

(*Received* 14 *December* 1984; *accepted* 28 *May* 1985)

Abstract—The amino acid conjugation of the phenoxyherbicides 2,4-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T) by animals was examined at the level of the enzymes catalyzing the reactions. The phenoxyherbicides were not substrates for the bile acid conjugating system but were substrates for the mitochondrial xenobiotic conjugating system. The two mitochondrial xenobiotic-CoA:amino acid *N*-acyltransferases (benzoyltransferase and phenylacetyltransferase) were separated and tested for activity towards 2,4-D-CoA and 2,4,5-T-CoA. The phenylacetyltransferase showed activity towards phenylacetyl-CoA, phenoxyacetyl-CoA and 2,4-D-CoA, but not 2,4,5-T-CoA. Benzoyltransferase conjugated both 2,4-D-CoA and 2,4,5-T-CoA. The overall rates of conjugation of the phenoxyherbicides were slow relative to the standard substrates with both enzymes. This slow rate was found to be due in both cases to a relatively high K_m for glycine, and a very slow catalytic rate constant. Both enzymes did, however, have a very high affinity for 2,4-D-CoA and 2,4,5-T-CoA so these compounds proved to be potent alternate substrate inhibitors of both enzymes. The data show that the inefficient *in vivo* conjugation of the phenoxyherbicides relative to structurally similar compounds can be understood in terms of the kinetic properties of the mitochondrial *N*-acyltransferases. Further, the potential for the interference of the phenoxyherbicides with the conjugation of other compounds is revealed.

2,4,5-Trichlorophenoxyacetate (2,4,5-T) and 2,4-dichlorophenoxyacetate (2,4-D) have been used extensively since the 1940s as herbicides. However, there is currently renewed interest in the potential for human toxicity associated with their use in agriculture and with their past use in the Vietnam conflict as defoliants. Studies of the biological fate of these compounds revealed that all animals readily absorb 2,4-D and 2,4,5-T, but rates of elimination differ greatly among species [1–7]. In all species, elimination is largely via the urine [1–8], and in several instances metabolites of 2,4-D and 2,4,5-T have been reported [1, 3, 7–9]. Grunow and Bohme [8] have shown that the primary metabolites of both 2,4-D and 2,4,5-T in rats and mice are the glycine and taurine conjugates. In marine species, 2,4-D is excreted almost exclusively in the form of the taurine conjugate [10].

Most interesting is the fact that, while only a small percentage of the administered 2,4-D or 2,4,5-T is excreted as a metabolite [1, 3, 7–9], the structurally similar molecule chlorophenylacetic acid is excreted almost exclusively in the form of an amino acid conjugate [11]. Similarly, phenylacetic acid and other aryl-substituted acetic acids are also excreted predominantly as amino acid conjugates [11, 12]. To determine the basis for the relatively poor metabolism of 2,4-D and 2,4,5-T, we conducted a study of the kinetic properties of the conjugating enzymes from bovine liver, comparing the phenoxyherbicides to phenylacetic acid and benzoic acid.

METHODS

2,4,5-T containing <1 ppb 2,3,7,8-tetrachlorodibenzodioxin was a gift of the Dow Chemical Co., Midland, MI. Phenoxyacetic acid was obtained from the Fluka Chemical Corp., Hauppauge, NY. Phenylacetyl-CoA, benzoyl-CoA, 2,4-D and CoA were obtained from the Sigma Chemical Co., St. Louis, MO. The CoA-thioesters of the phenoxyacetic acids were synthesized from the respective acid chlorides by the following procedure. One hundred micromoles of the phenoxyacetic acid was dissolved in 15 ml of methylene chloride (freshly distilled over calcium hydride). A 7-fold molar excess of thionyl chloride was added. The reaction tube was tightly stoppered, wrapped with aluminum foil, and placed in a 38° water bath for 4 hr. The solvent was removed by passing a stream of nitrogen gas into the tube. Fifty micromoles of the sodium salt of CoA was added to the reaction tube. Two milliliters of glass-distilled, nitrogen-purged water was added to

‡ Address all correspondence to: Dr. Donald A. Vessey, 151K, VA Hospital, 4150 Clement St., San Francisco, CA 94121.

the reaction tube. The solution was quickly adjusted to pH 7.8 to 8.0 with 0.5 N NaOH. Additional NaOH was added with stirring in order to maintain this pH range. After approximately 10 min the pH stabilized. The reaction tube was then purged with nitrogen gas, tightly stoppered, and placed in a 38° water bath for an additional 20 min. To purify the thioesters, the solution was applied onto four sheets of 23 cm × 57 cm Whatman no. 3 filter paper in bands 8 cm from the top of the paper. The paper was developed in a descending chromatography tank in 70% isopropanol until the solvent front reached the bottom of the paper. The papers were air dried and the bands were visualized with a short-wave ultraviolet light. The product band, which was broad and ran at an $R_f = 0.43$ to 0.53, was cut from the paper and eluted with 70% isopropanol. The eluate was evaporated to dryness using a rotary evaporator. The residue was redissolved in distilled water to make a 1 mM stock solution based on an $E^{M}_{260} = 16{,}800$ for coenzyme A.

The 2,4-D-glycine and 2,4,5-T-glycine standards were synthesized by a procedure similar to that of the coenzyme A adducts, except that the acid chlorides of the respective phenoxyacetates were dissolved in freshly distilled tetrahydrofuran and added to an equal volume of aqueous glycine solution; this mixture was then adjusted to pH = 9.5 and incubated for 30 min at 38°. The glycine conjugates were purified by high pressure liquid chromatography (HPLC) on a reversed phase C18-column. Polar components were eluted with aqueous 0.01 M trifluoroacetic acid (sequenal grade, Pierce Chemical Co.). The elution solvent was then changed to include 50% acetonitrile containing 0.01 M trifluoroacetic acid and the glycine-conjugate isolated. The structure of the conjugates was verified by mass spectrometry.

Bovine liver bile acid-CoA:glycine/taurine *N*-acyltransferase was purified from the soluble cell fraction as described previously [13]. Phenylacetyltransferase and benzoyltransferase were purified from bovine liver mitochondria as follows. Mitochondria, isolated by the method of Schnaitman and Greenawalt [14], were submitted to three freeze–thaw cycles, and the mitoplasts were removed by ultracentrifugation. The supernatant fraction was fractioned first at 4° with ammonium sulfate (0–40%). The precipitate was collected and fractionated on $Al(OH)_3$ gel as described by Nandi *et al.* [15]. The $Al(OH)_3$ gel supernatant fraction was brought to 75% saturation in ammonium sulfate. The precipitate was redissolved in cold 0.1 M KCl–20 mM Tris, pH 8, and layered onto a Sephacryl S-200 column (2.5 × 100 cm) equilibrated and eluted with 0.1 M KCl–20 mM Tris, pH 8 (4°). Benzoyltransferase activity eluted first, and fractions devoid of phenylacetyltransferase activity were collected and used without further purification. Phenylacetyltransferase activity was collected and rechromatographed on the S-200 column to remove contaminating benzoyltransferase activity.

N-Acyltransferase activity towards benzoyl-CoA, phenylacetyl-CoA, 2,4-D-CoA and 2,4,5-T-CoA was in most cases assayed by measuring CoA release. A continuous recording spectrophotometric assay for CoA release using DTNB [5,5′-dithiobis(2-nitrobenzoate)], validated by Webster *et al.* [16] and Nandi *et al.* [15], was used as described with the exception that bovine serum albumin (BSA) was omitted from the assay. The standard assay for both benzoyltransferase and phenylacetyltransferase contained 0.1 mM DTNB, 50 mM Tris, pH 8.0 (30°), 80 mM KCl and substrates. For both enzymes the assays were initiated by the addition of enzyme, and rates of reaction were corrected for any non-enzymatic rate of DTNB reaction. For the benzoyltransferase it was also necessary to correct for acyl-CoA thiolase activity present in the preparation. Benzoyltransferase was measured in some instances by following the disappearance of the benzoyl-CoA thioester absorbance at 280 nm. These assays were conducted in 50 mM Tris, pH 8.0, at 30°. The products of the reactions of 2,4-D-CoA and 2,4,5-T-CoA with glycine were identified as 2,4-D-glycine and 2,4,5-T-glycine by their identical HPLC retention times as compared to standards.

RESULTS

The conjugation of 2,4-D and 2,4,5-T with amino acids was investigated with both the mitochondrial xenobiotic conjugating system [16] and also the bile acid conjugating system [13]. A partially purified preparation of bovine liver bile acid-CoA:glycine/taurine *N*-acyltransferase was tested for activity towards 2,4-D-CoA and 2,4,5-T-CoA. No glycine or taurine conjugating activity was detectable with either. Thus, the remainder of the work presented in this paper details studies on the mitochondrial xenobiotic conjugating enzymes.

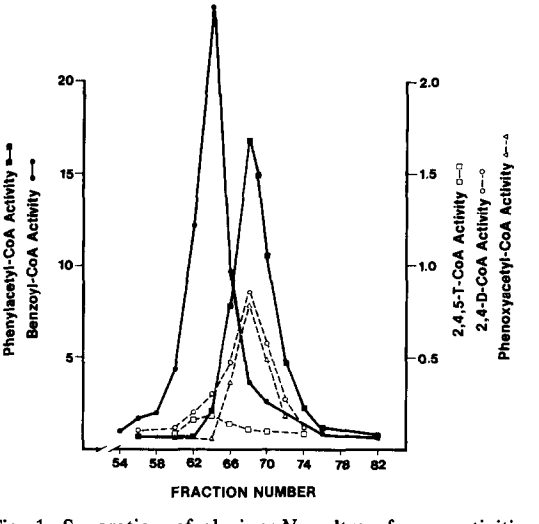


Fig. 1. Separation of glycine:*N*-acyltransferase activities by gel filtration chromatography. The supernatant fraction from the $Al(OH)_3$ gel treatment was chromatographed on a Sephacryl S-200 column as described in Methods. The column fractions were assayed for glycine:*N*-acyltransferase activity using the standard DTNB assay with 100 mM glycine and 200 μM of the following acyl-CoA substrates: benzoyl-CoA (●—●), phenylacetyl-CoA (■—■), phenoxyacetyl-CoA (△---△), 2,4-D-CoA (○---○) and 2,4,5-T-CoA (□---□). Rates are expressed as ΔO.D. per min per ml of fraction.

Nandi *et al.* [15] demonstrated that the xenobiotic conjugating activity of bovine liver mitochondria could be fractionated into two separate homogenous enzymes. The predominant enzyme form is generally referred to as benzoyltransferase, and it has activity towards benzoyl-CoA, salicylyl-CoA and short chain acyl-CoA thioesters. The other form is referred to as the phenylacetyltransferase and has activity towards phenylacetyl-CoA and indoleacetyl-CoA. To determine which of these forms of the enzyme was responsible for the conjugation of 2,4-D and 2,4,5-T, we separated the two forms on a Sephacryl S-200 column. Because the phenylacetyltransferase activity is less than 5% of the benzoyltransferase activity and because the separation between the two is not complete, it was actually necessary to collect the phenylacetyltransferase activity from a first S-200 column and rechromatograph it to obtain the profile shown in Fig. 1. The elution profile from the second S-200 column shows two well resolved peaks, one with all the benzoyltransferase activity and the other with the phenylacetyltransferase activity. These column fractions were assayed for glycine conjugating activity towards 2,4-D-CoA, 2,4,5-T-CoA phenylacetyl-CoA and phenoxyacetyl-CoA. Activity towards 2,4-D-CoA was found predominantly in the phenylacetyltransferase peak but, as can be seen clearly in fraction 62 (Fig. 1), it is also present in the benzoyltransferase peak. 2,4,5-T-CoA activity was only detected in the benzoyltransferase peak. Phenoxyacetyl-CoA activity was present exclusively in the phenylacetyltransferase peak. The dual localization of the herbicide conjugating activity necessitated separate kinetic characterizations of each form.

The kinetic analysis of the transferases was based on that conducted by Nandi *et al.* [15]. These investigators demonstrated a sequential ordered mechanism for the reaction which was of the form:

$$E + \text{Acyl-CoA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^{\text{Acyl-CoA}}$$

$$E^{\text{Acyl-CoA}} + \text{Gly} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E^{\text{Acyl-CoA}}_{\text{Gly}} \xrightarrow{k_p} \text{Products.}$$

The pattern of the double-reciprocal plots [15] does not conform to that of a rapid equilibrium ordered mechanism but rather to a steady-state ordered mechanism in that the Lineweaver–Burk plot of $1/v$ versus $1/\text{glycine}$ for several different concentrations of benzoyl-CoA did not intersect on the ordinate axis (cf. Ref. 17). Based on a steady-state ordered mechanism, we have chosen the following approach toward extracting meaningful constants from the kinetic data. Lineweaver–Burk plots of $1/v$ versus 1/acyl-CoA at several different fixed concentrations of glycine gave rise to a family of lines which intersected in the third quadrant. The projection of this intersection point onto the X-axis gives the value $-1/K_{\text{Acyl-CoA}}$ where $K_{\text{Acyl-CoA}} = k_{-1}/k_1$, i.e. $K_{\text{Acyl-CoA}}$ is the actual dissociation constant (K_D) for acyltransferase binding [17]. Replotting the Y-intercept versus the reciprocal of the respective glycine concentration ($1/V'_{\max}$ versus $1/\text{gly}$) gives a line with a Y-intercept that is the true $V_{\max}$ rate and with an X-intercept that is equal to $-1/K_m^{\text{gly}}$, the latter being the concentration of glycine which would yield a half-$V_{\max}$ rate at saturating acyl-CoA.

The kinetic constants for the conjugation of benzoyl-CoA with glycine by benzoyltransferase are shown in Table 1. The K_D for benzoyl-CoA was found to be 0.06 mM and the K_m for glycine was 15 mM. For phenoxyherbicide conjugation the K_D for 2,4-D-CoA was 0.095 mM and the K_D for 2,4,5-T-CoA was 0.060 mM. These values are similar to the K_D for benzoyl-CoA. However, the K_m for glycine in both the 2,4-D-CoA and 2,4,5-T-CoA reactions was approximately 100 mM which is 7-fold higher than the K_m for glycine in the benzoyl-CoA reaction. Further, the $V_{\max}$ values obtained from extrapolating to infinite concentrations of both substrates were approximately 200- to 300-fold lower for the 2,4-D-CoA and 2,4,5-T-CoA reactions as compared with benzoyl-CoA. Benzoyl-CoA was also conjugated with glutamine, but conjugation of 2,4-D-CoA and 2,4,5-T-CoA with glutamine was undetectable.

The K_D and $V_{\max}$ values in Table 1 suggest that 2,4-D-CoA and 2,4,5-T-CoA should actually be inhibitors of the benzoyl-CoA reaction, i.e. both 2,4-D-CoA and 2,4,5-T-CoA appear to have a high affinity for the enzyme as judged by the K_D values and yet very poor turnover rates as evidenced by the $V_{\max}$ values. The actual extent of this expected inhibition was determined in assays of benzoyltransferase based on the loss of absorbance at 280 nm (see Methods). As expected, 2,4-D-CoA and 2,4,5-T-CoA were both inhibitory, and 2,4,5-T-CoA was

Table 1. Kinetic constants for benzoyltransferase assayed with benzoyl-CoA, 2,4-D-CoA and 2,4,5-T-CoA as substrates

Substrates	$K_D^{\text{Acyl-CoA}}$ (mM)	K_m^{gly} (mM)	$V_{\max}$
Benzoyl-CoA/Glycine	0.060	15	215
2,4-D-CoA/Glycine	0.095	118	1.1
2,4,5-T-CoA/Glycine	0.060	100	0.74

Initial rates of reaction were determined for a range of acyl-CoA concentrations at each of several different fixed concentrations of amino acid. The values for $V_{\max}$ and for $K_D^{\text{Acyl-CoA}}$ (the dissociation constant for acyl-CoA binding to the enzyme) and K_m^{gly} (the Michaelis constant for glycine) were determined from Lineweaver–Burk plots as described in the text. Rates at $V_{\max}$ are expressed as μmoles of CoA released per min per ml of enzyme preparation.

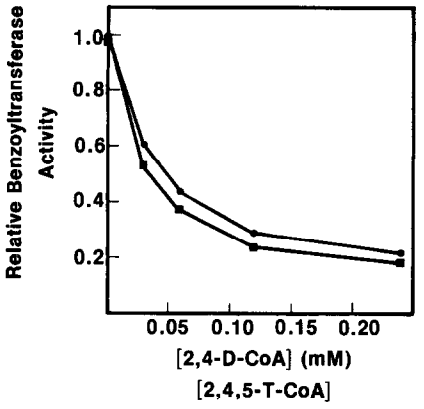


Fig. 2. Inhibition of benzoyltransferase by phenoxyherbicides. Benzoyltransferase activity was assayed with 40 μM benzoyl-CoA and 5 mM glycine using the assay at 280 nm described in Methods; the indicated concentration of either 2,4-D-CoA (●) or 2,4,5-T-CoA (■) was added to the assays. Rates are expressed as a fraction of the uninhibited rate.

the more potent inhibitor (Fig. 2). Dixon plots of the inhibition gave K_I values of 0.05 mM for 2,4-D-CoA and 0.04 mM for 2,4,5-T-CoA. Kinetic analysis of the inhibitions revealed that the inhibition was competitive with respect to benzoyl-CoA. When glutamine was used as the acceptor amino acid rather than glycine, comparable inhibition of benzoyl-CoA conjugation by 2,4-D-CoA and 2,4,5-T-CoA was obtained.

Since the CoA-derivatives of the phenoxyherbicides were effective alternate substrate inhibitors of the benzoyltransferase, it was of interest to determine if the phenoxyherbicides themselves were inhibitory. For assays containing 0.04 mM benzoyl-CoA, the addition of either 2,4-D or 2,4,5-T at concentrations up to 0.5 mM was without effect on enzyme activity. This was true for both 5 and 200 mM glycine or with 5 or 200 mM glutamine as the acceptor amino acid.

Using the spectrophotometric assay at 280 nm for benzoyltransferase, it was possible to test CoA as an inhibitor of benzoyl-CoA conjugation. CoA was found to inhibit the reaction with a K_I of 0.1 mM.

Kinetic analysis of the phenylacetyltransferase followed the same procedure as for benzoyltransferase. The results of this kinetic analysis are shown in Table 2. For phenylacetyl-CoA conjugation, the K_D for phenylacetyl-CoA was 0.007 mM and the K_m glycine was 9 mM. For 2,4-D-CoA conjugation, the K_D for 2,4-D-CoA was 0.015 mM and the K_m for glycine was 1000 mM. The conjugation of 2,4,5-T-CoA was not detectable with either glycine or glutamine as the acceptor amino acid. Since phenylacetyltransferase did not possess measurable activity towards 2,4,5-T-CoA, it was possible to test 2,4,5-T-CoA directly as an inhibitor of the enzyme. A Dixon plot of the inhibition of phenylacetyl-CoA conjugation by 2,4,5-T-CoA is shown in Fig. 3a. 2,4,5-T-CoA was an effective inhibitor with a K_I of 0.02 mM. The conjugation of 2,4-D-CoA by phenylacetyltransferase had a strikingly high K_m for glycine. As a result, 2,4-D-CoA could be tested as an inhibitor of phenylacetyl-CoA conjugation at 5 mM glycine (*ca.* K_m^{gly}) without 2,4-D-CoA conjugation contributing significantly to the over-all rate of CoA release. A Dixon plot of this data is shown in Fig. 3b. The K_I for 2,4-D-CoA was 0.02 mM.

DISCUSSION

Liver contains two distinct enzyme systems engaged in the conjugation of carboxylic acids with amino acids. The soluble cell fraction contains an enzyme designed to catalyze the reaction of glycine or taurine with the CoA-derivative of bile acids, the latter being synthesized by a microsomal enzyme. We have found that this bile acid-CoA:glycine/taurine *N*-acyltransferase from liver is without activity towards the phenoxyherbicides. The second conjugating system is located in the mitochondrial cell fraction and is engaged in the two-step synthesis of the amino acid conjugates of various xenobiotic carboxylic acids.

$$R{-}COO^- + ATP + CoA \rightarrow R{-}\overset{\overset{O}{\|}}{C}{-}S{-}CoA + AMP + PP_i \quad (1)$$

$$R{-}\overset{\overset{O}{\|}}{C}{-}S{-}CoA + gly \rightarrow R{-}\overset{\overset{O}{\|}}{C}{-}\underset{H}{N}{-}CH_2{-}COO^- + CoA \quad (2)$$

Table 2. Kinetic constants for phenylacetyltransferase assayed with phenylacetyl-CoA, 2,4-D-CoA and 2,4,5-T-CoA as substrates

Substrates	$K_D^{Acyl\text{-}CoA}$ (mM)	K_m^{gly} (mM)	V_{max}
Phenylacetyl-CoA/Glycine	0.007	9	0.33
2,4-D-CoA/Glycine	0.015	1000	0.15
2,4,5-T-CoA/Glycine			<0.01

Initial rates of reaction were determined for a range of acyl-CoA concentrations at each of several different fixed concentrations of amino acid. The values for V_{max} and for $K_D^{Acyl\,CoA}$ (the dissociation constant for acyl-CoA binding to the enzyme) and K_m^{gly} (the Michaelis constant for glycine) were determined from Lineweaver–Burk plots as described in the text. Rates at V_{max} are expressed as μmoles of CoA released per min per ml of enzyme preparation.

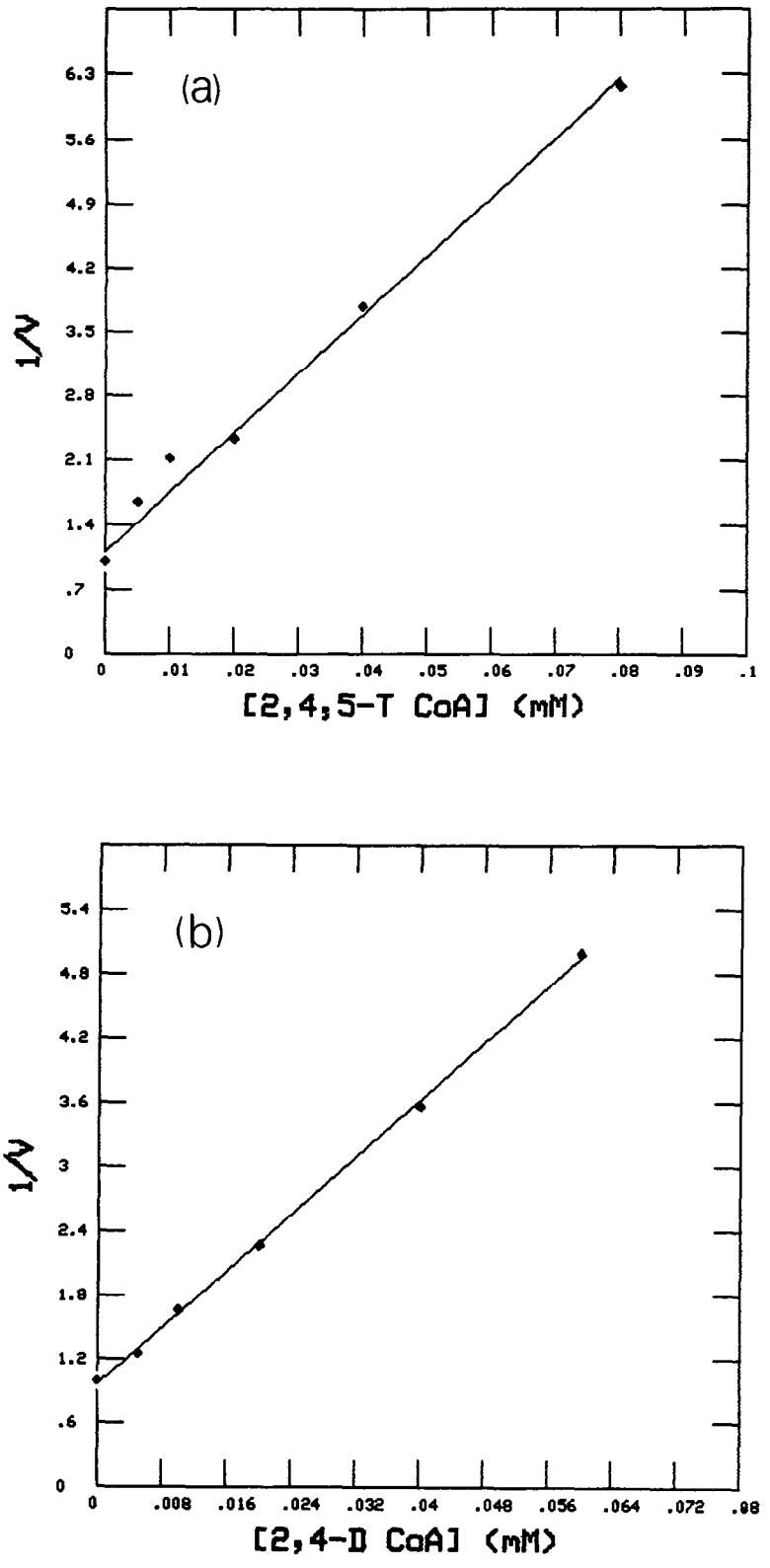
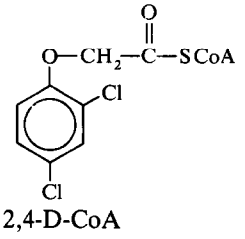


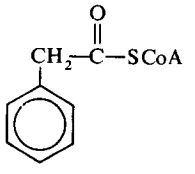
Fig. 3. Inhibition of phenylacetyltransferase by 2,4,5-T-CoA (a) and 2,4-D-CoA (b). Phenylacetyltransferase was assayed with 40 μM phenylacetyl-CoA and 5 mM glycine using the standard assay containing the indicated concentration of 2,4,5-T-CoA (a) or 2,4-D-CoA (b). Reaction velocities (V) are expressed as ΔO.D. per min per ml of enzyme preparation. The contribution of 2,4-D-CoA (and 2,4,5-T-CoA) to the overall rate of CoA release was negligible at 5 mM glycine.

The first step in the synthesis involves the ATP-dependent formation of the CoA-thioester. There appear to be two different acyl-CoA synthetase enzymes in mitochondria catalyzing reaction (1) for xenobiotics [18–20]. At least one of these enzymes has activity towards 2,4-D and in rats this activity is reported to be 30–50% of the activity seen with benzoic acid or phenylacetic acid [18]. Thus, the great deficiency in phenoxyherbicide conjugation does not seem to be solely explicable in terms of the activity of the acyl-CoA synthetase alone. For this reason we have examined extensively the enzymes catalyzing reaction (2), the *N*-acyltransferases.

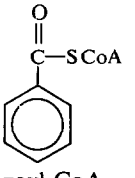
Mitochondria contain two distinct *N*-acyltransferases [15, 16]. One catalyzes the conjugation of phenylacetyl-CoA as well as indoleacetyl-CoA and is referred to as the phenylacetyltransferase. The other is referred to as the benzoyltransferase and it conjugates benzoyl-CoA and a wide variety of aromatic and acyl-CoA derivatives. Heretofore there had been no report of overlapping substrate specificity between these two *N*-acyltransferases. However, we found (Fig. 1) that 2,4-D-CoA was a substrate for both enzymes from bovine liver. Further, 2,4,5-T-CoA was a substrate predominantly for the benzoyltransferase. This was all unexpected. Based on the comparison of the



2,4-D-CoA

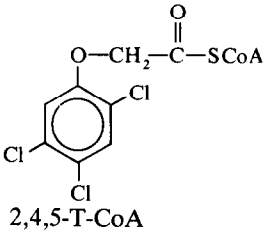


Phenylacetyl-CoA

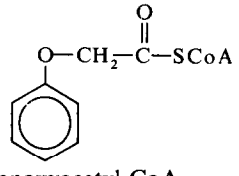


Benzoyl-CoA

structures of benzoyl-CoA, phenylacetyl-CoA and 2,4-D-CoA, this overlap would not be predicted. The methylene group between the ring and the thioester moieties is the sole feature that distinguishes phenylacetyl-CoA from benzoyl-CoA and must, therefore, also be the basis for the discrimination by the active sites of the two enzymes. The fact that phenoxyacetyl-CoA is a substrate for phenylacetyltransferase indicates that increasing the distance between the ring and the carbonyl by adding an ether link does not affect substrate specificity. Since 2,4-D-CoA is a substrate for both enzymes, and 2,4,5-T-CoA is a substrate predominantly for the benzoyltransferase, clearly



2,4,5-T-CoA



Phenoxyacetyl-CoA

the chlorine substituents on the phenoxy-ring are altering the active site chemistry in some way. We hypothesize that the chlorines on the aromatic ring prevent the binding of the ring at the active site of the benzoyltransferase. This allows the thioester link to be aligned by the CoA portion of the molecule. That this is possible is suggested by the strong binding of CoA to the enzyme (K_I = 0.1 mM) which indicates that the bulk of the binding energy for the acyl-CoA molecules is provided by interaction of the CoA portion with the enzyme. Thus, 2,4-D-CoA and CoA are high-affinity inhibitors, whereas 2,4-D does not inhibit at all. The same is true for 2,4,5-T-CoA. This chlorine-induced alteration of the binding geometry would allow catalysis, although predictably at a reduced rate as is seen. Altered binding geometry could also explain why the binding of either 2,4-D-CoA or 2,4,5-T-CoA to the enzyme greatly perturbs the subsequent binding of glycine relative to that for benzoyl-CoA or phenylacetyl-CoA (Table 1). The same argument applies to the binding of 2,4-D-CoA and 2,4,5-T-CoA to the phenylacetyltransferase (Table 2).

The kinetic constants for the conjugation of 2,4-D-CoA and 2,4,5-T-CoA by the benzoyltransferase (Table 1) and the phenylacetyltransferase (Table 2) provide an explanation for the failure of animals to efficiently conjugate the phenoxyherbicides. For both enzymes conjugating both herbicides, the K_m for the glycine is much higher than the values for the standard substrates and the catalytic rate constants are much lower. The data in Tables 1 and 2 can be used to approximate the effect of these differences in kinetic constants on the overall relatives rates of conjugation. The *N*-acetyltransferases are known to follow a sequential ordered mechanism [15] which has a rate equation of the form [17]:

Table 3. Comparative rates of conjugation of different acyl-CoA compounds

Enzyme	Substrate	*V*
Benzoyltransferase	Benzoyl-CoA	54
Benzoyltransferase	2,4-D-CoA	0.045
Benzoyltransferase	2,4,5-T-CoA	0.030
Phenylacetyltransferase	Phenylacetyl-CoA	0.12
Phenylacetyltransferase	2,4-D-CoA	0.00075
Phenylacetyltransferase	2,4,5-T-CoA	~0

Rates of conjugation were calculated from the data in Tables 1 and 2 using equation (4) and assuming a value of 5 mM for the concentration of glycine. Rates are expressed as μmoles of CoA released per min per ml of enzyme preparation.

$$V = \frac{V_{max}}{1 + \frac{K_{Acyl\text{-}CoA} K_m^{Gly}}{[Acyl\text{-}CoA][Gly]} + \frac{K_m^{Gly}}{[Gly]} + \frac{K_m^{Acyl\text{-}CoA}}{[Acyl\text{-}CoA]}} \quad (3)$$

Since the data in Tables 1 and 2 indicate that the primary differences between the phenoxyherbicides and the standard substrates are in the K_m^{gly} and V_{max} terms, a comparison of the rates should be relatively unaffected by the concentration of acyl-CoA chosen. We, therefore, choose the concentration of acyl-CoA to be very high so that equation (3) simplifies to:

$$V = \frac{V_{max}}{1 + (K_m^{Gly}/[Gly]} \quad (4)$$

The appropriate concentration of glycine to use for arriving at comparative rates is the *in vivo* concentration. The concentration of glycine in the whole liver is *ca.* 5 mM [21], and this value was used to calculate the rates shown in Table 3. From these comparative rates it can be seen that, based on *N*-acyltransferase rates alone, the rates of conjugation of the phenoxyherbicides will be predicted to be a hundred- to a thousand-fold slower than phenylacetic acid and benzoic acid respectively. It is not possible to directly compare the rates for benzoyltransferase to those for phenylacetyltransferase in Table 3 because the enzyme preparations were of unknown purity. However, the phenylacetyltransferase activity in mitochondria was less than 5% of the benzoyltransferase activity and thus quantitatively far less important for phenoxyherbicide conjugation. The acyl-CoA synthetase reaction [reaction (1)] may also make a contribution to the rate differences, although existing data [18] suggest that these differences will be small compared to those for the *N*-acyltransferase.

We found that 2,4-D-CoA and 2,4,5-T-CoA are potent alternate substrate inhibitors of both phenylacetyltransferase activity towards phenylacetyl-CoA and benzoyltransferase activity towards benzoyl-CoA (K_I values ranged from 0.02 to 0.05 mM). This is quite predictable from the kinetic data in Tables 1 and 2 which show that 2,4-D-CoA and 2,4,5-T-CoA had a high affinity for the *N*-acetyltransferases but a slow turnover rate. This means that they will be potent alternate substrate inhibitors by virtue of their ability to compete effectively for binding and then to occupy the enzyme active site for a *relatively* long time. The parent molecules, 2,4-D and 2,4,5-T, were not themselves inhibitory which indicates that the bulk of the binding energy for the CoA-analogues resides in the CoA portion of the molecules. Predictably, CoA itself was inhibitory to all activities ($K_I = 0.1$ mM). *In vivo*, one would expect that high doses of 2,4-D or 2,4,5-T would antagonize the metabolism of those xenobiotics conjugated by the mitochondrial *N*-acyltransferases, even though they are not conjugated extensively themselves.

In conclusion, we found that 2,4-D-CoA and 2,4,5-T-CoA were not conjugated by the bile acid conjugating enzyme of bovine liver. However, 2,4-D-CoA and 2,4,5-T-CoA were conjugated by the benzoyltransferase and phenylacetyltransferase enzymes of bovine liver mitochondria. A bisubstrate kinetic analysis revealed that the rate of conjugation of either phenoxyherbicide-CoA by either enzyme was very slow due to relatively low catalytic rate constants and extremely high K_m values for glycine. On the other hand, both enzymes possessed a high affinity for 2,4-D-CoA and 2,4,5-T-CoA which results in these compounds being potent alternate substrate inhibitors of the enzymes.

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