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Microsomal metabolism of *N*,*N*-diethyl-*m*-toluamide (DEET, DET): the extended network of metabolites

LUIS CONSTANTINO† and JIM ILEY‡*

† CECF, Faculdade de Farmácia, Avenida das For as Armadas, 1600 Lisbon, Portugal
‡ Chemistry Department, The Open University, Milton Keynes, MK7 6AA, UK

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1. The aim was to set out to establish the complete network of metabolites arising from the phenobarbital-treated rat liver microsomal oxidation of *N*,*N*-diethyl-*m*-toluamide (DEET). The products formed from DEET and all its subsequent metabolites were identified by HPLC retention times, UV spectroscopy, mass spectrometry and by comparison with authentic standards.

2. DEET (**1a**) produces three major metabolites, *N*-ethyl-*m*-toluamide (**1b**), *N*,*N*-diethyl-*m*-(hydroxymethyl)benzamide (**2a**) and *N*-ethyl-*m*-(hydroxymethyl)benzamide (**2b**), and, at low substrate concentrations or extended reaction times, two minor metabolites, toluamide (**1c**) and *N*,*N*-diethyl-*m*-formylbenzamide (**3a**). **1b** and **2a** are primary metabolites and their formation follows Michaelis-Menten-type kinetics. At low DEET concentrations, ring methyl group oxidation is favoured; at saturation concentrations, methyl group oxidation and *N*-deethylation proceed at similar rates. The rate of formation of **2b** *decreases* with increasing DEET concentration; **2b** is therefore a secondary metabolite of DEET and DEET acts as a competitive inhibitor of the metabolism of **1b** and **2a**.

3. Except for the primary amides, where *N*-dealkylation is impossible, metabolism of all subsequent compounds, **1b,c**, **2a-c**, **3a-c** and **4a,b**, involves an *N*-deethylation ($NEt_2 \rightarrow NHEt$ or $NHEt \rightarrow NH_2$) competitive with a ring substituent oxidation ($CH_3 \rightarrow CH_2OH$, $CH_2OH \rightarrow CHO$ or $CHO \rightarrow CO_2H$). Surprisingly, the aldehydes **3a-c** are also reduced to the corresponding alcohols **2a-c** ($CHO \rightarrow CH_2OH$); CO inhibits the oxidative metabolism of **3a-c**, but reduction to **2a-c** continues uninhibited.

4. The outcomes of this work are that (1) previously unreported aldehydes **3b** and **3c** form part of the DEET network of metabolites, (2) the reduction of the aldehydes **3a-c** has the potential to inhibit the formation of the more highly oxidized DEET metabolites, (3) amide hydrolysis was not observed for any substrate and (4) no evidence was obtained for *N*-(1-hydroxyethyl)amide intermediates.

Introduction

N,*N*-diethyl-*m*-toluamide (DEET) **1a** (figure 1) is generally considered the most effective insect repellent in world-wide use (Robbins and Cherniack 1986), and is active against the yellow fever mosquito (*Aedes aegypti*) and the malaria mosquito (*Anopheles quadrimaculatus*) (Gilbert 1966). DEET has been in extensive use for 40 years, and is generally considered of low toxicity. However, there have been several reports of toxic effects, including encephalopathy and anaphylaxis, related to the use of DEET (Robbins and Cherniack 1986), though it is unclear whether these effects are elicited by DEET or by one or more of its metabolites. DEET has at least two different, readily oxidizable groups, namely the amide *N*,*N*-diethyl substituents and the aromatic methyl substituent. Previous studies involving rat liver microsomes

* Author for correspondence: E-mail: j.n.iley@open.ac.uk
† Reprint requests to: Dr J. Iley, Chemistry Department, The Open University, Walton Hall, Milton Keynes MK7 6AA UK.

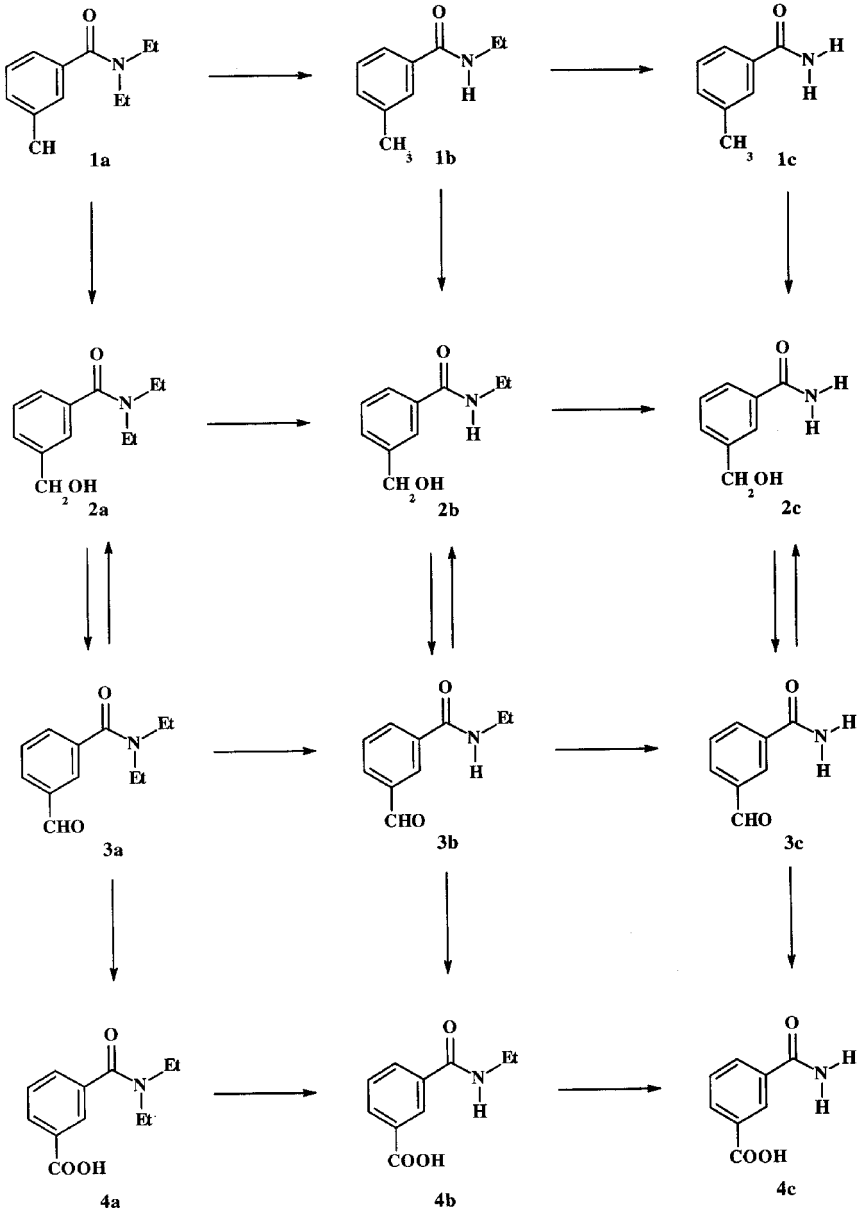

Figure 1. Pathways of DEET metabolism. The horizontal direction represents *N*-dealkylation; the vertical direction represents ring-methyl oxidation.

have identified compounds **1b**, **1c**, **2a**, **2b** and **3a** (figure 1) as metabolites (Taylor 1986, Yeung and Taylor 1988), and an *in vivo* study (Taylor and Spooner 1990) identified **4a-c**, as well as *m*-toluic acid, as urinary metabolites.

Our understanding of DEET metabolism, and of the metabolites that may contribute to its toxicological effects, remains incomplete. Consequently, to elucidate further the net of pathways of DEET metabolism, we have undertaken a study of the metabolism of DEET and each of its subsequent metabolites by phenobarbital-treated rat liver microsomes. Phenobarbital is a known inducer of

P450 isozymes that catalyse both amide dealkylation (Hall and Hanzlik 1990) and toluene benzylic oxidation (Wang and Nakajima 1991). The method has involved incubation of DEET with rat liver microsomes, identification of the metabolites so-formed, synthesis of the metabolites, subsequent incubation of each metabolite with rat liver microsomes and identification of the products formed. This process was repeated with each secondary metabolites until no further metabolism was observed. This approach has enabled us to identify (1) metabolites that are formed directly from DEET, (2) compounds that are formed from the subsequent transformation of DEET metabolites and (3) previously unknown metabolites. It has also enabled us to establish the different pathways of metabolite formation.

Materials and methods

Substrates and metabolites

All compounds were purified prior to use and were analytically pure by HPLC and gave satisfactory microanalyses (C, H, N± 0.3%). *N*,*N*-Diethyl-*m*-toluamide **1a**, *m*-toluamide **1c** and *m*-toluic acid were purchased from Aldrich Chemical Co. (Dorset, UK). *N*-Ethyl-*m*-toluamide **1b**, was synthesized by the Schotten-Baumann method (Furniss *et al*. 1978). *N*,*N*-Diethyl-*m*-(hydroxymethyl)benzamide **2a** and *N*-ethyl-*m*-(hydroxymethyl)benzamide **2b** were a kind gift from Dr Wesley Taylor (Canada Agriculture Research Station, Alberta) and purified by semipreparative reverse-phase HPLC. *m*-(Hydroxymethyl)benzamide **2c** was synthesized by sodium borohydride reduction of *m*-formylbenzamide **3c**. *N*,*N*-Diethyl-*m*-formylbenzamide **3a**, *N*-ethyl-*m*-formylbenzamide **3b** and *m*-formylbenzamide **3c** were obtained from *m*-formylbenzoic acid by activation with dicyclohexylcarbodiimide and subsequent addition of the corresponding amine (Taylor and Spooner 1990). *m*-Formylbenzoic acid was synthesized from *m*-toluic acid (Irrevedere *et al*. 1961).

m-(Diethylaminocarbonyl)benzoic acid **4a** was synthesized by the oxidation of *N*,*N*-diethyl-*m*-toluamide **1a** as follows. To a suspension of **1a** (7.65 g, 0.04 mol) in water (125 ml), $KMnO_4$ (3.17 g, 0.02 mol) was added. The suspension was heated at 80 °C for 1 h, after which the addition of permanganate and the heating were repeated. The suspension was then cooled and NaOH was added to dissolve the product. After filtration, the solution was extracted with dichloromethane and the aqueous phase was acidified. The product was extracted with chloroform, and the extract washed with water, dried (magnesium sulphate), and evaporated to afford **4a** in 31% yield: m.p. 128–129 °C; ν_{max} /cm^{-1} (KBr disc) 3090, 1716, 1609, 1434, 734, 708; δ_H ($CDCl_3$) 1.23 (6H, t), 3.43 (4H, m), 7.33–8.23 (4H, m), 11.8 (1H, s); *m*/*z* (%) 221 (29) (M^+), 220 (63), 150 (15), 149 (100), 121 (20), 76 (12), 65 (30). Found: C 65.3; H 6.8; N 6.1% $C_{12}H_{15}NO_3$ requires: C 65.1; H 6.8; N 6.3%.

m-(Ethylaminocarbonyl)benzoic acid **4b** was isolated in 10% yield as a secondary product from the synthesis of **4a**. Separation of the two from the reaction mixture proved possible because **4a** is preferentially soluble in chloroform whereas **4b** is soluble in ethyl acetate: m.p. 227–230 °C; ν_{max}/ cm^{-1} 3301, 1690, 1638, 1545, 1421, 730, 690; δ_H ($CDCl_3$) 1.16 (3H, t), 3.33 (2H, m), 7.33–8.66 (4H, m); *m*/*z* (%) 193 (24) (M^+), 192 (33), 166 (18), 165 (6), 149 (100), 121 (23), 76 (11), 65 (28), 50 (11), 28 (16). Found: C, 61.9; H, 5.4; N, 7.05%. $C_{10}H_{11}NO_3$ requires: C, 62.2; H, 5.7; N, 7.25 %.

m-(Aminocarbonyl)benzoic acid **4c** was synthesized in 41% yield by analogous $KMnO_4$ oxidation of **1c**: m.p. 287–289 °C (decomp.); ν_{max} /cm^{-1} 3474, 3190, 1700, 1634, 1558, 1398, 747, 690; δ_H ($CDCl_3$) 3.52 (2H, s br), 7.57–8.54 (4H, m), 13.22 (1H, s); *m*/*z* (%) 165 (71) (M^+), 150 (11), 149 (100), 121 (40), 76 (16), 75 (12), 65 (48), 50 (24), 44 (17). Found: C, 58.1; H, 4.3; N, 8.0%. $C_8H_7NO_3$ requires: C, 58.1; H, 4.3; N 8.5%.

Compounds **1b** and **4c** were recrystallized from ethanol. The acid **4a** was recrystallized from chloroform/*n*-hexane; **4b** was recrystallized from ethyl acetate:*n*-hexane. Compounds **2a** and **2b** were purified by semi-preparative HPLC using a C-18 column and an eluant comprising acetonitrile-water (85:25). Compound **2c** was purified by chromatography on silica using ethyl acetate as an eluant. The aldehydes **3a-c** were purified by chromatography on silica using dichloromethane-ethyl acetate (4:1) as eluant.

Microsomes

Ten-week-old male Wistar rats were injected intraperitoneally with phenobarbital (80 mg.kg^{-1}day^{-1}) for 4 days, starved for 1 day and killed by decapitation 24 h after the last injection. The livers were removed, immersed in ice-cold Tris-HCl buffer (50 mM) containing KCl (154 mM) minced, and washed with the same solution. The mince was homogenized with a Teflon-glass homogenizer immersed in ice, using 3 ml washing solution per g liver. The homogenate was centrifuged at 10 000*g* for 20 min at 4 °C

The pellet was discarded and the supernatant centrifuged again at 100000 *g* for 1 h at 4 °C. The resultant supernatant was discarded and the pellet resuspended in pH 7.4 phosphate buffer (68 mM) containing NaCl (74 mM) (PBS) and stored at −80 °C. Protein concentration was determined by the method of Lowry *et al*. (1951). A cytochrome P450 content of 1.50 nmol (mg protein)$^{-1}$ was determined using the CO difference method (Omura and Sato 1964a, b).

Product quantification

Microsomal incubations were performed in pH 7.4 PBS using 3.1 mg protein ml^{-1} microsomes, 6.25 mM glucose 6-phosphate, 1.25 mM $NADP^+$, 5 mM magnesium chloride and 2.5 U ml^{-1} glucose 6-phosphate dehydrogenase. Reactions were initiated by the addition of the substrate dissolved in pH 7.4 PBS. Initial substrate concentrations varied between 0.1 and 10 mM. Reactions were quenched by the sequential addition of 0.20 M zinc sulphate and 0.16 M barium chloride and centrifuged. The amide content of the supernatant was determined by HPLC using a Jones C-18 chromatography column (5 μm particle size, 25 cm) and an eluant consisting of either (1) 15% acetonitrile in pH 2.6 phosphate buffer (0.05 M) for compounds **1c**, **2b**, **2c**, **3c** and **4c**, or (2) a linear gradient starting with 15% acetonitrile in pH 2.6 phosphate buffer (0.05 M) reaching 42% acetonitrile in pH 2.6 phosphate buffer (0.05 M) after 22 min for the remaining compounds. Detection was achieved using a diode array detector, and quantification performed at 229 nm.

Reaction kinetics

Triplicate incubations were performed as above. Aliquots (100 μl) were withdrawn at timed intervals and quenched by the sequential addition of 0.2 mM zinc sulphate (400 μl) and of 0.16 mM barium chloride (400 μl). After centrifugation, the supernatant (750 μl) was treated with 1 M NaOH (200 μl) for 10 min, acidified with 1 M HCl (250 μl) and analysed by HPLC. Alternatively, for the presence of *N*-hydroxyalkyl-*N*-alkylamides, the supernatant was analysed directly by HPLC without NaOH treatment. Initial rates were determined from the linear phase of the reaction (the first 16 min) and correlated with substrate concentration via the Michaelis-Menten equation using a non-linear least-squares method. For reactions carried out in the presence of CO, CO was generated from formic and sulphuric acids (Furniss *et al*. 1978) and bubbled into the incubation mixture for 1 min prior to the addition of substrate.

Isolation of microsomal metabolites for mass spectrometric identification

Substrates (10 mM) were subjected to microsomal metabolism as described above. Reactions were monitored by HPLC and every 2 h further aliquots of glucose 6-phosphate, $NADP^+$ and glucose 6-phosphate dehydrogenase were added until 50% of the substrate had been consumed (between 6 and 8 h). After termination, by the addition of zinc sulphate and barium chloride and centrifugation, the supernatant solutions were extracted with ethyl acetate (3× 10 ml), the organic phase dried (sodium sulphate), evaporated, the residue redissolved in acetonitrile, and the metabolites separated by preparative HPLC using a C18 column with acetonitrile-water (85:15) as eluant.

Results and discussion

The microsomal incubation of DEET gives rise to three metabolites, the monoethylamide **1b**, derived from *N*-deethylation, the alcohol **2a**, derived from oxidation of the ring methyl group, and **2b**, derived from both ring methyl oxidation and *N*-deethylation. A plot of initial rate, v_i, for the formation of **1b, 2a** and **2b** versus DEET concentration is shown in figure 2. For **1b** and **2a** interpolation of the data gives rise to the V_{max} and V_{max}/K_m in table 1. Clearly, at low DEET concentrations oxidation of the methyl group is preferred, whereas at saturating concentrations of DEET both ring methyl oxidation and *N*-deethylation proceed at similar rates. In contrast with **1b** and **2a**, the profile for the formation of **2b** exhibits a *decrease* of v_i with increasing DEET concentration, consistent with **2b** being a secondary metabolite of DEET that can arise from **1b**, **2a** or from them both. Independent microsomal incubation of authentic samples of **1b** and **2a** reveals that **2b** is indeed formed from both compounds. The kinetic constants for the formation of **2b** from **1b** and from **2a** are shown in table 1. Either in terms of V_{max} or V_{max}/K_m these data reveal that DEET is metabolized more readily than either **2a** or **1b**. Thus

Table 1. Kinetic constants obtained from the microsomal oxidation of DEET.

	Product			
	2a (from DEET)	**1b** (from DEET)	**2b** (from **2a**)	**2b** (from **1b**)
V_{max} (nmol/min/mg protein)	10.3± 0.1	11.8± 0.1	2.5± 0.2	7.7± 0.5
k_m (10^{-1} mM)	1.5± 0.2	3.8± 0.5	10.2± 1.6	12.1± 0.2
V_{max}/k_m (10^{-5}/min/mg protein)	6.8	3.1	0.25	0.6

Data are the mean ± SEM for three determinations.

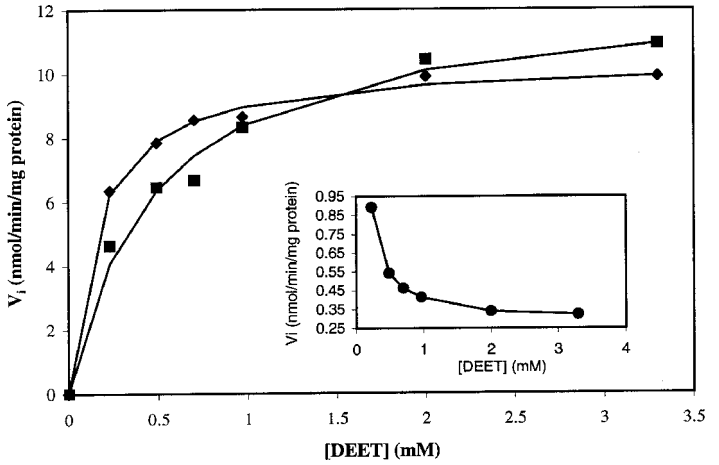

Figure 2. Substrate-dependency for the formation of **1b** (■), **2a** (◆) and (inset) **2b** (●) from DEET. At low DEET concentrations oxidation of the methyl group is preferred; at saturating concentrations of DEET, ring methyl oxidation and *N*-deethylation proceed at similar rates. Increasing DEET concentration inhibits the formation of **2b**.

DEET effectively acts as a competitive inhibitor of the subsequent oxidation reactions of **1b** and **2a**. Hence the decrease in the rate of formation of **2b** with increasing DEET concentration.

At low substrate concentrations (< 0.5 mM), or with longer incubations, two further minor metabolites, the aldehyde **3a** and the toluamide **1c,** were identified in microsomal incubations of DEET. In comparison with the oxidation of both **1b** and **2a** to **2b**, these compounds can arise from the subsequent microsomal oxidation of **2a** and **1b** respectively. Indeed, the incubation of an authentic sample of the alcohol **2a** leads to the formation of **3a** as well as **2b**. Similar incubation of the monoalkylamide **1b** produces the primary amide **1c** as well as **2b**. Thus, the origin of the three minor metabolites in the microsomal incubation of DEET involves the sequential metabolism of the primary metabolites **1b** and **2a**. Independent experiments revealed that DEET was metabolized at least three times faster than either **1b** or **2a** and the absence of **1c** and **3a** at the higher concentrations of DEET can be attributed to competitive inhibition of **1b** and **2a** by DEET itself. At low DEET concentrations, DEET metabolism depletes its concentration such that its ability to inhibit further metabolism of the primary metabolites is diminished.

Since the urinary metabolites of DEET are the acids, **4a-c,** we were interested to ascertain the pathways leading to these highly oxidized products, especially as the

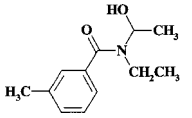

Figure 3. *N*-Ethyl-*N*-(1-hydroxyethyl)-*m*-toluamide.

Table 2. Initial rates† obtained from the independent incubations of 3.5 mM DEET, **2a** and **3a** in the absence or presence of CO‡.

	Initial rates (nmol product/min/mg protein)					
	Dealkylation		Ring substituent oxidation		Ring substituent reduction	
Substrate	−CO	+CO	−CO	+CO	−CO	+CO
1a	10.3 (± 0.4)	0.2 (± 0.04)	8.7 (± 0.6)	1.9 (± 0.2)	–	–
2a	1.9 (± 0.2)	0.08 (± 0.02)	4.6 (± 0.5)	0.2 (± 0.03)	–	–
3a	0.8 (± 0.2)	0.1 (± 0.03)	8.1 (± 1.7)	0.73 (± 0.1)	7.6 (± 0.7)	6.8 (± 0.6)

† Mean (± SD) of three determinations.

‡ Independent incubations were also performed in the absence of NADPH and all reactions, including the reduction reaction, were inhibited (data not shown).

aldehydes **3b** and **3c** have not previously been reported as DEET metabolites. Therefore we studied the incubation of authentic samples of the secondary metabolites **1c**, **2b** and **3a** as well as subsequent metabolites **2c**, **3b**, **3c**, **4a** and **4b**.

Incubation of the primary amide **1c** gave only one product, the alcohol **2c**. Taylor and Spooner (1990) have reported *m*-toluic acid in the urine of rat treated with DEET. Under the conditions of our experiments, we did not observe formation of this product, which could arise from hydrolysis of any of the amides **1a-c**, even when the cytochrome P450-catalysed reactions were inhibited by CO. We have previously reported that, for certain *N*,*N*-dialkylbenzamides, hydrolysis is a minor, competing pathway observed during microsomal incubations (Iley and Constantino 1994). However, the only amides that were found to hydrolyse at rates comparable with the microsomal oxidation were *N*-cyclopropyl-*N*-methylbenzamide and *N*-cyclopropylbenzamide.

Incubation of **2b** gave two products; **2c** (from further *N*-dealkylation) and **3b** (from oxidation of the hydroxymethyl group to aldehyde). Incubation of **2c** gave only one metabolite, the aldehyde **3c**. The aldehydes **3b** and **3c** are identified for the first time as potential DEET metabolites; the compounds isolated from the microsomal incubations had identical characteristics, (namely HPLC retention time, UV spectra and EI mass spectra, data not shown) to authentic samples.

Microsomal incubation of the aldehyde **3a** gave three products, **2a**, **3b** and **4a**. **3b** and **4a**, the products of *N*-dealkylation and aldehyde oxidation respectively, were expected. However, **2a**, the product of aldehyde reduction, was not. This reduction pathway was also observed during separate incubations of the aldehydes **3b** and **3c**, the products being **2b** and **2c** respectively. Incubation of **3a** with microsomes in the presence of CO reveals that the reduction pathway remains unaffected, whereas the rates of the *N*-dealkylation and ring methyl oxidation reactions are significantly reduced (table 2), as expected for cytochrome P450-mediated reactions. Aldo-keto

reductases are a class of cytoplasmic enzymes present in microsomes that possess broad substrate specificities; they require NADPH as a cofactor but, unlike cytochrome P450, are not inhibited by CO (Low and Castagnoli 1979). This reduction reaction may play an important role *in vivo* because it has the potential to retard the rate at which the alcohols **2a-c** are transformed into the corresponding carboxylic acids. Moreover, these alcohols are polar compounds that increases the possibility of their excretion either unchanged or in a conjugated form. Alternatively, if the alcohols are not excreted rapidly, then the partial reversal of the ring methyl oxidation route affords greater importance to the *N*-dealkylation pathway.

Oxidation of **3b** yields **3c** (from *N*-dealkylation), **4b** (from aldehyde oxidation) and **2b** (from aldehyde reduction). Similarly, microsomal oxidation of **3c** yields the carboxylic acid **4c** and the alcohol **2c**. Finally, microsomal incubation of **4a** yields **4b** via *N*-dealkylation while **4b** itself was further dealkylated to **4c**. No further transformations of **4c** upon incubation with microsomes was apparent.

Figure 1 summarizes the complete network of pathways for the oxidative metabolism of DEET by phenobarbital-treated rat liver microsomes obtained from this study. DEET metabolism is characterized by two main pathways: one (vertical) due to sequential oxidation of the aromatic substituent; the other (horizontal), due to amide dealkylation. We did not find any products resulting from the hydrolysis of the amides in our study, even from **4c** which might be expected to be a substrate for any hydrolytic pathway since the competing oxidation reactions are avoided.

Wu *et al*. (1979) proposed *N*-ethyl-*N*-(1-hydroxyethyl)-*m*-toluamide (figure 3) as an intermediate of DEET metabolism, but subsequently Taylor (1986) was unable to identify this compound in the microsomal incubation of DEET. We, too, have been unable to identify this or any other carbinolamide either as metabolites of DEET or of the *N*,*N*-diethylamides **2a**, **3a** or **4a**. Indeed, carbinolamides have only been observed as intermediates in the microsomal oxidation of *N*,*N*-di*methyl*amides (Constantino *et al*. 1992). For amides in which the *N*-alkyl groups are larger than methyl the derived carbinolamide is too unstable to be isolated (Ross *et al*. 1983, Bundgaard and Johansen 1984) and rapidly decomposes to the *N*-dealkylated amide.

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