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THE FORMATION AND METABOLISM OF *N*-HYDROXYMETHYL COMPOUNDS—IX

N-(ACETOXYMETHYL)-4-CHLOROBENZAMIDE: AN ELECTROPHILE BUT NOT A MUTAGEN IN *SALMONELLA TYPHIMURIUM*

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Abstract—The electrophilic properties of 4-chloro-*N*-(hydroxymethyl)benzamide as a model compound of carbinolamides formed during the metabolic oxidation of *N*-methylamides were investigated. 4-Chloro-*N*-(hydroxymethyl)benzamide did not react with nucleophiles such as cyanide or glutathione under physiological conditions. In contrast, *N*-(acetoxymethyl)-4-chlorobenzamide yielded the cyanomethylamide with KCN and *S*-(4-chlorobenzamidomethyl)glutathione with glutathione. Under non-aqueous conditions, *N*-(acetoxymethyl)-4-chlorobenzamide reacted avidly with ethanethiol, with methanol and with diethylamine in the presence of base, whereas 4-chloro-*N*-(hydroxymethyl)benzamide did not afford products under these conditions. These results show clearly that *N*-(acetoxymethyl)-4-chlorobenzamide is the precursor of reactive electrophilic methyleneimines. 4-Chloro-*N*-(hydroxymethyl)benzamide was not biotransformed to such electrophilic species when incubated with mouse hepatic microsomes or a microsomal supernatant with acetyl-CoA or a PAPS generating system. Neither 4-chloro-*N*-(hydroxymethyl)benzamide nor its acetate ester were mutagenic in the short term bacterial assay using *Salmonella typhimurium*. Nevertheless, esters of carbinolamides, such as *N*-(acetoxymethyl)-4-chlorobenzamide, might possess toxic or carcinogenic properties.

Compounds having *N*-methyl moieties are prone to oxidative metabolism to the corresponding *N*-hydroxymethyl analogues. Frequently, the latter compounds decompose to formaldehyde and the N–H derivative. However, in some xenobiotics, low electron density at nitrogen confers chemical stability on these *N*-hydroxymethyl metabolites [2]. Thus, for example, the *N*-hydroxymethyl metabolites of *N*-methylamides can either be excreted unchanged or undergo further oxidative metabolism [3].

We have recently reviewed the evidence which shows that metabolically generated carbinolamines and carbinolamides can be the precursors of electrophilic methyleneiminium or methyleneimine species, metabolites which may be toxins [4]. The formation of these species might be the consequence of the loss of hydroxide or the elimination of water, respectively. Alternatively, iminium ions or imines derived from *N*-methyl compounds may be the products of metabolically generated esters of the *N*-hydroxymethyl compounds rather than the *N*-hydroxymethyl compounds themselves [4]. In this report, work is described in which these hypotheses have been investigated. 4-Chloro-*N*-(hydroxymethyl)benzamide and its acetate ester, *N*-(acetoxymethyl)-4-chlorobenzamide, were synthesised as model compounds from which formaldehyde is not readily released [3]. In addition to a comparison of their ability to form reactive species, their genotoxic potential was investigated as measured by the *Salmonella* mutation assay. In this study, the acetate ester of 4-chloro-*N*-(hydroxymethyl)benzamide (a possible albeit unlikely metabolite) was chosen as a model ester for the sulphate analogue which is a difficult synthetic target. The results of these investigations help to explain certain toxic properties of *N*-methyl compounds such as the anticancer drug hexamethylmelamine [5], the herbicide Monuron [6] and some *N*-methylaniline derivatives [7, 8] which require metabolism to exert their toxicity and which are known or strongly suspected to undergo metabolism to their *N*-hydroxymethyl derivatives.

MATERIALS AND METHODS

Chemicals. The following substances were prepared generally according to published methods: 4-chloro-*N*-(hydroxymethyl)benzamide [2, 9], *N*-(acetoxymethyl)-4-chlorobenzamide [1], 4-chloro-*N*-(cyanomethyl)benzamide [10] and *S*-(4-chlorobenzamidomethyl)glutathione [11]. The latter compound is novel and was characterised by NMR spectroscopy: δ [400 MHz; $(CD_3)_2SO$] 2.1 (2H, m, glutamyl β-CH_2), 2.35 (2H, m, glutamyl γ-CH_2), 2.72 (1H, dd, *J* 13.5 and 10.0 Hz) and 3.07 (1H, dd, *J* 13.5 and 4.4 Hz) (cysteine β-CH_2), 3.77 (2H, d, *J*

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5.9 Hz, glycine CH_2), 3.9 (1H, m, glutamyl α-CH), 4.38 (1H, dd, *J* 13.7 and 2.2 Hz) and 4.48 (1H, dd, *J* 13.7 and 4.0 Hz) (NCH_2S), 4.61 (1H, m, cysteine α-H), 7.55 (2H, d, *J* 8.6 Hz) and 7.91 (2H, d, *J* 8.6 Hz) (ArH), 8.7 (7H, br, NH and OH) and 9.37 (1H, t, *J ca* 6 Hz, glycine NH).

$K^{14}CN$ was purchased from Amersham International (Amersham, U.K., specific activity: 59 mCi/mmol). The following materials were obtained from Sigma Chemical Co. (Poole, U.K.): acetyl-CoA, ATP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP. The benzamide derivatives were added to the incubation mixtures dissolved in DMSO or acetonitrile (25 μl).

Animals. Male BALB/c mice (19–24 g) were used for all metabolism experiments. In the bacterial mutagenicity tests, 9000 *g* supernatant fractions of livers from male Wistar rats (approximately 250 g) which had received Aroclor were used.

Studies of chemical reactivity. To investigate the reactivity of *N*-(acetoxymethyl)-4-chlorobenzamide with nucleophiles, the following chemical experiments were conducted. *N*-(Acetoxymethyl)-4-chlorobenzamide (341 mg, 1.5 mmol) was dissolved in methanol (5 ml). TLC analysis (silica gel, diethyl ether) showed no change after 15 min at ambient temperature or 5 min at 64°. Triethylamine (202 mg, 2 mmol) was added to the cooled mixture whereupon TLC showed quantitative conversion. Dichloromethane (20 ml) was added and the mixture was washed with aqueous toluene-4-sulphonic acid, water and saturated brine before being dried (Na_2SO_4). The solvents were evaporated under reduced pressure to give *4-chloro-N-(methoxymethyl)benzamide* (77%) as white crystals; NMR δ (60 MHz; $CDCl_3$) 3.35 (3H, s, OCH_3), 4.85 (2H, d, *J* 6 Hz, NCH_2O), 7.40 (2H, d, *J* 8 Hz) and 7.85 (2H, d, *J* 8 Hz) (ArH) and 8.0 (1H, br, NH). Treatment of a solution of *N*-(acetoxymethyl)-4-chlorobenzamide in dichloromethane with excess ethanethiol and *N,N*-diisopropylethylamine, followed by washing with water, drying (Na_2SO_4) and evaporation of the solvent under reduced pressure afforded *4-chloro-N-(ethylthiomethyl)benzamide* (85%) as a white powder; NMR δ (60 MHz; $CDCl_3$) 1.30 (3H, t, *J* 7 Hz, CH_3), 2.70 (2H, q, *J* 7 Hz, CH_2CH_3), 4.50 (2H, d, *J* 6 Hz, NCH_2O), 7.45 (2H, d, *J* 8 Hz) and 7.85 (2H, d, *J* 8 Hz) (ArH) and 8.65 (1H, t, *J* 6 Hz, NH). Similarly, *N*-(acetoxymethyl)-4-chlorobenzamide was allowed to react at ambient temperature with excess diethylamine in dichloromethane to give *4-chloro-N-(diethylaminomethyl)benzamide* (86%) as an off-white solid; NMR δ (60 MHz; $CDCl_3$) 1.10 (6H, t, *J* 7 Hz, CH_3), 2.58 (4H, q, *J* 7 Hz, CH_2CH_3), 4.35 (2H, d, *J* 6 Hz, NCH_2N), 7.2 (1H, br, NH), 7.30 (2H, d, *J* 8 Hz) and 7.75 (2H, d, *J* 8 Hz) (ArH).

N-(Acetoxymethyl)-4-chlorobenzamide (0.5 mM) was incubated in Earl's buffer, pH 7.4, (2.5 ml) with $K^{14}CN$ (1 mM, specific activity 3.2 mCi/mmol) at 37° for 1 hr under shaking. Incubations of *N*-(acetoxymethyl)-4-chlorobenzamide (1 mM) with glutathione (10 mM) were performed in buffer containing 3-(*N*-morpholino)propanesulphonic acid (MOPS, 0.4 M, pH 7.4).

In vitro *metabolism of 4-chloro-(N-hydroxymethyl)benzamide.* Livers were excised and a homogenate (25%) was prepared in sucrose solution (2.5 M, pH 7.4). Microsomes were obtained in the standard way involving centrifugation of the homogenate at 9000 *g* for 20 min and centrifugation of the 9000 *g* supernatant at 100,000 *g* for 1 hr. 4-Chloro-*N*-(hydroxymethyl)benzamide (1 mM) was incubated either with microsomes or microsomal supernatant equivalent to 0.3 g liver. Microsomal incubations were carried out for 1 hr in Earl's buffer (2.5 ml) containing $MgCl_2$ (5 mM) and a NADPH generating system (NADP, 1 mM, glucose-6-phosphate 4.5 mM, glucose-6-phosphate dehydrogenase 1.5 units). Incubations with microsomal supernatant were performed in MOPS buffer (0.4 M). These incubates also contained either $K^{14}CN$ (1 mM, specific activity 3.2 mCi/mmol) or glutathione (10 mM). In some experiments, a system which generated 3′-phosphoadenosine-5′-phosphosulphate (PAPS; K_2SO_4 2.4 mM, ATP, 8 mM, mercaptoethanol, 2.4 mM) or acetyl-CoA (2.5 mM) with ATP (5 mM) was added to the incubation. Incubations were terminated either by placing incubates on ice, or by precipitation of proteins via addition of 20% aqueous zinc sulphate (0.6 ml) followed by saturated aqueous barium hydroxide (0.6 ml). For the analysis of 4-chloro-*N*-(cyanomethyl)benzamide the incubation mixtures were passed through C_{18} Sep-Pak cartridges (Waters Associates, Northwich, U.K.), which were washed with water (5 ml) and eluted with methanol (5 ml). The methanol extract was evaporated to dryness under partial vacuum in a Savants Speed-vac concentrator. For the analysis of *S*-(4-chlorobenzamidomethyl)glutathione, the incubates were centrifuged after denaturation of proteins and aliquots of the supernatant were used immediately for analysis.

HPLC analysis. Separation of metabolites was performed using an Altex 100A pump (Anachem Ltd., Luton, U.K.) linked to a Pye LC-UU detector (λ = 254 nm) (Pye Unicam, Cambridge, U.K.) or a Waters trimodular system both fitted with Waters RCM-100 radial compression units and C_{18} 5-m reverse phase columns. For the analysis of the cyanomethylamide, the mobile phase was 60% methanol–40% 0.05 M sodium acetate in water (adjusted to pH 5); for the analysis of *S*-(4-chlorobenzamidomethyl)glutathione: 50% methanol–50% 0.05 M aqueous sodium acetate or 60% methanol–40% (1% trifluoroacetic acid in water). The flow rate was 1 ml/min.

Bacterial mutagenicity tests. The benzamide derivatives were evaluated using the plate incorporation assay as described by Maron and Ames [12] with *Salmonella typhimurium* strains TA 98 and TA 100. Some tests of 4-chloro-*N*-(hydroxymethyl)-benzamide involved preincubation of the compound with a liver 9000 *g* supernatant (10%) obtained from rats treated with Aroclor with or without K_2SO_4 (2.4 mM) and ATP (8 mM) or acetyl-CoA (2.5 mM) and ATP (5 mM) preceding the addition of the bacteria. The inclusion of acetyl-CoA was prompted by the observation that the mutagenicity of metabolites of the herbicide Monuron, a *N*-methyl compound related to the *N*-methylbenzamides, was increased in the presence of this cofactor [6].

RESULTS

Under physiological conditions, 4-chloro-*N*-(hydroxymethyl)benzamide did not react either with cyanide to afford the cyanomethylamide (Fig. 1) or with glutathione to yield the *S*-amidomethyl glutathione derivative (Fig. 2). In contrast, the acetate ester reacted with both cyanide (Fig. 1) and glutathione (Fig. 2). The product of the reaction between *N*-(acetoxymethyl)-4-chlorobenzamide and glutathione was isolated by HPLC. High-field proton NMR showed this material to be *S*-(4-chlorobenzamidomethyl)glutathione consistent with the spectrum of a sample of the compound prepared by the general method previously reported [11]. Under the incubation conditions used, 3% of the *N*-acetoxymethyl compound reacted with cyanide to give the cyanomethylamide, whereas the reaction with glutathione was almost quantitative.

In order to investigate the mechanisms whereby *N*-(acetoxymethyl)-4-chlorobenzamide reacts with nucleophiles, several experiments were conducted. Firstly, the pH dependence of the rate of hydrolysis of this compound to 4-chloro-*N*-(hydroxymethyl)benzamide in 0.02 M phosphate buffer was determined at 37° by HPLC analysis. The $t_{1/2}$ of hydrolysis was 13.2 min at pH 3, 16.9 min at pH 7 and less than 1 min at pH 9, which shows that the reaction was base-catalysed. Secondly, the substrate, in methanol, was treated with triethylamine. No reaction was observed by TLC during 10 min in the absence of base. Addition of successive aliquots of triethylamine caused very rapid methanolysis involving stoichiometric consumption of base. The isolated product was 4-chloro-*N*-(methoxymethyl)benzamide (see Fig. 4) in virtually quantitative yield, hence the electrophilic reactivity of the starting ester is at the methylene carbon exclusively rather than at the ester carbonyl. The reaction is stoichiometric in base, rather than catalytic. A similar reaction of *N*-(acetoxymethyl)-4-chlorobenzamide was observed with diethylamine and with ethanethiol in the presence of the non-nucleophilic base *N,N*-di*iso*propylethylamine, giving the diethylaminomethyl- and ethylthiomethylbenzamides, respectively. Under these experimental conditions, 4-chloro-*N*-(hydroxymethyl)benzamide did not react with methanol, ethanethiol or diethylamine.

When 4-chloro-*N*-(hydroxymethyl)benzamide was incubated with either mouse hepatic microsomes or microsomal supernatant with or without addition of acetyl CoA or a PAPS generating system, metabolism to precursors of methylene iminium ions or imines did not occur as indicated by the lack of evidence for the formation of a cyanomethylamide in the presence of cyanide or a glutathionyl derivative in the presence of glutathione (results not shown).

Whether *N*-(acetoxymethyl)-4-chlorobenzamide reacts with glutathione *in vivo* is unclear. The urine of mice which had received *N*-(acetoxymethyl)-4-

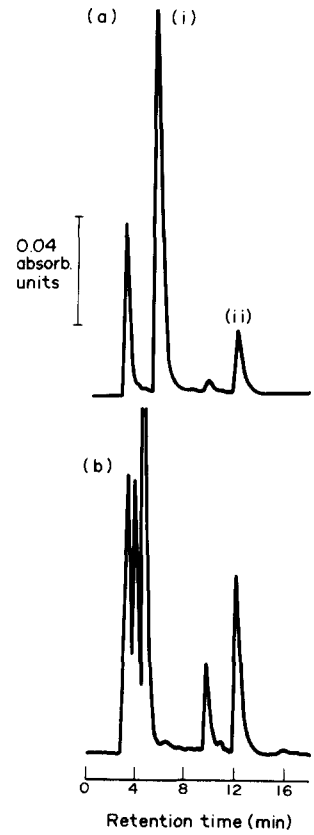

Fig. 2. High pressure liquid chromatogram of (a) a sample of the incubate of *N*-(acetoxymethyl)-4-chlorobenzamide with glutathione; (b) a sample of the incubate of 4-chloro-*N*-(hydroxymethyl)benzamide with a 9000 *g* supernatant and glutathione. Peak (i) co-elutes with *S*-(4-chlorobenzamidomethyl)glutathione and peak (ii) with 4-chloro-*N*-(hydroxymethyl)benzamide. A chromatographic trace very similar to the one shown in (b) was observed when the 9000 *g* supernatant was fortified with a PAPS generating system.

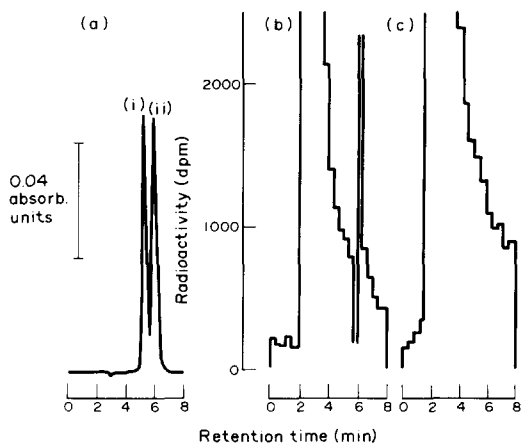

Fig. 1. High pressure liquid chromatogram of (a) reference compounds 4-chloro-*N*-(hydroxymethyl)benzamide (i) and 4-chloro-*N*-(cyanomethyl)benzamide (ii); (b) a sample of the incubate of *N*-(acetoxymethyl)-4-chlorobenzamide with $K^{14}CN$; and (c) a sample of the incubate of 4-chloro-*N*-(hydroxymethyl)benzamide with $K^{14}CN$. Detection by u.v. spectrophotometry (a) or radioactivity counting (b and c). Traces are representative of three experiments.

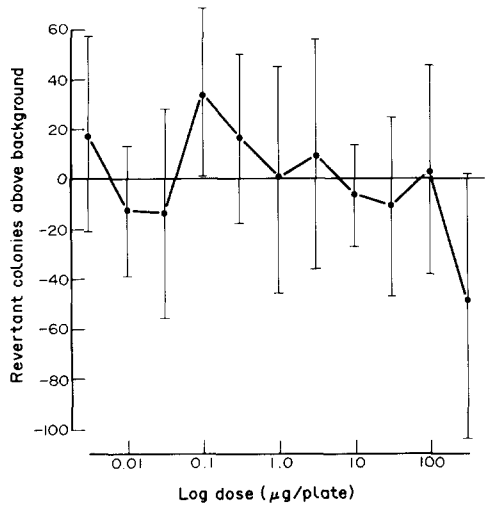

Fig. 3. Lack of mutagenicity of *N*-(acetoxymethyl)-4-chlorobenzamide to *Salmonella typhimurium* TA 100. Results are the mean ± SD of three plates.

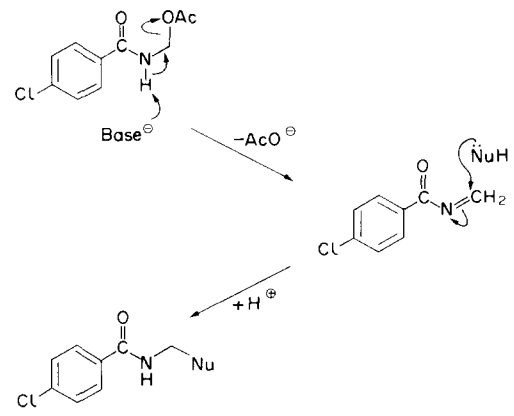

Fig. 4. Mechanism of the base-catalysed reaction between *N*-(acetoxymethyl)-4-chlorobenzamide and nucleophiles (Nu = CN^-, glutathione, MeO, EtS, Et_2N).

chlorobenzamide or 4-chloro-*N*-(hydroxymethyl)-benzamide contained 4-chlorohippuric acid and the HPLC chromatograms showed a peak which was possibly due to *S*-(4-chlorobenzamidomethyl)-*N*-acetylcysteine (results not shown). The small amount of this material formed and its chromatographic properties rendered its identification impossible.

Neither 4-chloro-*N*-(hydroxymethyl)benzamide nor its acetyl ester were genotoxic in the *Salmonella* assay (TA 100 strain) in the presence or absence of a 9000 *g* supernatant fraction obtained from rat (Table 1, Fig. 3). *N*-(Acetoxymethyl)-4-chlorobenzamide was, however, much more toxic towards the bacteria than was 4-chloro-*N*-(hydroxymethyl)-benzamide. At a concentration of 0.316 mg per plate of the acetate ester, 50% of the bacteria were killed whereas a concentration of 5 mg per plate 4-chloro-*N*-(hydroxymethyl)benzamide did not cause toxicity to the bacteria. Results obtained with the TA 98 strain were very similar to those observed with TA 100, with the rate of spontaneous reversion similar to that reported previously [12].

DISCUSSION

The results of this study demonstrate unequivocally that 4-chloro-*N*-(hydroxymethyl)benzamide does not form a methyleneiminium or methyleneimine under physiological conditions. This is not unexpected since the hydroxide group is a poor leaving group. However, the situation is very different in a molecule in which the *N*-hydroxymethyl group is esterified. In the model compound, *N*-(acetoxymethyl)-4-chlorobenzamide, the acetoxy group is a good leaving group. This renders the molecule capable of forming an imine under physiological conditions. Such species can react with cyanide to yield the cyanomethylamide or with glutathione to afford *S*-(4-chlorobenzamidomethyl)glutathione. In the presence of cyanide, the elimination–addition reaction with the electrophilic methylene was not the only reaction occurring, in that cyanide appeared

Table 1. Lack of mutagenicity of 4-chloro-*N*-(hydroxymethyl)benzamide to *Salmonella typhimurium* strain TA 100

Dose (μg/plate)	Number of revertant colonies above background, in the presence of 9000 *g* fraction	9000 *g* fraction and PAPS generating system	9000 *g* fraction and acetyl-CoA and ATP
50	−9*	3	16
100	−9 ± 22	−29 ± 25	−11 ± 21
500	−18 ± 16	−30	−21 ± 8
1000	20 ± 6	−29 ± 18	−42
5000	−12 ± 33	−21	−5
2-Acetylaminofluorene 50 μg/plate	2073 ± 219		

* Values are the mean of two or the mean ± SD of 3–5 tests. Background levels were: 123 ± 3 (9000 *g* supernatant), 98 ± 18 (9000 *g* fraction and PAPS generating system), 81 ± 18 (9000 *g* fraction and acetyl-CoA).

also to catalyse hydrolysis by acting as a base as well as a nucleophile. Therefore, the amount of the cyanomethylamide formed in the presence of cyanide was much less than the amount of the glutathionyl derivative generated during the reaction of the ester with glutathione. The pH dependence and the stoichiometry of base consumption of these substitution reactions suggests a mechanism implicating the methyleneimine rather than the methyleneiminium, as outlined in Fig. 4.

The results indicate that the metabolic conjugation of 4-chloro-*N*-(hydroxymethyl)benzamide with acetyl-CoA, activated sulphate or UDP-glucuronic acid is not a biochemical reaction *in vitro*. Reaction products of the *N*-hydroxymethyl compound with cyanide or glutathione could not be detected in incubations of liver fractions supplemented with either acetyl-CoA or a PAPS generating system. Furthermore, the presence of small amounts of a mercapturate derived from 4-chloro-*N*-(hydroxymethyl)benzamide was suspected in the urine of mice which had received this carbinolamide, but unequivocal identification was not possible. These observations are in accordance with our failure to detect either *S*-(*N*-(2,4,6-bis(dimethylamino)-1,3,5-triazin-5-yl)-*N*-methylaminomethyl)glutathione as a metabolite of *N*-(hydroxymethyl)pentamethylmelamine *in vitro* in liver preparations or *N*-acetyl-*S*-(*N*-(2,4,6-bis-(dimethylamino)-1,3,5-triazin-5-yl)-*N*-methylaminomethyl)cysteine as an *in vivo* metabolite of this compound in the urine of mice.*

Two recent reports describe the identification of NCH_2S conjugates of *N*-methyl compounds *in vivo*. A major biliary metabolite of the hepatocarcinogen 4-dimethylaminoazobenzene in the rat was shown to be *S*-((4-phenylazo)phenylaminomethyl)glutathione [7] and a major urinary metabolite of 4-cyano-*N,N*-dimethylaniline has been identified as *N*-acetyl-*S*-((4-cyanophenyl)aminomethyl)cysteine in rodents [8]. In both cases, the thioester metabolites were considered to be the products of the reaction between glutathione and either methyleneiminium ions or imines derived from the *N*-hydroxymethyl intermediates. As the hydroxymethyl moiety in the aniline derivatives is located in an environment of greater electron density than in the *N*-(hydroxymethyl)benzamide molecule, it is reasonable to assume that they undergo facile elimination of water, as do the aliphatic *N*-(hydroxymethyl)amines [13]. Alternatively, one could suggest that, in analogy to the results reported in this paper, metabolic esterification increases the leaving group ability of the hydroxy group in these *N*-(hydroxymethyl)aniline derivatives.

4-Chloro-*N*-(hydroxymethyl)benzamide was not a mutagen in the *Salmonella* mutation assay. This is not surprising in the light of its inability to form electrophilic species. It is, however, puzzling that *N*-(acetoxymethyl)-4-chlorobenzamide, which we have now shown unequivocally to be a precursor of electrophilic species, is not mutagenic. Two explanations can be tendered: either the methyleneimine formed by elimination of acetate is intrinsically unreactive with DNA to cause deleterious lesions or, more likely, the *Salmonella* test is incapable of detecting the potential mutagenicity of this kind of soft electrophile. It has been shown recently that *N*-(hydroxymethyl)chloroacetamide, an amide related to 4-chloro-*N*-(hydroxymethyl)benzamide, was without genotoxic properties in *Salmonella* [14]. However, it was shown to be clastogenic in a human lymphocyte cytogenic assay, both in the presence and absence of rodent liver 9000 *g* homogenate. It remains to be investigated whether or not *N*-(acetoxymethyl)-4-chlorobenzamide or, indeed, 4-chloro-*N*-(hydroxymethyl)benzamide are clastogenic in this genotoxicity test.

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* D. Ross, A. Gescher and M. D. Threadgill, unpublished results.

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