# Metabolism of 3,4-Dichlorobenzyl *N*-Methylcarbamate in the Rat

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The metabolism of *N*-methyl-$C^{14}$ and benzyl-$C^{14}$-labeled 3,4-dichlorobenzyl *N*-methylcarbamate was investigated in the rat. In 7 days the rat excreted 31 and 45% of the *N*-methyl label, respectively, in urine and as respiratory $CO_2$. Ninety-five per cent of the benzyl label was excreted in urine and none as respiratory $CO_2$. Fecal excretion of these labels amounted to only 3 to 5% of the dose. Tissue residues from the *N*-methyl label amounted to 7% of the dose after 7 days. The major urinary metabolites were identified as 3,4-dichlorobenzyl glucuronide (5%), 3,4-dichlorohippuric acid (63%), and 3,4-dichlorobenzoic acid (6%). A metabolite tentatively identified as 3,4-dichlorobenzoyl glucuronide (5%) was also found in urine.

Dichlorobenzyl *N*-methylcarbamates have shown promise as pre-emergent herbicides. Union Carbide is at present developing U.C. 22463, which is an 80 to 20 mixture of the 3,4-dichloro- and 2,3-dichloro- isomers, respectively. This study was undertaken to provide direct information on the metabolism and elimination by the rat of the 3,4-dichlorobenzyl *N*-methylcarbamate.

## METHODS

**Chemicals.** *N*-methyl-$C^{14}$- 0.269 mc. per mmole) and benzyl-$C^{14}$- (1.1 mc. per mmole) labeled 3,4-dichlorobenzyl *N*-methylcarbamate (Figure 1) were prepared by Bartley and Heywood (1967). The two labeled samples were acetylated using acetic anhydride and methane sulfonic acid and gas chromatographed according to the procedure of Sullivan *et al.* (1967). Ninety-eight per cent of the radioactivity from the two labeled samples chromatographed as 3,4-dichlorobenzyl *N*-methyl-*N*-acetyl carbamate. Samples of 3,4-dichlorobenzoic acid, nonlabeled and labeled in the carboxyl carbon (0.11 mc. per mmole), and 3,4-dichlorohippuric acid, nonlabeled and labeled (0.025 mc. per mmole) in the glycine moiety, were synthesized by Bartley and Heywood (1967). Nonlabeled 3,4-dichlorobenzyl *N*-methylcarbamate and 3,4-dichlorobenzyl alcohol were supplied by Olefins Division, Union Carbide, South Charleston, W. Va.

**Excretion Studies.** *N*-methyl-$C^{14}$- (0.036 mc. per mmole) and benzyl-$C^{14}$- (0.036 mc. per mmole) labeled 3,4-dichlorobenzyl *N*-methylcarbamate were dissolved in polyethylene glycol 400 and individually administered to rats (eight to 12 animals per labeled compound) at 100 mg. per kg., using the weighed syringe technique. Male rats (Wistar strain, Harlan Industries), weighing 150 grams, were maintained on a commercial synthetic diet and housed in all-glass metabolism cages (Knaak *et al.*, 1965). The individual urine and fecal samples were collected and analyzed daily for $C^{14}$ over a period of 7 days. Respiratory $CO_2$ from each animal was trapped on Ascarite and analyzed daily for $C^{14}$ over a period of 4 days. After 7 days, the animals were sacrificed, and the carcasses of four animals per labeled compound were analyzed for residual $C^{14}$. The analytical procedures were essentially those reported by Knaak *et al.* (1965).

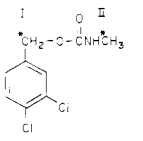

**Figure 1. Structural formula of 3,4-dichlorobenzyl *N*-methylcarbamate and position of $C^{14}$**

**Analysis of Urinary Metabolites.** The first day urines collected in the excretion studies were used as a source of metabolites for the following studies.

ANALYTICAL ION EXCHANGE CHROMATOGRAPHY. Two milliliters of urine containing the *N*-methyl-$C^{14}$ and benzyl-$C^{14}$ metabolites were individually chromatographed on a 1.5 × 24 cm. column of diethylaminoethyl (DEAE) cellulose according to the method of Knaak and Sullivan (1966). One hundred and forty 4-ml. fractions were collected, and 1 ml. of each fraction was analyzed for $C^{14}$ using scintillation counting techniques.

PREPARATIVE ION EXCHANGE CHROMATOGRAPHY. Forty milliliters of urine containing the benzyl-$C^{14}$-labeled metabolites were chromatographed on a 5 × 24 cm. column of DEAE-cellulose previously equilibrated with 0.02*M* ammonium acetate–acetic acid buffer, pH 7.0. A linear gradient of 0.02*M* ammonium acetate–acetic acid buffer, pH 7.0, to 0.05*M* ammonium acetate–acetic acid buffer, pH 7.0, was used to elute the metabolites. Twenty-four-hundred-milliliter volumes of the 0.02*M* and 0.05*M* buffers, respectively, were used in the mixing chamber and reservoir of the gradient device (Knaak *et al.*, 1965). Four hundred and eighty 10-ml. fractions were collected, and 0.2 to 1 ml. of every fifth fraction was analyzed for radioactivity using liquid scintillation counting techniques.

SILICA GEL CHROMATOGRAPHY. Metabolites B, C, and D as eluted from the preparative ion exchange column were individually concentrated to 5 ml. by vacuum distillation at 35° C. Residual water was removed by

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azeotropic distillation with acetonitrile. The residues containing the individual metabolites (B, C, and D) were separately dissolved in methanol, adsorbed onto 6 to 7 grams of silica gel (Davison Chemical Division, W. R. Grace & Co., Baltimore, Md., Grade 923), and air-dried. The adsorbed metabolites were then individually added to the top of a 2.5 × 24 cm. column of silica gel previously packed in acetonitrile and chromatographed using an acetonitrile-methanol gradient. Six hundred milliliters of acetonitrile were used in the mixing chamber, while an equal volume of methanol was used in the reservoir of the gradient device (Knaak *et al.*, 1965). Three hundred 4-ml. fractions were collected and 0.2 to 1 ml. of every fifth fraction was analyzed by liquid scintillation counting techniques.

HYDROLYSIS BY β-GLUCURONIDASE. The main radioactive peaks containing metabolites B, C, and D from the silica gel column were concentrated to 2 ml. by vacuum distillation. One milliliter of each of the samples containing metabolite B or C was evaporated to dryness, dissolved in 4 ml. of 0.1*M* sodium acetate–acetic acid buffer, pH 4.5, and incubated with 50 mg. (3000 units) of β-glucuronidase (Nutritional Biochemicals Corp., Cleveland, Ohio) for 24 hours at 37° C. At the end of the incubation period, the reaction mixture was adjusted to pH 7 and chromatographed on a 1.5 × 24 cm. DEAE-cellulose column and analyzed as previously described for the urine samples. The neutrals resulting from the hydrolysis of metabolite B were continuously extracted with diethyl ether over a 24-hour period at pH 7. The extract was concentrated by distillation to a volume of 2 ml. and set aside for analysis by gas chromatography.

GAS CHROMATOGRAPHY. A Barber-Colman 5000 gas chromatograph equipped with a radioactive monitor (RAM) and a hydrogen flame detector (10 to 1 split) was used throughout the study. Glass columns (8 feet × 5 mm. I.D.) were packed with either 2 or 5% SE-30 on Applied Science Gas-Chrom Q (80- to 100-mesh). The columns were temperature programmed at 3° C. per minute with an injection port temperature of 350° C. and a detector temperature of 320° C. A helium carrier gas flow of 120 ml. per minute was used throughout. The hydrogen flow was not optimized for maximum sensitivity.

One milliliter of the concentrated samples off the silica gel column containing metabolites B and C were individually evaporated to dryness and reacted for 0.5 hour at 100° C. with 2 to 3 ml. of acetic anhydride and one to two drops of methane sulfonic acid. The reaction mixture was poured into water and extracted with chloroform. After removal of the solvent, the residue was methylated with an ethereal solution of diazomethane and chromatographed. A 2% SE-30 column was used for the reaction products of metabolite B while a 5% SE-30 column was used for the products of metabolite C. The extracted neutrals resulting from the β-glucuronidase treatment of metabolite B were equally divided, and one portion was acetylated as previously described. Both samples were chromatographed on a 5% SE-30 column. In addition, the acetylated $C^{14}$ neutrals were cochromatographed with nonlabeled 3,4-dichlorobenzyl acetate on a 5% SE-30 column at 160° C.

One milliliter of the concentrated sample off the silica gel column containing metabolite D was evaporated to dryness, dissolved in 20 ml. of 0.01*M* HCl, and continuously extracted for 24 hours with diethyl ether. The ether extract was evaporated to dryness and methylated with 5 ml. of an ethereal solution of diazomethane. The excess diazomethane was removed after 10 minutes by distillation, and the remaining solution was concentrated to 1 ml. Ten microliters of this solution was gas chromatographed on a 5% SE-30 column. For identification purposes, the sample was cochromatographed with an authentic sample of methyl 3,4-dichlorohippurate on a 5% SE-30 column at 210° C.

## RESULTS

**Excretion Studies.** The results of the excretion studies are given in Figure 2 for the *N*-methyl-$C^{14}$- and benzyl-$C^{14}$-labeled 3,4-dichlorobenzyl *N*-methylcarbamate. The *N*-methyl-labeled carbamate gave rise to large quantities of respiratory $CO_2$ (about 45% of dose), while no $C^{14}O_2$ could be detected in the respired air from animals administered the benzyl-$C^{14}$ label. The benzyl label was excreted in urine (about 95% of dose), while only 31% of the dosed *N*-methyl label was found in urine. Fecal excretion of these labels was small as only 3 to 5%, respectively, of the dose for the *N*-methyl- and benzyl-labeled carbamate was excreted by this route. The excretion of the benzyl label was essentially complete in 4 days (about 99% of dose). The *N*-methyl label was excreted to the extent of 80% of

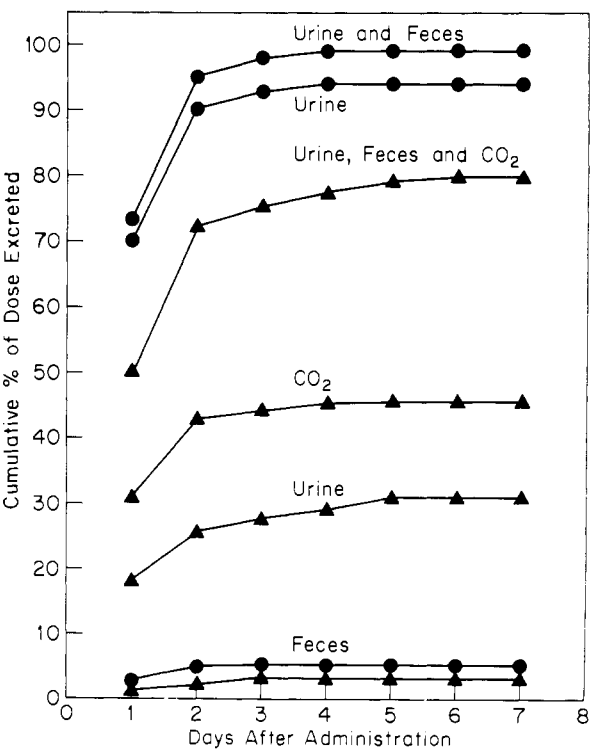


**Figure 2. Excretion of $C^{14}$ by the rat dosed with labeled 3,4-dichlorobenzyl *N*-methylcarbamate**

▲ Average of 12 rats administered 3,4 dichlorobenzyl *N*-methyl $C^{14}$ carbamate orally at 100 mg. per kg.
● Average of eight rats administered 3,4-dichlorobenzyl-$C^{14}$ *N*-methylcarbamate orally at 100 mg. per kg.

dose and tissue residues of approximately 7% of dose were found after 7 days.

**Urinary Metabolites of 3,4-Dichlorobenzyl *N*-Methylcarbamate.** Figure 3 gives the chromatographic results for the *N*-methyl-$C^{14}$- and benzyl-$C^{14}$-labeled metabolites on an analytical column (1.5 × 24 cm.) of DEAE-cellulose. Table I gives the approximate $C^{14}$ percentages found under each peak area for the benzyl label. Except for the neutrals (A), the *N*-methyl-labeled metabolites of the carbamate did not chromatograph identically with the benzyl-labeled metabolites. The *N*-methyl-labeled neutrals (A) amounted to 56% of the radioactivity recovered from the column. The two small peaks chromatographing in the region of B represent an additional 20% of the recovered

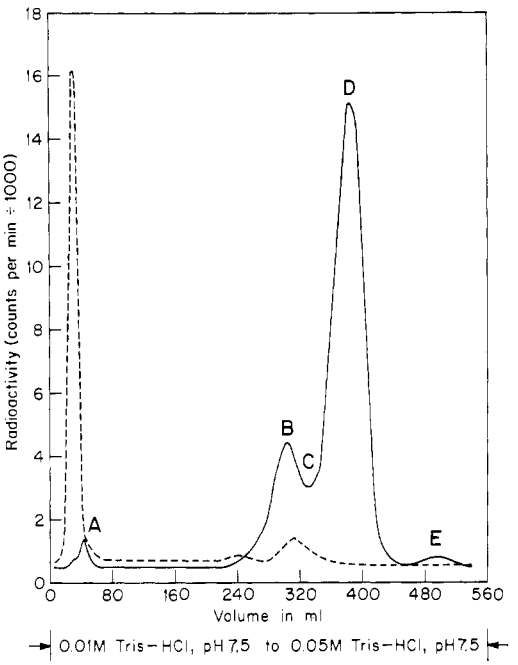


**Figure 3. DEAE-cellulose chromatogram of the urinary metabolites of $C^{14}$-labeled 3,4-dichlorobenzyl *N*-methylcarbamate on 1.5 × 24 cm. column**

——— Benzyl-$C^{14}$ label
– – – – *N*-methyl-$C^{14}$ label

Metabolites identified in text and Table I

**Table I. Urinary Metabolites of 3,4-Dichlorobenzyl-$C^{14}$ *N*-Methylcarbamate Excreted by the Rat[a]**

| | Metabolites[b] | Metabolites Expressed as % of Total[c] $C^{14}$ Recovered from Column I[d] | II |
|---|---|---|---|
| A | Neutrals | 2.1 | ... |
| B | 3,4-Dichlorobenzyl glucuronide and 3,4-dichlorobenzoyl glucuronide | 16.3[e] | 12.3 |
| C | 3,4-Dichlorohippuric acid | | 10.0 |
| D | 3,4-Dichlorohippuric acid | 63.0 | 78.0 |
| E | 3,4-Dichlorobenzoic acid | 6.2 | ... |

[a] All urines examined were pooled collections made from 4 to 10 animals, 24 hours after an oral dose of 100 mg. per kg.
[b] Listed in order of elution.
[c] DEAE-cellulose column I. 1.5 × 24 cm.
II. 5.0 × 24 cm.
[d] 12% recovered as high background.
[e] Average value for B and C.

radioactivity. The remaining $C^{14}$ recovered (about 24%) chromatographed before or after these *N*-methyl-labeled metabolites as background radioactivity.

Figure 4 gives the results obtained with the benzyl-$C^{14}$ metabolites on the preparative DEAE-cellulose column. The chromatogram is similar to that obtained on the analytical column, except for the ratio of the peak height to width. Metabolite B appears to be separated to a greater extent from the intermediate area designated as metabolite C. Table I gives the approximate percentages of these metabolites as recovered from the column. Metabolites A and E were not included in this chromatogram. Metabolite E cochromatographed on the 1.5 × 24 cm. DEAE-cellulose column with 3,4-dichlorobenzoic-$C^{14}$ acid.

Figure 5 gives the results obtained when the isolated metabolites (DEAE prep column, Figure 4, B, C, and D) were chromatographed on a silica gel column. The majority of the radioactivity from these metabolites (B about 85%; C about 70%; D about 84%) chromatographed as a single broad peak similar to the glucuronides prepared by Knaak *et al.* (1967). Metabolite B displayed a radioactive component (about 10%) which chromatographed just prior to the main peak, while metabolite C displayed a component (about 8.5%) which chromatographed at the beginning of the acetonitrile-methanol gradient.

Figure 6 illustrates the chromatographic results obtained with metabolites B and C (major components off silica gel column) on DEAE-cellulose after incubation with β-glucuronidase. Metabolite B was hydrolyzed to yield a neutral aglycone (about 56%) and an acidic aglycone or unhydrolyzed conjugate (about 44%). Metabolite C yielded a neutral aglycone (about 2%) and an acidic aglycone or unhydrolyzed conjugate.

Figure 7 is a composite chromatogram which illustrates the results obtained on temperature programmed runs of metabolites B, C, and D after the proper and individual treatment of each. The second peak is the neutral aglycone (Figure 6). It chromatographed on a 5% SE-30 column with the retention time of 3,4-dichlorobenzyl alcohol. The third peak is the acetylated neutral aglycone which has the retention time on this column of 3,4-dichlorobenzyl acetate. The first and fourth peaks are methyl 3,4-dichlorobenzoate and methyl 3,4-dichlorohippurate, respectively. The fourth peak was obtained from metabolite C after β-glucuronidase treatment and methylation. This metabolite, when acetylated and methylated without β-glucuronidase treatment, gas chromatographed as the methyl esters of 3,4-dichlorobenzoic acid and 3,4-dichlorohippuric acid. Since these two materials are separated by the ion exchange system, the 3,4-dichlorobenzoic acid must have been produced by treatment of the sample. Metabolite D was extracted at pH 2 and methylated. When chromatographed on the 5% SE-30 column, it chromatographed as methyl 3,4-dichlorohippurate.

Metabolite(s) B after acetylation and methylation chromatographed on a 2% SE-30 column as two components as indicated in Figure 7. The two components chromatographed similarly to methyl phenyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate (Knaak *et al.*, 1967), and they are believed to be methyl 3,4-dichlorobenzyl-2,3,4-tri-*O*-

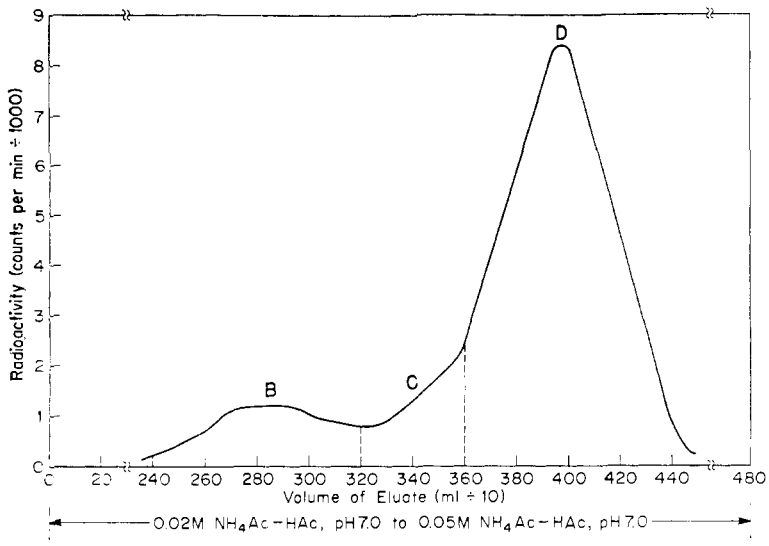


**Figure 4. DEAE-cellulose chromatogram of urinary metabolites of 3,4-dichlorobenzyl-$C^{14}$ *N*-methylcarbamate on 5.0 × 24 cm. column**

Metabolites identified in text and Table I

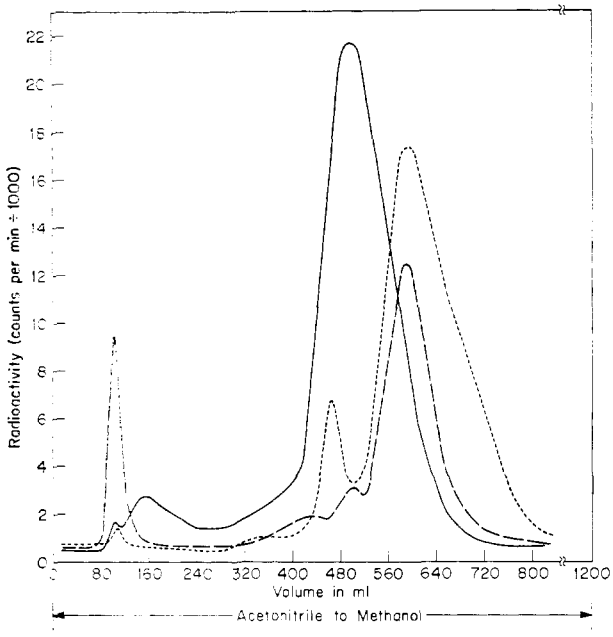


**Figure 5. Silica gel chromatogram of the metabolites of 3,4-dichlorobenzyl-$C^{14}$ *N*-methylcarbamate previously separated on 5.0 × 24 cm. DEAE-cellulose column**

– – – – – Metabolite B
— — Metabolite C
——— Metabolite D

acetyl-β-D-glucuronate and methyl 3,4-dichlorobenzoyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate. Treatment of metabolite(s) B with β-glucuronidase to produce 3,4-dichlorobenzyl alcohol and an acid further substantiated this assignment even though the acid upon extraction at pH 2 and methylation did not gas chromatograph.

Figure 8 gives the cochromatographic results obtained with nonlabeled 3,4-dichlorobenzyl acetate and the $C^{14}$ acetate of the neutral aglycone, derived from the enzymatic hydrolysis of B, on a 5% SE-30 column. To increase the

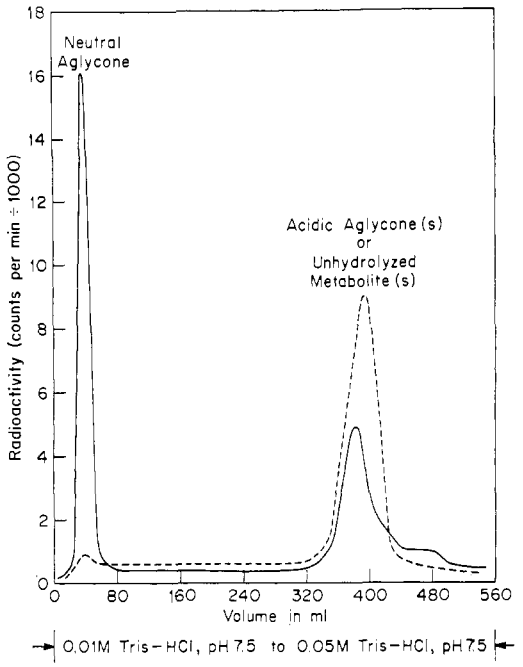


**Figure 6. DEAE-cellulose chromatogram of the aglycones obtained by the hydrolysis of metabolites B and C with β-glucuronidase preparation**

——— Metabolite B
– – – – Metabolite C
For conditions see text

probability that correct identification of the material had been made, the same sample was run on a 2% Carbowax 20-M column. Again, the neutral aglycone as its acetate cochromatographed on both polar and nonpolar columns.

Figure 9 shows the cochromatographic results obtained for a sample of nonlabeled methyl 3,4-dichlorohippurate and the methyl ester of metabolite D. Identical results were obtained on a 0.5% Carbowax 20-M column.

## DISCUSSION

3,4-Dichlorobenzyl *N*-methylcarbamate is absorbed from the gastrointestinal tract, metabolized, and excreted in urine and in respiratory $CO_2$ over a 4-day period. The benzyl label was excreted in urine (about 94% of dose), while the excreted *N*-methyl label was distributed between urine (about 31% of dose) and respiratory $CO_2$ (about 46% of dose). Small quantities of the *N*-methyl label (about 7% of dose) were found in the animal 7 days after oral administration, while no residues of the benzyl label could be found.

The proposed pathway for the metabolism of 3,4-dichlorobenzyl *N*-methylcarbamate is illustrated by Figure 10 and summarized in Table I. 3,4-Dichlorobenzyl *N*-methylcarbamate is metabolized and excreted as neutrals (about 2%), 3,4-dichlorobenzyl glucuronide (about 5%), 3,4-dichlorohippuric acid (about 63%), 3,4-dichlorobenzoic acid (about 6%), and a metabolite tentatively identified as 3,4-dichlorobenzoyl glucuronide (about 5%). No metabolites were isolated containing both labels. From Figure 3, peak B with the methyl label could be present as a conjugate in amounts not exceeding 3% of the dose. Any other intact carbamate conjugates would be present in substantially smaller percentages.

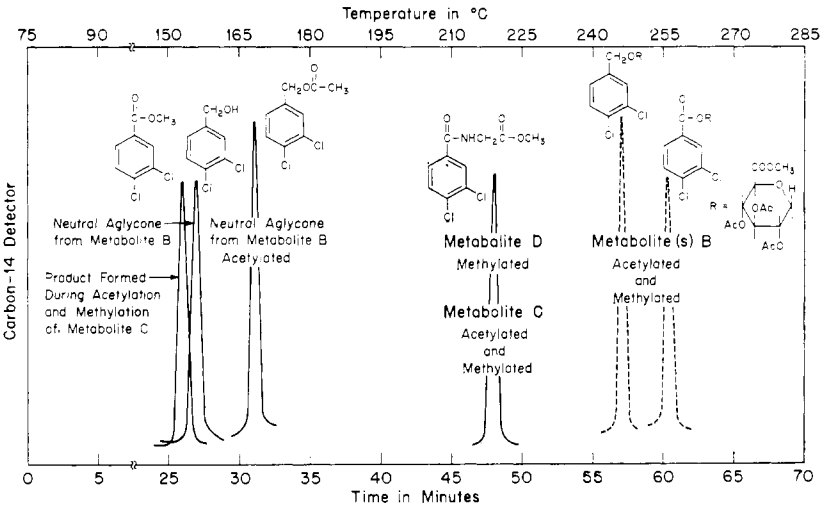


**Figure 7. Gas chromatogram of the urinary metabolites of 3,4-dichlorobenzyl-$C^{14}$ *N*-methylcarbamate**

Liquid phase ——5% SE-30
– – – – 2% SE-30
For conditions see text

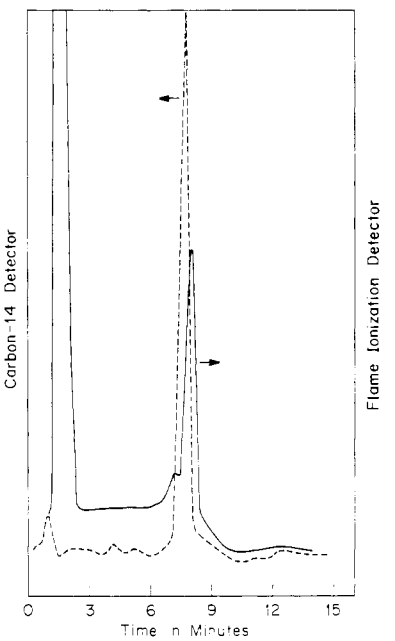


**Figure 8. Cochromatogram of the acetylated neutral aglycone from metabolite B and 3,4-dichlorobenzyl acetate on 5% SE-30 column**

Column temp. 160° C.
– – – – – $C^{14}$
——— Mass
For conditions see text

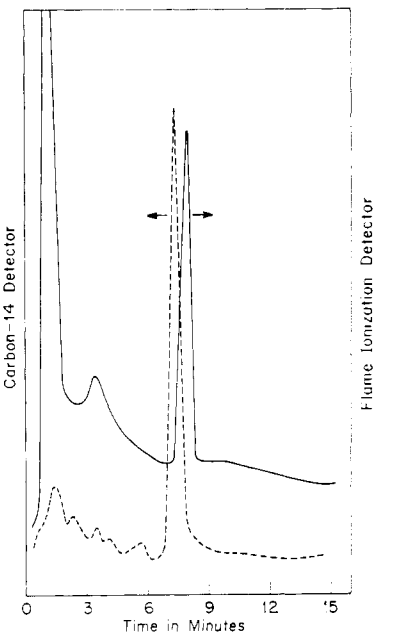


**Figure 9. Cochromatogram of the methyl ester of metabolite D and methyl 3,4-dichlorohippurate on 5% SE-30 column**

Column temp. 210° C.
– – – – $C^{14}$
——— Mass
For conditions see text

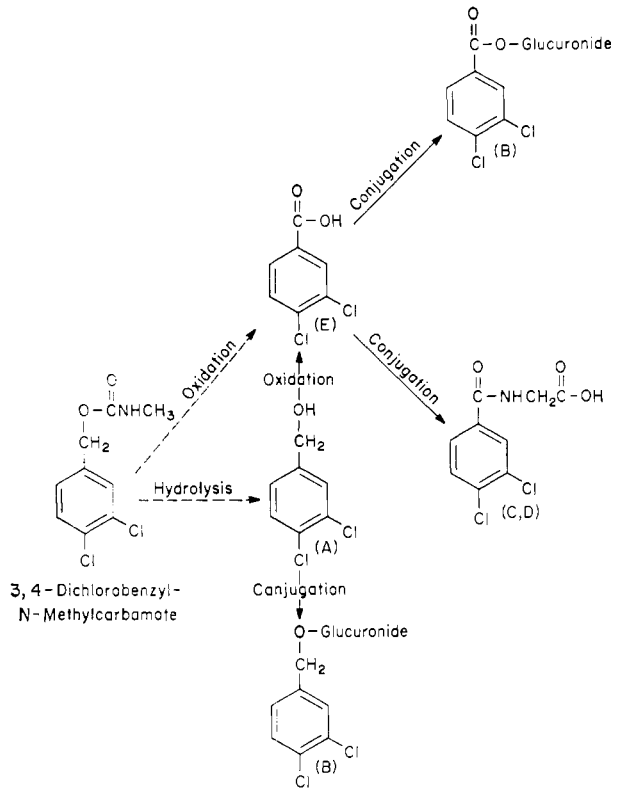


**Figure 10. Metabolic pathway for 3,4-dichlorobenzyl *N*-methylcarbamate in the rat**

Gas chromatography was used in combination with ion exchange and silica gel chromatography to identify and quantitate the metabolic products. The metabolites of the carbamate were incompletely resolved on the DEAE-cellulose column owing to small differences in their p$K$'s. 3,4-Dichlorohippuric acid could be readily separated from 3,4-dichlorobenzoic acid on a DEAE-cellulose column, while 3,4-dichlorohippuric acid could not be completely separated from the glucuronide conjugates. Metabolite B, suspected of being a glucuronide, was hydrolyzed to 3,4-dichlorobenzyl alcohol by a $\beta$-glucuronidase preparation and an acidic aglycone or unhydrolyzed conjugate. No evidence was obtained for methyl 3,4-dichlorobenzoate by gas chromatography of the acid after methylation. The $\beta$-glucuronidase preparation apparently had no effect on the acidic conjugate. Acetylation and methylation of metabolite B showed that this acid was most likely the ester glucuronide.

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