Enzymatic Degradation of 2-Chloro-4,5-dimethylphenyl *N*-Methylcarbamate by the Fat Bodies of *Blaberus giganteus*

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The mixed function oxidase character of an enzyme system responsible for the degradation of 2-chloro-4,5-dimethylphenyl *N*-methylcarbamate has been demonstrated using the fat body of the cockroach, *Blaberus giganteus*. Optimum conditions are reported for the microsomal fractions. Of at least three chloroform-soluble degradation products formed by the microsomal system, one has been tentatively identified as 2-chloro-4,5-dimethylphenyl *N*-hydroxymethylcarbamate.

Insect resistance to carbamate insecticides has led numerous researchers to study the causes. The findings by many workers that carbamate resistance could be reversed under the influence of methylenedioxy synergists, known inhibitors of several microsomal oxidative reactions, and the observations that *N*-methyl- and *N,N*-dimethylcarbamates are readily converted to several products of ring hydroxylation and/or epoxidation and *N*-methyl hydroxylation by insects have given strong support to the theory that rapid metabolism of carbamates results in the insect's ability to become resistant to them (*6, 7, 21*).

In vitro oxidative insect metabolism was suspected or demonstrated in the activation of schradan using desert locust fat body (*9*); in the hydroxylation of DDT using microsomes from German cockroaches (*1*); in the activation of azinphosmethyl using tissues obtained from American cockroaches (*15*), of methyl parathion using American cockroach fat body (*20*), and of parathion using American cockroach fat body microsomes (*16*); and in the demonstration of products of naphthalene hydroxylation and/or epoxidation when naphthalene was incubated with housefly and blow fly microsomes (*19*).

Despite these findings, little information exists as to the nature of the mixed function oxidase systems found in insects, especially those concerned with carbamate insecticide metabolism.

Materials

Adult *Blaberus giganteus*, reared at 26° C. and 55% relative humidity on Purina Laboratory Chow and water, were used for all the experiments.

2-Chloro-4-^{14}C, 5-dimethylphenyl *N*-methylcarbamate (4-methyl-^{14}C Banol, 4.76 mc. per mmole), *N*-methyl-^{14}C Banol (6.10 mc. per mmole), and carbonyl-^{14}C Banol (0.539 mc. per mmole) were obtained from the Upjohn Co., Kalamazoo, Mich. All samples assayed greater than 99% radiochemical purity.

Methods

Enzyme Preparation. Fat bodies of *B. giganteus* were removed from the adults (no care was exercised in trying to remove tracheols and Malphighian tubules), rinsed, and placed in ice-cold saline-buffer solution [0.05*M* sodium phosphate buffer (pH 7.30) containing 122 mmoles of NaCl, 10 mmoles of glucose, 3 mmoles of KCl, and 1.2 mmoles of $MgSO_4$ per liter of buffer] so that the final concentration was 1 fat body per 1.5 ml. (ca. 384 mg. of tissue per ml.). The fat bodies were homogenized in a Sorvall Omni-Mixer at 16,000 r.p.m. for 1 minute. Differential centrifugation was carried out using previously described procedures (*4*). Two modifications—i.e., the removal of the fat scum layer from the supernatant above the nuclear pellet and the centrifugation of the supernatant above the mitochondrial pellet at 122,500 G for 1 hour—were performed. The 122,500 G supernatant was decanted off and the microsomal pellet, loosely adhering to the clear gel pellet sedimenting out below it, was taken up in saline buffer. When an experiment called only for the use of microsomes, the original homogenate was centrifuged at 20,000 G for 10 minutes; the resulting supernatant, less the fat scum layer, was then centrifuged at 122,500 G for 1 hour. The above procedures were conducted at 0° to 2° C.

Enzyme assays were carried out in 25-ml. Erlenmeyer flasks. To each flask were added 0.28 μmole of Banol-^{14}C, 0.60 μmole of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), 0.65 μmole of reduced nicotinamide-adenine dinucleotide ($NADH_2$), the subcellular fraction equivalent to that obtained from one (only 4/10 of one in the case of the 122,500 G supernatant fraction) cockroach fat body, and sufficient saline buffer to bring the final volume up to 4 ml. The flasks were incubated for 1 hour at 35° C. and the reactions were stopped by acidification with 1*N* HCl followed by extraction with 10 ml. of acetonitrile. Following filtration, the procedure was repeated using 5 ml. of acetonitrile. The combined solutions were

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extracted with 15 ml. of chloroform and the organic layer was drawn off, dried with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. Previous experience had demonstrated that no significant differences resulted in determining the ratio between the products formed and the unreacted Banol recovered when this procedure was followed. The organic residue was taken up in 10 ml. of acetonitrile, filtered, and concentrated to a volume suitable for spotting (ca. 0.4 ml.) paper or thin-layer chromatographs. The extracts were lined on either Whatman No. 3 filter paper pretreated with a 5% acetone solution of Dow Corning 550 Fluid or 0.3-mm. silica gel G plates.

Paper chromatographs were developed ascendingly in a 4 to 1 water-acetonitrile system while the plates were developed twice in a 4 to 1 diethyl ether–*n*-hexane system. The separated radioactive products were located on a Vanguard Autoscanner, either cut or scraped off and eluted with scintillation solution (15 ml. of 0.3% w./v. 2,5-diphenyl-oxazole in toluene) and counted on a Packard Tri-Carb Semi-Automatic scintillation counter.

Product Characterization. The R_f values of the radiolabeled products were compared with those of suspected degradation products—i.e., 2-chloro-4,5-dimethylphenol, 2-chloro-4,5-dimethylphenyl *N*-hydroxy-*N*-methylcarbamate (*N*-hydroxy Banol), and 2-chloro-4,5-dimethylphenyl carbamate (*N*-demethylated Banol). The compounds were located by various color reagents—i.e., iodine vapor for all compounds, 1% $FeCl_3$ followed by 1% $K_3Fe(CN)_6$ and overspraying with glacial acetic acid for phenolic compounds, 0.2% ninhydrin plus 0.1% collidine in 1-propanol for Banol and *N*-demethylated Banol, and 1% $FeCl_3$ in 95% ethanol containing 0.1% HCl for *N*-hydroxy Banol; the radioactive compounds were located by scanning.

In a 7:2:1 2-propanol–water–NH_4OH developing system, Banol was readily hydrolyzed to the parent phenol and *N*-methylurea. The Banol degradation products as well as radiolabeled Banol were chromatographed in this system on Whatman No. 1 filter paper. Phenol, resorcinol, and phloroglucinol were also chromatographed in this system to try and reveal the behavior of multiple ring hydroxylation on migration. The radioactive compounds were located on the Autoscanner, while the other phenolic compounds were located by the $FeCl_3$–$K_3Fe(CN)_6$–acetic acid reagents.

N-methyl-^{14}C Banol and its degradation products were cut from paper chromatographs and placed in separate round-bottomed flasks. To each flask were added 20 ml. of 1*N* H_2SO_4 and 0.0013 ml. of 37% formaldehyde solution. The flask contents were distilled until 12 ml. of distillate were collected. A saturated solution of 5,5-dimethyl-1,3-cyclohexanedione (130 ml.) was added to the formaldehyde distillate. The contents were shaken and then held for 12 hours and the crystals harvested by vacuum filtration. Recrystallization from 50% ethanol was performed. The crystals were taken up in scintillation solution and counted to determine the presence of the radiolabeled methone derivative of formaldehyde.

Results and Discussion

Enzyme Studies. Earlier unpublished experiments had demonstrated that incubation of whole *B. giganteus* fat body with or without $NADPH_2$ in the presence of Banol-^{14}C always resulted in the formation of at least two radiolabeled, organosoluble products when assayed on paper chromatography (PC) and at least three when assayed on thin-layer chromatography (TLC). Despite the position of the ^{14}C- marker on Banol, the same number of degradation products cited above were observed at identical R_f values. Also despite the position of the ^{14}C -marker, the relative amount of each product formed, at an identical R_f value, was the same. The organosoluble metabolites found after various time intervals—i.e., up to 4 hours—after the injection of Banol-^{14}C into *B. giganteus* appear at R_f values identical to those found using the fat body preparations. No additional organosoluble metabolites were observed in these earlier in vivo experiments but over 80% of the metabolized products appeared in the water-soluble phase after 4 hours. At least two water-soluble products were formed (R_f values of 0.59 and 0.86 on paper chromatographs developed in a 250:60:250 butanol–acetic acid–water system); however, no attempt was made to study the enzymes involved in their formation or the chemical nature of these products.

Results of a number of in vitro experiments are reported in Table I. Over 93% of the radioactivity could be recovered from the reaction vessel; however, recoveries of between 35 and 75% were recorded following removal of the labeled areas from the plates. As no appreciable differences could be detected between the relative amount of each product formed in comparison with the undegraded Banol or between each product within this percentage range, it was assumed that the losses due to volatility were insignificant. The relative enzyme activity (column 5, Table I) was defined as the mean quotient of the counts per minute (c.p.m.) obtained from the organosoluble products to the total counts per minute found in the organosoluble phase adjusted such that the standard incubation mixture (SIM, test 6) had a value of 1000. This was equivalent to the degradation of 0.7% of the carbonyl-^{14}C Banol. In contrast with the in vivo experiment, only 12% of the product radioactivity could be recovered in the aqueous phase. The radiolabeled products and the undegraded carbonyl-^{14}C Banol (used exclusively in these experiments) were scraped off thin-layer plates for all of these analyses. Significance was determined by the Wilcoxon rank sum test (*11*) and applied where applicable.

The microsomal fraction was predominantly responsible for the degradation observed (at least 3.8 to 10.4 times more active than each of the remaining subcellular fractions) and its activity even exceeded that of the whole homogenate. The relative activity differential might have been greater if there were a zero time control for each of the subcellular assays, as there was for the microsomal assays. The number of organosoluble products and their respective R_f values were identical to those found using whole fat body preparations. This could not be demonstrated for all of the

Table I. Degradation of Carbonyl-^{14}C Banol Using Various Incubation Preparations

Test No.	Deletions from SIM[a]	Additions to SIM[a]	Replicates	Relative Enzyme Activity[b]
1	Microsomes	None	3	96
2	Microsomes	Nuclei	3	167
3	Microsomes	Mitochondria	3	257
4	Microsomes	122,500 G supernatant	3	280[c]
5	Microsomes	Whole homogenate	3	645
6	None	None	18	1000
7	60-min. incubation	0-min. incubation	3	270
8	60-min. incubation	120-min. incubation	3	838
9	60-min. incubation	240-min. incubation	3	1047
10	$NADH_2$	None	5	660
11	$NADPH_2$	None	5	182
12	$NADPH_2$ and $NADH_2$	None	3	150
13	$MgSO_4$	1.2 μmoles $FeSO_4$	3	529
14	$MgSO_4$	None	3	1190
15	None	0.375 μmole sesamex	3	379
16	None	0.375 μmole piperonyl butoxide	3	431
17	Air atmosphere	Nitrogen atmosphere	3	469
18	pH 7.30	pH 6.20	3	790
19	pH 7.30	pH 6.90	3	910
20	pH 7.30	pH 7.75	3	973
21	35° C. incubation	25° C. incubation	3	607
22	35° C. incubation	30° C. incubation	3	701
23	35° C. incubation	40° C. incubation	3	638

[a] Standard incubation mixture equivalent to 0.60 μmole of $NADPH_2$, 0.65 μmole of $NADH_2$, 60 μg. of carbonyl-^{14}C Banol, 4 ml. of pH 7.30 saline buffer (see text), and the microsomal equivalent of one cockroach fat body incubated at 35° C. for 60 min.
[b] For explanation see text.
[c] Value readjusted to equal one cockroach fat body.

assays, because of the lack of the sensitivity of the Autoscanner or of the product formation. The high level of microsomal activity in comparison to the other subcellular fractions has been reported for cockroach fat body schradan oxidase activity (*9*) and parathion-activating activity (*16*). Significant amounts of hydroxylating activity found in the nuclear and mitochondrial fractions of German cockroach preparations when DDT was used as a substrate (*1*) were not present in these fat body preparations when carbonyl-^{14}C Banol was used as a substrate.

The requirement for oxygen by the reaction was demonstrated by the fact that a 74% reduction in the production of degradation products occurred when the reaction was carried out in a nitrogen atmosphere. Observations of the thin-layer scans of these plates revealed that the amount of each product formed in the nitrogen atmosphere was in direct proportion to the amount formed when air was used as the reaction atmosphere. The fact that the 74% reduction was low in contrast to the reports of others (*1*, *8*, *15*) was, no doubt, attributable to the incomplete removal of oxygen from the reaction vessel.

The requirement for $NADPH_2$ is demonstrated when tests 10 and 11 are compared. Also demonstrated was the fact that $NADH_2$ contributed nothing to the reaction by itself. However, in conjunction with subminimal concentrations of $NADPH_2$ (test 6), an enhancement of activity was observed. Spectrophotometric measurements at 340 mμ indicated that an excess of $NADPH_2$ was present in the reaction vessel after 1 hour of incubation time; therefore, it must be concluded that it is unavailable for the reaction. $NADH_2$ presumably acted as a $NADPH_2$ mimic for competing receptors (probably proteins), thus appearing to potentiate the reaction. This would tend to explain the various reports that nicotinamide and/or $NADH_2$ enhanced the metabolic activity in certain systems yet $NADPH_2$ alone in other preparations was sufficient for complete activity (*4*, *9*, *19*).

The lack of any stimulation produced by the Mg^{+2} ions and the inhibition obtained by the use of Fe^{+2} ions cannot be accounted for, other than to point up the apparent dissimilarities of various oxidase systems. Enchancement of DDT hydroxylation activity has been reported when Mg^{+2} and Fe^{+2} ions were added to microsomal preparations of blow flies and German cockroaches (*1*, *19*). Activation of certain phosphorus-containing insecticides has not shown any requirement for either ion (*9*, *15*, *16*). The report that sesamex and piperonyl butoxide were both effective inhibitors of *N*-methyl hydroxylation reactions (*12*) and that several methylenedioxy synergists were inhibitors of the hydroxylation of naphthalene (*19*) points to the fact that the microsomal fat body enzyme(s) responsible for the oxidative degradation of Banol was characteristic

of mixed function oxidases when it was observed that sesamex and piperonyl butoxide inhibited the reaction 85 and 80%, respectively.

The rapid loss of enzyme activity as demonstrated by the lack of any appreciable build-up of products (the addition of $NADPH_2$ after 1 hour did not restore activity) after 2 and 4 hours and the inability to hold enzyme preparations at $-20°$ C. even in association with the $NADPH_2$ cofactor and the Banol substrate (90% loss in activity after 2 days) was in agreement with the finding that 96% of the insect microsomal naphthalene hydroxylating activity was lost in 8 days (*2*). Whether the loss in enzyme activity was due to the loss of the reduced state of the suspected metalloenzyme (*5*) or to an inhibitor released by the microsomal preparation (*3*) was not investigated.

The enzyme system demonstrated a characteristic maximum of activity around 35° C. when preparations were incubated at 5° C. intervals from 25° to 40° C. The observation that the LD_{50} of carbaryl to grasshoppers increased about 12-fold with a rise in temperature from 12° to 32° C., whereas the increase was only 3- to 6-fold when piperonyl butoxide was used as a synergist, supports this indirectly. Significance tests did not reveal a pH optimum between a pH range of 6.20 to 7.75 but one was suspected between pH 7.30 and 7.75.

Product Characterization. Reverse phase PC and TLC of the incubation extracts indicated presence of two (R_f 0.78 and 0.88) and three (R_f 0.09, 0.21, and 0.30) radiolabeled degradation products, respectively, despite the position of the radiolabel on Banol. The R_f 0.09 product contained 5% of the product radioactivity, the 0.21 product 36%, and the 0.30 product 59%. Evidence presented below indicates that the R_f 0.88 product on PC forms two products, R_f 0.09 and 0.21, on TLC. Banol-^{14}C had R_f values of 0.40 on PC and 0.68 on TLC. When the compounds were eluted and chromatographed on the opposite system, chromatographic patterns were identical to those cited above, except that no product could be detected at R_f 0.09 on TLC.

Comparison of the product R_f values to the known chromatographic behavior of 2-chloro-4,5-dimethylphenol, *N*-hydroxy Banol, and *N*-demethylated Banol indicated that none of these compounds was a detectable degradation product of Banol in the microsomal incubation preparations. Three oxidative degradation products of Banol have been demonstrated using rat liver microsome preparations along with substantial amounts of the parent phenol (*17*). This latter observation could not be duplicated using the cockroach system.

When the 4-methyl-^{14}C Banol product(s) found at R_f 0.88 was chromatographed in the 2-propanol–water–ammonia system most of the radioactivity was found at R_f 0.68 with a small amount located at 0.89. When *N*-methyl-^{14}C Banol was chromatographed in this system, one radiolabeled area was found at R_f 0.72. When the R_f 0.78 product was eluted and chromatographed in this system, the 4-methyl-^{14}C product produced a radio-labeled area at R_f 0.87 while the *N*-methyl-^{14}C product produced a radiolabeled area at R_f 0.70 and a shoulder at 0.72. As this system readily hydrolyzed Banol into its parent phenol (R_f 0.90) and *N*-methylurea (R_f 0.74), there was strong evidence that the phenolic portion of Banol had been modified in the degradation product(s) found at R_f 0.88 on PC. Despite its migration characteristics, which were similar to those encountered when polyhydroxylated aromatic rings were chromatographed, little else is known about it. Research results regarding the metabolism of Banol when applied to bean seedlings have demonstrated one major metabolite, a sugar conjugate of either a phenolic or benzylic character. The basic structure (ring and carbamate side chain) remained intact (*10*). The data concerning the hydrolysis of the product found at R_f 0.78 on PC indicated that this compound had an intact ring but a modified side chain. The radiolabeled products formed upon hydrolytic attack with ammonium hydroxide of this R_f 0.78 product are unknown (possibly *N*-methylamineurea and/or *N*-hydroxymethylurea), but the indication of at least two products using the *N*-methyl-^{14}C material pointed to the presence of a compound different from *N*-methylurea.

When *N*-methyl-^{14}C Banol and its degradation products were subjected to acidification with the eventual formation of a methone derivative of formaldehyde, about 400 mg. of derivative were obtained from each reaction. The results listed in Table II clearly demonstrated the presence of radioactive methone, pointing to the strong possibility that one of the products contained an *N*-hydroxymethyl function as *N*-methylol functions are readily cleaved in acid with the liberation of formaldehyde.

Little is known about the degradation product which appeared at R_f 0.09 on TLC. Upon heating the incubation mixtures containing 4-methyl-^{14}C Banol prior to extraction, it was observed that all of the unreacted Banol had disappeared along with over 85% of the radioactivity found at R_f 0.88 on PC. Prolonged heating did not destroy the remainder of this product, while the product found at R_f 0.78 on PC remained unchanged in amount. No radiolabeled phenol was present. The resistance of the degradation product found at R_f 0.78 and the remaining minor product at

Table II. Recovery of Radiolabeled Formaldehyde

No. of Recrystallizations	Weight of Crystals Counted, Mg.	Counts per Min.	Specific Activity, C.P.M./Mg.
0B[a]	45.7	304	5.85
3B[a]	90.0	62	0.28
4B[a]	62.2	47	0.16
0P[b]	44.1	1571	35.02
3P[b]	105.0	3084	25.02
4P[b]	73.0	2416	32.60

[a] Number preceding letter indicates number of recrystallizations—e.g., 0 refers to crystals initially obtained, 3 refers to crystals obtained after three recrystallizations. B refers to area removed from paper chromatographs representing undegraded radiolabeled Banol.
[b] P refers to area removed from paper chromatographs representing radiolabeled Banol degradation products.

R_f 0.88 to cleavage and/or steam volatility cannot be explained but points to the similarity in behavior between the two. As the R_f 0.78 product contains an *N*-hydroxymethyl function, it is suspected that the minor product at R_f 0.88 (believed to be 0.09 on TLC) might also contain this same functional group. The more polar character of the minor product as evidenced by its migration on thin-layer plates would suggest the existence of still another modification, presumably on the phenolic portion of the molecule.

The results of this study indicated the formation of *N*-hydroxymethyl Banol, a compound showing a modification of the phenolic portion of Banol, and a compound demonstrating both a modification of the phenolic portion and the carbamate side chain. A number of authors have indicated the possibility of these types of modifications occurring using different oxidative systems (*6, 7, 12, 17*). *N*-Hydroxy Banol has been demonstrated using rat liver microsome preparations but no information is afforded as to the nature of the two remaining metabolites (*18*). Though none of these authors demonstrated a metabolite displaying both ring and side chain modifications concurrently, the observation that *N,N*-dimethyl-2,2-diphenylacetamide was hydroxylated on the *N*-methyl position followed by either conjugation or loss of the hydroxymethyl group with the eventual hydroxylation of the aromatic ring (*14*) implies that such a compound might exist if the *N*-methylol function were stable as it appears to be in many carbamate insecticides.

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