Reports

Nonhydrolytic Pathway in Metabolism of N-Methylcarbamate Insecticides

Abstract. *Sevin (1-naphthyl N-methylcarbamate), when metabolized by rat liver microsomes and insects, yielded at least five carbamate metabolites involving modification of both the methyl group and ring. Certain of these metabolites appeared in milk when Sevin was fed to a goat. The metabolism of* o-iso*propoxyphenyl* N-*methylcarbamate by liver microsomes, insects, and plants was compared to that of Sevin.*

N,N-Dimethylcarbamates and particularly *N*-methylcarbamates are becoming increasingly important in insect control. It has been assumed that metabolism of the *N*-methylcarbamate inhibitors of cholinesterase generally involves initial esterase attack followed by degradation of the hydrolyzed fragments (for review, see *1*). However, the in vivo destruction of these insecticides proceeds more rapidly than can be explained on the basis of the esterases that have been found to effect their hydrolysis. Enzymes in rat liver microsomes, when fortified with reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), degrade *N,N*-dimethylcarbamate insecticides by oxidative attack on the methyl group (*2*). With the extensive use of Sevin it appeared important to re-examine the hypothesis that hydrolysis was the mechanism of its biological degradation in mammals, insects, and plants.

Three samples of C^{14} Sevin were prepared with labeling at different sites on the molecule. These included the *N*-methyl and carbonyl groups (*3*) and the 1-position of the naphthyl ring (*4*). Sevin was relatively stable in rat liver homogenates, unless these homogenates were fortified with $NADPH_2$ or the oxidized form of this cofactor. Tissue fractionation and cofactor addition experiments revealed that the microsomes, $NADPH_2$ and an oxygen-containing atmosphere were necessary for rapid loss of Sevin. Two chromatographic systems were developed to examine the nature of the metabolites. With chromatography on Florisil columns (*3*), Sevin was eluted with a 1 : 1 hexane-ether mixture, a first metabolite (I) with 1 : 3 hexane-ether, a second (II) with ether, and a third (III) with methanol. The three labeled samples of substrate gave similar results, with approximately 70 percent of the radioactivity recovered as Sevin, 15 percent as metabolite I, 9 percent as metabolite II, and 6 percent as metabolite III. Thin-layer chromatography on silica gel with mixtures of hexane and ether revealed that metabolite I from Florisil consisted of two major and one very minor component, while metabolite II from Florisil appeared to be a single compound. The nature and homogeneity of the metabolite (III) peak eluting from Florisil with methanol have not been ascertained. At least five metabolites with the C-O-C(O)-N-C skeleton intact were formed from Sevin, since these products were detected when each of the labeled samples of Sevin was used in separate but identical experiments. Degradation studies revealed modification of both the ring and methyl group within this series of carbamate metabolites. None of these metabolites were 1-naphthyl carbamate, 1-naphthyl *N*-hydroxycarbamate, or 1-naphthyl *N*-methyl, *N*-hydroxycarbamate, although one of the components of metabolite I decomposed under certain conditions to yield 1-naphthyl carbamate. In addition, 1-naphthol and two other metabolites lacking the carbonyl and methyl group were formed with naphthyl-1-C^{14}-Sevin.

Sevin fed to cows at up to 450 parts per million (ppm) of the dry matter intake yielded less than 0.01 ppm of Sevin residues in butterfat analyzed colorimetrically for both free and combined 1-naphthol (*5*). Carbonyl-C^{14}-Sevin (specific activity 1 mc/mmole) was administered orally to a lactating goat in a single dose of 1.3 mg/kg. Milk samples taken up to 96 hours after treatment were analyzed by extraction with acetonitrile and chloroform (*6*) and chromatography on Florisil. The following peak levels at times after treatment were found upon analysis of the samples: Sevin, 0.004 at 2 hours; metabolite I, always less than 0.001; metabolite II, 0.075 at 2 hours; a radiolabeled material eluting from the Florisil column with hexane-ether (1 : 1) slightly before Sevin, and not detected as a metabolite in other biological systems, 0.027 at 16 hours; and metabolite III eluted with methanol, 0.224 at 4 hours. This analytical procedure gave high percentage recovery of all metabolites except those eluted with methanol. Much of the radioactivity was not extractable from the milk and may have been similar chemically to the methanol (metabolite III) fraction. The level of total Sevin-C^{14}-equivalents appearing as metabolites in milk reached a peak of 0.93 ppm at 8 hours and diminished progressively to below 0.003 ppm by 60 hours. These data may partially explain the lack of Sevin residue detection in milk by colorimetric procedures with *p*-nitrobenzenediazonium fluoborate since: (i) the amounts of Sevin per se are too small to be detected by such procedures, (ii) the metabolite I fraction which yields 1-naphthol upon hydrolysis was not present in milk, (iii) the metabolite II fraction which was present in milk yields a phenolic material different than 1-naphthol on hydrolysis which fails to respond to the colorimetric analysis used for Sevin residue detection, and (iv) the major fraction of the further metabolites was not extractable into organic solvents. Analysis of urine from this goat gave results similar to those obtained with the milk. This indicates that analysis of urine for free and conjugated 1-naphthol, as for determination of occupational exposure (*7*), may detect only a portion of the metabolites excreted by this route.

Houseflies (*Musca domestica* L.) and cockroaches [*Periplaneta americana* (L.)] treated with each labeled Sevin sample yielded the same series of metabolites recovered from Florisil columns as with rat liver microsomes. Analysis of the cockroaches 4 hours after treatment with the carbonyl-C^{14} mate-

rial showed that Sevin constituted 20 percent of the injected radioactivity; metabolite I, 11 percent; metabolite II, 3 percent; and metabolite III, 4 percent. The remainder of the radioactivity appeared in the residue after acetone extraction and as expired carbon-14-O_2. The metabolite I fraction from roaches was further resolved into three components in the same manner as with this fraction from microsomes. Bean and cotton plants injected through the stem with Sevin slowly converted it to a metabolite(s) eluting from the Florisil column with methanol. This metabolite(s) accounted for about 90 percent of the labeled material remaining in the plant 28 days after treatment. There was no indication of metabolites I and II as detected with animals, or large loss of carbon-14 from the plants over a 28-day period.

A second carbamate insecticide, *o-iso*propoxyphenyl *N*-methylcarbamate, was subjected to the same studies with rat liver microsomes, insects, and plants utilizing carbonyl-C^{14} compound. The microsomes and insects yielded metabolites eluting from Florisil in a manner similar to metabolite I and metabolite III of Sevin. A microsome preparation yielded 67 percent of the carbamate precursor, 30 percent metabolite I, and 3 percent of the methanol fraction metabolite. Cockroaches after 4 hours contained 3 percent of the injected dose as the original carbamate, 12 percent as metabolite I, and 7 percent as the metabolite of the methanol fraction, and the remainder of the radioactivity was present in the residue or liberated as $C^{14}O_2$. As with Sevin, this metabolite I peak was resolved into two components by thin-layer chromatography. The metabolite I peak from the Florisil column was further purified on a celite-acetonitrile-hexane column to yield a material with an infrared spectrum nearly identical to that of *o-iso*propoxyphenyl *N*-methylcarbamate except for additional bands at 1030 and 3400 cm^{-1} which might be associated with a primary alcohol group. Degradation of this metabolite fraction yielded formaldehyde and *o-iso*propoxyphenol. Reaction of *o-iso*propoxyphenyl carbamate with formaldehyde in glacial acetic acid produced a 2-percent yield of a material identical to this metabolite fraction in chromatographic characteristics on Florisil and infrared spectrum. These observations suggest, but do not necessarily establish, that the major component of this metabolite I fraction was *o-iso*propoxyphenyl *N*-hydroxymethylcarbamate. The behavior of *o-iso*propoxyphenyl *N*-methylcarbamate in plants was similar to that of Sevin.

Certain carbamate metabolites from microsomes were separated on Florisil columns and the fractions eluted with hexane-ether and ether were assayed for anticholinesterase activity in vitro with homogenates of housefly heads. The metabolite I and II fractions of Sevin and the metabolite I fraction of the *o-iso*propoxyphenyl compound effected cholinesterase inhibition, although in each case these metabolite fractions were less than one-eighth as potent as their N-methylcarbamate precursors.

The mechanism of selective insecticidal activity, acquired resistance to carbamate insecticides, and synergism of insecticidal activity by methylenedioxyphenyl and other compounds appears to be at least partially related to the biological instability of these carbamates based on studies by many different investigators (for reviews see *1* and *8*). This instability may result from oxidative attack on the *N*-methyl group or the ring by enzymes in the microsomes (*9*).

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References and Notes

1. J. E. Casida, *Ann. Rev. Entomol.* **8**, 39 (1963).
2. E. Hodgson and J. E. Casida, *Biochim. Biophys. Acta* **42**, 184 (1960); *J. Biochem. Pharmacol.* **8**, 179 (1961).
3. J. G. Krishna, H. W. Dorough, J. E. Casida, *J. Agr. Food Chem.* **10**, 462 (1962).
4. W. J. Skraba and F. G. Young, *ibid.* **7**, 612 (1959).
5. G. G. Gyrisco, D. J. Lisk, S. N. Fertig, E. W. Huddleston, F. H. Fox, R. F. Holland, G. W. Trimberger, *ibid.* **8**, 409 (1960).
6. J. A. Timmerman, Jr., H. W. Dorough, J. R. Buttram, B. W. Arthur, *J. Econ. Entomol.* **54**, 441 (1961).
7. E. M. Best, Jr., and B. L. Murray, *J. Occupational Med.* **4**, 507 (1962); C. P. Carpenter, C. S. Weil, P. E. Palm, M. M. Woodside, J. H. Nair, III, H. F. Smyth, Jr., *J. Agr. Food Chem.* **9**, 30 (1961).
8. R. L. Metcalf, *Pest Control* **30**, (6), 20 (1962).
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Homologous Disease Reactivation by X-radiation

Abstract. *Hybrid* F_1 *mice* (BDF$_1$) *that failed to develop homologous disease after injection of parental (C57 Bl/6) spleen cells were treated with 400 roentgens of total body x-radiation 13 months later. Typical homologous disease promptly developed in these mice. This suggests that parental lymphoid cells may acquire immunologic tolerance to "foreign" host antigens and that the tolerant state is disrupted by sublethal doses of radiation.*

The administration of parental lymphoid cells to weanling F_1 hybrid mice results in a lethal disorder characterized by weight loss, dermatitis, diarrhea, hemolytic anemia with a positive antiglobulin test, leukopenia, thrombocytopenia, hyperglobulinemia, and splenomegaly (*1*). Homologous disease is due to an immunologic reaction of the grafted parental cells against antigens present in the host hybrid and hence has also been called the graft versus host reaction. In most parent-hybrid combinations the mortality rate due to the graft versus host reaction is high, and in many it is usually 100 percent.

In our laboratory the combination C57Bl/6→ (C57Bl/6 × DBA$_2$) F_1 usually kills 60 to 65 percent of recipients when 300 to 400 × 10^6 parental spleen cells are injected intraperitoneally into weanling F_1 hybrids. Why 35 to 40 percent of the recipients fail to develop homologous disease is not understood; "exhaustive sensitization" with the ultimate death of the parental cells has been proposed as a possible mechanism (*2*).

We recently had an opportunity to study this phenomenon in our laboratory. Seven BDF$_1$ mice that had been given approximately 300 × 10^6 C57Bl/6 spleen cells failed to develop homologous disease; they were observed for a period of 13 months. They were apparently healthy in every respect. At the end of this period of observation they were treated with 400 r of total body x-radiation. Within 2 weeks four of these mice had died of typical homologous disease. In each case there was weight loss, severe anemia, a strongly positive antiglobulin test, marked leukopenia, and splenomegaly. Two additional mice developed transiently positive antiglobulin tests, and slowly regained their preradiation