The Metabolism of 3,5-Di-*tert.*-Butylphenyl *N*-Methylcarbamate in Insects and by Mouse Liver Enzymes

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The oxidation of 3,5-di-*tert.*-butylphenyl *N*-methylcarbamate (Butacarb) has been studied in the flies *Musca domestica* and *Lucilia sericata*, grass grubs *Costelytra zealandica* and the mouse. In all species eleven oxidation products, which were formed by hydroxylation of the *tert.*-butyl groups and the *N*-methyl group, were detected.

Butacarb, 3,5-di-*tert.*-butylphenyl *N*-methylcarbamate, is an insecticide of particularly low mammalian toxicity but is extremely toxic to larvae of the sheep strike blowfly *Lucilia sericata* (Fraser, Greenwood, Harrison & Wells, 1967). It has, for an insecticide of this type, a long persistence on fleece and seemed a possible alternative to dieldrin or the more poisonous organophosphates in sheep dips. No information was available on its metabolism in vertebrates or insects, but it was of particular interest to us since its properties appeared to support the hypothesis put forward by Hook & Smith (1967) that the presence of alkyl substituents in the aromatic ring of an insecticide should be expected to induce a low vertebrate toxicity. It was also of interest since we had examined the comparative metabolism in several species of insects of a closely related compound, *m*-*tert.*-butylphenyl *N*-methylcarbamate, and had found a general inverse relationship between the rate of oxidation and toxicity (Douch & Smith, 1971).

We have therefore tried to assess the extent to which Butacarb is oxidized in insects and the mouse. Since the number and variety of different possible metabolites and conjugates is large, we have tried to assess only the different types of oxidation products and to compare the extent of this total oxidation with the toxicity of Butacarb in the different species.

MATERIALS AND METHODS

Compounds. 3,5-Di-*tert.*-butylphenyl *N*-methylcarbamate, m.p. 102°C, was prepared from 3,5-di-*tert.*-butylphenol and methyl isocyanate in ether (Fraser *et al.* 1967; Metcalf & Fukuto, 1965). A sample of [³H]Butacarb was that prepared by Douch & Smith (1971) and had a specific radioactivity of 0.1 Ci/mol. Treatment with 2M-NaOH

for 6 h at 60°C followed by acidification and extraction with ether gave a 99.7% recovery of the radioactivity, and it was concluded that the ³H was present in the aromatic ring and not labile in the aqueous solution.

Enzymes. Enzymes from mouse liver and flies were prepared as described previously (Douch & Smith, 1971; Jordan & Smith, 1970).

Insects. These were kept as described in the preceding paper (Douch & Smith, 1971) and dosed topically on the thorax with the [³H]Butacarb in 0.15 μl of acetone with a calibrated capillary tube. Insects were kept for up to 12 h before extraction as described in the preceding paper (Douch & Smith, 1971). In experiments with radioactive compounds recovery of dosed radioactivity was in the range 98-100%.

Chromatography and ionophoresis. These were carried out as described previously (Douch & Smith, 1971; Jordan, McNaught & Smith, 1970). Chromatography on Whatman no. SG81 paper was used in solvents *A* (ether-iso-octane, 5:1, v/v), *B* (toluene-ethyl formate-formic acid, 5:7:1, by vol.) and *C* (ether). Ionophoresis was normally carried out at 4000 V for 20 min (Table 1).

Colour reactions. Reactions used by Douch & Smith (1971) were employed to detect phenols, phenol esters, carbamates, *N*-substituted carbamates and *N*-hydroxymethyl derivatives. In addition 2,7-diaminofluorene (1% in acetic acid) gave yellow colours with aldehydes. A spray of a saturated solution of Rhodamine B in aq. 1% uranyl acetate was used to detect acids on chromatograms. Carboxylic acids increased the fluorescence whereas other compounds quenched fluorescence.

Gas-liquid chromatography. This was carried out as described in the preceding paper (Douch & Smith, 1971) with a 5% Carbowax 600 column and flame ionization detection (Table 1).

Measurement of ³H radioactivity. This was carried out in a Packard series 4000 scintillation spectrometer as described in the preceding paper (Douch & Smith, 1971). Extraction of Butacarb and its metabolites from enzyme mixtures and from whole insects was as described by Douch & Smith (1971), and extractions with ether were made repeatedly until at least 99% of radioactivity had been recovered.

Table 1. *Separation of phenols present in hydrolysed extracts of Butacarb*

Solvents were: *A*, ether-iso-octane (5:1, v/v), and *B*, toluene-ethyl formate-formic acid (5:7:1, by vol.); chromatograms were developed for 25 cm on Whatman no. SG 81 paper. Paper ionophoresis was at 4000 V for 20 min. Buffers were: pH 2, 0.01 M-HCl; pH 7, 0.1 M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$); pH 9, 0.1 M-borate buffer ($\text{Na}_2\text{B}_2\text{O}_7\text{--HCl}$); pH 12, 0.01 M-NaOH. G.l.c. was carried out on a 50 cm column with 5% Carbowax 600 on Celite at the temperatures indicated and with a gas flow rate of 100 ml/min. N.D. means not detected after 10 min.

Phenol spot	R_F value		Migration to anode (cm)				G.l.c. retention time (min)		
	Solvent <i>A</i>	Solvent <i>B</i>	pH 2	pH 7	pH 9	pH 12	140°C	150°C	160°C
I	0.06	0.30	0	6.1	7.3	9.7	N.D.	N.D.	N.D.
II	0.13	0.43	0	6.6	7.7	10.9	N.D.	N.D.	N.D.
III	0.18	0.61	0	0	6.2	6.3	N.D.	N.D.	4.3
IV	0.23	0.68	0	0	7.1	6.7	N.D.	N.D.	3.53
V	0.43	0.73	0	0	6.8	6.5	N.D.	3.90	2.5
VI	0.59	0.80	0	0	7.3	6.6	1.5	1.1	0.8
3,5-Di- <i>tert.</i> -butylphenol	0.61	0.81	0	0	7.4	6.5	1.5	1.1	0.8
3,5-Di- <i>tert.</i> -butylcatechol	0.80	0.87	0	0	6.2	5.8	N.D.	N.D.	N.D.

RESULTS

Oxidation of Butacarb by mouse enzymes

Mouse liver 10000g supernatant enzyme was incubated under the conditions used by Douch & Smith (1971) for 0.5 h with 1.0 mM-Butacarb. The unchanged material and metabolites were extracted with ether and hydrolysed with 1 M-sodium hydroxide as described in the preceding paper (Douch & Smith, 1971) to give a mixture of phenolic products. These were extracted into ether and chromatographed on Whatman no. SG 81 paper in solvents *B* and *C*.

Colour reactions with diazotized *p*-nitroaniline showed that six spots, I–VI, were present in each of these systems; one of the spots behaved chromatographically like the reference 3,5-di-*tert.*-butylphenol (Table 1). None of the six metabolic phenols reacted with ammoniacal silver nitrate and it was concluded that no 1,2- or 1,4-diphenols were present. No colour reaction was given with 2,7-diaminofluorene or di-*o*-anisidine in acetic acid and it was concluded that no aldehyde function was present in any of the spots. Two of the spots, I and II, had the increased fluorescence characteristic of carboxylic acids with the Rhodamine–uranyl acetate spray.

Each of the six phenolic spots was located by spraying a test strip on a similar chromatogram and the corresponding zones were eluted with ether. These eluates were subjected to paper ionophoresis at 4000 V for 20 min at pH 2, 7, 9 and 12 as described by Douch & Smith (1971) (Table 1). Only two spots, I and II, migrated at pH 7 and were similar in mobility to carboxylic acids studied

previously (see, e.g., Jordan *et al.* 1970; Douch & Smith, 1971).

These two compounds also exhibited the greater mobility at pH 12 expected of phenolic acids (Table 1). The other four spots, III–VI, behaved ionophoretically like simple phenols.

Each of the eluted phenolic hydrolysis products was also tested in the g.l.c. system described above. Only one peak was observed in the case of those compounds that gave a response within 10 min. One of these phenols, VI, was identical in chromatographic properties with 3,5-di-*tert.*-butylphenol. Spots I and II were not detected during the retention time examined, and spots V, IV and III had progressively longer retention times, considerably in excess of that of the monohydric phenol, VI.

The unhydrolysed extract from an oxidation experiment with the 10000g supernatant enzyme was also examined chromatographically in solvents *A* and *C* on Whatman no. SG 81 paper. When these chromatograms were sprayed with diazotized nitroaniline and 1 M-sodium hydroxide nine spots were visible on chromatograms in solvent *A* and eight on those in solvent *C*. Two-dimensional chromatography (solvent *A* followed by solvent *C*) showed at least eleven spots to be present (Fig. 1). Spots were eluted from one-dimensional chromatograms in solvent *A* with ether and the nine eluates, after evaporation, were hydrolysed to the phenols by standing them for 1 h in 1 M-sodium hydroxide. The phenols were then examined in solvent *B* and compared with the phenols I–VI described above. Similarly the eight spots found on chromatograms in solvent *C* were eluted, hydrolysed and also compared with phenols I–VI. The results are summarized in Table 2.

The eleven metabolite spots found in two-dimensional chromatograms in solvents *A* and *C* were examined with the colour reagent sprays described above. No reaction was obtained with ammoniacal silver nitrate or with the dianisidine

reagent for aldehydes. No reaction was obtained with the dimethylaminobenzaldehyde reagent used to detect carbamic acid esters. Only metabolites *e*, *h* and *k* gave a direct colour with diazotized *p*-nitroaniline characteristic of free phenols, but all the spots gave this colour when over-sprayed with 1M-sodium hydroxide. Only metabolites *f* and *i* gave the chromotropic acid reaction used to detect *N*-methylols. All the spots except *e*, *h* and *k* gave the ninhydrin–collidine colour reaction used to detect carbamates and *N*-substituted carbamates. Metabolites *a* and *b*, the only ones giving rise to the acidic phenols I and II, gave the Rhodamine B–uranyl acetate fluorescence reaction for acids. Probable structures for these metabolites are summarized in Table 2.

Fig. 1. Tracing of two-dimensional chromatogram on Whatman no. SG81 paper of products of enzymic oxidation of Butacarb. Mouse liver 10000g supernatant enzyme with glucose 6-phosphate and NADP⁺ was used as described in the text. Solvents were *A* (ether–iso-octane, 5:1, v/v) and *C* (dry ether) and the chromatogram was developed for 15 cm in each direction. Spots were detected with diazotized *p*-nitroaniline and 1M-NaOH as described in text.

Oxidation of Butacarb by fly enzymes

Housefly and blowfly enzymes were used as described by Douch & Smith (1971) to oxidize Butacarb. Extracts from fifty 10ml incubations were pooled and examined by chromatography, ionophoresis and colour reagents as described for the mouse liver experiments. All chromatograms from these experiments were qualitatively indistinguishable from those in the mouse liver experiments, and flies of both species appeared to form the same metabolites as the mouse.

Quantitative assays of the metabolites

In mouse liver and insect enzymes. Mouse liver and fly enzyme incubations, as described above,

Table 2. *R_F* values and probable structures of enzymic metabolites of Butacarb

Chromatography on Whatman no. SG81 paper was performed in solvent *A* (ether–iso-octane, 5:1, v/v) and solvent *C* (dry ether), and metabolites were detected as described in the text. Structures of the carbamoyl groups in the molecules were determined by colour reactions as described in the text. Alkaline hydrolysis of the eleven metabolites gave the six phenols I–VI, which were identified as shown in Table 1. Metabolites *a*–*k* are those shown in Fig. 1.

Metabolite	<i>R_F</i> value		Carbamoyl group	Phenol	<i>tert.</i> -Butyl groups metabolized by introduction of
	Solvent <i>A</i>	Solvent <i>C</i>			
<i>a</i>	0.06	0.04	O-CO-NH-CH ₃	I	Carboxyl group
<i>b</i>	0.17	0.21	O-CO-NH-CH ₃	II	Carboxyl group
<i>c</i>	0.10	0.30	O-CO-NH-CH ₃	III	Hydroxyl groups
<i>d</i>	0.17	0.48	O-CO-NH-CH ₃	IV	Hydroxyl groups
<i>e</i>	0.25	0.67	None	IV	Hydroxyl groups
<i>f</i>	0.30	0.67	O-CO-NH-CH ₂ -OH	V	Hydroxyl group
<i>g</i>	0.40	0.67	O-CO-NH-CH ₃	V	Hydroxyl group
<i>h</i>	0.50	0.67	None	V	Hydroxyl group
<i>i</i>	0.50	0.85	O-CO-NH-CH ₂ -OH	VI	Unchanged
<i>j</i>	0.64	0.90	O-CO-NH-CH ₃	VI	Unchanged (Butacarb)
<i>k</i>	0.74	0.90	None	VI	Unchanged (3,5-di- <i>tert.</i> -butyl-phenol)
Butacarb	0.64	0.90	—	—	—
3,5-Di- <i>tert.</i> -butylphenol	0.74	0.90	—	—	—

were made with $1\mu\text{Ci}$ of $[^3\text{H}]\text{Butacarb}/10\text{ml}$ of enzyme preparation, and the metabolites extracted as described above after 0.5h. Assessment of the relative amounts of different metabolites was made in three ways.

The extracts were hydrolysed and the phenols separated in solvent system *A* on Whatman no. SG81 paper, which was cut up and the radioactivities of the zones were counted as described above.

The acidic metabolites were assayed independently by paper ionophoresis of the unhydrolysed extracts at pH 7 at 4000V for 20min. The zone corresponding to the carboxylic acids with a migration of 5–8cm (see Table 1) was cut out, and its radioactivity was counted in the scintillation spectrometer and expressed as a percentage of the total radioactivity separated in the experiment.

The unhydrolysed extract was also separated on Whatman no. SG81 paper in solvent *A*. Zones were located by colour reactions, and parallel unsprayed chromatograms were cut up and the radioactivities of the zones counted to give values for each of the metabolites in Table 2 except metabolites *b* and *d*, which ran together, and metabolites *h* and *i*, which also gave a single spot. Values for metabolites *i* and *b* were obtained from a duplicate sample separated in solvent system *C* (Table 2), which permitted the calculation of values for metabolites *d* and *h*. Assays from these experiments are summarized in Table 3.

Where metabolites could be assayed by independent methods agreement was good; the acidic metabolites *a* and *b* in three experiments amounted to 0.95, 0.94 and 1.05% of the metabolism, as measured by the chromatographic assay and to

0.85, 1.03 and 0.98%, as measured by the ionophoretic assay.

The direct assay of phenolic metabolites also agreed satisfactorily with the value deduced from the separate values of the unhydrolysed metabolites; in three experiments with mouse enzyme the amount of phenol III was 1.6, 1.3 and 1.2% of the metabolism as measured by direct chromatographic assay of hydrolysed samples and 1.2, 1.1 and 1.9% from assay of metabolite *c*, which gives rise to phenol III on hydrolysis (see Table 2). The amount of phenol V was 12.2, 10.6 and 13.3% of metabolism as measured by direct assay of the hydrolysis mixture and 8.8, 10.6 and 12.6% by the sum of metabolites *f*, *g* and *h*.

In whole insects. Three-day-old adult houseflies, 5-day-old blowflies and third-instar larvae of the grass grub were each dosed topically with $0.1\mu\text{g}$ of $[^3\text{H}]\text{Butacarb}$ and duplicate groups of 50 were kept at 30°C . Flies were kept in large test tubes for 12h and grass grubs for 24h in the dark in Petri dishes before extraction.

Larvae of housefly and blowfly were also dosed topically with $0.01\mu\text{g}$ of $[^3\text{H}]\text{Butacarb}$ and duplicate batches of 50 were kept in Petri dishes in the dark for 12h.

Insects were extracted by grinding them with sand and ether, and the ether-extractable metabolites were collected in a separate fraction from the water-soluble fraction as described by Douch & Smith (1971). Preliminary chromatograms indicated that a very complex mixture of water-soluble and ether-soluble metabolites was present, and only the estimation of the hydrolysed phenolic metabolites was attempted.

Table 3. *Oxidation of $[^3\text{H}]\text{Butacarb}$ by enzyme from flies and mouse liver*

Metabolites were assayed after separation of primary metabolites (Unhyd.) or hydrolysed phenols (Hyd.) or by ionophoretic separation of acids (Ion.) as described in the text. Metabolites *a*–*k* are those described in text and Fig. 1 and that are hydrolysed to the phenols I–VI as described in text and Table 1. Each result is the mean of three separate experiments.

Source of enzyme ... Metabolite and derived phenol		% of substrate converted into metabolites								
		Mouse			Housefly			Blowfly		
		Unhyd.	Hyd.	Ion.	Unhyd.	Hyd.	Ion.	Unhyd.	Hyd.	Ion.
<i>a</i>	I	0.9	0.9	0.9	0.13	0.13	0.14	0.14	0.14	0.15
<i>b</i>	II	0.1	0.1		0.01	0.01		0.01	0.01	
<i>c</i>	III	1.4	1.4		0.17	0.16		0.14	0.14	
<i>d</i>	IV	5.4	6.2		0.04	0.34		0.03	0.23	
<i>e</i>	IV	0.6			0.29			0.19		
<i>f</i>	V	9.3	12.2		0.27	0.56		0.19	0.45	
<i>g</i>	V	1.1			0.17			0.16		
<i>h</i>	V	0.3			0.12			0.10		
<i>i</i>	VI	2.4			0.96			0.94		
<i>j</i>	VI	76.9	79.4		97.5	98.8		97.7	99.0	
<i>k</i>	VI	1.7			0.29			0.34		

The ether-soluble metabolites were hydrolysed with 1M-sodium hydroxide at room temperature for 1 h, and re-extracted into ether before separation in solvent A and ionophoresis at pH 7 to separate acidic products. The water-soluble fraction of the extract was refluxed with an equal volume of 10M-hydrochloric acid for 6 h before extraction into ether. The ether was evaporated and the residue hydrolysed with 1M-sodium hydroxide before re-extraction, chromatography and ionophoresis.

Assays of the phenolic metabolites were made on both fractions, but values quoted in Table 4 are the sums of the phenols I-VI found in these two fractions.

Some radioactivity remained in the insoluble residue in these experiments (1-4%), and some water-soluble residue (3.9-11.4%) remained unextractable after the hydrolysis. Values quoted in Table 4 are percentages of the ether-extractable material applied to the chromatograms.

In similar experiments groups of 50 blowflies or houseflies were dosed with 0.1 μ g of [^{3} H]Butacarb and extracts of the groups made at 4 h intervals for a day. Values obtained for the unchanged Butacarb from the chromatographic assays were plotted and its rate of disappearance was calculated from these graphs, which were linear up to 16 h. For both species of fly these rates were 0.005 μ g/h per fly. In similar experiments in which a dose of 0.01 μ g/fly was used the rates were 0.0006 μ g/h per fly in *Lucilia* and 0.0008 μ g/h per fly in *Musca*.

Maggots of both species, which had the same weight as the adult forms (14 mg for *Musca* and 15 mg for *Lucilia*), were killed within 2 h by the 0.1 μ g dose, and with the low dose (0.01 μ g) the

rates of disappearance of the compound were only half of those found in the two corresponding adult forms.

DISCUSSION

The metabolites of Butacarb in mice or insects form a complex mixture, but interpretation of the metabolism can be simplified by considering only the phenolic hydrolysis products of these metabolites and their conjugates formed by the whole animal. The hydrolysis products were similar in the three insect species and the mouse, and the striking species toxicity differences cannot be attributed to any qualitative difference in the metabolism of the insecticide in different species. In each organism the major phenolic product was 3,5-di-*tert*.-butylphenol, which accounted for more than half of the dose in the experiments with intact animals. Of the five other phenols present, one was found in larger amounts than the others in all the animals studied.

This phenol (V) was not hydroxylated in the aromatic ring, since this would have given rise to a 1,2- or a 1,4-dihydroxybenzene, which would have been readily detected by the colour reagents used. It is most likely to have been formed by oxidation in one of the *tert*.-butyl groups and to be analogous with the *m*-(β -hydroxy-*tert*.-butyl)phenol that is a major metabolite of *m-tert*.-butylphenyl *N*-methylcarbamate in mice and flies (Douch & Smith, 1971). The R_f values, which are lower than those of di-*tert*.-butylphenol in the adsorption chromatography systems used, suggest that it is a more polar molecule containing an additional hydroxyl function.

Table 4. Phenols present in fully hydrolysed extracts of insects dosed with [^{3} H]Butacarb

Insects were dosed with [^{3} H]Butacarb and homogenized and extracted with ether after 12 h (flies) or 24 h (grass grubs). Phenols I-VI are those indicated in Table 1 and were assayed as described in the text on the ether extract, and on the aqueous phase after acid and alkaline hydrolysis, as described in the text. Results are the totals of conjugated and free phenols except for those in parentheses beside the totals, which represent the percentages of the dose in a water-soluble ether-insoluble form before hydrolysis. A small amount of radioactivity was ether-insoluble after acid and alkaline hydrolysis (4-15% of the dose). Dose amounts (μ g/g) were: houseflies, 10; blowflies, 5; housefly larvae, 1; blowfly larvae, 0.5; grass grubs, 1.

Phenol	% of dose present in extracts				
	Houseflies		Blowflies		Grass grubs
	Adults	Larvae	Adults	Larvae	
I	6.1	1.8	7.0	2.5	10.6
II	0.2	0.4	2.1	1.4	0.9
III	2.1	1.4	2.9	5.7	2.1
IV	3.2	6.1	7.2	5.2	3.3
V	21.4	20.7	17.0	20.5	12.6
VI	55.9	63.7	50.9	56.2	60.4
	88.9 (28.2)	94.1 (52.5)	87.1 (24.5)	91.5 (45.0)	89.9 (31.6)

The longer retention times on Carbowax columns in g.l.c. (Table 1) also suggest that more than one hydroxyl group is present. The other two phenols, IV and III, which are more polar and are eluted still more slowly from chromatograms or the g.l.c. column, may be further hydroxylated. Since the phenols V, IV and III have neither aldehyde nor carboxyl groups possible structures may be as shown.

The two acidic metabolites, I and II, from their ionophoretic properties, appear to have only single carboxylic groups in their molecules. These were minor metabolites and may be presumed to have arisen through further oxidation of the primary alcohol groups of metabolites III, IV and V.

The major oxidation product of Butacarb is similar to that formed in the metabolism of *m*-*tert*-butylphenyl *N*-methylcarbamate. Similar oxidations of *tert*-butyl groups have been observed in rats, rabbits and houseflies treated with *tert*-butylbenzene (Robinson & Williams, 1955; Chakraborty & Smith, 1967) and with B.H.T. (3,5-di-*tert*-butyl-4-hydroxytoluene) (Dacre, 1961; Holder, Ryan, Watson & Wiebe, 1970). The *tert*-butyl group in B.H.T. is reported to be metabolized to an aldehyde derivative in man (Daniel, Gage, Jones & Stevens, 1967), but no evidence for this type of metabolite has been found with the *tert*-butyl groups of Butacarb, and the observation made by Daniel *et al.* (1967) has been questioned by Holder *et al.* (1970).

In contrast with *m*-*tert*-butylphenyl *N*-methylcarbamate, Butacarb does not appear to be extensively oxidized at the *N*-methylcarbamoyl group. Two of the eleven oxidation products formed in enzyme incubations gave colour reactions for the *N*-hydroxymethyl group and these, metabolites *f* and *i*, were derivatives of the major phenolic metabolite V and 3,5-di-*tert*-butylphenol respectively. No other metabolites that could have been derived from modification of the *N*-methylcarbamoyl group were detected by the procedures used by Douch & Smith (1971).

The correlation between low toxicity and ready oxidation of the insecticide that was observed with *m*-*tert*-butylphenyl *N*-methylcarbamate also

appears to hold with Butacarb. Both species of fly tolerated larger doses of the insecticide/g of insect than did the grass grub, which is less able to oxidize foreign organic compounds (Hook, Jordan & Smith, 1968) and which oxidized (metabolites I-V) a smaller percentage of a smaller dose in 24h than the flies were able to metabolize in 12h (Table 4).

The lower rate of oxidation of Butacarb by maggots is of some interest, since when it is used as a sheep dip the larval form of *Lucilia* is the target organism. Maggots tolerated only one-tenth of the topical dose that could be applied to adult *Lucilia*, and the lower rate of oxidation found in maggots would offer correspondingly less protection against the insecticide than that in adult flies.

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