Oxidation of Methyl Groups by Grass Grubs and Vertebrate Liver Enzymes

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1. Oxidation rates of *p*-nitrotoluene, *p*-acetamidotoluene and *p*-toluidine by intact grass grubs and vertebrate liver preparations were measured. 2. The effect of *p*-substitution in increasing the rate of conversion of the methyl into a carboxy group was in the order acetamido > nitro > amino in mice and grass grubs. 3. Rates of oxidation of the *N*-methyl group in some alkylaryl *N*-methylcarbamates was measured and the effect of ring substituents in increasing the rate was in the order hydrogen > *o*-methyl or *o*-isopropyl > *p*-methyl or *p*-isopropyl > *m*-methyl or *m*-isopropyl. 4. Rates of oxidation of the *N*-methyl groups were similar to those of the *p*-substituted toluenes.

Oxidation of alkyl *N*-methylcarbamates is a reaction of some importance in the detoxication of this type of insecticide (Metcalf & Fukuto, 1965; Casida, 1963) though as a rule the products of oxidation have not been fully characterized. It has been shown that the oxidation of the *N*-methyl group of this type of compound occurs in vertebrate liver preparations and there is evidence that a similar oxidation can take place in insects (Hodgson & Casida, 1960; Dorough & Casida, 1964). There is as yet little evidence to show whether or not the alkyl groups attached to the aromatic ring of a carbamate insecticide can be oxidized though these groups are readily oxidized when alkylbenzenes are administered to vertebrates or insects (Williams, 1959; Chakraborty & Smith, 1964, 1967).

The oxidation of an alkyl side chain to a carboxylic acid or to an alcohol that could be conjugated with glucose or glucuronic acid would be expected to facilitate the elimination of the molecule from the organism (Smith, 1962) and could have a profound effect on its effective toxicity. Oxidation of the *N*-methyl group, on the other hand, may not greatly affect the toxicity since the product could still react with cholinesterase (Dorough & Casida, 1964).

It was therefore decided to measure the relative rates of biochemical oxidation of methyl groups in some *N*-methylcarbamates and to compare these with the rate of oxidation of some substituted toluenes.

EXPERIMENTAL

Reference compounds. All melting points are uncorrected. *p*-Aminobenzyl alcohol, m.p. 64°, was prepared by reduction of *p*-nitrobenzyl alcohol with Adams platinum catalyst and hydrogen.

N-Methylcarbamates of phenols were prepared by leaving the phenol and a 10% excess of methyl isocyanate to stand in dry ether for 3 days. Phenyl *N*-methylcarbamate, m.p. 85°, *o*-cresyl *N*-methylcarbamate, m.p. 101°, *m*-cresyl *N*-methylcarbamate, m.p. 75°, *p*-cresyl *N*-methylcarbamate, m.p. 93°, *o*-isopropylphenyl *N*-methylcarbamate, m.p. 91°, and *p*-isopropyl *N*-methylcarbamate, m.p. 92°, were recrystallized from dry ether. *o*-Isopropoxyphenyl *N*-methylcarbamate, m.p. 92° (Bayer A.-G., Leverkusen, Germany) and *m*-isopropylphenyl *N*-methylcarbamate, m.p. 72° (Dow Chemical Co., Midland, Mich., U.S.A.), were commercial samples, as were the *o*-, *m*- and *p*-hydroxybenzaldehydes and the *o*-, *m*- and *p*-hydroxybenzyl alcohols.

Insects. Third-instar grass grubs (*Costelytra zealandica*) weighing about 0.1 g. were provided by the D.S.I.R. Entomology Division, Nelson, New Zealand, and were kept in moist earth at 5° for up to 3 weeks before use. Compounds (50 µg./grub) were injected into the haemocoel in 2.5 µl. of acetone or corn oil. Grubs were left on a small tuft of cotton wool in separate 6 in. × ½ in. homogenizer tubes until required for assay. Subsequent homogenization, extraction and centrifugations were carried out in the same tube to avoid transfer losses.

Liver homogenates. Livers from adult albino rats, guinea pigs or 6-week-old male mice were homogenized in 6 vol. of 0.25% sucrose at 0° and centrifuged at 10000g at 4° for 10 min. The supernatant was used as enzyme in the incubations, which were carried out at pH 7.4 in 150 ml. beakers in a shaking water bath in air at 37° for 0.5 hr. In the standard experimental conditions each beaker contained material from 0.5 g. of rat liver or 0.25 g. of mouse liver. The total volume was 5 ml., which contained 1 ml. of 0.1 M-tris buffer, pH 7.4, and cofactors to make 10 mM-nicotinamide, 15 mM-Mg²⁺, 0.1 mM-NADP, 0.1 mM-NAD and 4 mM-glucose 6-phosphate. Substrates (1.0 mg.) were added in 0.05 ml. of acetone to start the reaction and controls were prepared similarly except that substrate was added at the end of the incubation period.

Rates of oxidation per g. of tissue did not appear to be limited by the volume of incubation mixture and the same

rate was found when *p*-acetamidotoluene was incubated in a 150ml. beaker in total volumes between 5ml. and 20ml. provided that the concentrations of enzyme and cofactors were as stated above. In most rate experiments, however, mixtures equivalent to 2–3g. of liver in a total volume of 40ml. with the corresponding concentrations of cofactors were incubated in 1000ml. beakers and 5ml. portions were removed at intervals for analysis as described below.

Estimation of p-nitrobenzoic acid in homogenates and insects. This was carried out by the method of Chakraborty & Smith (1964).

Estimation of p-acetamidobenzoic acid. Enzyme incubation mixtures (5ml.) were deproteinized with 1ml. of 0.5N-NaOH and 1ml. of 10% (w/w) ZnSO₄. After shaking with 10ml. of CHCl₃ to remove unchanged substrate and centrifuging, 3ml. of supernatant was removed and acidified with 0.5ml. of 4N-HCl. This mixture was heated in stoppered tubes in a boiling-water bath for 1hr., cooled and treated successively with 0.4ml. of 0.1% NaNO₂, 0.4ml. of 0.2% ammonium sulphamate and 0.4ml. of aq. 0.1% *N*-1-naphthylethylenediamine dihydrochloride as described by Bratton & Marshall (1939). The colour was measured after 40min. at 540m μ in a Unicam SP.600 spectrophotometer and the amount of *p*-acetamidobenzoic acid calculated by reference to a calibration curve prepared from standard solutions of *p*-aminobenzoic acid.

Single grass grubs were homogenized in a Potter-Elvehjem homogenizer in 0.5ml. of 0.5N-NaOH, the pestle was rinsed with 3ml. of water and 0.5ml. of 10% (w/v) ZnSO₄, and, after shaking with 10ml. of CHCl₃ and centrifuging, 3ml. of supernatant was used for the colorimetric assay as described above. Recovery of *p*-acetamidobenzoic acid added to liver or grass-grub homogenate was 82s.e.m. $\pm$ 3.4% (22).

Estimation of *p*-aminobenzoic acid was made as described for *p*-acetamidobenzoic acid except that colorimetric assay was made directly on the deproteinized and acidified supernatant without heating to 100°. Recovery of *p*-aminobenzoic acid added to 5ml. of liver or grass-grub homogenate was 88s.e.m. $\pm$ 2.4% (10).

In experiments where *p*-nitrobenzyl alcohol and *p*-aminobenzyl alcohol were used as substrates these compounds were not completely removed by the CHCl₃ extraction and gave rise to azo dyes when measurements were made according to the procedure described above for *p*-nitrobenzoic acid or *p*-aminobenzoic acid. These interfering dyes were removed by extraction from alkaline solution; 0.5ml. of 5N-NaOH was added to the solution of azo dyes developed according to the standard procedure and the mixture shaken with 10ml. of CHCl₃. The CHCl₃ was removed, the aqueous layer neutralized with 0.5ml. of 5N-HCl and the azo colour derived from *p*-aminobenzoic acid measured as above. Recovery of *p*-aminobenzoic acid from 5ml. of homogenate containing 50–500 μ g. of *p*-aminobenzylalcohol was 80 s.e.m. $\pm$ 1.9% (18).

Estimation of formaldehyde. The homogenate (5ml.) containing supernatant from 0.25g. of liver was deproteinized with 0.5ml. of trichloroacetic acid (100g./100ml.) and centrifuged. A 4ml. portion of the supernatant was steam-distilled in a Markham Kjeldahl apparatus with a further 2ml. of the same trichloroacetic acid until 10ml. of distillate had collected. A 1.5ml. portion of the distillate was added to 5ml. of McFadyen's (1945) chromotropic acid-H₂SO₄ reagent and the mixture heated in a boiling-

water bath for 30min. After cooling the colour was measured at 570m μ in a Unicam SP.600 spectrophotometer. The amount of formaldehyde formed was calculated by reference to a calibration curve prepared from the reagent and known solutions of formaldehyde. Recovery of formaldehyde added to incubation mixtures was 80s.e.m. $\pm$ 2.8% (7) after deproteinization and steam-distillation.

Thin-layer chromatography. This was carried out on 20cm. $\times$ 20cm. glass plates after coating with a 0.25mm. layer of a suspension of 50g. of silica gel G (E. Merck A.-G., Darmstadt, Germany) in 120ml. of acetone–water (7:5, v/v) that was dried at 110° for 1hr. Solvents used are quoted in Table 1.

N-Hydroxymethyl derivatives were detected by the red colours produced when plates were sprayed with 0.2% chromotropic acid in 12N-H₂SO₄ and heated at 100° for 30min. Phenols were detected by the yellow colours produced when plates were sprayed with 0.1% *p*-nitrobenzenediazonium fluoroboride in saturated NaHCO₃. Carbamate esters of phenols were hydrolysed on the plates by spraying with 0.5N-NaOH and liberated phenols detected by spraying 1hr. later with 0.1% *p*-nitrobenzene diazonium fluoroboride in 0.5N-HCl. The yellow colours formed with this reagent were intensified by subsequent treatment with alkali. Aldehydes gave orange colours when sprayed with 1% 2,4-dinitrophenylhydrazine in 2N-HCl.

Inhibitors of acetylcholinesterase were detected by the procedure of Crosby, Leitis & Winterlin (1965) with human blood serum as a source of enzyme and bromocresol purple as indicator. Metabolites that inhibited the enzyme appeared as blue spots on a yellow background.

R_p values and colour reactions are summarized in Table 1.

RESULTS

Oxidation of substituted toluenes

Oxidation by grass grubs. Grass grubs were injected as described above with 50 μ g. of *p*-nitro-toluene, *p*-acetamidotoluene, *p*-toluidine, *p*-aminobenzyl alcohol and *p*-nitrobenzyl alcohol. Assays for the corresponding substituted benzoic acids were made as described above at intervals during the 2 days after dosing. Assays were carried out on individual grubs and six were taken at each time. Considerable individual variation in ability to oxidize the compounds was found and the results were plotted graphically to show the mean value and the extreme high and low values for each group (e.g. Fig. 1).

A rate of oxidation for each compound was estimated from the mean values at each station of the graph and an indication of the range of oxidation rates for a compound was obtained from lines drawn through the extreme values at each station (Fig. 1). Mean values and ranges assessed graphically in this manner are summarized in Table 2.

The rate of oxidation of *p*-nitro- and *p*-acetamidotoluene appeared to be linear with time for the 2 days of the experiment, but the initial high rate of oxidation of the substituted benzyl alcohols

Table 1. *Thin-layer chromatography of some N-methylcarbamates and their metabolites*

Silica gel G (Merck) was used on glass plates and solvents were run for 15 cm. Solvents were: I, diethyl ether-iso-octane (7:3, v/v); II, chloroform; III, xylene; IV, ethyl acetate-light petroleum (b.p. 60–80°) (3:1, v/v); V, acetone-*n*-hexane (3:5, v/v). Colour reagents were *p*-nitrobenzenediazonium fluoroborate (NBDF), 2,4-dinitrophenylhydrazine (DNPH) and chromotropic acid in 12*N*-H₂SO₄ (CTA).

	Solvents					Colour reagents		
	I	II	III	IV	V	CTA	NBDF	DNPH
Phenol	0.65	0.47	—	—	—	—	Yellow	—
Phenyl <i>N</i> -methylcarbamate	0.42	—	—	—	—	—	Nil	—
Metabolite A	0.19	—	—	—	—	Red	Nil	—
<i>o</i> -Cresol	0.57	0.47	0.15	—	—	—	Orange	—
<i>o</i> -Cresyl <i>N</i> -methylcarbamate	0.43	—	—	—	—	—	Nil	—
Metabolite B	0.11	—	—	—	—	Red	Nil	—
Metabolite C	0.20	—	—	—	—	Red	Nil	—
<i>o</i> -Hydroxybenzyl alcohol	0.31	0.10	0.01	0.72	0.33	Red	Orange	—
<i>o</i> -Hydroxybenzaldehyde	0.57	0.30	0.09	—	—	Red-grey	Yellow	Brown
<i>m</i> -Cresol	0.60	0.35	0.11	—	—	—	Orange	—
<i>m</i> -Cresyl <i>N</i> -methylcarbamate	0.43	—	—	—	—	—	Nil	—
Metabolite D	0.12	—	—	—	—	Red-grey	Nil	—
Metabolite E	0.23	—	—	—	—	Red-orange	Nil	—
<i>m</i> -Hydroxybenzyl alcohol	0.24	0.01	0	0.55	0.25	Green-grey	Orange	—
<i>m</i> -Hydroxybenzaldehyde	0.50	0.13	0.02	—	—	Grey	Brown	Orange
<i>p</i> -Cresol	0.60	0.33	0.1	—	—	—	Yellow	—
<i>p</i> -Cresyl <i>N</i> -methylcarbamate	0.43	—	—	—	—	—	Nil	—
Metabolite F	0.12	—	—	—	—	Red	Nil	—
Metabolite G	0.23	—	—	—	—	Red	Nil	—
<i>p</i> -Hydroxybenzyl alcohol	0.21	0.03	0	0.55	0.19	Red	Orange	—
<i>p</i> -Hydroxybenzaldehyde	0.30	0.14	0	—	—	—	Orange	Orange

diminished after 10 hr. and values quoted in Table 1 were taken from groups of insects assayed in the initial linear period of the experiments.

No substituted benzyl alcohols were detected as products of the oxidation of the substituted toluenes. The presence of *p*-aminobenzyl alcohol could readily be recognized in the diazotization and coupling reaction by its low rate of coupling. In all cases the azo dye reached its maximum colour intensity within 10 min. and the azo colour was not extractable from alkaline solution. Moreover, when *p*-amino- or *p*-nitro-benzyl alcohol was administered, it was oxidized much faster by grass grubs than the substituted toluenes.

Oxidation by liver homogenates. Cofactor concentrations used in most experiments were those described above and other concentrations tested were less satisfactory (Table 3).

The rates of oxidation of *p*-toluidine, *p*-acetamidotoluene and *p*-nitrotoluene were measured in the large-scale incubation mixtures described above from which 5 ml. portions were withdrawn at intervals for analysis. Rates of oxidation were calculated from the graphs obtained (e.g. Fig. 2), which for these substrates were linear for at least 1 hr. When the rate of oxidation diminished after this time it could not be restored by adding further NADPH₂.

When *p*-acetamidobenzoic acid was used as substrate, reaction velocity was proportional to amount of mouse enzyme only for amounts corresponding to less than 0.5 g. of liver. Experiments were therefore normally carried out with 0.2–0.3 g. of tissue to each 5 ml. of incubation mixture.

Oxidation of some N-methylcarbamates

Oxidation by grass grubs. Grass grubs were injected as described above with 50 µg. of phenyl *N*-methylcarbamate or with 50 µg. of *o*-cresyl *N*-methylcarbamate and kept in tubes on tufts of cotton wool until required for analysis. Twenty grubs were homogenized with 3 ml. of water, deproteinized with trichloroacetic acid and distilled as described above for the estimation of formaldehyde.

No formaldehyde was measurable after 3 hr. but after 24 hr. a trace of colour was measured that corresponded to the presence of 4 mµmoles of apparent formaldehyde/grub in grubs dosed with phenyl *N*-methylcarbamate and 2.5 mµmoles of apparent formaldehyde/grub in those dosed with *o*-cresyl *N*-methylcarbamate.

Oxidation by liver homogenates. Cofactor requirements for the oxidation of phenyl *N*-methylcarbamate were similar to those required for

p-acetamidotoluene and included NAD as well as NADP (Table 3).

In measurements of the rate of oxidation of phenyl *N*-methylcarbamate, linearity of the graphs relating product formed and time was usually maintained only for about 0.5 hr. even with samples

of enzyme in which the rate of oxidation of *p*-acetamidotoluene was sustained for longer periods (e.g. Fig. 2).

The linearity of these systems was not improved by the incorporation of 10 mM-semicarbazide in the incubation mixture to trap any free formaldehyde, and since this compound slightly diminished the rate of oxidation of both phenyl *N*-methylcarbamate and *p*-acetamidotoluene it was not used in subsequent experiments.

The apparent loss of activity of the enzyme towards phenyl *N*-methylcarbamate was not due to loss of substrate by hydrolysis since no free phenols could be detected in the incubation mixture after 0.5 hr. with tetra-azotized di-*o*-anisidine (Brentamine Fast Blue 2B salt) or by thin-layer chromatography of an extract of the mixture. Control experiments showed that a phenol concentration corresponding to the hydrolysis of 1% of the substrate used could readily be detected with this reagent.

The rate of oxidation of phenyl *N*-methylcarbamate was at maximum in 2 mM-solution (Fig. 3) and in experiments with other carbamates 2 mM-substrates were used. Different samples of mouse enzyme varied slightly in their activity towards phenyl *N*-methylcarbamate in the range 1–3 μ moles/hr./g. of liver, freshly prepared enzyme having the greater activity. A single sample of enzyme used was therefore used to compare the rates of oxidation of all the carbamates and these results are summarized in Table 4.

Chromatography of metabolites. Standard 5 ml. incubation mixtures of mouse enzyme and the *N*-methylcarbamates of *o*-, *m*- and *p*-cresol were made alkaline with 0.5 ml. of 0.5 *N*-sodium hydroxide after 1 hr. and left for 1 hr. to hydrolyse the carbamic esters. Zinc sulphate (0.5 ml. of 10%, w/v) was then added and the precipitate centrifuged off. The supernatant was acidified with 1 ml. of 2 *N*-hydrochloric acid and extracted with 5 ml. of ether. This was reduced to small volume and examined

Fig. 1. Oxidation of *p*-nitrotoluene by grass grubs. Grubs were injected with 50 μ g. in 2 μ l. of acetone-water (1:1, v/v) and analyses made for *p*-nitrobenzoic acid at intervals. Points indicate the mean of results from six individual grubs and the range.

Table 2. Oxidation of some substituted toluenes in insects and vertebrate liver

Oxidations were measured with intact grass grubs or 10000g supernatant of vertebrate liver homogenate as described in the text. Substrates 2, 3 and 4 were assayed simultaneously on the same sample of liver enzymes.

	Rate of oxidation. (μ moles/hr. per g. of insect or per g. of liver)				
	Rat liver	Mouse liver	Grass grubs	Locust fat body*	Fly*
1. Toluene	0.6	—	—	0.06	—
2. <i>p</i> -Nitrotoluene	0.6	1.73	0.01 (0.005–0.02)	0.06	0.2
3. <i>p</i> -Acetamidotoluene	0.43	2.84	0.02 (0.01–0.04)	—	—
4. <i>p</i> -Toluidine	0.02	0.12	0	0	—
5. <i>p</i> -Nitrobenzyl alcohol	—	—	0.16 (0.12–0.30)	—	—
6. <i>p</i> -Aminobenzyl alcohol	—	—	0.20 (0.10–0.30)	—	—

* Chakraborty & Smith (1967).

Table 3. *Effect of cofactors on the oxidation of some methyl groups*

The 10000g supernatants of liver homogenates in 0.25M-sucrose were incubated in air at 37° and pH 7.4 with the addition of the stated cofactors for 0.5 hr. Oxidation products of *p*-acetamidotoluene (PAAT), *p*-nitrotoluene (PNT) and phenyl *N*-methylcarbamate (PNMC) were measured as described in the text and rates are expressed as percentages of that under the best conditions found. Concentrations were: nicotinamide, 10mM; Mg²⁺, 15mM; NAD, 0.1 mM; NADP, 0.1 mM; glucose 6-phosphate, 4mM.

Cofactors added	Relative rates of oxidation of methyl groups			
	In PAAT		In PNMC	In PNT
	By guinea pig	By mouse	By mouse	By rat
None	12	0	0	0
Nicotinamide	13	—	—	—
Nicotinamide, Mg ²⁺	—	1	—	—
Nicotinamide, NAD	24	—	3	—
Nicotinamide, NAD, glucose 6-phosphate	—	—	4	—
NAD, glucose 6-phosphate	—	—	—	7
Nicotinamide, NADP	45	—	1	—
Nicotinamide, NAD, NADP	46	—	—	—
NAD, NADP	—	—	—	47
Nicotinamide, NADPH ₂	50	—	—	—
Nicotinamide, NADP, glucose 6-phosphate	59	—	67	—
NADP, glucose 6-phosphate	—	—	—	22
Nicotinamide, NADP, glucose 6-phosphate, Mg ²⁺	—	53	—	—
Nicotinamide, NADP, NAD, glucose 6-phosphate	100	82	92	—
NADP, NAD, glucose 6-phosphate	—	—	—	49
Nicotinamide, NADP, NAD, glucose 6-phosphate, Mg ²⁺	—	100	100	100
NADP, NAD, glucose 6-phosphate, Mg ²⁺	—	10	—	53

Fig. 2. Oxidation of phenyl *N*-methylcarbamate and *p*-acetamidotoluene by 10000g supernatant of mouse liver homogenate. Each beaker contained enzyme equivalent to 3.3g. of mouse liver in 40ml. of sucrose-tris medium, pH 7.4, containing 10mM-nicotinamide, 15mM-MgCl₂, 0.1mM-NADP, 0.1mM-NAD and 4mM-glucose 6-phosphate, and was incubated in air in a shaking water bath at 37°. Analyses for either formaldehyde or *p*-acetamidobenzoic acid were made on 5ml. portions withdrawn at intervals. Substrates were: ●, mm-phenyl *N*-methylcarbamate; ○, mm-*p*-acetamidotoluene; ▲, mm-*p*-acetamidotoluene in the presence of 10mM-semicarbazide.

Fig. 3. Effect of substrate concentration on rate of oxidation of phenyl *N*-methylcarbamate (●) and *p*-acetamidotoluene (○). Each beaker contained 5ml. of reaction mixture containing mouse liver homogenate, 10000g supernatant corresponding to 0.5g. of liver with cofactors and medium described in the text. Incubations were carried out in a shaking water bath in air at 37° and assays for formaldehyde or *p*-acetamidobenzoic acid were made after 20 min. Different samples of enzymes were used in the two experiments.

chromatographically in the systems of Table 1. Compounds were present that gave the colour reactions of phenols and that had *R_f* values

Table 4. *Oxidation of some aryl N-methylcarbamates by mouse liver enzymes*

Substrates were incubated in air at 37° with 10000g supernatant of a homogenate of mouse liver in 0.25M-sucrose. Cofactors were added to give 10mm-nicotinamide, 15mm-Mg²⁺, 5mm-glucose 6-phosphate, 0.1mm-NAD and 0.1mm-NADP. Portions of the mixture were analysed at intervals for formic acid as described in the text and rates of oxidation calculated from the initial linear range of the reaction/time graph.

<i>N</i> -Methylcarbamate	Rate of oxidation (μ moles/min./g. of liver)
Phenyl	46
<i>o</i> -Cresyl	30
<i>m</i> -Cresyl	8
<i>p</i> -Cresyl	9
<i>o</i> -Isopropoxyphenyl	24
<i>o</i> -Isopropylphenyl	22
<i>m</i> -Isopropylphenyl	6
<i>p</i> -Isopropylphenyl	10

corresponding to the isomeric cresols and, in each extract, a second substance was found having the same R_F as the corresponding *o*-, *m*- or *p*-hydroxybenzyl alcohol. No salicylic acid could be detected in the extract from *o*-cresyl *N*-methylcarbamate by the sublimation-fluorescence method of Feigl (1956).

Incubation mixtures were also extracted with 2 × 5ml. of ether without preliminary hydrolysis. The ether was evaporated to small bulk and examined chromatographically in solvent I and tested for salicylic acid derivatives by Feigl's (1956) sublimation procedure. No salicylic derivatives were detected in extracts of *o*-cresyl *N*-methylcarbamate incubations and no phenolic metabolites were found on the chromatographic plates, but two metabolites were present in each extract from cresyl *N*-methylcarbamate incubations in addition to the original carbamate. In extracts from phenyl *N*-methylcarbamate incubations only one metabolite was found, similar in R_F (0.2 in solvent I) to one of those found in the cresyl *N*-methylcarbamate extracts. These metabolites gave red colours with the chromotropic acid reagent and inhibited serum acetylcholinesterase in the chromatographic procedure of Crosby *et al.* (1965). These metabolites (A, C, E and G of Table 1) were separated from extracts of 5ml. incubations by chromatography on 0.75mm.-thick silica gel G plates and the approximate zones removed and extracted with 5 × 1ml. of ether. Each extract was evaporated to dryness and dissolved in 1ml. of 0.5N-sodium hydroxide. After 0.5hr. the solution was acidified, extracted with 1.0ml. of ether and examined chromatographically in solvents I, II and III. Only phenol, *o*-, *m*- or *p*-cresol was found in these extracts, depending on

whether the extract originated from experiments with phenyl or *o*-, *m* or *p*-cresyl *N*-methylcarbamate. It was therefore concluded that the metabolites A, C, E and G were probably formed by oxidation of the *N*-methyl group.

The second metabolites (B, D and F of Table 1) present in the extracts from the cresyl *N*-methylcarbamates had R_F values about 0.1 in solvent I. Those from the *o*- and *p*-isomers gave dull red colours with the chromotropic acid-sulphuric acid reagent similar to those given by the *o*- or *p*-hydroxybenzyl alcohol. When eluted and hydrolysed as described above the hydrolysates contained only materials with R_F values and colour reactions identical with those of the appropriate isomer of hydroxybenzyl alcohol. The metabolites B, D and F also inhibited serum acetylcholinesterase in the chromatographic procedure and it was concluded that these compounds resulted from oxidation of the ring methyl groups.

DISCUSSION

Metabolism of the phenyl and cresyl *N*-methylcarbamates in the liver preparations used appeared to be solely by oxidation. No evidence was obtained for the formation of any significant amounts of phenolic metabolites that might have been formed by hydrolysis. That oxidation occurred in the *N*-methyl group was shown by the presence of metabolites giving red colours with chromotropic acid. This reaction, which is given by formaldehyde and compounds giving rise to formaldehyde, indicated the presence of either an *N*-oxide or an *N*-methylol compound, since both these give the chromotropic acid reaction and may be formed consecutively in the oxidation of *N*-methyl groups (Fish, Johnson & Horning, 1956; Ziegler & Pettit, 1964). In similar oxidations of other carbamates (Hodgson & Casida, 1960; Dorough & Casida, 1964) the product has been assumed to be the *N*-methylol compound but its significance in the present context is as an index of the extent of oxidation occurring at this part of the molecule. No evidence for the oxidative removal of the *N*-methyl group was found.

Oxidation of the substituted toluenes occurred in both insects and vertebrate preparations and the detection of material with the chromatographic properties of the hydroxybenzyl alcohols suggests that this ring-methyl oxidation also took place in the cresyl *N*-methylcarbamates. However, whereas *p*-substituted toluenes were oxidized to carboxylic acids, the cresyl *N*-methylcarbamates only gave derivatives of hydroxybenzyl alcohols. If *o*- or *m*-hydroxybenzoic acid had been present in the chromatograms or in the deproteinized incubation mixtures, the colour reagents (diazotized *p*-nitroaniline or tetra-azotized di-*o*-ansidine) would have

been adequate to detect them. Attempts to demonstrate the oxidation of *o*- and *p*-cresyl *N*-methylcarbamates to carboxylic metabolites with sensitive spectrofluorometric assays for salicylic acid and *p*-hydroxybenzoic acid have also been unsuccessful (J. Chakraborty, personal communication).

The range of oxidation rates of the *N*-methyl groups in the carbamates was similar to that of the ring methyl groups and it seems possible that, in compounds where both types of methyl were present, the ring methyl could be oxidized as readily as the *N*-methyl group. Oxidation at the *N*-methyl position produced a compound that was still an effective anti-cholinesterase, but oxidation of the ring methyl groups to carboxyl would be expected to give a polar non-toxic compound capable of rapid excretion from an intact animal even though *in vitro* it could still be an effective inhibitor of acetylcholinesterase. The relative extent of oxidation at different sites in a molecule should therefore have a marked effect on its toxicity.

Attempts to correlate structure and toxicity *in vivo* in this series of compounds have not been entirely successful and the importance of the oxidative detoxication in giving rise to anomalies has been emphasized (Metcalf, Fukuto, & Winton 1960; Metcalf & Fukuto, 1965; Georgiou & Metcalf, 1962).

Several selective organophosphate insecticides also have structures containing methyl groups attached to the benzene ring and this may be partly responsible for the species differences in toxicity. Sumithion [dimethyl-(3-methyl-4-nitrophenyl) phosphorothionate] is an example that has excited recent interest (O'Brien, 1966) since it is much less toxic in vertebrates than in insects, whereas the closely related methylparathion [dimethyl-(4-nitrophenyl) phosphorothionate] has little or no selectivity. Very frequently the basis of selective toxicity lies in the ability of the resistant species to degrade a phosphorothionate more rapidly than the oxidation processes produce the toxic phosphate metabolite (Smith, 1964). In the light of the present observations on the oxidation of methyl groups and those of Chakraborty & Smith (1964, 1967) it seems probable that Sumithion would have an additional route of detoxication, by

oxidation of the ring methyl, not possessed by methylparathion. The differences in toxicity between these two insecticides does not seem explicable in terms of their hydrolytic breakdown (O'Brien, 1966) or in terms of target sensitivity since both the corresponding phosphates that are the 'activated' metabolites have about the same effect on cholinesterase (Nishizawa, Fujii, Kadota, Miyamoto & Sakamoto, 1961).

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