The Metabolism of 2,6-Di-*tert.*-butyl-4-hydroxymethylphenol (Ionox 100) in the Dog and Rat

By A. S. WRIGHT, D. A. A. AKINTONWA, R. S. CROWNE AND D. E. HATHWAY
Tunstall Laboratory, Shell Research Ltd., Sittingbourne, Kent

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1. A single oral dose of [^{14}C]Ionox 100 to rats is almost entirely eliminated in 11 days: 89.1–107.2% of the ^{14}C is excreted and $0.29 \pm 0.02\%$ of the dose is present in the carcass plus viscera after removal of the gut. Rats exhibit an individual variation in the elimination pattern, 15.6–70.8% of ^{14}C being excreted in the urine and 75.2–27.0% in the faeces during 11 days. 2. After the oral administration of [^{14}C]Ionox 100 to dogs, 87.1–90.3% of the ^{14}C is excreted in the faeces and urine during 4 days. 3. Dogs and rats do not show a species difference in this pattern of elimination. 4. The rate of elimination from dogs and rats given a single dose of Ionox 100 is not affected by the size of the dose and the presence of triglyceride fat in the diet. 5. Ionox 100 is completely metabolized in dogs and rats: unchanged Ionox 100 is absent from the urine and faeces, and from the carcass when elimination is complete. In rats, 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid accounts for 50–85% of a dose of Ionox 100 and (3,5-di-*tert.*-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid for 47–10%; in dogs, the unconjugated acid accounts for 85% and the ester glucuronide for 10–12%. 3,5-Di-*tert.*-butyl-4-hydroxyhippuric acid is not formed. Other metabolites, which have been detected in small quantity in the faeces and urine of animals dosed with Ionox 100, have not been identified. 6. 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid and (3,5-di-*tert.*-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid are also the major metabolites of Ionol (2,6-di-*tert.*-butyl-*p*-cresol) in rats. 7. The elimination of Ionox 100 metabolites from rats is faster than that of Ionol and its metabolites. Unlike Ionol, unchanged Ionox 100 could not be detected in the bodies of these animals.

2,6-Di-*tert.*-butyl-4-hydroxymethylphenol (Ionox 100) protects a variety of chemical and biological products against oxidative attack (Morris & Rocklin, 1962). Industrial application makes an understanding of the metabolism of this antioxidant desirable, because it may come into contact with food.

MATERIALS AND METHODS

General. Evaporations were carried out in N_2 under reduced pressure below 35° . Melting points were determined on a Kofler block. Specific rotations were determined in a 1 dm. micro-tube. Paper and thin-layer chromatography were carried out in a constant-temperature enclosure at 29° in all-glass apparatus. Chromatograms and thin-layer plates were dried at room temperature. The separation of lipids was effected in the dark.

Ionox 100. Technical Ionox 100 was prepared by the method of Rocklin & Morris (1965), described below for the preparation of the radioactive chemical, and was purified by chromatography on a column of acid-washed alumina of activity III (Brockmann & Schodder, 1941), on to which it was adsorbed from solution in light petroleum (b.p. $60\text{--}80^\circ$).

Elution with light petroleum–diethyl ether (7:3, v/v) gave Ionox 100, m.p. 141° , which was stored in the dark.

*3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid.* This compound, m.p. 210° , was prepared by Cannizzaro reaction of 3,5-di-*tert.*-butyl-4-hydroxybenzaldehyde (Yohe *et al.* 1956), which was itself obtained by bromine oxidation of Ionol (Coppinger & Campbell, 1953).

Ionol. 2,6-Di-*tert.*-butyl-*p*-cresol (Ionol) had m.p. 71° .

Bovine liver β -D-glucuronidase. This enzyme (1g., 560 000 units) (Fishman, Springer & Brunetti, 1948) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Reagents and solvents. All reagents and solvents were of AnalaR grade.

Radioactive chemical. 2,6-Di-*tert.*-butyl-4-hydroxy[^{14}C]methylphenol ([^{14}C]Ionox 100) (0.766 $\mu\text{C}/\text{mg.}$) was synthesized by adding a solution of potassium *tert.*-butoxide (3.5 mg.) in *tert.*-butyl alcohol (1 ml.) to a solution of 2,6-di-*tert.*-butylphenol (1.2 g.) and [^{14}C]paraformaldehyde (100 mg., 610 μC) in *tert.*-butyl alcohol (4 g.) under N_2 at 0° , and by maintaining the reaction mixture at 12° for 40 min. The catalyst was then poisoned with CO_2 and the solution neutralized by the addition of 2 ml. of aq. 10% (w/v) NH_4Cl . After evaporation, the residue was chromatographed on an alumina column, [^{14}C]Ionox 100 (600 mg.),

m.p. 141°, being eluted with light petroleum–diethyl ether (7:3, v/v).

Methyl 3,5-di-tert.-butyl-4-hydroxybenzoate. Excess of diazomethane in ether was added slowly with intermittent shaking to a solution of 3,5-di-tert.-butyl-4-hydroxybenzoic acid (100 mg.) in dry ether (25 ml.). Evaporation gave a pale-yellow solid (105 mg.). *Methyl 3,5-di-tert.-butyl-4-hydroxybenzoate* crystallized from light petroleum (b.p. 40–60°), forming large colourless plates, m.p. 165° (Found: C, 72.8; H, 8.9. $C_{18}H_{24}O_3$ requires C, 72.7; H, 9.1%).

3,5-Di-tert.-butyl-4-hydroxyhippuric acid. 3,5-Di-tert.-butyl-4-hydroxybenzoic acid (500 mg.) was refluxed with thionyl chloride (10 ml.) and dimethylformamide (2 drops) for 1 hr. Excess of thionyl chloride was evaporated, leaving a crystalline mass of the acid chloride (550 mg.). A cold solution of the 3,5-di-tert.-butyl-4-hydroxybenzoyl chloride in dimethylformamide (5 ml.) was added slowly to an ice-cold solution of glycine (500 mg.) in aq. 10% (w/v) NaOH (5 ml.) with intermittent shaking, and the mixture shaken continuously at 25° for 3 hr. The pH was then adjusted to 6.5 and most of the unchanged 3,5-di-tert.-butyl-4-hydroxybenzoic acid removed with ether. Ether extraction of the residual aqueous phase at pH 1.8 gave a yellow crystalline residue (500 mg.), from which an unidentified component and a trace of 3,5-di-tert.-butyl-4-hydroxybenzoic acid were removed by extraction with light petroleum (b.p. 40–60°)–ethanol (5:2, w/v). Recrystallization of the residue from aq. 50% (v/v) ethanol gave 3,5-di-tert.-butyl-4-hydroxyhippuric acid as colourless plates (50 mg.), m.p. 182° (Found: C, 66.4; H, 8.0; N, 4.4. $C_{17}H_{25}NO_4$ requires C, 66.4; H, 8.1; N, 4.6%). (The mass-spectrometer showed that the parent ion occurred at M/e 307; Calc. for $C_{17}H_{25}NO_4$: mol. wt. 307.) After refluxing 3,5-di-tert.-butyl-4-hydroxyhippuric acid (5 mg.) with 2N-HCl, paper chromatography separated glycine, 3,5-di-tert.-butyl-4-hydroxybenzoic acid and unchanged starting material.

Separation of (3,5-di-tert.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid. Urine (pH 6.3) from a rabbit treated with 3,5-di-tert.-butyl-4-hydroxybenzoic acid was continuously extracted with peroxide-free ether for 24 hr. to remove unconjugated phenols. The aqueous phase was adjusted with N-H₂SO₄ to pH 1.8 and was continuously extracted with peroxide-free ether for 24 hr. The dry extract (anhydrous Na₂SO₄) was evaporated to a yellow gum (3.5 g.), an ethereal solution of which (50 mg.) was spotted on Whatman no. 1 filter paper and chromatographed with butan-1-ol saturated with 2N-NH₃. Two strips from each chromatogram were sprayed with naphtharesorcinol, and regions on the main chromatograms coincident with the location of the ester glucuronide on the strips were eluted with methanol. Evaporation left a pale gum (35 mg.), which was purified by rechromatography and elution to give chromatographically homogeneous (3,5-di-tert.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid (25 mg.) as a pale gum.

The latter (5 mg.), dissolved in 0.15M-sodium acetate buffer, pH 4.2 (10 ml.), was incubated with β -glucuronidase (500 units) at 37° for 3 hr., and the mixture extracted with ether. 3,5-Di-tert.-butyl-4-hydroxybenzoic acid was detected on paper chromatograms of the ether extract run in butan-1-ol–2N-NH₃ with Gibb's reagent (Table 4). Free glucuronic acid was detected with naphtharesorcinol (Tollens, 1908, 1910), and it was identified on paper chromatograms of the concentrated aqueous phase run in butan-1-

ol–acetic acid–water (4:1:1, by vol.) with naphtharesorcinol and *p*-anisidine hydrochloride (Hough; Jones & Wadman, 1950).

Isolation of methyl (3,5-di-tert.-butyl-4-hydroxybenzoyl tri-O-acetyl- β -D-glucopyranosid)uronate. Repetition of the foregoing separation gave an ether extract from the residual aqueous phase at pH 1.8, which was shown by paper chromatography to contain mainly (3,5-di-tert.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid and a trace of the unconjugated acid. A solution of this residue (4.1 g.) in dry ether was methylated with excess of diazomethane in ether. The reaction mixture was kept at 0° for 16 hr., when excess of solvent and reagent were removed, and the residue was acetylated at 25° with acetic anhydride (25 ml.) and anhydrous pyridine (25 ml.). The mixture was triturated with ice–water to yield an amorphous solid, which crystallized from aq. methanol as colourless prisms (2.5 g.), m.p. 138–140°. This material was chromatographed on a silicic acid column (see Table 5). (3,5-Di-tert.-butyl-4-hydroxybenzoyl tri-O-acetyl- β -D-glucopyranosid)uronate was eluted with 50% (v/v) ether in light petroleum (b.p. 40–60°), and crystallized from 2% (v/v) ether in light petroleum, as rosettes of prisms (2.2 g.), m.p. 140–142°, $[\alpha]_D^{25} - 34.0^\circ$ (c 1.0 in methanol) (Found: C, 59.2; H, 6.8. Calc. for $C_{28}H_{38}O_{12}$: C, 59.4; H, 6.8%). This compound was described by Dacre (1961) as needles or prisms, m.p. 137–138° (decomp.); $[\alpha]_D^{20} - 35.0^\circ$ (c 1.0 in methanol).

Experiments with animals. Adult rats (approx. 2 months old, 200 g. body wt.) were used (Porton strain maintained as a specific pathogen-free colony in this Laboratory), and kept on sticks of food prepared by baking a standard pellet diet with eggs in cellophane tubing at 80–100° for 12 hr. The animals were checked for general physical well-being and kept without food for 16 hr. before experimental use. During experiments, the animals were kept singly in glass metabolism cages permitting the separate collection of urine, faeces and expired gases. The cages (Jencons of Hemel Hempstead, Herts.), designed for the study of the elimination pattern of radioactivity in rats dosed with a ¹⁴C- or ³H-labelled compound, make use of some features previously described by Roth, Leifer, Hogness & Langham (1948). A solution of [¹⁴C]Ionox 100 (16 mg., 0.202 μ Ci/mg.) in 3 ml. of olive oil was administered to each rat by stomach tube. Unrestricted food and water were supplied, and the urine and faeces were collected daily and stored at –29°. In this experiment, the rats were killed after 11 days, and the alimentary canal was removed. The carcass plus viscera, the alimentary canal and the skin and hair were stored separately at –29°. During the time when ¹⁴CO₂ was being collected in the expired gases, the cages were ventilated at 700 ml./min. and the exit was attached to two traps in series containing 4N-NaOH.

In another experiment with two rats, a solution of 25 mg. of Ionol in 2 ml. of olive oil was administered to each rat by stomach tube. Unrestricted food and water were supplied, and the urine and faeces were collected daily for 3 days.

Adult hounds (2–3 years old; 10–15 kg. body wt.) were used (beagles maintained for 2–3 years as a closed colony in this Laboratory). They were maintained on a standard dry diet (Mor-meat). The animals were kept under observations for 7 days before experimental use, during which time they were checked for general physical well-being and for solidity of faeces. During experiments, the animals were housed singly in stainless-steel metabolism cages.

After a period of training in the metabolism cages, each dog swallowed successively a gelatin capsule containing 13.24 mg. of [^{14}C]Ionox 100 (0.766 $\mu\text{C}/\text{mg.}$) in 1 ml. of olive oil and a gelatin capsule containing 200 mg. of Ionox 100 carrier. Unrestricted food and water were supplied, and the urine and faeces were collected separately at daily intervals.

Adult rabbits (1–2 years old; 2.5 kg. body wt.) were used (New Zealand White strain maintained as a closed colony in this Laboratory), and kept on a standard pellet diet and supplemented occasionally with cabbage leaves. During experiments, the animals were housed singly in metabolism cages. Each rabbit was intubated with a solution of 1 g. of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid in 10 ml. of olive oil on 3 consecutive days. Unrestricted food and water were supplied, and the urine was collected separately at daily intervals for 4 days. No ill effects were observed in rabbits given three doses of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid.

Chromatography on a silicic acid column. Mallinckrodt (100 mesh) silicic acid was washed successively with hot methanol, diethyl ether and light petroleum (b.p. 40–60°). This silicic acid was dried at room temperature and reactivated at 120°. Individually lipid components were successively eluted from the columns with solvents of increasing polarity. Typical fractionations are set out in Tables 5 and 6.

Paper chromatography. Phenolic compounds and their metabolites were separated by descending chromatography on Whatman no. 1 filter-paper sheets with the solvent systems used in Table 4. The compounds were detected and identified on paper by spraying with the following reagents: (1) Gibb's reagent, a solution of 2,6-dichloro-*p*-benzoquinone-4-chloroimine (0.05%, w/v) in ethanol followed by aq. 4.75% (w/v) $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$ buffer, pH 9.3; (2) SbCl_5 (20%, v/v) in CCl_4 (2,6-di-*tert.*-butylphenols, which do not have a carboxyl substituent, give red colours with this reagent); (3) Altman reagent, a freshly prepared solution of *p*-dimethylaminobenzaldehyde (4%, w/v) in acetic anhydride containing a few crystals of sodium acetate, followed by heating for 2–3 min. at 130° (Gaffney, Schreier, DiFerrante & Altman, 1954) (all hippuric acids react to yield orange or orange-red colours, which are stable indefinitely, and which possess a yellow fluorescence in ultraviolet light); (4) naphtharesorcinol-trichloroacetic acid reagent, prepared by the method of Elliott, Parke & Williams (1959) (this reagent does not react with acetylated-methylated glucuronides).

Thin-layer chromatography. Kieselgel G (30 g.) (E. Merck A.-G., Darmstadt, Germany) was stirred with 60 ml. of water and layered on glass plates (20 cm. $\times$ 20 cm.) at a thickness of 0.25 mm. After 15 min. at room temperature, the plates were heated to 120° for 30 min., cooled in a desiccator for 30 min., and then spotted with the faecal metabolites, various mixtures of metabolites from urine or with pure compounds, 2 cm. from the base. The plates were placed in glass tanks (25 cm. $\times$ 25 cm.) containing 100 ml. of solvent and sealed. The solvent was allowed to ascend for 18 cm. The compounds were detected and identified on thin plates with the reagents that were used for paper chromatography. The solvent systems used were light petroleum (b.p. 60–80°)-diethyl ether (3:2, v/v), light petroleum-diethyl ether (1:4, v/v) and ethyl acetate-benzene (3:1, v/v).

Thin-layer chromatography was useful as a rapid method for detecting unconjugated 2,6-di-*tert.*-butylphenols. Ionox 100 has R_F 0.6 in the first solvent system and R_F 0.95 in the

third, and 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid has R_F 0.1 in the first solvent system and R_F 0.5 in the third. The last fractions from column chromatography of the faecal metabolites (Table 5) were run with the second solvent system, when radioautography revealed traces of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid, R_F 0.8, and of three unidentified components with R_F 0.4, 0.3 and 0.

Measurement of radioactivity. A Tri-Carb spectrometer (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) was used for the measurement of ^{14}C by liquid-scintillation counting. For the measurement of radioactivity in urine and in the acetone extract of faeces, portions were mixed with NE221 gel scintillator [Nuclear Enterprises (G.B.) Ltd., Edinburgh]. This method gave an efficiency 70% of the theoretical. Residues from the solvent extraction of faeces contained less than 5% of total ^{14}C in the faeces, and portions were burnt in the Schöniger flask by the method of Dobbs (1963). The ^{14}C combustion products were absorbed in 2-phenethylamine, which was incorporated in liquid scintillator (Dobbs, 1963), and the resulting solutions were measured with an efficiency 60% of the theoretical. The $^{14}\text{CO}_2$ from the expired gases was absorbed successively in NaOH and in a solution of ethanolamine in β -methoxyethanol; this solution is suitable for measurement of ^{14}C in the foregoing methods. Methanol extracts of the gut and contents were also mixed with NE221 scintillator for measurement of ^{14}C . Residues from the solvent extraction of the gut, and dried samples of wet homogenates of the carcass plus viscera and of the skin plus hair, were burnt in the Schöniger flask for measurement of ^{14}C (Dobbs, 1963).

RESULTS

In an experiment with six adult rats of each sex, which had been treated orally with 2,6-di-*tert.*-butyl-4-hydroxy [^{14}C]methylphenol ([^{14}C]Ionox 100), 10.6–70.8% of the original label was excreted in the urine and 75.2–27.0% in the faeces during 11 days. A very small proportion of the ^{14}C was found in the expired air from the same animals during 0–96 hr.

The investigation of excretion and retention of radioactivity in rats after the oral administration of [^{14}C]Ionox 100 (see Tables 1 and 2) necessitated a large number of measurements (650) of ^{14}C , and the balances of radioactivity were as accurate as those in less extensive investigations in which other isotopically labelled foreign organic compounds had been administered to animals (e.g. Alleva, 1963; Clark, Young, Younger, Hunt & McLaren, 1964; Fang, George & Freed, 1964; Goldenthal, Nadkarni & Smith, 1958; Marco & Jaworski, 1964).

Some ^{14}C remained in the gut of some of the animals at the end of 11 days (Table 1). The small concentration of ^{14}C found in the carcass and viscera remaining after removal of the gastrointestinal tract is 0.29 ± 0.02 (11), expressed as the mean $\pm$ s.e.m. in terms of percentage of dose. A very small percentage of the original label is associated with the skin and hair at the end of the experiment (Table 1). This may be due to the

Table 1. *Excretion and retention of radioactivity in rats after oral administration of [¹⁴C]Ionox 100*

Male and female rats (200 g. body wt.) were treated by stomach tube with single doses (250 mg./kg.) of [¹⁴C]Ionox 100 (0.1936 μ C/mg.) dissolved in 3.0 ml. of olive oil. In dosing the sixth female rat sustained injury to a lung that was detected *post mortem*, during the course of the experiment.

Rat no.	Sex	Recovery of ¹⁴ C (% of dose)						Total
		Faeces (11-day collection)	Urine (11-day collection)	Expired gases (96 hr. collection)	Gut and contents	Carcass and remaining viscera	Skin and hair	
1	♂	75.2	16.8	0.4	0.2	0.2	<0.1	92.8
2	♂	40.1	53.4		0	0.2	0.1	94.2
3	♂	43.6	63.2		<0.1	0.2	0.3	107.7
4	♂	70.3	23.6		0.4	0.3	0.1	95.1
5	♂	67.0	31.5		0.3	0.3	0.2	99.7
6	♂	73.1	15.6		0.3	0.3	0.3	90.0
1	♀	29.8	70.3	<0.1	0	0.5	0.1	100.7
2	♀	33.1	57.2		0	0.2	0.3	90.8
3	♀	58.2	35.1		0.4	0.5	0.3	94.5
4	♀	55.1	34.5		0	0.2	0.1	89.9
5	♀	27.0	70.8		0	0.3	0.2	98.3

Table 2. *Rates of faecal and urinary elimination of radioactivity in rats after oral administration of [¹⁴C]Ionox 100*

Male and female rats were treated with [¹⁴C]Ionox 100 as described in Table 1. The concentrations in faeces refer to that proportion of the radioactivity extractable with acetone from wet faeces.

Rat no.	Sex	¹⁴ C eliminated (% of dose)													
		0-24 hr.		24-48 hr.		48-72 hr.		72-96 hr.		96-120 hr.		120-144 hr.		144-264 hr.	
		Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
1	♂	3.0	4.1	31.7	4.1	15.5	2.9	10.6	1.4	2.1	2.6	4.7	0.8	3.0	1.0
2	♂	12.1	20.5	20.6	11.8	2.4	17.1	0.4	2.2	0.6	1.4	0.3	0	0.2	0.4
3	♂	2.0	11.3	13.0	24.3	14.9	21.2	5.2	2.8	0.7	1.9	0.6	0.7	1.7	1.0
4	♂	17.5	5.1	25.3	6.6	11.1	5.1	4.3	2.0	4.1	2.6	2.1	0.8	2.0	1.3
5	♂	0	1.1	25.4	10.6	19.6	13.3	10.1	2.6	3.2	2.0	2.4	0.7	1.6	1.3
6	♂	16.2	5.4	24.6	4.3	13.1	2.6	6.6	1.1	1.3	1.4	4.3	0	1.9	0.8
1	♀	0.4	8.6	3.1	15.8	8.5	23.3	13.1	7.3	0.8	6.8	0.1	1.6	0.9	6.9
2	♀	3.6	10.3	9.3	14.9	3.0	12.6	8.8	10.0	3.9	4.5	1.1	2.5	0.8	2.4
3	♀	0.1	3.6	16.7	4.2	11.3	8.0	11.4	6.8	5.1	6.1	4.6	3.5	3.1	2.9
4	♀	10.2	12.5	12.5	15.0	20.1	7.3	5.2	3.8	1.5	3.6	0.7	1.2	3.1	1.1
5	♀	4.0	8.8	13.9	34.9	5.3	19.9	1.1	3.3	0.4	2.4	0.3	0.7	0.3	0.7

slight contamination of the hair and skin with either radioactive faeces or urine.

The rates of faecal and urinary elimination of radioactivity by rats given [¹⁴C]Ionox 100 are summarized in Table 2. During 96 hr., 12.5-66.9% of the ¹⁴C was excreted in the urine and 60.8-24.3% in the faeces. The big variation in the elimination pattern of individual animals suggested that predominantly the same primary metabolic product(s) is (are) eliminated by the faecal and urinary routes. Very small amounts of ¹⁴C are excreted in rat faeces and urine during 144-264 hr. and the animals then contain little ¹⁴C in the carcass and viscera. At the end of 4 days, the radioactivity eliminated from these rats was 72.5-94.7% of the dose compared with the 87.1-90.3%

eliminated from male and female dogs during the same period (Table 3). In dogs, excretion of ¹⁴C in the faeces is the main eliminative route: 68.5-78.8% of the original label was consistently eliminated in the faeces during 4 days.

After the oral administration of [¹⁴C]Ionox 100, the rate of elimination and recovery of radioactivity from animals does not seem to be affected by the size of the dose nor by the presence of triglyceride fat in the diet. Thus single doses of 250 mg./kg. and 20 mg./kg. were given to rats and approx. 17.62 mg./kg. to dogs. Rats were intubated with a solution of the antioxidant in 3 ml. of olive oil, and dogs were given a gelatin capsule containing unlabelled Ionox 100 immediately after swallowing a gelatin capsule containing [¹⁴C]Ionox 100 dissolved in

Table 3. Rates of faecal and urinary elimination of radioactivity in male and female dogs after oral administration of [^{14}C]Ionox 100

Each dog swallowed successively a gelatin capsule containing a weighed dose (13.24 mg.) of [^{14}C]Ionox 100 (0.766 $\mu\text{C}/\text{mg.}$) in 1 ml. of olive oil and a gelatin capsule containing 200 mg. of Ionox 100 carrier.

Dog no.	Body wt. (kg.)	Sex	¹⁴ C eliminated (% of dose)								Total
			0-24 hr.		24-48 hr.		48-72 hr.		72-96 hr.		
			Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	
1	14.8	♂	No faeces	6.6	51.4	3.5	18.8	2.0	4.0	0.8	87.1
2	13.0	♂	18.1	9.5	39.8	5.3	6.9	1.9	4.8	1.4	87.6
3	10.4	♀	19.7	13.2	29.2	3.0	16.5	2.4	4.4	1.0	89.3
4	10.2	♀	57.3	7.9	11.3	2.3	8.0	1.0	2.2	0.3	90.3

Table 4. R_F values on paper and colour reactions of Ionox 100 and its possible metabolites

R_F values in parentheses are those of Dacre (1961).

	R_F value in solvent system		Colour with reagent			
	Butan-1-ol-2N-NH ₃ (1:1, v/v)	Butan-1-ol-water (1:1, v/v)	Gibb's reagent	SbCl ₅	Altman reagent	Naphtha-resorcinol
Ionox 100	0.93	0.90	Magenta	Red	None	None
3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoic acid	0.70	0.90	Magenta	None	None	None
3,5-Di- <i>tert.</i> -butyl-4-hydroxyhippuric acid	0.63 (0.10)	0.60 (0.50-0.55)	Magenta	None	Orange*	None
(3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid	0.45	0.40	Magenta	None	None	Blue

* The orange colour has a yellow fluorescence in ultraviolet light.

1 ml. of olive oil. Absence of fat in the diet did not affect the rate of elimination of Ionox 100 metabolites from dogs.

Radioactivity was recovered quantitatively from the urine of rats that had been treated with [^{14}C]Ionox 100 by successive ether extraction at pH 6.0 and pH 1.5-2.0. The ether extract prepared at pH 6.0 from the 11-day urine contained half the amount of ^{14}C found in that prepared at pH 1.5-2.0. Paper chromatograms of representative samples of the ether extracts from each of the 24 hr. urines from each of the 11 rats were run in two solvent systems and examined for radioactivity. Ionox 100 was not present amongst the metabolites in the urine. The single peak of ^{14}C in extracts prepared at pH 6.0 corresponded to 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid. The major peak of ^{14}C (95% of radioactivity in extract) in extracts prepared at pH 1.5-2.0 corresponded to (3,5-di-*tert.*-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid, which was also located by Gibb's reagent and naphtharesorcinol on paper chromatograms (Table 4) of ether extracts similarly prepared from the

urine of rabbits dosed with 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid. There was also a small peak of ^{14}C (4% of radioactivity in extract) due to the uncombined acid and a peak of ^{14}C (1% of radioactivity in extract) due to an unknown component of R_F 0.1 in the butan-1-ol-ammonia solvent.

On paper chromatograms of extracts prepared from the urine of dogs dosed with [^{14}C]Ionox 100, nearly all the radioactivity corresponded to the ester glucuronide; traces of the unconjugated acid and of the unknown component of R_F 0.1 in the butan-1-ol-ammonia solvent were also present.

3,5-Di-*tert.*-butyl-4-hydroxybenz[^{14}C]oic acid was isolated from the ether extract of the urine (pH 6.0) of rats treated with [^{14}C]Ionox 100. In addition to (3,5-di-*tert.*-butyl-4-hydroxybenz[^{14}C]oyl β -D-glucopyranosid)uronic acid, the ether extract prepared from the urines at pH 1.5-2.0 contained natural urinary constituents and a variable amount of the unconjugated 3,5-di-*tert.*-butyl-4-hydroxybenz[^{14}C]oic acid, owing to the long standing of the urine, before extraction of the main portion for the isolation of metabolic products. The crude reaction

product from successive methylation and acetylation was accordingly chromatographed on a silicic acid column (Table 5). Two bands of radioactivity were separated, which contained respectively methyl 3,5-di-*tert*.-butyl-4-hydroxybenz[^{14}C]oate, and methyl (3,5-di-*tert*.-butyl-4-hydroxybenz[^{14}C]-oyl tri-*O*-acetyl- β -D-glucopyranosid)uronate (Table 5) identical with material obtained from the

metabolism of 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid in rabbits and isolated by column chromatography on silicic acid from the crude reaction product after methylation and acetylation. 3,5-Di-*tert*.-butyl-4-hydroxybenz[^{14}C]oic acid, which accounts for 96% of the ^{14}C in extracts of the faeces, was separated from the total lipid fraction of rat faeces by column chromatography on silicic acid

Table 5. *Separation by adsorption chromatography on silicic acid of the methylated and acetylated derivatives of the ether-soluble metabolites of [^{14}C]Ionox 100 from rat urine*

A portion of the reaction product from the methylation and acetylation of the ether-soluble fraction of the urine of rats (200g. body wt.) that had been treated orally with 250mg. of [^{14}C]Ionox 100 (0.1936 $\mu\text{C}/\text{mg.}$)/kg. was chromatographed on a silicic acid column (15mm. diam. $\times$ 15cm. long). Total recovery was 477.6mg. from a fraction weighing 492mg.

Fraction no. (100ml.)	Eluent	Residue (mg.)	Recovery of radioactive constituents
1-3	5% Ether in light petroleum (b.p. 40-60°)	22	m.p. 162°; 30% of ^{14}C added to the column: methyl 3,5-di- <i>tert</i> .-butyl-4-hydroxybenzoate crystallized from cyclohexane, forming plates (20mg.), m.p. and mixed m.p. with an authentic specimen 165°
4-6	25% Ether in light petroleum	77.4	7% of ^{14}C : trace amount of methyl (3,5-di- <i>tert</i> .-butyl-4-hydroxybenzoyl 2,3,4-tri- <i>O</i> -acetyl- β -D-glucopyranosid)uronate present
7	50% Ether in light petroleum	87.8	52% of ^{14}C : methyl (3,5-di- <i>tert</i> .-butyl-4-hydroxybenzoyl 2,3,4-tri- <i>O</i> -acetyl- β -D-glucopyranosid)uronate crystallized from ether in light petroleum, forming rosettes of prisms (26mg.), m.p. 137.5-138.5° and mixed m.p. with an authentic specimen (of m.p. 140-142°), 138-140° (Found: C, 59.2; H, 6.8. Calc. for $\text{C}_{28}\text{H}_{38}\text{O}_{12}$: C, 59.4; H, 6.8%)
8-10	50% Ether in light petroleum	55.4	8.2% of ^{14}C
11	Ether	215.9	2.8% of ^{14}C
12	Methanol	20	0.2% of ^{14}C

Table 6. *Separation of metabolites of [^{14}C]Ionox 100 from the acetone-soluble fraction from rat faeces by adsorption chromatography on silicic acid*

A proportion of the total lipid fraction from the faeces of rats (200g. body wt.) that had been treated orally with 250mg. of [^{14}C]Ionox 100 (0.1936 $\mu\text{C}/\text{mg.}$)/kg. was chromatographed on a silicic acid column (30mm. diam. $\times$ 25cm. long). Recovery was quantitative.

Fraction no. (500ml.)	Eluent	Residue (mg.)	Recovery of radioactive constituents
1	Light petroleum (b.p. 40-60°)	12.7	
2	2.5% Ether in light petroleum	990.4	
3	5% Ether in light petroleum	129.3	
4	7.5% Ether in light petroleum	45.4	1% of ^{14}C
5	10% Ether in light petroleum	78.8	95% of ^{14}C : 3,5-di- <i>tert</i> .-butyl-4-hydroxybenzoic acid, m.p. 210°, unchanged by admixture with an authentic sample, was separated with alkali
6	10% Ether in light petroleum		
7	15% Ether in light petroleum	44.3	
8	20% Ether in light petroleum	5.6	
9	50% Ether in light petroleum	94.3	
10	Ether	167.4	3% of ^{14}C : radioautograms of thin-layer plates show 3,5-di- <i>tert</i> .-butyl-4-hydroxybenzoic acid and three slow-moving constituents
11	Methanol	295.8	1% of ^{14}C : two slow-moving constituents

(Table 6). The late fractions contained traces of three slow-moving radioactive components. Unchanged Ionox 100 was not eliminated in the faeces.

The detection of small amounts of 3,5-di-*tert*.-butyl-4-hydroxyhippuric acid in the urine of rabbits treated orally with 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid has been claimed (Dacre, 1961), but after repetition of the work we could not find it. Some suspicion attached to the properties reported for this substance by Dacre (1961), since the glycine conjugate would have been expected to give a deep-orange colour with Altman's reagent, which should fluoresce yellow in ultraviolet light, and since the R_F of the glycine conjugate would have been expected to exceed that of the ester glucuronide in butan-1-ol-ammonia. By Schotten-Baumann reaction of 3,5-di-*tert*.-butyl-4-hydroxybenzoyl chloride and glycine in caustic alkali, we accordingly synthesized a small quantity of authentic 3,5-di-*tert*.-butyl-4-hydroxyhippuric acid, which had the colour reactions and R_F values (Table 4) that had been predicted for it; it was absent from the urine of rats dosed with Ionox 100.

In conclusion, among the metabolic products of Ionox 100 in the rat, (3,5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid accounts for 10–47% of the dose and 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid accounts for 85–50% of the dose. In the dog, (3,5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid accounts for 10–12% of a dose of Ionox 100 and 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid for 85% of the dose.

DISCUSSION

The expected general reaction of a primary aromatic alcohol in the animal body is oxidation to the corresponding aromatic acid, which is usually excreted as an ester glucuronide and a glycine conjugate. Benzyl (Bray, Thorpe & White, 1951), phenethyl (El Masry, Smith & Williams, 1956; Smith, Smithies & Williams, 1954) and hydratropyl alcohols (Gruneberg & Langecker, 1957; Robinson, Smith & Williams 1955) are metabolized in this way, and the metabolism of Ionox 100 is therefore typical of an aromatic primary alcohol. There is no evidence that the aromatic nucleus undergoes hydroxylation, or that a *tert*.-butyl substituent is oxidized. The ester glucuronide is excreted in the urine together with variable quantities of uncombined aromatic acid. No glycine conjugate is synthesized by herbivores (rabbits), omnivores (rats) or carnivores (dogs), and this may be due to the lack of reactivity of 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid for the free glycine in the first glycine pool (Arnstein & Neuberger, 1951).

The two structurally related antioxidants, Ionol (2,6-di-*tert*.-butyl-*p*-cresol) (I) and Ionox 100 (II) (Scheme 1), would be expected to have some metabolic products in common, and the present work shows that 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid (III) and the related ester glucuronide (IV) are the major metabolites of Ionol and Ionox 100 in rats. The fact that Ionol is oxidized *in vivo* at the 4-methyl group is contrary to the conclusion

Scheme 1. Metabolism of Ionox 100 (II) and Ionol (I) in the rat.

drawn from the work of Goldber, Ryan & Wright (1962) and Ladomery, Ryan & Wright (1963), who measured the elimination of ^3H and ^{14}C in the urine of rats dosed intraperitoneally with [^3H]Ionol and [^{14}C]Ionol respectively, but the design of these experiments would not have detected oxidation of a substantial proportion of the 4-methyl group. Scheme 1 shows the interrelationship of the metabolic pathways of Ionol and Ionox 100.

The elimination of the metabolites of Ionox 100 from rats is undoubtedly more rapid than that of Ionol and its metabolic products, and, unlike Ionol, unchanged Ionox 100 could not be detected in the bodies of these animals. Thus rats, given [^{14}C]Ionox 100 orally, eliminated ^{14}C equivalent to 72.5–94.7% of the dose during 4 days, whereas rats dosed with [^3H]Ionol excreted ^3H equivalent to 20.0–49.0% of dose in the urine during that time (Goldber *et al.* 1962). Ionox 100 is completely metabolized in rats and, when elimination was complete, no unchanged Ionox 100 could be detected in extracts of the organs and tissues. The lipid-soluble Ionol is more stable to oxidation *in vivo* than lipid-soluble Ionox 100, and some deposition of unchanged Ionol in adipose tissue has been reported after high dosage of Ionol to rats (Johnson, O'Halloran & Hewgill, 1958).

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