The Metabolism of Di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl) Ether (Ionox 201) in the Rat

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1. Up to one-third of a single oral dose of Ionox 201 was absorbed in rats. 2. In rats dosed with [^{14}C]Ionox 201 86.8–97.2% of the label is excreted in the faeces in 24 days (much of this is eliminated in the first 4 days after dosage), 5.6% in the urine and not more than 0.8% in the exhaled air; 5.0% of ^{14}C is present in the carcass and viscera after removal of the gut, and most of this is in the fatty tissues. 3. About 65.0% of ^{14}C in the faeces is due to unchanged antioxidant, 30.0% to 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid, 3.5% to unidentified polar constituent(s), 1.4% to 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde and 0.1% to 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone. A variable proportion of ^{14}C in the urine is due to 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid (40–60%) and the remainder (60–40%) to the ester glucuronide, when the animals were treated with different doses of antioxidant. In eight individual animals dosed with 6.78 mg. of [^{14}C]Ionox 201, one-third of ^{14}C in the bile is due to the free acid, 45% to the ester glucuronide, 20% to an unidentified constituent and 2% to unchanged antioxidant, and, in two animals dosed with 13.56 mg., there is a small proportion of free acid and a larger proportion of ester glucuronide. About 80% of ^{14}C in the body fat is due to unchanged antioxidant, 19% to the free acid and 1% to 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde. 4. At least 36.2% of a single oral dose of Ionox 201 is metabolized: 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid accounts for 30.2% of a dose, (3,5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid for 1.4%, 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde for 1.3%, 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone for 0.1% and unidentified polar metabolite(s) for 3.2%. 5. The metabolism of Ionox 201 *in vivo* is closely related to its antioxidant action *in vitro*.

Di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl) ether (I) (Fig. 1) (Ionox 201), which is an effective antioxidant in certain mineral oils, protects high-impact polystyrene and vulcanizates based on polyisoprene rubber. Ionox 201 is also effective in polyolefins, which may also be used as food-wrapping materials, and these potential commercial applications make an understanding of the metabolic fate of Ionox 201 desirable.

Ionox 201 is one of a number of structurally-related hindered phenolic antioxidants, of which 2,4,6-tri-(3,5-di-*tert*.-butyl-4-hydroxybenzyl)mesitylene (II) (Ionox 330) is not absorbed from the gastro-intestinal tract (Wright, Crowne & Hathway, 1965a), 2,6-di-*tert*.-butyl-4-hydroxymethylphenol (III) (Ionox 100) is quantitatively absorbed and metabolized (Wright, Akintonwa, Crowne & Hathway, 1965b) and di-(3,5-di-*tert*.-butyl-4-hydroxyphenyl)methane (IV) (Ionox 220) is partly absorbed and partly metabolized (Wright, Crowne & Hathway, 1966). Investigation of the alimentary absorption of another hindered phenolic anti-

oxidant, e.g. Ionox 201, with molecular properties intermediate between those of Ionox 100 and Ionox 330 is required to put the behaviour of the three antioxidants *in vivo* into perspective.

MATERIALS

General. All melting points, which were determined on a Kofler block, are uncorrected.

Ionox 201. This antioxidant was prepared by the method of Morris & Sullivan (1963), described below for the preparation of the radioactive chemical. Ionox 201 crystallized from *n*-hexane as chunky prisms, m.p. 137° [Morris & Sullivan (1963) give m.p. 137°] (Found: C, 79.3; H, 10.2. Calc. for $\text{C}_{30}\text{H}_{46}\text{O}_3$: C, 79.2; H, 10.2%).

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

Ionox 220. Di-(3,5-di-*tert*.-butyl-4-hydroxyphenyl)methane (Ionox 220) (Wright *et al.* 1966) had m.p. 155–156°.

*3,5-Di-*tert*.-butyl-4-hydroxybenzaldehyde.* This compound, prepared by bromine oxidation of Ionol (Coppinger & Campbell, 1953), was crystallized from *n*-hexane as needles, m.p. 190° (Found: C, 76.8; H, 9.3. Calc. for $\text{C}_{15}\text{H}_{22}\text{O}_2$: C, 76.9; H, 9.5%).

*3,5-Di-*tert*.-butyl-4-hydroxybenzoic acid.* This compound,

Fig. 1. Hindered phenolic antioxidants.

prepared by Cannizzaro reaction of 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde (Yohe *et al.* 1956), crystallized from cyclohexane as chunky prisms, m.p. 210°.

(3,5-Di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)-uronic acid. This compound, which was isolated from the urine of a rabbit that had been intubated with 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid by the method of Wright *et al.* (1965b), ran as a single spot in two solvent systems (Table 6) by using paper chromatography and naphtharesorcinol-trichloroacetic acid as location reagent (Elliott, Parke & Williams, 1959).

2,6-Di-*tert*.-butyl-p-benzoquinone. This compound, prepared by trifluoroacetic anhydride oxidation of 2,6-di-*tert*.-butylphenol (Chambers, 1959), crystallized from methanol in orange-yellow prisms, m.p. 68° (Found: C, 76.4; H, 9.0. Calc. for $C_{14}H_{20}O_2$: C, 76.3; H, 9.2%).

3,3',5,5'-Tetra-*tert*.-butyl-4,4'-diphenquinone. 2,6-Di-*tert*.-butylphenol was oxidized by nitric acid to 3,3',5,5'-tetra-*tert*.-butyl-4,4'-diphenquinone, m.p. 246° (Found: C, 82.5; H, 9.8. Calc. for $C_{28}H_{40}O_2$: C, 82.3; H, 9.9%) (Hart & Cassis, 1951). The spectral characteristics of this compound, in iso-octane, are as follows: λ_{max} . 254, 263, 272, 406 and 425 m μ (ϵ_{max} . 4700, 5500, 5000, 58800 and 84000 respectively) [Kharasch & Joshi (1957) give λ_{max} . 260, 270 and 418 m μ (ϵ_{max} . 4300, 4300 and 84000 respectively)].

3,3',5,5'-Tetra-*tert*.-butyl-4,4'-stilbenequinone. This compound was prepared by oxidation with molecular oxygen of an aqueous solution of 2,6-di-*tert*.-butyl-4-methyl-4-nitro-2,5-cyclohexadien-1-one, which was itself prepared by the action of cold HNO_3 on Ionol (Kharasch & Joshi, 1957). 3,3',5,5'-Tetra-*tert*.-butyl-4,4'-stilbenequinone had m.p. 317° (Found: C, 83.0; H, 9.7. Calc. for $C_{30}H_{42}O_2$: C, 82.9; H, 9.7%). Its spectral characteristics in ethanol are as follows: λ_{max} . 242, 422 and 450 m μ (ϵ_{max} . 7500, 75000 and 112000 respectively) [Kharasch & Joshi (1957) give λ_{max} . 442 m μ (ϵ_{max} . 72000)].

Di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl). 3,3',5,5'-Tetra-*tert*.-butyl-4,4'-stilbenequinone (20g.) in 500 ml. of cyclohexane was hydrogenated at 1250 lb./in.² at 90° with 10g. of Raney nickel as catalyst (J. L. Van Winkle, F. C. Davis & W. J. Sullivan, personal communication). Recrystallization of the crude reaction product from iso-octane and methanol successively afforded di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl) as colourless needles (18g.), m.p. 171° (Found: C, 82.2; H, 10.6. Calc. for $C_{30}H_{46}O_2$: C, 82.1; H, 10.6%).

2,6-Di-*tert*.-butyl-4-(3,5-di-*tert*.-butyl-4-oxocyclohexa-2,5-dienylidene)methylphenol. This stable phenoxy radical, prepared from Ionox 220 by the method of Coppinger (1957), was obtained from hexane as deep-blue crystals, m.p. 159°

(Found: C, 82.6; H, 9.6. Calc. for $C_{29}H_{41}O_2$: C, 82.6; H, 9.8%).

3,5-Di-*tert*.-butyl-4-hydroxyphenyl-(2,6-di-*tert*.-butyl-p-benzoquinone)methide (the quinonemethide). Coppinger's stable phenoxy radical was reduced with quinol in ether to the quinonemethide (Coppinger, 1957). Recrystallization of the quinonemethide from *n*-hexane afforded orange-yellow needles, m.p. 157–158° (Found: C, 82.4; H, 10.0. Calc. for $C_{29}H_{42}O_2$: C, 82.4; H, 10.0%).

Bovine liver β -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 A.R. grade. Diethyl ether was dried over sodium and redistilled over reduced iron immediately before use.

Radioactive chemicals. Di-(3,5-di-*tert*.-butyl-4-hydroxybenz[^{14}C]yl) ether ([^{14}C]Ionox 201) was synthesized by the action of a catalytic quantity (600 mg.) of aq. 55% (w/w) H_2SO_4 on 2,6-di-*tert*.-butyl-4-hydroxy[^{14}C]methylphenol (354 mg.; specific activity 1.75 μC /mg.) in methylene chloride (2 ml.). After 2.5 hr., the solvent was washed free from acid and dried (over $MgSO_4$). Evaporation under N_2 gave crude [^{14}C]Ionox 201 (343 mg.), which was purified by column chromatography on acid-washed alumina of activity III (Brockmann & Schodder, 1941). [^{14}C]Ionox 201 (specific activity 1.82 μC /mg.) was eluted with light petroleum (b.p. 40–60°)-diethyl ether (19:1, v/v) and crystallized from *n*-hexane in prisms (yield, 95% of the theoretical), m.p. 137°. The ^{14}C -labelled antioxidant behaved identically with an authentic specimen of unlabelled Ionox 201 on thin-layer plates of Kieselgel G run with light petroleum (b.p. 40–60°)-diethyl ether (19:1, v/v) and (3:2, v/v) solvents (Table 6). Radioautography did not reveal radioactive impurities. The ^{14}C purity of the radioactive chemical exceeded 99.9%.

2,6-Di-*tert*.-butyl-4-hydroxy[^{14}C]methylphenol ([^{14}C]Ionox 100) (specific activity 1.75 μC /mg.) was synthesized by treating a solution of 2,6-di-*tert*.-butylphenol in *tert*.-butyl alcohol with [^{14}C]paraformaldehyde in the presence of a catalytic amount of potassium *tert*.-butoxide, as described by Wright *et al.* (1965b).

L-[^{35}S]Cystine (specific activity 450 μC /mg.) was obtained from The Radiochemical Centre, Amersham, Bucks.

METHODS

Experiments with animals. Adult rats (approx. 3 months old; 200g. body wt.) were used for most of this work (Porton strain, maintained as a specific pathogen-free colony in this Laboratory), but one experiment was made with young axenic rats (approx. 1 month old; 50g. body wt.) (supplied by Charles River Mouse Farms Inc., Wilmington, Mass., U.S.A.), and another experiment was made with a male adult hound of 13.5 kg. body wt. selected from a beagle pack maintained for 5 years as a closed colony in this Laboratory.

For the metabolism experiments the rats were kept in all-glass metabolism cages under the conditions described by Wright *et al.* (1965b). In an experiment with adult rats a solution of [^{14}C]Ionox 201 (6.78 mg.; 1.82 μC /mg.) in 2 ml. of olive oil was administered to each animal by stomach tube. Unrestricted food and water were supplied, and the urine and faeces were collected daily and stored at -29° . The CO_2 , exhaled by a pair of these rats (one of each sex) during

the first 48 hr. after dosage, was trapped in 4N-NaOH. Pairs of animals (one of each sex) were killed 1, 4, 8, 16 and 24 days after dosage, when the entire alimentary canal and contents, the skin plus hair, the whole liver and a standard amount of intra-abdominal and subcutaneous fats were dissected from each cadaver. These organs and tissues, together with the carcass and remaining viscera, were stored at -29° .

In the experiment with two axenic rats of each sex, a solution of [^{14}C]Ionox 201 (6.78 mg.; 1.82 μC /mg.) in 1.0 ml. of olive oil was administered by stomach tube to each animal. Urine and faeces, which were collected daily for 4 days, were stored at -29° . Samples of faeces taken directly from the anus of these animals were examined for their bacterial flora. No growth was observed.

Biliary fistulae were established in rats that had previously been intubated with 2.0 ml. of an olive oil solution containing either [^{14}C]Ionox 201 (6.78 or 13.56 mg.; 1.82 μC /mg.) or [^{14}C]Ionox 100 (6.0 mg.; 1.75 μC /mg.). After recovery from the operation, the animals were placed in individual restraining cages, where they had access to unrestricted food and water. Bile was usually collected during 1.0–30.0 hr. after dosage in 2 ml. portions, by using an automatic fraction collector. These animals were killed at the end of the experiment. In an experiment with one male and one female rat, urine and bile were collected during 0.25–24.0 hr. after receiving the dose (6.78 mg. of [^{14}C]Ionox 201; 1.82 μC /mg.). The animals were killed at 24.0 hr. and the entire alimentary canal was carefully removed from the skinned carcasses, which were stored at -29° .

In another experiment, 2.0 ml. of aq. L-[^{35}S]cystine solution (10 μC /ml.) was administered, by stomach tube, to two male rats on 5 consecutive days. On the fifth day, each rat was intubated with 2.0 ml. of a solution of Ionox 201 (7 mg.) in olive oil. Biliary fistulae were established 1.0 hr. after dosage, and bile was collected as described above. The rats were killed at the end of the experiment. This experiment is based on work of Gutmann & Wood (1950) and Marsden & Young (1958).

In the dog experiment, a starved animal was given a gelatin capsule containing 250 mg. of Ionox 201, followed by a meal of Mor-meat. After 4 hr., the animal swallowed a second gelatin capsule containing 250 mg. of Ionox 201 and another meal of Mor-meat. The dog was killed, by an overdose of intravenously administered sodium pentobarbitone, 6 hr. after swallowing the first capsule, and the gall bladder containing 30 ml. of bile was promptly removed from the carcass.

Extraction of radioactivity. Extraction of ^{14}C from urine, bile, faeces and tissues was carried out as described by Wright *et al.* (1966); 98% of the ^{14}C was recovered from urine, 97% from the bile, 97% from faeces and more than 95% from tissues. Evaporations were carried out in N_2 under reduced pressure at below 35°.

Paper chromatography. Ionox 201 and its metabolites in bile and urine were separated by descending chromatography on Whatman no. 1 filter-paper sheets at 25° with the solvent systems shown in Table 6.

Thin-layer chromatography. Thin-layer chromatography on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) was carried out at 20° as described by Wright *et al.* (1965b). A Kieselgel G layer-thickness of 0.25 mm. was used, except for preparative thin-layer chromatography when the layer-thickness was 0.75 mm. Thin-layer chromatography was

used for the separation of Ionox 201 and its metabolites in faeces and tissues, as well as for the separation of PbO₂-oxidation products. The separation of lipids and lipid-soluble compounds was effected in the dark and the quinonemethide was purified in an atmosphere of N₂.

Detection of Ionox 201 and its metabolites on chromatograms. All ¹⁴C-labelled constituents were detected by radioactive scanning with a Nuclear-Chicago Actigraph II, equipped with a gas-flow cell utilizing a helium + *n*-butane (98.5:1.5) mixture. Radioautography was also used. The antioxidant, its metabolites and oxidation products were also detected on paper and thin-layer plates with the following location reagents: (1) Gibb's reagent, applied as described by Wright *et al.* (1965b); (2) carbon tetrachloride-SbCl₅ (Wright *et al.* 1965a); (3) the quinonemethide and Coppinger's stable phenoxy radical gave a characteristic magenta colour with NH₃; (4) naphtharesorcinol-trichloroacetic acid reagent, prepared by the method of Elliott *et al.* (1959), was used for the detection of the ester glucuronide on paper chromatograms.

Electrolyte solutions. Formic acid-acetic acid buffer, pH 2.0, was prepared by diluting a mixture of 98–100% formic acid (78 ml.) and acetic acid (148 ml.) with water to 2.5 l. Phosphate-NaOH buffer, pH 8.0, was made by mixing 500 ml. of 0.2 M-KH₂PO₄ and 468 ml. of 0.2 N-NaOH.

High-voltage electrophoresis. Electrophoresis (120 v/cm.) of the radioactive components from the bile of rats dosed with [¹⁴C]Ionox 201 was carried out in formic acid-acetic acid buffer, pH 2.0, and in phosphate-NaOH buffer, pH 8.0, by the method used by Griffiths, Moss, Rose & Hathway (1966).

Measurement of radioactivity. A Tri-Carb Spectrometer (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) was used for the measurement of ¹⁴C by liquid-scintillation counting. Measurements of radioactivity in urine, bile, faeces, gut and contents, carcass plus viscera, skin plus hair, liver, intra-abdominal and subcutaneous fats, eluates of excised spots from thin-layer plates and eluates of bands from paper chromatograms were made by the methods

described by Wright *et al.* (1965b). The ¹⁴CO₂ in the exhaled air, which had previously been trapped in 4 N-NaOH, was regenerated by the addition of acid and reabsorbed into a liquid scintillator (Dobbs, 1963) for the measurement of radioactivity.

Enzymic hydrolysis of 3,5-di-*tert*.-butyl-4-hydroxybenzoyl β-D-glucopyranosiduronic acid. This was carried out by using β-glucuronidase as described by Wright *et al.* (1966).

Oxidation of Ionox 201 by lead dioxide. A solution of Ionox 201 (5 g.) in *n*-hexane (200 ml.) was stirred with a suspension of PbO₂ (10 g.) for 6 hr. Lead oxides were removed and the filtrate was concentrated. Thin-layer chromatography revealed the presence of ten components including polar material remaining at the origin (see Table 1). About 90% of the major component, 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde, was removed by crystallization from the concentrate at 0°. 3,3',5,5'-Tetra-*tert*.-butyl-4,4'-diphenquinone, 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone, 2,6-di-*tert*.-butyl-*p*-benzoquinone, Ionox 201, 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde and 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid were separated in crystalline form by preparative thin-layer chromatography: 3,3',5,5'-tetra-*tert*.-butyl-4,4'-diphenquinone had m.p. 246° and mixed m.p. 246° with an authentic specimen (m.p. 246°) (Found: C, 82.5; H, 9.8%); 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone had m.p. 320° and mixed m.p. 318° with an authentic specimen (m.p. 317°) (Found: C, 83.0; H, 9.8%); 2,6-di-*tert*.-butyl-*p*-benzoquinone had m.p. 67° and mixed m.p. 67° with an authentic specimen (m.p. 68°) (Found: C, 76.4; H, 9.0%); Ionox 201 had mixed m.p. 136–137° with an authentic specimen (m.p. 137°); 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde had m.p. 190° and mixed m.p. 190° with an authentic specimen (m.p. 190°) (Found: C, 76.8; H, 9.4%); 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid had m.p. 210° and mixed m.p. 210° with an authentic specimen (m.p. 210°) (Found: C, 72.0; H, 8.7%. Calc. for C₁₅H₂₂O₃: C, 72.0; H, 8.9%).

Concentrated solutions of the quinonemethide and di-

Table 1. *Approximate proportions, R values on thin-layer plates and colour reactions of the products derived from Ionox 201 by lead dioxide oxidation*

At the end of the reaction period (6 hr.) there was less than 5% of the starting material. Only clear-cut responses to location reagents are shown.

Product	Yield of products (%)	<i>R_r</i> in light petroleum-ether (19:1, v/v)	Colour with reagent		
			NH ₃	Gibb's reagent	SbCl ₅
3,3',5,5'-Tetra- <i>tert</i> .-butyl-4,4'-diphenquinone	15	0.80			
Coppinger's stable phenoxy radical*	—	0.77	Magenta	Magenta	Red
3,3',5,5'-Tetra- <i>tert</i> .-butyl-4,4'-stilbenequinone	10	0.74			Red
Di-(3,5-di- <i>tert</i> .-butyl-4-hydroxybenzyl)	4	0.72		Dull yellow	Pale red
The quinonemethide	8	0.57	Magenta	Purple	Red
2,6-Di- <i>tert</i> .-butyl- <i>p</i> -benzoquinone	4	0.52		Pale grey-brown	Pale grey-green
Unknown	2	0.45		Magenta	Red
3,5-Di- <i>tert</i> .-butyl-4-hydroxybenzaldehyde	50	0.20		Magenta	Pale red
3,5-Di- <i>tert</i> .-butyl-4-hydroxybenzoic acid	3	0.05		Magenta	
Polar product(s)	4	0.00			

* This compound was detected in an interrupted reaction.

(3,5-di-*tert*.-butyl-4-hydroxybenzyl) were obtained by preparative thin-layer chromatography and these compounds were identified by isotope-dilution analysis involving PbO_2 oxidation of [^{14}C]Ionox 201. The identity of the quinonemethide was further established. The [^{14}C]-quinonemethide that had been crystallized to constant specific activity was oxidized to ^{14}C -labelled Coppinger's stable phenoxy radical, which was crystallized and found to possess the calculated specific activity. It has been established that Coppinger's stable phenoxy radical was an intermediate in the PbO_2 oxidation of Ionox 201, since this compound could be detected by thin-layer chromatography of the crude products from a reaction that was interrupted after 2 hr.

RESULTS

Alimentary absorption in animals dosed with Ionox 201. At the outset of the present work, it was important to establish in what proportion Ionox 201 is absorbed from the gut. We found that, when a pair of rats (one of each sex) equipped with biliary fistulae were treated orally with [^{14}C]Ionox 201, measurements of radioactivity in the bile, urine and in the carcass and viscera remaining after removal of the gut, together with measurements of $^{14}\text{CO}_2$ in the exhaled air from a pair of identically dosed non-fistulated rats, showed that up to 32% of the ^{14}C had been adsorbed (Table 2). This experimental design does not take into consideration radioactivity secreted into the alimentary canal by a route other than the biliary system. It is noteworthy that one-fifth of a single dose of the structurally related antioxidant, Ionox 220, was absorbed in rats (Wright *et al.* 1966). On the other hand, in an experiment in which a dog had been dosed orally with unlabelled Ionox 201 (38 mg./kg.), no unchanged antioxidant or its derivatives were detected in bile from the gall bladder 6 hr. after dosage. There is a strong supposition that Ionox 201 is not absorbed in dogs. In this connexion, rats and dogs showed a species difference in the degree of absorption following ingestion of another antioxidant, BHA (2- and 3-*tert*.-butyl-4-hydroxyanisole) (Astill,

Fassett & Roudabush, 1960; Astill, Mills, Fassett, Roudabush & Terhaar, 1962).

Elimination and retention of radioactivity in rats dosed with [^{14}C]Ionox 201. Throughout this work there is a lack of evidence of any significant sex difference except in the concentration of Ionox 201 in the tissues, and except in this case, the sexes are not therefore considered separately. Five adult rats of each sex were treated orally with [^{14}C]Ionox 201, and pairs of animals (one of each sex) were killed 1, 4, 8, 16 and 24 days after dosage. The investigation of elimination and retention of radioactivity in these animals is summarized in Table 3. Of the original label 86.8–97.2% was excreted in the faeces during 24 days. Up to 5.6% of the ^{14}C was excreted via the kidneys during this period and 0.8% in the exhaled air during 48 hr. Initial elimination in the faeces was slow, 20.2% of ^{14}C (two animals) in 24 hr., but 90.0% (72.0–97.2%) elimination (eight animals) during 4 days. Since there was little elimination of ^{14}C after 4 days, animals killed 4, 8, 16 and 24 days after dosage were treated as one group (Table 3).

The carcass and viscera remaining after removal of the gut and contents of animals, killed 24 hr. after dosage, contained 9.2% of the ^{14}C , and this value fell to 5.5% in animals killed 4 days after dosage. The fact that similar amounts of radioactivity, i.e. 5.0% (3.4–5.9%), were also found in the carcasses of animals killed 8, 16 and 24 days after dosage is indicative of storage. After an oral dose of [^{14}C]Ionox 201, more ^{14}C is stored in the fatty tissues than in the organs (Table 4). After 24 days, the concentration of ^{14}C in the liver of both male and female rats had fallen to 0.07 p.p.m. from concentrations of 0.75 p.p.m. (male rats) and 1.70 p.p.m. (females) at 4 days, whereas the concentrations of ^{14}C in the subcutaneous fat, intra-abdominal fat and pelts remained at a much higher level during 4–24 days. There is greater retention of ^{14}C in the fatty tissues of female rats than in those of males (Table 4).

Table 2. *Absorption of radioactivity from the alimentary canal in rats dosed with [^{14}C]Ionox 201*

A male and a female rat (200 g. body wt.) were each treated by stomach tube with a dose (6.78 mg./animal) of [^{14}C]Ionox 201 (1.82 $\mu\text{C}/\text{mg}$.) dissolved in 2.0 ml. of olive oil. Biliary fistulae were established 15 min. after dosage. Urine and bile were collected for 24 hr., when the animals were killed. Elimination of ^{14}C in the expired gases was measured in a separate experiment; a further 0.10% of ^{14}C was eliminated by this route during 24–28 hr.

Rat no.	Sex	Recovery of ^{14}C (% of dose)				Total
		Bile	Urine	Carcass and remaining viscera after removal of gut and contents	Expired gases	
1	♂	20.1	0.7	10.5	0.7	32.0
2	♀	17.8	1.5	8.7	0.7	28.7

Table 3. *Excretion and retention of radioactivity in rats after oral administration of [¹⁴C]Ionox 201*

Five male and five female rats (200g. body wt.) were each treated by stomach tube with a single dose (6.78 mg./animal) of [¹⁴C]Ionox 201 (1.82 μ C/mg.) dissolved in 2.0 ml. of olive oil. Pairs of animals (one male and one female) were killed 1, 4, 8, 16 and 24 days after dosage. No evidence for a sex difference except in the storage of Ionox 201 by fat (shown in Table 4) was found, and the sexes are not therefore considered separately. Since little elimination of ¹⁴C occurred after 4 days, animals killed 4, 8, 16 and 24 days after dosage were grouped together. Mean values are cited with the ranges in parentheses.

No. of rats	Animals killed after (days)	Recovery of ¹⁴ C (% of dose)				
		Faeces	Urine	Gut and contents	Carcass and remaining viscera	Total
2	1	20.2 (12.4, 28.0)	1.1 (0.7, 1.5)	70.9 (62.8, 78.9)	9.6 (8.7, 10.5)	101.8 (101.5, 102.0)
8	4, 8, 16 and 24	93.4 (86.8-97.2)	3.5 (0.8-5.6)	0.6 (0.1-1.6)	5.0 (3.4-5.9)	102.5 (96.7-106.6)

Table 4. *Tissue concentrations of radioactivity in rats after oral administration of [¹⁴C]Ionox 201*

Male and female rats (200g. body wt.) were treated with [¹⁴C]Ionox 201 as described in Table 3. A 5g. sample of subcutaneous fat, 5g. of intra-abdominal fat, the whole liver and the skin plus hair were taken from each animal for individual assay.

Rat no.	Sex	Animals killed after (days)	Concn. of ¹⁴ C (expressed in terms of p.p.m. of Ionox 201)			
			Liver	Subcutaneous fat	Intra-abdominal fat	Skin plus hair
1	♂	4	0.75	4.75	4.34	1.22
3	♂	8	0.04	1.22	1.22	1.49
5	♂	16	0.27	5.22	5.49	1.08
7	♂	24	0.07	5.34	6.78	1.29
2	♀	4	1.70	14.24	11.53	2.44
4	♀	8	0.14	5.36	5.29	3.19
6	♀	16	0.20	8.81	9.83	2.03
8	♀	24	0.07	11.53	10.85	2.58

About 25% of the ¹⁴C excreted in the faeces during 24 days originated in the bile: 15.5-24.3% (ten rats) was in the 30hr. bile and much of the additional 4-5% of ¹⁴C was eliminated from animals killed between days 1 and 24 (Table 3). A similar estimate is reached by subtracting the residual carcass ¹⁴C from that absorbed. Representative values for the collection of bile from eight male and two female rats dosed with [¹⁴C]Ionox 201 are summarized in Table 5; rats secreted 15.5-24.3% of the ¹⁴C in the 30hr. bile. Maximum secretion of ¹⁴C into bile occurred at approx. 6hr. after dosage. After 16hr., the concentration of ¹⁴C in the bile fell very slowly. These observations indicate a low degree of alimentary absorption and slow diffusion of ¹⁴C-labelled products from plasma to bile.

Separation and estimation of individual radioactive compounds in the urine, faeces, body fat and bile of rats dosed with [¹⁴C]Ionox 201. After radio-

assay, the urine, faeces and fatty tissues of rats that were killed 4, 8, 16 and 24 days after treatment with [¹⁴C]Ionox 201 (see Tables 3 and 4) were pooled according to sex for the estimation of relative proportions of the individual ¹⁴C-labelled metabolites (see Table 7). Radioactivity was recovered (98% of the theoretical) from the urines by successive ether extractions at pH 6.0 and pH 1.5-2.0. The ether extract prepared at pH 6.0 contained 1.5 times the amount of ¹⁴C as that prepared at pH 1.5-2.0. In an experiment in which a male and a female rat were given a larger dose of Ionox 201 (13.56 mg./animal; 1.82 μ C/mg.) this ratio was reversed. Paper chromatograms of representative samples of the ether extracts were run in two solvent systems and scanned for radioactivity. Ionox 201 was not present among the metabolites in the urine. The single peak of ¹⁴C in the extracts prepared at pH 6.0 corresponded to 3,5-di-*tert*.-butyl-4-hydroxybenz-¹⁴C]oic acid and the single peak of ¹⁴C in extracts

Table 5. *Time-course for the secretion of ^{14}C into the bile of rats dosed with [^{14}C] Ionox 201*

Single doses (6.78 mg./animal) of [^{14}C] Ionox 201 (1.82 μC /mg.) dissolved in olive oil (2.0 ml.) were administered orally to male and female rats (200 g. body wt.) at zero time, and biliary fistulae were established 1 hr. later. The post-operative animals were provided with unrestricted water and standard pellet diet, and bile was collected during 1.0–30 hr. after dosage in 2 ml. portions by using an automatic fraction collector. No sex difference was apparent, and the single set of results given was representative of ten animals: eight male and two female rats secreted 15.5–24.2% of the dose during this period.

Bile secreted (ml.)	Time (hr.)	Concn. of ^{14}C in bile (% of dose/ml.)	Amount of ^{14}C (% of dose)
2	2.00	0.39	0.78
4	3.25	0.64	2.06
6	4.50	1.39	4.84
8	6.00	1.47	7.78
10		1.11	10.00
12		1.06	12.12
14	9.50	0.65	13.42
16	11.00	0.53	14.48
18		0.35	15.18
20		0.21	15.60
22	16.00	0.18	15.96
24		0.16	16.28
26		0.16	16.60
28		0.15	16.90
30		0.22	17.34
32	21.00	0.15	17.64
34	24.00	0.14	17.92
36	26.00	0.16	18.24
38	28.00	0.13	18.50
40	30.00	0.11	18.72

prepared at pH 1.5–2.0 corresponded to (3,5-di-*tert.*-butyl-4-hydroxybenz[^{14}C]oyl β -D-glucopyranosid)uronic acid, which was also located with Gibb's reagent and naphtharesorcinol on paper chromatograms (Table 6) of ether extracts similarly prepared from the urine of rabbits dosed with 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid. Enzymic hydrolysis of this ester glucuronide gave 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid and glucuronic acid.

Radioactive components in the acetone extracts of the faeces were separated by preparative thin-layer chromatography (for details, see the Methods section). They were located by radioactive scanning and radioautography, estimated by liquid-scintillation counting of the appropriate eluates from thin-layer plates and identified by their chromatographic properties (Table 6) as well as by isotope-dilution analysis. Unchanged [^{14}C] Ionox 201 accounts for 65% of the ^{14}C in the acetone extracts of faeces, 3,5-di-*tert.*-butyl-4-hydroxybenz[^{14}C]oic acid for 30%, 3,5-di-*tert.*-butyl-4-hydroxybenz[^{14}C]aldehyde for 1.4% and 3,3',5,5'-tetra-*tert.*-butyl-4,4'-[^{14}C]stilbenequinone for 0.1%. It is noteworthy that the faeces of axenic rats dosed with [^{14}C] Ionox 201 contained an essentially similar range of ^{14}C -labelled metabolites to that of the other rats. This strongly suggests that 3,3',5,5'-tetra-*tert.*-butyl-4,4'-stilbenequinone, which was not detected in the animal tissues, results from antioxidant action in the faeces and not from transformation by the intestinal flora. A further 3.5% of the radioactivity was in an unidentified ^{14}C -labelled component(s) with polar properties

Table 6. *R_F values on paper and thin-layer plates, and colour reactions of Ionox 201 and its metabolites*

Only clear-cut colour responses with location reagents are shown.

	<i>R_F</i> on paper		<i>R_F</i> on thin-layer plates		Colour with reagent		
	Butan-1-ol satd. with 2N-NH ₃	Butan-1-ol satd. with water	Light petroleum-ether (19:1, v/v)	Light petroleum-ether (3:2, v/v)	Gibb's reagent	SbCl ₅	Naphtharesorcinol
Ionox 201	0.92	0.92	0.40	0.70	Magenta	Red	
3,3',5,5'-Tetra- <i>tert.</i> -butyl-4,4'-stilbenequinone	0.90	0.92	0.74	0.95		Red	
3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzaldehyde	0.90	0.92	0.20	0.55	Magenta	Pale red	
3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoic acid	0.70	0.90	0.05	0.20	Magenta		
(3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid	0.45	0.40			Magenta		Blue
Polar compound(s) from faeces			0.00	0.00			
Unidentifiable metabolite in bile	0.20	0.83			Blue		

Table 7. *Relative proportions of unchanged antioxidant and individual metabolites present in body fluids, faeces and body fat of rats treated orally with [¹⁴C]Ionox 201*

After radio-assay, the urine (at the lower dose), faeces and body fat of four pairs of rats, which were killed 4, 8, 16 and 24 days after treatment with [¹⁴C]Ionox 201 (see Tables 3 and 4), were pooled according to sex. Since the relative proportions of Ionox 201 metabolites found in the sample from each sex were identical, the results presented are considered to be representative of rats dosed with [¹⁴C]Ionox 201 irrespective of sex. On each of eight rats at the lower dose, measurement of ¹⁴C-labelled components was made in the bile, and on each of two animals at the higher dose in bile and in urine (30 hr. collections). Since there were only small variations in the proportions of the ¹⁴C-labelled metabolites in the body fluids, mean values are cited.

	Relative proportions of the ¹⁴ C-labelled components					
	Urine		Bile		Faeces	Body fat
	6.78	13.56	6.78	13.56	6.78	6.78
Dose (mg./animal)	2	2	8	2	2	2
No. of independent observations						
Ionox 201	None	None	2.0	3.0	65.0	80.0
3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzaldehyde	None	None	None	None	1.4	1.0
3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoic acid	60.0	40.0	33.0	17.0	30.0	19.0
3,3',5,5'-Tetra- <i>tert.</i> -butyl-4,4'-stilbenequinone	None	None	None	None	0.1	None
(3,5-Di- <i>tert.</i> -butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid	40.0	60.0	45.0	69.0	None	None
Unidentified polar compound(s) from faeces	None	None	None	None	3.5	None
Unidentified metabolites in bile	None	None	20.0	11.0	None	None

that was also excreted in the faeces of the treated rats (Table 7).

The ¹⁴C-labelled components of intra-abdominal and subcutaneous fats of rats dosed with [¹⁴C]Ionox 201 were separated by procedures used for the separation of such compounds from the faeces of these animals; the results shown in Table 7 are representative: 80% of the ¹⁴C was present as unchanged [¹⁴C]Ionox 201, 19% as 3,5-di-*tert.*-butylhydroxybenz[¹⁴C]oic acid and 1% as 3,5-di-*tert.*-butyl-4-hydroxybenz[¹⁴C]aldehyde.

Biliary fistulae were established in six male and two female rats that had previously been dosed with [¹⁴C]Ionox 201 (6.78 mg./animal), bile was collected during 1–30 hr. after dosage, and radioactivity was recovered from the bile of individual animals by ether extraction at pH 1.5–2.0 (for details, see the Methods section). Radioactive components in this solvent extract were separated by paper chromatography (Table 6) and estimated by scanning. Resolutions of ¹⁴C-labelled components were greatly improved by rechromatography on paper of radioactive zones that had been eluted with methanol. Only small differences in the relative proportions of ¹⁴C-labelled components were apparent in the bile of individual animals, and the results summarized in Table 7 are representative. 3,5-Di-*tert.*-butyl-4-hydroxybenz[¹⁴C]oic acid accounted for about 33% of the ¹⁴C in bile from the biliary-fistula rats, the ¹⁴C-labelled ester glucuronide for 45%, unchanged [¹⁴C]Ionox 201 for 2% and the unidentified ¹⁴C-labelled compound for 20%. The ester glucuronide and the unidentified metabolite were absent

from the faeces of treated rats. When the dose of [¹⁴C]Ionox 201 administered orally to two male rats was doubled (13.56 mg./animal), the proportion of the free ¹⁴C-labelled acid and unidentified ¹⁴C-labelled metabolite in the bile decreased, and that of the ¹⁴C-labelled ester glucuronide increased (30 hr. collection) (Table 7).

Identification of the unidentified ¹⁴C-labelled metabolite found in the bile of treated rats has been hampered by the small proportion secreted into rat bile. Since this component was absent from the faeces, it might be a conjugate, but enzymic hydrolysis showed that it was not a β -D-glucuronide. The possibility that this metabolite in bile may have been a sulphur-containing metabolite was tested by treating rats that had previously been dosed with [³⁵S]cysteine on several successive days with unlabelled Ionox 201 (see the Methods section; Griffiths *et al.* 1966; Gutmann & Wood, 1950; Marsden & Young, 1958). The experiment provided clear evidence for the absence from bile of a ³⁵S-labelled constituent with chromatographic and electrophoretic properties corresponding to that of the unidentified ¹⁴C-labelled constituent in the bile of animals dosed with the ¹⁴C-labelled antioxidant. That this metabolite in bile may be a hydrolysable conjugate was shown by hydrolysis with 4N-hydrochloric acid at 95° for 2 hr., when a complex mixture of ether-soluble products was obtained. The elucidation of the structure of this metabolite depends on finding an animal (not a dog) that secretes larger amounts of this compound into the bile.

Scheme 1. Metabolism of Ionox 201 in rats.

It was found that, when [^{14}C]Ionox 100 was administered orally to male and female rats, the ^{14}C secreted in the bile was accounted for by the presence of 3,5-di-*tert*.-butyl-4-hydroxybenz[^{14}C]oic acid (60% of ^{14}C) and (3,5-di-*tert*.-butyl-4-hydroxybenz[^{14}C]oyl β -D-glucopyranosid)uronic acid (40% of ^{14}C). The unidentified metabolite in the bile of rats dosed with Ionox 201 was absent from the bile of rats dosed with Ionox 100.

DISCUSSION

Approximately one-third of a single oral dose of Ionox 201 was absorbed in rats. Not all of the absorbed dose was metabolized, since some unchanged antioxidant was concentrated in the fatty tissues *in vivo* (Tables 4 and 7). A proportion of the dose was transformed into (metabolic) products in the intestinal tract, since 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone and unidentified polar compounds were found only in the faeces (see Table 7). From the results shown in Tables 3 and 7, obtained with rats killed 4, 8, 16 and 24 days after treatment with Ionox 201, we calculate that, among the

metabolic products of Ionox 201 (I) (Scheme 1) in the rat, 3,5-di-*tert*.-butyl-4-hydroxybenzoic acid (III) accounts for 30.2% of the dose, (3,5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucopyranosid)uronic acid (IV) for 1.4%, 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde (II) for 1.3%, 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone (V) for 0.1% and unidentified polar metabolite(s) for 3.2%. Thus 36.2% of a single oral dose was metabolized. However, this value is a low estimate of the total metabolism, since metabolites present in the gut of animals at death were not taken into consideration and since a small proportion of the dose was expired as carbon dioxide (see Table 2).

Metabolism data for dibenzyl ethers are unavailable, and it appears that simple ethers are stable in the body and are excreted unchanged (Williams, 1959). However, benzyl radicals are linked to the ether oxygen atom less tenaciously than ethyl and isopentyl radicals (Haworth, 1924; Schorigin, 1924), thus favouring greater reactivity of dibenzyl ethers. A scheme for the metabolism of di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl) ether (Ionox 201) *in vivo* is shown in Scheme 1. It may be relevant that, since

compounds (II), (III) and (V) can also be produced by mild oxidation *in vitro*, their formation *in vivo* need not necessarily be enzymic. However, conjugation of compound (II) *in vivo* to the ester glucuronide (IV) is undoubtedly an enzymic step. The metabolism of Ionox 201 *in vivo* is closely related to its antioxidant action *in vitro*. In the present work, the antioxidant action of Ionox 201, which involves a radical mechanism, was simulated by reaction with a single-electron oxidizing agent, lead dioxide. As well as compounds (II), (III) and (V) (Scheme 1), the following compounds were also produced: di-(3,5-di-*tert*.-butyl-4-hydroxybenzyl) (VI) (Scheme 2), 3,3',5,5'-tetra-*tert*.-butyl-4,4'-diphenylquinone (VII), 2,6-di-*tert*.-butyl-*p*-benzoquinone (VIII), the quinonemethide (IX) and Coppinger's stable phenoxy radical (X). All of the compounds (II)-(X), with the exception of the ester glucuronide (IV) (Scheme 1), can be derived from two radicals (see Cosgrave & Waters, 1951), (a) and (b) (Scheme 2), themselves generated in the oxidation reaction. It follows that the presence of 3,5-di-*tert*.-butyl-4-hydroxybenzaldehyde (II) and 3,3',5,5'-tetra-*tert*.-butyl-4,4'-stilbenequinone (V) in the faeces and of compound (II) in the fatty tissues probably arose by radical processes in the two biological situations. Since compounds (II) and (V) were also found in the faeces of axenic animals, they were not formed by the intestinal flora.

Although claims have been made that structurally related compounds are excreted in the urine (Akagi & Aoki, 1962*a,b*), neither lipid-soluble Ionox 201 nor lipid-soluble Ionox 220 (Wright *et al.* 1966) is excreted via the kidneys, and this agrees with the widely held view that lipid-soluble water-insoluble compounds are not ultrafiltered in the glomerulus (e.g. West & Todd, 1961).

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