The Comparative Metabolism of 2,6-Dichlorothiobenzamide (Prefix) and 2,6-Dichlorobenzonitrile in the Dog and Rat

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(Received 22 July 1965)

1. A single oral dose of either [^{14}C]Prefix or 2,6-dichlorobenzonitrile to rats is almost entirely eliminated in 4 days: 84.8–100.5% of ^{14}C from [^{14}C]Prefix is excreted, 67.3–79.7% in the urine, and 85.8–97.2% of ^{14}C from 2,6-dichlorobenzonitrile is excreted, 72.3–80.7% in the urine. Only $0.37 \pm 0.03\%$ of the dose of [^{14}C]Prefix and $0.25 \pm 0.03\%$ of the dose of 2,6-dichlorobenzonitrile are present in the carcass plus viscera after removal of the gut. Rats do not show sex differences in the pattern of elimination of the respective metabolites of the two herbicides. The rates of elimination of ^{14}C from the two compounds in the 24 hr. and 48 hr. urines are not significantly different ($P > 0.05$) from one another. 2. After oral administration to dogs, 85.9–106.1% of ^{14}C from [^{14}C]Prefix is excreted, 66.6–80.9% in the urine, and 86.8–92.5% of ^{14}C from 2,6-dichlorobenzonitrile is excreted, 60.0–70.1% in the urine. Dogs do not show sex differences in the pattern of eliminating the metabolites of either Prefix or 2,6-dichlorobenzonitrile. 3. Dogs and rats do not show species differences in the patterns of elimination of the two herbicides. 4. Prefix and 2,6-dichlorobenzonitrile are completely metabolized; unchanged Prefix and 2,6-dichlorobenzonitrile are absent from the urine and faeces, and from the carcasses when elimination is complete. In the hydrolysed urine of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzonitrile, 2,6-dichloro-3-hydroxybenzonitrile accounts for approx. 42% of the ^{14}C , a further 10–11% is accounted for by 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid, 2,6-dichloro-3- and -4-hydroxybenzoic acid and 2,6-dichloro-4-hydroxybenzonitrile collectively, and 25–30% by six polar constituents, of which two are sulphur-containing amino acids. 5. In the unhydrolysed urines of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzonitrile, there are present free 2,6-dichloro-3- and -4-hydroxybenzonitrile, their glucuronide conjugates, ester glucuronides of the principal aromatic acids that are present in the hydrolysed urines, and two sulphur-containing metabolites analogous to mercapturic acids or premercapturic acids. 6. Prefix is thus extensively transformed into 2,6-dichlorobenzonitrile: $\text{R} \cdot \text{CS} \cdot \text{NH}_2 \rightarrow \text{R} \cdot \text{CN} + \text{H}_2\text{S}$, where $\text{R} = \text{C}_6\text{H}_3\text{Cl}_2$. However, the competitive reaction: $\text{R} \cdot \text{CS} \cdot \text{NH}_2 + \text{H}_2\text{O} \rightarrow \text{R} \cdot \text{CO} \cdot \text{NH}_2 + \text{H}_2\text{S}$ takes place to a very limited extent.

2,6-Dichlorothiobenzamide (Prefix) (Yates, 1961) and 2,6-dichlorobenzonitrile (Belgian Patent, 1957) are promising herbicides with interesting biological properties (Barnsley, 1960; Koopman & Daams, 1960). Prefix is useful as a selective herbicide in transplanted rice. Agricultural application makes an understanding of the mammalian metabolism of the two herbicides desirable.

MATERIALS

2,6-Dichlorobenzonitrile. This was prepared by dehydration in boiling acetic anhydride of *anti*-2,6-dichlorobenzaldoxime (Koopman, 1961), which was itself prepared

by reaction of 2,6-dichlorobenzaldehyde (Fierz-David & Blangey, 1952; Reich, 1917) with hydroxylamine monosulphonate (Koopman, 1961). 2,6-Dichlorobenzonitrile had m.p. 145° (Koopman, 1961, gives $144\text{--}145^\circ$).

2,6-Dichlorothiobenzamide (Prefix). This was prepared by the addition of H_2S to a solution of 2,6-dichlorobenzonitrile in dimethylformamide at 60° , according to the method of Yates (1961). Prefix had m.p. 152° (Yates, 1961, gives m.p. 152°).

Purity of the herbicides. The two herbicides, which had been crystallized from benzene and ethanol to constant melting point, migrated as single substances on thin-layer plates of Kieselgel G run with benzene; phenoxyethanol- AgNO_3 spray (Mitchell, 1958) was used for locating the substance on the thin-layer plate. Infrared measurement

was used as a further criterion of purity. The purity of each compound exceeded 99.9%.

The unlabelled herbicides were used for dosing rats, in which the cysteine-cystine pools had been labelled with ^{35}S (see the Methods section). Unlabelled herbicides were also used as reference compounds to establish the presence or absence of unchanged ^{14}C -labelled herbicides in the urine and faeces of treated animals (see the Results section).

2,6-Dichlorobenzoic acid. This compound, m.p. 146–147°, was prepared from 2,6-dichlorobenzaldehyde (Fierz-David & Blangey, 1952; Reich, 1917) by oxidation with KMnO_4 in acetone solution (Davies, 1921).

2,6-Dichlorobenzamide. This compound, m.p. 202°, was prepared by treatment of 2,6-dichlorobenzonitrile with alkaline H_2O_2 (Reich, Salzmann & Kawa, 1917).

2,6-Dichloro-3-hydroxybenzonitrile. This was prepared from 2,6-dichloro-3-hydroxybenzaldehyde (Hodgson & Beard, 1926) as starting material. When the corresponding aldoxime (5.6g.) (Hodgson & Beard, 1926) was refluxed in 20 ml. of acetic anhydride for 2 hr. and poured into water, 2,6-dichloro-3-acetoxymethylbenzonitrile was obtained as an oil, which crystallized (5.1g., m.p. 106°). Hydrolysis, by refluxing for 1 hr. with 3*N*-KOH under N_2 , and acidification yielded 2,6-dichloro-3-hydroxybenzonitrile (4.1g., m.p. 196–198°) (Yates, 1962).

2,6-Dichloro-3-hydroxybenzoic acid. This was prepared by oxidation of 2,6-dichloro-3-hydroxybenzaldehyde (Hodgson & Beard, 1926) with freshly prepared Ag_2O according to the method of Pearl (1950). 2,6-Dichloro-3-hydroxybenzoic acid crystallized from water, forming prisms of the monohydrate, m.p. 126° (Mazzara & Bertozzi, 1900, give m.p. 123–124° for the monohydrate). **Methyl 2,6-dichloro-3-methoxybenzoate.** This compound, m.p. 57° (Mazzara & Bertozzi, 1900), was used as a reference compound in the gas-liquid chromatography (see the Methods section and Table 7). [The methyl ester and the two *O*-methyl ethers, which were also used in gas-liquid chromatography (see Table 7), were oils that failed to crystallize, and are not therefore described in the Materials section.]

2,6-Dichloro-4-hydroxybenzonitrile. This was synthesized from *p*-nitrosophenol as starting material. Reaction of an ethanolic solution of *p*-nitrosophenol with HCl yielded 2,6-dichloro-4-ethoxyaniline (Bargellini, 1929), which was converted into 2,6-dichloro-4-hydroxybenzonitrile by successive diazotization, Sandmeyer reaction and de-ethylation. This reaction sequence gave a better yield of final product from the same intermediate, 2,6-dichloro-4-ethoxyaniline, than the alternative sequence, de-ethylation, diazotization and Sandmeyer reaction, used by Strating & Backer (1943). 2,6-Dichloro-4-hydroxybenzonitrile crystallized from toluene, forming needles, m.p. 229–230° (Strating & Backer, 1943, give m.p. 228–230°).

2,6-Dichloro-3-hydroxythiobenzamide. This compound, m.p. 197–198°, was supplied by Dr J. Yates.

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., and was found to be free from hippuricase and sulphuric ester hydrolase activities.

Reagents and solvents. All reagents and solvents were of A.R. grade.

Radioactive chemicals. 2,6-Dichlorobenzonitrile was synthesized by reaction of 2,6-dichloriodobenzene (Körner & Contardi, 1913) with Cu^{14}CN and purified by column

chromatography on silicic acid. 2,6-Dichlorobenzonitrile (sp. activity 9.1 $\mu\text{C}/\text{mg.}$) was eluted with methylene dichloride.

2,6-Dichlorothiobenzonitrile (^{14}C Prefix) was prepared by addition of H_2S to 2,6-dichlorobenzonitrile (sp. activity 3.2 $\mu\text{C}/\text{mg.}$) and purified by column chromatography on silicic acid. ^{14}C Prefix (sp. activity 3.2 $\mu\text{C}/\text{mg.}$) was eluted with methylene dichloride.

The two ^{14}C -labelled herbicides behaved identically with authentic specimens of the unlabelled compounds on thin-layer plates of Kieselgel G run with benzene; radioautography did not reveal radioactive impurities. The radiochemical purity of both compounds exceeded 99.9%.

$\text{L-}^{35}\text{S}$ Cystine (sp. activity 424 $\mu\text{C}/\text{mg.}$) was obtained from The Radiochemical Centre, Amersham, Bucks.

METHODS

Experiments with animals. Adult rats (approx. 2 months old, 150–200g. body wt.) were used (Porton strain maintained as a specific pathogen-free colony in this Laboratory). For the metabolism experiments the animals were kept in all-glass metabolism cages under the conditions described by Wright, Akintonwa, Crowne & Hathway (1965). In the first experiment a solution of ^{14}C Prefix (1.8mg., 3.2 $\mu\text{C}/\text{mg.}$) in 2 ml. of aq. 5% (v/v) ethanol was administered to male and female rats by stomach tube, and in the second experiment 0.80mg. (males) or 1.3mg. (females) of 2,6-dichlorobenzonitrile (9.1 $\mu\text{C}/\text{mg.}$) in 2 ml. of arachis oil was administered to rats. Unrestricted food and water were supplied, the urine and faeces were collected daily and stored at -29° and the expired gases were monitored for $^{14}\text{CO}_2$. In these experiments the rats were killed after 4 days and the alimentary canal was removed. The carcass plus viscera, the alimentary canal and the skin plus hair were stored separately at -29° .

In another experiment 1.5 ml. of an aq. 2% (w/v) NaHCO_3 solution containing 13 μC of $\text{L-}^{35}\text{S}$ cystine/ml. was administered intraperitoneally to four rats of each sex on 5 consecutive days. On the fourth and fifth days two rats of each sex were each dosed by stomach tube with 1 ml. of a solution of Prefix (5mg.) in arachis oil and two rats of each sex were each dosed by stomach tube with 1 ml. of a solution of 2,6-dichlorobenzonitrile (5mg.) in arachis oil. Unrestricted food and water were supplied and the urine was collected on the fourth and fifth days and stored at -29° . This experiment is based on work of Gutmann & Wood (1950) and Marsden & Young (1958).

Adult hounds (7–15kg. body wt.) were used (beagles maintained for 2–3 years as a closed colony in this Laboratory). For the metabolism experiments the animals were kept in stainless-steel metabolism cages under the conditions described by Wright *et al.* (1965). In one experiment each dog swallowed a gelatin capsule containing a solution of 7.90mg. of ^{14}C Prefix (2.8 $\mu\text{C}/\text{mg.}$) in ethanol (1 ml.), and in another experiment each dog swallowed a gelatin capsule containing a solution of 0.92mg. of 2,6-dichlorobenzonitrile (8.52 $\mu\text{C}/\text{mg.}$) in 1 ml. of arachis oil. Unrestricted food and water were supplied and the urine and faeces were collected separately at daily intervals.

Fractionation of urines from treated animals. Solvent extracts of the pooled urines from 12 rats were made by continuous extraction at pH 1.5–2.0 in a Schacherl apparatus with ether and ethyl acetate successively. Evaporations

were carried out in N_2 under reduced pressure at below 35° .

Hydrolysis of urines from treated animals and fractionation of the hydrolysed urines. Preliminary investigations showed that hydrolysis of the ^{14}C -labelled metabolites in the urines of treated animals gave the same chromatographic and electrophoretic patterns of ether-soluble ^{14}C -labelled constituents irrespective of whether the hydrolysing agent was β -glucuronidase or $6N-HCl$ (3 hr. at 110°). The alternative hydrolytic procedures did, however, yield some different ^{14}C -labelled constituents in the residual aqueous phases after ether extraction. Thus the ^{14}C -labelled sulphur-containing metabolites (see below) were transformed by acid hydrolysis into ^{14}C -labelled sulphur-containing amino acids, whereas they were unaffected by enzymic hydrolysis. For the purpose of identifying ^{14}C -labelled constituents in urine hydrolysates, the pooled urines of 12 treated animals were hydrolysed with acid. The hydrolysed urines were exhaustively extracted by continuous liquid-liquid extraction (Schacherl apparatus) with ether and ethyl acetate successively, and the sum of the separate amounts of ^{14}C in the solvent extracts and residual aqueous phase accounted quantitatively for the original label in the unhydrolysed urine. Further, when the individual compounds in the solvent extracts and residual aqueous phase were separated on paper chromatograms and electrophoretograms (for details see below), which in turn were scanned and summed, an approximate balance of radioactivity was obtained. This showed that acid hydrolysis did not cause destruction of hydrolytic products with consequent loss of radioactivity as ^{14}C , $^{14}CO_2$ or ^{14}C -labelled precipitates. This work was reproducible; other batches of pooled urines from 12 rats dosed with the ^{14}C -labelled herbicides, when hydrolysed with acid in this way, also gave a consistent balance of ^{14}C and identical proportions of the same ^{14}C -labelled constituents.

Samples (5 mg.) of each of the ^{14}C -labelled constituents that were subsequently identified in the ether phase (2,6-dichlorobenzamide, 2,6-dichloro-3- and -4-hydroxybenzonitrile, 2,6-dichlorobenzoic acid and 2,6-dichloro-3-hydroxybenzoic acid) and 2,6-dichloro-3-hydroxythiobenzamide were heated separately with $6N-HCl$ (5 ml.) for 3 hr. at 110° . Paper chromatograms of the ether extracts of the separate reaction mixtures were run in butan-1-ol saturated with $2N-NH_3$, and these were identical in every respect with the chromatograms of the initial authentic compounds. In each case, unchanged starting material was recovered quantitatively from the reaction mixtures. These experiments demonstrate the great stability of the hindered amido, cyano and thioamido groups in these molecules and the lack of reactivity of the chloro substituents in 2,6-positions. Since these constituents were stable to acid and since, with the exception of 2,6-dichloro-3-hydroxythiobenzamide, they were formed by similar acid hydrolysis of the urines of animals dosed with the two ^{14}C -labelled herbicides, it follows that the reactions by which they were produced from the (conjugated) metabolites in urine were probably straightforward hydrolytic reactions.

Chromatography on a silicic acid column. Mallinckrodt (100 mesh) silicic acid was washed and reactivated as described by Wright *et al.* (1965). Ether-soluble ^{14}C -labelled metabolites were successively eluted with solvents of increasing polarity; a typical fractionation is set out in Table 7.

Paper chromatography. Unconjugated ^{14}C -labelled metabolites of [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile were separated by descending chromatography on Whatman no. 1 filter-paper sheets at 29° , and Table 8 shows a typical separation of the ether-soluble ^{14}C -labelled metabolites. Paper chromatography in the same solvent system was used for separating the more polar unconjugated ^{14}C -labelled metabolites and the ^{14}C -labelled conjugates in the unhydrolysed urines. All ^{14}C - or ^{35}S -labelled constituents were detected and identified on paper by radioactive scanning with a Nuclear-Chicago Actigraph II, equipped with a gas-flow cell utilizing a helium-*n*-butane (98.5:1.5, v/v) mixture. Phenoxyethanol- $AgNO_3$ (Mitchell, 1958) was used as location reagent, and the fluorescence of some unconjugated metabolites in ultraviolet light served as another means of location.

Thin-layer chromatography. This was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) as described by Wright *et al.* (1965). The ^{14}C -labelled metabolites were detected and identified on thin plates by radioactive scanning with a Desaga radiochromatogram scanner. Thin-layer chromatography was useful for separating ^{14}C -labelled aromatic acids and [^{14}C]phenols; acetone-*n*-hexane (1:1, v/v) was used as solvent system. The purity of the two ^{14}C -labelled herbicides was checked by thin-layer chromatography, benzene being the preferred solvent for development.

Gas-liquid chromatography. Chromatographs, each equipped with an electron-capture detector, were used for comparing retention times of ^{14}C -labelled metabolites with those of reference compounds. For the methyl esters, columns of working length 4 ft. were filled with Diatopore S that had been coated with 3.8% of SE30 silicone oil as stationary phase. The running conditions were: temperature, 120° ; inlet pressure, 20 lb./in.²; flow rate for N_2 , not known. For the *O*-methoxy and neutral constituents, columns of working length 2 ft. were filled with untreated Celite (100-120 mesh) that had been coated with 5% of phenyl diethanolamine succinate as stationary phase. The running conditions were: temperature, 188° ; flow rate for N_2 , 50 ml./min.

Electrolyte solutions. Formic acid-acetic acid buffer, pH 2.0, was prepared by diluting a mixture of 98-100% formic acid (78 ml.) and 99.6% acetic acid (148 ml.) with water to 2.5 l. Acetate-acetic acid buffer, pH 5.2, was made by mixing 0.2 *N*-acetic acid (200 ml.) and 0.2 *M*-sodium acetate (800 ml.). Veronal-HCl buffer, pH 9.0, was prepared by mixing 936 ml. of 0.1 *M*-veronal and 64 ml. of 0.1 *N*-HCl.

Low-voltage electrophoresis. After electrophoresis on strips of Whatman no. 1 filter paper in veronal-HCl buffer, pH 9.0, at 12 v/cm., [^{14}C]phenols and ^{14}C -labelled aromatic acids had migrated towards the anode; under these conditions, ^{14}C -labelled phenolic acids were separated from ^{14}C -labelled aromatic acids. Electrophoresis in acetate buffer, pH 5.2, at 12 v/cm. caused ^{14}C -labelled aromatic acids to migrate towards the anode. After electrophoresis the paper strips were infrared-heated in an air stream for 10 min. to remove most of the liquid and were subsequently dried at 80° for 15 min. Zones of radioactivity were scanned with the Nuclear-Chicago Actigraph 4 π system.

High-voltage electrophoresis. Electrophoresis on strips of Whatman no. 1 filter paper in formic acid-acetic acid buffer, pH 2.0, in 1 hr. at 120 v/cm. (Hathway, Mallinson & Akintonwa, 1965) caused movement of a ^{14}C - or ^{35}S -labelled

polar constituent towards the cathode with one-half of the speed of glycine, whereas low-voltage electrophoresis in veronal-HCl buffer, pH 9.0, caused the same ^{14}C - or ^{35}S -labelled polar constituent to migrate towards the anode. The dried electrophoretograms were scanned for radioactive zones.

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. Measurements of radioactivity in urine, faeces, gut and contents, carcass plus viscera and skin plus hair and of the $^{14}\text{CO}_2$ in the expired gases were made by the methods described by Wright *et al.* (1965).

Enzymic hydrolysis of the glucuronides of 2,6-dichloro-3- and -4-hydroxybenzonitrile. Solvent extracts of the urine of treated animals contained the glucuronides (R_F values on paper 0.15 and 0.45 in butan-1-ol-2*N*- NH_3) of 2,6-dichloro-3- and -4-hydroxybenzonitrile (see the Methods and Results sections). Separate elution of individual zones from suitable chromatograms provided material for enzymic hydrolysis. After evaporation of the solvent 5 mg. of the residue was dissolved in 0.15 M-sodium acetate buffer, pH 5.0 (10 ml.), and incubated with β -glucuronidase (500 units) at 37° for 3 hr., and the mixture extracted with ether. 2,6-Dichloro-3- or -4-hydroxybenzonitrile was detected on thin-layer plates of the ether extract run in acetone-*n*-hexane (1:1, v/v) with phenoxyethanol- AgNO_3 reagent (Mitchell, 1958). 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 (Elliott, Parke & Williams, 1959) and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

RESULTS

Excretion and retention of radioactivity in rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile. After oral treatment of six

adult male and six adult female rats with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile, the major eliminative route was via the kidneys and the remaining radioactivity was almost entirely eliminated by the faecal route. With the two ^{14}C -labelled herbicides, some ^{14}C was present in the gut of some of the animals at the end of 96 hr., especially when the animals were constipated on the third and fourth days (Tables 1 and 2). A single dose of either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile is almost entirely eliminated from rats in 4 days: 84.8–100.5% of ^{14}C from [^{14}C]Prefix and 85.8–97.2% of ^{14}C from 2,6-dichlorobenzo[^{14}C]nitrile are excreted, these values including the contents of the gut at 96 hr. Rats do not show a sex difference in the pattern of elimination of the respective metabolic products of either Prefix or 2,6-dichlorobenzo[nitrile].

Very small amounts of ^{14}C were found in the carcass and viscera remaining after removal of the gastrointestinal tract from animals dosed with [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile. The values for ^{14}C in the carcass of animals dosed with [^{14}C]Prefix take into consideration the skin and hair (Table 1), whereas the skin plus hair of animals dosed with 2,6-dichlorobenzo[^{14}C]nitrile was assayed separately for ^{14}C .

Excretion of ^{14}C in the urines of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile was very rapid, and the similar rates of elimination for the respective metabolic products of Prefix and 2,6-dichlorobenzo[nitrile] in rat urine do not preclude the possibility that 2,6-dichlorobenzo[nitrile] might be an intermediate in the metabolism of Prefix (Tables 3 and 4). Radioactivity was slowly

Table 1. *Excretion and retention of radioactivity in rats after oral administration of [^{14}C]Prefix*

Male and female rats (150–200 g. body wt.) were treated by stomach tube with 1.8 mg. of [^{14}C]Prefix (3.2 μC /mg.) in 2 ml. of aq. 5% ethanol.

		Recovery of ^{14}C during 4-day collection (% of dose)					
Rat no.	Sex	Faeces	Urine	Expired gases	Gut and contents	Carcass plus remaining viscera	Total
1	♂	20.4	68.0	< 0.05	0	0.4	88.8
2	♂	18.3	79.7		2.5*	0.5	101.0
3	♂	16.0	74.5		0	0.5	91.0
4	♂	17.1	74.8		0.5	0.5	92.9
5	♂	15.6	79.7		0.1	0.3	95.7
6	♂	17.1	67.3		0.4	0.4	85.2
7	♀	16.1	71.8	< 0.05	0	0.3	88.2
8	♀	18.0	71.4		0.4	0.2	90.0
9	♀	18.1	71.8		1.6	0.4	91.9
10	♀	20.9	74.7		0.7	0.3	96.6
11	♀	17.9	72.0		0.1	0.4	90.4
12	♀	9.2	76.0		8.6*	0.3	94.1

* Constipated animals (see Table 3).

Table 2. *Excretion and retention of radioactivity in rats after oral administration of 2,6-dichlorobenzo[¹⁴C]nitrile*

Rats (150–200 g. body wt.) were treated by stomach tube with 0.80 mg. (males) or 1.3 mg. (females) of 2,6-dichlorobenzo[¹⁴C]nitrile (9.1 μ C/mg.) in 2 ml. of arachis oil.

Rat no.	Sex	Recovery of ¹⁴ C during 4-day collection (% of dose)						
		Faeces	Urine	Expired gases	Gut and contents	Skin and hair	Carcass plus remaining viscera	Total
1	♂	19.0	74.3	< 0.02	0.1	0.1	0.1	93.6
2	♂	19.2	73.2		0.9	0.3	0.3	93.9
3	♂	18.5	72.7		0.1	0.1	0.1	91.5
4	♂	13.4	72.3		0.1	0.2	0.2	86.2
5	♂	11.6	76.0		4.6	0.2	0.5	92.9
6	♂	19.1	74.6	< 0.03	1.2	0.2	0.2	95.3
7	♀	13.6	76.9		4.0	0.8	0.3	95.6
8	♀	15.1	80.7		0.6	0.6	0.2	97.2
9	♀	17.7	74.8		0.2	0.7	0.3	93.7
10	♀	16.3	78.6		0.1	0.5	0.3	95.8
11	♀	19.7	77.3		0.2	0.5	0.2	97.9
12	♀	11.5	77.1		6.1*	0.5	0.3	95.5

* Constipated animal (see Table 4).

Table 3. *Rates of faecal and urinary elimination of radioactivity in rats after oral administration of [¹⁴C]Prefix*

Male and female rats were treated with [¹⁴C]Prefix as described in Table 1.

Rat no.	Sex	Elimination of ¹⁴ C (% of dose)							
		0–24 hr.		24–48 hr.		48–72 hr.		72–96 hr.	
		Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
1	♂	15.3	66.1	4.7	1.3	0.3	0.4	0.1	0.3
2	♂	11.8	76.3	5.3	2.3	1.2	0.6	No faeces	0.5
3	♂	9.5	72.4	4.3	1.5	2.0	0.4	0.3	0.3
4	♂	13.8	72.1	2.6	1.8	0.3	0.6	0.4	0.4
5	♂	4.2	75.1	5.9	3.2	4.94	0.8	0.51	0.6
6	♂	10.4	65.2	1.5	0.5	3.8	1.1	1.3	0.5
7	♀	No faeces	70.0	15.4	0.9	0.5	0.6	0.1	0.4
8	♀	0.01	67.2	12.3	2.7	5.1	0.6	0.6	0.8
9	♀	9.6	67.5	3.6	2.5	1.7	0.8	3.2	1.0
10	♀	9.2	68.6	4.5	4.6	2.4	1.1	4.7	0.5
11	♀	No faeces	67.3	9.8	3.3	7.7	0.9	0.4	0.4
12	♀	6.0	71.2	3.2	3.6	No faeces	0.7	No faeces	0.5

eliminated in the faeces, and some irregularity in faecal elimination may be due to coprophagy. Alimentary absorption is virtually complete and the two herbicides are rapidly metabolized. A big proportion of the products of metabolism of each herbicide enters the circulating blood and is rapidly cleared in the kidneys; the low rate of elimination of metabolites in the faeces is probably due to enterohepatic circulation.

Elimination of radioactivity in dogs dosed with either [¹⁴C]Prefix or 2,6-dichlorobenzo[¹⁴C]nitrile. A single oral dose of either [¹⁴C]Prefix or 2,6-dichlorobenzo[¹⁴C]nitrile in dogs is almost entirely eliminated in 4 days: 85.9–106.1% of ¹⁴C from [¹⁴C]Prefix and 86.8–92.5% of ¹⁴C from 2,6-dichlorobenzo[¹⁴C]nitrile are excreted. The major eliminative route was via the kidneys and the remainder of the original label was almost entirely

Table 4. *Rates of faecal and urinary elimination of radioactivity in rats after oral administration of 2,6-dichlorobenz[¹⁴C]nitrile*Male and female rats were treated with 2,6-dichlorobenz[¹⁴C]nitrile as described in Table 3.

Rat no.	Sex	Elimination of ¹⁴ C (% of dose)							
		0-24 hr.		24-48 hr.		48-72 hr.		72-96 hr.	
		Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
1	♂	10.8	69.1	3.7	3.6	2.8	1.2	1.7	0.3
2	♂	1.5	63.6	14.1	7.7	2.4	1.3	1.3	0.6
3	♂	1.6	63.9	14.9	7.3	1.6	1.1	0.4	0.4
4	♂	9.5	66.7	3.3	2.6	0.4	1.2	0.2	1.8
5	♂	2.9	64.9	5.0	7.8	3.1	1.7	0.6	1.6
6	♂	5.9	67.6	12.4	3.8	0.4	1.0	0.4	2.2
7	♀	2.0	65.7	1.5	8.6	2.8	1.7	7.3	0.9
8	♀	No faeces	71.0	7.1	6.7	3.9	1.6	4.1	1.4
9	♀	4.0	68.5	5.7	4.1	5.2	1.5	2.8	0.7
10	♀	2.3	71.5	3.7	5.6	9.8	1.0	0.5	0.5
11	♀	4.4	70.0	6.4	6.0	6.3	0.9	2.6	0.4
12	♀	11.4	70.1	0.1	5.2	No faeces	1.1	No faeces	0.7

Table 5. *Rates of faecal and urinary elimination of radioactivity in male and female dogs after oral administration of either [¹⁴C]Prefix or 2,6-dichlorobenz[¹⁴C]nitrile*Each dog swallowed a gelatin capsule containing a solution of either 7.90 mg. of [¹⁴C]Prefix (2.8 μ C/mg.) in ethanol (1 ml.) or 0.92 mg. of 2,6-dichlorobenz[¹⁴C]nitrile (8.52 μ C/mg.) in arachis oil (1 ml.).

Dog no.	Body wt. (kg.)	Sex	Elimination of ¹⁴ C (% of dose)							
			0-24 hr.		24-48 hr.		48-72 hr.		72-96 hr.	
			Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
			[¹⁴ C]Prefix							
1	14.8	♂	No faeces	33.5	No faeces	18.5	24.4	11.9	1.6	2.7
2	14.5	♂	6.6	33.0	No faeces	16.2	9.2	14.6	2.2	4.1
3	10.4	♀	No faeces	55.1	No faeces	20.8	24.5	4.1	0.7	0.9
4	10.2	♀	0.6	51.3	3.8	18.2	No faeces	6.4	11.6	1.6
2,6-Dichlorobenz[¹⁴ C]nitrile										
5	11.5	♂	16.8	50.7	4.3	10.7	1.5	2.3	0.4	0.8
6	12.3	♂	15.1	44.8	10.2	11.2	1.5	2.9	0.4	1.1
7	12.7	♀	16.6	59.7	1.8	6.6	3.8	3.2	0.2	0.6
8	7.2	♀	11.2	44.1	5.3	13.7	2.6	6.2	1.4	2.3

eliminated by the faecal route (Table 5). Dogs do not show a sex difference in this pattern of elimination of the metabolites of either Prefix or 2,6-dichlorobenzonitrile. Dogs and rats do not show species differences in the patterns of elimination of the metabolic products of either Prefix or 2,6-dichlorobenzonitrile. Excretion of ¹⁴C in the urines of treated dogs was slower than in rats. The elimination of ¹⁴C in the urines of dogs treated with [¹⁴C]Prefix was irregular and the rate of elimination of ¹⁴C was different for the two sexes. This experiment was complicated by irregular defaecation of the animals, which were ill-adjusted to the diet, and the number of observations on the output of

¹⁴C in the urine was too small for a conclusion on the difference in rate to be made.

Separation of fractions of chemically similar substances from the hydrolysed urines of rats dosed respectively with [¹⁴C]Prefix and 2,6-dichlorobenz[¹⁴C]nitrile. Rats treated with Prefix or 2,6-dichlorobenzonitrile do not eliminate either herbicide unchanged. 2,6-Dichlorobenzonitrile may be a metabolite of Prefix with a high turnover, since 2,6-dichlorobenzonitrile is itself completely metabolized in rats, and to test this supposition the metabolic products of the two herbicides were investigated.

By successive extraction at pH 1.5-2.0 with ether

and ethyl acetate, 80% of the radioactivity was recovered from the hydrolysed urines of rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile. In both cases the ethyl acetate extract contained one-half of the amount of ^{14}C found in the ether extract. Scanning revealed six radioactive zones on paper chromatograms of the ether-soluble material that had been run in butan-1-ol saturated with 2*N*-ammonia, and the fact that they reacted with phenoxyethanol-silver nitrate (Mitchell, 1958) was regarded as evidence that the main ^{14}C -labelled metabolite in each of the zones contained chlorine. These ether-soluble ^{14}C -labelled constituents were fractionated by column chromatography into aromatic acids, neutral constituents and phenols (Table 6). The ethyl acetate extracts of hydrolysed urines contained the more polar ^{14}C -labelled constituents, which also reacted with the phenoxyethanol-silver nitrate reagent.

A solution of each phenolic fraction in sodium

hydroxide was purified by solvent extraction. Electrophoresis in veronal-hydrochloric acid buffer, pH 9.0, caused phenolic ^{14}C -labelled constituents to migrate towards the anode, whereas in acetate buffer, pH 5.2, they did not move. Major ^{14}C -labelled constituents that were separated by thin-layer chromatography were identified as 2,6-dichloro-3- and -4-hydroxybenzonitrile by their fluorescences in the ultraviolet and chromatographic properties (Table 7).

A solution of each neutral fraction was purified by extraction with 2*N*-sodium hydroxide, and electrophoresis of these fractions in buffers, pH 2.0–9.0, did not cause movement. A single ^{14}C -labelled constituent was identified as 2,6-dichlorobenzamide by its chromatographic properties (Table 7).

The acidic fractions were purified either by solvent extraction of their solution in aqueous sodium hydrogen carbonate or by thin-layer chromatography (Table 7). After electrophoresis in acetate

Table 6. *Separation of ether-soluble ^{14}C -labelled constituents of the hydrolysed urines into fractions of chemically similar substances*

Proportions of the ether-soluble material from the hydrolysed urines of rats (150–200 g. body wt.) that had each been dosed with 1.8 mg. of [^{14}C]Prefix (3.2 $\mu\text{C}/\text{mg.}$) and 0.8 mg. of 2,6-dichlorobenzo[^{14}C]nitrile (9.1 $\mu\text{C}/\text{mg.}$) were chromatographed on a silicic acid column (10 mm. diam. $\times$ 10 cm. long). Recovery was quantitative.

Fraction no. (10 ml.)	Eluent	Recovery of radioactive constituents
1–3	20% Acetone in <i>n</i> -hexane	Phenolic fraction (80% of ^{14}C)
4–6	50% Acetone in <i>n</i> -hexane	Neutral fraction (1% of ^{14}C)
7–9	Acetone	Aromatic acids (19% of ^{14}C)

Table 7. *Fluorescences, R_F values on paper and thin plates, and retention times on gas-liquid chromatographs for the ether-soluble constituents in hydrolysed urines of rats dosed with either Prefix or 2,6-dichlorobenzonitrile*

The labelled constituents were identified by comparing their chromatographic and fluorescence properties with those of the authentic unlabelled compounds.

Constituents in hydrolysed urines	R_F		Fluorescence in u.v.	Retention times for the methylated derivatives* (min.)
	Paper in butan-1-ol satd. with 2 <i>N</i> - NH_3	Thin-layer plates in acetone- <i>n</i> -hexane (1:1, v/v)		
2,6-Dichlorobenzamide	0.92	0.7	Dull violet	15.0
2,6-Dichloro-3-hydroxybenzonitrile	0.66	0.9	Bright violet	4.0
2,6-Dichloro-4-hydroxybenzonitrile	0.72	0.7	Dull violet	3.5
2,6-Dichlorobenzoic acid	0.45	0.02	Dull violet	4.0
2,6-Dichloro-3-hydroxybenzoic acid	0.06	0.02	Dull violet	17.6
Unknown aromatic acid (2,6-Dichloro-4-hydroxybenzoic acid)	0.16	0.02	—	—

* After their separation, the acid, the two phenols and the phenolic acid respectively were exhaustively methylated with excess of diazomethane in ether solution, and *n*-hexane solutions of the methylated derivatives were used for gas-liquid chromatography: (a) Stationary phase: 5% (w/w) phenyl diethanolamine succinate on Celite; flow rate, 50 ml./min.; temperature, 188°. (b) Stationary phase: 3.8 (w/w) SE30 silicone oil on Diatopore S; chromatograph not equipped with flow-meter; temperature, 120°.

buffer, pH 5.2, acidic ^{14}C -labelled constituents migrated towards the anode. Major ^{14}C -labelled components in the acidic fractions that were separated by paper chromatography were identified as 2,6-dichlorobenzoic acid and 2,6-dichloro-3-hydroxybenzoic acid by their chromatographic properties (Table 7). An unidentified ^{14}C -labelled component was possibly 2,6-dichloro-4-hydroxybenzoic acid. After electrophoresis in veronal-hydrochloric acid buffer, pH 9.0, 2,6-dichloro-3-hydroxybenz[^{14}C]oic acid migrated twice as far as 2,6-dichlorobenz[^{14}C]oic acid.

Polar ^{14}C -labelled constituents, which account for the radioactivity in the residual aqueous phase after ether extraction, could be quantitatively extracted with butan-1-ol, but the resulting solution was contaminated with large amounts of the components of normal urine, including electrolytes and water. A solution, containing major proportions of these ^{14}C -labelled constituents and smaller amounts of the contaminating substances, especially electrolytes and water, was prepared by exhaustive extraction with ethyl acetate; four main radioactive zones and two minor ones were present on paper chromatograms. At least one ^{14}C -labelled component was adsorbed on to a column of cation-exchange resin in the H^+ form and eluted with basic buffers. Electrophoresis (120 v/cm.) in acetic acid-formic acid buffer, pH 2.0, caused a single radioactive peak to migrate towards the cathode with one-half of the speed of glycine. In acetate buffer, pH 5.2, there was no movement in an electric field, and electrophoresis in veronal-hydrochloric acid buffer, pH 9.0, caused a single radioactive peak to move towards the anode. The presence of at least one deacetylated [^{14}C]mercapturic acid in the hydrolysed urines would account for these observations, and this supposition was tested by labelling the cysteine-cystine pools of rats with ^{35}S and treating these animals with the unlabelled herbicides (see the Methods section; Gutmann & Wood, 1950; Marsden & Young, 1958). This experiment provides clear evidence for the presence in the hydrolysed urines of two ^{35}S -labelled constituents, R_f 0.37 and 0.47 on paper. These ^{35}S -labelled constituents were absent from paper chromatograms of the hydrolysed urine of the control animals that had been given [^{35}S]cystine. Neither of these ^{35}S -labelled constituents could have been confused with $^{35}\text{SO}_4^{2-}$, which ran entirely differently in the butan-1-ol-2*N*-ammonia solvent system employed, and neither of them was due to organic [^{35}S]sulphate esters which would have been hydrolysed by the acid hydrolysis. These ^{35}S -labelled hydrolysis products were ninhydrin-positive, and they migrated in an electric field (120 v/cm.) in acetic acid-formic acid buffer, pH 2.0, towards the cathode and in veronal-

hydrochloric acid buffer, pH 9.0, towards the anode. The foregoing chromatographic and electrophoretic properties of the ^{35}S -labelled constituents corresponded to those of two ^{14}C -labelled constituents present in the hydrolysed urines of animals dosed with the ^{14}C -labelled herbicides. The conclusion is drawn that the hydrolysed urines of animals dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile and the hydrolysed urines of animals treated with [^{35}S]cystine and the unlabelled herbicides contained two sulphur-containing constituents derived from metabolite(s) of the two herbicides, and that the physical and chemical properties of these constituents are consistent with the possession of an associated amino acid residue. From the conspectus of knowledge on the mammalian metabolism of foreign organic compounds, it is known that there are two common types of sulphur-containing metabolites, mercapturic acids and sulphate esters (see Young & Maw, 1958). For the hydrolytic products, we are not concerned with sulphate esters and the sulphur-containing compounds in the hydrolysed urines may have been produced from mercapturic acid(s) or premercapturic acid(s) by hydrolysis. The nature of the amino acid residue in each sulphur-containing constituent has not been investigated, and the structures of the two sulphur-containing constituents have not been elucidated; this would require the isolation of pure compounds followed by careful degradative studies. However, our knowledge of the chemistry of the two herbicides indicates that the chloro substituents would not be expected to undergo nucleophilic replacement, and that the reactivities of the cyano group of 2,6-dichlorobenzonitrile and of the thioamido group of Prefix are very hindered indeed. Thus the addition of hydrogen sulphide to 2,6-dichlorobenzonitrile takes place under forcing conditions, whereas the addition of mercaptans does not take place under any conditions (Dr J. Yates, personal communication). The two sulphur-containing compounds in hydrolysed urines of treated animals may therefore be substituted 2,6-dichlorobenzonitriles, and this was tested by dethiolation with Raney nickel catalyst, when each of the sulphur-containing constituents generated a compound with the chromatographic properties of 2,6-dichlorobenzonitrile. The data seem to be consistent with the partial-type formula (VII) (Scheme 1) suggested for these compounds.

In the work-up of the hydrolysed urine from animals dosed with the ^{14}C -labelled herbicides, the previously mentioned ^{14}C that remained in the aqueous phase after exhaustive extraction with ether and ethyl acetate, and that could be completely extracted with butan-1-ol, was shown to be almost entirely due to further amounts of the two sulphur-containing constituents, R_f 0.37 and 0.47

Table 8. Proportions of ^{14}C -labelled constituents in the hydrolysed urines of rats dosed respectively with $[^{14}\text{C}]\text{Prefix}$ and 2,6-dichlorobenzo $[^{14}\text{C}]$ nitrile

The labelled constituents were identified by comparing their chromatographic and fluorescence properties with those of the authentic unlabelled compounds.

Constituents in hydrolysed urine	Rats dosed with ...	Proportions of ^{14}C -labelled metabolites (% of total ^{14}C in the urines)	
		$[^{14}\text{C}]\text{Prefix}$	2,6-Dichlorobenzo- $[^{14}\text{C}]$ nitrile
2,6-Dichlorobenzamide		< 1.0	< 1.0
2,6-Dichlorobenzoic acid		2.3	< 1.0
2,6-Dichloro-3-hydroxybenzoic acid		2.8	5.0
2,6-Dichloro-3-hydroxybenzonitrile		42.0	41.7
2,6-Dichloro-4-hydroxybenzonitrile		2.8	4.0
Unknown aromatic acid (R_f 0.16)		2.1	1.3
Ethyl acetate-soluble metabolites: (I)		3.0	3.0
(II)		4.9	6.8
(III)		8.1	9.0
(IV)		9.4	6.5
(V)		< 1.0	2.2
(VI)		< 1.0	1.5
Recovery ...		> 77.4	> 81.0

Scheme 1. Transformations of Prefix and 2,6-dichlorobenzonitrile in the rat.

on paper. Greater proportions of these compounds than those shown in Table 8 were therefore present in the hydrolysed urines, from which they were incompletely extracted with ethyl acetate. The two sulphur-containing constituents are accordingly important to the transformations of Prefix and 2,6-dichlorobenzonitrile in the rat.

The structures of the ^{14}C -labelled constituents that have been investigated in the hydrolysed urine are related to those of the two herbicides in such a way that they may have been formed direct from the ^{14}C -labelled metabolites by hydrolysis.

Relative proportions of the various constituents in the hydrolysed urine of rats dosed with Prefix and 2,6-dichlorobenzonitrile. Quantitative aspects of the comparative metabolism of Prefix and 2,6-dichlorobenzonitrile in rats are summarized in Table 8. Pooled urines of 12 rats dosed with [^{14}C]Prefix and pooled urines of 12 rats dosed with 2,6-dichlorobenzonitrile were separately hydrolysed in the way that has been described, and radioactivity corresponding to the different ^{14}C -labelled constituents present in the various fractions was measured. These results were reproducible, the experiment being repeated with two further sets of 24 rats with identical results. Metabolism of the two herbicides yields the same constituents in the hydrolysed urine in strikingly similar proportions. For Prefix (I) (Scheme 1), *m*-hydroxylation accounts for 44.8% of the constituents (III and IV), and, on the assumption that the unknown aromatic acid is 2,6-dichloro-4-hydroxybenzoic acid (VI), *p*-hydroxylation accounts for 4.9% of the constituents (V and VI). For 2,6-dichlorobenzonitrile (II), the corresponding values are 46.7 and 5.3%. Metabolism of the two herbicides also yields approximately similar proportions of polar constituents, of which two of the ethyl acetate-soluble constituents are sulphur-containing compounds of partial-type formula (VII).

Since Prefix and 2,6-dichlorobenzonitrile have essentially similar biological properties and herbicidal activities (Barnsley, 1960), and since 2,6-dichlorobenzonitrile is the main metabolite of Prefix in plants and unsterilized soil (Barnsley, 1960; Milborrow, 1965), the possibility arises that Prefix might be transformed into 2,6-dichlorobenzonitrile in animals, and the fact that the metabolism of the two herbicides has given the same constituents in the hydrolysed urine in strikingly similar proportions strongly suggests an interrelationship of the metabolic pathways of the two herbicides. Prefix therefore appears to be extensively transformed into 2,6-dichlorobenzonitrile:

There is slight evidence that the competitive reactions:

take place to a very limited extent, since the hydrolytic product, 2,6-dichlorobenzoic acid (IX) (Scheme 1), is formed *in vivo* in greater quantity from Prefix than from 2,6-dichlorobenzonitrile; but the same quantity of 2,6-dichlorobenzamide was measured in parallel experiments with the two herbicides.

The results are not consistent with direct hydroxylation of Prefix and subsequent hydrolysis of thioamido products. The stability of 2,6-dichloro-3-hydroxythiobenzamide to boiling 6*N*-hydrochloric acid, and its absence from the hydrolysed urines of animals dosed with Prefix, is strong evidence that hydroxylated thiobenzamides were not formed *in vivo*.

Scheme 1 shows the transformations of Prefix and 2,6-dichlorobenzonitrile in rats.

^{14}C -labelled metabolites in the unhydrolysed urines of rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzonitrile. Free 2,6-dichloro-3- and -4-hydroxybenzonitrile were extracted in small quantity from the urine of rats dosed with either Prefix or 2,6-dichlorobenzonitrile. These phenols may have been excreted as their sulphate esters, which would probably have been hydrolysed under the prevailing acidic conditions of our ether extraction (see Young & Maw, 1958). In other experiments, urine at pH 6.0 was extracted with ether, and the material in the residual aqueous phase was submitted to paper electrophoresis (13 v/cm.) in 0.025*M*-phthalate buffer, pH 5.9, for various times. The rapidly migrating radioactive area from +14 to +20 cm. (Fig. 1) of strongly anionic substances was tentatively ascribed to strongly acidic sulphate esters; in another context similar assignments have also been made (Williams, Meikle & Redemann, 1964). The relative proportion of strongly anionic substances, when urines were extracted at pH 6.0, was almost the same as that of the free phenols, when the urines were extracted under more acid conditions.

Glucuronides of 2,6-dichloro-3- and -4-hydroxybenzonitrile had R_F 0.15 and 0.45 on paper in the butan-1-ol-2*N*-ammonia solvent system. Such compounds were also present in the urine of treated animals, and separation by paper chromatography followed by hydrolysis of individual components with β -glucuronidase generated 2,6-dichloro-3- and -4-hydroxybenzonitrile and free glucuronic acid (for details see the Methods section).

The aromatic acids that had previously been found in the hydrolysed urines of rats dosed with the two herbicides appear to be present as ester

Fig. 1. Electrophoretic separation of ^{14}C -labelled metabolites in the residual aqueous phase after ether extraction of urine at pH 6.0. Metabolites were separated from the residual aqueous phase by electrophoresis on Whatman no. 1 filter papers in 0.025M-phthalate buffer, pH 5.9, at 13 v/cm. for 2.5 hr. (—) or 1.0 hr. (----).

glucuronides in the original urines. Hydrolysis with β -glucuronidase caused the disappearance of radioactive spots from the paper chromatograms of ether-extracted urines and the simultaneous generation of other radioactive spots due to the ^{14}C -labelled aromatic acids that had been found in the hydrolysed urines.

The urine of rats in which the cysteine-cystine pools had been labelled with ^{35}S and where the animals were dosed with unlabelled herbicides contained two ^{35}S -labelled metabolites that were absent from the urine of control rats that had been given [^{35}S]cystine. One of these substances could be extracted by ether (R_f 0.20 on paper in butan-1-ol-2N-ammonia) and the other by ethyl acetate (R_f 0.27 on paper in butan-1-ol-2N-ammonia). These metabolites did not migrate in an electric field (120 v/cm.) towards the cathode in acetic acid-formic acid buffer, pH 2.0, and they did not give a colour reaction with ninhydrin. The chromatographic and electrophoretic properties of the foregoing ^{35}S -labelled metabolites corresponded to those of two ^{14}C -labelled metabolites present in the urines of rats dosed with the ^{14}C -labelled herbicides. The conclusion is drawn that the urines of rats dosed with [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile contained two sulphur-containing metabolites. Hydrolysis of these metabolites with 6N-hydrochloric acid gave the two ^{14}C -labelled sulphur-containing amino acid constituents, which migrated in an electric field towards the cathode in buffers of pH 2.0 and towards the anode in buffers of pH 9.0. The generation of a single sulphur-containing amino

acid from each of the sulphur-containing metabolites, in the way that has been described, may have been brought about by elimination of an acyl group from the amino nitrogen atom of each sulphur-containing acylamino acid metabolite. The conditions selected for hydrolysis of the urine were those that would have been sufficiently drastic to effect *N*-deacetylation of *N*-acetylcysteyl-containing metabolites (Thomson, Barnsley & Young, 1963). The structures of the two sulphur-containing metabolites have not been elucidated; at first sight, they may be analogous to 2,6-dichlorobenzonitrile mercapturic acids or premercapturic acids.

Quantitative assessments were made on the unhydrolysed urines of rats dosed with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile to give information for whole urines of the sort reported in Table 8 for hydrolysed urines. Not much would be gained by the inclusion of detailed information, since reference would have to be made to unknown metabolites and partially elucidated metabolites. Valuable confirmation was obtained of the amounts of ^{14}C -labelled constituents present in the various solvent extracts of hydrolysed urines by the preparation of solvent extracts of hydrolysates of the corresponding solvent extracts of whole urine.

DISCUSSION

Treatment of animals with Prefix or 2,6-dichlorobenzonitrile has given rise to the expected metabolic products.

3- and 4-Hydroxylation of 2,6-dichlorobenzonitrile has now been demonstrated *in vivo*; hydroxylation is predominantly *meta*, owing to the inductive effect of the 2,6-dichloro atoms. The 2,6-dichloro substituents contribute to the rapid rate of hydroxylation of 2,6-dichlorobenzonitrile *in vivo*, since the slowness with which the metabolic products of benzonitrile and nitrobenzene are eliminated from the body has been attributed to difficulty associated with the hydroxylation of aromatic compounds that have *meta*-directing groups (Bray, Hybs & Thorpe, 1951; Smith, 1950; Smith & Williams, 1950). By analogy with chlorobenzene (Smith, Spencer & Williams, 1950) and naphthalene (Corner & Young, 1955), the possibility that hydroxylation of 2,6-dichlorobenzonitrile *in vivo* may involve intermediate formation of 2,6-dichloro-3,4-dihydro-3,4-dihydroxybenzonitrile is attractive, since this diol might itself be formed from an epoxide precursor, which would also be expected to react with the thiol group of cysteine, glutathione or tissue proteins (Parke & Williams, 1958) to yield the two sulphur-containing acylamino acid metabolites that have been discussed.

In general, aryl nitriles are metabolized to only a

small extent by reactions involving the cyano group:

The reaction is a minor one, and its extent depends on the nature of the substituents in the benzene nucleus. With benzonitrile and *o*-tolunitrile, oxidation of the aromatic nucleus is the major pathway of metabolism, but at least 10% of benzonitrile and 25% of *o*-tolunitrile are metabolized by hydrolysis of the cyano group to carboxyl (Bray *et al.* 1951). Less than 2% of the sterically hindered 2,6-dichlorobenzonitrile is metabolized *in vivo* to 2,6-dichlorobenzamide plus 2,6-dichlorobenzoic acid. These compounds cannot be produced *in vitro* by drastic acid hydrolysis, and it is noteworthy that the amide is stable to prolonged refluxing with 6*N*-hydrochloric acid. The hydroxylation products, 2,6-dichloro-3- and -4-hydroxybenzonitrile, are also partially hydrolysed *in vivo* to 2,6-dichloro-3- and -4-hydroxybenzoic acid.

In agreement with the known behaviour of other aryl nitriles, no evidence was found for the formation of hydrogen cyanide from 2,6-dichlorobenzonitrile *in vivo*. Thus when 2,6-dichlorobenzonitrile was administered orally, LD₅₀ values (expressed as mg./kg.) were more than 3000 (rats), 866 (mice) and more than 1250 (chickens). The toxicological properties and symptomatology of acute poisoning are incompatible with release of hydrogen cyanide.

The authors thank their colleagues Mr P. A. Harthoorn for synthesizing the radioactive herbicides, Mr A. Richardson for help with the gas-liquid chromatography and Dr J. Yates for providing some intermediates and reference compounds. They are also grateful to Dr J. G. Wit, Instituut voor Veterinaire Farmacologie der Rijksuniversiteit te Utrecht, for a gift of 2,6-dichloro-4-hydroxybenzonitrile.

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