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Metabolism of the Herbicide 2,6-Dichlorobenzonitrile in Rabbits and Rats

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1. The metabolism of 2,6-dichlorobenzonitrile was studied in rabbits and rats. Oral administration caused an increased urinary excretion of glucuronides and ethereal sulphates. There was also an indication of mercapturic acid formation. 2,6-Dichloro-3-hydroxybenzonitrile and its 4-hydroxy analogue were identified as metabolites in the urine. A small amount of the unchanged substance was recovered from the faeces. 2. By using 2,6-dichlorobenzo[^{14}C]nitrile the phenolic metabolites were determined quantitatively and some other possible metabolic routes were excluded. 3. Incubation of 2,6-dichlorobenzonitrile with enzyme preparations (papain and high-speed supernatant of rat-liver homogenate plus glutathione) gave no indications for a reaction with thiol compounds.

Since the discovery of the naturally occurring plant growth hormone indol-3-ylacetonitrile by Jones, Henbest, Smith & Bentley (1952), synthetic organic nitriles have been screened for their biological effects on plants. 2,6-Dichlorobenzonitrile (Dichlobenil, Casoron 133) was the first powerful herbicide among a series of substituted benzonitriles to be reported (Koopman & Daams, 1960). Additional studies on the effects on plants were made by Barnsley (1960), Barnsley & Rosher (1961) and Daams & Barnsley (1961). The ability of plants and soil micro-organisms to convert 2,6-dichlorobenzaldoxime into 2,6-dichlorobenzonitrile was studied by Milborrow (1963). Massini (1961) reported that, after treatment of plants with 2,6-dichlorobenzonitrile, extracts contained a residue of the original compound and an unknown substance that was not 2,6-dichlorobenzoic acid. Pate, Funderburk & Davis (1965) indicated that 2,6-dichlorobenzoic acid was a metabolite of 2,6-dichlorobenzonitrile in plants.

The compound is used as a weed-killer and, because of its relatively low toxicity in warm-blooded animals (mice), its application as an anti-sprouting agent for potatoes promised to be of value. Additional toxicological studies have been made by H. G. Verschuuren, R. Kroes & G. J. van Esch (personal communication). Surprisingly, it appeared in this work that rabbits are far more sensitive to the acute effects of the compound than rats. The acute oral LD_{50} in rabbits was about one-tenth of the corresponding value for rats. In rabbits an oral dose of 250mg./kg. body wt. or higher may produce hepatic damage resulting in death. These observations initiated a metabolic study with both rats and rabbits to find a correlation between metabolic events and toxicological effects.

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EXPERIMENTAL

Materials and reference compounds. All melting points are uncorrected.

A batch of the technical product 2,6-dichlorobenzonitrile was purified by boiling an acetone solution with activated charcoal, filtering and precipitation by adding water. The purified compound had m.p. 143–144°. For isotope-dilution experiments, the compound was recrystallized from methanol, m.p. 144–145°. Samples of 2,6-dichlorobenzo-[^{14}C]nitrile (m.p. 144–145°), 2,6-dichlorobenzoic acid (m.p. 142–144°), 2,6-dichlorobenzamide (m.p. 201–201·5°), 2,6-dichloro-3-hydroxybenzonitrile (m.p. 197–199°), 2,6-dichloro-4-hydroxybenzonitrile (m.p. 226–228°), 2,6-dichloro-3-hydroxybenzamide (m.p. 182·5–183°) and 2,6-dichloro-4-hydroxybenzamide (m.p. 251–253°) were obtained from Philips Duphar Research Laboratories, Weesp, The Netherlands.

Scintillation liquids. One scintillation liquid was prepared as described by Meade & Stiglitz (1962), as modified by Houtman (1965), and consisted of 830ml. of toluene, 140g. of Hyamine 10X (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), 55ml. of methanol, 7·0g. of 2,5-diphenyloxazole and 0·050g. of 1,4-bis-(5-phenyloxazol-2-yl)-benzene. This liquid is referred to as T.H.M.

A second liquid was prepared according to the method of Jeffay & Alvarez (1961): 5·5g. of 2,5-diphenyloxazole was dissolved in 1l. of toluene–ethylene glycol monomethyl ether (2:1, v/v). This solution is referred to as Jeffay's liquid.

Animal experiments. Rats [Wistar and Choclat (one

group)] of about 2 months old and 150–200g. body wt. were used. Rabbits (F_1 generation of Danish Rabbit × Flemish Giant) of about 4 months old and about 2kg. body wt. were used. For the metabolism experiments the animals were kept in metabolism cages on a pellet diet. The daily amounts of food and water both before and during the experimental period were rationed to stabilize as much as possible the amount of conjugates in the urine before and after oral administration of the test material. The animals, deprived of food for about 16–18hr., were dosed with a water suspension of the compound by stomach tube (rabbits about 100ml., rats about 1ml./100g. body wt.).

Conjugates in urine. Glucuronides, ethereal sulphates and mercapturic acids were determined as described by Read, Smith & Williams (1958), Bray & Thorpe (1957) and Spencer & Williams (1950) respectively. Quantitative glycine-conjugate determinations as described by El Masri, Smith & Williams (1956) and Umberger & Fiorese (1963) failed in our hands.

Metabolites in urine. Rat and rabbit urines were collected for 48hr. and acidified to pH4–5. Samples (100–200ml.) were steam-distilled. The residue was mixed with 35% (w/w) HCl (⅓vol.) and refluxed for 3hr. After cooling, K_2CO_3 was added to adjust the pH to 4–5, followed by continuous ether extraction for 8hr., giving the 'phenolic ether' extract. Then HCl was added to lower the pH to about 1 and another continuous ether extraction (8hr.) was carried out, yielding the 'acid ether' extract. The ether extracts were dried separately over anhydrous Na_2SO_4 and evaporated to a small volume or a syrup. No crystallization occurred. Samples of the concentrates were diluted with acetone and used for chromatographic analysis.

Analysis of the faeces. Faeces, and occasionally the contents of the digestive tract of rabbits that had died from poisoning within 24hr. after oral administration, were homogenized in water and steam-distilled.

Chromatography. Acetone dilutions of the concentrated urinary ether extracts were applied to Whatman no. 1 paper. Descending chromatograms were developed at room temperature until the solvent front was about 20cm. from the application sites. The solvent systems used are listed in Table 3. The dried chromatograms were examined under u.v. light (366mμ) and then sprayed with: (1) a dilute aqueous solution of Brentamine Fast Blue B Salt (British Drug Houses Ltd., Poole, Dorset), followed by aq. NH_3 (sp.gr. 0·88) (phenol reagent); (2) 0·1M-$K_2Cr_2O_7$-acetic acid (1:1, v/v), followed by 0·1M-$AgNO_3$ (Knight & Young, 1958) (mercapturic acid reagent); (3) *p*-dimethylaminobenzaldehyde in acetic anhydride (El Masri *et al.* 1956) (glycine-conjugate reagent); (4) 1% KIO_3, KI and starch in water (acid reagent).

Thin-layer chromatography on alkaline Al_2O_3 (Fluka A.-G., Basle, Switzerland) was carried out with ethanol–5N-acetic acid (23:2, v/v) as developing solvent system. To detect chlorine-containing organic compounds, the chromatogram was sprayed with a 2-phenoxyethanol–$AgNO_3$ mixture followed by exposure to u.v. light (254mμ), as described by Mitchel (1958) (see Table 3).

Radioactivity determinations. All radioactivity determinations were performed by liquid-scintillation counting in a Tri-Carb Counter (Packard Instruments Co.).

(*a*) Urine. The radioactivity of the (pooled) urines was measured by adding 0·5ml. to 15ml. of T.H.M. After repeated countings, 0·5ml. of a standard solution of 2,6-dichlorobenzo[^{14}C]nitrile [0·02% (w/v) in toluene–methanol (16:1, v/v)] was added and radioactivity was counted again. The total amount of urinary radioactivity was expressed as μg. of 2,6-dichlorobenzonitrile equivalents.

(*b*) Faeces. A 0·5ml. sample of the (pooled) acetone extracts was mixed with 0·5ml. of toluene and the acetone was allowed to evaporate at room temperature. Scintillation liquid (T.H.M.) was added. Counting and calculation were the same as described above. The solid residue, after acetone extraction, was dried at 60° and samples (100mg.) were burned in a combustion flask in an O_2 atmosphere. The $^{14}CO_2$ was captured in 5ml. of ethanolamine–ethylene glycol monomethyl ether (1:4, v/v). Samples were taken and added to 15ml. of Jeffay's scintillation liquid. Counting and calculation were carried out as described above.

Isotope dilutions. (*a*) 2,6-Dichlorobenzonitrile. Urine (pooled sample, pH about 3) was added to an ether solution containing carrier 2,6-dichlorobenzonitrile and shaken by hand. The ether was separated, dried over anhydrous Na_2SO_4 and evaporated. The crystalline material was steam-distilled. The distillate was extracted by ether. The ether was dried and evaporated. The crystals were purified by recrystallization first from boiling dichloroethane by adding a small volume of light petroleum (b.p. 40–60°) and then twice from methanol.

For acetone extracts of faeces (individual samples), the carrier was dissolved and the acetone evaporated. The residue was steam-distilled. The distillate was treated as described for urine.

(*b*) 2,6-Dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile. Carrier amounts were added to individual samples of urine (pH above 8) and dissolved. Conc. HCl was added to about 4N and the mixture was boiled for 3hr. After cooling, the mixture was made alkaline to pH above 8 and centrifuged to precipitate solid materials. The clear solution was decanted and treated with conc. HCl, giving a precipitate. The precipitate was collected by centrifuging. This cycling through alkalinization and acidification was repeated. The precipitate was dissolved in acetone and evaporated until dry. The residue was dissolved in boiling benzene, filtered through cotton wool and chromatographed on silicic acid columns (10cm. × 1cm.). The compounds were eluted with benzene–ethanol (40:1, v/v). Recrystallization was carried out from methanol–water (40:1, v/v) and once or twice from dichloroethane. 2,6-Dichloro-4-hydroxybenzonitrile yielded glittering needles and the 3-hydroxy analogue was granular.

For assay with acetone extracts of faeces the carriers were dissolved and the solvent was evaporated. The residue was suspended in 4N-HCl and boiled for 3hr. Isolation procedures were the same as those described for urine.

(*c*) 2,6-Dichlorobenzamide. Urine (pooled samples) was adjusted to pH6–7 and carrier was dissolved. The urine was extracted continuously with ether for 6hr. The ether extracts were dried and the solvent was evaporated, yielding a crystalline solid. The solid was dissolved in boiling benzene, filtered, chromatographed on silicic acid columns and eluted with benzene–ethanol (40:1, v/v). Recrystallization was carried out from dichloroethane. 2,6-Dichlorobenzamide yielded glittering needles.

To acetone extracts of faeces (pooled samples) carrier amounts of the amide were added and the acetone was evaporated. The solid residue was dissolved in benzene

and chromatographed on a silicic acid column. Further purification was the same as described for urine.

(*d*) 2,6-Dichloro-3-hydroxybenzamide and 2,6-dichloro-4-hydroxybenzamide. The carriers were dissolved in the urine (pH 6–7) and conc. HCl was added to 4 N. The mixture was boiled for 3 hr., followed by continuous ether extraction (pH 5–6) for 8 hr. The ether extract was dried and evaporated. Solids separated readily from the remaining syrup of the 2,6-dichloro-4-hydroxybenzamide extract. Crystallization of the 3-hydroxy analogue extract took longer. The solids were collected and twice recrystallized from dichloroethane and twice from methanol–water. The specific radioactivity was not constant and a further purification was carried out by chromatography on silicic acid columns. The compounds were eluted with benzene–ethanol (40:3·7, v/v). The eluates were evaporated to a small volume and the crystals so obtained were collected.

After dissolving the carriers in acetone extracts of faeces the acetone was removed by evaporation. The remaining solid was suspended in 4 N-HCl and boiled for 3 hr. Isolation and purification procedures were the same as described for urine.

(*e*) 2,6-Dichlorobenzoic acid. An alkaline solution of the carrier was added to pooled samples of urine that had been made alkaline. After 60 min. the mixture was centrifuged. The clear liquid was decanted, acidified to pH below 1, boiled for 3 hr. and extracted with ether continuously for 3 hr. The ether extract was shaken with N-NaOH. The aqueous phase was acidified and extracted with ether. The ether extract was dried and evaporated, leaving a syrupy residue. The residue, dissolved in boiling benzene, was chromatographed on a silicic acid column. The compound was eluted with benzene–ethanol (100:11, v/v). The coloured eluate gave a syrup after evaporation and the chromatographic step was repeated. After evaporation of the eluate, the syrupy residue was dissolved by boiling in a small volume of dichloroethane. Crystals began to separate during cooling. The solids were collected and recrystallized several times from dichloroethane to which a small volume of light petroleum (b.p. 40–60°) had been added. The acid was twice recrystallized from light petroleum (b.p. 40–60°). The last crystallization was carried out from dilute HCl.

Carrier amounts of 2,6-dichlorobenzoic acid were dissolved in the pooled acetone extract of faeces and the acetone was removed by evaporation. The residue was suspended in 0·1 N-HCl and boiled for 3 hr. Isolation and purification procedures were the same as for urine.

Samples (2–10 mg.) of the recovered and purified isotope dilutions were dissolved in the scintillation liquid T.H.M. and counted. To assure a constant specific activity, crystallizations were repeated until the differences in net counts/min./mg. of solid did not exceed 4%.

Enzyme assays. (*a*) Papain. The determination of the milk-clotting power of a commercial papain preparation was performed as described by Balls & Hoover (1937) and Bray, Thorpe & Vallance (1951*b*). Benzyl chloride was used to compare inhibitory effects on papain.

(*b*) Glutathiokinase. The enzyme activity of rat-liver high-speed supernatant (Booth, Boyland & Sims, 1961) was used to investigate enzymic formation of *S*-substituted glutathione derivatives. Glutathione before and after incubation was measured by the method of Woodward & Fry (1932). 3,4-Dichloronitrobenzene was used to compare the glutathione addition.

RESULTS

During the experiments two main difficulties were encountered. The appetite of the animals, especially rabbits, was affected for about 24 hr. Secondly, most of the rabbits of the non-radioactive experiments died from poisoning within 24 hr. at doses we expected from earlier toxicological studies to be harmless. Gross examination revealed severe hepatic damage (centrilobular necrosis). These interfering circumstances introduce some uncer-

Table 1. *Daily excretion of conjugates in urine before and after oral administration of 2,6-dichlorobenzonitrile to rabbits*

The values for the conjugates in the control period are given as means ± S.D.

Rabbit.....	A	B	C	D	E	F
Weight (kg.)	2·50	2·40	1·65	2·20	2·60	2·60
Dose (mg./kg.)	130	135	200	200	200	200
Time of death after administration (hr.)	24	—	—	24	24	30
Control period (days)	8	8	6	6	1	1
pH of urine			8·6 ± 0·3	8·8 ± 0·4		
Glucuronides (mg. of glucuronolactone)	95 ± 45	151 ± 73	140 ± 45	102 ± 17	150	130
Ethereal sulphates (mg. of SO_4^{2-})	40 ± 11	47 ± 8	26 ± 4	26 ± 2	38	17
Mercapturic acids (m-equiv. of I^-)	0·16 ± 0·03	0·24 ± 0·06	0·17 ± 0·03	0·22 ± 0·01	0·12	0·03
Experimental period (days)	1	1	1	1	1	1
pH of urine			6·3	6·2		
Glucuronides (mg. of glucuronolactone)	140	250	220	230	470	260
Ethereal sulphates (mg. of SO_4^{2-})	101	59	49	46	67	92
Mercapturic acids (m-equiv. of I^-)	0·48	0·68	0·48	0·59	0·65	0·65

tainty in the results. Nevertheless, sufficient information for further analysis of the metabolic pathway could be obtained. Rats were far less sensitive to the compound and no animal died from poisoning. The administration of the compound to rats was rather troublesome. The insoluble substance frequently clogged the stomach tube and spills occurred.

Urinary excretion of conjugates. When 2,6-dichlorobenzonitrile was given to rabbits, an enhanced excretion of glucuronides and ethereal sulphates was observed (Table 1). Mercapturic acid formation was positive when screened (Table 1). Occasional pH determinations of the urine showed a decrease of 2 units 24hr. after administration, indicating an increased output of acidic materials (Table 1).

Treated rats showed an increased urinary excretion of ethereal sulphates, but that of glucuronides did not vary from the control amounts. Mercapturic acid formation was less when compared with the rabbits (Table 2). The acidification of the urine occurred more gradually.

The results of the conjugate determinations gave strong evidence for the presence of conjugated phenolic metabolites and mercapturic acid formation.

Metabolites in urine (rabbits and rats). Steam-distillates of the urine did not contain 2,6-dichlorobenzonitrile, as shown by thin-layer chromatography. 2,6-Dichloro-3-hydroxybenzonitrile was demonstrated by a positive phenol reaction (Brentamine Fast Blue B Salt), blue fluorescence in u.v. light (paper chromatography) and a positive chlorine reaction (thin-layer chromatography). Other phenol reagents used, e.g. diazotized sulphanilic acid, Brentamine Fast Red B Salt and Brentamine Fast Red GG, gave negative results on paper chromatograms with the pure reference substance.

The presence of 2,6-dichloro-4-hydroxybenzonitrile in ether extracts sometimes seemed doubtful, but in several instances was clearly demonstrated, notwithstanding the small differences in R_F value between the monophenols. Also, this compound did not give positive reactions with the other

Table 2. *Excretion of conjugates in rat urines, pooled for 5 consecutive days, before and after oral administration of 2,6-dichlorobenzonitrile*

Group of rats.....	A	B	C
No. of animals	8	8	7
Total weight (kg.)	1·369	1·422	1·169
Dose (mg./kg.)	502	525	540
Control period (days)	5	5	5
Glucuronides (mg. of glucuronolactone)	664	730	729
Ethereal sulphates (mg. of SO_4^{2-})	75	58	54
Mercapturic acids (m-equiv. of I^-)	0·28	0·22	0·36
Experimental period (days)	5	5	5
Glucuronides (mg. of glucuronolactone)	598	850	660
Ethereal sulphates (mg. of SO_4^{2-})	100	99	86
Mercapturic acids (m-equiv. of I^-)	0·46	0·57	0·55

Table 3. *R_F values on paper chromatograms (Whatman no. 1) and on thin-layer chromatograms (alkaline alumina)*

The detection of the compounds is described in the Experimental section.

	Paper chromatography		Thin-layer chromatography
	Propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (8:1:1, by vol.)*	Butan-1-ol–pyridine–satd. NaCl–aq. NH_3 (sp.gr. 0·88) (4:8:5:1, by vol.)†	96% Ethanol–5N-acetic acid (23:2, v/v)
2,6-Dichlorobenzonitrile	—	—	0·84
2,6-Dichloro-3-hydroxybenzonitrile	0·85	0·73	0·63
2,6-Dichloro-4-hydroxybenzonitrile	0·87	0·77	0·63
2,6-Dichlorobenzoic acid	0·68	0·64	0·40

* Armstrong, Shaw & Wall (1956). † Bray, Hybs & Thorpe (1951*a*).

phenol reagents mentioned above for 2,6-dichloro-3-hydroxybenzonitrile. Fluorescence was insignificant.

No evidence could be obtained from paper or thin-layer chromatography for the presence of 2,6-dichlorobenzamide or 2,6-dichlorobenzoic acid in ether extracts of the urine. Also, no other glycine conjugate besides hippuric acid was found, which might indicate the formation of 2,6-dichlorobenzoic acid.

Ether extracts of rat and rabbit urine did not show the presence on the chromatograms of compounds containing bivalent sulphur groups.

The results thus far obtained from the analysis of the urine only revealed the presence of both monophenols of 2,6-dichlorobenzonitrile. The absence of the amide and the corresponding acid indicates that hydrolysis of the nitrile group is insignificant. 2,6-Dichlorobenzamide and 2,6-dichlorobenzoic acid could be readily detected in the urine of rabbits, when these compounds were given orally. 2,6-Dichlorobenzoic acid, however, was not excreted by a rabbit dosed with 2,6-dichlorobenzamide (450mg./kg. body wt.).

The absence of mercapturic acid from urinary ether extracts is at variance with the positive indications obtained by the general screening method for this type of conjugate.

Metabolites in faeces. Pulverized faeces or the contents of the digestive tract contained 2,6-dichlorobenzonitrile, sometimes in rather large amounts, notably in fatal cases, probably owing to the occurrence of diarrhoea. It was identified by thin-layer chromatography and comparison of the infrared spectrum with the original compound.

Excretion of ^{14}C-labelled 2,6-dichlorobenzonitrile. As shown in Tables 4 and 5 both species excrete most of the radioactivity in the urine and the faeces within 24hr. For rabbits, the urinary elimination is the major route, whereas for rats the urinary and faecal excretion equal each other. The faecal activity is to be divided into an acetone-soluble fraction and a part tightly bound to the solid residue.

Isotope analysis was restricted to the urine and the acetone extracts. The results obtained by the chromatographic analysis were confirmed (Tables 6 and 7). The monophenolic metabolites are largely excreted in the urine. The ratio between both analogues varies with the species (the 3-hydroxy/4-hydroxy ratio is about 10 for rabbits and about 1 for rats). Hydrolysis of the nitrile group is insigni-

Table 4. *Excretion of radioactivity in urine and faeces of male rabbits after an oral dose of 2,6-dichlorobenzo[^{14}C]nitrile*

The values represent the percentages of the dose.

Rabbit.....	I*	II	III	IV	V†	VI	VII	VIII
Weight (kg.)	1·80	1·90	2·00	2·20	2·05	2·05	1·85	2·20
Dose (mg./kg.)	103·2	111·7	164·4	101·9	103·9	104·8	100·8	100·4
Specific activity (μc/g.)	50	50	50	250	250	250	250	250
Urine								
0–24hr.	65·6	78·4	60·0	88·5	25·8	83·6	87·6	60·9
24–48hr.	1·3	2·0	8·9	3·1	40·3	0·7	1·5	6·8
48–72hr.	0·8	0·5	0·2	0·9	9·7	0·9	0·5	1·1
72–96hr.	—	—	0·2	0·2	1·0	0·1	0·3	0·3
Total	67·7	80·9	69·5	92·7	76·8	85·3	89·9	69·1
Faeces (acetone extracts)								
0–24hr.	12·3	12·9	17·9	2·2	2·1	8·0	3·6	12·8
24–48hr.	—	—	0·6	0·3	2·1	1·0	0·8	0·9
48–72hr.	—	—	—	1·1	1·7	0·6	0·2	0·7
Total	12·3	12·9	18·5	3·6	5·9	9·6	4·6	14·4
Faeces (solid residue)								
0–24hr.	6·2	3·9	4·9	1·4	1·6	7·9	4·8	4·3
24–48hr.	2·5	2·8	8·0	5·0	2·1	3·7	2·0	4·0
48–72hr.			1·3					
72–96hr.			0·2					
Total	8·7	6·7	14·4	6·4	3·7	11·6	6·8	8·3
Excretion in urine + faeces	88·7	100·5	102·5	102·7	86·4	106·5	101·3	91·8

* Spills occurred during administration.

† Rabbit was injured in the back during administration, and became incontinent.

Table 5. *Excretion of radioactivity in urine and faeces of male rats after an oral dose of 2,6-dichlorobenzo[^{14}C]nitrile*

The values represent the percentages of the recovered radioactivity.

Group of rats.....	I	II*	III	IV	V
Wt. of five animals (kg.)	0·870	0·650	0·945	1·075	1·065
Dose (mg./kg.)	61·2	50·3	91·0	95·0	99·3
Dose (mg.)	53·3	32·7	86·0	102·2	105·8
Specific activity (μc/g.)	50	50	250	250	250
Recovered radioactivity (as mg. of dosed compound)	51·5	32·7	72·9	53·7	68·8
Urine					
0–24 hr.	45·6	37·6	43·9	43·9	49·6
24–48 hr.	1·0	1·2	5·1	13·4	7·8
48–72 hr.	—		0·7	1·4	0·7
72–96 hr.	—		0·6	0·8	0·5
Total	46·6	38·8	50·3	59·5	58·6
Faeces (acetone extracts)					
0–24 hr.	34·3	43·4	26·9	17·9	19·8
24–48 hr.	0·5	1·2	3·2	0·5	2·6
48–72 hr.			1·4	0·9	0·1
72–96 hr.			—		
Total	34·8	44·6	31·5	19·3	22·5
Faeces (solid residue)					
0–24 hr.	13·3	15·6	11·0	15·3	13·2
24–48 hr.	2·1	0·6	7·4	6·0	5·6
Total	15·4	16·2	18·4	21·3	18·8

* This group contained four animals (Choclat variety). One animal had to be removed because of a failure in administration. The dose is calculated on recovered radioactivity.

Table 6. *Isotope-dilution analysis of the rabbit urines* (0–72 *hr.*) *and acetone extracts of rabbit faeces* (0–48 *hr.*)

The values represent the percentages of the dose given orally (see Table 4).

Rabbit.....	IV		V		VI		VII		VIII	
	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.
2,6-Dichlorobenzonitrile	0·09	0·33	0·08	0·21	0·09	7·19	0·09	1·73	0·06	0·32
2,6-Dichloro-4-hydroxybenzonitrile	2·41	0·06	1·67	0·10	2·56	0·21	1·47	0·10	1·79	0·32
2,6-Dichloro-3-hydroxybenzonitrile	27·10	0·30	22·36	0·50	23·86	1·08	20·87	0·53	19·33	1·64
2,6-Dichlorobenzoic acid	<0·001	<0·01	<0·001	<0·05	<0·001	<0·05	<0·001	<0·05	<0·001	<0·05
2,6-Dichlorobenzamide	<0·01	<0·01	<0·01	<0·05	<0·01	<0·05	<0·01	<0·05	<0·01	<0·05
2,6-Dichloro-3-hydroxybenzamide	<0·7	—	<0·5	—	<0·6	—	<0·6	—	<0·5	—
2,6-Dichloro-4-hydroxybenzamide	<0·1	0	<0·1	0	<0·1	0	<0·1	0	<0·1	0
Sum	29·60	0·69	24·11	0·81	26·51	8·48	22·43	2·36	21·18	2·28

ficant. A large fraction of the urinary and acetone-soluble faecal radioactivity is still unidentified.

Enzyme assay. The positive results obtained by the general screening for mercapturic acid, the unsuccessful attempts to demonstrate the presence of mercapturic acid by chromatographic analysis and the unidentified fraction of soluble radioactive material in the urine are puzzling.

Table 7. *Isotope-dilution analysis of rat urines* (0–72 *hr.*) *and acetone extracts of rat faeces* (0–48 *hr.*)

The values represent the percentages of the recovered radioactivity after oral administration (see Table 5).

Group of rats.....	III		IV		V	
	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.	Urine 0–72 hr.	Faeces 0–48 hr.
2,6-Dichlorobenzonitrile	0·13	1·93	0·15	3·50	0·15	8·92
2,6-Dichloro-4-hydroxybenzonitrile	11·78	1·66	13·94	1·02	4·48	1·23
2,6-Dichloro-3-hydroxybenzonitrile	17·99	5·12	15·35	3·15	32·59	3·80
2,6-Dichlorobenzoic acid	<0·3	<0·1	<0·3	<0·1	<0·3	<0·1
2,6-Dichlorobenzamide	<0·05	<0·2	<0·05	<0·1	<0·05	<0·2
2,6-Dichloro-3-hydroxybenzamide	<0·5	<0·5	<0·6	<0·2	<0·6	<0·5
2,6-Dichloro-4-hydroxybenzamide	<0·5	<0·1	<0·5	<0·1	<0·5	<0·1
Sum	29·90	8·71	29·44	7·67	37·22	13·95

The ability of 2,6-dichlorobenzonitrile to react with thiol compounds was investigated in incubation mixtures with glutathione and the high-speed supernatant of rat-liver homogenate (glutathiokinase). No formation of an *S*-substituted glutathione derivative was observed. Also, no reactivity to a thiol enzyme (papain) was found. The milk-clotting power of this enzyme was not affected.

DISCUSSION

Because of its molecular structure, it seems likely that the metabolism of 2,6-dichlorobenzonitrile will have some aspects in common with the metabolic patterns of chloro- and cyano-benzenes. Similarities in toxicological effects may also exist.

In general, the metabolism of halogenated aromatic hydrocarbons in the animal body is characterized by the formation of phenols, catechols and mercapturic acids (Williams, 1959). Since the toxic effects of some halogenated benzenes are thought to be associated with the utilization of sulphur-containing amino acids for mercapturic acid formation, the metabolism and toxicology of dichlorobenzene is of special interest. Both 1,3-dichlorobenzene and 1,2-dichlorobenzene were largely metabolized to monophenols and to a small extent to catechol derivatives. Mercapturic acid formation occurred in rabbits (oral dose) to 11% and to 5% for 1,3-dichlorobenzene and 1,2-dichlorobenzene respectively (Williams, 1959). Little is known about the toxicology of 1,3-dichlorobenzene. The toxic effects of 1,2-dichlorobenzene (see Patty, 1962) are relatively mild, but exposure of small animals to the fumes produced severe hepatic lesions (Cameron *et al.* 1937). Rabbits fed with both dichlorobenzenes tolerate a dose of 0·5 g./kg. body wt. (Williams, 1959).

The metabolism of benzonitrile has been studied by Smith & Williams (1950) and Bray *et al.* (1951*a*). Hydroxylation appeared to be the main metabolic route, yielding *o*-, *m*- and *p*-cyanophenol. Mercapturic acid formation as a minor metabolic reaction was suggested (Smith & Williams, 1950). However, attempts to isolate this compound were not successful. The investigators did not agree about the hydrolysis of the nitrile group. Only few toxicological data on benzonitrile are available, some of them dating back to 1899 (see Spector, 1956). Smith & Williams (1950) reported that an oral dose of 250 mg./kg., dissolved in olive oil, can be fatal for rabbits. More toxicological details were given by Bray *et al.* (1951*a*). Although the last-mentioned authors considered benzonitrile to be extremely toxic for the rabbit, no hepatic injury was recorded. In a few experiments we administered a Tween 80–water emulsion of benzonitrile to rabbits by stomach tube. A dose of 600 mg./kg. body wt. was fatal within 36 hr. Gross visual examination revealed degeneration of the liver, which had a strong smell of benzonitrile. Rabbits as well as rats survived an oral dose of 200 mg./kg. body wt. With regard to the chlorinated benzonitriles, Adeline, Cerecedo & Sterwin (1926) studied the metabolism of *p*-chloro- and 2,4-dichloro-benzonitrile in rabbits and dogs. No evidence for urinary excretion of chlorinated benzoic acid was obtained.

With these observations in mind, we expected hydroxylation and mercapturic acid formation to be the major metabolic reaction of 2,6-dichlorobenzonitrile. Hydrolysis of the nitrile group might be a minor metabolic route. There is also a possibility that after hydroxylation the nitrile group might become more liable to hydrolysis, and so chlorinated amides or their acid analogues might be present as urinary excretion products.

With regard to the results of the conjugates in urine, our expectations seemed to be substantiated. For rabbits, the glucuronide and ethereal sulphate content of the urine was enhanced. Mercapturic acid formation could be deduced from the increased iodine consumption in alkali hydrolysates. Rats

gave positive results for ethereal sulphates and mercapturic acid formation. Two monophenolic metabolites were detected by chromatography. No further support for the presence of mercapturic acid was provided by paper chromatography nor by assays with a glutathione-conjugating enzyme preparation (glutathiokinase). Also, no reactivity of the compound to papain, a thiol enzyme, was observed. Balance studies with ^{14}C-labelled 2,6-dichlorobenzonitrile demonstrated a rapid absorption, metabolism and excretion of the compound. By isotope dilution both monophenolic metabolites were estimated. These compounds proved to be major metabolic products. Rabbits converted 2,6-dichlorobenzonitrile to an extent of approx. 2% and 23% into 2,6-dichloro-4-hydroxybenzonitrile and 2,6-dichloro-3-hydroxybenzonitrile respectively. Both phenols appeared in the faeces to a small extent. In rat urine about 9% of the recovered radioactivity was excreted as 2,6-dichloro-4-hydroxybenzonitrile and 22% as the 3-hydroxy analogue. In faeces 1% of the recovered activity was due to 2,6-dichloro-4-hydroxybenzonitrile and 4% to the 3-hydroxy derivative.

The hydrolysis of the nitrile group of the molecule appears to occur to a very minor extent. Only insignificant amounts of the amide and benzoic acid derivatives were found. This is supported by our own observation that a rabbit did not convert 2,6-dichlorobenzamide into the corresponding acid. The resistance of the nitrile group to hydrolysis *in vivo* is in accord with the stability of this group *in vitro*. *ortho*-Disubstituted benzonitriles are converted into the corresponding amides by treatment with 80% sulphuric acid (Sudborough, 1895) or by concentrated alkaline-ethanol solutions. The corresponding acid of several *ortho*-disubstituted benzamides can be obtained by treatment with nitrous acid (Sudborough, 1895). The nitrile group of the monophenols is also resistant to chemical hydrolysis.

In general, our results agree qualitatively with those published by Griffiths, Moss, Rose & Hathway (1966). In their study the presence of two additional metabolites of 2,6-dichlorobenzonitrile, containing sulphur amino acid residues, was demonstrated. These compounds could be extracted by butan-1-ol from acid-hydrolysed urines, but not by ether. This fact may explain why we did not detect mercapturic acids in ether extracts by paper chromatography, whereas the general screening method for mercapturic acid in urine by iodometric titration in alkali-hydrolysed urines gave a positive indication. It seems to us that mercapturic acid formation probably occurs via an epoxy intermediate. This route was suggested by Boyland (1962) for monohalogenated benzenes and aromatic hydrocarbons.

In further studies an attempt has been made to correlate the results of the present metabolic studies with the hepatotoxic action of 2,6-dichlorobenzonitrile in the rabbit. In the next paper (Wit & van Genderen, 1966) we consider the interference of the phenolic metabolites with energy metabolism as a possible mode of action of 2,6-dichlorobenzonitrile.

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