The Monophenolic Metabolites of the Herbicide 2,6-Dichlorobenzonitrile in Animals as Uncouplers of Oxidative Phosphorylation

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1. Both monophenolic metabolites of 2,6-dichlorobenzonitrile (2,6-dichloro-3-hydroxybenzonitrile and its 4-hydroxy analogue) added to starved yeast cells incubated with a limited quantity of glucose cause a significant rise in oxygen consumption of the cells. 2. The same compounds induce adenosine-triphosphatase activity in isolated intact rat-liver mitochondria. 3. The possible role of the hydroxylation of 2,6-dichlorobenzonitrile in mammals in relation to hepatic injury is discussed.

At toxic doses the herbicide 2,6-dichlorobenzonitrile (Dichlobenil, Casoron 133) produces liver-cell necrosis in rabbits. Wit & van Genderen (1966) found that the compound is partly metabolized by rats and rabbits to 2,6-dichloro-3-hydroxybenzonitrile and its 4-hydroxy analogue. Several authors have drawn attention to the effects of chlorophenols on oxidative-phosphorylation processes (Loomis, 1949; De Deken, 1955; Weinbach, 1956; Parker, 1958).

By analogy, the monophenolic metabolites of 2,6-dichlorobenzonitrile in mammals might also possess uncoupling activity. 2,6-Dichloro-3-hydroxybenzonitrile and its 4-hydroxy analogue were investigated, together with some other compounds structurally related to these phenols. The results of these investigations are now reported.

EXPERIMENTAL

Materials. The following test compounds were obtained as samples from Philips Duphar Research Laboratories, Weesp, The Netherlands: 2,6-dichlorobenzonitrile, 2,6-dichlorobenzoic acid, 2,6-dichlorobenzamide, 2,6-dichloro-3-hydroxybenzonitrile, 2,6-dichloro-4-hydroxybenzonitrile, 2,6-dichloro-3-hydroxybenzamide and 2,6-dichloro-4-hydroxybenzamide (see Wit & van Genderen, 1966). 3-Hydroxybenzonitrile was prepared by the method of Goldberg & Wragg (1958), m.p. 79–80° (uncorr.). 4-Hydroxybenzonitrile was prepared as described by Ashley, Barber, Ewins, Newberry & Self (1942), m.p. 111–112° (uncorr.). 1,3-Dichlorobenzene, benzonitrile and 2,4-dinitrophenol were obtained from British Drug Houses Ltd., Poole, Dorset.

Methods. The influence of the test compounds on yeast-cell respiration was determined manometrically as described by Brostoff, Moses & Smith (1961). Starved yeast-cell

suspensions, in Warburg flasks, were exposed for 45 min. to the compounds before measurement of the initial rate of oxygen consumption due to endogenous respiration. A limited quantity of glucose (8 μ moles) was added, which in untreated yeast cells is enough to be consumed completely by respiration and assimilation processes within about 60 min. The moment of exhaustion of the substrate is clearly seen in the graphs by the sudden fall in oxygen uptake to the level of endogenous respiration. The compounds were dissolved (final concn. 34 μ M) in phosphate buffers (67 mM) with different pH values. Determinations were made at 32°.

The ability of the compounds to stimulate the ATPase† activity of freshly prepared rat-liver mitochondria (Myers & Slater, 1957; Hülsmann, 1958; Hemker, 1963) was determined by adding mitochondria to incubation mixtures containing ATP and various concentrations of the test compounds (see legend to Fig. 1). The inorganic phosphate liberated was measured by the method of Fiske & Subbarow, as modified by Sumner (1944). The protein content of the enzyme preparation was determined by the biuret method, as applied to mitochondrial preparations by Cleland & Slater (1953). The effects obtained were compared with those produced by 2,4-dinitrophenol.

RESULTS

Experiments with yeast cells. The experimental curves for total oxygen consumption could be divided into four main types as follows: A, no effect; B, prolonged respiration (providing evidence for uncoupling activity; Simon, 1953; Brostoff *et al.* 1961); D, inhibited respiration; C, intermediate between B and D (Fig. 1).

In Table 1 the type of curves obtained after exposure of the yeast cells to the test compounds are given in relation to the pH. Positive effects, i.e. prolonged respiration, were obtained with 2,6-dichloro-3-hydroxybenzonitrile (34 μ M) at

† Abbreviation: ATPase, adenosine triphosphatase.

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pH 2.6–6.2. At higher pH values this compound had no effect (type A). 2,6-Dichloro-4-hydroxybenzonitrile ($34\mu\text{M}$), however, produced ambiguous effects at pH 4.5–6.2, which may be due to variations in the concentration since these phenols have an extremely low solubility. At higher pH values respiration was slightly prolonged or not affected.

Both compounds inhibited yeast-cell respiration at a concentration of 0.34 mM (pH 4.5), as did 2,4-dinitrophenol. The effects observed indicate the phenomenon of uncoupling oxidative processes from substrate assimilation.

Experiments with mitochondria. Both the monophenols of 2,6-dichlorobenzonitrile induced ATPase

Fig. 1. Stimulation and inhibition of oxygen consumption of starved baker's yeast cells in the presence or absence of $34\mu\text{M}$ -2,6-dichloro-3-hydroxybenzonitrile at pH 7.2 (a) or pH 4.5 (b) or $34\mu\text{M}$ -2,6-dichloro-4-hydroxybenzonitrile at pH 4.5 (c) or pH 2.6 (d) before and after addition of a limited quantity of glucose. Each Warburg flask contained 0.1 ml. of yeast suspension (20%, w/v) and 0.8 ml. of the test substance dissolved in phosphate buffer (Δ), or phosphate buffer (control) ($\circ$), in the main compartment. The centre well contained 0.1 ml. of 10% (w/v) KOH. The side arm contained 0.1 ml. of 0.08 M-glucose. The yeast suspension was exposed for 45 min. to the test solution before measurement of the endogenous respiration. Endogenous oxygen consumption was measured for 1 hr. Then (arrows) glucose was added from the side arm to the yeast suspension. The incubation temperature was 32° . Measurements were carried out in triplicate. A change of 15% from the corresponding control was considered to represent a significant effect. Types of curves: A, no effect (a); B, stimulation (b); D, inhibition (d); C, intermediate between B and D (c).

Fig. 2. Induction of ATPase activity in relation to concentration at pH 7.0. The induction of ATPase was investigated in a reaction mixture containing tris-HCl buffer (40mm), KCl (75mm), EDTA (0.5mm), ATP (2mm), sucrose (50mm) and mitochondrial suspension (about 0.15 mg. of protein N/ml.) in a total volume of 1.5 ml. Test substances were: (a) 2,6-dichloro-4-hydroxybenzonitrile; (b) 2,6-dichloro-3-hydroxybenzonitrile; (c) 2,6-dichloro-4-hydroxybenzamide; (d) *p*-cyanophenol; (e) *m*-cyanophenol. They were added in 0.25M-sucrose or in ethanolic solution (final concn. of ethanol less than 3%). The incubation temperature was $20 \pm 2^\circ$ and the incubation time 15 min. The reaction was stopped by adding an equal volume of 10% (w/v) trichloroacetic acid. Determinations were performed in duplicate.

activity over a wide range of concentrations. 2,6-Dichloro-4-hydroxybenzamide and *m*- and *p*-cyanophenol also induced ATPase activity. Their ability to do so was far less than with the phenols of 2,6-dichlorobenzonitrile (Fig. 2). In Table 2 ATPase induction is given in relation to pH. The compounds 2,6-dichlorobenzonitrile, 2,6-dichlorobenzoic acid, 2,6-dichlorobenzamide, 2,6-dichloro-3-hydroxybenzamide, 1,3-dichlorobenzene and

benzonitrile did not induce ATPase at a concentration of 0.1mm over the pH range 5-8.

DISCUSSION

The experiments with yeast-cell suspensions as well as with isolated intact rat-liver mitochondria demonstrate the uncoupling activity of both monophenolic metabolites of 2,6-dichlorobenzonitrile. In mitochondria the 4-hydroxy derivative is about three times as active as the 3-hydroxy analogue. 2,6-Dichloro-4-hydroxybenzamide and *m*- and *p*-cyanophenol also induced ATPase activity in the mitochondria. No activity was found with the other substances tested (2,6-dichlorobenzonitrile, 2,6-dichlorobenzoic acid, 2,6-dichlorobenzamide, benzonitrile and 1,3-dichlorobenzene). The peculiarities in the results for 2,6-dichloro-4-hydroxybenzonitrile (type D curve) can be explained if the uncoupling agent can act as an inhibitor of respiration at relatively high concentrations (Brostoff *et al.* 1961; Hemker, 1963).

2,6-Dichlorobenzonitrile is converted into the 3- and 4-hydroxy derivatives in rats and rabbits (Wit & van Genderen, 1966). Toxic doses of 2,6-dichlorobenzonitrile produce hepatic injury, particularly in rabbits. Since normally the hydroxylation of such foreign compounds occurs in the liver, it seems that the production of the uncoupling phenols in the liver cell could explain the damage at this site. To produce this effect the concentration of the free phenols should exceed a certain threshold. The concentration of the free phenols is determined by the rate of hydroxylation and the rate of conjugation with glucuronic acid and sulphuric acid to give harmless conjugates. This concentration will rise gradually with increasing doses of the parent substance up to a level where the degree of uncoupling starts to interfere with the conjugation process, which itself is directly or indirectly ATP-dependent. This initiation of a positive-feedback element in the formation-detoxication pathways could give rise to a sudden increase in the concentration of free phenols after a toxic dose of 2,6-dichlorobenzonitrile, resulting in necrosis of the liver.

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Table 1. *Relation between pH and the type of oxygen consumption curves (see Fig. 1) of starved baker's yeast cells exposed to 2,6-dichlorobenzonitrile, its phenolic metabolites and structurally related compounds*

Compound	pH... ..	Type of curve				
		2.6	4.5	6.2	7.2	8.1
Concn. 34 μ M						
2,6-Dichlorobenzonitrile		A	A	A		A
2,6-Dichloro-3-hydroxybenzonitrile		B	B	B	A	A
2,6-Dichloro-4-hydroxybenzonitrile		D	C-D	C-D	B-C	A
2,6-Dichlorobenzamide		A	A	A		A
2,6-Dichlorobenzoic acid			A	A	A	A
1,3-Dichlorobenzene			A	A	A	A
Benzonitrile			A	A	A	A
<i>m</i> -Cyanophenol			A	A	A	A
2,4-Dinitrophenol		C	B	B	A	A
Concn. 0.34 mM						
2,6-Dichloro-3-hydroxybenzonitrile			D			
2,6-Dichloro-4-hydroxybenzonitrile			D			
2,4-Dinitrophenol			D			

Table 2. *Induction of ATPase in rat-liver mitochondria*

The ranges of molar concentrations of compounds inducing ATPase activity (at least 10 μ moles of P_i/mg. of N/hr.) at a chosen pH are given as the negative logarithms of the concentrations.

Compound	pH... ..	-log (Molar concn.)				
		5.0	6.0	7.0	8.0	9.0
2,6-Dichloro-3-hydroxybenzonitrile		5.5-2.8	5.2-2.8	5.0-2.5	4.5-2.5	3.8-2.8
2,6-Dichloro-4-hydroxybenzonitrile		6.2-3.1	5.8-2.8	5.7-2.5	5.2-2.2	4.8-2.7
2,6-Dichloro-4-hydroxybenzamide			4.3-2.1	3.3-2.1		
<i>m</i> -Cyanophenol			3.1-2.1	3.0-2.1		
<i>p</i> -Cyanophenol			3.3-2.1	3.3-2.1		

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