SHORT COMMUNICATIONS

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The inhibitory action of 2,6-dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile on the beating of heart cells in tissue culture Antagonism of oligomycin

HARARY AND SLATER[1] studied the effects of an uncoupling agent (dinitrophenol), an inhibitor of oxidative phosphorylation (oligomycin) and an inhibitor of glycolysis (iodoacetate) on the beating rate of heart cells *in vitro*. Two interesting conclusions were drawn: (a) dinitrophenol does not affect directly the contractile mechanism, but acts by decreasing the ATP concentration in the cell; (b) the beating of the cells is not supported by high-energy intermediates of oxidative phosphorylation, but by ATP as such, derived either from oxidative phosphorylation or from glycolysis. The decrease of cellular ATP, due to the dinitrophenol-induced ATPase activity is selectively antagonized by oligomycin.

The classification of chemicals as "uncouplers" depends on their effects on reactions occurring in isolated mitochondria. Beating heart cells *in vitro* offer a good opportunity to investigate whether the biological activity on the intact living cell is due to the same mode of action.

With this in mind, we studied the effects of 2,6-dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile. Both phenols are metabolites of the herbicide 2,6-dichlorobenzonitrile (Dichlobenil, Casoron 133*) in rats and rabbits[2]. The phenols are able to uncouple oxidative phosphorylation, as indicated by the induction of ATPase activity in isolated rat-liver mitochondria and by their effects on the oxygen consumption of starved baker's yeast cells incubated with limited amounts of glucose[3]. Also, in other experiments, similarities in biological activity of dinitrophenol and both metabolites were found, *e.g.*, a rapid post-mortem rigidity after intraperitoneal injections in mice and the induction of contracture in isolated rat diaphragm preparations, with and without (in)direct electric stimulation[4].

The experimental technique was carried out as described by HARARY AND SLATER[1], except that 2,4-dinitrophenol, 2,6-dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile were dissolved in 96% ethanol to 25 mM. The beating frequency was determined by visual counting of the contractions in the cultures, maintained at 25°. The initial volume of medium was 4 ml/dish.

Potency of 2,6-dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile

The means of the final concentrations of the compounds needed to arrest the beating were found to be for 2,4-dinitrophenol (9 dishes) 0.41 mM $\pm$ 0.10, (S.D.) for 2,6-dichloro-3-hydroxybenzonitrile 0.31 $\pm$ 0.055 mM (10 dishes) and for 2,6-dichloro-4-hydroxybenzonitrile 0.24 $\pm$ 0.025 mM (9 dishes). The differences in biological activity between the 3 compounds are statistically significant ($P < 0.01$; Wilcoxon test and variance analysis).

Antagonism of oligomycin

Oligomycin (20 μg/ml) was added 5 min after 2,4-dinitrophenol , 2,6-dichloro-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile had arrested the

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beating of the cells. The cells resumed beating without exception. Tissue cultures, pretreated with 2,4-dinitrophenol (5 dishes) and 2,6-dichloro-3-hydroxybenzonitrile (6 dishes), did not regain their original beat frequency. Cells, inhibited by 2,6-dichloro-4-hydroxybenzonitrile (5 dishes) and treated with oligomycin, almost regained their original frequency. Comparison between the frequencies (beats/min) immediately before the phenol treatment and 8 min after the oligomycin addition, respectively, gave for 2,4-dinitrophenol 47.8 ± 19.9 and 24.4 ± 10.3; for 2,6-dichloro-3-hydroxybenzonitrile 40.3 ± 18.7 and 19.8 ± 10.0, and for2, 6-Dichloro-4-hydroxybenzonitrile 32.0 ± 14.9 and 31.4 ± 10.4.

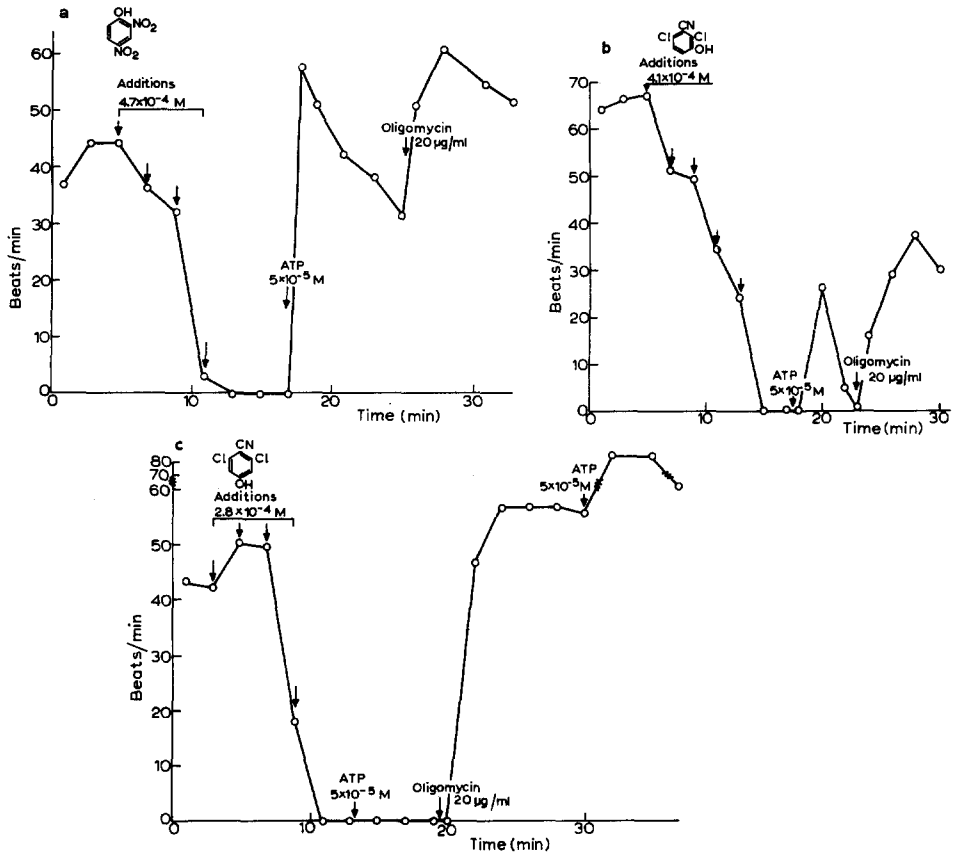

Fig. 1. Inhibition of the beating of heart cells *in vitro* by a, 2,4-dinitrophenol; b, 2,6-dichloro-3-hydroxybenzonitrile and c, 2,6-dichloro-4-hydroxybenzonitrile. Restoration by ATP and oligomycin.

Effect of ATP on pretreated cells

To 12 preparations, arrested in their beating by the phenols (4 dishes/compound), ATP was added (0.2 µmole/dish). A difference in response to ATP was observed. 2,4-Dinitrophenol-treated cells started beating within 60 sec after the addition. Cells treated with 2,6-dichloro-3-hydroxybenzonitrile began to beat 2–3 min after ATP addition. Cultures pretreated with 2,6-dichloro-4-hydroxybenzonitrile did not

respond to ATP during the time of observation (6 min). When 2,6-dichloro-4-hydroxybenzonitrile-inhibited cells were treated with oligomycin (20 μg/ml), 6 min after addition of ATP, they started to beat again. An additional amount of ATP increased the frequency. The rise in frequency by ATP additions after oligomycin treatment was also observed on 2,6-dichloro-3-hydroxybenzonitrile and 2,4-dinitrophenol-inhibited cells (Fig. 1).

So far the qualitative picture of the biological activity of both monophenolic metabolites of Dichlobenil is in close agreement with the action of 2,4-dinitrophenol on beating heart cells. The differences in response to ATP additions by cells, pretreated with 2,6-dichloro-4-hydroxybenzonitrile, its 3-hydroxy analogue and 2,4-dinitrophenol, probably reflect the quantitative differences in ATPase activities niduced in the living cells. The antagonism of oligomycin to the monophenols of 2,6-dichlorobenzonitrile is in agreement with the conclusion of HARARY AND SLATER[1] for 2,4-dinitrophenol, that both metabolites act on the mitochondria and not on the cytoplasmic constituents of the living cell.

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