The Biochemical Mode of Action of the Fasciolicides Nitroxylin, Hexachlorophane and Oxyclozanide

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There is little information on the biochemical mode of action of compounds for controlling *Fasciola hepatica* L. The chemicals used can be divided into two groups: (a) carbon tetrachloride and other compounds containing the trichloromethyl group; (b) substituted phenols and salicylanilides. We have investigated the latter group, taking nitroxylin (4-hydroxy-3-iodo-5-nitrobenzonitrile), hexachlorophane [2,2'-methylenebis-(3,4,6-trichlorophenol)] and oxyclozanide (3,3',5,5',6-pentachloro-2'-hydroxysalicylanilide) as structurally representative and commercially significant examples.

It is well known that substituted phenols and salicylanilides are uncouplers of oxidative phosphorylation, and symptoms of uncoupling of the host's metabolism have been shown for several fasciolicides of this group. However, the obvious hypothesis that the fasciolicidal effect is due to uncoupling does not seem to have been suggested. This communication gives our evidence that the uncoupling hypothesis is correct.

Using an oxygen electrode with rat liver mitochondria we found the following minimum uncoupling concentrations: nitroxylin, 27–33 μM ; hexachlorophane, 0.6–0.8 μM ; oxyclozanide, 0.3–0.4 μM .

Although the adult fluke is thought to live anaerobically in the bile duct it will take up oxygen from air. Using Warburg flasks with glucose as a food source we found that the compounds stimulated oxygen uptake by some 20–40% over the previous untreated rate at the following concentrations: nitroxylin, 10–100 μM , hexachlorophane, 1–10 μM ; oxyclozanide, 0.1–1 μM . There is therefore an approximate correlation between the concentration causing stimulation of oxygen uptake and the minimum uncoupling concentration. After the stimulation the oxygen uptake decreases to a rate much lower than that of control flukes, which received solvent only, presumably owing to the progressive death of the flukes treated with fasciolicides.

A measure of the effect of the three compounds *in vitro* on fluke was gained by keeping flukes in Tyrode's solution plus bovine serum and examining the animals 24 h after treatment. The following concentrations were required to cause apparent death (i.e. lack of visible movement): nitroxylin, 170–350 μM ; hexachlorophane, 3.5–7 μM ; oxyclozanide, 3.5–7 μM . This result is complicated by the

presence of serum, which might bind the compounds to a differing extent, but there is a reasonable correlation with the minimum uncoupling concentrations of the fasciolicides, suggesting that death is indeed due to uncoupling.

The Effect of Organic Anions on the Biliary Excretion of Tri[^{14}C]methyl-(3-hydroxyphenyl)ammonium O-Glucuronide

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In previous experiments differences between the biliary excretion of tri[^{14}C]methyl-(3-hydroxyphenyl)ammonium iodide in normal Wistar animals and in Gunn strain of jaundiced rats were observed (Calvey, Somani & Wright, 1970). Both strains of animals mainly excreted the quaternary amine as a O-glucuronide. In the normal rat small amounts of this metabolite (equivalent to 3% of the dose in 4 h) were eliminated in bile; in contrast homozygous Gunn rats excreted large amounts of the glucuronide in bile (26% of the dose in 4 h). It was suggested that the diminished excretion in normal animals was dependent on competition between bilirubin glucuronide and tri[^{14}C]methyl-(3-hydroxyphenyl)ammonium O-glucuronide for a common excretory pathway in the liver cell. In the homozygous Gunn rat competition for transport did not occur, so that large amounts of the exogenous glucuronide were transferred from liver to bile.

In the present experiments the effect of [^3H]bilirubin and [^3H]bilirubin glucuronide on the biliary excretion of tri[^{14}C]methyl-(3-hydroxyphenyl)ammonium O-glucuronide was studied in normal Wistar animals and in homozygous Gunn rats. In all experiments the quaternary amine was administered as the iodide salt (2.0 $\mu\text{mol/kg}$ body wt.). ^3H and ^{14}C radioactivity was simultaneously assayed in samples of bile by liquid-scintillation spectrometry.

In normal Wistar animals [^3H]bilirubin or [^3H]bilirubin glucuronide (1.0 $\mu\text{mol/kg}$ body wt.) decreased the biliary excretion of tri[^{14}C]methyl-(3-hydroxyphenyl)ammonium O-glucuronide by 0–20%; a similar decrease was induced by other organic anions (e.g. sulphobromophthalein, phenol-sulphonphthalein and dehydrocholate). Different results were obtained in the homozygous Gunn rat. [^3H]Bilirubin (1.0 $\mu\text{mol/kg}$ body wt.) has no effect on the biliary elimination of the ^{14}C -labelled quaternary glucuronide; in contrast [^3H]bilirubin glucuronide (1.0 $\mu\text{mol/kg}$ body wt.) decreased its excretion by 80–90%, and a similar but less marked effect was induced by sulphobromophthalein and phenolsulphonphthalein.

These studies were considered to support the view that tri[¹⁴C]methyl-(3-hydroxyphenyl)-ammonium *O*-glucuronide is normally transferred from liver cell to bile in competition with bilirubin glucuronide, and that the enhanced excretion of the quaternary metabolite in the homozygous Gunn rat is due to the absence of conjugated bilirubin from the liver cell.

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Biochemical Effects of the Hypoglycaemic Compound Diphenyleneiodonium in Rat Liver Mitochondria: Inhibition of Adenosine Triphosphate Synthesis

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Diphenyleneiodonium nitrate is a very active hypoglycaemic compound (Stewart & Hanley, 1969). We therefore investigated its effects on some reactions in rat liver mitochondria, since several unrelated hypoglycaemic compounds impair gluconeogenesis secondarily to inhibition of energy metabolism (see Senior & Sherratt, 1968; Sherratt, 1969).

Diphenyleneiodonium (10–30 μ M) strongly inhibits State 3 respiration (with succinate as substrate) stimulated by ADP or 2,4-dinitrophenol in a standard medium containing 130 mM-Cl⁻. Since diphenyleneiodonium catalyses a Cl⁻-OH⁻ exchange across the inner mitochondrial membrane (Holland & Sherratt, 1971) two Cl⁻-free media were also used: one in which glycerol 2-phosphate²⁻ was substituted for Cl⁻ (based on Aldridge, 1957) and which gave similar respiratory rates and ADP/O ratios (with succinate as substrate) as in standard medium; and 0.3 M-sucrose buffered with 2 mM-*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate (cf. Stockdale, Dawson & Selwyn, 1970). In these Cl⁻-free media diphenyleneiodonium had little effect on respiration stimulated by ADP or by 2,4-dinitrophenol.

The effects of trialkyltin compounds on mitochondria resemble those of diphenyleneiodonium in that they inhibit State 3 respiration stimulated by ADP or 2,4-dinitrophenol in Cl⁻-containing medium (Aldridge, 1958) and they catalyse a Cl⁻-OH⁻ exchange across the inner mitochondrial membrane (Selwyn, Dawson, Stockdale & Gains, 1970). By contrast they inhibit State 3 respiration stimulated by ADP but not by 2,4-dinitrophenol in a Cl⁻-free sucrose medium (Stockdale *et al.* 1970) or in glycerol 2-phosphate-containing medium.

Stockdale *et al.* (1970) suggested that inhibition of respiration stimulated by ADP is due to an oligomycin-like effect of the trialkyltin compounds and that by contrast their inhibition of 2,4-dinitrophenol-stimulated respiration in Cl⁻-containing media is indirectly dependent on the Cl⁻-OH⁻ exchange. Our results suggest that diphenyleneiodonium does not have a strong oligomycin-like effect and that its inhibition of State 3 respiration in Cl⁻-containing media depends on its ability to catalyse a Cl⁻-OH⁻ exchange. Indeed, in Cl⁻-containing media 10 μ M-diphenyleneiodonium inhibits the 2,4-dinitrophenol-stimulated mitochondrial adenosine triphosphatase by only 20% while completely suppressing the State 4 to State 3 transition. These results also suggest that diphenyleneiodonium may not sufficiently impair the synthesis of ATP *in vivo* to account for its hypoglycaemic effects, since the intracellular Cl⁻ concentration is very low.

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Biochemical Effects of the Hypoglycaemic Compound Diphenyleneiodonium in Rat Liver Mitochondria: Anion-Hydroxyl Ion Exchange

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The possibility that diphenyleneiodonium blocks State 3 respiration (Holland & Sherratt, 1971) by inhibiting entry of substrate anions into mitochondria was investigated by using the technique of P_i-induced swelling of mitochondria suspended in ammonium succinate or malate solutions buffered with 5 mM-tris-HCl (Chappell, 1968). No inhibition was found, but it was noticed that some swelling was caused by diphenyleneiodonium and that this was dependent on the Cl⁻ present. Rapid and ex-