Inhibition by sporidesmin of hepatocyte bile acid transport

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Exposure of isolated rat hepatocytes (approx. 2×10^7 – 5×10^7 cells/10 ml of incubation mixture) to 0.5 mg of the mycotoxin sporidesmin for 30–60 min at 37°C produced loss of plasma-membrane microvilli with some disruption of organelle distribution in the sub-surface region. There was accompanying inhibition of [14 C]cholate and [14 C]taurocholate transport, but bile acid conjugation was not altered. Inhibition of cholate uptake was maximal after exposure of hepatocytes to sporidesmin for 1 min, and was not reversed by washing cells free of extracellular sporidesmin. *N*-Ethylmaleimide (0.1 mM) or dithiothreitol (1 mM) partially protected hepatocytes from sporidesmin inhibition of bile acid uptake. Significant protection was not given by other thiols or by zinc sulphate, cholesterol, ascorbate or α -tocopherol. The results are discussed in terms of sporidesmin action on cell membranes and the toxin's effect on bile secretion.

The dioxodithiopiperazine mycotoxin sporidesmin (Fig. 1) is responsible for major production losses in New Zealand livestock as the causative agent of the photosensitizing disorder facial eczema. The primary lesion involves liver and bile-duct damage, manifested in sheep as transient hepatocyte degeneration followed by intra- and extra-hepatic bile-duct injury (Mortimer, 1963). Rats are less susceptible to hepatobiliary injury, but within 1 h of perfusion of 2 mg of sporidesmin to isolated rat liver there was inhibition of bile flow and of bile acid and bilirubin secretion, accompanied by degeneration of the hepatocyte canalicular membrane (Bullock *et al.*, 1974). Administration of sporidesmin (6 mg/kg intraperitoneally) to intact rats led to transitory cessation of bile flow within 18–30 h (Slater & Griffiths, 1963), and we have observed 50% diminution of bile flow in bile-duct-cannulated rats 1 h after the administration of sporidesmin

(2 mg/kg intraperitoneally) (S. J. Cordiner & T. W. Jordan, unpublished work). The diminution of bile flow in sheep exposed to the oral administration of 1 mg of sporidesmin/kg occurred more slowly, reaching a minimum at about 2 weeks after dosing (Mortimer & Stanbridge, 1969).

Kroker *et al.* (1977) reported that sporidesmin inhibited bile acid synthesis but not bile acid secretion in isolated hepatocytes. Depression of bile acid synthesis may in part account for the diminution of bile flow in sporidesmin-poisoned animals, as a major component of flow is coupled to maintenance of hepatic bile acid pools, with subsequent bile acid secretion across canalicular membranes. To investigate the cholestatic action of sporidesmin further we have analysed the effects of sporidesmin on bile acid uptake, conjugation and efflux in isolated hepatocytes. Bile acid uptake from portal blood is a rapid process catalysed by an Na^+

Fig. 1. Structures of sporidesmin and gliotoxin

K⁺-dependent ATPase located on the hepatocyte sinusoidal membrane (Blitzer & Boyer, 1982), and in isolated hepatocytes has been characterized as ouabain-sensitive active transport (Schwarz *et al.*, 1975) inhibited by microfilament-disrupting agents (Reichen *et al.*, 1981). Schwarz *et al.* (1976) characterized taurocholate efflux from isolated hepatocytes as a carrier-mediated process that differed from bile acid uptake and possessed kinetics similar to those of bile acid secretion in whole animals. Gallagher (1964) and Middleton (1974*a,b*) demonstrated that sporidesmin altered the metabolic and permeability properties of isolated mitochondria, and Middleton (1974*b*) postulated a direct interaction of mitochondrial membranes with the disulphide group of sporidesmin. Similarly Bullock *et al.* (1974) reported that perfused liver exposed to sporidesmin developed ultrastructural changes including modification of the bile canaliculus, and postulated interaction of sporidesmin with the pericanalicular microfilament web. Inhibition of bile secretion by direct interaction of sporidesmin with hepatic bile acid-transporting membranes thus seemed worth investigating, particularly as a number of other cholestatic agents have been shown to act by modifying hepatic plasma-membrane fluidity (Keefe *et al.*, 1980) or microfilament actin organization (Prentki *et al.*, 1979).

Experimental

Materials

Sporidesmin (mol.wt. 473, unsolvated) was provided by Ruakura Agricultural Research Centre (Hamilton, New Zealand). Gliotoxin was a gift from American Cyanamid Co. (Pearl River, NY, U.S.A.). Sodium [*carboxy*-¹⁴C]cholate (sp. radioactivity 59.5 Ci/mol) from The Radiochemical Centre (Amersham, Bucks., U.K.) and [24-¹⁴C]taurocholic acid (sp. radioactivity 52.0 Ci/mol) from New England Nuclear (Boston, MA, U.S.A.) were greater than 97% pure when chromatographed in three separate t.l.c. systems. Bile acids, collagenase (type IV), carbonyl cyanide *m*-chlorophenylhydrazone, Trypan Blue and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A.R.-grade chemicals for perfusion and incubation media were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Hepatocyte preparation

Isolated hepatocytes were prepared from 160–240 g female rats by collagenase perfusion (Berry & Friend, 1969). The perfusion buffer was maintained at 37°C, continuously gassed with O₂, and contained 140 mM-NaCl, 5.4 mM-KCl, 0.8 mM-MgSO₄, 0.8 mM-NaH₂PO₄, 25 mM-Hepes, 12.5 mM-glucose,

3.3 mM-sodium pyruvate and 2.3 mM-sodium glutamate, and was adjusted to pH 7.4 with NaOH. Rats were anaesthetized with thiopentone sodium (80 mg/kg intraperitoneally), and 1000 units of heparin were injected intraperitoneally. After exposure of the liver by abdominal incision, the hepatic portal vein was cannulated, the flow of perfusion buffer (15 ml/min) was started and the vena cava was severed above and below the liver. The liver was dissected free and transferred to a liver dish for recycling perfusion in 30 ml of perfusion buffer containing 15 mg of collagenase and 1.5 mM-CaCl₂. After about 15–30 min, when perfusate was leaking through the surface capsule, the cannula was removed and the liver was transferred to a Petri dish for mechanical separation of cells by combing. The crude cell suspension was passed through 300 μm-mesh Nybolt (Swiss Silk Bolting Cloth, Zürich, Switzerland), and the cells were washed two or three times by centrifugation in perfusion media at 50 g for 1 min. The washed-cell pellet was passed through 60 μm-mesh Nybolt and the filtrate was used for incubation experiments. Yields were about 5 × 10⁷ cells/g of liver, with viability greater than 90% as measured by Trypan Blue exclusion. The purified cell suspension contained at least 95% hepatocytes when examined by phase-contrast and electron microscopy.

Electron microscopy

Cells were fixed for 30 min at room temperature in 0.03 M-OsO₄/0.05 M-sucrose/0.02 M-K₄Fe(CN)₆/0.08 M-cacodylate, adjusted to pH 7.4 with HCl. After dehydration, tissue was embedded in Araldite and examined as previously described (Lim & Jordan, 1981).

Bile acid uptake and efflux

Isolated hepatocytes were suspended in O₂-gassed incubation medium similar to the initial perfusion buffer but also containing 2.5 mM-CaCl₂.

For measurement of bile acid uptake approx. 2 × 10⁷–5 × 10⁷ hepatocytes suspended in 10 ml of medium were incubated in an O₂-gassed atmosphere at 37°C with 2.5 μM-[¹⁴C]cholate (approx. 2 × 10⁴ d.p.m.) in a shaking water bath at 60 oscillations/min with 3 cm amplitude. At intervals up to 30 min 1 ml portions of the suspension were removed and immediately centrifuged at 1000 g for 15 s to sediment the cells. Within 1 min 500 μl samples of the supernatant were transferred to vials with 6 ml of a liquid-scintillation 'cocktail' for radioactivity measurement (Lim & Jordan, 1981). Cholate uptake was thus assayed as radioactivity lost from cell-free supernatants of hepatocyte suspensions.

Bile acid efflux was measured as taurocholate release from hepatocytes previously loaded by

EXPLANATION OF PLATE 1

Electron micrographs of control hepatocytes

Isolated rat hepatocytes were incubated for 30 min at 37°C before preparation for electron microscopy. Cells (magnification $\times 7200$) show extensive plasma-membrane microvilli, with inset (magnification $\times 40000$) showing detail of the plasma membrane.

EXPLANATION OF PLATE 2

Electron micrographs of cells after exposure to sporidesmin

Isolated hepatocytes were incubated with 0.5 mg of sporidesmin in 10 ml of medium for 30 min at 37°C before preparation for electron microscopy. Cells (magnification $\times 14\,400$) show loss of plasma-membrane microvilli, with inset (magnification $\times 40\,000$) showing detail of changes at the surface and in the adjacent cytoplasm.

preincubation with 25 μM - ^{14}C taurocholate at 37°C for 30 min. Taurocholate uptake during the preincubation period was 40–70% of the added radioactivity. Efflux analysis was started by transferring 1 ml of the loaded hepatocyte suspension (approx. 10^7 cells; 8×10^4 d.p.m.) to a flask containing 9 ml of incubation buffer. At intervals up to 10 min 1 ml samples were removed, centrifuged and processed for scintillation counting of radioactivity.

Cholate conjugation

Conjugation of added ^{14}C cholate was measured by t.l.c. Samples of cell-free supernatants and cell pellets prepared by centrifugation of incubation mixtures were each added to 1 ml of conc. HCl and extracted twice with water-saturated butan-1-ol. Radioactive conjugates in the butanol extracts were measured as previously described (Lim & Jordan, 1981).

Results

Hepatocyte viability

Viability of isolated hepatocytes was always rapidly checked by using the Trypan Blue exclusion test. Cells that showed slightest traces of staining were classed as non-viable. Other viability criteria used were those of Zahlten & Stratman (1974) and Schwarz *et al.* (1975). Isolated hepatocytes had basal rates of glucose efflux approx. 2–3 nmol/min per mg wet wt., which were stimulated 2-fold by 1 μM -adrenaline or by 1 nM-glucagon and were depressed by 50% by 0.5 μM -insulin. Cells with less than 85% Trypan Blue exclusion had lower basal rates of glucose efflux and showed only 0–30% stimulation of glucose output in the presence of 1 μM -adrenaline. Electron microscopy of cell populations that excluded Trypan Blue revealed glycogen retention and good ultrastructural preservation (Plate 1), but Trypan Blue-staining cell populations had major structural disruptions, including membrane breaks and distortions and extensive mitochondrial darkening. Hepatocyte populations that had greater than 90% Trypan Blue exclusion showed 50–80% increase in respiration rates on the addition of 20 μM -carbonyl cyanide *m*-chlorophenylhydrazone and less than 20% stimulation of respiration by 1 mM-succinate. Freshly isolated hepatocyte suspensions with greater than 90% Trypan Blue exclusion always had high viabilities as assessed by biochemical and ultrastructural criteria. Exposure of isolated hepatocytes to sporidesmin (0.5 mg of toxicant/ 2×10^7 – 5×10^7 cells/10 ml of incubation mixture) for 30 min at 37°C did not significantly alter Trypan Blue staining, succinate stimulation of respiration, respiratory control or leakage of the

cytosolic enzyme glutathione *S*-transferase (Lim & Jordan, 1981) into the incubation medium.

Electron microscopy

When 0.5 mg of sporidesmin was incubated with approx. 2×10^7 – 5×10^7 hepatocytes in 10 ml of medium for 30–60 min, consistent changes could not be observed by phase-contrast light-microscopy, but electron-microscopic examination showed changes particularly at the plasma membrane and in the adjacent cytoplasmic region. Control cells not exposed to sporidesmin were rounded up, with extensive plasma-membrane microvilli, numerous organelles penetrating the cytoplasm and many glycogen deposits (Plate 1). Exposure to sporidesmin rapidly led to loss of surface microvilli, with thinning of organelles from the sub-surface region (Plate 2). Some disorganization of the endoplasmic reticulum was also apparent in sporidesmin-treated cells.

Bile acid uptake

When rates of bile acid uptake or efflux were compared in control hepatocyte populations of various degrees of viability, measured as Trypan Blue exclusion and adrenaline stimulation of glucose release, maximum rates of bile acid transport occurred in high-viability preparations. There was a positive relationship between viability and bile acid uptake. Hepatocytes with less than 20% Trypan Blue exclusion, separated from normal suspensions by centrifugation on metrizamide, did not have significant rates of cholate extraction. Uptake was not altered by preincubation of hepatocytes for up to 60 min at 37°C before exposure to ^{14}C cholate or by storage of hepatocytes for 16 h at 4°C. Cell viabilities decreased by less than 10% during the course of these experiments. Uptake was inhibited almost completely when one of the mitochondrial uncouplers 2,4-dinitrophenol (2 mM) and carbonyl cyanide *m*-chlorophenylhydrazone (20 μM) was present in the incubation media.

Bile acid uptake was inhibited when 0.1–1.0 mg of sporidesmin in 10 μl of redistilled ethanol was added to the hepatocyte suspension 10 min before the addition of ^{14}C cholate (Fig. 2). Slight inhibition, equivalent to that produced by 0.01 mg of sporidesmin, was given by ethanol alone. Sporidesmin solid added without solvent to flasks containing hepatocyte suspensions inhibited bile acid uptake to the same extent as sporidesmin added dissolved in ethanol. Sporidesmin has low water-solubility (30 p.p.m.) but readily dissolved in cell suspensions. Additional inhibition of cholate uptake was not observed when up to 1 mg of sporidesmin was stored at 4°C with isolated hepatocytes overnight. Hepatocytes stored overnight in the presence or in the absence of sporidesmin excluded Trypan Blue and

Fig. 2. Effect of sporidesmin on cholate uptake by hepatocytes

Hepatocytes were incubated with [14 C]cholate, and uptake was measured at intervals as loss of supernatant radioactivity after sedimentation of cells. Results are means for five experiments. Bars show standard deviations. ■, Control (no sporidesmin); □, 10 μ l of ethanol; ●, 0.01 mg of sporidesmin; ○, 0.1 mg of sporidesmin; ▲, 1.0 mg of sporidesmin.

accumulated [14 C]cholate, but when examined by phase-contrast microscopy considerable blebbing of the plasma membrane was apparent. Preincubation of hepatocytes for 1–30 min at 37°C with sporidesmin before the addition of [14 C]cholate gave equivalent inhibition of bile acid uptake to that observed with 10 min preincubation. When 0.5 mg of sporidesmin was preincubated with hepatocytes for 2, 10 or 30 min and the cells were washed twice by resuspension and centrifugation in incubation buffer to remove extracellular sporidesmin, inhibition of cholate uptake was equivalent to that achieved when sporidesmin was present throughout the incubation and preincubation periods. Trypan Blue exclusion and cholate extraction in cells similarly treated in the absence of sporidesmin were not affected by the washing procedure. Increasing the extracellular cholate concentration to 25 μ M did not protect against sporidesmin inhibition. To investigate further the effect of sporidesmin on bile acid uptake, cholate extraction was measured at several cell concentrations. The rate of cholate uptake was

Fig. 3. Effect of cell concentration on sporidesmin inhibition of cholate uptake by hepatocytes

Incubation mixtures (10 ml) contained 10^7 (□ and ■), 3×10^7 (○ and ●) or 6×10^7 (△ and ▲) hepatocytes in the absence (○, □ and △) or in the presence (●, ■ and ▲) of 0.5 mg of sporidesmin. Results are means for two or three experiments, the results of which varied by less than 10%.

greatest at the higher cell concentrations, but sporidesmin inhibition was greatest when fewer hepatocytes were present (Fig. 3), indicating that the cellular rather than the aqueous concentration of sporidesmin determined the extent of inhibition. This effect was reflected in variability among hepatocyte preparations, so that comparable rates of bile acid uptake and extents of inhibition were only obtained from incubation mixtures containing similar numbers of viable cells.

Bile acid efflux

When small amounts of sporidesmin (5–50 μ g) were incubated with taurocholate-loaded hepatocytes, bile acid efflux was stimulated, but 0.5 mg of sporidesmin inhibited bile acid efflux (Fig. 4). Similarly bile acid efflux was stimulated 2–3-fold by 2 mM 2,4-dinitrophenol or 20 μ M carbonyl cyanide *m*-chlorophenylhydrazone. Cell viabilities assessed as Trypan Blue exclusion were not decreased during the course of these experiments. The apparent stimulation of taurocholate efflux by mitochondrial uncouplers may be attributed to inhibition of re-uptake of secreted taurocholate, as uptake (Schwarz *et al.*, 1975) was more sensitive than efflux

(Schwarz *et al.*, 1976) to inhibition by these compounds. It was not clear whether the stimulation of taurocholate efflux at low sporidesmin concentration was also due to blocked re-uptake of excreted taurocholate or whether under these conditions sporidesmin facilitated export of taurocholate from hepatocytes.

Cholate conjugation

Although sporidesmin decreased bile acid uptake by hepatocytes, cholate conjugation to glycocholate and taurocholate was not altered (Fig. 5) when conjugate formation over a 30 min period was

Fig. 4. Effect of sporidesmin on taurocholate efflux from hepatocytes

Results are means for two experiments, the results of which varied by less than 10%. Hepatocytes were loaded with [14 C]taurocholate by incubation at 37°C and transferred to taurocholate-free medium for efflux analysis. ■, Control (no sporidesmin); □, 10 µl of ethanol; ●, 0.005 mg of sporidesmin; ○, 0.05 mg of sporidesmin; △, 0.5 mg of sporidesmin.

Fig. 5. Effect of sporidesmin on bile acid conjugation by hepatocytes

Hepatocytes were incubated with [14 C]cholate for 30 min at 37°C. Cells and supernatants were separated by centrifugation at 500 g for 5 min. Proportions of cholate and conjugates in butan-1-ol extracts were analysed after t.l.c. in butan-2-one/water/acetic acid (40:20:1, by vol.). Results are typical of three separate experiments. Migration of reference compounds is shown by bars: I, cholate; II, glycocholate; III, taurocholate. (a) Supernatant from control incubation mixtures; (b) cells from control incubation mixtures; (c) supernatant from suspensions exposed to sporidesmin; (d) cells from suspensions exposed to sporidesmin.

examined in the presence and in the absence of 0.5 mg of sporidesmin. Similar proportions of taurocholate and glycocholate were found in cell and supernatant fractions from control and sporidesmin-incubated hepatocytes.

Effects of other compounds on cholate uptake

When the effects of sporidesmin, gliotoxin, dithiothreitol and 2,3-dimercaptopropanol (each 1 μ mol) on bile acid uptake were compared (Fig. 6), sporidesmin was a better inhibitor than the other compounds. When 10 μ mol of 2,3-dimercaptopropanol was included in 10 ml incubation mixtures bile acid uptake was substantially inhibited without decreasing cell viability as assessed by Trypan Blue exclusion. Addition of 1–10 μ mol of other thiol compounds, including reduced glutathione, cysteine, methionine, thioglycolic acid and mercaptoethanol, did not significantly decrease rates of cholate uptake. Because of the potential for sporidesmin interaction with cellular thiols, we investigated the effect of *N*-ethylmaleimide on bile acid uptake. Similarly to 2,3-dimercaptopropanol, 10 μ mol of *N*-ethylmaleimide almost completely abolished cholate uptake,

Fig. 6. *Inhibition by sulphur-containing compounds of cholate uptake by hepatocytes*

[14 C]Cholate uptake was measured in the presence of: ●, 0.1 mM-sporidesmin; ○, 0.1 mM-gliotoxin; □, 0.1 mM-dithiothreitol; ■, 0.1 mM-2,3-dimercaptopropanol; ▽, 1 mM-2,3-dimercaptopropanol; ▲, 1 mM-glutathione. △, Control incubation mixtures. Results are means for three experiments, the results of which varied by less than 10%.

but 1 μ mol only slightly diminished the extraction process.

In an attempt to elucidate the mechanism by which sporidesmin altered hepatocyte bile acid transport, we investigated possible antagonists of sporidesmin action. Unless otherwise indicated, none of the compounds alone interfered with cholate uptake and none decreased cell viability as assessed by Trypan Blue exclusion. Potential antagonists were incubated with hepatocytes for 15 min before addition of 0.5 mg (approx. 1 μ mol) of sporidesmin, and after a further 15 min [14 C]cholate was added and the rate of bile acid uptake was measured. ZnSO₄ partially protects livestock against sporidesmin-induced liver damage (Towers & Smith, 1978). However, preincubation of hepatocytes with 0.05 mM- or 0.5 mM-ZnSO₄ did not alter the rate of bile acid uptake and did not protect against sporidesmin inhibition of cholate uptake in isolated hepatocytes. Stacey & Klaassen (1981) have shown that ZnCl₂ is rapidly taken up by hepatocytes under these conditions. Similarly, (+)- α -tocopherol acetate (Sigma type III) and ascorbate (each 10 μ mol) showed no protectant action. Nor did 1–10 μ mol of cholesterol, 2,3-dimercaptopropanol, cysteine, reduced glutathione, methionine, thioglycolic acid or mercaptoethanol significantly protect against sporidesmin inhibition of bile acid uptake. Partial protection (30–50%) was afforded by 10 μ mol of dithiothreitol and by 1 μ mol of *N*-ethylmaleimide.

Discussion

Sporidesmin did not diminish hepatocyte viability as measured by processes that assess plasma-membrane permeability. There were insignificant effects on Trypan Blue exclusion, succinate permeability and leakage of the cytosolic enzyme glutathione *S*-transferase.

However, sporidesmin altered hepatocyte morphology, particularly at the plasma membrane and adjacent cytoplasm, and affected transport of bile acids across the plasma membrane. Loss of hepatocyte microvilli and 'ballooning' of the cell membrane has been seen in liver of sheep exposed to sporidesmin (Bertaud & Mortimer, 1966), and loss of canalicular microvilli occurred in rat liver exposed to the mycotoxin (Bullock *et al.*, 1974). We have previously reported the effect of sporidesmin on hepatocyte glucose efflux and adrenaline-stimulated glucose output (Cordiner *et al.*, 1983), and in the present work have used changes in bile acid transport as a measure of the mycotoxin's membrane action. The significance of bile acid uptake and efflux in isolated hepatocytes was complicated by loss of plasma-membrane structural features in single cells that did not show (Plate 1) the distinct sinusoidal and canalicular membrane faces respon-

sible for bile acid uptake and secretion *in vivo*. The possibility that isolation altered plasma-membrane transport processes must therefore be accepted. However, transport properties were apparently retained in isolated hepatocytes because cholate uptake but not taurocholate efflux was readily blocked by mitochondrial uncouplers, and *in vivo* uptake is an active-transport process whereas secretion is probably by facilitated diffusion (Blitzer & Boyer, 1982). Schwarz *et al.* (1976) also obtained evidence for preservation of the polarity of bile acid excretion in isolated hepatocytes, and Wisher & Evans (1977) demonstrated that the enzyme and protein distributions of plasma-membrane subfractions prepared from isolated hepatocytes were similar to those prepared from whole-liver homogenates. It was not obvious whether the effect of sporidesmin on bile acid transport reflected general disruption of membrane morphology or specific interaction with bile acid-transport components. However, as sporidesmin affected bile acid uptake and efflux and altered glucose output and its hormonal stimulation (Cordiner *et al.*, 1983), multiple functional lesions at the plasma membrane were indicated. The nature of microvilli loss deserves further study. Possible mechanisms include shedding or internalization of surface components with deletion of bile acid-transport systems from the plasma membrane. Such explanations, however, may not simply account for the concentration-dependent inhibition of bile salt transport by sporidesmin or the selective inhibition of cholate uptake compared with taurocholate efflux at low sporidesmin concentrations.

Our results show that 0.1–1.0 mg of sporidesmin per 5×10^7 cells (equivalent to 1 g of liver) depressed bile acid uptake and efflux without significantly altering bile acid conjugation. The amount of sporidesmin required to inhibit bile acid transport in isolated hepatocytes was relatively large compared with the amounts (1–6 mg/kg body wt.) used to decrease bile flow in live animals, but accumulation of sporidesmin in the liver and bile *in vivo* (Mortimer & Stanbridge, 1968) may achieve local concentrations of the toxin sufficient to inhibit bile acid transport. Our demonstration that inhibition of cholate uptake was produced by brief exposure to sporidesmin and was not reversed by washing exposed cells in incubation medium indicated that transitory high concentrations of sporidesmin may be sufficient to alter hepatocyte function. A similar situation has been encountered with cholestatic steroids, which in small amounts depress bile acid secretion in intact animals but require concentrations in the order of those used in our present studies to modify isolated hepatocyte bile acid transport (Schwarz *et al.*, 1977). Eakins (1978) demonstrated that sporidesmin did not inhibit liver

plasma-membrane $\text{Na}^+ + \text{K}^+$ -dependent ATPase *in vitro*, and Bullock *et al.* (1974) reported that sporidesmin did not significantly interfere with ATP production in isolated perfused liver. Bile acid conjugation is an ATP-dependent process, and the normal course of conjugate formation in sporidesmin-exposed hepatocytes (Fig. 5) also indicated that ATP supplies were not substantially diminished in our preparations. Assuming that sporidesmin did not block bile acid uptake by interfering with ATP metabolism, other mechanisms of sporidesmin action must be sought. Earlier observations of sporidesmin-induced mitochondrial swelling (Gallagher, 1964; Middleton, 1974a,b) provide the basis for a model of sporidesmin action on hepatocyte membranes. Sporidesmin altered adrenaline stimulation of glucose output from isolated hepatocytes in a concentration-dependent manner, with slight activation given by 10–50 μg of sporidesmin per 10^7 hepatocytes and substantial inhibition when sporidesmin concentrations were 10-fold greater (Cordiner *et al.*, 1982). Similar behaviour has been observed with agents, including chlorpromazine (Keefe *et al.*, 1980) and benzyl alcohol (Dipple & Houslay, 1978), that are thought to modify plasma-membrane fluidity.

Sporidesmin is rapidly taken up by isolated hepatocytes, converted into sporidesmin dithiol and in part associates with membrane proteins (C. H. Sissons, J. R. Ronaldson & S. J. Cordiner, unpublished work), and Munday (1982) has indicated that sporidesmin dithiol is rapidly generated by chemical reaction of the sporidesmin disulphide with reduced glutathione at pH 7.4. A role of thiols in bile acid transport was suggested by the inhibitory action of *N*-ethylmaleimide and 2,3-dimercaptopropanol, so that interaction of the disulphide group of sporidesmin with membrane thiols, as postulated by Middleton (1974b), may account for the effects that we have observed. However, in view of the reactivity of thiols with carbonyl and carboxy groups, the potential for nucleophilic attack on double bonds (Jocelyn, 1972) and the possibility of hydrophobic association of sporidesmin with membrane proteins and lipids, mechanisms of toxin action other than by disulphide exchange with membrane thiols (Middleton, 1974b) or by superoxide-mediated damage (Munday, 1982) are feasible. Lack of protection by α -tocopherol suggested that sporidesmin-initiated free-radical attack did not account for the inhibition of bile acid transport. Absence of protective action by reduced glutathione and ascorbate was not surprising, as their normal hepatocyte concentrations (Smith *et al.*, 1982) are similar to those used in our present studies. The partial protection afforded by dithiothreitol and *N*-ethylmaleimide may be due to interaction of those compounds with sporidesmin

sulphur atoms. Our results parallel those obtained by Middleton (1974b), who found that dithiothreitol, but not cysteine or reduced glutathione, blocked the toxic action of sporidesmin on mitochondrial membranes and that among a range of sulphur compounds dithiothreitol had significantly the greatest ability to react chemically with sporidesmin. Although the sporidesmin sulphur atoms are implicated in the toxic mechanisms, a related dioxo-dithiopiperazine gliotoxin (Fig. 1) had less effect on bile acid uptake (Fig. 6) and was 50-fold less toxic than sporidesmin to cultured cells (Mortimer & Collins, 1968). The substituted indole moiety or adjacent hydroxy groups of sporidesmin apparently have an important role in toxicity, perhaps related to the molecule's cellular and membrane distribution or susceptibility to metabolic transformation.

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References

- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Bertaud, W. S. & Mortimer, P. H. (1966) *Proc. Int. Congr. Electron Microscopy 6th* 619–620
- Blitzer, B. L. & Boyer, J. L. (1982) *Gastroenterology* **82**, 346–357
- Bullock, G., Eakins, M. N., Sawyer, B. C. & Slater, T. F. (1974) *Proc. R. Soc. London Ser. B* **186**, 333–356
- Cordiner, S. J., Moore, C. M., Vintiner, S. K. & Jordan, T. W. (1983) *Proc. Congr. Int. Soc. Human and Animal Mycology 8th*, 1982, in the press
- Dipple, I. & Houslay, M. D. (1978) *Biochem. J.* **174**, 179–190
- Eakins, M. N. (1978) *Chem.-Biol. Interact.* **21**, 117–124
- Gallagher, C. H. (1964) *Biochem. Pharmacol.* **13**, 1017–1026
- Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, pp. 63–93, Academic Press, London and New York
- Keefe, E. B., Blankenship, N. M. & Scharschmidt, B. F. (1980) *Gastroenterology* **79**, 222–231
- Kroker, R., Anwer, M. S. & Hegner, D. (1977) *Zentralb. Veterinärmed. Reihe A* **24**, 205–209
- Lim, W. C. & Jordan, T. W. (1981) *Biochem. J.* **197**, 611–618
- Middleton, M. C. (1974a) *Biochem. Pharmacol.* **23**, 801–810
- Middleton, M. C. (1974b) *Biochem. Pharmacol.* **23**, 811–820
- Mortimer, P. H. (1963) *Res. Vet. Sci.* **4**, 166–185
- Mortimer, P. H. & Collins, B. S. (1968) *Res. Vet. Sci.* **9**, 136–142
- Mortimer, P. H. & Stanbridge, T. A. (1968) *J. Comp. Pathol.* **78**, 505–512
- Mortimer, P. H. & Stanbridge, T. A. (1969) *J. Comp. Pathol.* **79**, 267–275
- Munday, R. (1982) *Chem.-Biol. Interact.* **41**, 361–374
- Prentki, M., Chaponnier, C., Jeanrenaud, B. & Gabbiani, G. (1979) *J. Cell Biol.* **81**, 592–607
- Reichen, J., Berman, M. D. & Berk, P. D. (1981) *Biochim. Biophys. Acta* **634**, 126–133
- Schwarz, L. R., Burr, R., Schwenk, M., Pfaff, E. & Greim, H. (1975) *Eur. J. Biochem.* **55**, 617–623
- Schwarz, L. R., Schwenk, M., Pfaff, E. & Greim, H. (1976) *Eur. J. Biochem.* **71**, 369–373
- Schwarz, L. R., Schwenk, M., Pfaff, E. & Greim, H. (1977) *Biochem. Pharmacol.* **26**, 2433–2437
- Slater, T. F. & Griffiths, D. B. (1963) *Biochem. J.* **88**, 60P–61P
- Smith, M. J., Loveridge, N., Willis, E. D. & Chayen, J. (1982) *Biochem. J.* **182**, 103–108
- Stacey, N. H. & Klaasen, C. D. (1981) *Biochim. Biophys. Acta* **640**, 693–697
- Towers, N. R. & Smith, B. L. (1978) *N.Z. Vet. J.* **26**, 199–202
- Wisher, M. H. & Evans, W. H. (1977) *Biochem. J.* **164**, 415–422
- Zahlten, R. N. & Stratman, F. W. (1974) *Arch. Biochem. Biophys.* **163**, 600–608