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THE INVOLVEMENT OF THE DISULPHIDE GROUP OF SPORIDESMIN IN THE ACTION OF THE TOXIN ON SWELLING AND RESPIRATION OF LIVER MITOCHONDRIA

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Abstract—The disulphide group of the mycotoxin sporidesmin appears to be involved in the action of the toxin on the swelling and increased respiration of mitochondria. The requirements of the swelling produced by sporidesmin and gliotoxin, a toxic antibiotic which also contains the epidithiapiperazinedione moiety, are similar. The swelling produced by oxidized glutathione is quite different as there is no requirement for substrate or phosphate and much higher concentrations of the disulphide are required. Dithiothreitol but not a number of mono- or other dithiols inhibits the action of 70 μM sporidesmin, 50 per cent reduction in the rate of swelling being observed at dithiothreitol concentrations of 42 μM. Dithiothreitol but not the other mono- and dithiols reacts with sporidesmin to form a number of products. It is suggested that effects of sporidesmin on mitochondria *in vitro* may involve a reaction between the disulphide group of the toxin and reactive thiols in the membrane and that sporidesmin may exert its primary effect *in vivo* by reacting with specific thiol groups of certain hepatic membranes.

IN THE preceding paper[1] it was shown that under the appropriate conditions the disulphide containing mycotoxin, sporidesmin, produces rapid and reversible swelling of liver mitochondria. The conditions include a supply of oxidizable substrate and phosphate, and the occurrence of electron transport and coupled respiration. The requirement for phosphate appears to be related to the ability of this anion to alter the conformation of the mitochondrial membrane rather than to its ability to penetrate the membrane. Sporidesmin also stimulates state 4 respiration of well coupled mitochondria but is without effect on sonicated or Triton X-100 treated preparations. The above effects of sporidesmin could be explained by an interaction of the toxin with the mitochondrial membrane.

It has been known for some time that disulphide containing compounds can alter the permeability of mitochondrial membranes. Lehninger and Neubert[2,3] reported that certain disulphides and disulphide containing hormones produce rapid swelling of isolated mitochondria. Skrede[4] has reported that small uncharged disulphides penetrate the mitochondria and react with the membrane in a manner that alters its permeability. Mercurial reagents can also produce swelling of mitochondria by reacting with thiols in the membrane.[5] This reaction is dependent on the lipid solubility of the mercurial and on the metabolic state of the mitochondria.[6]

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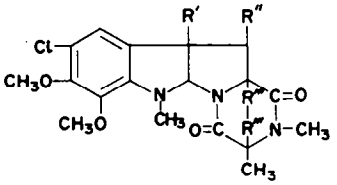
Evidence is presented in favour of the hypothesis that sporidesmin, as a lipid soluble uncharged disulphide, may alter the permeability of the mitochondrial membrane by reaction with specific thiol groups in the membrane, perhaps by a disulphide interchange reaction.



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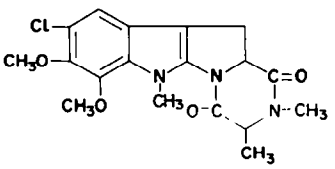

(II)

MATERIALS AND METHODS

Sporidesmin (I; R′ = OH, R″ = OH, R‴ = S), sporidesmin B (I; R′ = OH, R″ = H, R‴ = S), sporidesmin diacetate (I; R′ = OCH_3, R″ = OCH_3, R‴ = S), sporidesmin D (I; R′ = OH, R″ = OH, R‴ = SCH_3) and anhydrodesthiosporidesmin (II) were supplied by Dr. E. P. White and Mr. J. W. Ronaldson (Ruakura Animal Research Station, Hamilton, New Zealand). [^{36}S] sporidesmin, prepared as described by Towers and Wright, was kindly supplied by Dr. D. E. Wright of Ruakura Animal Research Station. Gliotoxin was a kind gift from Dr. A. Taylor (Atlantic Regional Laboratory, Halifax, Canada). Dithiothreitol, α-lipoic acid, bovine albumin (crystallized and lyophillized), rotenone and most other organic chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other reagents were of AnalaR grade.

Methods. The isolation of mitochondria, measurements of swelling and oxygen consumption, and determination of respiratory control indices were carried out as described in the previous paper.[1] The measurements of ADP:O ratios were carried out as described by Estabrook[7] but as this is an inaccurate method for estimating these ratios in loosely coupled mitochondria, measurements under these conditions have been termed apparent ADP:O ratios. The composition of the suspending media and other experimental conditions are presented in the text with the appropriate experiments.

Sporidesmin solutions were irradiated by light from a Hanovia UVS 500 mercury lamp which had been filtered through a thin glass plate. This filter transmitted less than 3 per cent of the incident radiation at 297 nm and 10 per cent at 304 nm.[8] Other experimental conditions are described in the text with the appropriate experiments.

RESULTS

Solutions of sporidesmin, which had been irradiated with u.v. light in which the shorter wavelengths were filtered out, showed no decrease in extinction at 252 nm (Table 1). The azide–iodine titres, however,—some measure of the organic sulphur content of the toxin[9]—were decreased. These solutions had reduced ability to produce swelling compared with non-irradiated ones (Table 1) but the reductions did not obviously correlate with the decrease in azide–iodine titre. The azide–iodine titre, however, does not distinguish between sulphur in sporidesmin or in its degradation products which might themselves promote swelling.

An involvement of the disulphide group of sporidesmin in producing swelling and the increase in state 4 respiration of mitochondria is suggested from Table 2. Deriva-

TABLE 1. COMPARISON OF THE ORGANIC SULPHUR TITRE OF AQUEOUS SOLUTIONS OF SPORIDESMIN, WHICH HAVE BEEN SUBJECTED TO MERCURY ARC RADIATION FILTERED THROUGH GLASS, WITH THEIR ABILITY TO CAUSE SWELLING OF MITOCHONDRIA

Exposure (min)	Extinction (at 252 nm)	Azide–iodine titre (μmoles of organic sulphur)	Rate of swelling (max. linear decrease of extinction at 520 nm/min)
0	2·58	3·13	0·175
60	2·42	2·12	0·134
120	2·50	1·43	0·130
180	2·46	0·87	0·137

The experimental details were as described in Table 2 except that the Petri dishes were covered by a thin glass plate which removed most of the radiation with a wavelength below 310 nm. The azide–iodine titre (an estimate of the organic sulphur in sporidesmin) was performed as described in the Methods.

tives of sporidesmin in which the disulphide group was either absent (anhydrodesthio sporidesmin) or was blocked by methylation (sporidesmin D) were inactive, while those containing the disulphide group had an activity similar to that of sporidesmin. Brewer *et al.*[10] and Mortimer and Collins[11] have evidence which implicates the disulphide group of sporidesmin in the toxin's action on cells in culture.

Some characteristics of the swelling produced by sporidesmin have been compared with those of three other disulphide containing compounds (Table 3).

Compounds containing the 2,5-epidithia-3,6-dioxopiperazinedione moiety, namely sporidesmin and gliotoxin (a toxic antibiotic), produced swelling of mitochondria at similar molar concentrations and had a similar dependence on succinate, phosphate and electron transport. The swelling produced by α-lipoic acid had similar requirements but this compound was only active at higher concentrations—350 μM α-lipoic acid produced similar rates of swelling to those by 70 μM sporidesmin. The swelling produced by oxidized glutathione had quite different characteristics from the above. No requirement for succinate or phosphate could be demonstrated. In fact

TABLE 2. EFFECTIVENESS OF SPORIDESMIN DERIVATIVES AS STIMULATORS OF MITOCHONDRIAL SWELLING AND OF STATE 4 RESPIRATION

Sporidesmin derivative	Rate of swelling (max. linear decrease of extinction at 520 nm/min)	Stimulation of state 4 respiration (%)
Sporidesmin (I; R′ = OH, R″ = OH, R‴ = S)	0·19	32·0
Sporidesmin B (I; R′ = OH, R″ = H, R‴ = S)	0·14	—
Sporidesmin diacetate (I; R′ = OCH_3, R″ = OCH_3, R‴ = S)	0·15	36·4
Anhydrodesthio sporidesmin (II)	0·01	3·5
Sporidesmin D (I; R′ = OH, R″ = OH, R‴ = S-CH_3)	0·01	2·9

The reaction medium contained 80 mM-KCl, 20 mM Tris–chloride buffer pH 7·2, 4 mM-succinate (Tris salt), 1·25 mM phosphate (Tris salt), and 2 μM-rotenone. Sporidesmin (70 μM) or one of its derivatives, dissolved in ethanol, was then added (giving an ethanol concentration of 1% v/v) followed by the mitochondria (0·31 mg of protein/ml).

TABLE 3. DEPENDENCE OF THE MITOCHONDRIAL SWELLING PRODUCED BY SPORIDESMIN, GLIOTOXIN, α-LIPOIC ACID AND OXIDIZED GLUTATHIONE ON SUCCINATE, SUCCINATE PLUS PHOSPHATE, AND ANTIMYCIN

		Rate of swelling (max. linear decrease extinction 520 nm/min)			
		Complete system	Complete system plus antimycin	Complete system minus succinate	Complete system minus succinate and phosphate
Sporidesmin	70	0·130	0·010	0·046	0·012
Gliotoxin	70	0·174	0·010	0·042	0·015
α-Lipoic acid	350	0·158	0·010	0·013	0·011
Glutathione (oxidized)	1670	0·882	—	0·011	0·520*

The reaction medium was as described in Table 1. Sporidesmin, gliotoxin and α-lipoic acid were added as ethanolic solutions. Oxidized glutathione was added as an aqueous solution. In all cases the final concentration of ethanol was 0·3% (v/v).

* The total decrease of extinction at 520 nm produced by compounds in the complete system was about 0·45. For oxidized glutathione in the system minus succinate and phosphate, the total decrease in extinction was about 0·80.

in the absence of these compounds the rate of swelling was much greater and the total decrease in extinction was greater than that for compounds having a requirement for succinate or phosphate. Sporidesmin, α-lipoic acid and oxidized glutathione all decreased the respiratory control indices of mitochondria (Table 4) by increasing the rate of state 4 and decreasing the rate of state 3 respiration. All three compounds also decreased the apparent ADP:O ratios. Concentrations of sporidesmin of between 40 and 80 μM produced similar changes to those produced by 190 μM α-lipoic acid and 1130 μM oxidized glutathione.

Known inhibitors of mitochondrial swelling such as Mg^{2+} and EDTA inhibited the swelling produced by sporidesmin (Table 5). Bovine albumin was a potent

TABLE 4. EFFECTS OF SPORIDESMIN, α-LIPOIC ACID AND OXIDIZED GLUTATHIONE ON THE RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA

Addition	Concn (μM)	Decrease of state 3 respiration (%)	Increase in state 4 respiration (%)	Respiratory control index	Apparent ADP:O
Sporidesmin	0	0·0	0·0	3·1	1·5
	40	9·2	37·9	2·3	1·3
	80	17·7	34·4	2·1	1·3
	160	29·6	52·9	1·3	1·1
α-Lipoic acid	190	13·6	45·1	2·1	1·3
	380	24·4	49·1	1·7	1·2
	760	30·0	55·0	1·6	1·0
Oxidized glutathione	1130	28·5	31·4	2·0	1·4

The basic reaction medium contained 0·225 M-sucrose, 10 mM-potassium phosphate, 5 mM-$MgCl_2$, 20 mM-KCl, 10 mM-Tris-chloride buffer, pH 7·4, and 3 μM-rotenone. Mitochondria (0·75 mg of protein/ml) were pre-incubated with sporidesmin, α-lipoic acid or oxidized glutathione for 3 min in the basic medium before addition of 5 mM-succinate (state 4 respiration), followed, after 5 min. by 3 mM-ADP (state 3 respiration). Respiratory rates were measured with the oxygen electrode. The apparent ADP:O ratio was determined as described in the Methods.

TABLE 5. INHIBITION OF THE MITOCHONDRIAL SWELLING PRODUCED BY SPORIDESMIN BY CERTAIN COMPOUNDS

Addition	Concn (mM)	Inhibition of the swelling produced by sporidesmin (%)
—	—	0
EDTA	0·33	86
$MgCl_2$	4·0	92
Bovine plasma albumin	0·03	61
	0·001	35
NAD	3·0	0
Cysteine	3·0	0
Glutathione (reduced)	3·0	0
Dithiothreitol	0·1	87

The reaction medium was as described in Table 1 except for the addition of the various compounds at the concentrations stated below. The concentration of sporidesmin throughout was 70 μM and the mitochondria (0·35 mg of protein/ml) were added last.

inhibitor and, although complete inhibition of swelling was never achieved, 1 μM bovine albumin caused 35 per cent inhibition of the swelling produced by 70 μM sporidesmin. The inhibitory effect of bovine albumin on sporidesmin produced swelling was present whether or not the toxin was added to the mitochondria immediately before or after the bovine albumin. The thiol compounds cysteine and

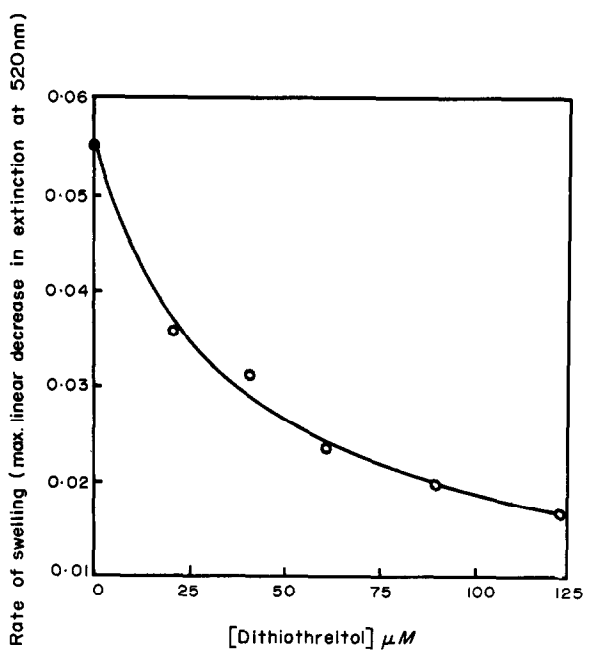

FIG. 1. The effect of dithiothreitol on the rate of mitochondrial swelling produced by sporidesmin. The basic medium was as described in Table 1 except that various concentrations of dithiothreitol were included and the concentration of sporidesmin was 70 μM. Mitochondria (0·29 mg of protein/ml) were added last. The rate of swelling was measured as the maximum linear decrease of extinction at 520 nm/min.

reduced glutathione were without any protective action. However, low concentrations of dithiothreitol, a dithiol reagent which has a lower redox potential that these monothiols, inhibited the swelling produced by sporidesmin (Fig. 1). Forty-two μM dithiothreitol caused a 50 per cent reduction in the rate of swelling produced by 70 μM sporidesmin.

Dithiothreitol altered the radiochromatographic profile of [^{35}S] sporidesmin and [^{35}S] containing compounds were produced which had lower mobilities in a benzene–ethyl acetate solvent system (Fig. 2). The product(s) were not inorganic sulphur, which, under these conditions, runs at the solvent front. Increasing amounts of dithiothreitol resulted in an increasing conversion to the new product(s). Sporidesmin as assayed by the extinction at 260 nm (Fig. 3a) is eluted from a column of Sephadex LH-20 in one essentially symetrical peak,[12] When the toxin was pre-incubated with a 10 M excess of dithiothreitol there was a decrease in the sporidesmin peak and peaks appeared which were eluted earlier. These earlier peaks were not separated from excess dithiothreitol.

Gliotoxin, another disulphide containing toxin with the epidithiapiperazinedione moiety, reacted in a similar manner (Fig. 3b) but a greater conversion to peaks of lower mobility was obtained at the same molar excess of dithiothreitol. On thin layer chromatograms, increasing concentrations of dithiothreitol resulted in a greater conversion of sporidesmin to product(s) with altered mobility (Fig. 4). There was little if any loss of sulphur as hydrogen sulphide. A number of monothiols and some other dithiols in a 10 M excess were without detectable effect on sporidesmin (Table 6). Dimercaptopropanol was only partly effective and 33 per cent of the applied counts were lost.

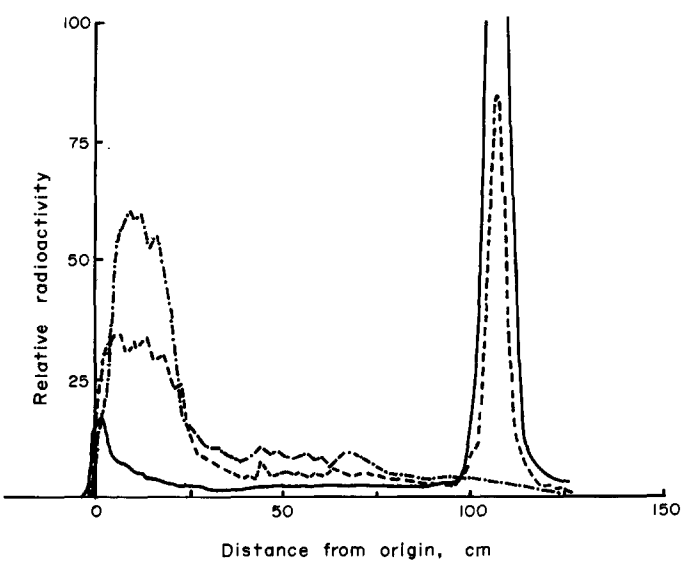

FIG. 2. The effect of dithiothreitol on the radiochromatographic profile of sporidesmin on thin layer silica gel chromatograms. Sporidesmin (0·6 μmoles containing 0·05 μCi of [^{35}S] sporidesmin) was incubated for 3 min in ethyl acetate (10–15 μl) with or without dithiothreitol. The samples were quantitatively applied to Eastman Chromagram silica gel strips (sheet 6060) and developed in benzene-ethyl acetate (1:1 by vol.). The strips were scanned for radioactivity on a Packard Radiochromatogram Scanner (Model 7201). The amounts of dithiothreitol in the incubations were (———) 0 μmoles; (- - - -) 1·8 μmoles; and (·····) 6·0 μmoles.

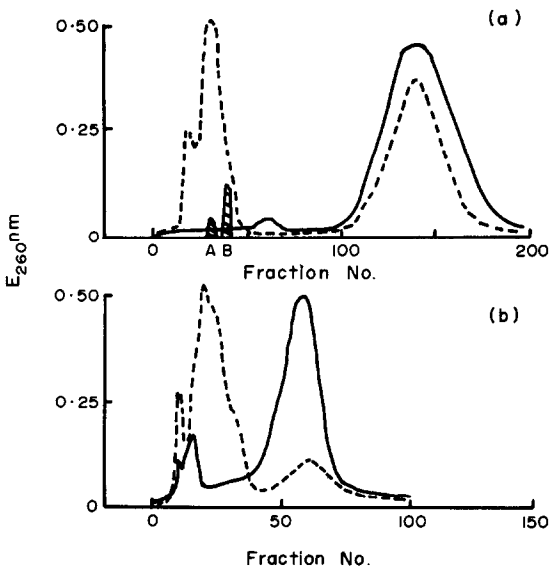

FIG. 3. The effect of dithiothreitol on the elution profiles of (a) sporidesmin and (b) gliotoxin from a column of Sephadex LH-20. (a) Sporidesmin (1·8 μmoles) was incubated for 3 min with or without dithiothreitol (18·0 μmoles). The sample (1 ml) was applied to a column (20 × 1·1 cm dia) of Sephadex LH-20 and eluted with ethanol (20%, v/v). A and B respectively indicate the elution profiles of 20 μmoles of reduced and oxidized dithiothreitol. (b) Gliotoxin (5·0 μmoles) was incubated for 3 min with or without dithiothreitol (50·0 μmoles) and then treated as in (a). (———) Profile of untreated sporidesmin or gliotoxin; (– – – –) profile of sporidesmin or gliotoxin treated with dithiothreitol.

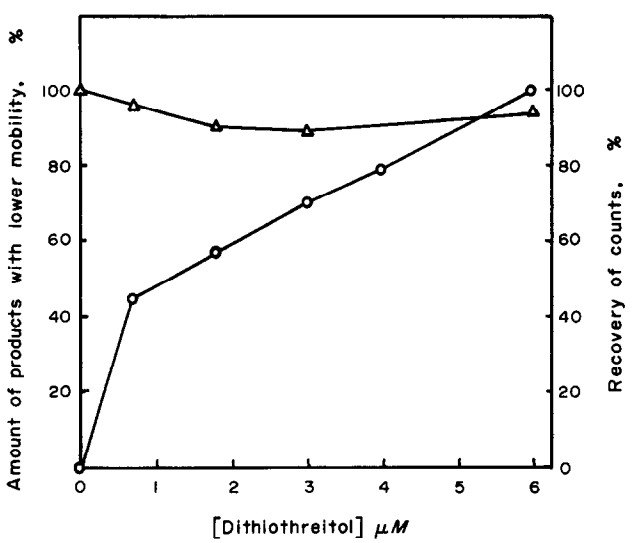

FIG. 4. The effect of the concentration of dithiothreitol on the conversion of sporidesmin to product(s) with lower mobilities. The experimental details were as described in Fig. 1. The amount of sporidesmin for each incubation was 0·6 μmoles containing 0·05 μCi: of [^{35}S] sporidesmin. The percentage recovery of counts (△) is based upon the counts in the sample without DTT. The amount of altered product(s) (○) was estimated by comparing the area of the peak of altered product(s) with the total area of the peaks of sporidesmin plus altered product(s).

TABLE 6. EFFECTIVENESS OF A NUMBER OF MONO- AND DITHIOL CONTAINING COMPOUNDS FOR CONVERTING SPORIDESMIN TO PRODUCT(S) WITH LOWER MOBILITY

Thiol compound	Recovery of counts (%)	Amount of product(s) with lower mobility (%)
—	100	0
Mercaptoethanol	91	0
Thioglycollic acid	87	0
Cysteine	100	0
2,8-Dithio-6-oxypurine	91	0
2,6-Dithiopurine	74	0
2.4-Dithiopyrimidine	93	0
2,3-Dimertcaptopropanol	66	34
1.4-Dithiothreitol	95	95

Sporidesmin (0·6 μmoles containing 0·05 μCi of [^{35}S] sporidesmin) was incubated for 3 min with the various thiol compounds (6·0 μmoles) in ethyl acetate (20–30 μl). The samples were quantitatively applied to Eastman Chromogram silica gel strips (sheet 6060) which were developed and assayed for [^{35}S] as described in Fig. 2. The amount of altered product(s) was estimated by comparing the area of the peak of altered product(s) with the total area of the peaks of sporidesmin plus altered product.

DISCUSSION

A large number of disulphide containing compounds are known to cause swelling of isolated mitochondria. They include oxidized glutathione,[3] certain disulphide containing hormones,[2] cystamine and tetramethyl cystamine.[4] These compounds are thought to react with certain thiol groups in the mitochondrial membrane and so alter its permeability. This view is supported by the fact that mercurial reagents, which combine readily with thiol groups, are also swelling agents.[13] Moreover, during the swelling produced by oxidized glutathione, Riley and Lehninger[14] detected a decrease in mitochondrial thiol groups.

The decreased ability of sporidesmin derivatives which lack the disulphide group and of sporidesmin solutions with a reduced organic sulphur titre to produce the swelling and increased state 4 respiration of mitochondria suggests that the disulphide group of sporidesmin is involved in the toxin's action on mitochondria. The inhibition by dithiothreitol of the effect of sporidesmin on swelling and respiration of mitochondria also implicates the toxin's disulphide group.

Allison[15] stressed the importance of the lipid solubility of drugs for their penetration into cells and Scott *et al.*[6] reported an increasing ability of mercurials to react with mitochondrial thiol groups with increasing lipid solubility of the reagents. Mitochondria also have a permeability barrier against positively charged disulphides.[4] Sporidesmin, being lipid soluble and uncharged and having its disulphide group in an exposed position in the molecule, could, therefore, readily penetrate the inner mitochondrial membrane and react with membrane thiol groups whether they are orientated externally or internally.

The characteristics of the swelling produced by sporidesmin, gliotoxin and α-lipoic acid were similar, but the two toxins were active in lower concentrations. The swelling produced by oxidized glutathione had quite different characteristics and this compound was only active at much higher concentrations. The greater activity of

the toxins compared with α-lipoic acid may be related to the lipid solubility of the toxins and/or to the properties of the epidithiapiperazinedione ring which results in a disulphide group of relatively low reactivity towards thiols (J. W. Ronaldson, personal communication).

The mechanism of dithiothreitol inhibition of the swelling produced by sporidesmin is of interest because this dithiol is not a general inhibitor of mitochondrial swelling. It does not, for example, inhibit the swelling produced by gramicidin (M. C. Middleton, unpublished results). Dithiothreitol could act either by protecting essential thiol groups in the membrane or by reacting with the disulphide groups of sporidesmin to form products which are inactive. The elution profile of sporidesmin on both thin layer chromatography and Sephadex LH-20 is altered by dithiothreitol and the disulphide group of the toxin can be reduced by compounds of sufficiently low redox potential (e.g. $LiAlBH_4$).[16] It is therefore suggested that dithiothreitol reacts with the disulphide group of sporidesmin to form products which do not produce swelling or increased respiration of mitochondria. The disulphide group of gliotoxin is chemically more readily reduced and the greater degree of conversion of this toxin by dithiothreitol to products of altered mobility is in agreement with the above mechanism.

As 50 per cent conversion of sporidesmin to products of lowered mobility was observed at 2·5 moles of dithiothreitol/mole of sporidesmin and half-maximum inhibition of the swelling process required only 0·6 mole dithiothreitol/mole of sporidesmin, chemical reaction of dithiothreitol with the toxin cannot be the complete explanation for inhibition of swelling. Moreover, there is no evidence that the products of altered mobility do not themselves cause swelling. Attempts to purify the products by preparative thin layer chromatography were unsuccessful because the products, once separated from excess dithiothreitol, were extremely unstable.

The inability of a number of other thiols to alter the radiochromatographic profile of sporidesmin may be taken as evidence that the disulphide group of the toxin will only react with "reactive" thiols. The thiol groups in dithiothreitol may be termed "reactive" because of their low redox potential and because the reduced product is stable.[17] This ability to react only with "reactive" thiols could explain the toxin's specificity and mechanism of action *in vitro*. Riley and Lehninger[13] have distinguished certain thiol groups in the mitochondria which are more reactive than others and these appear to be involved in maintaining the selective permeability of the mitochondrial membrane. For example, swelling of mitochondria was observed when only 12 per cent of the total thiol groups were titrated with Ag^{2+}. As the authors reported that mitochondria contain a total of 90 nmoles of thiol groups/mg of protein, then swelling can be initiated when only 11 nmoles of thiols have reacted. Sporidesmin has a detectable effect on mitochondrial swelling at 2 nmoles/mg of protein.[1] It is therefore active at sufficiently low concentrations for reaction with specific reactive mitochondrial thiol groups to be a possible explanation of the toxin's action *in vitro*.

If sporidesmin only interacts with those specific thiol groups in the mitochondrial membrane that are important in maintaining the selective permeability of the membrane, this toxin may provide a useful tool for investigating these essential thiols.

The reaction of sporidesmin with specific thiol groups in the mitochondria may reflect a general reaction of the toxin with specific thiol groups in other membranes.

It is of interest that sporidesmin has recently been demonstrated to alter membranes of the bile cannaliculi in the perfused liver 30 min after addition of the toxin to the perfusing medium.[18] It is therefore tentatively suggested that sporidesmin produces the primary biochemical changes *in vivo* by altering the permeability of the bile cannalicular membranes and that these changes lead to the biliary damage which eventually produces the symptoms of facial eczema.

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