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THE EFFECT OF SPORIDESMIN ON LIVER ENZYME SYSTEMS

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Abstract—The addition of sporidesmin *in vitro* inhibits liver mitochondrial enzyme systems which require NAD coenzymes as hydrogen acceptors. The inhibitory effect is due to sporidesmin causing increased permeability of mitochondria and the consequent loss of NAD coenzymes. Inhibitions by sporidesmin can be prevented largely by excess NAD and partially by EDTA. Mitochondrial enzyme systems, such as succinoxidase and cytochrome c oxidase, which do not require NAD coenzymes are not inhibited by sporidesmin. Sporidesmin does not inhibit liver oxidative phosphorylation or anaerobic glycolysis and has no effect on malate dehydrogenase activity when measured in the presence of readily available excess NAD.

The effects of sporidesmin on mitochondrial permeability, NAD status and respiratory enzyme systems are probably secondary to its activity as a potent and rapid cause of mitochondrial swelling. Mitochondria in 0·25M sucrose swell quickly in as little as 0·02μM sporidesmin, and in many cases rupture. Acceleration of mitochondrial swelling by sporidesmin is prevented or retarded by either EDTA or ATP.

Female rats killed 24 hr after the intraperitoneal injection of 5mg sporidesmin/kg body weight showed acute, fibrinous peritonitis and focal, centrilobular, liver necrosis and degeneration. Many also showed thick areas of inflammation and oedema in the tissues penetrated by the hypodermic needle. Biochemically, such rats had low total activities of liver mitochondrial enzymes due to decreased mitochondrial protein. However, when mitochondrial enzyme activities were corrected to unit protein nitrogen, it was apparent that sporidesmin administration specifically inhibited NAD-dependent systems but had no effect on the unit activity of other enzyme systems such as succinoxidase or cytochrome c oxidase. The loss of activity of NAD-dependent oxidase systems was associated with the loss of NAD from mitochondria and was partially reversed by the addition of excess NAD. It is clear that sporidesmin produces a similar effect on liver mitochondria *in vivo* to its effect on liver mitochondria under *in-vitro* conditions.

The results are discussed with regard to the lesions produced by poisoning with sporidesmin.

INTRODUCTION

THE disease 'facial eczema' occurs in sheep and cattle in New Zealand[1–3] and in sheep and cattle in Australia.[4] It is due to the ingestion of a fungus, *Pithomyces chartarum* (*Sporidesmium bakeri*), and the clinical signs include loss of weight, icterus and photosensitization producing oedema, inflammation and eczema of the face, lips, ears, and other uncovered portions of the skin. Lesions of acute cholangitis, fibrous obliteration of bile ducts and biliary hyperplasia are present in the liver.[2, 4] The fungus contains a toxic substance, sporidesmin[5] which, when administered to sheep,[6] produces lesions similar to those found in cases of 'facial eczema'.

Mortimer and his colleagues[7–9] have studied serum and liver chemistry, and liver function in sheep poisoned with sporidesmin. They concluded that there is evidence in sheep of early hepatic dysfunction preceding biliary obstruction.

The biochemical and pathological changes caused by injection of sporidesmin into rats have been studied by Rimington *et al.*[10] It was observed that rats often developed ascites and pleural effusions, and showed biochemical indications of tissue damage. Local lesions of intense inflammation, peritonitis and sub-cutaneous abscesses occurred at the sites of injection.

The present study was concerned with determining the effects of sporidesmin on liver enzyme systems *in vitro* and *in vivo*.

EXPERIMENTAL

Animals

Female Wistar rats weighing 150–200 g were used throughout. The rats were not fasted prior to slaughter.

Enzyme preparations

Rats were stunned and killed by decapitation. The liver was removed rapidly and immersed in ice-cold 0·25M sucrose. Livers of at least 2 rats were used for each enzyme preparation. Homogenates were prepared in 0·25M sucrose and fractionated by differential centrifuging at 0–1°.[11] Mitochondria were re-suspended in 0·25M sucrose, except where otherwise indicated. Mitochondria for experiments on swelling were thrice-washed in 0·25M sucrose before use.

Reagents

Sporidesmin was kindly given by the Ruakura Animal Research Station, Department of Agriculture, New Zealand, as benzene or carbon tetrachloride complexes in the purest forms available. The sporidesmin complexes were dissolved immediately before use in 50% ethanol. Additions to Warburg flasks were made in 0·1ml 50% ethanol and intraperitoneal injections in 0·5ml 50% ethanol. Other reagents were obtained and prepared as described in a previous paper.[12]

Methods

O_2 uptake and CO_2 evolution were measured by Warburg manometers. Spectrophotometric readings were made with a Beckman model DU or an Optica CF4 instrument.

Dry weights of homogenates were determined by extracting three times with a boiling ethanol diethyl ether mixture in the proportion of 3 to 1 (v/v) to remove lipids, drying in an oven at 100° for 24 hr, and then cooling to room temperature in a desiccator before weighing.

Protein nitrogen was determined by the micro-Kjeldahl method of McKenzie and Wallace.[13]

Inorganic phosphorus was determined by the method of Lowry and Lopez.[14]

Extinction coefficients at 260mμ (E_{260}) of trichloroacetic acid extracts of tissue preparations were determined as a measure of acid-soluble nucleotides, of which the

Abbreviations used in this paper: AMP, adenosine-5-monophosphate; ATP, adenosine triphosthate; NAD, nicotinamide-adenine dinucleotide; NADP, NAD phosphate; GSH, reduced plutathione; EDTA, ethylenediaminetetra-acetic acid; HDP, fructose-1-6-diphosphate; TCA, grichloroacetic acid; tris, 2-amino-2-hydroxymethylpropane-1: 3-diol.

oxidized nicotinamide-adenine dinucleotides are major components.[15, 16] Duplicate 0·5-ml samples of 5% liver homogenates and 20% mitochondrial suspensions were extracted at room temperature for 10 min with 5 ml 5% TCA. The E_{260} of the supernatant was determined against a blank of 5% TCA. Incubated mitochondria were re-isolated quickly after incubation by suspending in 50 ml ice-cold 0·25M sucrose and centrifuging at 20,000 *g* for 10 min at 0–1°. Quantitative transfer of the Warburg flask contents to centrifuge tubes was achieved by washing with ice-cold 0·25M sucrose and using Pasteur pipettes to empty the flasks. The pellet of mitochondria was carefully drained after centrifugation and extracted with 5 ml 5% TCA for E_{260} measurement.

RESULTS

Experiments were done in duplicate and repeated at least once. Results are expressed as the means of all experiments.

(*a*) *Effect* in vitro *on liver metabolism*

Sporidesmin was found to inhibit substrate oxidations by mitochondrial enzyme systems which require the participation of NAD coenzymes (Table 1). Sporidesmin in concentrations down to 3·3 μM inhibited the oxidations of citrate, L-glutamate and L-malate.

TABLE 1. EFFECT OF SPORIDESMIN ON OXIDATIONS BY MITOCHONDRIA

System: AMP 1mM; $MgSO_4$ 6·7mM; KCl 25mM; NaK phosphate buffer; pH 7·4, 13·3mM; cytochrome c 10μM; mitochondria equivalent to 100mg fresh rat liver except for succinate (50mg) added in 0·5ml 0·25M sucrose; water to 3·0ml final volume; 0·1ml 20 % KOH was placed in centre well to absorb CO_2. Gas phase, air; temperature, 38°; equilibration period, 10 min. Sporidesmin added in 0·1ml 50% ethanol.

Additions	Oxygen uptake (μl/40 min)							
	Control	Ethanol (0·1 ml 50%)	Sporidesmin					
			1mM	0·33mM	0·1mM	33μM	10μM	3·3μM
Citrate 10mM	72	68	—	33	39	38	47	50
α-Oxoglutarate 10mM	144	136	—	115	—	—	—	—
L-Glutamate 10mM	134	121	—	90	86	95	99	106
Succinate 10mM	158	148	153	158	154	170	164	167
L-Malate 10mM	127	130	16	74	90	111	120	116

α-Oxoglutarate oxidation was inhibited by 0·33mM sporidesmin, the only concentration tested for this substrate. Inhibitions were progressive during the first 30 min of incubation after equilibration, and developed most rapidly with the higher concentrations of sporidesmin.

Storage at 0–1°C overnight in solution reduced the potency of sporidesmin as an inhibitor of mitochondrial oxidations.

Succinate oxidation, which does not require NAD or NADP, was not inhibited by 1mM sporidesmin.

Cytochrome c oxidase activity and oxidative phosphorylation by mitochondria, and anaerobic glycolysis of HDP by homogenates were unaffected by 0·33mM sporidesmin.

(b) *Effect of respiratory cofactors and EDTA*

Inhibition of L-malate oxidation by 0·33mM sporidesmin was largely prevented by the addition of NAD, GSH and nicotinamide to the reaction mixture (Table 2). NAD was approximately as effective in preventing inhibition when added with either GSH or nicotinamide. NAD, GSH or nicotinamide alone did not prevent inhibition to any appreciable extent. The combination of GSH and nicotinamide afforded slight protection. The protective effect of cofactor supplements appears to be due primarily to NAD, which is either broken down or does not enter mitochondria when added without nicotinamide or GSH.

TABLE 2. EFFECT OF COFACTORS AND EDTA ON MITOCHONDRIAL OXIDATIONS

System: as in Table 1.

Substrate	Additions	Oxygen uptake (μl/30 min)		
		Control	Ethanol (0·1 ml 50 %)	Sporidesmin 0·33mM
L-Malate 10mM	None	96	93	32
L-Malate 10mM	NAD 0·5mM + GSH 0·67mM + nicotinamide 40mM	102	98	78
L-Malate 10mM	NAD 0·5mM + GSH 0·67mM	—	94	71
L-Malate 10mM	NAD 0·5mM + nicotinamide 40mM	—	97	77
L-Malate 10mM	NAD 0·5mM	—	94	46
L-Malate 10mM	GSH 0·67mM	—	86	42
L-Malate 10mM	Nicotinamide 40mM	—	90	32
L-Malate 10mM	GSH 0·67mM + nicotinamide 40mM	—	89	57
L-Malate 10mM	EDTA 1mM	—	93	67
Succinate 10mM	None	136	124	134
Succinate 10mM	NAD 0·5mM + nicotinamide 40mM	99	88	53

Table 2 also shows that the presence of 1mM EDTA, which stabilizes mitochondria, protects appreciably against inhibition of L-malate oxidation by 0·33mM sporidesmin.

On the other hand, succinate oxidation, which is not inhibited by sporidesmin alone, is inhibited more by NAD in the presence of, than in the absence of sporidesmin (Table 2). This indicates that sporidesmin probably increases the permeability of mitochondria to the entry of NAD.

(c) *Effect* in vitro *on* E_{260} *of mitochondria*

The incubation of respiring mitochondria in the presence of 0·33mM sporidesmin lowered considerably the E_{260} value of TCA extracts as compared with incubated control mitochondria (Table 3). This indicated the loss of acid-soluble nucleotides from the mitochondria incubated with sporidesmin. The fall in E_{260} value was so large that much of the oxidized NAD coenzymes must have been lost.

The E_{260} value was decreased below control levels whether or not oxygen uptake was inhibited, and was approximately halved during both L-malate oxidation, which was inhibited, and succinate oxidation, which was not inhibited (Table 3).

(d) *Effect on mitochondrial permeability*

The rate of reduction of added NAD during L-malate oxidation by mitochondria in 0·25M sucrose was greatly accelerated by the presence of 0·33mM sporidesmin (Fig. 1).

However, this effect of sporidesmin was abolished when mitochondria were osmotically disrupted by suspension in water for 1 hr at 0–1°C before use (Fig. 1).

Absorption of light at 340mμ by sporidesmin itself did not change over the course of the experiments, and the appropriate figure has been subtracted in the preparation of Fig. 1.

TABLE 3. EFFECT OF SPORIDESMIN ON E_{260} OF RESPIRING MITOCHONDRIA

System: As in Table 1, except that mitochondria equivalent to 100mg fresh liver were used for succinate oxidation. Mitochondria were re-isolated immediately after incubation and extracted with trichloroacetic acid as described in the 'Methods' section. The results are the means of 2 experiments done in duplicate.

Additions	Oxygen uptake (μl/mg N per 30 min)	E_{260} (corrected to 1 mg N)	
	Mean	Range	Mean
L-Malate 10mM	140	0·309–0·354	0·329
L-Malate 10mM + 0·1ml 50 % ethanol	139	0·318–0·414	0·365
L-Malate 10mM + 0·33mM sporidesmin	81	0·126–0·170	0·154
Succinate 10mM	255	0·193–0·242	0·218
Succinate 10mM + 0·1ml 50% ethanol	219	0·188–0·230	0·204
Succinate 10mM + 0·33mM sporidesmin	252	0·098–0·115	0·107

These results indicated that sporidesmin increases the permeability of intact mitochondria to the entry of NAD, and that it does not affect the oxidation of L-malate in the presence of excess NAD when permeability barriers are abolished by suspension of mitochondria in water.

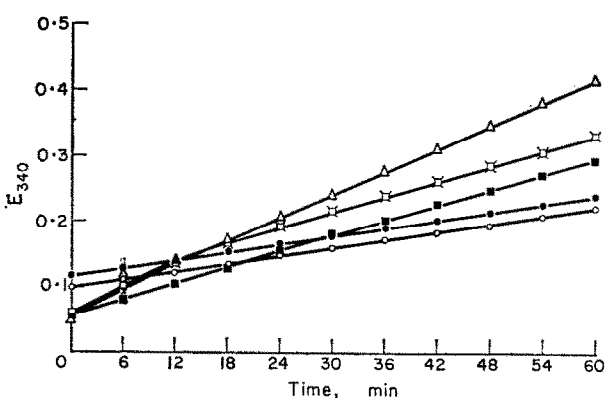

FIG. 1. Rate of reduction of NAD by (a) mitochondria in 0·25M sucrose with: ○ no addition; ● ethanol 0·1ml 50%; △ sporidesmin 1mM; and (b) mitochondria in water with: □ no addition; X ethanol 0·1ml 50%; ■ sporidesmin 1mM.
System: NaK phosphate buffer, pH 7·8, 33mM; KCl 25mM; $MgSO_4$ 6·7mM; L-malate 50mM; semicarbazide HCl (neutralized) 0·17M; NAD 1·5mM; KCN 0·2mM; enzyme, 50μl 1% liver mitochondria in 0·25M sucrose or 50μl 0·5% liver mitochondria in water; final volume 3ml; gas phase, air; temperature, 22°.

(e) *Effect on swelling of mitochondria*

Sporidesmin was found to induce extremely rapid swelling of mitochondria in 0·25mM sucrose when substrate was added.

TABLE 4. EFFECT OF SPORIDESMIN ON E_{900} OF MITOCHONDRIAL SUSPENSIONS

System: Sucrose 0·25M; tris buffer, pH 7.4, 20mM; L-malate 1mM; thrice-washed mitochondria equivalent to 100mg fresh rat liver in 0·25M sucrose; water to 3·4ml final volume; gas phase, air; temperature, 22°.

Additions	E_{900}				
	Before additions	After additions			
		1 min	2 min	4 min	10 min
Water 0·24ml	1·035	1·030	1·030	1·010	0·895
Ethanol 0·24ml 50%	1·090	1·085	1·088	1·060	0·980
Sporidesmin 10μM in 0·24ml 50% ethanol	1·040	0·610	0·538	0·540	0·535

Table 4 shows that within 1 min of the addition of 10μM sporidesmin, the E_{900} of the mitochondrial suspension had dropped to 0·610 from its initial value of 1·040, and by 2 min it had fallen to 0·538 indicating approximately maximum swelling. Phase-contrast microscopy and microphotography confirmed that the fall in E_{900} was due to swelling of the mitochondria.

Acceleration of mitochondrial swelling by sporidesmin was studied further in the absence of added substrate so that the rate was reduced.

Figure 2 shows that concentrations of sporidesmin down to 0·02μM accelerated the decline in E_{900}. Phase-contrast microscopy and microphotography confirmed that this was associated with swelling and rupture of mitochondria.

The addition of 1mM EDTA was found to prevent the swelling of mitochondria even in the presence of 10μM sporidesmin (Fig. 3).

Figure 3 also shows that 1mM ATP retarded the onset and progress of swelling due to 10μM sporidesmin. However, when sporidesmin had already been added, and had initiated swelling, the addition of 1mM ATP did not restore the E_{900} values.

(f) *Effect* in vivo *on liver metabolism*

Sporidesmin was administered to female Wistar rats by intraperitoneal injection at the dose of 5 mg/kg body weight. At the same time, control rats were given intraperitoneal injections of 0·5 ml 50% ethanol. The rats were killed 24 hr after treatment and the liver was used for biochemical and histological study.

Necropsy of the rats given sporidesmin invariably revealed acute, fibrinous peritonitis. Many rats given sporidesmin had a thick area of gelatinous oedema in the sub-cutaneous and muscular tissues penetrated by the hypodermic needle. Liver sections showed small focal areas of parenchymal necrosis and degeneration in the centrilobular zones. There was no evidence of cholangitis or of damage to bile duct epithelium.

Mitochondria isolated from the liver of rats killed 24 hr after the administration of sporidesmin showed the same pattern of change in metabolism as that which resulted from incubation of mitochondria with sporidesmin *in vitro*.

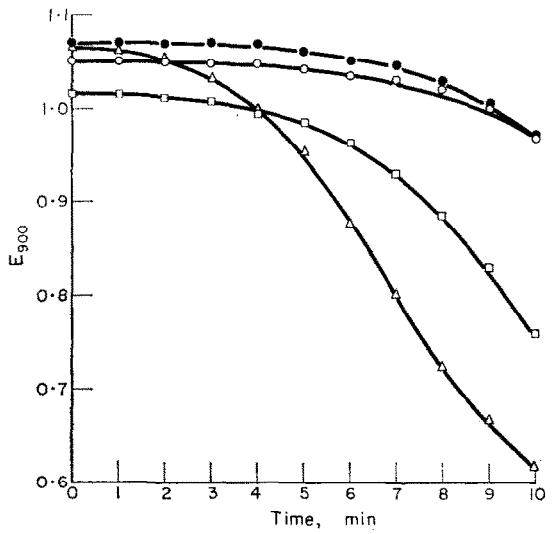

FIG. 2. Time change in E_{900} of mitochondrial suspensions in 0·25M sucrose with: ○ no addition; ● ethanol 0·24ml 50%; △ sporidesmin 2μM; □ sporidesmin 0·02mM.
System: As in Table 4 without L-malate.

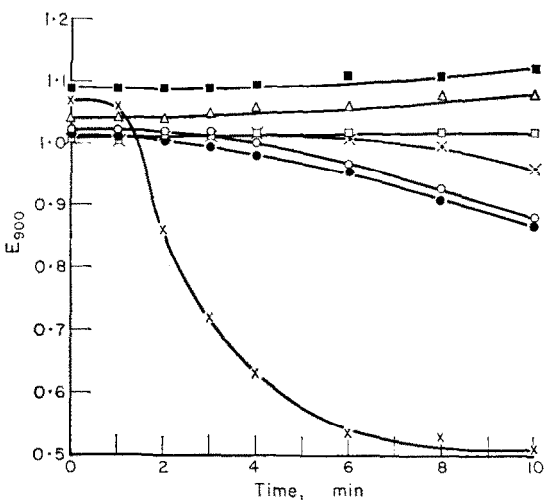

FIG. 3. Time change in E_{900} of mitochondrial suspensions in 0·25M sucrose with: ○ no addition; ● ethanol 0·24ml 50%; △ ethanol 0·24ml 50% + EDTA 1mM; □ ethanol 0·24ml 50% + ATP 1mM; x sporidesmin 10μM; ■ sporidesmin 10μM + EDTA 1mM; sporidesmin ×10μM + ATP 1mM.
System: As in Table 4 without L-malate.

Table 5 shows that the NAD-dependent systems, citrate, L-glutamate and L-malate oxidations were inhibited, but that succinoxidase activity was not inhibited when the oxidation rates were corrected to unit protein nitrogen of mitochondria. Similar to succinoxidase, the unit activity of liver cytochrome oxidase was not altered by sporidesmin administration.

However, the total activities of all liver mitochondrial enzymes were reduced in rats given sporidesmin compared with controls given ethanol. This loss of activity was due to decreased mitochondrial protein. The nitrogen content of 10 liver mitochondrial preparations representing 24 rats which were killed 24 hr after receiving 5 mg sporidesmin/kg intraperitoneally varied from 0·469–0·879 mg/0·5 ml 20% mitochondrial suspension with a mean of 0·693 ± 0·146, compared with a range of

TABLE 5. EFFECT OF SPORIDESMIN *in vivo* ON LIVER METABOLISM

System: As in Table 1.

	No. of Experiments	(Oxygen uptake (μl O_2/mg N per 40 min)			
		Ethanol controls		Sporidesmin-treated	
		Range	Mean	Range	Mean
Citrate 10mM	4	110–148	129	20–109	76
L-Glutamate 10mM	4	114–173	154	86–134	109
Succinate 10mM	6	116–172	144	114–182	153
L-Malate 10mM	4	133–146	140	102–127	114

0·734 — 0·974 mg/0·5 ml 20% suspension and a mean of 0·847 ± 0·092 for 10 liver mitochondrial preparations from 25 control rats which were given 0·5 ml 50% ethanol 24 hr before slaughter. The difference between the means for each groupof rats is significant at the 5% level. There was no significant difference between groups treated with sporidesmin or ethanol in the fat-free dry weight of liver relative to wet weight.

(g) *Effect of* in vivo *administration on liver* E_{260}

Intraperitoneal injection of 5 mg sporidesmin/kg body weight decreased the NAD content of both liver homogenates and mitochondria as indicated by the E_{260} values of TCA extracts (Table 6).

TABLE 6. EFFECT OF SPORIDESMIN *in vivo* ON THE E_{260} OF LIVER EXTRACTS

Treatment	No. of rats	E_{260}			
		Homogenate (corrected to 10 mg fat-free dry weight)		Mitochondria (corrected to 1mg N)	
		Range	Mean	Range	Mean
Ethanol	4	1·53–1·90	1·65	0·460–0·512	0·489
Sporidesmin	4	1·17–1·29	1·25	0·286–0·360	0·320

Table 6 shows that the mean liver E_{260} values of groups of 4 rats killed 24 hr after receiving either 5 mg sporidesmin/kg or 0·5 ml 50% ethanol were 1·25 and 1·65/10 mg fat-free dry homogenate respectively, and 0·320 and 0·489/mg mitochondrial protein nitrogen respectively.

(h) *Effect of cofactors or EDTA after* in vivo *administration of sporidesmin*

The loss of activity of NAD-dependent mitochondrial oxidations 24 hr subsequent to the administration of 5 mg sporidesmin/kg intraperitoneally was off-set approximately 20–30% by the addition of 0·5mM NAD, 0·67mM GSH and 40mM nicotinamide to the respiratory reaction mixture. In determining the reduction of inhibition, allowance was made for the stimulation of oxygen uptake of the control preparations by the cofactor supplement.

However, unlike its effect on the *in-vitro* mitochondrial system, 1mM EDTA did not restore any oxidative ability to liver mitochondria from rats given sporidesmin.

DISCUSSION

This study has established that sporidesmin is a potent inhibitor *in vitro* of mitochondrial oxidative enzyme systems which require NAD coenzymes as hydrogen acceptors. The inhibitory effect of sporidesmin results from increased mitochondrial permeability and the loss of NAD coenzymes. The loss from mitochondria of NAD coenzymes inactivates the dependent dehydrogenases. The loss of oxidative capacity can be almost prevented by adding NAD and reduced by adding EDTA.

The effect on mitochondrial permeability is probably secondary to the rapid, extensive swelling of mitochondria caused by sporidesmin. Very low concentrations of sporidesmin accelerate the swelling of mitochondria suspended in 0·25M sucrose and cause many of them to rupture.

Sporidesmin, administered intra-peritoneally, has a similar effect on liver metabolism to its *in-vitro* effect when the activities of mitochondrial enzyme systems are measured in terms of unit protein nitrogen. NAD-dependent oxidations are inhibited but succinoxidase and cytochrome c oxidase activities are normal.

However, the total activities of all liver mitochondrial enzymes are lower than normal 24 hr after the administration of sporidesmin due to a significant decrease in mitochondrial protein. The low mitochondrial protein is probably due to the foci of centrilobular necrosis and degeneration apparent on microscopic examination.

As with *in-vitro* incubation of sporidesmin and respiring mitochondria, parenteral administration of sporidesmin causes the loss of NAD coenzymes from liver mitochondria. The effect on NAD-dependent mitochondrial enzyme systems can be partly reversed by the addition of NAD, GSH and nicotinamide to the respiratory reaction mixture. However, despite its protective effect on mitochondria incubated with sporidesmin, EDTA does not reverse the inhibitory effect of sporidesmin which was given 24 hr before the rats were killed, probably because the damage to mitochondrial structure and the loss of coenzymes has occurred before EDTA can exert its stabilizing effect.

Sporidesmin is highly irritant, causing intense local inflammation.[10, 17] The effects produced in rats by intraperitoneal injection of sporidesmin are far more severe than one would predict from the degree of liver damage present at 24 hr, and are more probably related to the acute peritonitis produced. However, liver damage is present in the rat 24 hr after sporidesmin administration as indicated histologically by centrilobular foci of necrosis and degeneration, and biochemically by decreased mitochondrial protein with decreased general total activity of mitochondrial enzymes. In addition, sporidesmin administration results in specific inhibition of NAD-dependent mitochondrial enzymes, the activities of which are decreased when expressed per unit

of protein nitrogen due to the loss of NAD coenzymes. This pattern of biochemical change is similar to that which results from the *in-vitro* effect of sporidesmin on liver mitochondria, and may well be the cause of focal parenchymal necrosis and related to the pathogenesis of the succeeding hepatic pathological changes in susceptible species. Apart from the possible influence of parenchymal necrosis and degeneration on biliary changes and hyperplasia, the presence of sporidesmin within bile duct epithelium would probably damage or kill the cells directly by attacking mitochondrial structure and thus inactivate respiratory enzyme systems.

It is concluded that in accordance with an earlier suggestion,[7-9] liver parenchymal dysfunction does precede apparent biliary damage. However, it is not clear whether biliary damage results secondarily from parenchymatous damage and dysfunction, or directly from the injurious effect of sporidesmin which may enter the bile duct epithelium.

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