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EFFECTS OF THE MYCOTOXIN SPORIDESMIN ON SWELLING AND RESPIRATION OF LIVER MITOCHONDRIA

MARSHALL C. MIDDLETON*

Ruakura Animal Research Station, Hamilton, New Zealand

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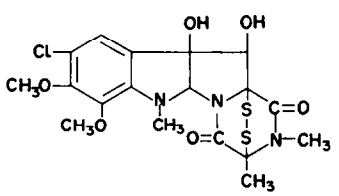
Abstract – In the presence of both oxidizable substrate and phosphate, low concentrations of sporidesmin produced rapid swelling of mitochondria isolated from guinea-pig liver. There is no requirement for alkali metal ions. Uncoupling agents or respiratory inhibitors inhibited the swelling. Mitochondria that had been swollen in the presence of sporidesmin could be contracted by addition of ATP, magnesium ions and bovine serum albumin. The rate of swelling was dependent on the concentration of both sporidesmin and mitochondrial protein and half-maximum effect was observed at 140 nmoles of sporidesmin per mg of protein. A lag period occurred before the maximal effect of sporidesmin and this period was also dependent on the concentration of both sporidesmin and mitochondrial protein. Sporidesmin decreased the respiratory control index of mitochondria by increasing the rate of state 4 respiration and decreasing that of state 3. As sporidesmin had no effect on the respiration of mitochondrial preparations that had either been treated with Triton X-100 or been subject to sonication, the toxin does not directly inhibit the respiratory chain. It is suggested that sporidesmin produces the effects described above by altering the permeability of the mitochondrial membrane.

UNDER certain weather conditions, the fungus *Pithomyces chartarum* heavily contaminates the pastures in New Zealand. Ingestion of fungal spores by cattle and sheep can give rise to the disease facial eczema. The toxic agent, sporidesmin, is a metabolite of the fungus. The toxin has been isolated and its structure (I) determined.[1,2] In



(I)

sheep, at least, liver dysfunction appears to be the primary cause of the disease[3,4] and Towers,[5] has demonstrated high levels of radioactivity in the livers of guinea pigs and rats a few hours after intraperitoneal injection of [^{35}S] sporidesmin. From the morphological and chemico-pathological evidence, Mortimer,[4] and Slater *et al.*[6] suggested that sporidesmin may act by altering capillary permeability.

Gallagher,[7] reported that sporidesmin caused rapid swelling of mitochondria isolated from rat liver and this swelling was not dependent on oxidizable substrate.

* Present address: Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Macclesfield, Cheshire.

Slater *et al.*[6] and Wright and Forrester,[8] however, did not observe any marked effect of sporidesmin on the swelling of mitochondria from either guinea-pig or rat liver. Gallagher,[7] and Wright and Forrester,[8] reported that sporidesmin inhibited the respiration of isolated mitochondria oxidizing substrates requiring NAD but slightly stimulated the oxidation of succinate. Slater *et al.*[6] however, observed a stimulation of respiration from NAD requiring substrates. All the above results were obtained by Warburg manometry which necessitates lengthy incubation times. More recently, D. E. Wright (personal communication) using the oxygen electrode, which allows shorter incubations, observed a stimulation of succinate oxidation from guinea pig liver mitochondria in the presence of sporidesmin.

The results reported here to some extent resolve the earlier apparently conflicting results and indicate that under the appropriate conditions (viz. a supply of oxidizable substrate and phosphate), sporidesmin produces a rapid and reversible swelling of mitochondria from guinea-pig liver and decreases the respiratory control index of these mitochondria. Mitochondria isolated from sheep, rabbit and rat liver are similarly affected.

MATERIALS AND METHODS

Materials. Sporidesmin was obtained from Dr. E. P. White (Ruakura Animal Research Station, Hamilton, New Zealand) as the crystalline sporidesmin–benzene (1:1) complex. It was dissolved in absolute ethanol immediately before use to give a 21 mM solution and addition of 0–30 μl of this stock solution gave the required final concentrations. Control samples contained benzene and ethanol in the appropriate concentrations. The quoted concentrations of sporidesmin are based on an unsolvated molecular weight of 474. Antimycin A, rotenone, ADP (disodium salt), and most other organic chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sucrose and inorganic chemicals were of AnalaR grade.

Methods. Sporidesmin solutions were irradiated by light from a Hanovia UVS 500 mercury lamp. Guinea-pig liver mitochondria were isolated essentially by the method of Mustafa *et al.*[9] in a medium consisting of 0·25 M-sucrose, 25 mM-mannitol and 0·1 mM-EDTA, pH 7·5. The mitochondria were washed three times and the pellet stored at 0° for not more than 2 hr before use. The mitochondria were then resuspended in the preparative buffer except when varying amounts of mitochondrial protein were added. In this case, to avoid adding increasing amounts of EDTA (which inhibits mitochondrial swelling), the mitochondria were diluted in 0·25 M sucrose containing 25 mM-mannitol, pH 7·5.

Swelling of mitochondria. The suspending media, which have previously been utilized by Chappel and Crofts,[10,11] the composition of the media and other experimental conditions are presented in the text with the appropriate experiments. Swelling and contraction of mitochondria were followed by the change in extinction at 520 nm.

Oxygen consumption. Oxygen uptake was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Ohio, U.S.A.). The suspending medium was that utilized by Estabrook,[12] and is described in the text with the appropriate experiment. Determination of rates of state 4 and state 3 respiration and of the respiratory control indices were carried out as described by Estabrook.[12] Adenosine triphospha-

tase activity was determined in a medium consisting of 3 mM-ATP (Tris salt), 3 mM-$MgCl_2$ and 30 mM-Tris chloride buffer, pH 7·0. The inorganic phosphate released was estimated essentially by the colorimetric procedure of Neufeld and Levy,[13] involving an isobutanol extraction of the phosphomolybdate complex.

All results are for representative preparations of mitochondria.

RESULTS

Effect on swelling of mitochondria. In the presence of succinate (4 mM) and phosphate (1·25 mM), sporidesmin (70 μM) produced a rapid decrease in the extinction of mitochondrial suspensions prepared from livers of guinea-pig, rat, rabbit and

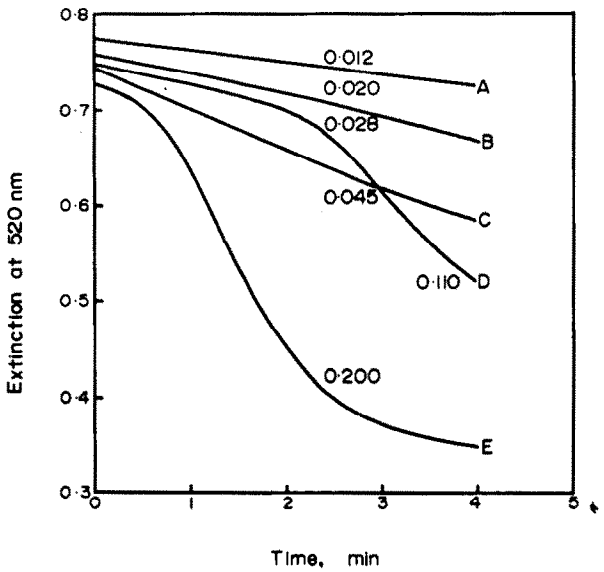


FIG. 1. Dependence of the mitochondrial swelling produced by sporidesmin on succinate and on phosphate. The complete medium (3·0 ml) contained 80 mM-KCl, 20 mM-Tris–chloride buffer pH 7·2, 4 mM-succinate (Tris salt), 1·25 mM-phosphate (Tris salt), 2 μM-rotenone, 70 μM-sporidesmin and mitochondria (0·30–0·33 mg of protein/ml). The mitochondria were added last. The values given represent maximum rates of swelling measured as the maximum linear decrease of extinction at 520 nm/min. (A) illustrates the results obtained from the complete medium minus sporidesmin and succinate or phosphate; (B) from complete medium minus sporidesmin; (C) from complete medium minus succinate; (D) from complete medium minus phosphate and (E) from complete medium.

sheep. Results from a representative preparation of mitochondria from guinea-pig liver are shown in Fig. 1. Low rates of swelling occurred in the absence of added succinate. In the absence of added phosphate, low rates of swelling occurred initially followed, after about 2 min, by an increased rate which was typically about 50 per cent of that observed in the presence of added phosphate. The added phosphate did not itself cause swelling throughout the experiment. Mitochondria that had been depleted of endogenous phosphate by incubation with glucose, hexokinase, ADP and succinate showed similar behaviour towards added phosphate. Formaldehyde at a concentration of 4 μmoles/mg of mitochondrial protein caused a 60 per cent reduction in the rate of swelling produced by 0·35 μmoles of sporidesmin/mg of protein (equivalent to 70 μM sporidesmin in the assay medium).

The characteristics of swelling were unaltered when 0·25 M-sucrose was substituted for the 80 mM-KCl of the suspending medium or when glutamate (4 mM) plus malate (4 mM) replaced succinate as substrate. However, the rates of swelling from glutamate plus malate were only 15 per cent of those observed from succinate.

Solutions of sporidesmin that had been irradiated with ultra-violet light from a mercury lamp which is known to degrade its aromatic nucleus,[14] had a decreased extinction at 252 nm and a reduced swelling ability compared with non-irradiated solutions (Table 1). This evidence taken together with the observations that no metal

TABLE 1. COMPARISON OF THE EXTINCTION AT 252 nm OF AQUEOUS SOLUTIONS OF SPORIDESMIN, WHICH HAVE BEEN SUBJECTED TO MERCURY ARC RADIATION, WITH THE ABILITY OF THESE SOLUTIONS TO CAUSE SWELLING OF MITOCHONDRIA

Exposure (min)	Extinction (252 nm)	Rate of swelling (max linear decrease of extinction at 520 nm/min)
0	2·22	0·232
15	2·37	0·095
30	2·11	0·125
60	1·83	0·108
120	1·08	0·102
200	0·36	0·102

The reaction medium consisted of 80 mM-KCl, 20 mM-Tris–chloride buffer pH 7·2, 2% (v/v) ethanol and 210 μM-sporidesmin. Ten ml samples of this medium, in Petri dishes partly immersed in a water bath at 20°, were exposed to the direct radiation from a mercury arc lamp at a distance of 33 cm. After the stated exposures, the volumes were made up to 10 ml with 80 mM-KCl, 20 mM-Tris–chloride buffer pH 7·2 and the extinction at 252 nm determined. One ml aliquots of these solutions were added to 2 ml of a reaction medium which contained 54 mM KCl, 13 mM-Tris–chloride buffer pH 7·2, 4 mM-succinate (Tris salt), 125 mM phosphate (Tris salt), 2 μM-rotenone and mitochondria (0·33 mg of protein/ml) and the rates of swelling were determined.

ions could be detected in sporidesmin, within the limits of atomic absorption spectrophotometry, and that recrystallized preparations of sporidesmin were equally as potent swelling agents, indicates that it is sporidesmin and not a metal ion contaminant which produces the effects on mitochondria.

In addition to the requirement for oxidizable substrate and phosphate, the swelling produced by sporidesmin was also dependent on electron transport and coupled respiration. Thus a respiratory inhibitor (antimycin) or an uncoupling agent (dinitrophenol) prevented the swelling process (Fig. 2). Even after swelling was initiated, it was inhibited by the addition of antimycin. The antimycin inhibition was overcome by adding ascorbate plus tetramethylphenylene diamine.

Once the sporidesmin produced swelling had occurred, it could be reversed by adding ATP together with $MgCl_2$ (Fig. 3). The inclusion of bovine serum albumin as well resulted in a more rapid and almost total reversal of the swelling process. Antimycin (2 μM) only partially inhibited the reversal.

The swelling produced by sporidesmin was not dependent on alkali metal ions and the rate of swelling was essentially unaltered by them (Table 2). Both ammonium and

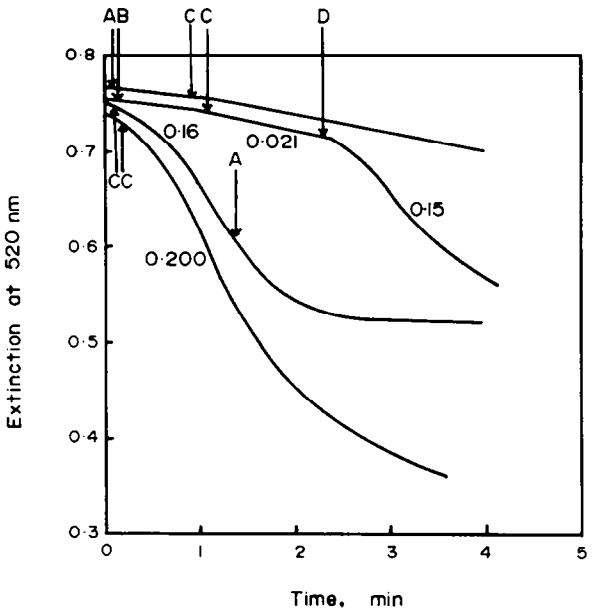


FIG. 2. Effects of dinitrophenol, antimycin and ascorbate plus tetramethyl-*p*-phenylenediamine on swelling in the presence of sporidesmin. Basic conditions, except for the omission of sporidesmin, were as given in Fig. 1. Additions were made as follows: (A) dinitrophenol (40 μM); (B) antimycin (2 μM); (C) sporidesmin (70 μM); (D) ascorbate (2 mM) together with tetramethyl-*p*-phenylenediamine (0·2 mM). The values given are the maximum linear decrease of extinction at 520 nm/min.

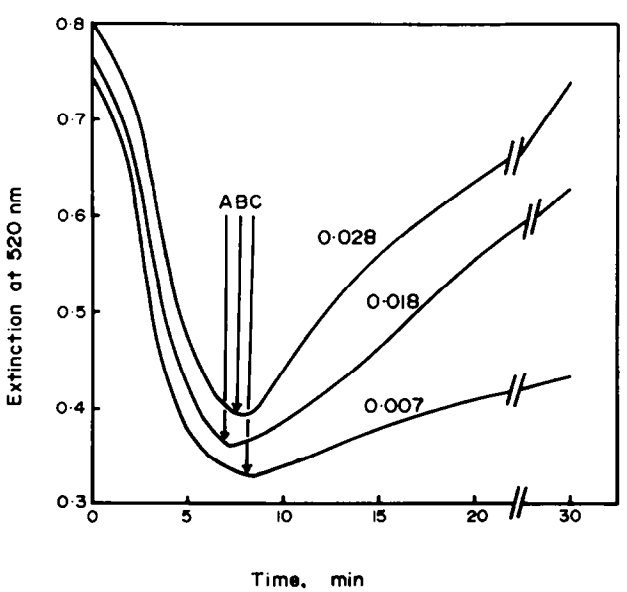


FIG. 3. Reversal of the swelling produced by sporidesmin in the presence of ATP, magnesium ions and bovine plasma albumin. Basic conditions were as given in Fig. 1. Additions were made, as follows: (A) ATP (5 mM) plus $MgCl_2$ (5 mM); (B) as (A) plus bovine plasma albumin (0·2 mg/ml); (C) as (B) plus antimycin (2 μM). The values given are the maximum linear increase in extinction at 520 nm/min.

TABLE 2. EFFECTS OF ALKALI-METAL, AMMONIUM AND MAGNESIUM IONS ON THE RATE OF SWELLING PRODUCED BY SPORIDESMIN

Additions	Maximum rate of swelling (max linear decrease in extinction at 520 nm/min)
—	0·140
NH_4Cl (3·0 mM)	0·074
LiCl (3·0 mM)	0·115
NaCl (3·0 mM)	0·117
KCl (3·0 mM)	0·100
RbCl (3·0 M)	0·100
$MgCl_2$ (5·0 mM)	0·020

Mitochondria (0·3 mg of protein/ml) were suspended in a medium (3·0 ml) containing 0·20 M-sucrose, 20 mM-Tris–chloride buffer pH 7·2, 4 mM succinate (Tris salt), 1·25 mM phosphate (Tris salt), 2 μM-rotenone and 70 μM-sporidesmin. The rates of swelling have been corrected for the low rates observed in the absence of sporidesmin.

magnesium ions, however, markedly decreased this rate. Sporidesmin did not alter the rate of swelling of mitochondria which were suspended in a medium consisting of 0·14 M sodium acetate, 10 mM-Tris chloride buffer, pH 7·2 or one consisting of 80 mM-KCl, 10 mM-Tris chloride buffer, pH 7·2 and 4 mM-sodium acetate.

Although different preparations of mitochondria varied in their susceptibility to sporidesmin, in typical preparations (Fig. 4) stimulation of swelling could be detected at 0·7 μM sporidesmin (2·1 nmoles/mg of mitochondrial protein). Half-maximum swelling occurred at sporidesmin concentrations of 36 μM (140 nmoles/mg of protein).

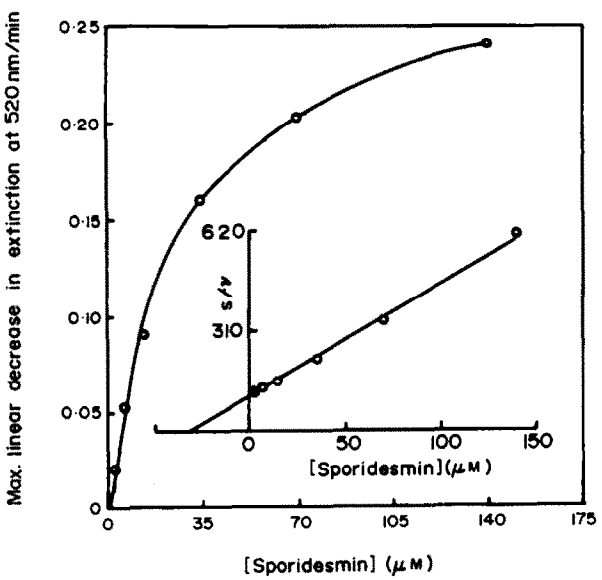


FIG. 4. Effect of sporidesmin on the rate of mitochondrial swelling. The reaction medium (3·0 ml) contained 80 mM-KCl, 20 mM Tris–chloride buffer pH 7·2, 4 mM-succinate (Tris salt), 1·25 mM-phosphate (Tris salt), 2 μM-rotenone and varying concentrations of sporidesmin. The mitochondria (final concentration 0·26 mg of protein/ml) were added last. Rates were measured as the maximum linear decrease of extinction at 520 nm/min.

A lag period occurred between the addition of sporidesmin and the attainment of the maximum rate of swelling. The reciprocal of this lag period was directly proportional to the concentration of sporidesmin (Fig. 5).

The concentration of mitochondrial protein also affected the rate of swelling and the lag period as shown in Fig. 6. At a constant concentration of sporidesmin, increasing concentrations of mitochondrial protein increased the lag period and the rate of swelling. Above a certain limiting value, however, a further increase in protein caused a decreased rate of swelling.

Incubating mitochondria under conditions in which sporidesmin produces swelling resulted in increased leakage from the mitochondria of material absorbing at 260 nm. Ion exchange chromatography indicated that this material consisted of

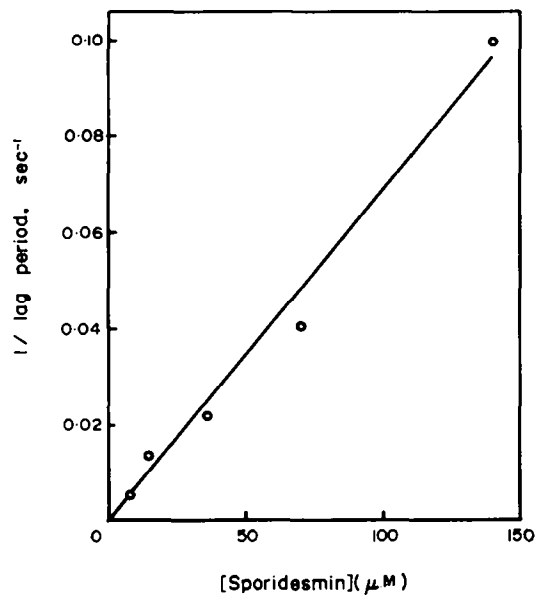


FIG. 5. Effect of sporidesmin on the reciprocal of the lag period before the maximum rate of swelling. The experimental details were as described in Fig. 4. The lag period was the time between the addition of the mitochondria and the onset of the maximum rate of swelling.

adenine nucleotides and both nicotinamide and flavin containing coenzymes. When inhibitors of swelling were added, the leakage of material was similar to that from the control samples incubated without sporidesmin.

Effect on respiration of mitochondria. Sporidesmin decreased the respiratory control indices of mitochondria from guinea-pig liver (Fig. 7). The decrease was compounded of a stimulation of state 4 respiration (with succinate as substrate) and a decrease of state 3 respiration (with succinate and ADP as substrate). Typical K_m values for the stimulation of state 4 respiration were about 140 μM sporidesmin (160 nmoles/mg protein).

Mitochondria isolated from sheep and rat liver behaved in a similar manner. Sporidesmin stimulated state 4 respiration when α-ketoglutarate (5 mM), pyruvate (4 mM) plus malate (4 mM), or glutamate (4 mM) plus malate (4 mM) replaced succinate as substrate. Unlike the swelling process, the stimulation of state 4 respiration by sporidesmin was not dependent on added phosphate.

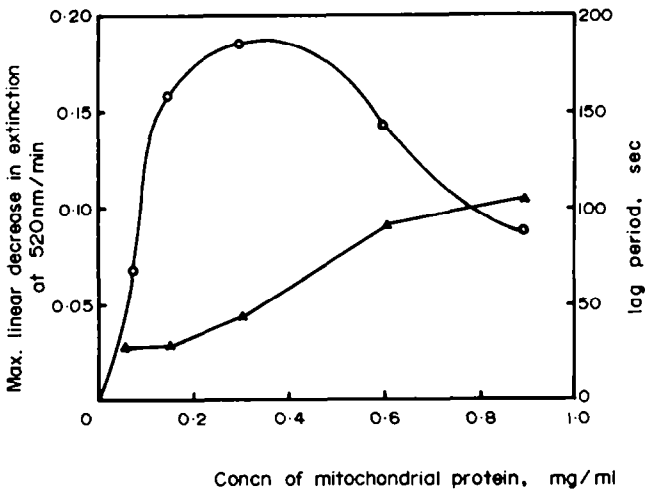


FIG. 6. Effect of mitochondrial protein concentration on the rate of swelling produced by sporidesmin and on the lag period. Basic conditions were as described in Fig. 1 except that the mitochondria, which were added in different concentrations, had been resuspended in 0·25 M sucrose with no EDTA present—see Methods. The lag period was the time between the addition of the mitochondria and the onset of the maximum rate of swelling. (○), rate of swelling; (Δ) lag period.

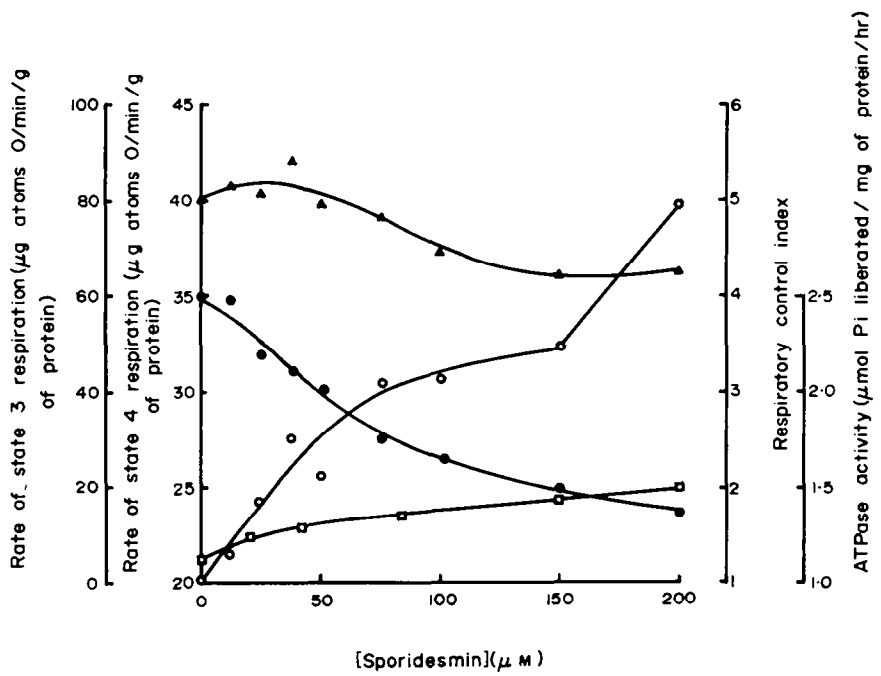


FIG. 7. Effect of sporidesmin on respiration and adenosine triphosphatase activity of respiring mitochondria. The basic reaction medium (4·0 ml) contained 0·225 M-sucrose, 10 mM-potassium phosphate, 5 mM-$MgCl_2$, 20 mM-KCl, 10 mM-Tris–chloride buffer, pH 7·4 and 3 μM-rotenone. Mitochondria (0·86 mg of protein/ml) were pre-incubated with sporidesmin for 3 min in the assay 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. Adenosine triphosphatase activity was estimated as described in the Methods. (○), state 4 respiration; (Δ), state 3 respiration; (□), adenosine triphosphatase activity; (●), respiratory control index.

The stimulation of mitochondrial respiration by sporidesmin cannot be directly attributed to swelling of the mitochondria because mitochondria do not markedly swell in the respiratory assay buffer described in Fig. 7. This is probably a result of the magnesium ions present in the buffer which are known to inhibit swelling (see Table 1).

Sporidesmin stimulated mitochondrial adenosine triphosphatase but the degree of stimulation is low when compared with classical uncouplers. 4 μM-DNP produced a similar stimulation to 200 μM-sporidesmin.

Sporidesmin did not stimulate state 4 respiration of mitochondria that had been either sonicated or treated with Triton X-100 (0·02 per cent) and that had respiratory control indices of 1·0.

DISCUSSION

Sporidesmin, at concentrations greater than 2 nmoles/mg of mitochondrial protein, can under appropriate conditions produce a rapid decrease in the extinction of suspensions of mitochondria from livers of guinea-pigs, rats, rabbits and sheep. Changes in extinction are only qualitatively related to swelling of the mitochondria[15] because of leakage of compounds into the suspending medium and because of changes in shape of the mitochondria. The former process, however, is unlikely to be significant within the short incubation times necessary for the sporidesmin produced swelling. With these limitations in mind, the changes in extinction can be taken to indicate that sporidesmin, in the presence of substrate and phosphate, produces rapid swelling of mitochondria.

In the presence of formaldehyde, an inhibitor of phosphate uptake,[16] swelling was inhibited but the requirement for phosphate is not merely for permeant anion because acetate did not substitute for phosphate. Phosphate is required for the penetration of cystamine—a positively charged disulphide—into mitochondria[17] and in this case the ability of phosphate to affect the metabolic state of the mitochondria appeared to be as important as its ability to penetrate the membrane. The requirement of the sporidesmin induced swelling for phosphate could be related to the metabolic effect of this anion.

The apparently conflicting results of earlier workers[6–8] are probably a result of the different preparative and assay media used rather than species differences. Neither Slater *et al.*[6] nor Wright and Forrester[8] included oxidizable substrate or phosphate in their assay media.

The swelling produced by sporidesmin was dependent on coupled respiration and on electron transport. The latter process was required not only to promote swelling but also to maintain it since antimycin inhibited the swelling even after it had begun. The characteristics of the sporidesmin swelling resemble those of the swelling produced by the ionophorous antibiotics. However, unlike these antibiotics, sporidesmin produces swelling in the absence of alkali metal ions.

The dependence of the rate of swelling on the concentration of mitochondria-protein as well as of sporidesmin and the occurrence of a lag period before maximum effect are suggestive of a time dependent interaction between sporidesmin and sites on the mitochondria. As the swelling can be reversed under the appropriate conditions, the interaction cannot cause any drastic or irreversible changes. The concentration of mitochondrial protein must be taken into account when comparing the

susceptibility of mitochondria from different preparations and from different species to sporidesmin. The K_m value for the stimulation of respiration (140 μM sporidesmin) at first sight appears to be greater than that for stimulating swelling (35 μM). However, when the concentrations are expressed per mg of mitochondrial protein, the value for stimulation of respiration (160 nmoles/mg of protein) is similar to that for the stimulation of swelling (140 nmoles/mg of protein).

A decrease in controlled respiration resulted from mitochondrial swelling[18] but the decreased respiratory control produced by sporidesmin cannot be directly attributed to this mechanism because the Mg^{2+} present in the respiratory assay medium inhibits the swelling process (Table 2). In addition, the effects of sporidesmin on swelling and respiration had different requirements for phosphate. Although both processes were affected at similar concentrations of sporidesmin (when the concentrations were expressed per mg of protein), the results were obtained in media of different composition and thus cannot be compared.

Both the increased swelling and state 4 respiration which is observed in the presence of sporidesmin may be a result of the toxin increasing the permeability of the inner mitochondrial membrane. As increased uptake of ions or sucrose from the suspending medium could then result in swelling and an increased uptake of substrate, if this is a rate limiting process,[18] could result in increased respiration. Alternatively, the mechanism of action of sporidesmin on respiration may be quite different from that on swelling.

It is suggested that the effects of sporidesmin on mitochondrial permeability *in vitro* may reflect a general action of the toxin with membranes and, if this is so, the mitochondria may be a good model system for characterizing this action.

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