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EFFECT OF FILIPIN ON RAT-LIVER AND YEAST MITOCHONDRIA

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SUMMARY

1. Under the appropriate conditions intact yeast and mammalian mitochondria exhibit a heretofore unobserved sensitivity to the polyene antibiotic, filipin. The activity of the "filipin complex" (Filipins I, II, III and IV) is shown to be primarily due to the component designated Filipin II.

2. Yeast mitochondria treated with filipin complex, or purified Filipin II, exhibit "uncoupled" succinate oxidation and inhibited α-ketoglutarate oxidation. Maximum filipin effect is observed at a concentration of 4 mM Filipin II. Rat-liver mitochondria are more sensitive to filipin than yeast mitochondria, and respiratory inhibition is observed regardless of substrate.

3. In liver mitochondria filipin-inhibited respiration is not relieved by Mg^{2+}, K^+, Ca^{2+} or 2,4-dinitrophenol, but is reversed by cytochrome *c*.

4. It is proposed that filipin treatment leads to altered membrane permeability and that respiratory inhibition is due to a loss of endogenous respiratory cofactors or an inactivation of primary dehydrogenases. The filipin-uncoupled yeast respiration may likewise be attributed to an altered phosphate permeability of the yeast mitochondrial membranes.

INTRODUCTION

Recent investigations[1-3] indicate that the action of polyene antibiotics, such as filipin, is dependent upon the phospholipid and sterol content of biological and artificial membranes. The *in vivo* interaction of polyenes with sterol-containing plasma membranes alters permeability so that phosphate, potassium, organic acids, and esterified phosphates leak out of the treated cell[4,5]. The resulting decrease in metabolic activity has been attributed to a secondary effect of polyene action, *i.e.* an unfavorable alteration of the intracellular milieu[6].

However, polyene antibiotics interact with membranes other than the plasma membrane. In particular, polyenes are extensively bound by mitochondria from *Saccharomyces cerevisiae*[7] and from *Neurospora crassa*[6]. In spite of this, KINSKY, GRONAU AND WEBER[6] and GOTTLIEB *et al.*[4], using fungal mitochondria, and LARDY, JOHNSON AND MCMURRAY[8], and PRESSMAN[9], using mammalian mitochondria, demonstrated no significant physiological effect of various polyenes on isolated mito-

chondria. Such results are unexpected, since polyenes cause extensive structural changes in membranes[1], and particularly since mitochondria must retain their structural integrity for optimal physiological function.

This report demonstrates that under appropriate conditions filipin does alter the oxidative metabolism of both rat-liver and yeast mitochondria. The recent fractionation of filipin into four components[10] (Filipins I, II, III and IV), has also permitted us to reconcile many of the apparently conflicting observations of several laboratories.

MATERIALS AND METHODS

Suspensions of yeast mitochondria were prepared in 0.6 M mannitol according to the method of BALCAVAGE AND MATTOON[11]. Rat-liver mitochondria were prepared and suspended in 0.25 M sucrose as described by SCHNEIDER[12]. Filipins I, II, III and IV and filipin complex, which has been reported to consist of a mixture of 4 % Filipin I, 25 % Filipin II, 53 % Filipin III and 18 % Filipin IV (ref. 10) were gifts of the Upjohn Co. As received from the manufacturer, filipin complex (originally thought to be a single compound) contained a large quantity of insoluble residue which gave a non-specific absorption pattern in the Beckman DBG scanning spectrophotometer. Since filipin is rather unstable, the complex was recrystallized from methanol, propanol, or ethyl acetate to remove oxidation products and other insoluble material, and stored under N_2 at $-20°$. Solutions of filipin were prepared daily in dimethylformamide and kept in the dark. Equal aliquots of serial dilutions were added to the mitochondrial suspensions to yield the desired final filipin concentrations. Suspensions of yeast or rat-liver mitochondria (35 mg protein/ml) were incubated with filipin, as described in the figure legends, at 15° for 15 and 5 min, respectively. In some cases lower concentrations of filipin and longer incubation times were used with equivalent results. Aliquots were removed from the incubation mixtures and polarographic respiratory assays were performed as previously described[11,13]. Final apparent concentrations of filipin in the incubation mixtures ranged from $8.6 \cdot 10^{-4}$ M to $9.5 \cdot 10^{-3}$ M, corresponding to 16 μg filipin per mg mitochondrial protein to 178 μg filipin per mg mitochondrial protein. The 2,4-dinitrophenol (a-grade) (Fisher Scientific Co.) was prepared in aqueous solution. Horse-heart cytochrome *c* (Type III) was purchased from Sigma Chemical Co. All other reagents and substrates were the sodium salts of reagent grade materials.

RESULTS

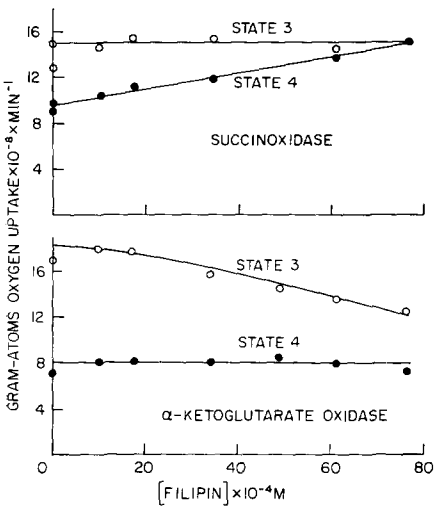
Initially it was found that filipin had little or no effect on yeast mitochondria when added to the respiring organelles in a polarographic reaction chamber. However, when yeast mitochondria were preincubated with recrystallized filipin complex (presumably containing a mixture of Filipins I–IV), they exhibited the anticipated alterations in respiratory activity, and as shown below (see Figs. 3 and 4), it may be assumed that the observed "filipin activity" is in fact caused primarily by the Filipin II component of the filipin complex. As shown in Fig. 1, filipin treatment leads to respiratory stimulation (uncoupling) or inhibition depending upon the respiratory substrate employed. Thus, with succinate as substrate, a State 4 (ADP-limited)

stimulation by filipin was observed with no apparent effect on the State 3 (ADP-stimulated) respiratory rate. However, with α-ketoglutarate as substrate, the energy-coupling apparatus appeared unaffected, since State 4 respiratory rate was unaltered over a wide range of filipin concentrations. Conversely, with α-ketoglutarate as substrate a pronounced inhibition of State 3 activity was induced by filipin.

Similar results were obtained with filipin complex recrystallized from methanol or propanol (originally thought to be a single compound). However, in these cases the concentration of antibiotic required for 50 % activity varied, presumably as a consequence of alteration in the proportions of Filipins I–IV in the various re-crystallized products.

Rat-liver mitochondria were found to be more sensitive to filipin than yeast mitochondria, since shorter incubation times were required to effect equivalent inhibition of α-ketoglutarate oxidase. In addition, filipin produced only an inhibition of State 3 respiration of rat-liver mitochondria regardless of respiratory substrate. Thus, succinate-supported State 3 respiration was inhibited as shown in Fig. 2. Similar results were obtained when α-ketoglutarate was used as substrate.

The inhibition of rat-liver mitochondria by filipin cannot be reversed by Mg^{2+},



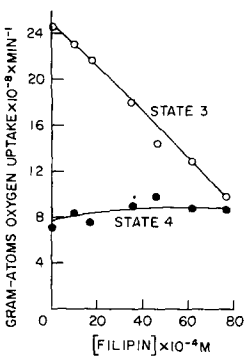


Fig. 1. The effect of filipin complex on succinate and on α-ketoglutarate oxidase activities of yeast mitochondria. Filipin complex was recrystallized from ethyl acetate. Mitochondria (35 mg protein/ml) were incubated with filipin at 15°. Aliquots containing 2.7 mg of protein for α-ketoglutarate oxidase assays or 0.86 mg of protein for succinoxidase assays were removed after 15 min and introduced into the 3.0-ml reaction vessel which contained either 16.6 mM succinate or 33.2 mM α-ketoglutarate and reaction medium. State 3 respiratory rates (○) were initiated by addition of 0.4 μmole of ADP. State 4 respiratory rates (●) were obtained after ADP exhaustion.

Fig. 2. The effect of filipin complex on succinoxidase activity of rat-liver mitochondria. Filipin complex was recrystallized from ethyl acetate. Freshly prepared mitochondria (35 mg protein/ml) were incubated with filipin at 15°. After 5 min polarographic respiratory assays were performed on aliquots of the incubation mixture. Each aliquot contained 3.4 mg protein. The 3.0-ml polarography vessel contained reaction medium (B. CHANCE AND G. WILLIAMS[13]) and 16.6 mM sodium succinate at 25°. The State 3 rates (○) were initiated by the addition of 0.58 μmole of ADP to the respiring mitochondria. State 4 rates (●) were those observed after the exhaustion of ADP in the reaction mixture.

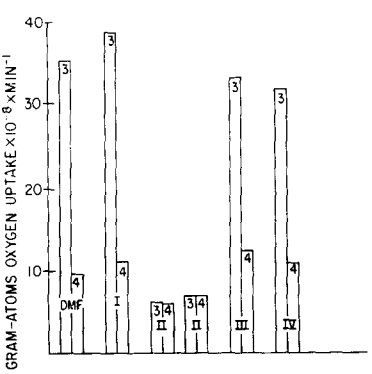
K^+, orthophosphate, or uncoupling concentrations of 2,4-dinitrophenol or valinomycin. Recently we have observed that increased ionic strength of the reaction medium accentuates the effect of filipin on rat-liver mitochondria. As shown in Table I, the State 3 inhibition can be released, and respiration largely restored by addition of cytochrome *c* to the reaction medium.

TABLE I

THE REVERSAL OF FILIPIN INHIBITION BY CYTOCHROME *c*

Rat-liver mitochondria (35 mg protein/ml) were incubated with filipin complex in dimethylformamide at the concentrations indicated. Incubation conditions were 15° for 5 min. Aliquots containing 5 mg of protein were removed and examined for respiratory capacity with 16.6 mM succinate as substrate. Cytochrome *c*, when used, was present in the 3.0-ml respiratory vessel before addition of mitochondria. 195 μM ADP was added to the reaction mixture to initiate State 3. Filipin was recrystallized from ethyl acetate.

Incubation treatment	*Cytochrome c concn. (μM)*	*State 3 Q_{O_2} (natoms/min per mg)*
4.3 mM filipin + 5 % dimethylformamide		6.8
3.6 mM filipin + 4.7 % dimethylformamide		16.6
3.6 mM filipin + 4.7 % dimethylformamide	5.2	29.0
3.6 mM filipin + 4.7 % dimethylformamide	13.3	34.7
3.6 mM filipin + 4.7 % dimethylformamide	26.6	34.9
4.7 % dimethylformamide		46.7



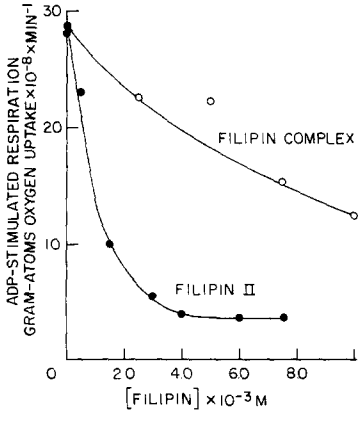


Fig. 3. Relative respiratory inhibition by various purified filipins. Rat-liver mitochondria were incubated as described in Fig. 2 with Filipins I–IV. All filipins were present in the incubation mixture at 3.5 mg/ml. Bars labeled 3 and 4 represent the succinate-supported respiratory rates in States 3 and 4, respectively. Dimethylformamide (DMF) indicates solvent control. State 3 respiration was initiated with 130 μM ADP.

Fig. 4. Relative activities of Filipin II and filipin complex. Rat-liver mitochondria were incubated as described in Fig. 2, with pure Filipin II and filipin complex crystallized from propanol. Aliquots were removed from the incubation mixture after 5 min and tested for succinoxidase activity by the polarographic method. Respiratory assays were performed under the conditions described in Fig. 2.

The preceding studies were performed with a material which contained a mixture of Filipins I, II, III and IV. However, the recent availability of the purified filipins has led to the identification of the active component of the complex mixture. Fig. 3 illustrates the fact that Filipin II alone possesses a significant capacity to inhibit State 3 respiration of rat-liver mitochondria.

Similar results were obtained with yeast mitochondria with α-ketoglutarate as substrate. A direct comparison of Filipin II and the complex mixture (Fig. 4) illustrates the apparent enhanced potency of pure Filipin II, and permits the definition of the 50 % inhibition level at about $8 \cdot 10^{-4}$ M Filipin II.

DISCUSSION

The apparent concentrations of filipin used in these studies are significantly higher than have heretofore been observed to cause *in vivo* inhibition of fungal respiration[4,14]. However, similar milligram ratios of filipin to mitochondrial protein have previously been employed[6] with no apparent detrimental effect on the treated mitochondria. Furthermore, while high concentrations of filipins have been routinely employed in this study, lower concentrations can lead to the same effects upon prolonged incubation. Since filipins have a very low solubility in aqueous media, effective incorporation of the antibiotic into non-polar membranes is rather slow, and probably reflects the need for transfer of filipin from an emulsion into the membrane. During the course of the present investigation MORTON AND LARDY[15] reported an *in vivo* effect of filipin on epididymal sperm cells. In LARDY's report, filipin concentrations similar to those employed here did effect an *in vivo* respiratory inhibition. Although GOTTLIEB *et al.*[4] also reported an *in vivo*, filipin-mediated, inhibition of yeast respiration, they could detect no filipin effect on isolated yeast or mammalian mitochondria. KINSKY[6] has interpreted such results to mean that *in vivo* the mitochondria are damaged by a filipin-induced cytoplasmic dilution rather than by a direct effect of filipin on the mitochondrial membrane. In contrast, the studies reported here indicate that filipin does indeed interact with mitochondria to effect an alteration in respiratory capacity.

Initially, our interest in filipin as an inhibitor of oxidative phosphorylation stemmed from certain physical and chemical similarities to oligomycin. Although the complete structure of oligomycin is not known, it is believed to be a macrocyclic lactone. The ultraviolet spectrum of oligomycin indicates that it contains a conjugated system[16]. The more complete structural studies of filipin[17] show that this antibiotic is also a macrocyclic lactone with a higher degree of unsaturation than oligomycin. Consideration of these analogous chemical features raised the question of whether filipin might not act like oligomycin in fungal mitochondria. If this proved to be the case, it would follow that oligomycin, like filipin, might act by binding sterols in the mitochondrial membrane. In this connection it is relevant to note the sterol-like structure of atractyloside and related compounds which inhibit oxidative phosphorylation[18]. Although the observed filipin-induced inhibition of State 3 respiration mimics the effect of oligomycin, the much higher relative concentrations required and the lack of respiratory release by 2,4-dinitrophenol indicate that the two antibiotics probably act by different mechanisms, or at different "sterol" sites.

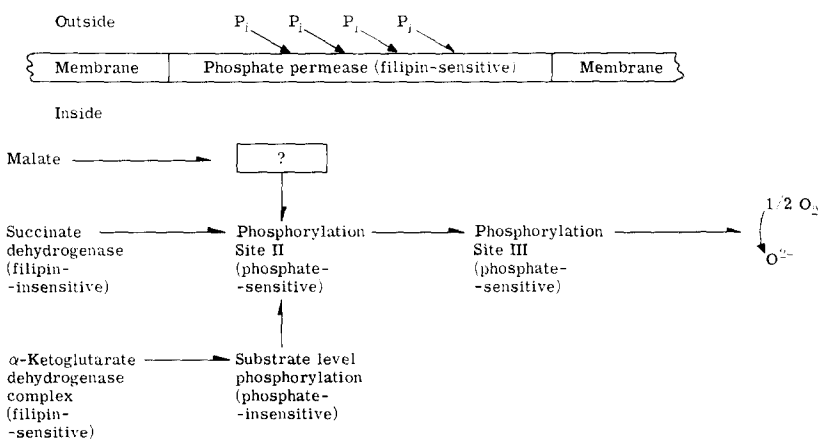
Since filipin inhibition was not relieved by Mg^{2+}, K^+, valinomycin, orthophosphate or ADP, the effects of added cytochrome *c* were tested. The resulting reversal of inhibition indicates that filipin treatment caused a change in the rate-limiting step in electron transport. Thus, the permeability of the mitochondrial membrane may be altered so that cytochrome *c* becomes rate-limiting as it leaks into the assay medium. Alternatively, it is possible that added cytochrome *c* binds filipin, thus removing it from its inhibitory site on the mitochondrial membrane. However, since excess cytochrome *c* does not completely reverse the filipin effect, and since electron microscopy indicates a filipin-induced destruction of rat-liver mitochondrial outer membranes (C. SCHNAITMAN, personal communication) the former suggestion seems more likely. It is likely, then, that at least in liver mitochondria, filipin acts by attacking the sterol-rich outer membrane[19], thus exposing the inner membrane to the effects of salts in the assay medium which are known to extract cytochrome *c* readily[20].

The results with yeast mitochondria, on the other hand, cannot be so simply explained, since filipin acts as an uncoupler when succinate is the respiratory substrate.

These differential effects of filipin on the succinoxidase and pyridine nucleotide-linked oxidase systems resemble the differential effects of phosphate concentration observed by BALCAVAGE AND MATTOON[11]. In the latter investigation it was observed that while succinoxidase activity was uncoupled (increased oxidation rate) at low phosphate concentrations, malate oxidation showed a typical Michaelis–Menten dependency on phosphate concentration.

Scheme I presents a tentative model consistent with the observations on yeast mitochondria made in this laboratory.

It has been shown that filipin has the capacity to alter the permeability characteristics of membranes[1,5]. It may thus be postulated that filipin reduces phosphate



SCHEME I

Tentative identification of the filipin and phosphate sensitive sites in Saccharomyces mitochondria. Arrows indicate the pathway of electron flow. The question mark indicates the uncertainty of our knowledge of "Site I" processes in Saccharomyces.

permeability of yeast mitochondria, thereby lowering phosphate concentration at Sites II and III. This, in turn, induces uncoupling analogous to that observed when succinate is the electron donor. Since substrate-level phosphorylation is not uncoupled at low phosphate concentration*, the inhibition of the α-ketoglutarate oxidase activity may be attributed to a limitation of succinyl thiokinase activity by lowered phosphate in the mitochondria.

Alternatively, filipin may act on α-ketoglutarate oxidase by causing loss of NAD^+, or the results may be rationalized by postulating the existence of two independent types of respiratory assemblies, one which supports succinate oxidation and the other which supports the oxidation of NAD-linked substrates.

Two alternative explanations are offered for the inability of previous investigators to observe an *in vitro* effect of filipin on fungal mitochondria: (a) With yeast mitochondria we have shown that filipin induces an apparent uncoupling of succinate-supported respiration, rather than an inhibition. Since fungal mitochondria, as prepared by GOTTLIEB and KINSKY, undoubtedly would be uncoupled or loosely-coupled, these authors would not have observed a filipin effect on succinoxidase other than a slight increase in respiratory rate in the more tightly-coupled preparations. In fact, KINSKY[6] has reported just such an increase. (b) In this report we have also shown that cytochrome *c* can reverse the effect of filipin. Respiratory assays which monitor electron transport as a function of the rate of reduction of exogenous cytochrome *c* (ref. 6) are thus performed under reconstitutive conditions, and would not permit an observation of the filipin effect.

It should be noted that none of the previous attempts to demonstrate filipin interaction with mitochondria employed pure Filipin II, but rather filipin complex. The much lower potency of the latter, demonstrated here, and the instability of filipins in light and air, may also have contributed to previous failures to detect effects.

In this connection it is important to emphasize that the observed filipin inhibition is primarily due to the action of Filipin II. Thus, while filipin complex at high concentrations interacts with oleic acid monolayers[21], this non-physiological effect is probably not the effect observed in the studies reported here, since Filipin II, but not the complex at the same concentration, alters the respiratory activity of mitochondria. In monolayer studies, however, it is likely that all of the filipins are equally effective. Moreover, intimate contact between non-polar filipin and a monolayer is much more readily obtained than between a filipin micelle and mitochondria in aqueous 0.6 M mannitol.

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* W. X. BALCAVAGE, unpublished results.

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