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EFFECTS OF INHIBITORS ON THE PLASMA MEMBRANE AND MITOCHONDRIAL ADENOSINE TRIPHOSPHATASES OF *NEUROSPORA CRASSA*

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Summary

A comparative study has been made of the effects of a variety of inhibitors on the plasma membrane ATPase and mitochondrial ATPase of *Neurospora crassa*. The most specific inhibitors proved to be vanadate and diethylstilbestrol for the plasma membrane ATPase and azide, oligomycin, venturicidin, and leucinostatin for mitochondrial ATPase. *N,N'*-Dicyclohexylcarbodiimide, octylguanidine, triphenylsulfonium chloride, and quercetin and related bioflavonoids inhibited both enzymes, although with different concentration dependences. Other compounds that were tested (phaseolin, fusicoccin, deoxycorticosterone, alachlor, salicyclic acid, *N*-1-napthylphthalamate, triiodobenzoic acid, cyclic AMP, cyclic GMP, theobromine, theophylline, and histamine) had no significant effect on either enzyme. Overall, the results indicate that the plasma membrane and mitochondrial ATPases are distinct enzymes, in spite of the fact that they may play related roles in H^+ transport across their respective membranes.

Introduction

In the fungi, which have served as useful model organisms for the study of a variety of membrane transport processes, it is now generally believed that the primary transport system in the plasma membrane is an ATP-driven H^+ pump, which transports H^+ out of the cell and thereby generates a large electrochemical gradient for H^+ (predominantly a membrane potential [1—4]). Coupled to this primary H^+ transport process are secondary H^+-dependent cotransport

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N,N'*-bis-(2-ethanesulfonic acid); alachlor, 2-chloro-2',6'-diethyl-*N*-(methoxy-methyl) acetaniide.

systems for sugars, amino acids, and inorganic ions [2,5—10]. Initial attempts to demonstrate plasma membrane ATPase activity directly in the fungi were complicated by the lack of a specific inhibitor for this enzyme and by the difficulty of preparing pure plasma membranes free of contamination by mitochondria (and mitochondrial ATPase). Recently, however, improved methods of cell fractionation have been developed [11—13], and ATPase activity with distinctive properties has been identified in plasma membranes from *Neurospora crassa* [11,14,15], *Saccharomyces cerevisiae* [12,13,16—18], and *Schizosaccharomyces pombe* [19].

The present paper returns to the question of the inhibitor sensitivity of the *Neurospora* plasma membrane ATPase, as part of an attempt to understand the structural and functional relationship of this enzyme to other energy-coupling ATPases. Of special interest is its relationship to mitochondrial ATPase, both for practical reasons (to permit the plasma membrane ATPase to be distinguished unambiguously from contaminating mitochondrial ATPase) and for theoretical ones (because mitochondrial ATPase, like the plasma membrane ATPase of fungi, is postulated to be involved in H^+ transport [20]). We have therefore determined the sensitivity of the *Neurospora* plasma membrane ATPase to a variety of inhibitors known to affect other membrane-bound ATPases, and in each case have performed parallel measurements with *Neurospora* mitochondrial ATPase, both in its membrane-bound form and as purified F_1.

Methods

Growth of cells. Wild-type strain RL21a of *N. crassa* was used in these experiments. Conidia were inoculated at a density of 10^6/ml into Vogel's minimal medium [21] containing 2% sucrose as carbon source, and grown with vigorous aeration at 25° for 13 h. Control experiments have shown that the yield and specific activity of both plasma membrane and mitochondrial ATPase are maximal in mid-exponential phase (at 13 h), and decline steadily from that point on.

Cell fractionation. Cells were harvested, treated with β-glucuronidase (type H-2; Sigma Chemical Co., St. Louis, Mo.), and disrupted by homogenization; and mitochondria were isolated by two cycles of differential centrifugation [22]. The remaining 10 000 × *g* supernatant was centrifuged once more at 10 000 × *g* for 30 min to remove any residual mitochondria and then at 48 000 × *g* for 30 min to obtain a high-speed particulate fraction. This fraction contained oligomycin-insensitive ATPase activity (assayed at pH 6.0 as described previously [14]) averaging 1.5 μmol/min per mg protein, a value equivalent to that found for plasma membranes isolated from the slime mutant of *Neurospora* [14]. Furthermore, the membrane-bound ATPase from the wild-type strain was indistinguishable from the slime plasma membrane ATPase in pH optimum, K_m for MgATP, specificity for nucleoside triphosphates and divalent cations, and sensitivity to inhibitors (Bowman, B.J. and Slayman, C.W., unpublished experiments), consistent with the view that it is plasma membrane ATPase. Mitochondrial contamination of the wild-type 48 000 × *g* fraction was somewhat less than in the case of slime membranes, with residual

oligomycin-sensitive ATPase activity at pH 8.2 averaging 0.2 and 0.5 μmol/min per mg protein in the two strains. For these reasons, and because the 48 000 × *g* fraction from the wild-type strain can be obtained conveniently and in good yield, we have used it in the present experiments.

Purification of F_1 ATPase. Mitochondria were sonicated as described previously [23], and the resulting submitochondrial particles were suspended at a final density of 5 mg protein/ml in 20 mM Tris/sulfate/1 mM EDTA, pH 7.5. All steps from this point on were carried out at room temperature. The submitochondrial particles were then extracted with chloroform by the method of Beechey et al. [24], and F_1 ATPase was purified from the extract by ammonium sulfate fractionation and chromatography on DEAE-cellulose. The resulting F_1 ATPase had a specific activity of 30—120 μmol/min per mg protein. It was cold-labile, insensitive to oligomycin (100 μg/mg protein), and upon SDS-acrylamide gel electrophoresis, gave two major protein bands and one minor band at the position of the α, β, and γ subunits of *Neurospora* F_1 (ref. 25; Mainzer, S.E. and Slayman, C.W., unpublished observations).

ATPase assays. Unless otherwise specified, ATPase activity was measured at 30°C by the liberation of inorganic phosphate in 0.5 ml of reaction mixture as follows: (i) For the assay of mitochondrial ATPase (membrane-bound or solubilized) at its pH optimum, the reaction mixture contained 5 mM Na_2ATP (Sigma), 5 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, 25 μg pyruvate kinase, and 10 mM Tris/glycylglycine buffer, pH 8.2 (23). (ii) For the assay of plasma membrane ATPase at its pH optimum, the reaction mixture contained 5 mM Na_2ATP (Sigma), 5 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, 25 μg pyruvate kinase, 0.5 mM EDTA, 5 mM KN_3 (to inhibit residual mitochondrial ATPase), and 10 mM Tris/PIPES buffer, pH 6.7. The EDTA in this reaction mixture functioned to reverse the inhibitory effect of traces of vanadate present in Sigma ATP (ref. 26; Bowman, B.J. and Slayman, C.W., unpublished observations). In the presence of EDTA, the specific activity of the plasma membrane ATPase increased and the pH optimum of the enzyme shifted from 6.0 (the value reported previously [14]) to 6.7; an identical pH profile has been obtained subsequently with Boehringer-Mannheim ATP (which is free of vanadate) in the absence of EDTA (Bowman, B.J. and Slayman, C.W., unpublished results). (iii) Mitochondrial ATPase was also assayed at pH 6.7 in the same reaction mixture (as described in part ii) except that azide was omitted. In each case, the reaction was started by the addition of enzyme and, after 10 min, was stopped by the addition of 100 μl 50% trichloroacetic acid. The tubes were then centrifuged at 2000 × *g* for 5 min, and inorganic phosphate was measured in a portion of the supernatant by the method of Dryer et al. [27].

In the case of certain inhibitors (quercetin, myricetin, fisetin, morin, rutin) which interfere with the colorimetric determination of inorganic phosphate, the release of $^{32}P_i$ from [γ-^{32}P]ATP was measured as described previously [14].

Estimation of protein. Protein was assayed by the method of Lowry et al. [28] with bovine serum albumin as standard.

Reagents. Na_2ATP (purified from equine muscle), phospho*enol*pyruvate, pyruvate kinase (type II), oligomycin, *N,N'*-dicyclohexylcarbodiimide (DCCD), diethylstilbestrol, deoxycorticosterone, cyclic AMP, theobromine, theophylline, and histamine were purchased from Sigma Chemical Corp., St. Louis, Mo.;

quercetin, myricetin, fisetin, morin, and rutin, from Aldrich Chemical Co., Milwaukee, Wisc.; venturicidin (a mixture of venturicidins A and B prepared by British Drug Houses Ltd.), from Gallard-Schlesinger, Carle Place, N.Y.; KN_3, from J.T. Baker Co., Phillipsburg, N.J.; sodium orthovanadate, from Fisher Scientific Co., Springfield, N.J.; and salicylic acid, from Mallinckrodt, St. Louis, Mo. Leucinostatin was a gift from Dr. R.L. Hamill, Eli Lilly Pharmaceutical Co., Indianapolis, Ind.; octylguanidine, from Dr. B.C. Pressman, University of Miami; phaseolin, from Dr. H. Van Etten, Cornell University; fusicoccin, from Dr. A. Ballio, University of Rome; alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide; Monsanto), from Dr. P. Day, Connecticut Agricultural Experiment Station; *N*-1-napthylphthalamate (Uniroyal) and triiodobenzoic acid, from Dr. M.H.M. Goldsmith, Yale University; and cyclic GMP and [γ-^{32}P]ATP ($4.4 \cdot 10^7$ dpm/nmol), from Dr. P. Greengard, Yale University.

Of the inhibitors, oligomycin, venturicidin, leucinostatin, DCCD, octylguanidine, diethylstilbestrol, deoxycorticosterone, phaseolin, and triiodobenzoic acid were dissolved in absolute ethanol and added to the reaction mixture such that the final ethanol concentration was less than 0.5%; the same concentration of ethanol was added to the control tubes in these experiments. Quercetin, myricetin, fisetin, morin, and rutin were dissolved directly in the reaction mixture at alkaline pH, and the mixture was then titrated back with HCl to the appropriate pH (8.2 or 6.7). KN_3, sodium orthovanadate, cyclic AMP, cyclic GMP, theobromine, theophylline, histamine, salicylic acid, fusicoccin, *N*-1-naphthylphthalamate, and alachlor were dissolved in water.

Results

Oligomycin, venturicidin, DCCD, leucinostatin

Oligomycin [19,29—32], venturicidin [30—34], DCCD [30—32], and leucinostatin [36—38] are potent inhibitors of mitochondrial ATPase in its membrane-bound form, acting on the F_o portion of the complex from beef heart, rat liver, and yeast mitochondria. Figs. 1 and 2 illustrate that, as expected, they also inhibit the membrane-bound mitochondrial ATPase from *Neurospora*, with half-maximal effect (at the pH optimum, 8.25) produced by 1 μg oligomycin/mg protein, 0.3 μg venturicidin/mg protein, 0.5 μM DCCD, and 0.2 μM leucinostatin (Table I). They have little effect on solubilized F_1 ATPase, even at much higher concentrations (Figs. 1 and 2).

The same four compounds fall into three groups with respect to their action on the plasma membrane ATPase. Oligomycin and venturicidin are not significantly inhibitory, even at 100 μg/mg protein (Fig. 1). Leucinostatin does inhibit, but only at concentrations substantially higher than those effective against mitochondrial ATPase (Fig. 2A). DCCD, however, is a potent inhibitor of the plasma membrane ATPase, producing a half-maximal effect at 3 μM (Fig. 2B; Table I).

Azide

Azide is known to inhibit mammalian mitochondrial ATPase by acting on the F_1 portion of the complex [39]. Fig. 3 illustrates that it is similarly active against both the F_1 and membrane-bound forms of the corresponding *Neuros-*

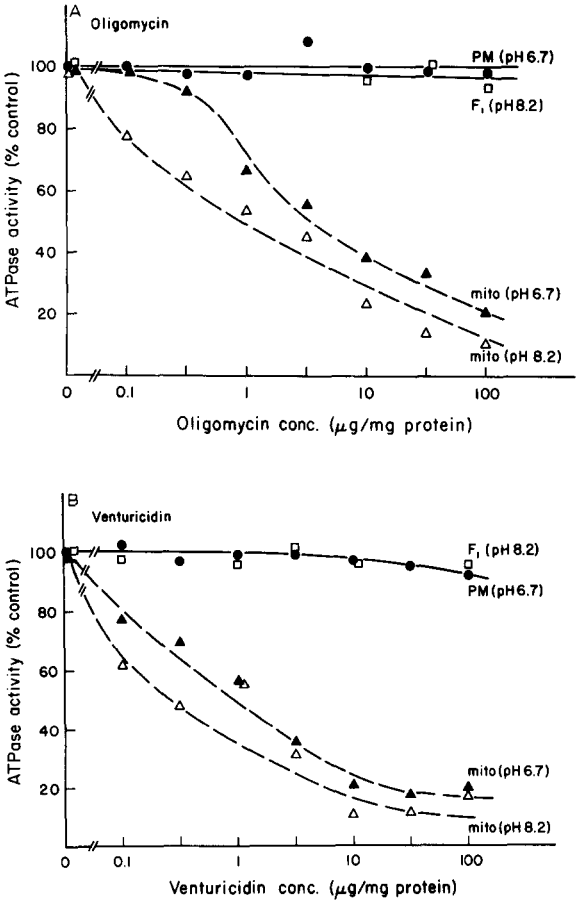

Fig. 1. Effect of oligomycin (A) and venturicidin (B) on ATPase activity. •, plasma membrane ATPase (30 μg protein/0.5 ml) assayed at pH 6.7; ▲, membrane-bound mitochondrial ATPase (60 μg protein/0.5 ml) assayed at pH 6.7; △, membrane-bound mitochondrial ATPase (60 μg protein/0.5 ml) assayed at pH 8.2; □, solubilized mitochondrial ATPase (F_1) (1.6 μg protein/0.5 ml) assayed at pH 8.2. Reaction mixtures are described in Methods. In each case, the reaction was started by the addition of enzyme and terminated after 10 min by the addition of 0.1 ml 50% trichloroacetic acid; and the results are expressed as percent of control activity in the absence of inhibitor. Control rates were, for the oligomycin and venturicidin experiments, respectively, 1.67 and 1.69 μmol/min per mg protein for the plasma membrane ATPase at pH 6.7, 0.65 and 0.81 μmol/min per mg protein for mitochondrial ATPase at pH 6.7, 1.55 and 1.09 μmol/min per mg protein for mitochondrial ATPase at pH 8.2, and 57.8 and 57.4 μmol/min per mg protein for F_1 ATPase at pH 8.2.

pora enzyme, with somewhat lower concentrations required for half-maximal inhibition at pH 8.2 (0.009 mM for F_1, 0.03 mM for the membrane-bound enzyme) than at pH 6.7 (0.03 and 0.1 mM, respectively) (Table I). Azide has no significant effect on the plasma membrane ATPase, however (Fig. 3); the small amount of inhibition that was seen (less than 10%) can be accounted for by contaminating mitochondrial ATPase, since oligomycin also gave 10% inhibition of this particular preparation (data not shown).

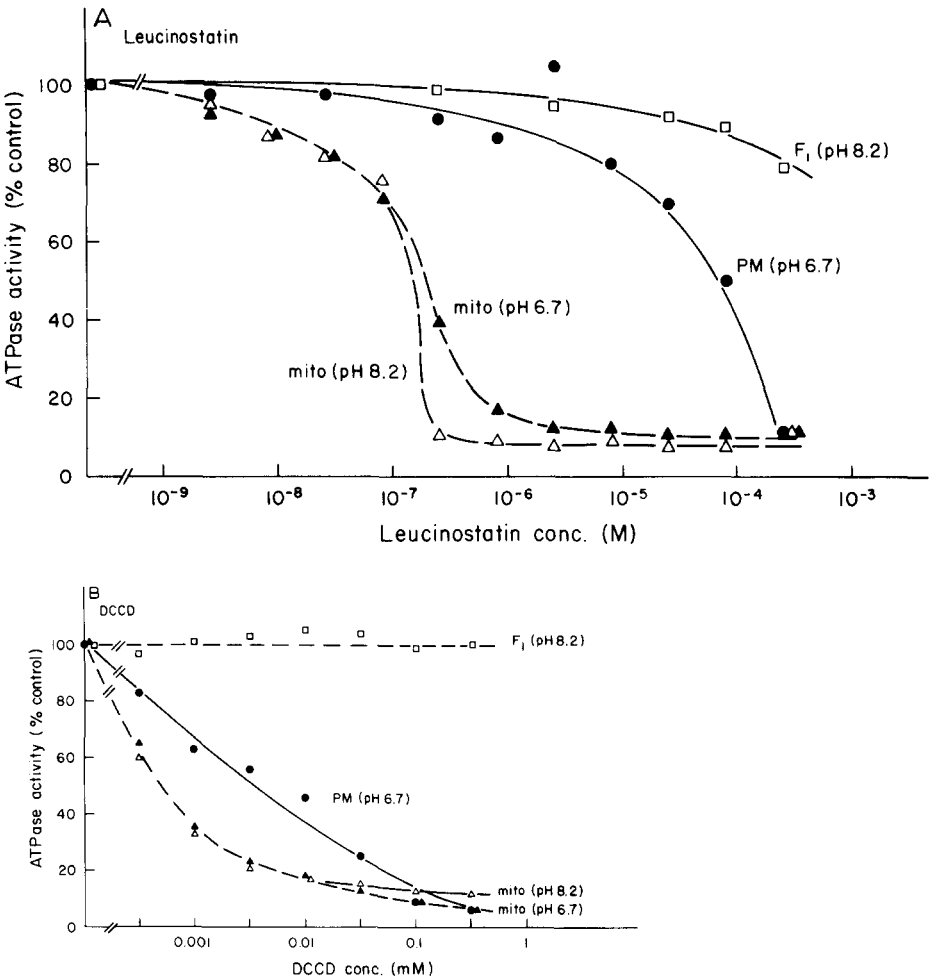

Fig. 2. Effect of DCCD (B) and leucinostatin (A) on ATPase activity. Symbols and reaction conditions for the leucinostatin experiment are described in the legend to Fig. 1. In the DCCD experiment, enzyme (5 μg protein for the plasma membrane ATPase, 30 μg for mitochondrial ATPase, and 0.8 μg for F_1 ATPase) was added to 0.4 ml of reaction mixture, which lacked the ATP-regenerating system and also lacked EDTA. DCCD was added in 1 μl of ethanol to give the appropriate final concentration (0—0.3 mM), and the mixture was incubated at 30°C for 5 min. The reaction was then started by the addition of 0.1 ml of 25 mM ATP (Boehringer-Mannheim), and terminated after 10 min by the addition of 0.1 ml 50% trichloroacetic acid. Control rates were, for the DCCD and leucinostatin experiments, respectively, 1.09 and 2.09 μmol/min per mg protein for the plasma membrane ATPase at pH 6.7; 0.66 and 0.63 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 1.10 and 1.17 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 34.0 and 51.7 μmol/min per mg protein for F_1 ATPase at pH 8.2.

Octylguanidine, triphenylsulfonium chloride

These two lipophilic cations are known to be taken up by mitochondria, and to inhibit both oxidative phosphorylation (by acting on the F_1 portion of the ATPase) and electron transport (in the NAD-cytochrome *b* region of the respiratory chain [40—43]). Octylguanidine has also been reported to inhibit potassium transport across the plasma membrane in yeast [44] and in barley roots

TABLE I

EFFECTS OF INHIBITORS ON PLASMA MEMBRANE AND MITOCHONDRIAL ATPase

Inhibitor	Concentration giving 50% inhibition			
	Plasma membrane ATPase (pH 6.7)	Mitochondrial ATPase (pH 6.7)	Mitochondrial ATPase (pH 8.2)	F_1 ATPase (pH 8.2)
Oligomycin	n.i. *	3 μg/mg protein	1 μg/mg protein	n.i.
Venturicidin	n.i.	1 μg/mg protein	0.3 μg/mg protein	n.i.
Leucinostatin	90 μM	0.2 μM	0.2 μM	n.i.
DCCD	3 μM	0.5 μM	0.5 μM	n.i.
Azide	n.i.	0.1 mM	0.03 mM	0.009 mM
Octylguanidine	0.015 mM	0.5 mM	0.09 mM	0.09 mM
Triphenylsulfonium chloride	0.03 mM	0.2 mM	0.08 mM	0.02 mM
Quercetin	0.2 mM	0.9 mM	2 mM	2 mM
Diethylstilbestrol	0.009 mM	n.i.	0.1 mM	n.i.
Vanadate	1 μM	n.i.	n.i.	n.i.

* n.i. means not significantly inhibitory at the highest concentration tested; see Figs. 1—7.

[45] and $(Na^+ + K^+)$ATPase activity in microsomes from cardiac tissue [46].

In the present study, octylguanidine and triphenylsulfonium chloride were found to inhibit both the mitochondrial and plasma membrane ATPases of *Neurospora*. Fig. 4 illustrates that octylguanidine was somewhat more effective

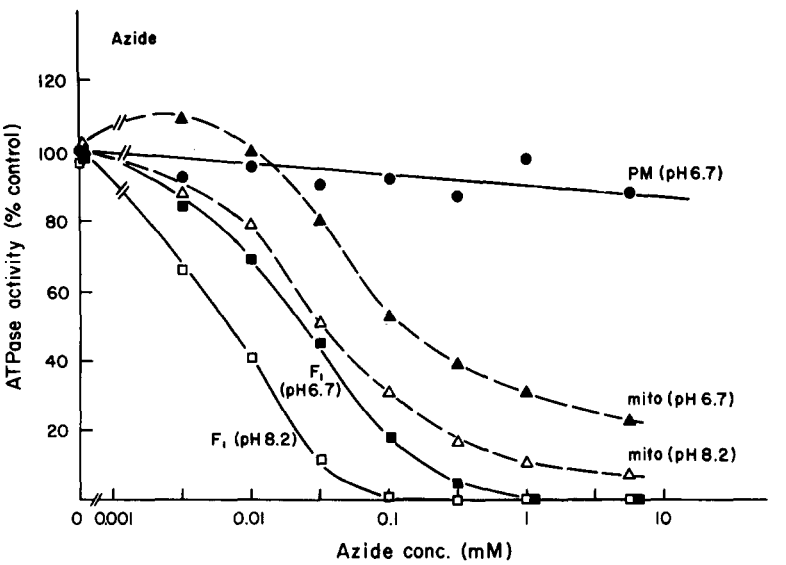

Fig. 3. Effect of azide on ATPase activity. Symbols and reaction conditions are described in the legend to Fig. 1. Control activities were 1.62 μmol/min per mg protein for plasma membrane ATPase at pH 6.7; 0.72 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 1.69 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 39.3 μmol/min per mg protein for F_1 ATPase at pH 8.2.

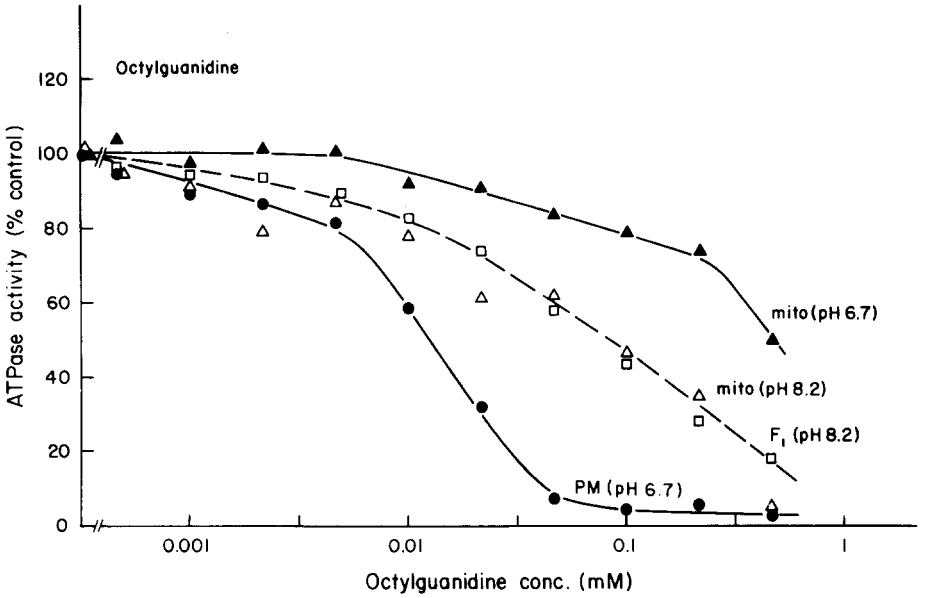

Fig. 4. Effect of octylguanidine on ATPase activity. Symbols and reaction conditions are described in the legend to Fig. 1. Control activities were 1.52 μmol/min per mg protein for plasma membrane ATPase at pH 6.7; 0.73 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 1.47 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 33.6 μmol/min per mg protein for F_1 ATPase at pH 8.2.

against the plasma membrane ATPase (half-maximal inhibition at 0.015 mM) than against mitochondrial ATPase (half-maximal inhibition at 0.09—0.5 mM, depending upon the pH) (Table I). By contrast, triphenylsulfonium chloride inhibited in all cases (plasma membrane ATPase at pH 6.7; membrane-bound mitochondrial ATPase at pH 6.7 and 8.2; F_1 ATPase at pH 8.2) over the same concentration range (half-maximal inhibition at 0.02—0.20 mM; Table I).

Quercetin and other flavonoids

Special attention has been paid during the past few years to bioflavonoids, which are widely distributed in plants and which inhibit the F_1 ATPase of some mammalian mitochondria (refs. 47 and 48; but see ref. 49), CF_1 ATPase from chloroplasts [50], the ATPase of *Escherichia coli* [51], the $(Na^+ + K^+)$ATPase of the plasma membrane of animal cells [52,53], and the Ca^{2+}-ATPase of sarcoplasmic reticulum [54]. Fig. 5 and Table II show that the bioflavonoids also inhibit the plasma membrane ATPase and the mitochondrial ATPase of *Neurospora.* The five compounds tested (fisetin, morin, myricetin, quercetin, and rutin) were nearly equally effective against the plasma membrane ATPase, with half-maximal inhibition in the range 0.1—0.3 mM (Fig. 5; Table II; additional data not shown). They were less effective against mitochondrial ATPase, in some cases failing to produce half-maximal inhibition even at the highest concentration tested (1.0 mM).

Diethylstilbestrol

Diethylstilbestrol has been reported to alter the membrane current and mem-

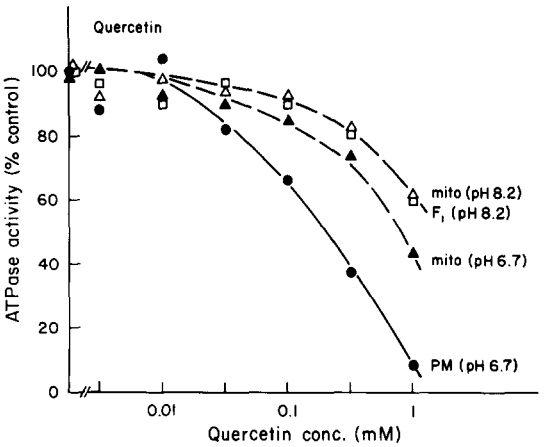

Fig. 5. Effect of quercetin on ATPase activity. Symbols are described in the legend to Fig. 1. ATPase activity was assayed by the release of $^{32}P_i$ from [γ-^{32}P]ATP as described previously [14]. Control activities were 1.72 μmol/min per mg protein for plasma membrane ATPase at pH 6.7; 0.80 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 1.96 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 51.5 μmol/min per mg protein for F_1 ATPase at pH 8.2.

TABLE II

INHIBITION OF ATPase ACTIVITY BY BIOFLAVONOIDS

Reaction conditions are described in the legend to Fig. 5. Control activities were 1.31—1.89 μmol/min per protein for plasma membrane ATPase, 0.49—0.81 μmol/min per mg protein for mitochondrial ATPase at pH 6.7, 0.92—1.97 μmol/min per mg protein for mitochondrial ATPase at pH 8.2, and 34.9—51.5 μmol/min per mg protein for F_1 ATPase at pH 8.2.

Inhibitor (mM)	Inhibition (%)			
	Plasma membrane ATPase (pH 6.7)	Mitochondrial ATPase (pH 6.7)	Mitochondrial ATPase (pH 8.2)	F_1 ATPase (pH 8.2)
Fisetin				
0.3	58.8	8.6	0	0
1.0	80.5	24.8	1.6	15.3
Morin				
0.3	81.3	17.3	10.6	28.0
1.0	97.4	74.9	58.8	63.5
Myricetin				
0.3	68.8	46.4	16.0	0
1.0	92.3	71.5	13.3	n.d.
Quercetin				
0.3	62.4	25.6	17.6	18.5
1.0	91.2	56.5	37.7	39.5
Rutin				
0.3	49.8	1.4	8.2	1.0
1.0	90.2	25.8	0	21.7

n.d., not determined.

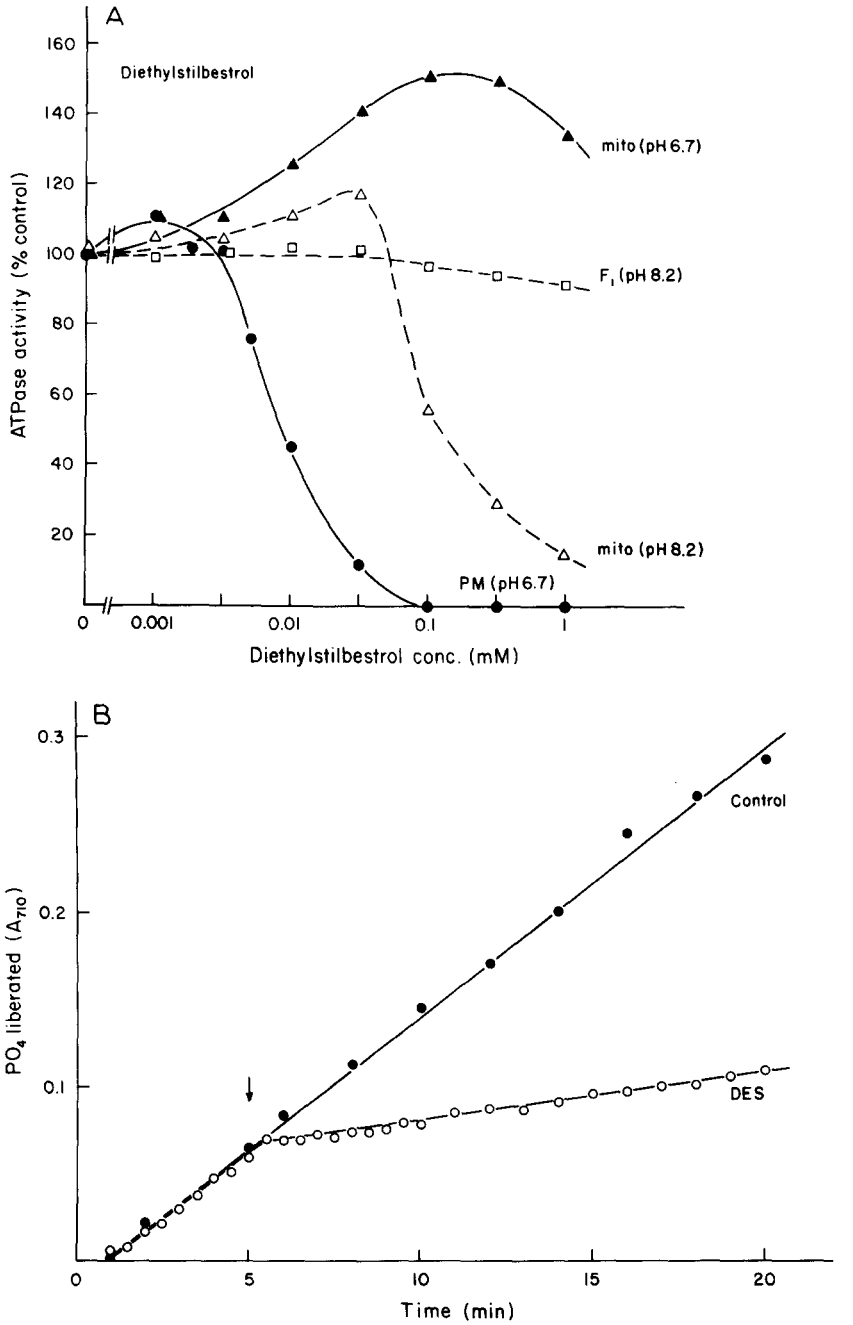

Fig. 6. Effect of diethylstilbestrol on ATPase activity. Symbols and reaction conditions are as described in the legend to Fig. 1. In A, control activities were 1.45 μmol/min per mg protein for plasma membrane ATPase at pH 6.7; 0.69 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 1.54 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 37.5 μmol/min per mg protein for F_1 ATPase at pH 8.2. In B, the reaction mixture contained 0 or 10 μM diethylstilbestrol (added at arrow). The reaction was started by the addition of plasma membrane ATPase, and was terminated after 0 to 20 min by the addition of 0.1 ml 50% trichloroacetic acid. Inorganic phosphate was assayed in a portion of the supernatant by the method of Dryer et al. [27], and the liberation of P_i (A_{710nm}) is plotted as a function of time.

brane conductance of voltage-clamped *Chara* cells [55], and the membrane potential of *Nitella* [56], in a manner consistent with a direct effect on the electrogenic pump of the plasmalemma it is also a potent inhibitor of plasma membrane ATPase activity in higher plants [57,58]. Fig. 6A shows that it is similarly effective against the *Neurospora* plasma membrane ATPase, producing half-maximal inhibition at approx. 0.009 mM (considerably below the concentration at which it causes generalized perturbation of artificial lipid films; ref. 59). Because the effect of diethylstilbestrol in the whole-cell *Chara* experiments was quite slow [55], the time-course of inhibition of the *Neurospora* plasma membrane ATPase was determined; the results, illustrated in Fig. 6B, indicate that inhibition of ATPase activity in isolated membranes was rapid, occurring in about 0.5 min.

By contrast with its straightforward inhibition of plasma membrane ATPase activity, diethylstilbestrol had complex effects on the membrane-bound form of mitochondrial ATPase (see also ref. 60). At pH 6.7, it stimulated this activity by 50% as the concentration was raised to 0.1 mM, and then began to inhibit as the concentration was further increased to 1 mM. At pH 8.2, the same pattern was observed but was shifted to lower concentrations, with stimulation up to 0.03 mM and sharp inhibition above this point. Diethylstilbestrol had very little effect on solubilized F_1 ATPase.

Vanadate

By far the most effective inhibitor of the plasma membrane ATPase turned out to be sodium vanadate, recently found to be a contaminant in Sigma ATP prepared from equine muscle and to be a potent inhibitor of the $(Na^+ + K^+)$-ATPase of animal cells [26,61]. Fig. 7 illustrates that vanadate produced 50% inhibition of the *Neurospora* plasma membrane ATPase at a concentration of

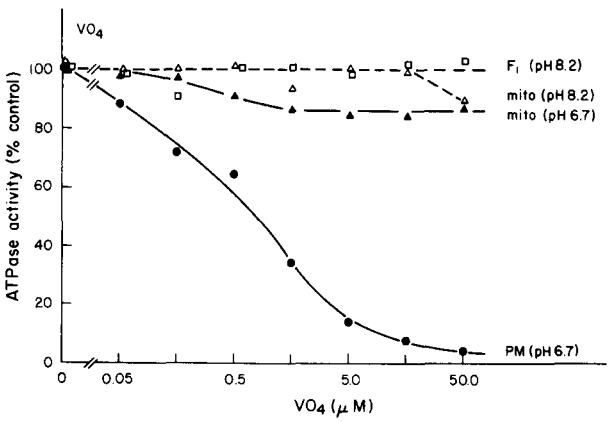

Fig. 7. Effect of vanadate on ATPase activity. Symbols and reaction conditions are described in the legend to Fig. 1, except that Boehringer-Mannheim ATP (instead of Sigma ATP) was used, and the reaction mixture for the plasma membrane ATPase at pH 6.7 lacked EDTA. The amounts of enzyme protein were (in 0.5 ml) 70 μg for the plasma membrane ATPase, 35 μg for mitochondrial ATPase, and 1.6 μg for F_1 ATPase. Control activities were 2.30 μmol/min per mg protein for plasma membrane ATPase at pH 6.7; 1.03 μmol/min per mg protein for mitochondrial ATPase at pH 6.7; 2.36 μmol/min per mg protein for mitochondrial ATPase at pH 8.2; and 52.0 μmol/min per mg protein for F_1 ATPase at pH 8.2.

approx. 1 μM and greater than 95% inhibition at 50 μM. Under the conditions used (pH 6.7; concentration less than 0.1 mM), it exists predominantly as the orthovanadate ion, $H_2VO_4^-$ [62], which is believed to be the inhibitory species for the $(Na^+ + K^+)$ATPase as well [26].

Over the same range of concentrations, vanadate had no significant effect on either membrane-bound or solubilized mitochondrial ATPase. (The apparent 15% inhibition of mitochondrial ATPase at pH 6.7, shown in Fig. 7, occurs in the concentration range that is effective against plasma membrane ATPase, and probably reflects contamination of this particular mitochondrial preparation by plasma membrane fragments.)

Other compounds tested

A number of other compounds were found to have no significant effect on the plasma membrane ATPase (Table III). These compounds include: phaseolin, a phytoalexin which causes rapid depolarization of the *Neurospora* membrane and which had been proposed as a possible inhibitor of the plasma membrane ATPase [63]; deoxycorticosterone, which inhibits K^+ transport in *Neurospora* [64,65]; cyclic AMP, cyclic GMP, theobromine, theophylline, and histamine, tested because of indirect evidence linking cyclic nucleotides to regulation of the membrane potential in *Neurospora* [66,67]; fusicoccin, which has been reported to stimulate the K^+-activated ATPase of higher plants [68]; and alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide), salicylic acid, sodium *N*-1-naphthylphthalamate, and triiodobenzoic acid, four compounds that inhibit metabolite transport in higher plants [69—71]. Previous work has shown that ouabain is also without effect on the plasma membrane ATPase of *Neurospora* [14,15].

TABLE III

EFFECTS OF OTHER COMPOUNDS ON THE PLASMA MEMBRANE ATPase

Reaction conditions are described in the legend to Fig. 1. Control activities were 1.08—2.39 μmol/min per mg protein. The numbers in parentheses refer to stimulation of activity.

Inhibitor	Concentration tested	Inhibition (%)
Phaseolin	0.12 mM	0.0
Deoxycorticosterone	0.1 mM	6.8
	1.0 mM	11.1
Cyclic AMP	1.0 mM	12.0
Cyclic GMP	1.0 mM	0.9
Theobromine	1.0 mM	(8.5)
Theophylline	1.0 mM	(6.3)
Histamine	1.0 mM	(19.0)
Fusicoccin	6 μg/ml (0.1 μg/mg protein)	0.7
Alachlor	0.17 mM	9.2
	1.0 mM	30.5
Salicyclic acid	0.17 mM	13.6
	1.0 mM	3.1
Triiodobenzoic acid	0.5 mM	0.0
N-1-Naphthylphthalamate	1.0 mM	5.0

Discussion

A specific aim of the work presented in this paper was to examine the relationship between the plasma membrane ATPase and the mitochondrial ATPase of *Neurospora*. It seems clear, on the basis of inhibitor sensitivities, that these are distinct enzymes; while this work was in progress, a report appeared reaching the same conclusion for the yeast *S. pombe* [19]. Some of the compounds tested in the present study (DCCD, octylguanidine, quercetin and related bioflavonoids) were active against both the plasma membrane ATPase and mitochondrial ATPase of *Neurospora*, but many affected either one or the other fairly selectively. In practical terms, the most useful inhibitors appear to be vanadate in the case of the plasma membrane ATPase and azide in the case of mitochondrial ATPase (azide being preferable to oligomycin in purification studies because it is active against solubilized F_1 as well as against the membrane-bound form of mitochondrial ATPase). By choosing the appropriate concentration (15 μM vanadate; 5 mM azide), each of these compounds can be made to inhibit more than 95% of one ATPase but less than 5% of the other.

In theoretical terms, the results with inhibitors which act upon the F_o basepiece of mitochondrial ATPase are of particular interest. Both oligomycin and DCCD have recently been demonstrated to interact with a proteolipid component of F_o, which can be extracted from the mitochondrial membrane with chloroform/methanol [72,73] and which has been designated subunit 9 (of the yeast mitochondrial ATPase complex [74]). Treatment of mitochondria with [^{14}C]DCCD leads to the covalent labeling of subunit 9 [75,76], and mutation of either yeast or *Neurospora* to oligomycin resistance is correlated with a change in the amino acid sequence of subunit 9 [77]. Subunit 9 appears to play a physiologically important role in proton translocation through the mitochondrial membrane, since removal of F_1 ATPase greatly increases the proton permeability of mitochondrial vesicles, and the addition of oligomycin or DCCD reverses this increase [78]. Furthermore, in recent studies with artificial phospholipid membranes, subunit 9 was shown to discharge the potential difference that had been generated across the membrane (a photopotential resulting from the action of light on bacteriorhodopsin); and the addition of oligomycin caused the potential difference to be reestablished [79]. All of these results can be interpreted to mean that the subunit 9 proteolipid somehow mediates the transmembrane movement of H^+ that is intimately linked to the synthesis or hydrolysis of ATP by the mitochondrial ATPase complex [78]. An analogous proteolipid, sensitive to DCCD but not to oligomycin, has been demonstrated in the F_o basepiece of the proton-translocating bacterial ATPase [80—82]. One must therefore ask whether the plasma membrane ATPase of *Neurospora* also possesses a proteolipid subunit that functions in proton translocation. The inhibitor results presented above are certainly consistent with this possibility. Although the plasma membrane ATPase is not sensitive to oligomycin or venturicidin, thus ruling out the idea of a single proteolipid common to the mitochondrial and plasma membranes, it is extremely sensitive to DCCD, being inhibited at concentrations not much greater than those effective against mitochondrial and bacterial ATPases. Further studies, making use of ^{14}C-labeled DCCD and chloroform/methanol extraction, should reveal whether or not a

DCCD-binding proteolipid is present in the *Neurospora* plasma membrane.

Finally, it will be interesting to explore in a more general way the relationship between fungal plasma membrane ATPases and membrane-bound ATPases that have been studied in animal and plant cells. Preliminary results indicate that the purified plasma membrane ATPases of *S. pombe* and *Neurospora* contain a single large polypeptide subunit of molecular weight 100 000—105 000 (Goffeau, A., personal communication; Bowman, B.J., Blasco, F. and Slayman, C.W., unpublished experiments), thus resembling the $(Na^+ + K^+)$ATPase of animal cells (which contains a large polypeptide of 89 000—135 000 daltons and a glycoprotein of 35 000—57 000 daltons [83]) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (with a subunit of 102 000 daltons [84]). In this regard, it is perhaps significant that the *Neurospora* plasma membrane ATPase, although insensitive to ouabain (the classical inhibitor of $(Na^+ + K^+)$ATPase), is extremely sensitive to vanadate, recently identified as a contaminant in Sigma ATP and found to be a potent inhibitor of $(Na^+ + K^+)$ATPase (but not of Ca^{2+}-ATPase or mitochondrial ATPase) [26,61]. Studies are now in progress to investigate the mechanism of vanadate inhibition in greater detail.

The relationship of the fungal plasma membrane ATPase to ATPases found in the plasma membranes of algae and higher plants will be equally important to investigate, although little direct information is available at the present time. Considerable indirect evidence points to the existence of an electrogenic proton pump in the giant algae *Chara* and *Nitella* [55,85—87]; and if the pump is driven by ATP, as it is in *Neurospora*, algal plasma membranes should contain an ATPase related functionally (and perhaps structurally) to the fungal ATPase. The problem of isolating reasonable amounts of algal plasma membranes free of contamination by mitochondrial and chloroplast membranes is a difficult one, however, and no relevant cell fractionation studies have yet been reported. In the case of higher plants, a substantial body of data exists concerning ATPase activity in isolated plasma membranes, and has been interpreted in terms of a K^+-stimulated ATPase (believed to be involved in K^+ transport) and possibly other ion-stimulated ATPases as well [88]. The plasma membrane ATPase from corn roots is quite sensitive to DCCD [89] and to diethylstilbestrol [54], however. These data, together with the fact that ionic stimulation of ATPase activity can be unrelated to transport (e.g. ref. 23), raise the possibility that at least a portion of the plasma membrane ATPase activity of higher plants may function in proton pumping as in the fungi. Further inhibitor studies of the plant ATPases, coupled with purification of these enzymes and determination of their subunit structure, will ultimately reveal the extent to which plant and fungal ATPases are homologous.

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