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The Inhibition of Mitochondrial Energized Processes by Fluorescein Mercuric Acetate*

Martin J. Lee,† Robert A. Harris, ‡§ T. Wakabayashi° and David E. Green

Institute for Enzyme Research, University of Wisconsin
Madison, Wisconsin 53706

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Abstract

Fluorescein mercuric acetate (FMA) has been shown to be a potent inhibitor of energized processes in both beef heart mitochondria and ETP_H particles. FMA reacts preferentially with a small number of specific sulfur atoms and inhibits the phosphate-dependent configurational transition. FMA enhances the anaerobic to aerobic pH changes observed in intact mitochondria and submitochondrial particles, and also enhances nonenergized swelling in 0·15 M sodium or potassium chloride. The results are interpreted in terms of a model whereby FMA, in reacting with the mitochondrion, modifies its conformation. The resulting conformational changes which occur upon energization are therefore different from those conformational changes which would occur in the absence of FMA. The net result of this process is the inhibition of some processes (e.g., oxidative phosphorylation, ATP-$^{32}P_i$ exchange, etc.) and the enhancement of other processes (the proton shift and nonenergized swelling in chloride salts).

Introduction

Fluorescein mercuric acetate (FMA)‖ has been shown to be a potent reagent for modulating the conformational¶ and configurational cycle of beef heart mitochondria.[1] At low concentration levels (10 nmoles/mg protein) FMA can largely suppress oxidative phosphorylation, ATP-$^{32}P_i$ exchange and a whole set of coupled processes that depend upon inorganic phosphate. The reversibility of these inhibitions by dithiothreitol

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† Postdoctoral trainee of the University of Wisconsin.

‡ Established investigator of the American Heart Association.

§ Present address: Department of Biochemistry, Indiana University Medical Center, Indianapolis, Indiana 46202.

° On leave of absence from the Department of Pathology, Nagoya University School of Medicine, Nagoya, Japan.

‖ Abbreviations used in this work: *m*-ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DTT, dithiothreitol; FMA, fluorescein mercuric acetate; ETP_H, phosphorylating submitochondrial particles; HBHM, heavy beef heart mitochondria.

¶ Conformation is the term we are applying to describe the tertiary and quarternary structure of the repeating units. Configuration is the term applied to describing the geometric and ultrastructural features of the cristal membrane itself.

establishes that FMA is reacting in a reversible manner with groups that modulate coupling.

The present study suggests that the reaction of FMA with groups in the repeating units of the cristal membrane alters the conformation of these units. The conformational cycle which the mitochondrion undergoes in the presence of FMA may then be a modified cycle in which certain processes are arrested (e.g., phosphorylation) while other processes are enhanced (e.g., the anerobic to aerobic proton jump). With the present results as a starting point it may be possible to analyze the effects of various reagents in terms of how they modify the conformational transitions inherent to the energization process.

Materials and Methods

Preparation of Mitochondria and Mitochondrial Fractions

Heavy beef heart mitochondria were prepared by the method of Crane[2] as described by Hatefi and Lester,[3] except that 10 mM Tris-Cl (pH 7·4) was substituted for the phosphate buffer and 1 mM EGTA was included in the isolation medium. The phosphorylating submitochondrial particle, ETP_H, was prepared from HBHM by the method of Hansen and Smith.[4] Protein was determined colorimetrically by the biuret method of Gornall *et al.*[5]

Assay of Enzymatic Activities

Reaction mixtures for determination of ATP-$^{32}P_i$ exchange activity contained in a final volume of 1 ml, 250 μmoles sucrose, 20 μmoles Tris-Cl (pH 7·4), 10 μmoles $MgCl_2$, 2 μg rotenone, 10 μmoles ATP, 10 μmoles potassium phosphate-$^{32}P_i$, 1 mg mitochondrial protein and the indicated quantity of FMA. Samples were incubated for 2 min at 30° before starting the reaction by addition of ATP and $^{32}P_i$. The reaction was stopped after 2 min by the addition of 0·1 ml perchloric acid (6 N). The precipitated protein was removed by centrifugation, and a 0·2 ml sample of the supernatant solution was extracted with solvent by the method of Lindberg and Ernster[6] to separate organic from inorganic phosphate. After extraction, 0·5 ml of the aqueous phase (containing the organic phosphate) was withdrawn and the radioactivity determined in a Packard Tri-Carb scintillation counter. Where indicated, dithiothreitol (5 mM) was added to the reaction mixtures after the first minute of incubation, i.e., 1 min before starting the reaction by the addition of ATP and $^{32}P_i$.

ATP synthesis was measured as described by MacLennan *et al.*[7] The reaction mixtures contained in a final volume of 2·5 ml, 690 μmoles sucrose, 58 μmoles glucose, 100 μg hexokinase, 11·5 μmoles $MgCl_2$, 7 μmoles ADP, 23 μmoles potassium phosphate labelled with $^{32}P_i$, 20 μmoles potassium succinate and 4 mg mitochondrial protein. Oxygen consumption was followed with a Clark electrode connected to a Beckman Oxygen Analyzer and recorder. Aliquots (0·1 ml) were removed after 1 min and assayed for organic phosphate.[6]

ATPase activity was measured at 30° in reaction mixtures (final volume 1 ml) containing 250 μmoles sucrose, 20 μmoles Tris-Cl (pH 7·4), 10 μmoles $MgCl_2$, 2 μg rotenone, 10 μmoles ATP and 1 mg mitochondrial protein. Samples were preincubated for 2 min at 30° before addition of ATP. Reactions were terminated after 2 min. Inorganic phosphate was separated from organic phosphate by the method of Lindberg and Ernster[6]

and determined colorimetrically by the method of Martin and Doty.[8] Where indicated, DTT (95 μmoles) or rutamycin (2 μg) was added after 1 min of incubation and allowed to react for 1 min before the addition of ATP.

The energized uptake of calcium ions was measured by a modification of the method of Penniston *et al.*[9] Reaction mixtures for ATP-energized calcium translocation (final volume 3 ml) contained 750 μmoles sucrose, 10 μmoles imidazole chloride (pH 7·0), 30 μmoles $MgCl_2$, 5 μg rotenone, 10 μmoles potassium phosphate (pH 7·0), 3 μmoles $^{45}CaCl_2$, 10 μmoles ATP and 2·0 mg mitochondrial protein. When substrate-energized translocation was measured, potassium succinate (25 μmoles) and rutamycin (4 μg) were included in the reaction medium. Reaction mixtures were incubated for 3 min at 30° before starting the reaction by addition of $^{45}CaCl_2$. Reactions were stopped after 1 min by the addition of 100 μg of either antimycin (for substrate-energized translocation) or rutamycin (for ATP-energized translocation). The reaction mixtures were layered over 0·88 M sucrose and centrifuged at 106,000 *g* for 20 min. The pellet was homogenized in 1 ml water, and an aliquot counted in a Packard Tri-Carb scintillation counter.

Measurement of Light Scattering

Light scattering at 90° to the incident beam was measured in a Brice Phoenix series 2000 light-scattering photometer as described by Harris *et al.*[10] Reaction mixtures for measuring light scattering by beef heart mitochondria contained (final volume 3 ml) 750 μmoles sucrose, 15 μmoles Tris-Cl (pH 7·5), 20 μg rotenone (in ethanol) and 3·2 mg mitochondrial protein. Where indicated the following additions were made: 15 μmoles potassium succinate (pH 7·5), 30 μmoles potassium phosphate (pH 7·5), 32 nmoles FMA (pH 7·5), 15 μmoles dithiothreitol and 5×10^{-9} moles *m*-ClCCP (in ethanol).

Reaction mixtures for measuring light scattering by ETP_H (final volume 3 ml) contained 750 μmoles sucrose, 15 μmoles Tris-Cl (pH 7·5), 30 μmoles potassium phosphate (pH 7·5) and 1·5 mg submitochondrial particles, as described by Harris *et al.*[11] Where indicated, the following additions were made: 15 μmoles ATP, 3 μg antimycin (in ethanol), 30 nmoles FMA (pH 7·5) and 15 μmoles dithiothreitol.

Measurement of Proton Shift

Reaction mixtures for measuring the pH change (hereafter called the proton shift) which occurs during the anaerobic to aerobic transition contained in a final volume of 6 ml, 1500 μmoles sucrose, 50 μmoles tetramethyl ammonium succinate (pH 7·4), 50 μg rotenone (in ethanol), 600 μg catalase and 18 mg submitochondrial particles or beef heart mitochondria. The following additions were made where indicated: hydrogen peroxide (0·02 ml of 0·3%), 90 μmoles dithiothreitol and varying amounts of the tetramethyl ammonium salt of fluorescein mercuric acetate. Changes in pH were measured with a Sargent model 5-30070-10 combination pH electrode coupled to a Beckman expandomatic pH meter.

Electron Microscopy

Samples for electron microscopy were fixed exactly as described by Allmann *et al.*,[12] except that glutaraldehyde was added to a final concentration of 2%. Sections were examined in a Hitachi HU-11B electron microscope.

Results

Fluorescein mercuric acetate inhibits ATP synthesis and related coupled processes at the same concentrations that it accelerates ATPase activity. The following experiments illustrate these points.

Inhibitory Action of FMA on ATP-$^{32}P_i$ Exchange

Fluorescein mercuric acetate inhibited ATP-$^{32}P_i$ exchange in both beef heart mitochondria and submitochondrial particles. Figure 1 shows the ATP-$^{32}P_i$ exchange

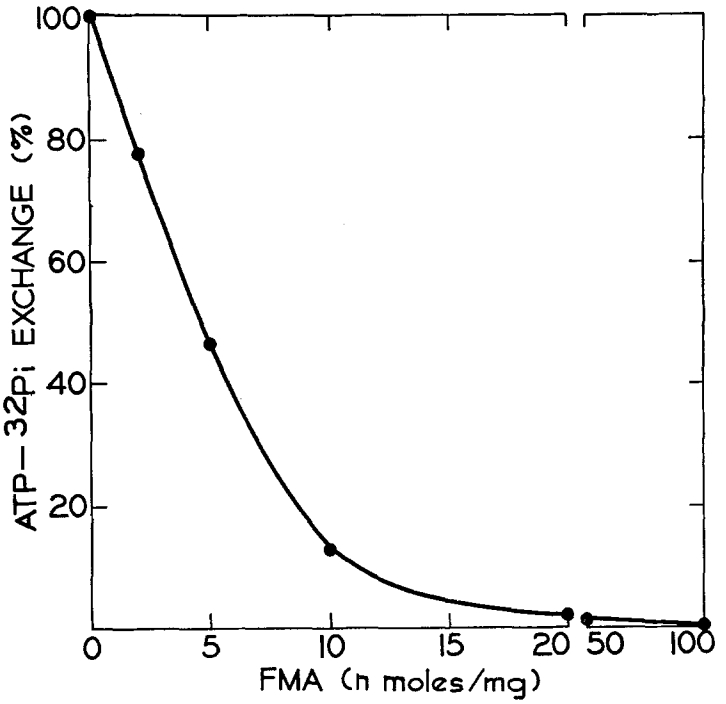


Figure 1. Effect of FMA on ATP-$^{32}P_i$ exchange of ETP_H. Reaction mixtures contained in a final volume of 1 ml, 250 μmoles sucrose, 20 μmoles Tris-Cl (pH 7·4), 10 μmoles $MgCl_2$, 2 μg rotenone, 10 μmoles ATP, 10 μmoles $^{32}P_i$, 1 mg ETP_H and the indicated quantity of FMA. One hundred percent activity corresponds to 140 nmoles of exchange activity per minute per milligram of protein.

activity of ETP_H as a function of the concentration of FMA. Exchange activity was inhibited 50% by FMA at a concentration of 4·4 nmoles/mg protein. Figure 2 shows that dithiothreitol at a concentration of 5 μmoles/mg protein effectively reversed this inhibitory action of FMA. At higher levels of dithiothreitol, reversal of the inhibition of exchange activity is less complete though still pronounced.

Table I contains a comparison of the effect of FMA on ATP-$^{32}P_i$ exchange activities in both submitochondrial particles and beef heart mitochondria. ATP-$^{32}P_i$ exchange

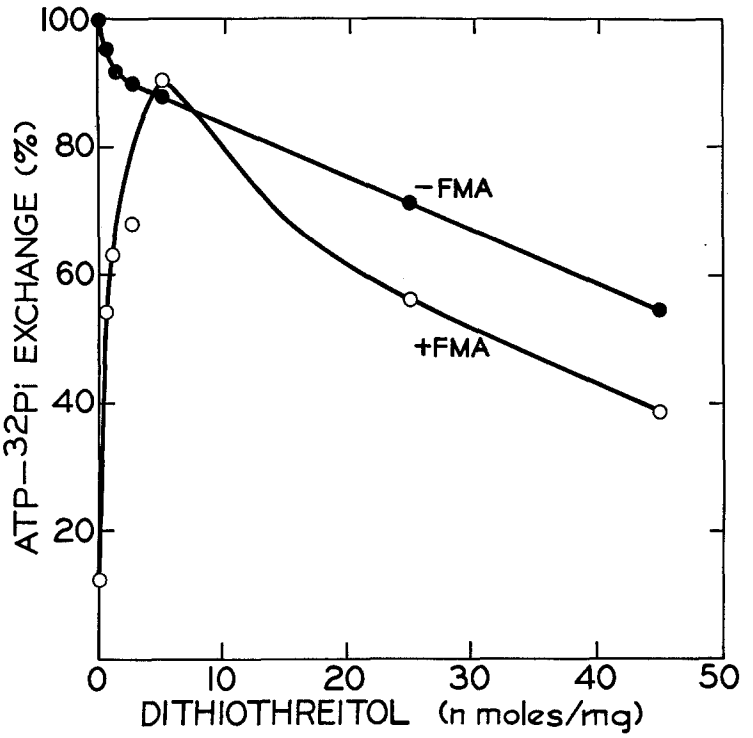


Figure 2. Reversal of the inhibition by FMA of ATP-$^{32}P_i$ exchange. Dithiothreitol was added one minute after the addition of FMA (10 nmoles/mg) and allowed to react for one additional minute before addition of ATP and $^{32}P_i$ to the medium. Other conditions were the same as those described in the legend of Fig. 1.

activity was inhibited in both preparations. While the reversal of inhibition by dithiothreitol is not very dramatic in beef heart mitochondria, significant reversal is nonetheless apparent.

TABLE I. Effect of FMA on ATP-$^{32}P_i$ exchange* (nmoles P_i exchanged per minute per milligram of protein)

Additions	HBHM	ETP_H
None	164	140
FMA (10 nmoles/mg protein)	1	7
Dithiothreitol (5 μmoles/mg protein)	152	129
FMA + dithiothreitol	44	148

* Reaction mixtures for determination of ATP-$^{32}P_i$ exchange rates contained in a final volume of 1 ml, 250 μmoles sucrose, 20 μmoles Tris-Cl (pH 7·4) 10 μmoles $MgCl_2$, 2 μg rotenone, 10 μmoles potassium phosphate-$^{32}P_i$ and 1 mg mitochondrial protein. Other additions were as indicated above.

TABLE II. Effect of FMA on ATPase activity* (nmoles P_i released per minute per milligram of protein)

Additions	HBHM	ETP_H
None	137	404
FMA (10 nmoles/mg protein)	189	493
Dithiothreitol (5 μmoles/mg protein)	149	342
FMA + dithiothreitol	142	361

* Reaction mixtures for measuring ATPase rates contained in a final volume of 1 ml, 250 μmoles sucrose, 20 μmoles Tris-Cl (pH 7·4), 10 μmoles ATP and 1 mg mitochondrial protein. Other additions were as indicated above.

Effect of FMA on ATPase Activity

Table II shows the effect of FMA (10 nmoles/mg protein) on the ATPase activity of both HBHM and ETP_H. A 38% stimulation of the ATPase activity of beef heart mitochondria and a 22% stimulation of the ATPase activity of submitochondrial particles was observed. This enhancement does not appear great enough to account for the inhibition by FMA of ATP-$^{32}P_i$ exchange activity. While the data are not shown in Table II, FMA did not affect the oligomycin sensitivity of ATPase activity. The sensitivity of ATPase activity to oligomycin but not to FMA shows that these two inhibitors have different actions.

Effect of FMA on Oxidative Phosphorylation

The effect of FMA on the P/O ratio and rate of phosphorylation was examined next (Table III) in order to confirm that coupled processes generally are sensitive to the inhibitor. The substrate in these experiments was potassium succinate (4 mM). FMA, at a concentration of 10 nmoles/mg protein, inhibited phosphorylation in both ETP_H and intact mitochondria. It did not, however, affect the efficiency of that small portion of the total phosphorylation capability of ETP_H which remained. In mitochondria, however, FMA induced a partial modification of the residual phosphorylating capability as indicated by the lowering of the P/O ratio from 0·94 to 0·20. The mechanism of

TABLE III. Effect of FMA on ATP synthesis and P/O ratio*. Values are expressed as nmoles esterified phosphate per minute per milligram of protein

	HBHM		ETP_H	
Additions	ATP Synthesis	P/O	ATP Synthesis	P/O
None	153	0·94	110	0·76
FMA	10·5	0·20	34·6	0·91
FMA + dithiothreitol	144	1·0	138	0·99
Dithiothreitol	155	1·1	141	0·74

* Reaction mixtures contained in a final volume of 2·5 ml, 625 μmoles sucrose, 75 μmoles glucose, 125 μg hexokinase, 12·5 μmoles $MgCl_2$, 7·5 μmoles ADP, 25 μmoles potassium phosphate-$^{32}P_i$ and 5 mg mitochondrial protein. The concentration of FMA, where present, was 10 nmoles/mg, and that of dithiothreitol 5 μmoles/mg.

action of FMA, however, cannot be directly on respiration, since FMA inhibits ATP-$^{32}P_i$ exchange and both substrate and ATP-driven Ca^{2+} translocation (see below).

Effect of FMA on Calcium Translocation

The effect of FMA on coupled processes was extended further by the study of calcium ion translocation (in beef heart mitochondria) energized by succinate or ATP. Table IV shows that FMA suppressed almost completely both ATP- and substrate-driven uptake of calcium ions. In both cases the addition of dithiothreitol reversed the action of FMA and increased by 40–50% the net rate of Ca^{2+} translocation.

TABLE IV. Effect of FMA on calcium ion translocation

Additions	Rate of substrate-driven translocation*	Rate of ATP-driven translocation*
None	228	101
FMA (22·5 nmoles/mg protein)	73	7
Dithiothreitol (7·5 μmoles/mg protein)	218	97
FMA + dithiothreitol	325	151

* Values are expressed as nmoles Ca^{2+} accumulated per minute per milligram of protein. Reaction mixtures contained in a final volume of 3 ml, 750 μmoles sucrose, 10 μmoles imidazole chloride (pH 7·0), 30 μmoles $MgCl_2$, 5 μg rotenone, 10 μmoles potassium phosphate (pH 7·0), 3 μmoles $^{45}CaCl_2$, 10 μmoles ATP and 2 mg mitochondrial protein. Other additions were as indicated above.

We next undertook to determine which aspects of the conformational cycle were particularly sensitive to FMA. The configurational cycle of beef heart mitochondria[13] was examined extensively as a possible reflection of conformational events. The following studies show that FMA inhibits primarily the generation of the energized-twisted configuration from the nonenergized or energized configuration.

Effect of FMA on Configurational Change

In order to correlate the inhibitory action of FMA on configurational changes in the cristal membrane a series of electron microscopic investigations was initiated. The reaction mixture for this study contained both succinate and inorganic phosphate so that the majority of the mitochondria were in the energized-twisted state (Fig. 3A). Addition of FMA to nonenergized mitochondria prevented the subsequent generation of the energized-twisted configuration (Fig. 3B). The population of mitochondria, though mixed, was predominantly in the energized configuration, with a lower frequency of nonenergized configurations.

During the course of these studies we observed what appeared to be a change in the ultrastructural appearance of nonenergized mitochondria induced by FMA. Fig. 4A shows a typical field of nonenergized mitochondria. In Fig. 4B the mitochondria have been modified by the addition of 10 nmoles FMA per milligrams of protein. In a significant number of the mitochondria found in any field the cristal membranes were somewhat thicker and also somewhat straighter than were the cristae in a comparable field of

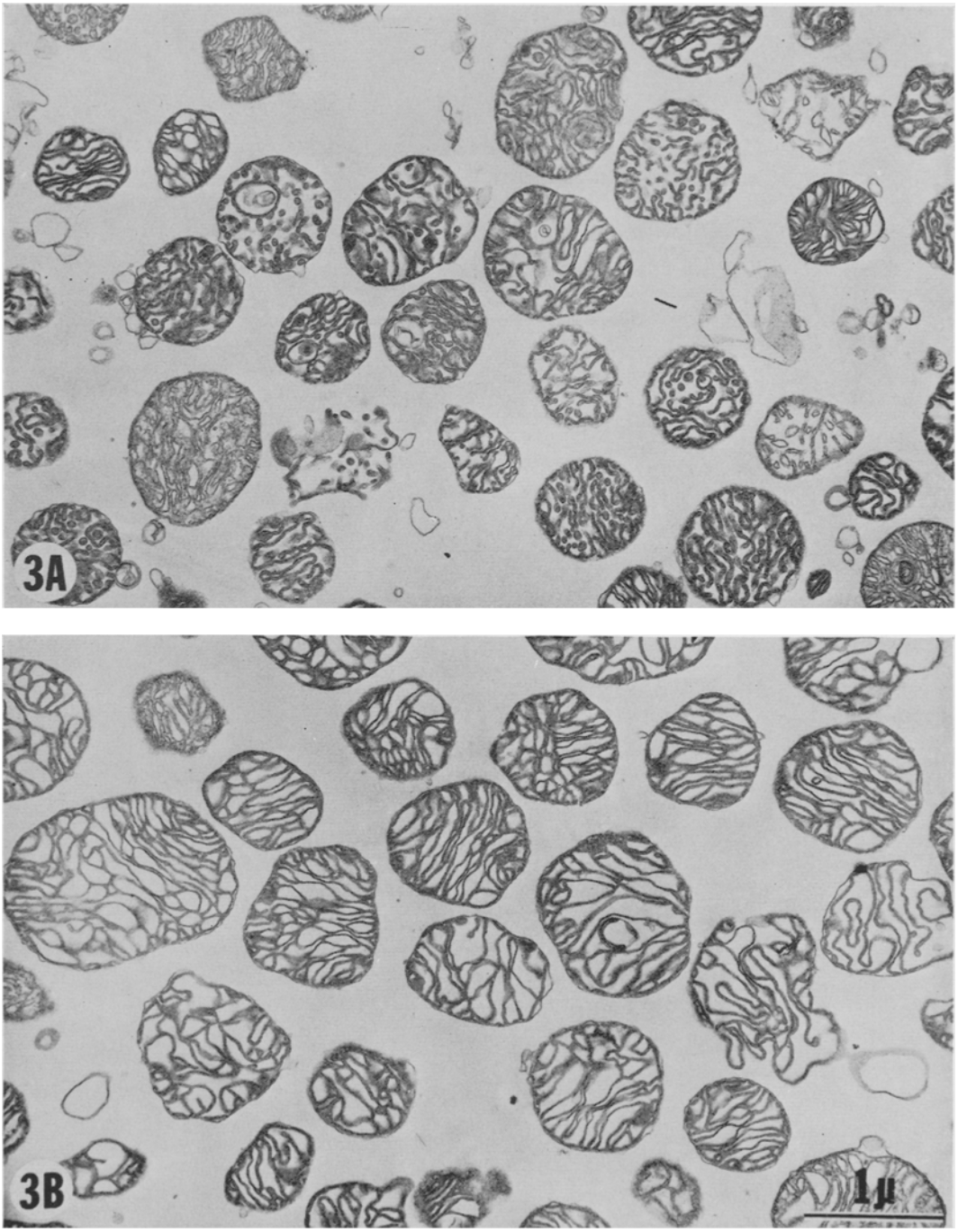


Figure 3. Effect of FMA on the generation of the energized-twisted configuration. The reaction mixture for the experiments described in Fig. 3A (final volume 9 ml), contained sucrose (0·25 M), Tris-Cl (5 mM, pH 7·4), rotenone (2 μg/mg protein), potassium succinate (5 mM), potassium phosphate (10 mM) and 1 mg/ml mitochondrial protein. The reaction mixture for the experiment described in Fig. 3B was identical with the above except for the inclusion of 10 nmoles/mg FMA. Mitochondria were preincubated with FMA 1 min before initiating electron transfer by addition of succinate.

untreated mitochondria. Figure 4C shows that, at a concentration of 40 nmoles/mg protein, FMA induces a marked thickening and straightening of the cristae. The frequency and extent of the alteration is much higher than at the lower concentration level.

Effect of FMA on Energized Configurational Changes as Measured by Light Scattering

The above studies suggested that FMA prevented formation of the energized-twisted configuration. In another set of experiments (not shown) it was demonstrated that FMA was equally effective in discharging the energized-twisted configuration. A study of the effect of FMA on light-scattering changes was, therefore, undertaken in order to confirm the results of electron microscopy.

Figure 5A shows a typical 90° light-scattering trace for beef heart mitochondria. The addition of succinate produced a small light-scattering change that was, in our hands, somewhat variable. The subsequent addition of phosphate, however, resulted in a large-amplitude light-scattering decrease which has been shown to correlate with generation of the energized-twisted configuration.[13] When FMA was present in the reaction mixture (Fig. 5B) the phosphate-induced light-scattering change was completely inhibited. While dithiothreitol alone had no effect on light scattering (not shown), addition of dithiothreitol after FMA (Fig. 5C) completely reversed the inhibitory action. As shown in Fig. 5D, FMA when introduced into the reaction mixture after addition of phosphate, reversed, as well as prevented, the large-amplitude light-scattering changes exhibited by beef heart mitochondria during the energy cycle.

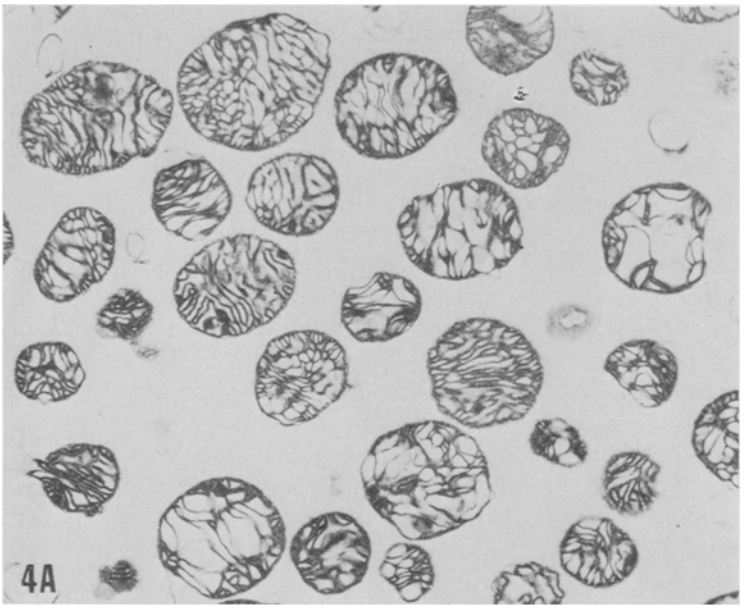
A typical light-scattering response for submitochondrial particles is shown in Fig. 6A. In this experiment, the medium in which the ETP_H particles were suspended already contained inorganic phosphate as described by Harris *et al.*[11] Addition of ATP induced an increase in light scattering which could be largely reversed by addition of rutamycin. Subsequent addition of succinate resulted in a light scattering increase which was abolished by addition of antimycin.

As is evident from Fig. 6B, addition of FMA caused a light-scattering increase which mimicked that induced by ATP or succinate. After addition of FMA, however, no further light-scattering changes could be observed. Addition of dithiothreitol reversed the action of FMA (Fig. 6C) and allowed the normal ATP and succinate-induced light-scattering changes to occur. The action of FMA in producing a light-scattering increase is somewhat curious. While the details remain to be clarified, it seems reasonable to suggest that the reaction of ETP_H with FMA resulted in a conformational change in the repeating units of the particles which induced a configurational change in the membrane as monitored by light scattering.

It was clear from these experiments the FMA prevented the normal energy-dependent light-scattering changes of both intact mitochondria and ETP_H particles. Thus, both electron microscopy and light-scattering measurements show that FMA can inhibit the usual phosphate-dependent configurational cycle. It soon became apparent to us, however, that not all energized processes are inhibited by FMA.

Effect of FMA on the Proton Jump

In aged mitochondria a characteristic release of protons occurs during the transition from anaerobic to aerobic conditions, providing a suitable substrate is present. A similar proton shift, but in the opposite direction (proton uptake) occurs when oxygen is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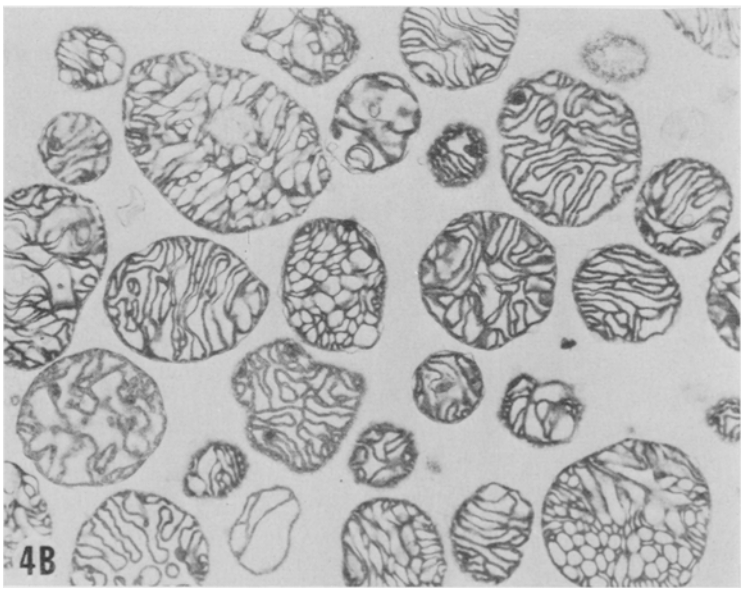
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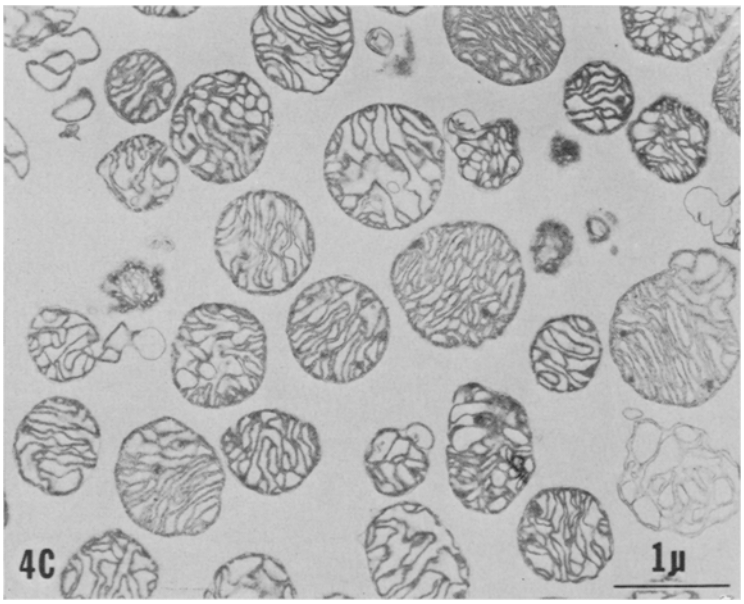

Figure 4. Effect of FMA on the configuration of nonenergized mitochondria. The reaction mixtures (final volume 9 ml) contained sucrose (0·25 M), Tris-Cl, (5 mM, pH 7·4), rotenone (2 μg/mg) and 1 mg/ml mitochondrial protein. The reaction mixture for the experiment described in Fig. 4B contained 10 nmoles/mg FMA in addition to the reactants listed above, while that for the experiment described by Fig. 4C contained 40 nmoles/mg FMA.

introduced into suspensions of ETP_H in the presence of substrate. Figure 7 shows the effects of the tetramethyl ammonium salt of FMA on the proton shift of both HBHM and ETP_H. Addition of 10 nmoles FMA per milligram of protein produced in these experiments a nine-fold stimulation in the proton shift of beef heart mitochondria and a two-fold stimulation in ETP_H particles. If, as we would like to suggest, the energy-dependent uptake or release of protons reflects a conformational transition independent of the binding of inorganic phosphate, FMA actually enhanced the manifestation of this transition.

It appeared at this point that FMA inhibited some coupled processes while enhancing at least one other process (the proton shift). The effect of suboptimal levels of FMA on the proton shift and light-scattering changes was investigated further in the hope of elucidating this apparent discrepancy.

Effect of Sub-Optimal Levels of FMA on the Proton Shift

In the proton-shift experiments reported above, FMA produced its maximum stimulation at a concentration of 10 nmoles/mg. This is the concentration that prevented ATP-$^{32}P_i$ exchange (Fig. 1). The effect of sub-optimal concentrations of FMA on the anaerobic to aerobic proton shift of beef heart mitochondria is revealing (Fig. 8). At a concentration of 6·7 nmoles/mg, FMA produced only a partial stimulation of the proton shift reaction. Furthermore, the amount of protons released never reached an equilibrium value, suggesting that FMA was producing a sub-optimal response at this concentration.

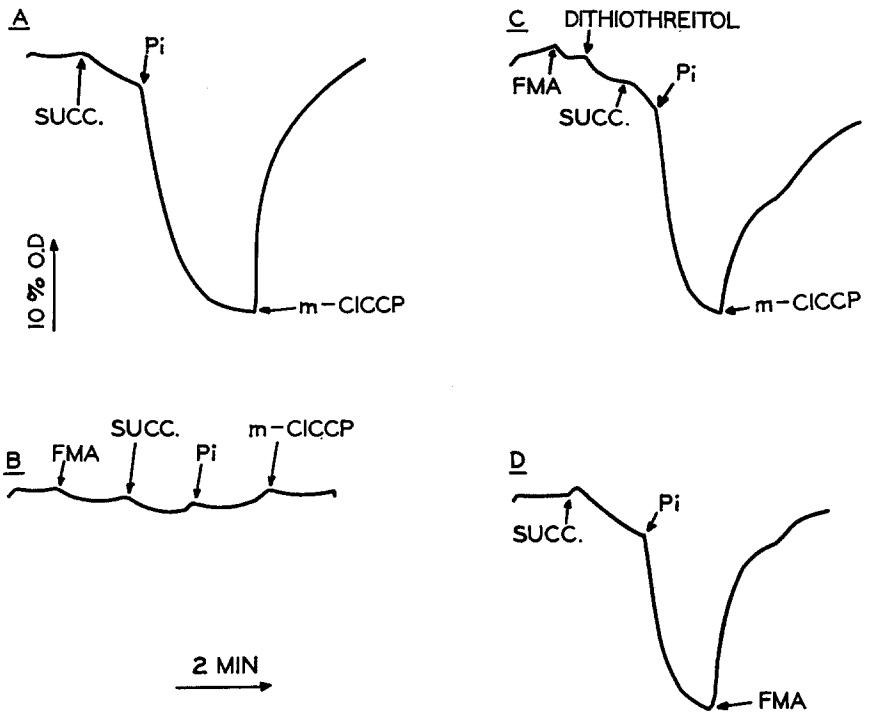

Figure 5. Effect of FMA on the 90° light-scattering response of HBHM. Conditions were as described in the section on methods. Where indicated, the following additions were made: 32 nmoles FMA, 15 μmoles dithiothreitol and 5×10^{-9} moles *m*-ClCCP.

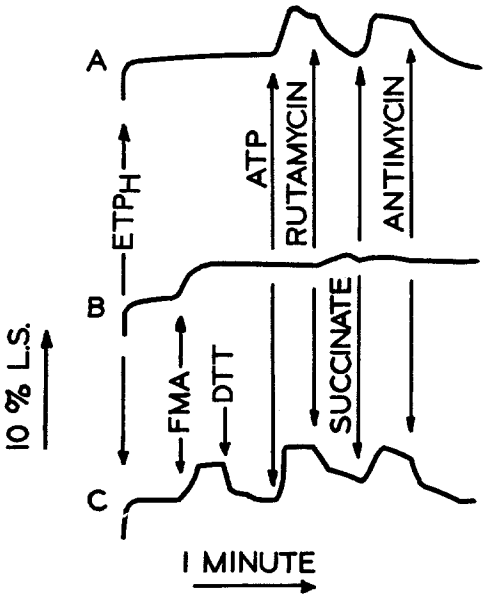

Figure 6. Effect of FMA on the 90° light-scattering response ot ETP_H. Reaction mixtures contained in a final volume of 3 ml, 750 μmoles sucrose, 15 μmoles Tris-Cl (pH 7·5), 30 μmoles potassium phosphate (pH 7·5) and 1·5 mg ETP_H protein. Where indicated, the following additions were made: 3 μmoles ATP, 3 μg rutamycin, 7·5 μmoles potassium succinate, 3 μg antimycin, 30 nmoles FMA and 15 μmoles DTT.

Effect of Sub-Optimal Levels of FMA on Light Scattering

An investigation into the action of sub-optimal levels of FMA shed some light on the mechanism of the inhibition of the light scattering response. In Fig. 9, curves (a) through (e) show the effect of decreasing concentrations of FMA. The amount of FMA used in the experiment described by curve (a) has already been shown to inhibit the rate of ATP-$^{32}P_i$ exchange to the extent of 90%. In the experiment described by curve (e) of Fig. 9, FMA at a sub-optimal level did not inhibit the large-amplitude light-scattering decrease, but the light-scattering change was immediately reversed. The light-scattering change subsequently peaked, started to decrease, reached a trough, and increased again

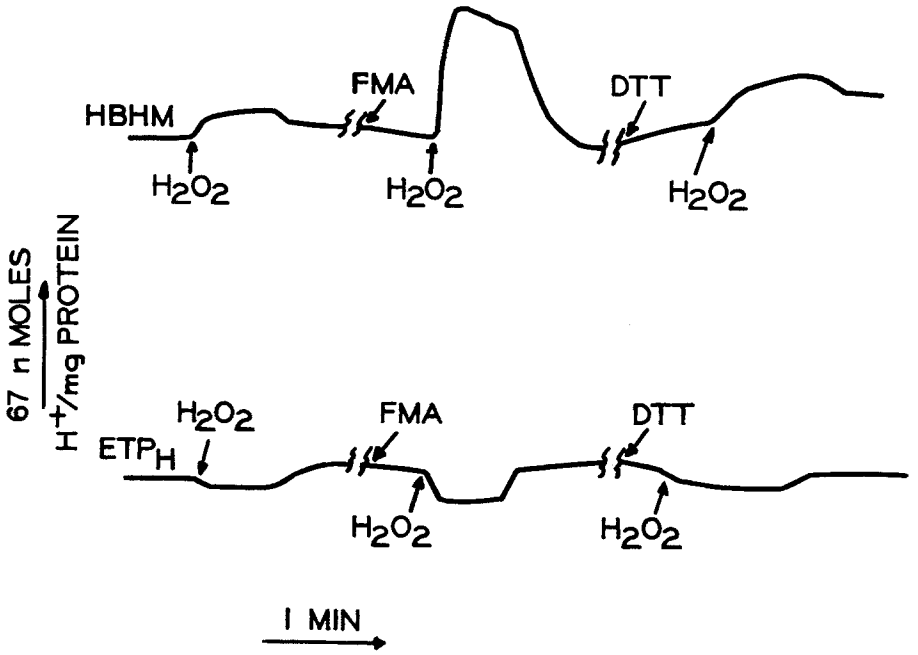

Figure 7. Effect of FMA on the energized proton jump. Conditions were as described in the section on methods. Where indicated, the following additions were made: 0·02 ml hydrogen peroxide (0·3%), 180 nmoles FMA and 90 μmoles dithiothreitol.

until the media became anaerobic. This behavior is not seen in beef heart mitochondria under normal conditions, i.e., in absence of ionophores such as valinomycin.[10]

Specificity of the Action of FMA

The action of sub-optimal levels of FMA in inducing a light-scattering oscillation was reminiscent of the action of valinomycin at appropriate concentration levels in inducing an oscillation[10] of light scattering in beef heart mitochondria. The oscillation, therefore, suggested a possibility not previously considered that the inhibitory action of FMA on the light-scattering response was caused by a moiety which did not interact with sulfhydryl groups and which could participate in ion movements.

The effect of fluorescein and fluorescein amine on the light-scattering response of

beef heart mitochondria was, therefore, investigated. Both reagents which contain no mercury were found to be completely without effect at concentrations up to 100 nmoles/mg protein. While these experiments do not rigorously rule out the possibility that the unreacted form of FMA is the species which inhibits the light-scattering response, they show, nonetheless, that the reactive mercurial groups of FMA are requisite for inhibitory activity.

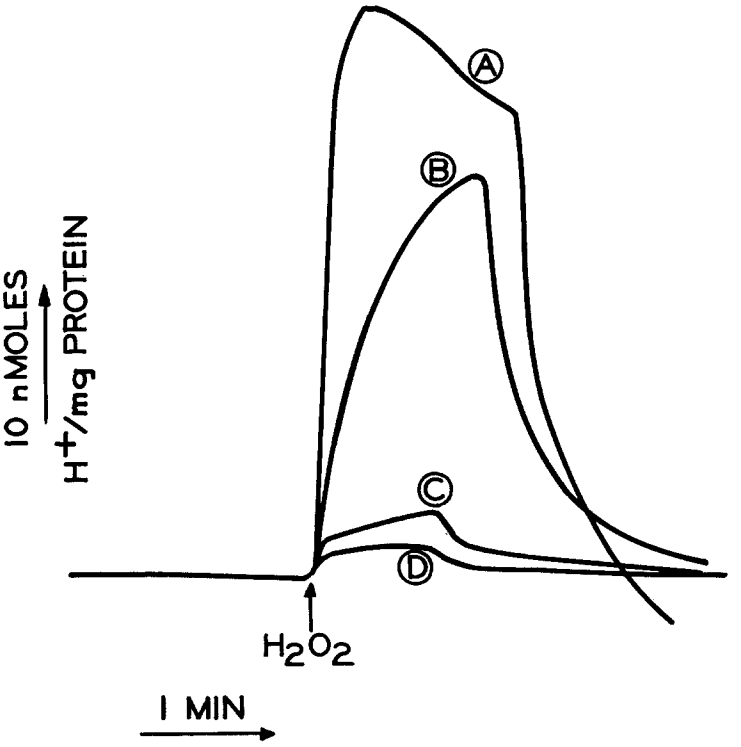

Figure 8. Effect of sub-optimal levels of FMA on the energized proton jump reaction of beef heart mitochondria. The following amounts of FMA were added: curve a, 10 nmoles/mg; curve b, 6·7 nmoles/mg; curve c, 3·3 nmoles/mg; curve d, control without added FMA.

Binding of FMA to HBHM

Figure 10 shows the results of an experiment to determine the number of binding sites for FMA and the binding constant for the interaction of FMA with HBHM. Mitochondria were exposed for 10 min* to FMA and the treated suspension was then sedimented in a Misco high-speed centrifuge. The amount of free FMA was determined from the optical density at 496 mμ of the supernatant solution, and the data plotted by the method of Scatchard.[14] Fluorescein mercuric acetate was found to have an absorption peak with a molar extinction coefficient, ϵ_{496}, of $6{\cdot}5 \times 10^4$ at pH 7·5. It is clear from the data of Fig. 11 that FMA reacted in a biphasic manner—binding very tightly to about 10 nmoles of sulfhydryl per milligram of mitochondrial protein, and

* Separate studies showed that no change in the amount of free FMA occurred after this time.

more loosely to an additional 110–120 nmoles/mg protein. Although the quantitation is subject to some error as no consideration was given to the possibility that some FMA was dissolved in the phospholipid phase, these experiments showed explicitly that FMA reacts in a specific and selective manner with a small number of sulfur atoms.

The binding experiments, the experiments with FMA analogues, and the quite general reversibility with dithiothreitol suggested very strongly that the deleterious action of FMA depended on the interaction of FMA with sulfhydryl groups in the protein.

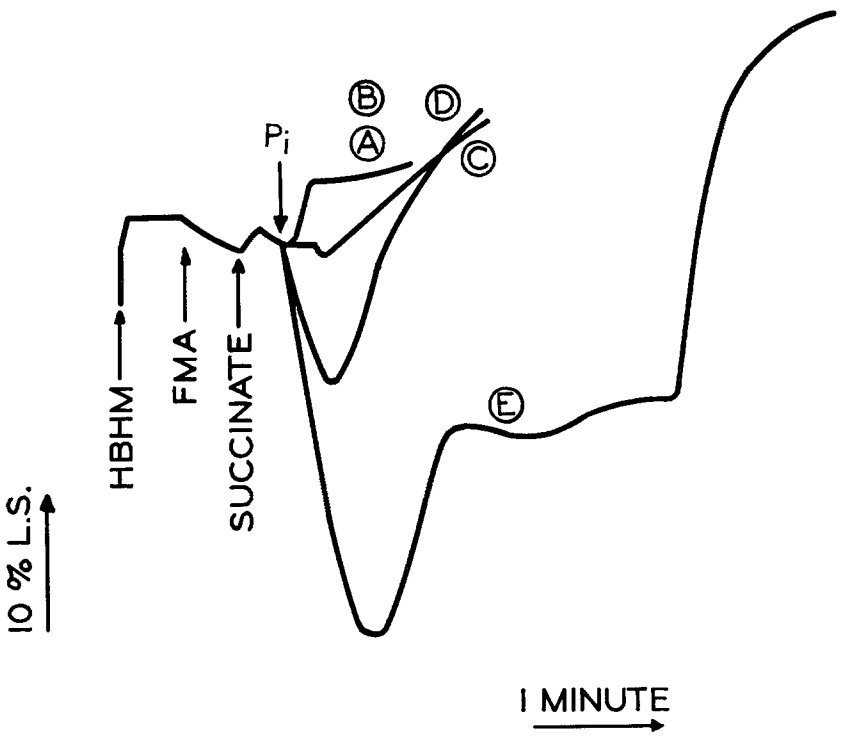

Figure 9. Effect of sub-optimal levels of FMA on the 90° light scattering of beef heart mitochondria. Conditions were as described in the section on methods. Where indicated, the following amounts of FMA were added: curve a, 10 nmoles/mg; curve b, 6·7 nmoles/mg; curve c, 6·0 nmoles/mg; curve d, 5·0 nmoles/mg; curve e, 3·3 nmoles/mg.

A study of the effects of FMA on swelling was therefore initiated in order to determine whether this process was inhibited or enhanced by FMA.

Effect of FMA on Nonenergized Swelling

In order to clarify the action of FMA on ion movements, the capability of FMA to induce pseudoenergized swelling was examined. Figure 11 describes swelling of mitochondria (as measured by changes in density of the medium) in 0·15 M NaCl and KCl under nonenergizing conditions. The driving force for this nonenergized swelling is attributed to the membrane potential generated by the asymmetric distribution of negative changes on two sides of the inner mitochondrial membrane.[15] The potentiating

effect of valinomycin on swelling in the KCl-containing medium[16,17] and of gramicidin on swelling in the NaCl-containing medium is well known.[16,17] What is quite striking, however, was the large increase in both the rate and amount of swelling in these two media when FMA was added to the reaction medium. Maximum swelling induced by the NaCl-containing medium was achieved at an FMA concentration of 10 nmoles/mg

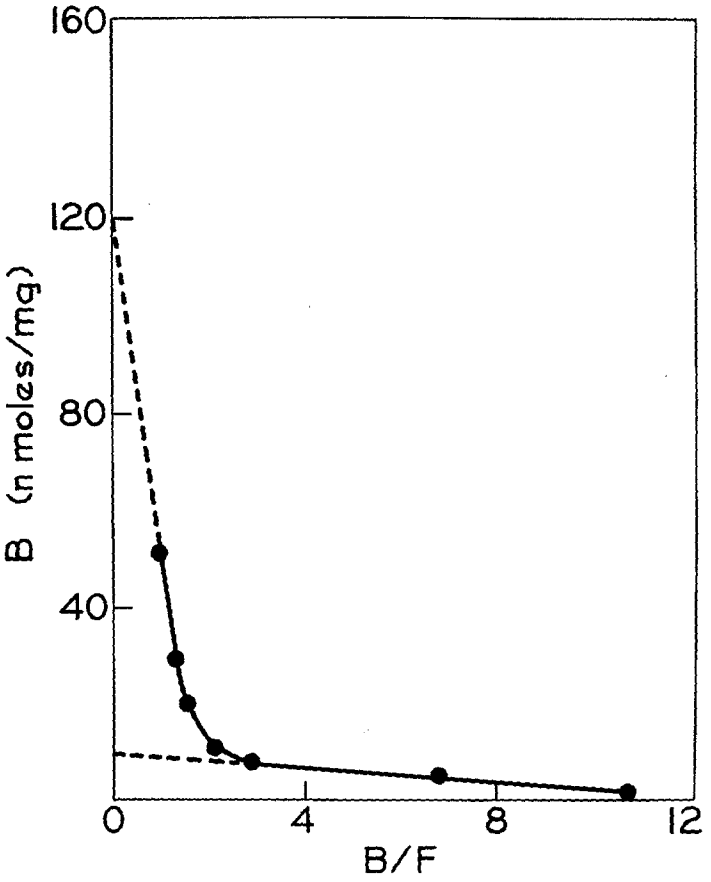

Figure 10. Scatchard plot of the binding of FMA of beef heart mitochondria. Reaction mixtures contained in a final volume of 2·5 ml, 650 μmoles sucrose, 25 μmoles Tris-Cl (pH 7·5), 2·5 mg mitochondrial protein and a varying amount of FMA. The amount of bound FMA, (B) is plotted on the ordinate versus B/F, the ratio, bound FMA/free FMA, on the abcissa.

protein. FMA at a concentration of 2 nmoles/mg had almost no effect, while FMA at concentrations greater than 10 nmoles/mg had a gradually decreasing effect. The potentiating effect of FMA on swelling in sodium or potassium chloride was completely reversed by the subsequent addition of dithiothreitol (5 μmoles/mg). Furthermore, neither fluorescein, 2,7-dichlorofluorescein nor fluorescein amine had any effect on pseudoenergized swelling. Thus, there is little alternative to the conclusion that FMA which has reacted with sulfhydryl groups in the protein is responsible for this enhancement of swelling.

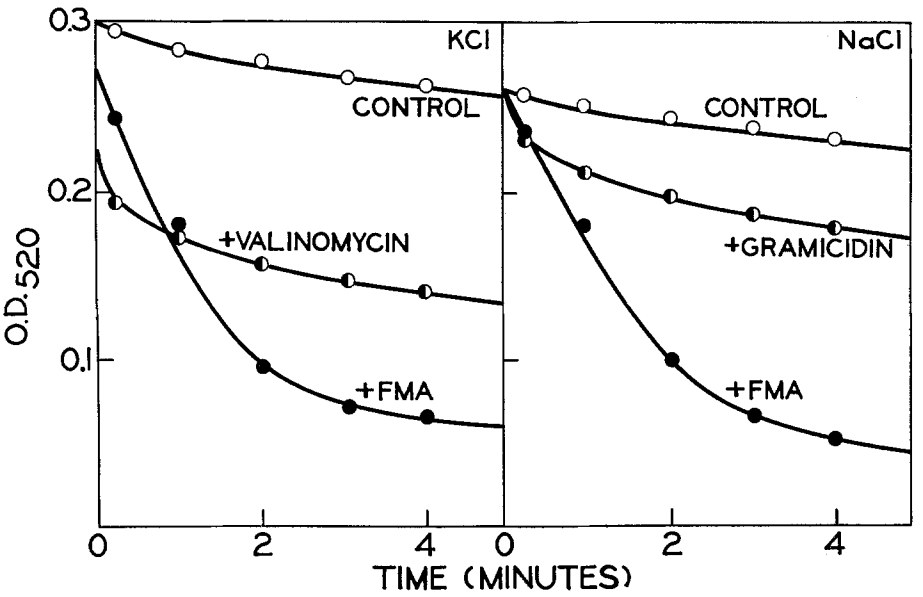

Figure 11. Effect of FMA on pseudoenergized swelling in the presence of alkali metal salts of chloride. Reaction mixtures contained in a final volume of 3 ml, 450 moles of either sodium or potassium chloride, 15 μmoles Tris-Cl (pH 7·4), 1 μg rotenone, 1 μg antimycin and 0·5 mg mitochondrial protein. Where indicated 3 nmoles valinomycin, 3 nmoles gramicidin or 5 nmoles FMA were also present.

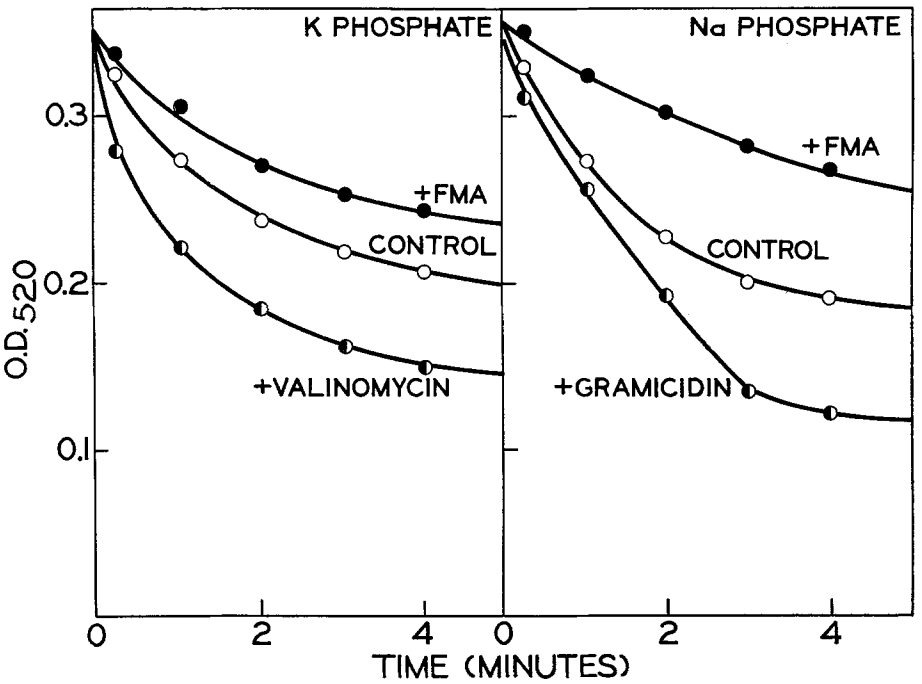

Figure 12. Effect of FMA on pseudoenergized swelling in the presence of alkali metal salts of phosphate. Conditions were the same as in the experiment described by the legend of Fig. 9, except that Tris-Cl was omitted and 450 μmoles sodium or potassium phosphate (pH 7·4) were used to replace the chloride salts employed in the previous study.

A parallel series of experiments are shown in Fig. 12 in which pseudoenergized swelling was induced with either the sodium or potassium salt of inorganic phosphate (pH 7·5). In contrast to the results of the previous experiment, FMA (10 nmoles/mg) inhibited swelling in media containing phosphate salts, and this was true whether the cation was Na^+ or K^+.

Discussion

The present study was initiated with the thought that FMA might prove to be a useful fluorescent probe of energy-linked conformational changes. This proved not to be the case, however (Lee, M. J. unpublished studies), and the mechanism responsible for FMA's dramatic and unexpected effects upon coupled mitochondrial processes were therefore studied systematically. It is clear from the experiments presented here that FMA is a potent inhibitor of oxidative phosphorylation. It reacts with about 10 nmoles of sulfhydryl groups per milligram of protein and inhibits phosphorylation, energized uptake of inorganic phosphate, phosphate-induced configurational transitions, and phosphate-induced pseudoenergized swelling. It is, however, a potentiator of certain other processes, increasing the magnitude of the proton-shift reaction, and of pseudoenergized swelling in chloride salts of sodium or potassium.

The action of FMA is strikingly similar in many respects to that reported by Brierley *et al.*,[18] for *p*-chloromercuriphenylsulfonate as well as other mercurial reagents. Thus, *p*-chloromercuriphenylsylfonate was shown by Brierley *et al.*,[18] to interact with 10–20 nmoles of "fast-reacting" thiol groups, to increase the apparent "permeability" of the mitochondrial inner membrane to cations, especially K^+ and Li^+, and to stimulate the mitochondrial ATPase activity. In this report FMA is shown in several ways to influence ion-dependent processes, as evidenced (a) by the increase in the proton jump reaction, (b) by the increase in the rate of nonenergized swelling in KCl and NaCl media, and (c) by the oscillation of the energy-linked light-scattering response induced by low levels of potassium phosphate.

Mercurials have also been demonstrated to bring about inhibition of the capacity of the mitochondrion to accumulate inorganic phosphate.[19–23] FMA is like other mercurials in this respect as shown by its inhibitory action on the light-scattering response of mitochondria to inorganic phosphate under energizing conditions; its inhibitory action on nonenergized swelling in Na^+ and K^+ phosphate media; and its inhibitory action upon phosphate accumulation, *per se*.[1]

FMA selectively inhibited a wide array of energized processes all dependent in some fashion on uptake of inorganic phosphate. Included in this group was ATP synthesis, ATP-$^{32}P_i$ exchange, calcium translocation, phosphate-induced light-scattering changes, generation of the energized-twisted configuration and energized phosphate uptake.[1]

We would like to suggest that the binding of FMA in and of itself modifies the conformation of the repeating units. Here we invoke the argument that a change in any component parameter will affect all the parameters of the system. Thus we could say that the binding of FMA generates a new, modified nonenergized conformation. This system could undergo an energy conserving conformational transition to a new, modified energized conformation. According to this interpretation, then, the proton shift may not be a property of well-coupled systems but only of one or more modified conforma-

tional cycles which may not lie on the direct pathway of oxidative phosphorylation. It may be, then, that well-coupled mitochondria undergo a conformational cycle in which the proton change during energizing is minimal and that in fact ATP synthesis cannot proceed unless this condition is fulfilled. Various ways of altering the protein conformations, however, may induce modified conformational cycles which inhibit ATP synthesis and result in maximal ion translocation.

Acknowledgements

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