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Biogenesis of Mitochondria*

THE EFFECTS OF ALTERED MEMBRANE LIPID COMPOSITION ON CATION TRANSPORT BY MITOCHONDRIA OF *SACCHAROMYCES CEREVISIAE*

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1. The fatty acid composition of the membrane lipids of a fatty acid desaturase mutant of *Saccharomyces cerevisiae* was manipulated by growing the organism in a medium containing defined fatty acid supplements. 2. Mitochondria were obtained whose fatty acids contain between 20% and 80% unsaturated fatty acids. 3. Mitochondria with high proportions of unsaturated fatty acids in their lipids have coupled oxidative phosphorylation with normal P/O ratios, accumulate K^+ ions in the presence of valinomycin and an energy source, and eject protons in an energy-dependent fashion. 4. If the unsaturated fatty acid content of the mitochondrial fatty acids is lowered to 20%, the mitochondria simultaneously lose active cation transport and the ability to couple phosphorylation to respiration. 5. The loss of energy-linked reactions is accompanied by an increased passive permeability of the mitochondria to protons. 6. Free fatty acids uncouple oxidative phosphorylation in yeast mitochondria and the effect is reversed by bovine serum albumin. 7. The free fatty acid contents of yeast mitochondria are unaffected by depletion of unsaturated fatty acids, and free fatty acids are not responsible for the uncoupling of oxidative phosphorylation in organelles depleted in unsaturated fatty acids. 8. It is suggested that the loss of energy-linked reactions in yeast mitochondria that are depleted in unsaturated fatty acids is a consequence of the increased passive permeability to protons, and is caused by a change in the physical properties of the lipid phase of the inner mitochondrial membrane.

The lipid composition of a fatty acid desaturase mutant of *Saccharomyces cerevisiae* that cannot synthesize unsaturated fatty acids may be extensively manipulated by growing the organism in media containing defined supplements of fatty acids (Proudlock *et al.*, 1971). Such studies have demonstrated that mitochondria containing 20% or less of their total fatty acids as unsaturated fatty acids, retain respiratory activity, but lose the capacity for coupled oxidative phosphorylation both *in vivo* and *in vitro* (Proudlock *et al.*, 1969, 1971; Haslam *et al.*, 1971). The loss of coupling ability is reversed *in vivo* by the incorporation of unsaturated fatty acids into mitochondrial membranes, and since recoupling is not prevented by inhibitors of protein synthesis, the loss of oxidative phosphorylation appears to be purely a lipid lesion (Haslam *et al.*, 1971).

The present report concerns two further areas of investigation into the nature of the lesion. First, since active cation transport is intimately related to oxidative phosphorylation in mitochondria and has been proposed by Mitchell (1966) to be the primary event in energy conservation (for review see Greville, 1969), the effects of unsaturated fatty acid depletion on cation transport by yeast mitochondria was investigated. Three distinct types of cation transport were studied: (1) the active accumulation of K^+ ions in the presence of valinomycin, energized either by respiration or by ATP hydrolysis; (2) the active ejection of protons energized by respiration; (3) the passive uptake of protons by non-respiring mitochondria. Secondly, since free fatty acids uncouple oxidative phosphorylation in mammalian mitochondria (Hülsmann *et al.*, 1960; Borst *et al.*, 1962), the ability of free fatty acids to uncouple oxidative phosphorylation in yeast mitochondria was investigated, and the effects of unsaturated fatty acid depletion on the free-fatty acid content of isolated mitochondria was determined.

* This paper is no. 33 in the series. No. 32 is Haslam *et al.* (1973).

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Experimental

Preparation of mitochondria

The fatty acid desaturase mutant of *S. cerevisiae* was grown as described by Proudlock *et al.* (1971) to

give cells whose fatty acids are between 20% and 80% unsaturated. Protoplasts were prepared by treatment with snail gut enzyme, and mitochondria were isolated in a medium containing sorbitol (0.5mM), EDTA (0.5mM), Tris–maleate buffer (10mM, pH6.5) as described by Watson *et al.* (1970).

Lipid analyses

Fatty acid analyses were performed as described by Proudlock *et al.* (1971). In the determination of the free fatty acid content of mitochondria the organelles were purified on a discontinuous sorbitol gradient as described by Watson *et al.* (1970). A known weight of pentadecanoic acid was added to a measured amount of mitochondrial protein, and the total lipids were extracted and fractionated by using silicic acid chromatography as described by Lukins *et al.* (1968). The free fatty acid fraction was then converted into methyl esters, and analysed by g.l.c. as described by Proudlock *et al.* (1971). Measurement of the proportion of pentadecanoic acid in the free fatty acids allows the quantitative measurement of the other free fatty acids.

Measurement of K^+ transport

The uptake of K^+ by yeast mitochondria was measured by two independent methods, which each have separate advantages.

(*a*) *Direct chemical analysis of the K^+ content of mitochondria.* Mitochondria (3–6mg of protein) were incubated in small flasks containing 10ml of medium consisting of sorbitol (0.5M), Tris–maleate (10mM), KCl (1mM), EDTA (0.5mM), dialysed bovine serum albumin (2mg/ml) and Tris–acetate (3mM). Other additions where indicated were valinomycin (0.02μg), Tris–ATP (10mM), Tris–succinate (3mM), 2,4-dinitrophenol (0.1mM) and oligomycin (100μg/ml). The pH of all reagents was adjusted to 6.5. Flasks were vigorously shaken at 30°C, 1ml samples were removed at intervals and filtered on to Sartorius cellulose nitrate Millipore filters (0.45μm pore size) in less than 5s, followed by a 5s wash with 5ml of ice-cold medium. The filters were digested with 0.1ml of conc. HNO_3 by heating gently for 15min to 120°C, care being taken not to let the digest become dry. Filters were prewashed with 2M-NaCl followed by water to remove adsorbed K^+. After this treatment the Millipore filters contain approx. 10ng-ions of K^+/filter, and this amount is subtracted from experimental values of mitochondrial K^+ content. Samples were made up to 2ml in 4mM-CsCl to suppress interference by other univalent cations, and the K^+ concentration was measured by using a Techtron model 4 atomic-absorption spectrometer at a wavelength of 766.6nm with an air–acetylene flame. The readings were calibrated by using standard KCl solutions (0.01–0.1mM, containing 4mM-CsCl). This method has the advantage that the initial and final K^+ contents of the mitochondria are measured directly. However, since samples were removed from the incubation for assay at 1- or 2-min intervals, it is difficult to measure accurately rapid rates of K^+ movement. Further, the necessity for removing mitochondria from the incubation medium by Millipore filtration causes some variation in results owing to the 5±2s required for filtration, and the correction that has to be applied for the K^+ content of the filter. In practice the accuracy of the assay was improved by taking duplicate samples at 15s intervals, and each experimental point represents the average of two such samples.

(*b*) *Measurement of changes in K^+ concentration by a K^+-sensitive microelectrode.* Changes in K^+ concentration were measured by using an EIL (Richmond, Surrey, U.K.) GM 23/B K^+-sensitive microelectrode connected to a Pye Unicam (Cambridge, Cambs., U.K.) 292 pH-meter. A mercury–calomel electrode with a saturated-KCl salt bridge was used as reference electrode. Incubations were at 30°C in 3ml of medium containing mitochondria (4–10mg of protein), sorbitol (0.5M), Tris–acetate (3mM), EDTA (0.5mM), dialysed K^+-free bovine serum albumin (2mg/ml) and ethanol (50mM), final pH6.5. Other additions where indicated were antimycin A (2μg), Tris–ATP (10mM), valinomycin (0.5μg), and carbonyl cyanide *m*-chlorophenylhydrazone (10μM). The response of the electrode was calibrated by adding standard solutions of KCl (100 or 200nmol), and was recorded on a Rikadenki B24 recorder. This method has the advantage that rapid rates of K^+ uptake or release can be followed from the continuous recording, but has the disadvantage that it does not give the initial mitochondrial K^+ content, and the K^+ concentration of the incubation medium has to be lower than 0.2mM to obtain a sensitive recording.

Measurement of the respiration-dependent ejection of protons by mitochondria

The respiration-dependent ejection of protons by yeast mitochondria was measured essentially as described by Mitchell & Moyle (1967*b*). Mitochondria (3–6mg of protein) were incubated at 30°C in 3.0ml of medium containing KCl (150mM), glycylglycine buffer (2mM, pH6.5), dialysed bovine serum albumin (4mg), EDTA (0.5mM, pH6.5) and ethanol (50mM). The incubations were performed in a thermostatically controlled glass vessel fitted with a plastic plunger through which two holes had been drilled. The pH of the incubation was measured by using an EIL 23B pH microelectrode inserted in the side of the glass vessel and connected to a Pye Unicam 292 pH-meter and Rikadenki B 24 recorder. Changes in proton concentration were calibrated by

the addition of standard solutions of O_2-free HCl (10 or 20μl of 0.5mM).

A cannula was placed through one of the holes in the lid of the incubation cell and dissolved O_2 was removed by flushing with O_2-free N_2. Mitochondria were then added through the second hole on the lid of the vessel by using a microsyringe. The mitochondria were incubated for up to 20min until the drift of the baseline ceased or was very slight. Mitochondrial respiration was then activated by the addition of an oxygenated solution of KCl (150mM) that had been equilibrated against air at 30°C. The efficiency of proton ejection is expressed as the ratio of protons ejected to O atoms consumed (Mitchell & Moyle, 1965).

Proton permeability of mitochondria

The response of mitochondria to added acid was determined as described by Mitchell & Moyle (1967*a*,*c*). Mitochondria (4–12mg of protein) were incubated aerobically at 30°C in 3.0ml of medium containing KCl (125mM), glycylglycine buffer (2mM, pH7.0), EDTA (0.5mM, pH7.0), bovine serum albumin (2mg/ml) and antimycin A (5μg). Other additions where indicated were valinomycin (0.2μg), carbonyl cyanide *m*-chlorophenylhydrazone (10μM) and HCl (20 or 40μl of a 25mM solution). The same incubation vessel, pH-measuring and recording equipment was used as described above for the measurement of respiration-dependent proton ejection.

Protein assay

Protein was determined by the method of Gornall *et al.* (1949), by using as standard bovine serum albumin (fraction V, Commonwealth Serum Laboratories, Parkville, Vic., Australia).

Measurement of respiratory control and ADP/O ratios

Respiratory-control indices and ADP/O ratios of mitochondria (1–2mg of protein) were determined in a Clark electrode cell at 30°C in 2.5ml of medium containing sorbitol (0.5M), KH_2PO_4 (10mM), Tris–maleate and (12mM), EDTA (1–2mM), adjusted to pH6.5 with Tris base. Ethanol (50mM) was used as substrate, and State 3 respiration was initiated by the addition of ADP (0.5μmol) (Chance & Williams, 1955).

Materials

Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Calbiochem, San Diego, Calif., U.S.A. Tris–ATP and oligomycin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Results and Discussion

Effects of changed fatty acid composition on the active transport of K^+ ions by yeast mitochondria

The effects of changed fatty acid composition on the initial K^+ content and the energy-dependent uptake of K^+ ions by yeast mitochondria are illustrated in Figs. 1, 2 and 3. In these experiments the K^+ content of mitochondria was measured by atomic-absorption spectrometry. Mitochondria from mutant yeast cells grown in the presence of excess of unsaturated fatty acid supplements have an approximately normal fatty acid composition (unsaturated fatty acids = 70–80% of total fatty acids; Proudlock *et al.*, 1971), and contain about 80ng-ions of K^+·mg of protein^{-1} after preincubation for 10min in an incubation medium containing KCl (2mM) plus a

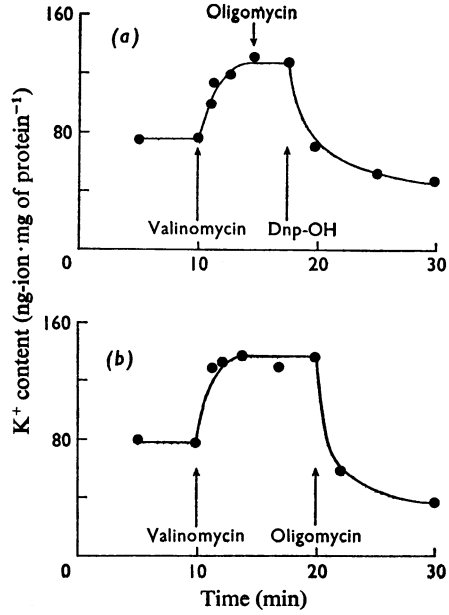

Fig. 1. *Uptake of K^+ ions by yeast mitochondria supplemented with unsaturated fatty acids measured by chemical analysis*

The K^+ content of mitochondria supplemented with unsaturated fatty acids (unsaturated fatty acids = 72% of fatty acids) is measured by atomic-absorption spectrometry as described in the Experimental section. (*a*) Energy source is succinate (3mM); (*b*) energy source is ATP (10mM). Other additions where indicated are valinomycin (0.2μg), 2,4-dinitrophenol (Dnp-OH; 0.1mM) and oligomycin (100μg/ml). Each point represents the average of two samples taken at intervals of 15s.

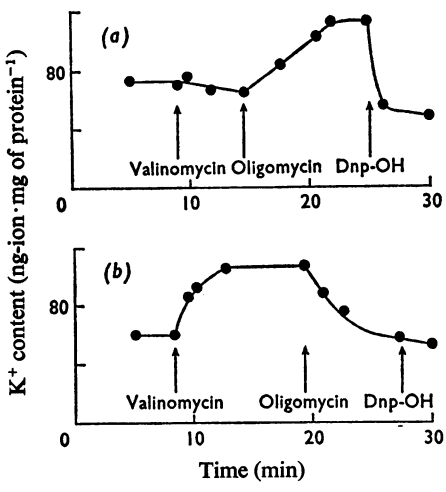

Fig. 2. *Uptake of K^+ ions by yeast mitochondria partially depleted in unsaturated fatty acids measured by chemical analyiss*

The mitochondrial fatty acids contain 38% unsaturated fatty acids. Incubation conditions are otherwise as in Fig. 1. (*a*) Energy source is succinate (3mM); (*b*) energy source is ATP (10mM).

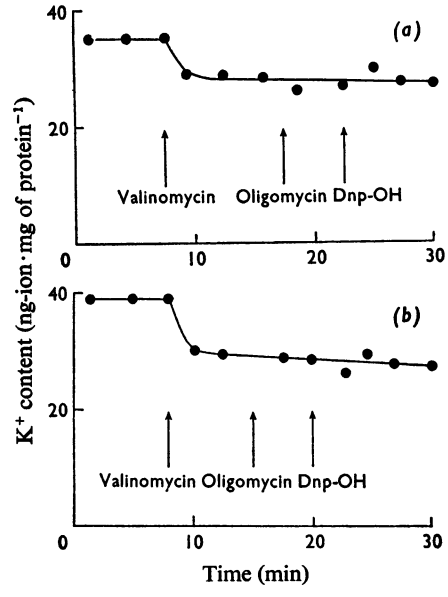

Fig. 3. *Uptake of K^+ ions by yeast mitochondria extensively depleted in unsaturated fatty acids measured by chemical analysis*

Mitochondrial fatty acids contain 21% unsaturated fatty acids. Incubation conditions are otherwise as in Fig. 1. (*a*) Energy source is succinate (3mM); (*b*) energy source is ATP (10mM).

source of energy supplied either by succinate oxidation (Fig. 1*a*) or by ATP hydrolysis (Fig. 1*b*). The amount of K^+ in the isolated organelles is similar to that reported for mammalian mitochondria (Amoore & Bartley, 1958). The addition of valinomycin stimulates the rapid accumulation of approx. 45ng-ions of K^+·mg of protein^{-1} with either succinate or ATP as energy source. The uncoupler 2,4-dinitrophenol causes the rapid release of all the actively accumulated K^+ plus an extra 25–35ng-ions of K^+·mg of protein^{-1}, suggesting that even in the absence of valinomycin much of the mitochondrial K^+ is maintained in the organelle by an energy-dependent mechanism. Oligomycin has no effect on K^+ uptake energized by succinate oxidation, but causes release of the actively accumulated K^+ when ATP is the energy source. Thus the accumulation of K^+ in the presence of valinomycin proceeds as in mammalian mitochondria (Moore & Pressman, 1964), and is a typical energy-linked function of mitochondria.

Yeast mitochondria that are partially deficient in unsaturated fatty acids (Fig. 2) contain somewhat lower initial concentrations of K^+ (60–70ng-ions·mg of protein^{-1}), and the addition of valinomycin plus ATP as energy source causes the accumulation of K^+ at somewhat lower rates than in mitochondria that are fully supplemented with unsaturated fatty acids (Fig. 2*b*). In contrast, succinate oxidation supports accumulation of K^+ only if oligomycin is also present (Fig. 2*a*). The stimulation of K^+ accumulation by oligomycin is similar to the effects of the antibiotic on the energy-linked reactions of submitochondrial particles whose energy-conservation mechanism has been damaged by mechanical disruption (Lee & Ernster, 1965). However, the organelles deficient in unsaturated fatty acids that are used in the present experiments are morphologically intact (Haslam *et al.*, 1971), and the partial loss of energized cation transport appears to be a direct effect of unsaturated fatty acid depletion.

Yeast mitochondria that have been extensively depleted in unsaturated fatty acids are shown in Fig. 3 to contain relatively low initial concentrations of K^+ (about 40ng-ions·mg of protein^{-1}), and the addition of valinomycin in the presence of either succinate or ATP as energy source causes the further loss of about 10ng-ions of K^+·mg of protein^{-1}. Even the addition of oligomycin to the succinate-energized system fails to stimulate K^+ uptake. The lower K^+ content of freshly isolated mitochondria depleted in unsaturated fatty acids incubated in the absence of

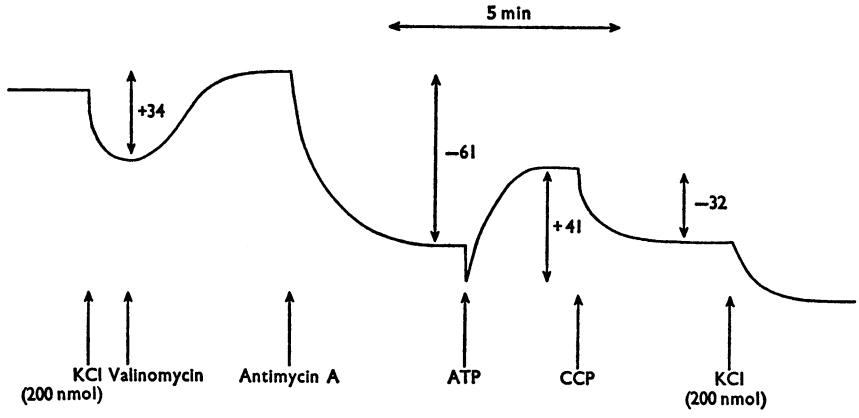

Fig. 4. *Transport of K^+ ions by yeast mitochondria supplemented with unsaturated fatty acids measured by a K^+-sensitive microelectrode*

Mitochondria supplemented with unsaturated fatty acids (unsaturated fatty acids = 73% of fatty acids; 8mg of protein) were incubated at 30°C, and changes in the concentration of K^+ in the medium were measured as described in the Experimental section. Other additions where indicated are antimycin A (1μg), valinomycin (1μg), ATP (10mM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCP; 10μM). The electrode is calibrated at two points by adding a standard solution of KCl (0.1 ml of 2.00mM; i.e., 200nmol). The values above each phase of the response curve indicate uptake or release of K^+ ions (nmol·mg of protein^{-1}).

valinomycin suggests that the mechanism for the energy-dependent maintenance of normal intramitochondrial K^+ concentrations is impaired *in vivo*. An increased permeability of the inner mitochondrial membrane to K^+ ions would allow the cations to leak out passively, faster than they can be accumulated in the energy-dependent reaction, and could account for the observed results. However, the stimulation of the release of K^+ ions from mitochondria depleted in unsaturated fatty acids by valinomycin shows that the mitochondria retain a normal permeability to K^+. Thus the results are not simply due to an abnormally high endogenous K^+ permeability, but are the consequence of a lesion in energy coupling.

As an independent check on these results transport of K^+ by yeast mitochondria supplemented with unsaturated fatty acids and depleted in unsaturated fatty acids was also measured by using a K^+-sensitive microelectrode. Fig. 4 shows that respiring yeast mitochondria fully supplemented with unsaturated fatty acids rapidly accumulate K^+ after the addition of valinomycin. The new equilibrium content of K^+ represents an accumulation of 34ng-ions·mg of protein^{-1}, and is reached after about 2min. The addition of antimycin A completely inhibits respiration, and causes the release of not only the K^+ accumulated subsequent to the addition of valinomycin, but also a further 27ng-ions of K^+·mg of protein^{-1}, which is explained by a requirement for energy to maintain intramitochondrial K^+ concentrations even in the absence of valinomycin. If the mitochondria are subsequently energized by the addition of ATP, most of the K^+ is reaccumulated, and this is subsequently reversed by the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. These results agree with those obtained for normal *Saccharomyces* mitochondria by Kovac *et al.* (1972), with a similar assay system. Fig. 5 shows that respiring mitochondria depleted in unsaturated fatty acids do not accumulate K^+ ions, but release a small amount of K^+ after the addition of valinomycin. Thus two independent methods show that unsaturated fatty acid depletion causes the loss of active valinomycin-dependent K^+ accumulation by yeast mitochondria.

Effects of depletion in unsaturated fatty acids on the energy-dependent ejection of protons by yeast mitochondria

When a small sample of oxygenated KCl solution is added to anaerobic yeast mitochondria that are fully supplemented with unsaturated fatty acids, there is a short period of respiration accompanied by a rapid ejection of protons. Fig. 6 shows that, if a relatively large amount of O_2 is added, proton ejection reaches a maximum plateau value of 10ng-ions of H^+·mg of protein^{-1}. This value is very similar to that obtained by Mitchell & Moyle (1967*b*,*c*) for rat liver mitochondria. If the H^+/O ratio is directly calculated it gives a value of 1.5, but if the extent of proton ejection is corrected for the decay of the proton gradient,

as indicated in Fig. 6, an extrapolated H^+/O ratio of 2.2 is obtained. If smaller samples of O_2 are added, the plateau value of proton ejection is not reached, and higher H^+/O ratios approaching 3.0 are obtained with ethanol as substrate. *S. cerevisiae* mitochondria apparently have two sites of phosphorylation with NADH-linked substrates such as ethanol (for review see Linnane *et al.*, 1972), and by Mitchell's (1966) hypothesis optimum H^+/O ratios of 4.0 are possible. In the present experiments, the maximal H^+/O ratios of 3.0 are satisfactory, as the experimentally determined P/O ratios obtained with our mitochondria rarely exceed 1.7 with NADH-linked substrates. If the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone is added, the ability of the mitochondria supplemented with unsaturated fatty acids to eject protons is lost, as occurs in mammalian mitochondria (Mitchell & Moyle, 1967*a*,*b*). Mitochondria extensively depleted in unsaturated fatty acids (unsaturated fatty acids = 20% of total fatty acids) behave like uncoupled mitochondria and completely lack the ability to eject protons in an energy-dependent manner, whereas mitochondria whose lipids contain intermediate amounts of unsaturated fatty acids eject protons with a lower efficiency.

Effects of depletion in unsaturated fatty acids on the passive permeability of yeast mitochondria to protons

Intact well-coupled mammalian mitochondria are highly impermeable to protons (Mitchell & Moyle, 1967*a*). If acid is added to non-respiring mitochondria suspended in a lightly buffered medium the pH falls abruptly and then partially recovers in a time-dependent fashion. The overshoot in acidity of the

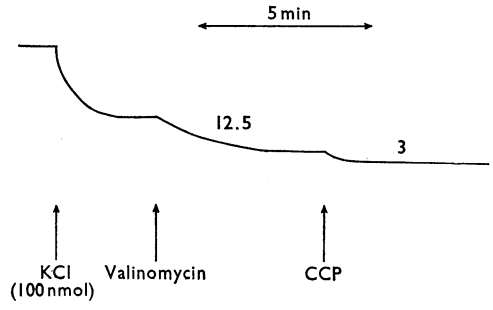

Fig. 5. *Transport of K^+ ions by yeast mitochondria depleted in unsaturated fatty acids measured by a K^+-sensitive microelectrode*

Mitochondria depleted in unsaturated fatty acids (unsaturated fatty acids = 22% of fatty acids; 4mg of protein) were incubated, and K^+ concentrations measured as described in Fig. 4. Other additions are valinomycin (0.5μg) and carbonyl cyanide *m*-chlorophenylhydrazone (CCP; 10μM). The values above each phase of the response curve indicate K^+ released (nmol·mg of protein^{-1}). KCl (100nmol was added for calibration.

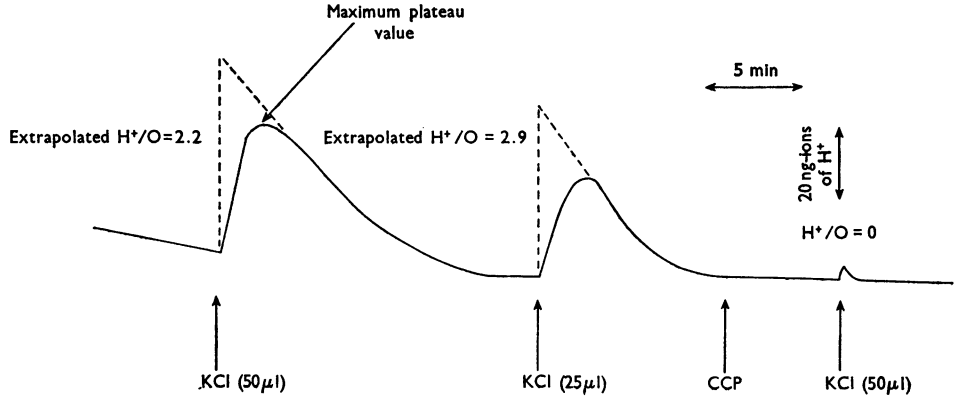

Fig. 6. *Respiration-dependent ejection of protons by yeast mitochondria*

The respiration-dependent ejection of protons by yeast mitochondria supplemented with unsaturated fatty acids (unsaturated fatty acids = 78% of fatty acids) is measured as described in the Experimental section. Respiration is initiated by the addition of 50μl (23ng-atoms of O) or 25μl (11.5ng-atoms of O) of aerated KCl (150mM). Total protein ejection is extrapolated from the decay curves as indicated, and the efficiency of proton ejection is calculated as the H^+/O ratio, and is given on the figure for two consecutive additions of O_2. The maximum plateau value is 10ng-ions of H^+·mg of protein^{-1}. Another addition where indicated is carbonyl cyanide *m*-chlorophenylhydrazone (CCP; 10μM). Yeast mitochondria extensively depleted in unsaturated fatty acids (unsaturated fatty acids = 20% of fatty acids) give no response at all in the same system on addition of aerated KCl. Mitochondria whose unsaturated fatty acid content is decreased from 70% to 20% give progressively lower H^+/O ratios.

medium is due to the impermeability of the inner mitochondrial membrane, and the subsequent time-dependent alkalinization of the medium is a measure of passive proton entry into the mitochondrial matrix. If a chemical uncoupler is added before the acid, the overshoot in acidity is almost abolished and, if the uncoupler is added after the acid, the rate of entry of the protons into the mitochondria is increased. Valinomycin added together with an uncoupler abolishes the membrane potential that opposes the entry of more protons and the rate of proton entry is further stimulated (Mitchell & Moyle, 1967*a*,*c*). Fig. 7(*a*) shows that yeast mitochondria supplemented with unsaturated fatty acids behave in exactly the same way as mammalian mitochondria. On addition of acid there is a large overshoot in aciditiy, followed by a very slow alkalinization of the medium with a half-life of 160s. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (10μM) decreases the half-life of the alkalinization to 60s, and the further addition of valinomycin decreases it to 10s. In parallel experiments shown in Fig. 7(*b*) mitochondria depleted in unsaturated fatty acids behave quite differently; the overshoot in acidity is very much less, and the rate of the subsequent alkalinization is unaffected by carbonyl cyanide *m*-chlorophenylhydrazone, indicating that the inner mitochondrial membrane is very permeable to protons. However, since valinomycin is still required to collapse the membrane potential and enable maximal rates of proton entry, the inner mitochondrial membrane apparently retains a low permeability to K^+ ions.

All the main hypotheses for the mechanism of oxidative phosphorylation agree that there is a close link between cation transport and energy conservation (for review see Greville, 1969). Since yeast mitochondria are capable of using the energy of oxidative phosphorylation to build a proton gradient, any change in the inner mitochondrial membrane that greatly increases proton permeability will tend to collapse the proton gradient and drain off the energy that would otherwise be used to drive the energy-linked reactions of mitochondria. Thus the increased permeability of the inner mitochondrial membrane to protons that accompanies depletion in unsaturated fatty acids provides a satisfactory explanation of the loss of oxidative phosphorylation and of energized cation transport.

Possible role of free fatty acids in the uncoupling of oxidative phosphorylation in yeast mitochondria depleted in unsaturated fatty acids

It is well established that free fatty acids, particularly unsaturated fatty acids, are uncouplers of oxidative phosphorylation, this action being reversed by bovine serum albumin (Hülsmann *et al.*, 1960; Borst *et al.*, 1962). Further, free fatty acids act as natural uncouplers of oxidative phosphorylation *in vivo* in brown-fat tissue (Bulychev *et al.*, 1972). It has also been reported that isolated yeast mitochondria contain much higher amounts of free fatty acids than do mammalian mitochondria (Lukins *et al.*, 1968),

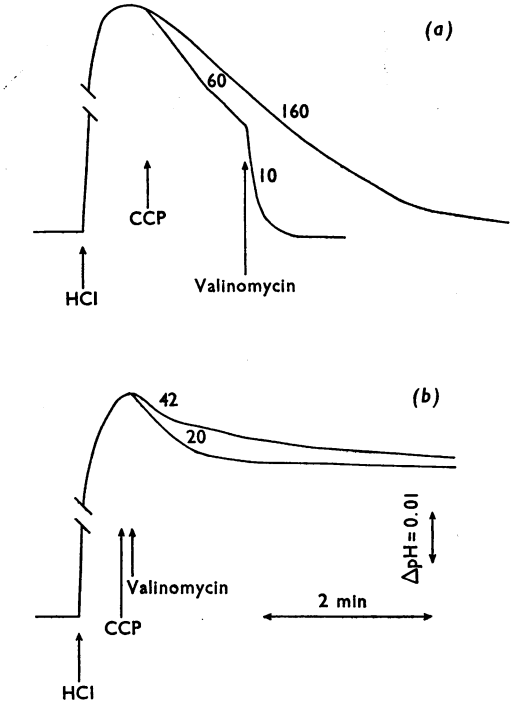

Fig. 7. *Effects of depletion in unsaturated fatty acids on the passive permeability of yeast mitochondria to protons*

(*a*) Mitochondria supplemented with unsaturated fatty acids (unsaturated fatty acids = 72% of fatty acids; 10mg of protein). (*b*) Mitochondria depleted in unsaturated fatty acids (unsaturated fatty acids = 19% of fatty acids; 7mg of protein). Mitochondria are preincubated for 10min at 30°C in the medium described in the Experimental section. Further additions where indicated are HCl (0.5μmol), carbonyl cyanide *m*-chlorophenylhydrazone (10μM) and valinomycin (1μg). The values above the curves are the half-times (s) for the passive equilibration of protons. The top curve in both (*a*) and (*b*) represents a control incubation in the absence of carbonyl cyanide *m*-chlorophenylhydrazone or valinomycin. Carbonyl cyanide *m*-chlorophenylhydrazone (CCP) added at the point indicated had no effect on the uptake of protons by mitochondria depleted in unsaturated fatty acids, and a curve identical with the control was obtained. The ΔpH scale (see *b*) is the same in (*a*) and (*b*).

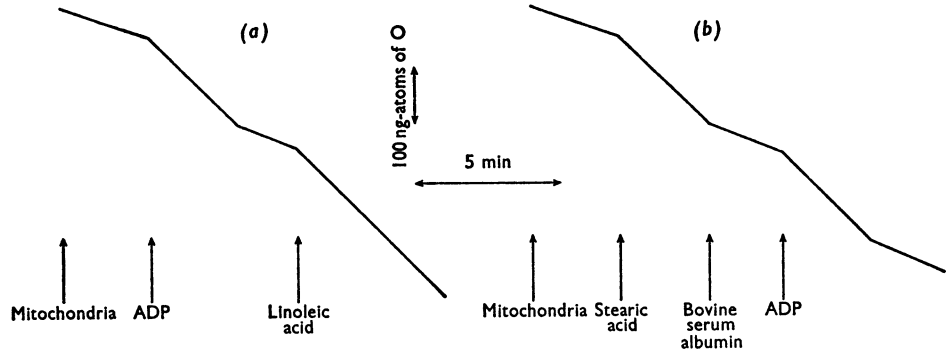

Fig. 8. *Effects of free fatty acids on the respiratory control of yeast mitochondria*

The figure shows polarographic recordings of the respiration of yeast mitochondria supplemented with unsaturated fatty acids (72% unsaturated fatty acids) in two experiments, (*a*) and (*b*). Incubation media are as described in the Experimental section, and the additions indicated are mitochondria (1 mg of protein), ADP (0.5 μmol), linoleic acid (30 μg), stearic acid (130 μg) and bovine serum albumin (5 mg). The initial respiratory-control index of the mitochondria is 2.5 and the ADP/O ratio is 1.65. The minimal amounts of free fatty acids required for partial uncoupling of the mitochondria are 5 μg of linoleic acid or 30 μg of stearic acid, and maximal uncoupling is obtained with 30 μg of linoleic acid or 130 μg of stearic acid. Bovine serum albumin (5 mg) totally reverses the uncoupling effects of 20 μg of linoleic acid or 125 μg of stearic acid.

Table 1. *Free fatty acid content of yeast mitochondria at different extents of depletion in unsaturated fatty acids*

Mitochondria were prepared and lipids analysed as described in the Experimental section. The results are for four individual preparations of mitochondria.

Unsaturated fatty acid content of mitochondria (% of total fatty acids)	Free fatty acids (μg/mg of protein)	
	Total	Unsaturated
72	7.3	2.9
51	6.2	2.0
29	5.7	1.2
20	8.2	1.0

and the presence of free fatty acids probably accounts for the very high Mg^{2+}-stimulated adenosine triphosphatase and the relatively poor respiratory-control ratios of *Saccharomyces* mitochondria in the absence of bovine serum albumin (Kovac *et al.*, 1968). Accordingly, we investigated the possibility that depletion in unsaturated fatty acids causes perturbations in fatty acid metabolism leading to the presence of uncoupling concentrations of free fatty acids in the mitochondria.

The effects of free fatty acids on the respiratory control of yeast mitochondria supplemented with unsaturated fatty acids are shown in Fig. 8. This particular preparation is well-coupled even in the absence of bovine serum albumin, having a respiratory-control ratio of 2.5 with ethanol as substrate and an ADP/O ratio of 1.65. The addition of either 30 μg of linoleic acid or 130 μg of stearic acid·mg of mitochondrial protein^{-1} produces maximal uncoupling (respiratory-control ratio 1.0), but these effects are completely reversed by 5 mg of bovine serum albumin.

In view of the above results, the free fatty acid contents of mitochondria depleted in unsaturated fatty acids and supplemented with unsaturated fatty acids were determined. In these studies the mitochondria were gradient purified and then immediately extracted with chloroform–methanol (2:1, v/v) to minimize any breakdown of mitochondrial lipids caused by contaminating lipases. The results in Table 1 show that mitochondria contain 6–8 μg of free fatty acids·mg of protein^{-1}. These values are much lower than those reported by Lukins *et al.* (1968), but probably reflect the greater care taken in the preparation of the mitochondria and in the lipid analyses of the organelles in the present study. There is very little difference between the free fatty acid content of mitochondria, whose fatty acids contain between 20% and 70% unsaturated fatty acids. Further, the amounts of free unsaturated fatty acids, which are more effective than saturated fatty acids in uncoupling oxidative phosphorylation (Borst *et al.*, 1962), are lowest in the mitochondria most extensively depleted in unsaturated fatty acids. The amounts of free fatty acids present in mitochondria both supplemented with

unsaturated fatty acids and depleted in unsaturated fatty acids are much smaller than the concentrations required to uncouple phosphorylation in the standard assay medium containing 5mg of bovine serum albumin (Haslam *et al.*, 1971). Further experiments (J. M. Haslam, unpublished work) show, moreover, that mitochondria depleted in unsaturated fatty acids still have negligible phosphorylation in the presence of bovine serum albumin at a concentration of 20mg/ml. It is therefore concluded that the loss of oxidative phosphorylation in yeast mitochondria depleted in unsaturated fatty acids is not due to increased concentrations of free fatty acids.

Nature of the lesion in energy conservation produced by depletion in unsaturated fatty acids of yeast mitochondria

Depletion in unsaturated fatty acids could cause an increased fragility of mitochondrial membranes and lead to extensive mechanical damage of the organelles during isolation. This would explain the loss of oxidative phosphorylation and the absence of energy-linked cation transport in mitochondria depleted in unsaturated fatty acids *in vitro*. However, we discard this explanation, as previous studies indicate that the isolated mitochondria are intact and that coupling of oxidative phosphorylation is also lost *in vivo* (Proudlock *et al.*, 1971; Haslam *et al.*, 1971). The further possibility that free fatty acids, acting as uncouplers, cause the loss of energy-linked reactions in mitochondria depleted in unsaturated fatty acids is also eliminated by the present report. The evidence that the lesion is mediated purely by lipids is strong, because recoupling is induced by the incorporation of unsaturated fatty acids in the absence of protein synthesis (Haslam *et al.*, 1971). We propose that the primary lesion is a change in the physical properties of the lipid core of the inner mitochondrial membrane, leading to an increased permeability of the membrane to protons. The investigation of the effects of changed fatty acid composition on other physical parameters of inner membrane organization may reveal the detailed reasons for the changes in proton permeability.

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