Bioenergetics (1971) **2**, 351–370

Biogenesis of Mitochondria 20
The Effects of Altered Membrane Lipid Composition on Mitochondrial Oxidative Phosphorylation in *Saccharomyces cerevisiae*

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Received 2 September 1971

Abstract

1. The lipid composition of mitochondria isolated from a fatty acid desaturase mutant of *Saccharomyces cerevisiae* may be extensively manipulated by growing the organism on defined supplements of unsaturated fatty acid (UFA).

2. The fatty acid composition of the mitochondrial lipids closely follows that of the whole cells from which the mitochondria are isolated. UFA-depleted mitochondria contain normal levels of sterols, neutral lipids and total phospholipids, but have much lower levels of phosphatidyl inositides.

3. UFA-depleted mitochondria possess a full complement of cytochromes, oxidase both NAD-linked and flavoprotein-linked substrates at normal rates, and have levels of succinate and malate dehydrogenases similar to those of UFA-supplemented mitochondria. However, UFA-depletion has a marked effect on the ability of cytochrome *c* to reactivate the NADH oxidase activity of cytochrome *c*-depleted mitochondria.

4. The efficiency of oxidative phosphorylation decreases progressively with the UFA content of the mitochondria, and oxidative phosphorylation is completely lost in mitochondria containing approximately 20% UFA.

5. The incorporation of UFA into the lipids of UFA-depleted mitochondria *in vivo* results in a recoupling of oxidative phosphorylation. Recoupling is insensitive to both chloramphenicol and cycloheximide, indicating that all the proteins necessary for oxidative phosphorylation are present in UFA-depleted mitochondria, and that the less of oxidative phosphorylation is a purely lipid lesion.

6. ATPase activity is apparently unaffected by UFA-depletion, but $^{32}P_i$–ATP exchange activity is lost in mitochondria which have been extensively depleted in UFA.

7. Valinomycin stimulates the respiration of UFA-supplemented mitochondria in media containing potassium, but has no effect on the respiration of UFA-depleted mitochondria, suggesting that active transport of potassium is lost as a result of UFA-depletion.

Introduction

In the preceding companion paper[1] it has been shown that manipulation of the unsaturated fatty acid (UFA) composition of a fatty acid desaturase mutant of *Saccharomyces cerevisiae* leads to profound changes in the energy metabolism of the cell. Cells containing less than approximately 20% of their total fatty acids as UFA lose the ability to grow on

non-fermentable carbon sources, although these substrates are still oxidized at normal rates. The cells can, however, continue to grow by fermentation of glucose until they contain only 5% UFA. These results suggested that the changes in membrane lipid composition may result in a specific loss of the ability of the mitochondria to couple oxidation to the formation of ATP. This communication describes the effects of UFA-depletion on the membrane lipid composition of the mutant yeast mitochondria, and on the energy-linked reactions of the isolated mitochondria. It is shown that mitochondria oxidative phosphorylation is lost as a direct consequence of changes in membrane lipid composition. A preliminary account of some of this work has already appeared elsewhere.[2]

Methods

Methods were as described in the preceding companion paper,[1] but with the following additions.

Growth of cells. In the initial studies strain KD115 was grown on a medium containing 1% yeast extract, *Saccharomyces* salts, 0·4% glucose and UFA at either 50 μg/ml to give UFA-depleted cells or at 1 mg/ml to give UFA-supplemented cells. However, cells obtained on this medium have rather variable respiratory activities due to some catabolite repression. In later experiments cells with reproducibly high respiratory activities were obtained by growth on either 1% ethanol or 2% galactose media; the cells cease growing on these substrates when their fatty acids contain approximately 20% UFA. Media containing UFA-supplements in the range 50 μg/ml to 1 mg/ml give cells whose fatty acids contain 20 to 75% UFA.

Preparation of mitochondria. Mitochondria were prepared essentially as described by Lamb *et al.*[3] except that protoplasts were broken by passing through a French pressure cell at 50 p.s.i. in a medium containing 0·5 M sorbitol, 0·5 mM EDTA, 10 mM tris-HCl, pH 7·4 and 2 mg/ml bovine serum albumin. Mitochondrial preparations that were used for lipid analyses were further purified on a discontinuous sorbitol gradient as described by Watson *et al.*[4] Cytochrome *c* deficient mitochondria were prepared by breaking protoplasts by osmotic shock in a medium containing 0·1 M sorbitol, 0·5 mM tris-EDTA (pH 7·4).

Respiratory control ratios were determined in a polarograph cell at 30° in a medium containing 0·60 M mannitol, 5 mM $KHPO_4$, 0·5 mM EDTA, 10 mM tris-maleate, yeast mitochondria (1–2 mg protein) and substrates: 4 mM pyruvate plus 4 mM L-malate, 4 mM succinate or 5 mM α-oxoglutarate. The pH of all solutions was adjusted to 6·5.

P.O. ratios were determined manometrically at 30° as described by Haslam.[5] The main compartment of the Warburg flasks contained in 6·62 ml: 0·48 M mannitol, 1·1 mM EDTA, 10 mM tris-maleate, 10 mM $KHPO_4$, 5 mM Mg Cl_2, 0·4 mM ATP, 25 mM glucose, 1 mg hexokinase, 5 mg bovine serum albumin and yeast mitochondria (2–4 mg protein), final pH 6·5. Further additions where indicated were 0·1 mM 2:4-dinitrophenol and substrates: 4 mM pyruvate plus 4 mM L-malate, 4 mM succinate and 4 mM α-oxoglutarate. Inorganic phosphate was determined by the method of Gomori.[6]

Since *yeast mitochondrial ATPase* has two pH optima,[7–9] the enzyme was assayed at both optima of pH 6·2 and pH 9·4. The specific protein inhibitor of mitochondrial

ATPase from beef heart mitochondria was isolated and assayed against yeast mitochondrial ATPase as described by Monroy and Pullman.[10] ATPase was measured essentially as described by Monroy and Pullman[10] using an ATP-regenerating system but with certain minor modifications. Incubations were at 30° for 10 min in 1·82 ml containing 1 mM ATP, 3 mM phosphoenol-pyruvate, 3 mM $MgCl_2$, 8 units of rabbit muscle pyruvate kinase, and either 50 mM tris-maleate (pH 6·2) or 50 mM tris-HCl (pH 9·4). Reactions were stopped by the addition of 0·3 ml of 0·9 M $HClO_4$, 0·6 M Na_2SO_4. Precipitated protein was removed by centrifugation and 1·0 ml aliquots of the supernatant were assayed for inorganic phosphate by the method of Gomori.[6]

Succinate dehydrogenase activity was assayed by the method of Arrigoni and Singer,[11] and *malate dehydrogenase* as described by Vary *et al.*[12] *NADH oxidase* activity was assayed spectrophotometrically by the decrease in absorbance at 340 nm in a medium containing 0·1 mM NADH, 0·25 sucrose, 10 mM tris-HCl, pH 7·4. *$^{32}P_i$–ATP exchange* activity was assayed essentially as described by Pullman.[13] Incubations were for 10 min at 30° in a medium containing 10 mM ATP, 10 mM Mg Cl_2, 20 mM $^{32}P_i$ (0·01 $\mu C_i/\mu$mole), pH 7·4. The mitochondrial sample was adjusted to contain 0·2–0·3 units of ATPase activity so that not more than 30% of the ATP was split during the incubation. *Energy-linked transhydrogenase* activity of yeast submitochondrial particles was measured as described by Lee, Azzone and Ernster.[14] *Calcium uptake* by yeast mitochondria was investigated under the conditions described by Carafoli and Lehninger[15] for massive loading with Ca^{2+}. *The effects of valinomycin on the respiration of yeast mitochondria* were measured polarographically at 30° in a medium containing 0·25 M sucrose, 10 mM tris-maleate, 1 mM KCl, 3 mM tris-acetate and 0·3 to 0·5 mg/ml mitochondrial protein. Other additions where indicated were 2 mM ATP plus 3·6 mM succinate, 10 mM ATP, 0·1 mM 2:4 dinitrophenol and 0·02 μg/ml valinomycin dissolved in 2 μl of methanol. *Protein* was assayed by the method of Lowry *et al.*[16]

Cytochrome spectra. Difference spectra were determined in a Carey Model 14 spectrophotometer using sodium dithionite as the reductant and potassium ferricyanide as the oxidant, as described by Chance.[17]

Materials

Oligomycin, ADP, ATP, NADH, NAD and NADP were obtained from Sigma Chemical Company, St. Louis, USA. Valinomycin and pyruvate kinase were purchased from Calbiochem. Inc. Los Angeles, California, and bovine serum albumin Fraction V from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Alcohol dehydrogenase was obtained from C.F. Boehringer and Soehne G.m.b.H. Mannheim, Germany. $^{32}P_i$ was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia, and ^{45}Ca from the Radiochemical Centre, Amersham, England. All other reagents were the best available reagent grades. The cyclohexylammonium salt of phosphoenolpyruvate was synthesized by Dr. G. M. Kellerman, and converted to the potassium salt prior to use as described by Clark and Kirby.[18]

Results

Fatty Acid Composition of Mitochondria Isolated from Cells Containing Different Levels of UFA

The fatty acid composition of mitochondria and cells grown at three levels of UFA supplementation are given in Table I. The fatty acid composition of the mitochondrial

TABLE I. Fatty acid composition of mitochondria and whole cells of strain KD115 containing different levels of UFA

Preparation	Fatty acid composition (mg/100 mg total fatty acid)										% $C_{8:0}$–$C_{14:0}$	% $C_{16:0}$ + $C_{18:0}$	% UFA
	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{18:0}$	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$			
A Whole cells	0·3	TR	0·7	2·2	TR	16·9	7·3	0·4	28·2	44·0	3·2	24·2	72·6
Mitochondria	0·6	0·6	2·1	3·0	0·8	14·2	5·0	1·5	32·6	39·6	6·3	19·2	73·7
B Whole cells	0·3	7·9	7·0	7·5	3·1	38·6	4·1	TR	3·2	28·3	22·7	42·7	31·4
Mitochondria	1·1	6·8	7·1	8·0	3·5	41·4	3·7	TR	3·4	25·0	23·0	45·1	28·4
C Whole cells	1·1	9·6	10·5	12·3	2·3	40·2	4·9	TR	3·3	15·8	33·5	45·1	19·2
Mitochondria	2·3	15·3	13·0	13·6	2·7	32·3	3·9	TR	2·9	14·0	44·2	36·2	16·9

Cultures were grown to early stationary phase on 1% yeast extract, *Saccharomyces* salts medium containing 0·4% glucose and the following UFA supplements: Preparation A, 1 mg/ml; preparation B, 70 μg/ml; and preparation C, 50 μg/ml. The fatty acids are denoted by the convention: number of carbon atoms: number of double bonds. TR = trace (<0·2%).

lipids closely resembles that of the whole cells from which the mitochondria were isolated. No preferential accumulation of UFA into the mitochondrial lipids is observed, although there appears to be a slight preponderance of the short chain saturated fatty acids ($C_{8:0}$–$C_{14:0}$) in the mitochondria. Traces of pentadecanoic acid are also detected in these preparations, and these increase in amount as the UFA content of the cells and mitochondria decreases. The source of this acid is attributed to the Tween 80, as the particular batch of Tween 80 used in these experiments was found to contain 4% pentadecanoic acid.

Total Lipid Content and Composition of Mitochondria Isolated from Cells Grown on High and Low Levels of UFA

Table II shows that mitochondria whose fatty acids contain 73, 78 and 20% UFA are remarkably similar in their contents of total lipids, neutral lipids, phospholipids and sterols. However, there is a marked drop in the content of phosphatidyl inositides;

TABLE II. Lipid composition of mitochondria isolated from cells grown on 0·4% glucose and various UFA supplements

UFA supplement to growth medium (μg/ml)	UFA content % total fatty acid	Total lipid content	Neutral lipid content	Sterol content	Phospho-lipid content	Phospholipid fraction (μg/mg protein)				
						DPG	PC	PE	PI	PS
50	20	528	305	63	223	34	85	79	9	13
70	38	535	320	70	215	37	69	88	15	7
1000	73	594	345	75	249	39	104	67	33	7

Cultures were grown on 1% yeast extract, 0·4% glucose, *Saccharomyces* salts medium plus the level of UFA supplementation indicated into early stationary phase of growth (24–30 hours). Mitochondria were isolated and lipid analyses were performed as detailed in Methods. The following abbreviations have been used. DPG = diphosphatidyl glycerol; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PI = phosphatidyl inositol; PS = phosphatidyl serine.

in the mitochondria containing 20% UFA the phosphatidyl inositide content is reduced to about 30% of that in the mitochondria containing 73% UFA. Changes in the other classes of phospholipids are apparent as the mitochondrial UFA content is decreased, but these are not so extreme.

Cytochrome Spectra and Respiratory Activities of Normal and UFA-depleted Mitochondria

Curves A and B of Fig. 1 show the difference spectra of mitochondria whose fatty acids contain 18% and 73% UFA. No obvious differences are apparent between the two preparations. The effect of UFA-depletion on the respiratory activity of isolated mitochondria oxidizing three different substrates is given in Table III together with the activities of the mitochondrial enzymes malate and succinate dehydrogenases. Although there is a wide variation in the respiratory activities of individual preparations, there is essentially no difference between the activities of mitochondria containing low and high levels of UFA. The wide range of activities probably reflects varying degrees of catabolite repression in the individual preparations of mitochondria. The activities of the

tricarboxylic acid cycle enzymes malate and succinate dehydrogenases are also little affected by UFA-depletion. These results agree with the observation of normal respiration and cytochrome spectra in whole cells whose fatty acids contain as low as 12% UFA.[1]

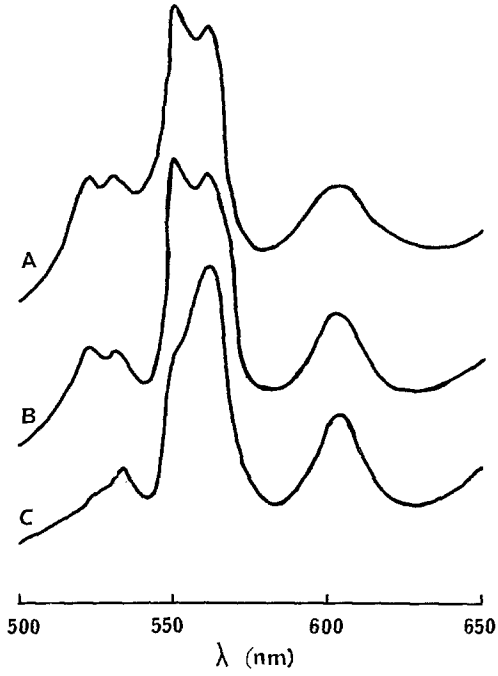

Figure 1. Difference spectra of KD115 mitochondria. Mitochondrial suspensions were adjusted to approximately 5 mg protein/ml and spectra were recorded on a Carey Model 14 spectrophotometer as detailed in Methods. A. Mitochondria isolated from cells grown on 1% yeast extract, *Saccharomyces* salts medium plus 0·4% glucose and 50 μg/ml UFA; UFA-content is 18% of the total fatty acids. B. Mitochondria isolated from cells grown on 1% yeast extract, *Saccharomyces* salts medium plus 0·4% glucose and 2 mg/ml UFA, UFA content is 73% of the total fatty acids. C. Mitochondria from cells grown as in B but prepared by the osmotic shock procedure as described in Methods.

TABLE III. Enzyme activities of mitochondria containing high and low levels of UFA

UFA content of mitochondria (% total fatty acid)	State 3 respiratory activity (μg atoms oxygen/min/mg protein)			Malate dehydrogenase (μmoles/min/mg protein)	Succinate dehydrogenase (μmoles/min/mg) protein
	Pyruvate + malate	α-Oxoglutarate	Succinate		
15–24	0·14–0·40	0·10–0·22	0·07–0·15	7·8–8·4	0·15–0.21
67–74	0·17–0·42	0·11–0.19	0·08–0·18	7·9–8·6	0·16–0·22

Mitochondria were isolated from cells grown on 1% yeast extract, *Saccharomyces* salts medium plus low (50 μg/ml) and high (2 mg/ml) levels of UFA. Respiratory activities were measured manometrically under the conditions described in Methods for the determination of P:O ratios. Malate dehydrogenase and succinate dehydrogenase activities were determined as described in Methods. The results presented are grouped into low and high mitochondrial UFA levels and each group is composed of five separate experiments.

Effect of UFA-depletion on the Activation of Mitochondrial NADH Oxidase Activity in Cytochrome c

Intact yeast mitochondria, unlike their mammalian counterparts, are able to oxidize externally added NADH at a rapid rate, and the oxidation is tightly coupled to phosphorylation.[19,20] These observations were confirmed, and it was further found that if yeast mitochondria are depleted of cytochrome *c* by osmotic shock, NADH oxidase activity is lost but can be fully restored by the addition of horse heart cytochrome *c*.

Phospholipid has been shown to be important in the interaction and binding of cytochrome *c* to cytochrome oxidase, and in the complexing of lipid with cytochrome *c* (for review see Green and Fleischer[21]). Furthermore, Das *et al.*[22,23] have shown that the complex formed between phospholipid and cytochrome *c* is affected by the UFA content of the phospholipid. Thus it is possible that UFA-depletion affects the interaction of cytochrome *c* with other components in the respiratory chain. In order to investigate this possibility, mitochondria were purposely depleted of cytochrome *c* by subjecting them to osmotic shock as described in Methods. Mitochondria from both UFA-depleted and UFA-supplemented cells obtained by this method lose cytochrome *c* compared to intact mitochondria prepared by the normal procedure. Curve C in Fig. 1 shows a typical difference spectra of these cytochrome *c*-depleted mitochondria. Cytochrome *c* appears only as a shoulder at 550 nm, but its presence can be unequivocally demonstrated by spectroscopy at the temperature of liquid nitrogen which shows a peak at 547 nm. The

TABLE IV. Effect of UFA-depletion on the activation of mitochondrial NADH oxidase by cytochrome *c*

UFA content of mitochondria % (total fatty acids)	NADH oxidase Initial activity (nmoles/min/mg protein)	V_{max}	Apparent K_m for cytochrome *c* ($\times 10^{-7}$M)
20	69	400	17
26	67	416	14
44	94	276	5
73	50	310	1.1
76 (KD115-1)	63	280	1·2

NADH oxidase activity was determined spectrophotometrically as described in Methods. Initial activity is the NADH oxidase activity in the absence of added cytochrome *c*. Activity is independent of the mitochondrial concentration in the range 50–200 μg mitochondrial protein/ml. K_m (apparent) and V_{max} are calculated from double reciprocal plots of activity against cytochrome *c* concentration. NADH oxidase activity is inhibited >98% by antimycin A (4 μg/ml) or KCN (1 mM) in the presence or absence of added cytochrome *c*.

effects of added cytochrome *c* on the NADH oxidase activity of mitochondria containing different levels of UFA are given in Table IV. The oxidation of both succinate and NADH by the cytochrome *c*-deficient mitochondria is stimulated three to six-fold on the addition of cytochrome *c*. The degree of stimulation is dependent on cytochrome *c* concentration only, and independent of the mitochondrial protein concentration over the four-fold range used in the assays. Antimycin A and potassium cyanide completely inhibit the activity even in the presence of excess cytochrome *c* indicating that cytochrome *c* is functioning at its normal site in the electron transport chain. Although NADH oxidase activity in the absence of added cytochrome *c* varies between preparations, UFA-content does not appear to affect the total activity of the fully activated enzyme. However, there is a direct relationship between the apparent Michaelis–Menten constant (K_m) for the stimulation of NADH oxidase activity by cytochrome *c* (obtained by a double reciprocal plot), and the UFA-content of the mitochondrial lipids. UFA-depleted mitochondria exhibit an apparent K_m for cytochrome *c* which is fifteen times greater than that of mitochondria containing high levels of UFA. Cytochrome *c*-depleted mitochondria prepared

TABLE V. Respiratory control and phosphorylation efficiencies of mitochondria containing different levels of UFA

Mitochondrial UFA (% total fatty acids)	P : O ratios						Respiratory control ratio (αOG)
	Pyruvate + malate	Pyruvate + malate + DNP	Succinate	Succinate + DNP	αOG	αOG + DNP	
67–80	1·1–1·3	<0·1	1·1–1·3	<0·1	1·6–2·5	0·8–1·1	2–5
50–60	0·6–1·0	<0·1	0·6–1·0	<0·1	1·0–1·5	0·6–1·0	1·5–2·5
33–43	0·2–0·6	<0·1	0·3–0·5	<0·1	0·8–1·5	0·3–1·0	1·3–2·0
15–28	<0·1	<0·1	<0·1	<0·1	0·4–0·8	0·8–0·4	0·9–1·3

Cells were grown to the stationary phase of growth on media containing 1% yeast extract, *Saccharomyces* salts, 0·4% glucose and UFA supplements in the range 50 μg/ml to 2 mg/ml. Mitochondria were isolated and their UFA content estimated as described in Methods. P : O ratios were determined manometrically as described in Methods with the substrates: 4 mM pyruvate + 4 mM malate, 4 mM succinate and 4 mM α-oxoglutarate (αOG). Respiratory control ratios were determined polarographically as described in Methods with 4 mM α-oxoglutarate as substrate. Respiratory control ratios with succinate or pyruvate plus malate were generally lower than with α-oxoglutarate as substrate. 2:4-Dinitrophenol (DNP) was added where indicated at 0·2 mM. The data are the range in values observed in at least four sets of experiments.

from the prototrophic strain KD115-1 give an apparent K_m for the restoration of respiratory activity by added cytochrome *c* close to that of the mutant containing high levels of UFA. These results suggest that the binding of cytochrome *c* to its site in the electron transport chain is affected by the level of UFA in the mitochondrial lipids and is less efficient in the UFA-depleted mitochondria.

Effect of UFA-depletion on Mitochondrial Oxidative Phosphorylation

The phosphorylation efficiencies of mitochondria exhibiting a wide range of UFA-contents are given in Table V. Mitochondria containing high levels of UFA (67–80%) have P : O ratios that are in the normal range for yeast mitochondria which lack the first (NADH dehydrogenase) site of phosphorylation.[24, 25] Depletion of the UFA-content of the mitochondrial fatty acids causes a concomitant fall in the P : O ratios and in the respiratory control ratios. Mitochondria containing between 15 and 28% UFA possess no detectable phosphorylation and show a complete lack of respiratory control. P : O ratios with α-oxoglutarate as substrate fall to 0·6–0·8 in the UFA-depleted mitochondria, but as this ratio is unaffected by 2:4-dinitrophenol the remaining phosphorylation is that catalyzed by succinate thiokinase. The level of UFA at which oxidative phosphorylation is essentially abolished corresponds to the level of UFA at which cells were found previously to stop growing on non-fermentable substrates. Furthermore, the partial loss of oxidative phosphorylation at intermediate levels of UFA (33–60%) accounts for the gradual fall observed in growth rate and growth efficiency at these intermediate levels of UFA-supplementation.[1]

Beck *et al.*[26] have shown that oxidative phosphorylation in the mutant p_9 is restored by addition of 5 mM ADP, the lesion apparently affecting the adenine nucleotide translocase and hence the K_m for ADP in oxidative phosphorylation. This possibility was tested in UFA-depleted mitochondria, but ADP at concentrations as high as 10 mM still gives negligible phosphorylation.

The Recoupling of Oxidative Phosphorylation in UFA-depleted Mitochondria in vivo

It has previously been observed that UFA-depleted cells readily incorporate added UFA and recover the ability to grow on non-fermentable substrates.[1] This suggests that UFA-depleted mitochondria *in vivo* can regain the ability to couple phosphorylation to respiration on incorporation of UFA into their membrane lipids. It is, however, also possible that UFA-depletion produces secondary effects on the synthesis of mitochondrial proteins required for oxidative phosphorylation, and that the recovery of cells from UFA-depletion requires not only changes in lipid composition but also the synthesis of proteins. Specific inhibitors were, therefore, used to investigate whether protein synthesis is required during the recovery process. Chloramphenicol was used as a specific inhibitor of the mitochondrial protein synthesizing system, and cycloheximide as a specific inhibitor of the cytoplasmic ribosomal protein synthesizing system.[3] Chloramphenicol completely inhibits the formation of particulate cytochrome by cells of KD115 at 2 mg/ml of medium, and in these experiments it was assumed that the antibiotic completely inhibits the mitochondrial protein synthesizing system *in vivo* at a concentration of 4 mg/ml of medium. Cycloheximide was used in the experiments at a concentration of 10 μg/ml of medium as it inhibits the incorporation of ^{14}C leucine into whole cells of KD115 by more than 99% at this concentration. The effects of added UFA and

antibiotics on the growth of UFA-depleted cells are shown in Fig. 2. The cells stop growing after approximately 20 hours when their fatty acids contain 20% UFA. These galactose-grown cells have normal cytochrome spectra and a high respiratory activity (150 ng atoms oxygen/min/mg dry weight with glucose as substrate). UFA was added at 23 hours and cells in the control incubation without antibiotics (curve A) start to grow approximately 7 hours later, indicating that oxidative phosphorylation has become recoupled. The extent of growth of the cells has doubled by 15 hours after the addition of UFA, but the cells still have spectra and respiratory activities identical to those of the initial UFA-depleted cells. In the presence of chloramphenicol (curve B) the cells start to grow at approximately the same time as in the control incubation, but

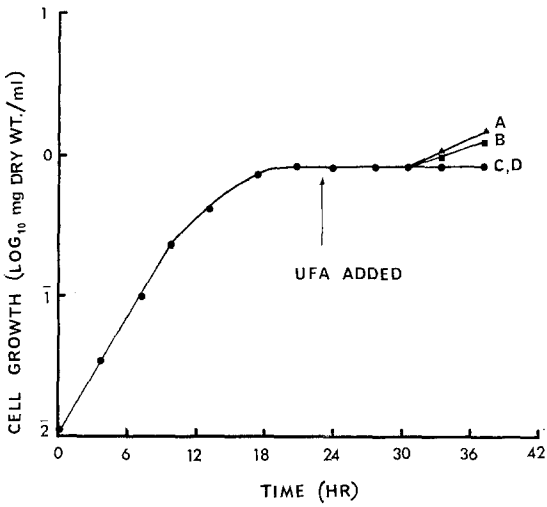

Figure 2. The effects of antibiotics on the recovery of KD115 cells from UFA-depletion. Cells of KD115 were incubated aerobically until growth ceased in a medium containing 1% yeast extract, *Saccharomyces* salts, 2% galactose and UFA (50 μg/ml). The cells were then divided into four batches, and at 23 hours the following additions were made: A. Control incubation, UFA, 0·1%; B UFA, 0·1%; chloramphenicol, 4 mg/ml; C. UFA, 0·1%; cycloheximide, 10 μg/ml; D. UFA, 0·1%; chloramphenicol, 4 mg/ml; and cycloheximide, 10 μg/ml.

their growth after 15 hours exposure to added UFA is significantly less. Although the total respiratory activity of the chloramphenicol inhibited cells remains approximately constant, there is a decrease in the concentrations of cytochromes *a* and *b* and in the specific activity of respiration. This is because chloramphenicol prevents the synthesis of new cytochromes, but pre-existing respiratory enzymes are unaffected and are merely diluted in concentration by cellular growth. Furthermore, the recommencement of growth indicates that the electron transport chain present in the cells before the addition of chloramphenicol has become recoupled. In the presence of cycloheximide the cells are incapable of growth due to the inhibition by this antibiotic of protein synthesis on the cytoplasmic ribosomes. The viability of the cycloheximide-inhibited cells starts to fall

and after 12 hours approximately 60% of the cells are incapable of dividing even after the removal of the antibiotic, indicating that irreversible changes are occurring in the cells. However, many metabolic processes are virtually unaffected including respiration, which only falls by approximately 20% after 12 hours exposure to cycloheximide.

In order to examine the effects on mitochondria of the addition of UFA to UFA-depleted cells, mitochondria were isolated at various stages of the incubations illustrated in Fig. 2. The effects of added UFA and antibiotics on the UFA-composition and P : O

TABLE VI. The effects of added UFA and antibiotics on the fatty acid composition and oxidative phosphorylation of UFA-depleted mitochondria

Time (h)	Additions	% UFA	Ratio with O:P pyruvate + malate
0	None	17–24	<0·1
5	None	39	0·37
7	None	61	0·85
7	Chloramphenicol	60	0·85
7	Cycloheximide	50	0·15
7	Cycloheximide + chloramphenicol	43	0·11
10	None	74	1·20
10	Chloramphenicol	64	0·85
10	Cycloheximide	62	0·35
10	Chloramphenicol + cycloheximide	64	0·12
12	None	71	1·22
12	Chloramphenicol	72	1·28
12	Cycloheximide	67	0·55
12	Cycloheximide + chloramphenicol	64	0·22
12	UFA omitted	18	<0·1
15	Cycloheximide + chloramphenicol	67	0·58

Cells were grown as described in Fig. 2. and reached the stationary phase of growth after 20–22 hours at a cellular concentration of approximately 1 mg dry weight/ml. At this stage a small sample of cells was removed for fatty acid analysis, and the incubation was divided into four portions of about 2 litres. The four samples were then incubated with UFA (0·1%) in the presence of chloramphenicol (4 mg/ml) and/or cycloheximide (10 μg/ml) where indicated. Cells were harvested at 0, 5, 7, 10, 12 and 15 hours after the addition of UFA. Mitochondria, were isolated and their fatty acid compositions and phosphorylative abilities with pyruvate (4 mM) plus L-malate (4 mM) were determined as described in Methods. The table summarizes the results of ten such experiments, and each value represents the average of at least two determinations.

ratios of UFA-depleted mitochondria are shown in Table VI. In the absence of antibiotics the added UFA is incorporated into the mitochondria relatively slowly, and takes about 10 hours to reach the normal level of 70–75% of total fatty acids. This is similar to the rate of incorporation of UFA into the lipids of the whole cells.[1] Accompanying the incorporation of UFA the mitochondria become recoupled, and at 7 hours the P : O ratio indicates approximately 50% recoupling; this is the time at which the cells start to grow, presumably as a consequence of the recoupling. Thus the loss of oxidative phosphorylation in UFA-depleted mitochondria can be reversed in a non-growing

system by changes in mitochondrial lipid composition. The incorporation of UFA and recoupling of oxidative phosphorylation are unaffected by chloramphenicol, indicating that all the products of mitochondrial protein synthesis required for oxidative phosphorylation are present in the UFA-depleted cells.

In the presence of cycloheximide the incorporation of UFA is slower. This is not surprising in view of the decrease in viability produced by this antibiotic. However, it is possible to demonstrate the recoupling of oxidative phosphorylation in cycloheximide-inhibited cells. The recoupling is much slower than in the control experiment, but is significantly measurable after 10 hours. In the presence of both chloramphenicol and cycloheximide the delay in recoupling is even longer, and it requires 15 hours before oxidative phosphorylation reaches a moderate efficiency. These results clearly demonstrate that all the proteins required for oxidative phosphorylation pre-exist in the UFA-depleted mitochondria, and that the loss or oxidative phosphorylation is purely a lipid lesion.

Effects of UFA-depletion on the Energy-linked Reactions of Yeast Mitochondria

In addition to oxidative phosphorylation the inner mitochondrial membrane of mammalian mitochondria catalyses a number of other endergonic reactions. These energy-linked reactions can be driven by ATP hydrolysis or the energy of electron transport, and include the reduction of NADP (energy-linked transhydrogenase), the reduction of NAD by succinate (energy-linked reversed electron transport), the transport and accumulation of divalent cations, the transport and accumulation of monovalent cations in the presence of the antibiotics valinomycin or gramicidin, the ejection of hydrogen ions, and conformational changes observable by electron microscopy or light-scattering measurements. The energy-linked reactions are all closely linked to oxidative phosphorylation; it is, therefore, of considerable interest to determine the effects of UFA-depletion on these reactions. Unfortunately, mitochondria for *S. cerevisiae* lack most of the easily measurable energy-linked reactions present in mammalian mitochondria. Several investigators have shown that the first phosphorylation site in *S. cerevisiae* is lacking,[20, 24, 25] and consequently the energy-linked reduction of NAD^+ by succinate. We have confirmed the observation that submitochondrial particles also lack the energy-linked transhydrogenase,[28] but the particles do exhibit a slow non-energy dependent transhydrogenase reaction (5–10 nmoles/min/mg protein).

Very little is known about ion transport, and conformational changes in yeast mitochondria. Carafoli *et al.*[19] have recently reported that *S. cerevisiae* mitochondria lack energy-dependent Ca^{2+} transport, but bind 40–50 nmoles of Ca^{2+} per mg protein independently of metabolism. We have investigated the effects of UFA-depletion on this phenomenon and the results are presented in Table VII. The binding of Ca^{2+} is independent of energy in mitochondria containing either high or low levels of UFA. The amount of calcium bound (20–25 nmoles/mg mitochondrial protein) is less than reported by Carafoli *et al.*[29] but was measured under different experimental conditions.

Carafoli *et al.*[29] also reported that the respiration of mitochondria from *S. cerevisiae* is stimulated by valinomycin in the presence of KCl, suggesting that energy-linked transport of K^+ occurs. The effect of valinomycin plus KCl on the respiration of mitochondria containing high and low levels of UFA is presented in Fig. 3. The UFA-supplemented mitochondria in these experiments are tightly coupled and have a respira-

TABLE VII. ^{45}Ca uptake by mitochondria containing high and low levels of UFA

UFA content of mitochondria (% total fatty acid)	^{45}Ca uptake (μmoles/10 min/mg protein)		
	No additions	ATP added	ATP + DNP added
25	25	26	22
73	20	19	17

^{45}Ca uptake was measured as described by Carafoli and Lehninger[15] at 30° in a medium containing 10 mM tris-HCl (pH 7·2), 10 mM $MgCl_2$, 4 mM K–PO_4 (pH 7·2). 80 mM NaCl (0·05 μCi/mole), and mitochondrial protein (1 mg/ml). ATP (15 mM, pH 7·2) and DNP (2:4-dinitrophenol, 0·2 mM) were added where indicated. The results are the average of three experiments. In the same system liver mitochondria take up about 760 nmoles ^{45}Ca/10 min/mg protein.

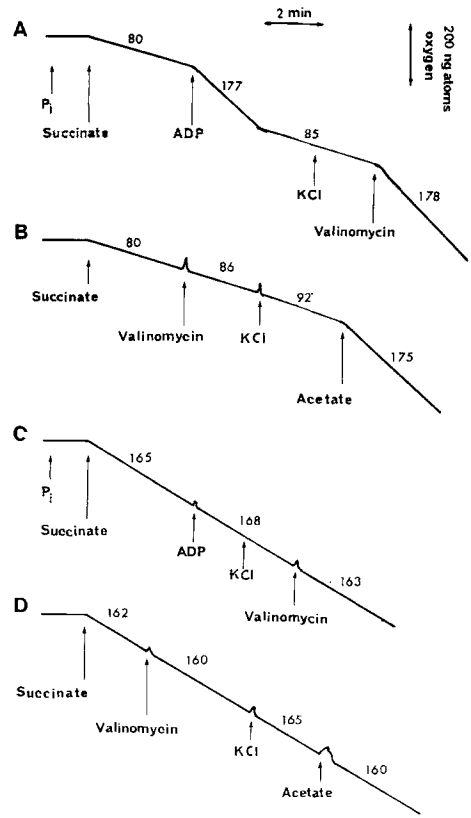

Figure 3. The effects of valinomycin plus KCl on the respiration of yeast mitochondria containing high and low levels of UFA. Mitochondria were isolated as described in Methods from cells grown on 1% yeast extract, *Saccharomyces* salts medium containing 2% galactose and either excess UFA (4 mg/ml) in experiments A and B, or growth limiting UFA (50 μg/ml) in experiments C and D. The mitochondrial fatty acids contain 71% in experiments A and B and 21% UFA in experiments C and D. The figure shows polarographic recordings of respiratory activity at 30° in a medium containing in an initial volume of 2·20 ml, 0·25 M sucrose, 10 mM tris-HCl pH 6·7. Other additions where indicated are 7 mM tris-succinate (pH 6·7), 1 mM tris-phosphate (pH 6·7), 1 mM KCl, 2 mM tris-acetate (pH 6·7), 0·2 μg valinomycin, 0·4 mM tris-ADP (pH 6·7). The numbers above the polarograph traces indicate respiratory activity (ng atoms oxygen/min/mg) protein). Experiments A and B contain 0·45 mg mitochondrial protein and experiments C and D contain 0·38 mg mitochondrial protein.

tory control ratio of 2·2 and an ADP : O ratio of 1·7 with succinate as substrate. Respiration is uncoupled by the addition of KCl and valinomycin, and increases to the rate obtained in the presence of ADP. Addition of valinomycin plus KCl in the absence of

a permeant anion has only a slight effect on the rate of respiration (Trace, B) but maximal stimulation is subsequently obtained on addition of 1·5 mM acetate or 1·5 mM phosphate. Traces C and D show that UFA-depleted mitochondria lack respiratory control, and that respiration is unaffected by the addition of valinomycin plus KCl.

Effects of UFA-depletion on the Terminal Reactions of Oxidative Phosphorylation

The terminal steps of oxidative phosphorylation are catalysed by the mitochondrial ATPase designated F_1 by Racker.[27] In order to further define the nature of the lesion in UFA-depleted mitochondria, the activity of F_1–ATPase in mitochondria containing high and low levels of UFA was determined. Table VIII shows that the activity of mitochondrial ATPase in UFA-depleted and UFA-supplemented mitochondria is very similar. Furthermore, the ATPase activity in both preparations is 85–90% inhibited

TABLE VIII. ATPase activity of mitochondria containing high and low levels of UFA

UFA content of mitochondria (% total fatty acid)	ATPase activity (μmoles ATP/min/mg protein)		
	No addition	+F_1 inhibitor	+Oligomycin
21	1·65	0·20	0·33
73	1·72	0·17	0·38

Cells were grown to stationary phase on 1% yeast extract *Saccharomyces* salts 2% galactose medium containing high (1 mg/ml) and low supplements (50 μg/ml) of UFA. Mitochondria were isolated, and ATPase activity and sensitivity to inhibitors determined at pH 6·2 as described in Methods; similar results were obtained at pH 9·5. The results are the average of five determinations. The protein ATPase inhibitor from beef heart mitochondria (F_1 inhibitor) and oligomycin were used at concentrations of 16 and 60 μg/mg mitochondrial protein respectively.

by the specific protein inhibitor isolated from beef heart mitochondria by the method of Monroy and Pullman,[10] and 70–75% inhibited by oligomycin at 60 μg/mg mitochondrial protein.

The $^{32}P_i$–ATP exchange activity of mitochondria requires F_1–ATPase and, furthermore, is generally regarded as reflecting the intactness of the terminal coupling sequence of oxidative phosphorylation, as it is abolished by oligomycin, uncouplers of oxidative phosphorylation or mechanical disruption of the mitochondria (for review see 27). Accordingly, the effects of UFA-depletion on $^{32}P_i$–ATP exchange activity were investigated, and the results are given in Table IX. Mitochondrial $^{32}P_i$–ATP exchange activity decreases progressively as the UFA-content falls, but activity is still present in mitochondria whose fatty acids contain as low as 20% UFA. The activity is also sensitive to oligomycin and dinitrophenol. However, if cells are more extensively depleted in UFA by growth on 1% glucose, isolated mitochondria whose fatty acids contain less than 17% UFA have insignificant exchange activity.

TABLE IX. $^{32}P_i$–ATP exchange activity of mitochondria containing different levels of UFA

UFA content of mitochondria (% total fatty acids)	Additions to growth medium	Exchange activity (nmoles $^{32}P_i$ exchanged/min /mg protein)		
		No addition	+DNP	+Oligomycin
78	2·0% ethanol, 0·4% UFA	60	2	3
72	0·4% glucose, 0·4% UFA	25	8	3
37	0·4% glucose, 100 μg/ml UFA	27	4	5
24	2·0% ethanol, 50 μg/ml UFA	32	10	2
22	2·0% galactose, 50 μg/ml UFA	14	2	0·5
20	0·4% glucose, 50 μg/ml UFA	12	3	2
18	1·0% glucose, 50 μg/ml UFA	2·0	0·8	1·1
17	1·0% glucose, 50 μg/ml UFA	0·6	0·6	0·6
13	1·0% glucose, 50 μg/ml UFA	0·5	0·4	0·2

Cells were grown on media containing 1% yeast extract, *Saccharomyces* salts and the substrates and UFA supplements indicated. Mitochondria were isolated and assayed for $^{32}P_i$–ATP exchange activity as described in Methods. DNP represents 2:4-dinitrophenol (250 μM), and oligomycin was used at a concentration of 50 μg/mg mitochondrial protein. The results are those of typical preparations of mitochondria.

Discussion

Effects of UFA-depletion on the Lipid Composition of Yeast Mitochondria

A decrease in the UFA-content of whole cells of the mutant is paralleled by a similar depletion of UFA in the mitochondrial lipids. UFA-depleted mitochondria do not differ greatly in their total lipid content compared to mitochondria containing high levels of UFA, but there is a 70% decrease in the phosphatidyl inositide content. This decrease in the level of mitochondrial phosphatidyl inositides is more marked than that observed in the whole cell lipids.[1] The reason for the deficiency of phosphatidyl inositides both in whole cells and in the mitochondria is not known, but may be significant in relation to the loss of oxidative phosphorylation. A number of authors have previously implicated phosphatidyl inositides in the energy-linked processes of mitochondria. Vignais and colleagues[30, 32] have shown that phosphatidyl inositides are required for the contraction of aged rat liver mitochondria. Moreover Hill *et al.*[33] have reported the incorporation of $^{32}P_i$ into an unidentified lipid fraction by a particulate ATPase isolated from beef heart mitochondria. The labelled lipid was extractable by organic solvents under conditions that extract polyphosphoinositides. However, it is unlikely that this lipid is an intermediate in oxidative phosphorylation both from the point of view of its high chemical stability and its slow rate of formation. Furthermore, evidence against the involvement of phosphatidyl inositides in oxidative phosphorylation comes from fractionation studies performed on mammalian mitochondria. Phosphatidyl inositides are found predominantly in the outer mitochondrial membrane (14–17% of the total phospholipid), whereas only a small amount is found in the inner membrane (2–4% of the total phospholipid[34, 35]). The phospholipid composition of inner and outer membranes of yeast mitochondria have not been reported. It is, nonetheless, still possible that a quantitatively minor phosphatidyl inositide fraction has a specific role in the function of the inner mitochondrial membrane.

Effects of UFA-depletion on the Respiratory Enzymes of Mitochondria

Mitochondria containing either high or low levels of UFA contain essentially normal levels of cytochromes $a + a_3$, b and c_1, succinate and malate dehydrogenases, and oxidize both NADH-linked and flavoprotein-linked substrates at normal rates. Although UFA-depletion has no effect on the NADH oxidase activity of intact mitochondria, it markedly affects the kinetics of the reactivation of NADH oxidase by cytochrome *c* in mitochondria whose cytochrome *c* has been removed by mild disruption using osmotic shock. The apparent K_m for the stimulation of NADH oxidase activity by cytochrome *c* is fifteen times greater in UFA-depleted than in normal mitochondria. This result clearly illustrates the importance of the UFA moiety of the mitochondrial lipids in the interaction of the components of the respiratory chain.

The importance of phospholipids in the respiratory chain has long been recognized,[21] but few studies have been reported for phospholipids differing in their fatty acid composition. De Pury and Collins[36,37] studied the reactivation of succinate-cytochrome *c* reductase activity of acetone-extracted rat liver mitochondria by phospholipids isolated from rats deficient in essential polyunsaturated fatty acids (EFA), and found that reactivation was equally effective as with phospholipids rich in essential fatty acids. However, the rate of binding of the phospholipid to the mitochondrial protein was affected by EFA-depletion. Green and colleagues[38–41] have shown that D(−)-3-hydroxybutyrate dehydrogenase has a specific requirement for phosphatidyl choline which contains unsaturated fatty acid groups. Das *et al.*[22,23] have shown that cytochrome *c* forms an isooctane soluble complex with phospholipids, phosphatidyl ethanolamine being the most effective. Furthermore, the nature of the complex is determined by the level of UFA in the phospholipid; highly unsaturated phospholipids readily form isooctane soluble complexes, whereas phospholipids low in UFA content form loose complexes essentially insoluble in isooctane and showing low activity with cytochrome oxidase. In view of these findings it is suggested that UFA-depletion lowers the ability of the cytochrome *c* to complex in a spatially ideal arrangement within the phospholipid milieu of the mitochondrial membrane, and thus the affinity for cytochrome oxidase is decreased.

The Effects of UFA-depletion on Oxidative Phosphorylation

UFA-depletion causes a loss of oxidative phosphorylation and respiratory control in the isolated mitochondria. The residual phosphorylation obtained with α-oxoglutarate as substrate is insensitive to 2:4-dinitrophenol, indicating that it is due to the substrate level phosphorylation catalysed by succinate thiokinase. The loss of oxidative phosphorylation closely parallels the decrease in UFA composition of the mitochondria; phosphorylation capacity is reduced by about 50% in cells containing 33–45% UFA, and approaches zero when the cells contain 15–28% UFA. A similar correlation is noted with the decrease in phosphatidyl inositide content which is halved in mitochondria containing 20% UFA. The possibility that the loss of oxidative phosphorylation is due to an increased fragility of the mitochondria during isolation lending to damage is discounted by the intact appearance of the mitochondria in electron micrographs and by the observations that mitochondrial energy metabolism is affected *in vivo*.[1]

A study that at first consideration has some features in common with the one presented in this paper is the effect of essential fatty acid (EFA) deficiency on mammalian mito-

chondria.[42–47] EFA-deficiency is characterized by a decrease in linoleic and arachidonic acids and a rise in palmitoleic, oleic and docosatrienoic acids,[45] and affects the mitochondria both structurally and functionally. EFA-deficient mitochondria are larger in size and possess a more irregularly organized inner membrane than normal mitochondria.[47] They also exhibit different swelling properties than normal mitochondria, and oxidative phosphorylation, respiratory control and $^{32}P_i$–ATP exchange activity are much more readily lost.[42–46] However, EFA-deficient mammalian mitochondria differ from UFA-deficient yeast mitochondria in two important respects. Firstly, EFA-deficient mammalian mitochondria are unlike UFA-deficient yeast mitochondria in having a phosphorylation efficiency not significantly different from that of normal mitochondria.[45, 47] Secondly, the changes in fatty acid composition of the mammalian mitochondria are much more complex than those in the yeast mitochondria, as they involve both the nature and amount of monounsaturated and polyunsaturated fatty acids. Thus the effect of UFA-depletion on oxidative phosphorylation by yeast mitochondria appears to be a much more clearly defined phenomenon.

The Recoupling of Mitochondrial Phosphorylation in vivo

The non-phosphorylating mitochondria of UFA-depleted cells recoupled upon incorporation of UFA even in the presence of both chloramphenicol and cycloheximide. This demonstrates that neither cytoplasmic ribosomal nor mitochondrial protein synthesis are required for recoupling, and that the UFA-depleted mitochondria contain all the necessary proteins for coupled electron-transport. The loss of oxidative phosphorylation, therefore, appears to be a specific lipid lesion.

Cycloheximide significantly delays the rate of incorporation of UFA into mitochondrial lipids. This may represent a diminution of the enzymes required for *de novo* lipid synthesis or for the transacylation of lipids; significant turnover in the enzymes could be expected in the 12–15 hour period required for recoupling, and the enzymes would not be replaced in the presence of cycloheximide. An alternative hypothesis is provided by the work of Schiefer[48] who found that protein synthesis is necessary for the synthesis of new phospholipid by rat liver, and suggested that phospholipid synthesis is regulated by the amount of protein available for the binding of the newly synthesized phospholipid. It is possible that the partial inhibition by cycloheximide of the incorporation of UFA into mitochondrial lipids is due to the loss of certain mitochondrial proteins that are not replaced in the presence of the antibiotic; this would also explain the lower extent of recoupling in the presence of cycloheximide.

The loss of viability in the presence of cycloheximide presumably reflects the irreversible loss of certain important proteins required for cell division, for example, proteins of the cytoplasmic membrane. The demonstration of extensive recoupling of oxidative phosphorylation in non-viable cells is remarkable, but metabolic activity and the ability to divide are not conditionally linked.

Whether the incorporation of UFA during recoupling represent the synthesis of new lipid or the replacement of the saturated fatty acid variety in existing lipid was not determined. Experiments with rat liver microsomes and mitochondria suggest that acylation may contribute significantly to the renewal and turnover of the fatty acids of phospholipids in membranes.[49, 50]

Effects of UFA-depletion on the Terminal Reactions of Oxidative Phosphorylation

UFA-depleted mitochondria contain normal levels of ATPase which is fully sensitive to oligomycin and to the ATPase inhibitor from beef heart mitochondria. The presence of the enzyme is not surprising as the recoupling experiments demonstrate that all the proteins necessary for oxidative phosphorylation are present in UFA-depleted mitochondria. However, the activity of the enzyme in the *in vitro* assay does not necessarily reflect its activity in the cell, and it is possible that the ATPase of UFA-depleted mitochondria has an aberrantly high activity thus preventing the net synthesis of ATP.

$^{32}P_i$–ATP exchange activity is present in mitochondria containing high levels of UFA and decreases only slowly as the UFA-content of mitochondrial fatty acids drops from 78% to 20%. The activity is even present in the mitochondria from cells that have stopped growing on ethanol, galactose or low concentrations of glucose (0·4%) because of the lesion in oxidative phosphorylation. This is surprising as conditions which cause a loss of oxidative phosphorylation normally result in the simultaneous loss of $^{32}P_i$–ATP exchange activity.[27] However, mitochondria containing slightly lower levels of UFA ($\leqslant$17% of total fatty acids), obtained by depleting cells further on a higher concentration of glucose (1%), have negligible $^{32}P_i$–ATP exchange activity. Thus it appears that P_i–ATP exchange activity is abruptly lost at a critical level of UFA slightly lower than that which leads to the complete loss of oxidative phosphorylation. The presence of exchange activity in the non-phosphorylating mitochondria whose fatty acids contain 20% UFA presumably reflects the retention of a marginally intact terminal coupling sequence which allows $^{32}P_i$–ATP exchange but not the net synthesis of ATP.

The Effects of UFA-depletion on the Energy-linked Reactions of Mitochondria

Investigations into the effects of UFA-depletion on energy-linked reactions in mitochondria are severely hampered by the lack of readily measurable reactions in *S. cerevisiae*. The measurement of energy-dependent potassium transport in the presence of valinomycin is a promising approach, as the stimulation of respiration observed in UFA-supplemented mitochondria strongly indicates that active potassium transport is occurring. Equally, the failure of valinomycin plus potassium to stimulate respiration in UFA-depleted mitochondria suggests that potassium transport is absent in these mitochondria. Direct measurement of potassium movements in these experiments are currently being undertaken, and preliminary results confirm the above conclusions.

The Nature of the Lesion Affecting Oxidative Phosphorylation in UFA-depleted Mitochondria

The present studies support the conclusion that the lesion affecting oxidative phosphorylation in UFA-depleted mitochondria is a direct consequence of changes in lipid composition, as the UFA-depleted mitochondria contain all the proteins necessary for oxidative phosphorylation. The nature of the lesion is not yet fully understood, but two major possibilities are open to investigation. Firstly, the large changes in lipid composition would be expected to affect the physical properties of the mitochondrial membranes, in particular the permeability of the inner mitochondrial membrane to protons may increase. This would lead to the collapse of the proton motive force proposed in Mitchell's chemiosmotic hypothesis,[51] and the loss of oxidative phosphorylation and $^{32}P_i$–ATP exchange activity. Experiments to investigate this possibility are currently in progress in our laboratory. Alternatively, a deficiency of UFA or phosphatidyl inositides in the

lipids of specific lipoprotein enzymes may alter the ability of the lipid to confer the correct spatial arrangement to the enzymes in their multienzyme complexes in the membrane, thus leading to inhibited or aberrant enzyme activities. Such an effect is apparent in the decreased ability of cytochrome *c* to activate the NADH oxidase activity of UFA-depleted mitochondria. Disorganization of the complexes of the electron transport chain or of the F_i–ATPase could lead to the loss of oxidative phosphorylation in a number of ways. For example, the loops of the electron transport chain postulated in the chemiosmotic hypothesis might be altered leading to the loss of proton translocation,[51] or the ability of the electron transport complexes to undergo conformational changes as suggested by Green[52,53] might be lost. Alternatively, the formation or stability of the high energy intermediates postulated by certain authors (for review see 54) could be affected.

References

1. J. W. Proudlock, J. M. Haslam and A. W. Linnane, *J. Bioenergetics*, **2** (1971) 327.
2. J. W. Proudlock, J. M. Haslam and A. W. Linnane, *Biochem. Biophys. Res. Commun.* **37** (1969) 847.
3. A. J. Lamb, G. D. Clark-Walker and A. W. Linnane, *Biochim. Biophys. Acta*, **161** (1968) 415.
4. K. Watson, J. M. Haslam, B. Veitch and A. W. Linnane, in: *Autonomy and Biogenesis of Mitchondria and Chloroplasts*, N. K. Boardman, A. W. Linnane and R. M. Smillie (eds.), North Holland Publishing Co., Amsterdam, 1971, p. 162.
5. J. M. Haslam, *Biochim. Biophys. Acta*, **105** (1965) 184.
6. G. Gomori, *J. Lab. Clin. Med.*, **27** (1942) 955.
7. L. Kovac, H. Bednarova and M. Greksak, *Biochim. Biophys. Acta*, **153** (1968) 32.
8. G. Schatz, *J. Biol. Chem.*, **243** (1968) 2192.
9. M. Somlo, *Europ. J. Biochem.*, **5** (1968) 276.
10. G. C. Monroy and M. E. Pullman, in: *Methods in Enzymology*, R. W. Estabrook and M. E. Pullman (eds.), Academic Press, New York, **10** (1967) 510.
11. O. Arrigoni and T. P. Singer, *Nature*, **193** (1962) 1256.
12. M. J. Vary, C. L. Edwards, and P. R. Stewart, *Arch. Biochem. Biophys.*, **130** (1969) 235.
13. M. E. Pullman, in: *Methods in Enzymology*, R. W. Estabrook and M. E. Pullman (eds.), Academic Press, New York, **10** (1967) 57.
14. C-P., Lee, G. F. Azzone and L. Ernster, *Nature*, **201** (1964) 152.
15. E. Carafoli and A. L. Lehninger, in: *Methods in Enzymology*, R. E. Estabrook and M. E. Pullman (eds.), Academic Press, New York, **10** (1967) 745.
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193** (1952) 1265.
17. B. Chance, in: *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan (eds.), Academic Press, New York, **4** (1957) 273.
18. V. M. Clark and A. J. Kirby, *Biochem. Prep.*, **11** (1966) 101.
19. E. A. Duell, S. Inoue and M. Utter, *J. Bacteriol.*, **88** (1964) 1762.
20. T. Ohnishi, K. Kawaguchi and B. Hagihara, *J. Biol. Chem.*, **241** (1966) 1797.
21. D. E. Green and S. Fleischer, *Biochim. Biophys. Acta*, **79** (1963) 554.
22. M. L. Das, H. Hiratsuka, J. M. Machinist and F. L. Crane, *Biochim. Biophys. Acta*, **60** (1963) 433.
23. M. L. Das, D. E. Myers and F. L. Crane, *Biochim. Biophys. Acta*, **84** (1964) 618.
24. E. Vitols and A. W. Linnane, *J. Biophys. Biochem. Cytol.*, **9** (1961) 701.
25. G. Schatz and E. Racker, *Biochem. Biophys. Res. Commun.*, **22** (1966) 579.
26. J. C. Beck, J. R. Mattoon, D. C. Hawthorne and F. Sherman, *Proc. Natl. Acad. Sci., U.S.*, **60** (1968) 186.
27. E. Racker, *Mechanisms in Bioenergetics*, Academic Press, New York, 1965.
28. G. Schatz, in: *Methods in Enzymology*, R. W. Estabrook and M. E. Pullman (eds.), Academic Press, New York, **10** (1967) 197.
29. E. Carafoli, W. X. Balcavage, A. L. Lehninger and J. R. Mattoon, *Biochim. Biophys. Acta*, **205** (1970) 18.
30. P. M. Vignais, P. V. Vignais and A. L. Lehninger, *J. Biol. Chem.*, **239** (1964) 2011.
31. P. V. Vignais, P. M. Vignais and A. L. Lehninger, *J. Biol. Chem.*, **239** (1964) 2002.
32. P. V. Vignais and P. M. Vignais, in: *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 368.
33. R. D. Hill, S. Ford, K. H. Byington, A. Tzagaloff and P. D. Boyer, *Arch. Biochem. Biophys.*, **627** (1968) 756.
34. D. Parsons, G. Williams, W. Thompson, D. Wilson and B. Chance in: *Mitochondrial Structure and Compartmentation*, E. Quagliariello, A. Papa, E. C. Slater and J. M. Tager (eds.), Adriatica Editrice, Bari, 1967, p. 29.
35. W. Stoffel and H. G. Schiefer, *Hoppe-Seylers Z. Physiol. Chemie*, **349** (1968) 1017.
36. G. G. de Pury and F. D. Collins, *Chem. Phys. Lipids*, **1** (1966) 1.
37. G. G. de Pury and F. D. Collins, *Chem. Phys. Lipids*, **1** (1966) 20.
38. I. Sekuzu, P. Jurtshuk and D. E. Green, *Biochem. Biophys. Res. Commun.*, **6** (1961) 71.

39. I. Sekuzu, P. Jurtshuk and P. E. Green, *J. Biol. Chem.*, **238** (1963) 975.
40. P. Jurtshuk, I. Sekuzu and D. E. Green, *Biochem. Biophys. Res. Commun.*, **6** (1961) 76.
41. P. Jurtshuk, I. Sekuzu and D. E. Green, *J. Biol. Chem.*, **238** (1963) 3595.
42. P. D. Klein and R. N. Johnson, *J. Biol. Chem.*, **211** (1954) 103.
43. E. Levin, R. N. Johnson and S. Albert, *J. Biol. Chem.*, **228** (1957) 15.
44. T. Ito and R. N. Johnson *J. Biol. Chem.*, **239** (1964) 3201.
45. L. A. Biran, W. Bartley, C. W. Carter and A. Renshaw, *Biochem. J.*, **94** (1965) 247.
46. Y. Kagawa, T. Takaoka and H. Katsuta, *J. Biochem.*, **65** (1969) 799.
47. R. C. Stancliff, M. A. Williams, K. Utsumi and C. Parker, *Arch. Biochem. Biophys.*, **141** (1969) 629.
48. H. G. Schiefer, *Hoppe-Seylers Z. Physiol. Chemie*, **350** (1969) 235.
49. G. L. Scherphof and L. L. N. van Deenan, *Biochim. Biophys. Acta*, **113** (1966) 417.
50. L. L. N. van Deenan, H. van den Bosch, L. M. V. van Golde, G. L. Scherphof and B. M. Waite, in: *Cellular Compartmentalisation and Control of Fatty Acid Metabolism*, F. C. Gran (ed.), Universitetforlaget, Oslo, 1968, p. 89.
51. P. Mitchell, *Biol. Rev., Cambridge*, **41** (1966) 455.
52. D. E. Green, J. Asai, R. A. Harris and J. T. Penniston, *Arch. Biochem. Biophys.*, **125** (1968) 684.
53. J. T. Penniston, R. A. Harris, J. Asai and D. E. Green, *Proc. Natl. Acad. Sci., U.S.*, **59** (1968) 624.
54. E. C. Slater, in: *Comprehensive Biochemistry*, M. Florkin and E. H. Stotz (eds.), Elsevier, Amsterdam, **14** (1967) 327.