Biochem. J. (1977) **166**, 287–298
Printed in Great Britain

The Effects of Altered Membrane Sterol Composition on Oxidative Phosphorylation in a Haem Mutant of *Saccharomyces cerevisiae*

By ANNE M. ASTIN* and J. M. HASLAM
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

(*Received* 1 *February* 1977)

1. The sterol, unsaturated fatty acid and cytochrome contents of cells of a δ-aminolaevulinate synthase mutant of *Saccharomyces cerevisiae* are manipulated by growing the organism in media containing defined supplements of δ-aminolaevulinate and other porphyrin intermediates. 2. If unsaturated fatty acids are added to the growth medium as Tween 80, sterol content and respiratory cytochromes alone are manipulated. 3. In the presence of δ-aminolaevulinate (10–50 mg/l) cells exhibit moderate to high respiratory activity, but growth yields are low, indicating a loss of oxidative phosphorylation. This is associated with the depletion of membrane lipids, either unsaturated fatty acids and sterols together or sterols alone. 4. Sterol depletion leads to the loss of coupled mitochondrial oxidative phosphorylation *in vitro*. 5. The lesion in oxidative phosphorylation is associated with an increase in the passive permeability of sterol-depleted mitochondria to protons. 6. Arrhenius plots of mitochondrial permeability to protons indicate that the activation energy for proton entry increases as the sterol content of the membranes decreases. 7. Studies on a cytoplasmic *petite* mutant isolated from strain *ole-3*, which lacks a functional membrane-bound protein-translocating adenosine triphosphatase, indicate that proton permeability of the *petite* mitochondria varies as a function of sterol composition in the same way as that of *ole-3 grande* mitochondria. This indicates that sterols alone are probably directly responsible for the increased proton entry, owing to a reorganization of the lipid in the membrane. 8. Supplemented *ole-3* cells with a normal lipid composition and normal or higher than normal respiratory activities have a growth efficiency only 65% of that of the wild-type, indicating that a further lesion in energy metabolism may be present.

In the preceding paper (Astin *et al.*, 1977), it was shown that the sterol and unsaturated fatty acid contents and respiratory activity of cells of the *ole-3* mutant of *Saccharomyces cerevisiae* can be extensively manipulated by growing the organism with defined supplements of δ-aminolaevulinate. If the cells are also supplemented with Tween 80, a source of oleic acid, the effects of changes in sterol content on energy metabolism can be investigated.

In the present work the effects of lipid depletion, and in particular of sterol depletion alone, on the efficiency of oxidative phosphorylation in mutant *ole-3* are investigated. Two approaches were used. The first uses the method of Bauchop & Elsden (1960) for determining the efficiency of energy transformations by estimating molar growth yields of growing cells *in vivo*, i.e. the g dry wt. of cells produced per mol of substrate oxidized, and from this calculating the dry weight of cells formed per mol of ATP, which is defined as the ATP coefficient. The second method involves the determination of phosphorylation efficiencies *in vitro* of mitochondria isolated from the mutant cells.

* Present address: Department of Biochemistry, Monash University, Clayton, Vic. 3168, Australia.

The results of these studies indicate that depletion of unsaturated fatty acids and sterols in combination or of sterols alone cause a decrease in the efficiency of oxidative phosphorylation both *in vivo* and *in vitro*. The loss of oxidative phosphorylation is partly explained by an increase in the passive permeability of the mitochondria to protons.

Materials and Methods

Methods for the growth of cells and determination of lipids and ethanol are as in the preceding paper (Astin *et al.*, 1977). The following additional methods were used.

Yeast strains

The wild-type strain S288C and the mutant *ole-3* were used as described previously (Astin *et al.*, 1977). A spontaneous cytoplasmic *petite* mutant was also derived from *ole-3* cells by the following procedure. About 200 cells of strain *ole-3* were plated on medium containing Difco yeast extract (2 g/litre), glucose (1 g/litre), glycerol (50 g/litre), Tween 80 (10 g/litre), *Saccharomyces* salts and δ-aminolaevulinate (50 mg/

litre), and were incubated for 72h at 28°C. Three small mutant colonies were detected, and identified as cytoplasmic *petites* by their inability to grow on non-fermentable substrates and the absence of respiratory cytochromes *a*, a_3 and *b*, even in the presence of δ-aminolaevulinate (50mg/litre).

Preparation of mitochondria

Mitochondria were prepared from cells grown under various conditions essentially as described by Lamb *et al.* (1968). After the preparation of sphaeroplasts by using snail-gut enzyme (Lamb *et al.*, 1968), the cells were washed three times in resuspension buffer (0.9M-sorbitol / 10mM-Tris/HCl / 0.5mM-EDTA, final pH7.4) and broken in the same medium in an Aminco French pressure cell by using the minimum pressure necessary to obtain a reasonable yield of intact mitochondria. The breaking pressure varied according to the yeast strain, and growth conditions used to obtain the cells, but was within the range of 2–8MPa. Mitochondria were then purified by differential centrifugation as described by Lamb *et al.* (1968). Two criteria of mitochondrial intactness were used.

(a) *Cytochrome spectra.* Reduced minus oxidized difference spectra of isolated mitochondria were determined in an SP.1800 spectrophotometer at room temperature (18–20°C) as described by Chance (1957), at a protein concentration of 5mg/ml.

(*b*) *Sucrose-impermeable space.* The sucrose-impermeable space was determined as described by Haslam *et al.* (1973*a*). Mitochondria of strains *ole-3* and *ole-3 petite* appeared to be intact by this criterion, having a sucrose-impermeable space of 1.6–2.4μl/mg of protein.

Assay of mitchondrial oxidative phosphorylation

The efficiency of oxidative phosphorylation by isolated mitochondria was determined manometrically at 30°C as described by Haslam (1965), but by with the following media. The main compartment of the Warburg flasks contained, in 2.9ml: sorbitol (0.6M); EDTA (1.2mM); K_2HPO_4 (6mM); $MgCl_2$ (6mM); ATP (0.48mM); bovine serum albumin (6mg); Tris/maleate (24mM); yeast mitochondria (3–5mg of mitochondrial protein) in the presence and absence of 2,4-dinitrophenol (0.1mM), final pH6.5. The first side arm of the Warburg flasks contained 0.2ml of hexokinase (activity 80μmol/min; Sigma type V St. Louis, MO, U.S.A.) +37.5mM-glucose, and 0.1ml of the substrates, succinate (100mM) or ethanol (10%, w/v). The second side arm contained 0.3ml of stopping mixture (0.8M-$HClO_4$/0.6M-Na_2SO_4). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and P_i was determined by the method of Gomori (1942).

Proton permeability of mitochondria

The passive permeability of non-respiring mitochondria to added protons was determined essentially as described by Mitchell & Moyle (1967), as modified by Haslam *et al.* (1973*b*). Mitochondria (5–10mg) were incubated aerobically at 30°C in 3.0ml of medium containing KCl (150mM), glycylglycine buffer (2mM), EDTA (0.5mM), bovine serum albumin (2mg/ml) and antimycin A (5μg), final pH7.2. Other additions, where indicated, were valinomycin (0.2μg), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (3μM), and HCl (1.0μmol). The incubations were performed in a thermostatically controlled glass vessel containing a magnetic stirrer. The pH of the incubation was measured by using a GK2301C (Radiometer, Copenhagen, Denmark) pH electrode inserted into the top of the vessel and connected to a PHM62 (Radiometer) pH-meter and Servoscribe 1S recorder. Changes in proton concentration were calibrated by the addition of HCl (50nmol), and the full-scale deflexion of the recorder was adjusted to 0.2pH unit.

Materials

Chemicals and yeast strains were as described by Astin *et al.* (1977). ADP, valinomycin and oligomycin were obtained from Sigma Chemical Co. [^{3}H]H_2O and [^{14}C]sucrose were from The Radiochemical Centre, Amersham, Bucks., U.K. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Calbiochem, San Diego, CA, U.S.A. 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole was a gift from Professor R. B. Beechey, Shell Research Ltd., Sittingbourne, Kent, U.K.

Results

Molar growth yields and phosphorylation efficiencies of S288C and ole-3 cells grown on ethanol medium in the absence and presence of δ-aminolaevulinate and Tween 80

Cells were grown to late exponential phase (48h) on ethanol medium with or without supplements of unsaturated fatty acids. Molar growth yields were calculated from cell growth yields by dividing by the ethanol that had disappeared from the medium during the growth period. ATP coefficients and phosphorylation efficiencies were calculated from molar growth yields by the method of Kormancikova *et al.* (1969). The results of the studies on wild-type and mutant cells are presented in Table 1(*a*). All the cultures had respiratory activities in excess of 150ng-atoms of oxygen/min per mg dry wt. of cells, and characteristic absorption bands of mitochondrial respiratory cytochromes $a+a_3$, *b* and *c* were present. Wild-type cells had a fatty acid composition that is 61% un-

Table 1. *Molar growth yields and phosphorylation efficiency of S288C and ole-3 cells grown on ethanol medium with and without unsaturated fatty acids plus δ-aminolaevulinate*

Cultures were grown for 48h with or without Tween 80 (1%, w/v) as a source of unsaturated fatty acids on ethanol (10g/l) media with the concentrations of δ-aminolaevulinate indicated. Cell growth, ethanol and lipids were determined as described in the Materials and Methods section. Sterol contents are given in μg of sterol/mg of mitochondrial protein. Molar growth yields are estimated as g dry wt. of cells formed/mol of ethanol oxidized. ATP coefficients are calculated from molar growth yields and the theoretical yield of 12mol of ATP formed from the catabolism of 1mol of ethanol, and are given in g dry wt. of cells/mol of ATP. Phosphorylation efficiencies are determined from ratio of molar growth yield and the Eldsen constant (Bauchop & Eldsen, 1960), and are given in mol of ATP formed from the catabolism of 1mol of ethanol.

Strain	δ-Aminolaevulinate concn. (mg/l)	Unsaturated fatty acids (% of total)	Sterol content	Molar growth yields	ATP coefficient	Phosphorylation efficiency
(a) Growth in the absence of unsaturated fatty acid						
S288C	0	61	6.42	73.6	6.13	7.00
	50	70	6.36	75.4	6.28	7.18
ole-3	0	22	3.06	3.7	0.31	0.35
	5	30	3.12	5.5	0.46	0.50
	10	50	3.64	18.4	1.53	1.75
	20	78	4.38	34.5	2.87	3.29
	50	79	6.38	40.5	3.38	3.85
	100	80	7.06	45.5	3.79	4.34
	500	84	10.90	47.5	3.95	4.51
(*b*) Growth in the presence of Tween 80 (1%, w/v)						
S288C	0	75	6.29	72.7	6.06	6.92
	50	78	6.53	76.3	6.36	7.27
ole-3	0	84	3.00	4.6	0.38	0.44
	5	80	3.08	5.2	0.43	0.50
	10	79	3.64	10.6	0.88	1.01
	20	82	4.45	26.3	2.19	2.50
	50	84	6.11	32.7	2.72	3.11
	100	83	7.12	38.6	3.22	3.68
	500	80	11.40	45.5	3.79	4.33

saturated, and a total sterol content of 6μg/mg dry wt. of cells, and produced 7mol of ATP/mol of ethanol consumed. Addition of δ-aminolaevulinate to the wild-type cultures did not significantly affect phosphorylation efficiency.

At the lowest δ-aminolaevulinate supplement (5mg/l), mutant *ole-3* had a phosphorylation efficiency that was only 7% of that of the wild-type. Increasing δ-aminolaevulinate supplements cause a progressive increase in efficiency from 0.4 to 4.5mol of ATP formed/mol of ethanol used, which is associated with a rise in unsaturated fatty acid and sterol content. At a δ-aminolaevulinate concentration of 50mg/l, unsaturated fatty acid and sterol contents in the mutant are the same as those of the wild-type, but even at the highest δ-aminolaevulinate supplement of 500mg/l, the efficiency of coupling during ethanol oxidation appears to be only 65% of that obtained in wild-type cells.

Cells were also grown with Tween 80 supplements as a source of unsaturated fatty acids to examine the effects of sterol depletion alone on the phosphorylation efficiencies *in vivo*. The results presented in Table 1(*b*) show that over the whole range of δ-aminolaevulinate supplements tested (0–500mg/l), the Tween 80-supplemented cells contain about 80% unsaturated fatty acids, but the phosphorylation efficiencies are similar to those obtained with δ-aminolaevulinate in the absence of Tween 80 and given in Table 1(*a*). Thus the low efficiency of oxidative phosphorylation is mainly associated with sterol depletion, and is reversed by 65% by increasing the concentration of δ-aminolaevulinate in the growth medium to 500mg/l.

Lipid composition of mitochondria isolated from cells grown on ethanol medium with supplements of δ-aminolaevulinate

The fatty acid composition of isolated mitochon-

Table 2. *Fatty acid composition of mitochondria isolated from wild-type and mutant cells grown on ethanol media with and without unsaturated fatty acid supplement and δ-aminolaevulinate*

Cultures were grown to late exponential phase (48h) on ethanol medium as indicated in the text. The results are average values for at least two experiments. Tween 80 (1%, w/v) and δ-aminolaevulinate were added where indicated.

Strain	Supplements	δ-Aminolaevulinate concn. (mg/l)	Fatty acids (%)								Totals (%)		
			$C_{12:0}$	$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{12:0}$ to $C_{15:0}$	$C_{16:0}$ + $C_{18:0}$	Unsaturated fatty acid
S288C	None	0	0.9	1.7	0	3.2	12.6	31.2	3.0	4.6	42.8	5.8	20.2	74.0
	None	50	0.7	1.1	0	2.1	6.6	37.1	2.2	3.7	45.9	4.5	12.5	83.0
ole-3	None	10	4.2	1.8	7.9	12.6	23.5	17.4	0	8.6	24.0	26.5	32.0	49.3
	None	20	1.4	5.2	8.6	6.6	22.0	12.0	0	5.1	39.1	21.8	27.1	59.7
	None	50	0.8	2.4	0	0.9	7.4	33.9	0.7	6.9	47.0	4.1	15.0	80.9
S288C	Tween 80	0	0.7	1.3	0	2.2	11.6	31.7	1.7	2.2	48.6	4.2	15.5	80.3
ole-3	Tween 80	10	0.2	3.9	1.0	0.8	14.8	19.2	0	5.3	54.8	5.9	20.1	75.0
	Tween 80	20	0.9	3.0	0.2	0.6	16.1	16.0	0	5.4	57.8	4.7	21.5	74.0
	Tween 80	50	0.6	0.9	0	0.8	18.2	20.6	1.3	2.6	65.0	2.3	12.1	85.6

dria from cells grown on ethanol with and without unsaturated fatty acid supplements in the presence of increasing concentrations of δ-aminolaevulinate are given in Table 2. The fatty acid pattern of the mitochondria closely follows the whole-cell fatty acid composition (Astin *et al.*, 1977), and there is no preferential incorporation of unsaturated fatty acids into the mitochondrial fatty acids. However, decanoic acid is not detected in the mitochondria, even at low concentrations of δ-aminolaevulinate, where high concentrations of other shorter-chain fatty acids (C_{12}–C_{15}) are present. In the mitochondria, from wild-type cells, and in mitochondria isolated from mutant cells grown with high supplements of δ-aminolaevulinate, the long-chain saturated fatty acid heptadecanoate was detected, and in mitochondria isolated from cells grown with low supplements of δ-aminolaevulinate the short-chain fatty acid pentadecanoate accumulated. These latter fatty acids probably originated from the Tween 80, which contains mainly oleic acid as the major constituent together with smaller quantities of $C_{15:0}$, $C_{16:0}$ and $C_{17:0}$ acids.

The results of sterol analyses on several mitochondrial preparations from cells grown on ethanol media in the absence and presence of unsaturated fatty acid supplements with increasing δ-aminolaevulinate concentrations are given in Table 3. Changes in mitochondrial sterol content and composition closely parallel the changes observed in cellular sterol (Astin *et al.*, 1977). However, a relatively wide range of sterol content is obtained in the mitochondria (10-fold) compared with that in whole cells (3-fold). In addition, an exogenous supply of unsaturated fatty acids to the mutant cultures increases sterol contents in the mitochondria of cells grown with low supplements of δ-aminolaevulinate (10 and 20mg/l), but at the highest concentrations of added δ-aminolaevulinate (50mg/l), the addition of unsaturated fatty acids has little effect.

Cytochrome spectra of mitochondria isolated from cells grown on ethanol medium with supplements of δ-aminolaevulinate

Mitochondria prepared from cells grown on ethanol with δ-aminolaevulinate supplements of 10, 20 and 50mg/l have normal cytochrome spectra. Fig. 1 shows difference spectra of dithionite-reduced *ole-3* mitochondrial preparations containing a range of lipid contents. Characteristic absorption bands of cytochrome $a+a_3$ (603nm), cytochrome *b* (560nm) and cytochromes *c* and c_1 (550 and 554nm respectively) were present in all the mitochondrial preparations, indicating that the mitochondria were reasonably intact, and that the cytochromes can bind to the inner mitochondrial membrane even in cases of severe lipid depletion.

Table 3. *Sterol content and composition of mitochondria isolated from wild-type and mutant cells grown on ethanol media with and without unsaturated fatty acids and δ-aminolaevulinate*

Sterols were determined or described in the Materials and Methods section. The results are the average values for at least two experiments. Retention times relative to cholestan-3β-ol (RRT) are: 0.52, squalene; 1.21, ergosterol; 1.40, 24(28)-dehydroergosterol; 1.56, episterol; 1.63, lanosterol. Tween 80 (1%, w/v) and δ-aminolaevulinate were added where indicated.

Strain	Supplements	δ-Aminolaevulinate concn. (mg/l)	Sterol content (μg/mg of mitochondrial protein) RRT ...	0.52	1.21	1.40	1.56	1.63	Total
S288C	None	0		0.90	8.62		0.56		9.18
	None	50		0.55	11.47		0.12		11.59
	Tween 80	0		1.21	9.59		0.25		9.84
ole-3	None	10		2.31	0.30	0.12		0.37	0.79
	None	20		1.52	1.67	0.30		1.02	2.99
	None	50		0.55	5.01	3.74		1.09	9.84
	Tween 80	10		0.83	1.19	0.70		0.30	2.19
	Tween 80	20		0.63	3.56	1.30		0.42	5.28
	Tween 80	50		0.82	6.69	3.15		1.05	10.89

Effect of lipid depletion on the efficiency of mitochondrial oxidative phosphorylation

The phosphorylation efficiencies of mitochondria isolated from cells grown to late exponential phase on ethanol with low, intermediate and high supplements of δ-aminolaevulinate are presented in Table 4. Supplements of Tween 80 are added where indicated to determine the effects of sterol depletion alone on mitochondrial oxidative phosphorylation. Mitochondria with high lipid contents have P/O ratios that are almost as high as those of wild-type mitochondria. *S. cerevisiae* lacks the Site-I phosphorylation normally found in animal mitochondria (Vitols & Linnane, 1961; Schatz & Racker, 1966; Kormancikova *et al.*, 1969), and has a maximum P/O ratio of 2 with either succinate or ethanol as substrate. The values obtained with mitochondria of strain S288C are within the range normally found in yeast mitochondria (Kovac *et al.*, 1968; Jollow *et al.*, 1968; Marzuki *et al.*, 1975), but the value of 1.4 is somewhat less than the theoretical value of 2. In our laboratory we have never obtained P/O ratios greater than 2, even under the conditions claimed by Mackler & Haynes (1973) and Ohnishi (1970) to induce Site-I phosphorylation. The low values indicate a relatively loose coupling between respiration and phosphorylation in the mitochondria, as is indicated by the molar growth yields *in vivo* (Tables 1*a* and 1*b*).

Depletion of both unsaturated fatty acids and sterols causes a concomitant fall in the P/O ratios in mutant *ole-3*, and at contents of between 40% and 50% unsaturated fatty acids and sterol contents of less than 3 μg/mg of protein the mitochondria had very low phosphorylation efficiencies with either succinate or ethanol as substrates. This confirms the results found in the whole-cell studies. P/O ratios fell to between 0.20 and 0.34 with ethanol as substrate, but there was still some coupling in the mitochondria, since 2,4-dinitrophenol gave P/O ratios of less than 0.1. When unsaturated fatty acid contents are returned to normal values of 70–80% by including Tween 80 in the culture medium, P/O ratios of the sterol-depleted mitochondria containing between 2 and 5 μg of sterol/mg of protein increase slightly to between 0.5 and 0.8. Under these circumstances, the mitochondria are partially recoupled, but the decrease in sterol content alone still causes considerable uncoupling. However, with δ-aminolaevulinate supplements of 50–500 mg/l, the efficiency of oxidative phosphorylation approaches normal values.

Effects of altered membrane lipid composition on the passive permeability to protons of mitochondria isolated from ole-3 cells

The effects of lowering the sterol content on the passive uptake of protons by *ole-3* mitochondria were examined, as Haslam *et al.* (1971, 1973*b*, 1974; Haslam & Fellows, 1975) demonstrated that the lesion in energy coupling produced by depletion of unsaturated fatty acid in yeast mitochondria is due to an increased passive permeability of the membrane to protons. To test the possible involvement of the proton-translocating adenosine triphosphatase in the proton permeability of *ole-3* mitochondria, a cytoplasmic *petite* strain was isolated from mutant *ole-3*, and its mitochondria were investigated.

The results obtained for the permeability to protons of non-respiring mutant and *petite* mitochondria with high unsaturated fatty acid and sterol contents are presented in Fig. 2. The traces resemble those previously observed in yeast mitochondria (Haslam *et al.*, 1973*b*). When a pulse of acid is added to non-respiring mitochondria suspended in a lightly buffered

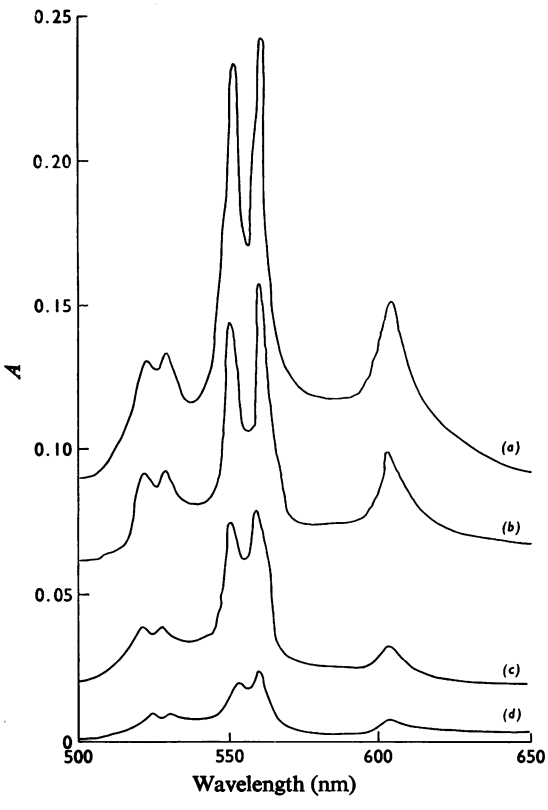

Fig. 1. *Difference spectra of mitochondria isolated from cells grown on ethanol medium in the presence of δ-aminolaevulinate*

Cells were grown on ethanol medium (1%, w/v), mitochondria were isolated and difference spectra determined as described in the Materials and Methods section at a protein concentration of 5mg/ml. (*a*) Mitochondria from S288C cells; unsaturated fatty acids, 74%; sterols, 9.20μg/mg of mitochondrial protein. (*b*) Mitochondria from *ole-3* cells grown with δ-aminolaevulinate (50mg/l); unsaturated fatty acids, 81%, sterols, 9.48μg/mg of mitochondrial protein. (*c*) Mitochondria from *ole-3* cells grown with δ-aminolaevulinate (20mg/l); unsaturated fatty acids, 51%, sterols, 2.99 μg/mg of mitochondrial protein. (*d*) Mitochondria from *ole-3* cells grown with δ-aminolaevulinate (10mg/l). Unsaturated fatty acids, 42%; sterols, 0.79μg/mg of mitochondrial protein.

medium there is a large 'overshoot' in acidity. This overshoot in acidity is due to the impermeability of the inner mitochondrial membrane to protons, and it is followed by a slow time-dependent alkalinization of the medium, which gives a measure of passive proton entry into the mitochondrial matrix (Mitchell & Moyle, 1967). In *ole-3* mitochondria containing relatively high concentrations of unsaturated fatty

Table 4. *Phosphorylation efficiencies of mitochondria isolated from wild-type and mutant cells grown on ethanol media with and without unsaturated fatty acids and δ-aminolaevulinate*

P/O ratios are determined manometrically as described in the Materials and Methods section. Sterol contents are measured as μg/mg of mitochondrial protein. Tween 80 (1%, w/v), 2,4-dinitrophenol (0.1mM) and δ-aminolaevulinate were added where indicated.

Strain	Supplements	δ-Aminolaevulinate concn. (mg/l)	No. of expts.	Unsaturated fatty acids (% of total)	Sterol content	P/O ratios			
						Succinate			
						Succinate	+2,4-dinitrophenol	Ethanol	Ethanol +2,4-dinitrophenol
S288C	None	0	2	72–74	8.9–9.2	1.35–1.40	<0.1	1.16–1.20	<0.1
	Tween 80	50	2	80–83	10.8–11.6	1.42–1.49	<0.1	1.48–1.54	<0.1
	Tween 80	0	2	78–80	9.8–10.2	1.29–1.35	<0.1	1.20–1.24	<0.1
ole-3	None	10	5	41–44	0.7–0.8	0.28–0.40	<0.1	0.20–0.34	<0.1
	None	20	3	49–53	2.6–3.0	0.40–0.57	<0.1	0.37–0.56	<0.1
	None	50	3	80–83	9.7–9.8	1.16–1.42	<0.1	1.10–1.44	<0.1
	Tween 80	10	4	65–74	2.2–2.6	0.60–0.70	<0.1	0.50–0.60	<0.1
	Tween 80	20	4	70–74	5.0–5.5	0.56–0.84	<0.1	0.52–0.82	<0.1
	Tween 80	50	3	82–86	10.7–10.9	1.37–1.56	<0.1	1.22–1.44	<0.1

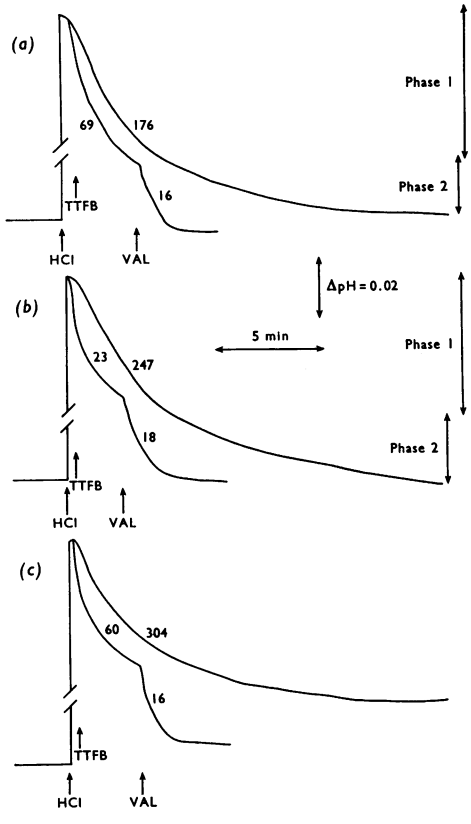

Fig. 2. *Passive entry of protons into mitochondria with a normal fatty acid and sterol composition*

Mitochondria were preincubated for 10min at the temperature indicated in the medium described in the Materials and Methods section. Further additions where indicated were HCl (0.5μmol), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) (20μM) and valinomycin (VAL) (1μg) (total volume 3.0ml). HCl (1.0μmol) was added at the time indicated and produced a fall in pH of approx. 0.3pH unit of which the 'overshoot' was 0.12–0.14pH units. (*a*) *ole-3* mitochondria at 30°C containing 68% unsaturated fatty acids and 11.2μg of sterol/mg of mitochondrial protein. (*b*) *Petite ole-3* mitochondria at 30°C containing 80% unsaturated fatty acids and 15.0μg of sterol/mg of mitochondrial protein. (*c*) *Petite ole-3* mitochondria at 10°C containing 80% unsaturated fatty acids, and 15.0μg of sterol/mg of mitochondrial protein. The numbers above the curves are the half-times ($t_{\frac{1}{2}}$) for the passive entry of protons in seconds. The upper curves represent control incubations. The lower curves show the effects of uncoupler (TTFB 20μM) and valinomycin (1μg). The 30°C traces show a biphasic response as indicated by Phase 1 and Phase 2; the low-temperature profile is an exponential curve.

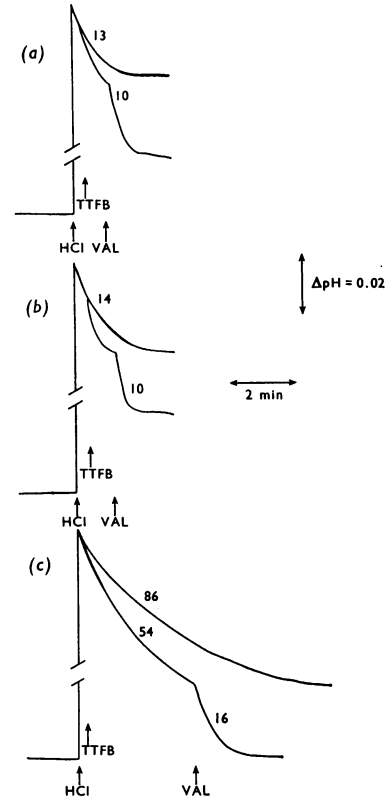

Fig. 3. *Passive entry of protons into sterol-depleted mitochondria*

Conditions are as in Fig. 2. (*a*) *ole-3* mitochondria at 30°C containing 72% unsaturated fatty acids and 1.9μg of sterol/mg of protein. (*b*) *Petite ole-3* mitochondria at 30°C containing 62% unsaturated fatty acids and 2.5μg of sterol/mg of protein. (*c*) *Petite ole-3* mitochondria at 9°C containing 62% unsaturated fatty acids and 2.5μg of sterol/mg of protein. Half-times of proton entry are indicated by the numbers above the curves.The total pH change was 0.3pH unit in each experiment, of which the overshoot was 0.05pH unit in (*a*) and (*b*), and 0.08 unit in (*c*). The upper curves represent control incubations. The lower curves show the effects of uncoupler (TTFB, 20μM) and valinomycin (VAL, 1μg).

acids and sterols (Figs. 2*a* and 2*b*), the half-time of proton entry at 30°C is 3–4min, and in those of the *petite* mutant (Fig. 2*c*) it is approx. 5min. If a chemical uncoupler is added before the acid, the overshoot in acidity is abolished, and when the uncoupler is added after the acid, the half-time of proton entry into the mitochondria decreased to 69s in mitochondria of mutant *ole-3* and 23s in those of the *petite* mutant.

The addition of valinomycin together with uncoupler to the medium further decreased the half-time of proton entry to 16s in both types of mitochondria.

In sterol-depleted mitochondria of mutant *ole-3*, the overshoot in acidity is very much diminished, as shown in Fig. 3(*a*). These organelles had a normal fatty acid content of 72% unsaturated fatty acids, but the sterol content was only 1.9 μg of sterol/mg of protein, of which 18% was ergosterol. The sterol-depleted mitochondria are 14 times as permeable to protons as the sterol-supplemented mitochondria, with a half-time of proton entry of only 15s at 30°C. An equally low value of 14s is also obtained for sterol-depleted mitochondria of the *petite* mutant (Fig. 3*b*), indicating that the increased rate of proton entry into the matrix is not dependent on the membrane-bound subunits of the adenosine triphosphatase coded for by the mitochondria. The addition of uncoupler to the medium does not significantly affect the half-time of proton entry, but valinomycin is still required to abolish the membrane potential completely and enable maximal rates of proton entry. One as yet unexplained observation in the traces is that, at temperatures above 15°C in sterol-supplemented mitochondria and above 20°C in sterol-depleted organelles, passive proton entry exhibits biphasic kinetics as shown in Figs. 2(*a*) and 2(*b*), but below these temperatures it has a simple first-order decay curve (Fig. 2*c*).

For a first-order reaction, $t_{\frac{1}{2}} = \ln 2/k$, where $t_{\frac{1}{2}}$ is the half-time of the reaction, and k is the rate constant. At lower temperatures the plot of the logarithm of the rate against time is linear, indicating a first-order process, but at higher temperatures the entry of protons appeats to be biphasic, with a second phase occurring at 5 min and later. However, the deviations from first-order kinetics are not extreme, and, for the purposes of comparison, empirical half-time values were compared over the whole temperature range, 6–30°C, for the first phase of proton entry.

The rates of passive proton entry into mitochondria of strains S288C, *ole-3* and the *petite* mutant, and their Arrhenius activation energies, are presented in Table 5. Fully supplemented mitochondria from wild-type and mutant strains with high contents of sterols are highly impermeable to protons over the whole range of temperatures tested (6–30°C).

When the sterol content of mitochondria from mutant *ole-3* cells was progressively decreased from 15 to 2.0 μg/mg of protein while unsaturated fatty acid concentrations were maintained at 62–80%, the membrane became more permeable to protons, and Arrhenius activation energies increased dramatically from 18 to 52 kJ/mol. At the lowest supplements of δ-aminolaevulinate and in the absence of Tween 80 supplements, proton entry into organelles depleted of sterol and unsaturated fatty acid is very rapid; the rate of entry is unaffected by the addition of uncoupler and the Arrhenius activation energy is increased to 65 kJ/mol. The permeability of *petite ole-3* mitochondria to protons is identical with those of wild-type and *ole-3* mitochondria, which have comparable sterol contents. The addition of uncoupler immediately after the acid stimulates proton entry into mitochon-

Table 5. *Effects of sterol depletion on the passive entry of protons into wild-type and mutant mitochondria*

Cells were grown on ethanol (1%, w/v) media plus appropriate supplements of Tween 80 (1%, w/v) or δ-aminolaevulinate (10–50 mg/l) to manipulate lipid composition as described in Tables 2 and 3. Mitochondria were isolated and half-times of passive proton entry were determined as described in Figs. 2 and 3. Arrhenius plots of proton entry over the temperature range 6–37°C were linear, from which Arrhenius activation energies were determined.

Mitochondria from strain	Sterol content (μg/mg of protein)	Unsaturated fatty acid content (% of total)	Arrhenius activation energy (kJ/mol)	$t_{\frac{1}{2}}$ of passive proton entry at 30°C (s)
S288C	11.4	70	22	183
ole-3	11.2	70	18	176
ole-3	4.5	62	26	75
ole-3	2.0	66	52	28
ole-3	1.8	48	65	15
petite ole-3	15.0	80	9	247
petite ole-3	12.4	74	14	206
petite ole-3	10.5	80	33	110
petite ole-3	5.7	65	37	69
petite ole-3	2.6	72	54	32
petite ole-3	2.5	52	62	14
*petite ole-3**	12.4	80	36	21
*petite ole-3**	10.6	80	44	17
*petite ole-3**	2.5	62	53	13

* +4,5,6,7,-Tetrachloro-2-trifluoromethylbenzimidazole (20 μM).

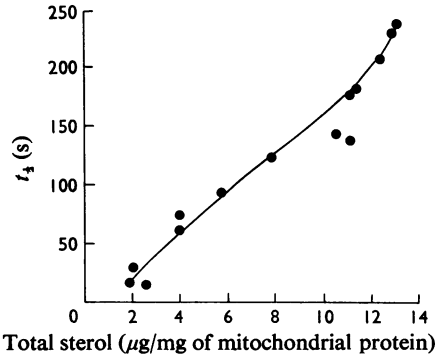

Fig. 4. *Relationship between mitochondrial sterol content and the activation energies and half-times of passive proton entry into mitochondria*

The sterol composition of *ole-3* cells was manipulated as described in Table 1(*b*). The half-times of the passive entry of protons at 30°C into isolated mitochondria were determined as in Fig. 2.

dria. The kinetics of the uncoupler-stimulated proton entry were measured for *petite ole-3* mitochondria, with different sterol contents. The Arrhenius plots are linear, and over the range of sterol contents tested (2.5–15 μg/mg of protein) the permeability of the sterol-depleted mitochondria after the addition of uncoupler only slightly increased, but the activation energies increased from 36 to 53 kJ/mol as the sterol content was lowered. The effects of the chemical uncoupler on the permeability of lipid-supplemented organelles are comparable with the effects of lowering the sterol content. This raised the possibility that the uncoupling of oxidative phosphorylation and the stimulation of proton entry could be caused by natural chemical uncouplers such as free fatty acids, particularly unsaturated fatty acids (Bulychev *et al.*, 1972; Hülsmann *et al.*, 1960). However, the amounts of bovine serum albumin in both the isolation medium (2 mg/ml) and the mitochondrial incubation medium (2 mg/ml) are sufficient to counteract the effects of much larger amounts of free fatty acids than are present in the yeast mitochondria (Haslam *et al.*, 1973*b*). It was concluded that the loss of oxidative phosphorylation in *ole-3* mitochondria depleted of sterols is not due to the presence of free fatty acids.

Relationship between mitochondrial sterol contents and the half-times and activation energies of passive proton entry into mitochondria

Examination of Arrhenius activation energies for proton permeability of wild-type, mutant and *petite* mitochondria showed that the activation energy of proton entry is related to the sterol content of mitochondria (Table 5). Activation energies appear to vary inversely with sterol content, but the two parameters are not directly proportional, as a plot of the data deviates from linearity. As sterol concentrations increase from 2 to 15 μg/mg of protein, the half-times of proton entry decrease from 15 to 250 s (Table 5). The correlation between rate of entry and sterol content is good, except at the highest sterol concentrations (Fig. 4).

Discussion

Manipulation of mitochondrial lipid and cytochrome composition in ole-3 cells by growth in the presence of δ-aminolaevulinate

If *ole-3* cells are grown in medium containing Tween 80, the cells and mitochondria have a normal fatty acid composition, but the sterol composition of the mitochondria can be extensively manipulated by varying the δ-aminolaevulinate concentration in the growth medium. At a given concentration of δ-aminolaevulinate the mitochondrial ergosterol composition is considerably enhanced by addition of Tween 80 to the growth medium, particularly at the lower concentrations of δ-aminolaevulinate. This increase in ergosterol content may be due to a decrease in the need for haematin precursors of cytochrome b_5, the prosthetic group of fatty acid desaturase, with a consequent increase in the supply of haematin compounds which act as the prosthetic group of the sterol demethylase. An alternative explanation of the increased ergosterol contents in the mitochondria is that high unsaturated fatty acid concentrations in the mitochondria may allow the insertion of more sterols, because the phospholipids are esterified with unsaturated fatty acids in the 2-position, which is optimal for the integration of the planar $\Delta^{5,7}$ sterol nucleus into the lipid bilayer (Darke *et al.*, 1972).

Effect of lipid depletion on whole-cell energy metabolism

Bauchop & Elsden (1960) showed that in a rich growth medium micro-organisms synthesize approx. 10.5 g dry wt. of cell material per mol of ATP produced by catabolism of an energy source, and this value was used to calculate phosphorylation efficiencies *in vivo*. The resultant calculated efficiency of 7 mol of ATP formed/mol of ethanol oxidized by wild-type cells in the present work is in good agreement with the yield of 6.7 mol of ATP/mol reported by Kormancikova *et al.* (1969). This value is much lower than the theoretical yield of 12 mol of ATP/mol calculated by assuming that *S. cerevisiae* lacks the first phosphorylation site of oxidative phosphorylation corresponding to Site I in animal mitochondria (Vitols & Linnane, 1961). However, under certain conditions, incomplete coupling of oxidative phosphorylation may occur *in*

vivo (Rottenberg & Caplan, 1967; Stouthamer & Bettenhausen, 1973), and ethanol may not be utilized purely as an energy source, but could be incorporated into cellular components or required for cellular maintenance.

The results of molar growth yields for strain *ole-3* indicate that mitochondrial oxidative phosphorylation becomes progressively uncoupled as the unsaturated fatty acid and sterol contents are decreased below normal values. Previous studies by Proudlock *et al.* (1971) and Marzuki *et al.* (1975) have shown that depletion of unsaturated fatty acids uncouples oxidative phosphorylation. The present work shows that sterol depletion has a similar effect. When unsaturated fatty acid composition is maintained at normal values by growing *ole-3* cells in media containing Tween 80 (1%, w/v), the phosphorylation efficiencies both *in vivo* and *in vitro* are low.

Effects of sterol depletion on the passive permeability to protons of mitochondria isolated from wild-type, mutant and cytoplasmic petite strains of S. cerevisiae

Lowering of the unsaturated fatty acid and sterol content or of sterol content alone in *ole-3* mitochondria progressively increased the permeability of non-respiring organelles to protons. However, valinomycin is still required in conjunction with uncoupler or lipid depletion to collapse the membrane potential and pH gradient across the membrane completely, and thus allow maximal rates of proton entry, indicating that sterol depletion does not markedly affect the permeability of the mitochondrial membrane to K^+. All the current models for the role played by proton translocation in energy transduction agree that respiration can be linked osmotically to phosphorylation through cyclic proton translocation across the inner mitochondrial membrane (for reviews see Papa, 1976; Hanstein, 1976). The energy-coupling activity of the respiratory carriers requires a closed membrane structure and specific orientation of the catalytic sites in the membrane. Thus any perturbations in membrane structure, for example, depletion of unsaturated fatty acid and sterol content, that render the membrane more permeable to protons, will tend to collapse the proton gradient produced by transfer of electrons along the respiratory chain and prevent the synthesis of ATP. The indications in mutant *ole-3* are that the loss of coupled mitochondrial oxidative phosphorylation *in vivo* and *in vitro* under conditions of sterol depletion is mainly due to an increased passive permeability of the inner membrane to protons.

The possibility also arises that additional proton entry was occurring through a malfunctional adenosine triphosphatase, since the activity of this enzyme is affected by the lipid composition of the membrane (Haslam *et al.*, 1974; Cobon & Haslam, 1973). To test this possibility a cytoplasmic *petite* strain was derived from mutant *ole-3* and the permeability characteristics of its mitochondria were determined, as the membrane-bound subunits of the enzyme, which are synthesized on mitochondrial ribosomes, are absent, owing to the inability of the *petite* mutant to carry out mitochondrial protein synthesis (Tzagaloff *et al.*, 1973). Mitchell (1973, 1974) has shown that the lipophilic CF_0 factor of the mitochondrial adenosine triphosphatase, which is continuous with the lipid phase of the coupling membrane, has a specific proton-conducting pathway through it that can be blocked by oligomycin or dicyclohexylcarbodi-imide, and the component F_1 which catalyses synthesis of ATP, is known to close the proton-conducting pathway through the F_0F_1 adenosine triphosphatase complex.

The results show that there was no difference between the passive permeability to protons of *petite* and *ole-3* mitochondria, indicating that the additional proton entry was not catalysed by the adenosine triphosphatase. A decrease in the sterol content of the *petite* mutant also caused a progressive increase in passive proton entry through the mitochondrial membrane. Furthermore this increase could not be attributed to extensive disruption of the organelles during isolation, since the cytochrome *c* content of mutant *ole-3* and the sucrose-impermeable space of the *petite* mitochondria showed that these mitochondria are intact, and retained their selective permeability. The lesion in energy metabolism is apparently due to an involvement of sterol in the impermeability of the lipid part of the membrane to protons. Similar observations have been made under conditions of unsaturated fatty acid depletion in the *ole-1* mutant of *S. cerevisiae* (Haslam *et al.*, 1973*b*, 1974).

However, cells and mitochondria of mutant *ole-3* with normal unsaturated fatty acid and sterol compositions, and normal or higher than normal respiratory activities, do not exhibit the same efficiency of oxidative phosphorylation as wild-type cells and organelles, being only 60–70% coupled. The possibility exists that, in addition to the enhanced proton permeability induced by sterol depletion, there is a further lesion in *ole-3* mitochondria related to the porphyrin deficiency.

The Arrhenius plots of the passive permeability of wild-type, mutant and *petite* mitochondria are entirely different from those which have been determined for membrane-bound enzymes, including the components of the respiratory chain of strains S288C and *ole-3* (Haslam *et al.*, 1974). The plots are linear, indicating that in the range of temperatures examined any phase changes that occur in the membrane lipids do not alter the membrane permeability to protons. Mitochondria with a low sterol content were very permeable to protons, and became pro-

gressively more impermeable as the sterol contents of the membranes were increased, with a corresponding lowering of the Arrhenius activation energies. The low activation energies obtained with mitochondria containing more than 6μg of sterol/mg of protein suggest that the process could be diffusion-limited. Work concerned with the permeability properties of lipids has indicated that such properties are related to the head-group composition of the phospholipid moiety, (McLaughlin *et al.*, 1970; Lelievre & Rich, 1973; Hopfer *et al.*, 1970), the length of the acyl chains and the degree of unsaturation of the chains (de Gier *et al.*, 1968; Bangham *et al.*, 1967; McElhaney *et al.*, 1973). It has also been shown that the degree of packing of lipid membranes is determined either by chain length and degree of unsaturation of the fatty acid chains of phospholipid molecules, or by their specific interactions with other lipid molecules, such as sterols (Demel *et al.*, 1972; Chapman, 1972). Studies on wheat endosperms with different sterol compositions (Carbonero *et al.*, 1975) show that endogenous sterol modifies membrane permeability; this is consistent with the results presented above. Further evidence for the direct correlation between sterol contents and the passive permeability of mitochondria to protons is shown in Fig. 4 and Table 5, where the Arrhenius activation energy increases and the half-time of proton entry decreases as the sterol content of the mitochondrial membrane is lowered.

High activation energies and increased proton entry were also observed in the presence of a chemical uncoupler (Table 5). In mitochondria with a high sterol content(22μg/mg of protein), the permeability of the membrane was increased more than 10-fold by the addition of uncoupler. In sterol-depleted mitochondria the passive entry of protons was only slightly affected by uncoupler. However, the half-times of proton entry at 30°C in the presence of uncoupler is significantly increased by sterol depletion, as is the Arrhenius activation energy, suggesting that the two effects could be mediated in different ways. Uncoupling of oxidative phosphorylation has been extensively studied, and various theories have been proposed for the mechanism of this process (for review see Hanstein, 1976), but the molecular mechanism is still not understood. Mitchell (1968) proposes that proton-conducting uncouplers pass one way through the coupling membrane in a protonated form, this being lipid-soluble, and the other way as the anion. Evidence that uncouplers cause protons to pass through phospholipid bilayers rather than creating pores in the membrane has been presented by Lieberman & Topaly (1968). In the present system sterol depletion could be acting by either increasing the number of pores in the lipid bilayer or modifying the activity of a natural proton carrier, but the results with the *petite* mitochondria rule out the possibility that the latter is a membrane-bound subunit of the adenosine triphosphatase, the so-called protonophore of the adenosine triphosphatase complex. The present work shows that sterols help to maintain the impermeability of yeast mitochondria to protons, and hence the ability to couple phosphorylation to oxidation. This may also be true in mammalian mitochondria, but in bacteria, which possess little or no sterol, other lipids may perform this function.

We thank Mr. N. F. Fellows and Mrs. J. Jones for technical assistance. This work was supported by a Medical Research Council scholarship to A. M. A. and Science Research Council grant B/RG/45754 to J. M. H.

References

Astin, A. M., Haslam, J. M. & Woods, R. A. (1977) *Biochem. J.* **166**, 275–285
Bangham, A. D., de Gier, J. & Greville, G. D. (1967) *Chem. Phys. Lipids* **1**, 225–246
Bauchop, T. & Elsden, S. R. (1960) *J. Gen. Microbiol.* **23**, 457–469
Bulychev, A., Kramar, B., Drahota, Z. & Lindberg, O. (1972) *Exp. Cell Res.* **72**, 169–187
Carbonero, P., Torres, J. V. & Garcia-Olmedo, F. (1975) *FEBS Lett.* **56**, 198–201
Chance, B. (1957) *Methods Enzymol.* **4**, 273–329
Chapman, D. (1972) *Ann. N.Y. Acad. Sci.* **195**, 179–201
Cobon, G. S. & Haslam, J. M. (1973) *Biochem. Biophys. Res. Commun.* **52**, 320–326
Darke, A., Finer, E. G., Flook, A. J. & Phillips, M. C. (1972) *J. Mol. Biol.* **63**, 265–279
de Gier, J., Mandersloot, J. G. & Van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* **150**, 666–675
Demel, R. A., Geurls van Kassel, W. S. M. & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **266**, 26–40
Gomori, G. (1942) *J. Lab. Clin. Med.* **27**, 955–960
Hanstein, W. G. (1976) *Biochim. Biophys. Acta* **456**, 129–148
Haslam, J. M. (1965) *Biochim. Biophys. Acta* **105**, 184–187
Haslam, J. M. & Fellows, N. F. (1975) *Biochem. Soc. Trans.* **3**, 772–775
Haslam, J. M., Proudlock, J. W. & Linnane, A. W. (1971) *J. Bioenerg.* **2**, 351–370
Haslam, J. M., Perkins, M. & Linnane, A. W. (1973*a*) *Biochem. J.* **134**, 935–947
Haslam, J. M., Spithill, T. W., Linnane, A. W. & Chappell, J. B. (1973*b*) *Biochem. J.* **134**, 949–957
Haslam, J. M., Cobon, G. S. & Linnane, A. W. (1974) *Biochem. Soc. Trans.* **2**, 207–209
Hopfer, U., Lehninger, A. L. & Lennarz, W. J. (1970) *J. Membr. Biol.* **3**, 142–155
Hülsmann, W. C., Elliott, W. B. & Slater, E. C. (1960) *Biochim. Biophys. Acta* **39**, 267–276
Jollow, D., Kellerman, G. M. & Linnane, A. W. (1968) *J. Cell Biol.* **37**, 221–250
Kormancikova, V., Kovac, L. & Vidova, M. (1969) *Biochim. Biophys. Acta* **180**, 9–17
Kovac, L., Bednárová, H. & Greksak, M. (1968) *Biochim. Biophys. Acta* **153**, 32–42

Lamb, A. J., Clark-Walker, G. D. & Linnane, A. W. (1968) *Biochim. Biophys. Acta* **161**, 415–427

Lelievre, J. & Rich, G. J. (1973) *Biochim. Biophys. Acta* **298**, 15–26

Lieberman, E. A. & Topaly, V. P. (1968) *Biochim. Biophys. Acta* **163**, 125–136

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mackler, B. & Haynes, B. (1973) *Biochim. Biophys. Acta* **292**, 88–91

Marzuki, S., Cobon, G. S., Haslam, J. M. & Linnane, A. W. (1975) *Arch. Biochem. Biophys.* **169**, 577–590

McElhaney, R. N., de Gier, J. & van der Neut-Kok, E. C. M. (1973) *Biochim. Biophys. Acta* **298**, 500–512

McLaughlin, S. G. A., Szabo, G., Eisenman, G. & Ciani, S. M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1268–1275

Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall

Mitchell, P. (1973) *FEBS Lett.* **33**, 267–274

Mitchell, P. (1974) *FEBS Lett.* **43**, 189–194

Mitchell, P. & Moyle, J. (1967) *Biochem. J.* **104**, 588–600

Ohnishi, T. (1970) *Biochem. Biophys. Res. Commun.* **41**, 344–352

Papa, S. (1976) *Biochim. Biophys. Acta* **456**, 39–84

Pederson, P. L. (1975) *J. Bioenerg.* **6**, 243–275

Proudlock, J. W., Haslam, J. M. & Linnane, A. W. (1971) *J. Bioenerg.* **2**, 327–349

Rottenberg, H. & Caplan, S. R. (1967) *Nature (London)* **216**, 610–611

Schatz, G. & Racker, E. (1966) *Biochem. Biophys. Res. Commun.* **22**, 579–586

Stouthamer, A. H. & Bettenhausen, C. (1973) *Biochim. Biophys. Acta* **301**, 53–70

Tzagaloff, A., Rubin, M. S. & Sierra, M. F. (1973) *Biochim. Biophys. Acta* **301**, 71–104

Vitols, E. & Linnane, A. W. (1961) *J. Biochem. Biophys. Cytol.* **9**, 701–710