Biochem. J. (1977) **166**, 565–570
Printed in Great Britain

The Effects of Unsaturated Fatty Acid Depletion on the Proton Permeability and Energetic Functions of Yeast Mitochondria

By J. M. HASLAM and NORMAN F. FELLOWS
Department of Biochemistry, University of Liverpool, P.O. Box 147, *Liverpool L*69 3*BX, U.K.*

(*Received* 11 *March* 1977)

1. The fatty acid composition of the *ole-1* and *ole-1 petite* mutants of *Saccharomyces cerevisiae* was manipulated by growing the organism in the presence of defined supplements of Tween 80 or by allowing cells that had first been grown in the presence of Tween 80 to deplete their unsaturated fatty acids by subsequent growth in the absence of Tween 80. 2. The transition temperature of Arrhenius plots of mitochondrial ATPase (adenosine triphosphatase) increases as the unsaturated fatty acid content is lowered. 3. Cells require larger amounts of unsaturated fatty acids to grow on ethanol at lower temperatures. 4. Cells that stop growing owing to unsaturated fatty acid depletion at low temperatures are induced to grow further by raising the temperature and this results in a further depletion of unsaturated acids. This is due to a higher rate, but not a greater efficiency, of mitochondrial ATP synthesis. 5. Arrhenius plots of the passive permeability of mitochondria to protons between 4 and 37°C are linear. The rate and the Arrhenius activation energy of proton entry increase greatly as the unsaturated fatty acid content is lowered. 6. Unsaturated fatty acid depletion has the same effects on the proton permeability of *ole-1 petite* mitochondria, indicating that the mitochondrially synthesized subunits of the ATPase are not involved in the enhanced rates of proton entry. 7. The adenylate energy charge of depleted *ole-1* cells is greatly decreased by growth on ethanol medium. 8. The adenylate energy charge of isolated mitochondria is also lowered by unsaturated fatty acid depletion. 9. The results confirm that unsaturated fatty acid depletion uncouples oxidative phosphorylation in yeast both *in vivo* and *in vitro*, and is a consequence of changes in the lipid part of the membrane.

Previous work has shown that the fatty acid composition of the *ole-1* mutant of *Saccharomyces cerevisiae* (Resnick & Mortimer, 1966) may be manipulated by growing the organism in the presence of defined supplements of Tween 80 as a source of unsaturated fatty acids (Proudlock *et al.*, 1971). This system enabled us (Haslam *et al.*, 1971, 1973*a*,*b*; Haslam & Fellows, 1975) to show that unsaturated fatty acid depletion specifically uncouples oxidative phosphorylation, and is associated with an increased permeability of the mitochondrial membranes to protons. The present work extends these observations by investigating the effects of temperature on the coupling of oxidative phosphorylation and proton permeability, and the possible involvement of the mitochondrial ATPase* in uncoupling. The results show that uncoupling is directly related to changes in lipid composition, is not related to temperature, and does not appear to involve the mitochondrial ATPase.

Experimental

General

Growth media, growth of cells and fatty acid analyses were as described by Proudlock *et al.* (1971).

* Abbreviation: ATPase, adenosine triphosphatase.

The determination of protein, isolation of mitochondria and measurement of P/O ratios were as described by Haslam *et al.* (1971). The measurement of the passive permeability of isolated mitochondria to protons was as given previously (Haslam *et al.*, 1973*b*; Astin & Haslam, 1977). ATPase activity was measured as described by Cobon & Haslam (1973).

Yeast strains

The *ole-1* mutant strain KD115 (Resnick & Mortimer, 1966) was that used previously (Proudlock *et al.*, 1971). A cytoplasmic *petite* derivative of strain *ole-1* was selected by treating *ole-1* cells in liquid growth medium with ethidium bromide (50mg/litre) for 16h. This procedure results in the quantitative conversion of the cells into cytoplasmic *petite* mutants (Perlman & Mahler, 1971), of which the *rho*^{0} type predominates. A single clone of the *ole-1 petite* mutant was selected, and was shown to lack respiration and particulate mitochondrial cytochromes.

Determination of adenine nucleotides

Adenine nucleotides were extracted from whole cells as described by Somlo (1970). A minimum of

16h was required for complete extraction at 0°C. Adenine nucleotides were extracted from isolated mitochondria (10–20mg of protein) by mixing with an equal volume of ice-cold $HClO_4$ (0.8M) plus Na_2SO_4 (0.6M). The denatured protein was removed by centrifugation at 600*g* for 5min and a sample of the supernatant was carefully neutralized by adding the appropriate volume of KOH (1M). $KClO_4$ was removed by centrifugation at 600*g* for 5min and the supernatant analysed for AMP, ADP and ATP as described by Adam (1965) and Lamprecht & Trautschold (1965).

Materials

Pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3), lactate dehydrogenase (EC 1.1.1.27) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer, Mannheim, W. Germany. Phosphoenolpyruvate, ATP and ADP were from Sigma Chemical Co., St. Louis, MO, U.S.A. 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole was the gift of Professor R. B. Beechey, Shell Research, Sittingbourne, Kent, U.K. Tween 80 (polyoxyethylene sorbitan mono-oleate) was obtained from BDH Chemicals, Poole, Dorset, U.K.

Results and Discussion

Effects of temperature on mitochondrial ATPase

Manipulation of the unsaturated fatty acid content of mitochondrial membranes has profound effects on the temperature-dependence of mitochondrial ATPase. Arrhenius plots of mitochondrial ATPase have sharp dicontinuities, and as the unsaturated fatty acid content of the membranes is lowered, the temperature of the discontinuity increases (Haslam *et al.*, 1973*a*). The results of 25 such experiments are summarized in Fig. 1, which shows that, as the proportion of unsaturated fatty acids in the membrane is lowered from 82 to 12%, there is an increase in the transition temperature from 5 to 35°C.

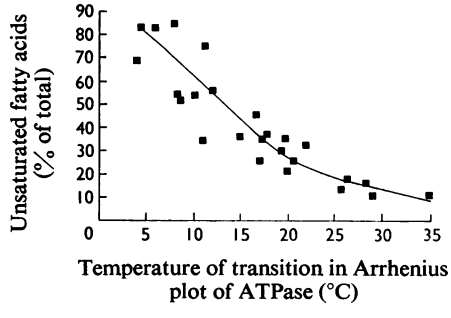

Fig. 1. *Relationship between fatty acid composition and the Arrhenius transition temperature of mitochondrial ATPase*
Mitochondria of different fatty acid composition were isolated as described by Haslam *et al.* (1971). ATPase activity was measured as described by Cobon & Haslam (1973), and Arrhenius plots of the activity were drawn. The graph shows the relationship between the transition temperature of the Arrhenius plots and membrane fatty acid composition in 25 separate experiments. Transition temperatures were accurate to ±1°C.

It was noted that cells of the *ole-1* mutant stop growing on non-fermentable substrates when 20% of the fatty acids of the mitochondrial membrane lipids are unsaturated, and the temperature of the transition in the Arrhenius plot is just below that of the growth temperature (28°C). This could indicate that the increased proton permeability of the unsaturated fatty acid-depleted mitochondria (Haslam *et al.*, 1973*b*) and the loss of oxidative phosphorylation are a consequence of changes in the mitochondrial proton-translocating ATPase, and are also related to temperature.

Effects of temperature on the growth-limiting unsaturated fatty acid composition of yeast cells

To test the hypothesis that temperature affects the consequences of unsaturated fatty acid depletion on the coupling of mitochondrial oxidative phosphorylation *in vivo*, cells were grown to stationary phase at different temperatures between 4 and 40°C. The cellular fatty acids were initially 80% unsaturated owing to the inoculum being grown in the presence of Tween 80 (1%, w/v). However, the growth medium contained no unsaturated fatty acids, and growth resulted in a dilution of the unsaturated fatty acids in the membrane by newly formed saturated fatty acids. It was previously known that at 28°C the cells continue to grow on ethanol medium until their unsaturated fatty acid content is 20%, when growth ceases because of the uncoupling of oxidative phosphorylation (Haslam *et al.*, 1971). However, on glucose medium energy is obtained by fermentation, and growth proceeds until the unsaturated fatty acid content is 5–7% at 28°C. Fig. 2 shows the relationship between growth temperature and the growth-limiting unsaturated fatty acid content on both ethanol (1%, w/v) and glucose (5%, w/v) media. At temperatures between 10 and 36°C cells stop growing at a higher unsaturated fatty acid content on ethanol medium than on glucose medium, indicating a specific lesion in mitochondrial oxidative phosphorylation. Below 10°C and above 36°C there is very little growth and hence only small changes in fatty acid composition on either medium. If Fig. 2 is compared with Fig. 1, there is a good correlation between the growth-limiting proportion of unsaturated fatty acids and the transition temperature of the mitochondrial ATPase, between 5 and 30°C, but above 30°C there is no relationship between the two parameters. Nevertheless, the latter could be

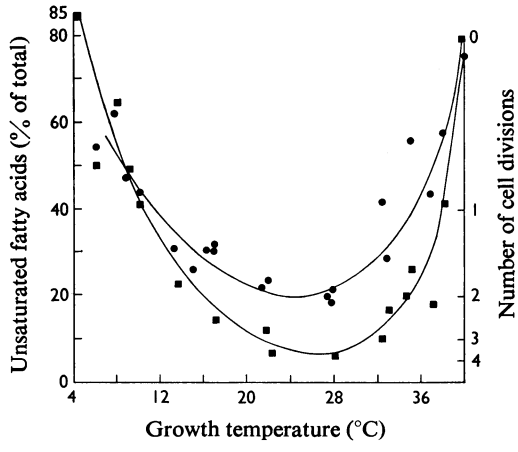

Fig. 2. *Relationship between growth temperature and limiting unsaturated fatty acid composition*
ole-1 cells were grown in the presence of Tween 80 (1%, w/v), harvested and resuspended in either ethanol (1%, w/v) (●) or glucose (5% w/v) (■) medium in the absence of added unsaturated fatty acids, and grown at various temperatures until growth ceased (48–90h).

due to other destructive effects of high temperature on yeast cells.

If the loss of oxidative phosphorylation in unsaturated fatty acid-depleted cells is related to the change in the temperature-dependence of the mitochondrial ATPase, it should be possible to recouple oxidative phosphorylation in the cells by raising the growth temperature. Table 1 shows an experiment in which cells were depleted for up to 69h at 17°C, at which temperature the minimum proportion of unsaturated fatty acid is 32%. The cells were removed at intervals and placed in an incubator at 28°C for 24h. If oxidative phosphorylation was restored, this would result in further growth and further depletion of the unsaturated fatty acids. Table 1 shows that apparent recoupling of oxidative phosphorylation does occur because the cells grow and deplete their unsaturated fatty acids. The amount of growth is proportional to the extent of depletion of the unsaturated fatty acids, provided that the cells are not left for more than 32h at 17°C. Longer periods at the lower temperature progressively diminish the subsequent ability of the cells to grow at 28°C, and the viability of cells left for 69h at 17°C was only 3–5%.

Effects of temperature on the efficiency of oxidative phosphorylation by isolated mitochondria

The effects of temperature on the efficiency of oxidative phosphorylation of unsaturated fatty acid-depleted mitochondria were measured directly *in vitro*

Table 1. *Re-initiation of the growth of unsaturated fatty acid-depleted cells by raising the temperature*
Cells were grown on ethanol (1%, w/v) medium as described by Proudlock *et al.* (1971) in the presence of Tween 80 (1%, w/v). Their fatty acids were initially 80% unsaturated. The cells were then resuspended in ethanol medium in the absence of Tween 80. In the initial incubation, cells were incubated at 17°C for the times indicated, until their fatty acid composition was that given after 1.1–1.2 cell divisions. For the secondary incubation, cells were harvested at the times indicated in the initial incubation and incubated for a further 24h. The final growth-limiting fatty acid composition is given in the fourth column. The growth of the cells is calculated as follows: (80/final percentage of unsaturated fatty acids) × initial inoculum.

Initial incubation		Secondary incubation	
Time at 17°C (h)	Unsaturated fatty acids (% of total)	Time at 28°C (h)	Unsaturated fatty acids (% of total)
24	36	24	19
32	33	24	23
45	32	24	26
52	32	24	28
69	32	24	31

Table 2. *Effects of temperature and fatty acid composition on P/O ratios*
Cells were grown on ethanol medium (1%, w/v) and fatty acids determined as described by Proudlock *et al.* (1971). Mitochondria were isolated as described by Haslam *et al.* (1971). ATPase activities and the transition temperature of Arrhenius plots were determined as described by Cobon & Haslam (1973). P/O ratios were determined at 17 and 28°C as described by Haslam *et al.* (1971), with pyruvate (1.7mM) plus L-malate (1.7mM) as substrate.

Unsaturated fatty acids (% of total)	ATPase transition temperature (°C)	P/O ratios at: 17°C	28°C
25 ± 1	22 ± 1	0.44	0.33
27 ± 1	21 ± 1	0.44	0.36
28 ± 1	20 ± 1	0.54	0.31
30 ± 1	20 ± 1	0.80	0.60
31 ± 1	19 ± 1	0.53	0.60
32 ± 1	19 ± 1	0.83	0.83

and Table 2 summarizes the results of a large number of such experiments. P/O ratios were determined at 17 and 28°C, which correspond to temperatures 2–5°C below and 6–9°C above the transition temperatures of the mitochondrial ATPase. The hypothesis that higher temperatures could recouple the system was conclusively disproved. Indeed, the P/O ratios at 28°C were significantly lower than at 17°C. The explanation of the re-initiation of growth by raising

the temperature, and the apparent greater requirement for unsaturated fatty acids for growth on ethanol at lower temperatures, is explained by the greater rate of respiration. The efficiency of oxidative phosphorylation is related directly to unsaturated fatty acid content, but the rate at which ATP is generated determines whether the cells can grow at the higher temperature. Thus in raising the temperature from 17 to 28°C the rate of respiration is doubled and this provides more ATP per min, even though the efficiency of oxidative phosphorylation is slightly lower at the higher temperature and declines further as the cells start to grow and diminish their unsaturated fatty acid content.

Effects of temperature on the proton permeability of yeast mitochondria

Preliminary work of Haslam & Fellows (1975) showed in two experiments that Arrhenius plots of $1/T$ against $\log(t_{\frac{1}{2}}^{-1})$ for the passive permeability of yeast mitochondria to protons are linear between 4 and 37°C; depletion of unsaturated fatty acid increases the rate of proton entry and increases the Arrhenius activation energy of the process. Fig. 3 summarizes the results of 13 experiments, and shows a clear relationship between decrease in unsaturated fatty acid composition and an increase in the permeability of the mitochondrial membrane to protons, and an inverse relationship between the Arrhenius activation energy of proton entry and fatty acid composition.

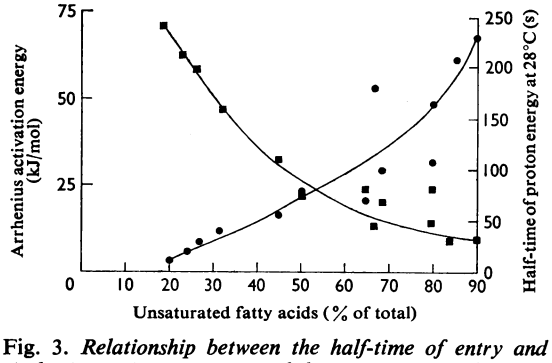

Fig. 3. *Relationship between the half-time of entry and Arrhenius activation energy of the passive permeability of mitochondria to protons and membrane fatty acid composition*

ole-1 mitochondria were isolated and their passive permeability to added protons was determined as described by Haslam *et al.* (1973*b*) and is shown in Fig. 4. The graph shows the relationship between fatty acid composition and the half-time of proton entry at 28°C (●). Arrhenius plots of $\log(t_{\frac{1}{2}}^{-1})$ against $1/T$(K) gave straight lines in each of the 12 experiments as shown previously (Haslam & Fellows, 1975). Arrhenius activation energies were calculated from the slopes of the plots (■).

Possible role of mitochondrial ATPase in the uncoupling effects of unsaturated fatty acid depletion

The Arrhenius plots of mitochondrial-membrane proton permeability are linear, whereas those of the ATPase show discontinuities. Furthermore the proton permeability of unsaturated fatty acid-

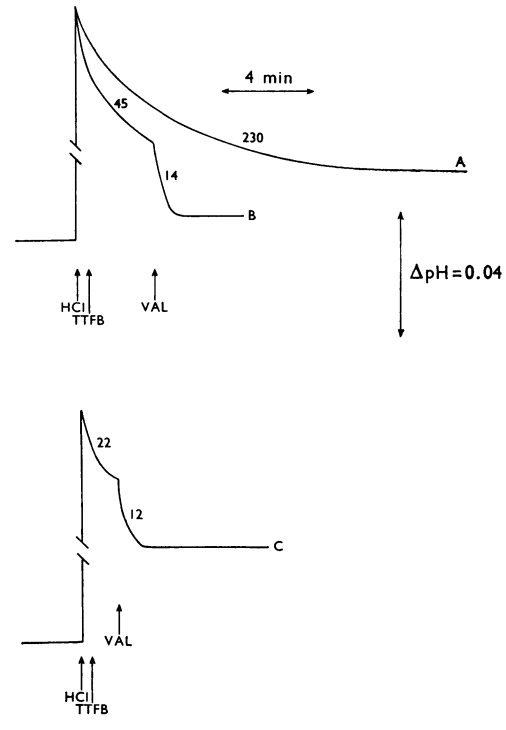

Fig. 4. *Effects of changed fatty acid composition on the rate of passive entry of protons into mitochondria of the petite ole-1 mutant*

The experimental system is as described by Haslam *et al.* (1973*b*). Mitochondria of the *petite ole-1* mutant (5mg of protein) were preincubated for 10min at 28°C in 3.0ml of medium containing KCl (150mM), glycylglycine buffer (2mM, pH6.5), dialysed bovine serum albumin (4mg), EDTA (0.5mM, pH6.5) and antimycin A (5μg). At the points indicated, HCl (1.0μmol), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB; 10μM) or valinomycin (VAL; 1μg) were added. Curves A and B, mitochondrial fatty acids were 82% unsaturated; curve C, mitochondrial fatty acids were 23% unsaturated. In curves B and C, TTFB and valinomycin were added. In curve A no further additions were made after the HCl. The total immediate decrease in pH after addition of HCl was approx. 0.3pH unit in all three experiments, and is not shown in full; the graphs indicate the subsequent slow entry of protons into the mitochondria. Numbers above the curves indicate the half-times of proton entry.

depleted mitochondria is unaffected by the ATPase inhibitor oligomycin (Haslam & Fellows, 1975), which would be expected to inhibit proton entry via an aberrant ATPase. Both these factors argue against the involvement of the ATPase in the enhanced proton permeability of unsaturated fatty acid-depleted organelles. As a further test, the cytoplasmic *petite* mutant of strain *ole-1* was investigated. This mutant lacks the four membrane-bound mitochondrially synthesized subunits of the ATPase (Tzagaloff *et al.*, 1973), which are thought to be involved in the proton-translocating function of the enzyme. Fig. 4 shows that *petite* mitochondria with a high unsaturated fatty acid content have a normal low permeability to protons, whereas depleted organelles have a greatly enhanced proton permeability. It is thus concluded that the effect of unsaturated fatty acid depletion on proton permeability and oxidative phosphorylation is probably a lipid lesion that does not involve the ATPase.

Effects of unsaturated fatty acid depletion and uncouplers on the adenylate energy charge of yeast cells and mitochondria

As a further test of the effects of unsaturated fatty acid depletion on the energetics of cells *in vivo*, the adenylate energy charge of whole cells and of isolated mitochondria of the *ole-1* mutant was measured, and the results are presented in Table 3. The energy charge, defined by Ball & Atkinson (1975) as ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP]), has a value of between 0.8 and 0.9 in growing cells, and only declines on prolonged starvation in *S. cerevisiae*. Table 3 shows that the energy charge of cells grown on glucose (2%, w/v) medium and then with aeration on ethanol (1%, w/v) medium depends on their fatty acid composition. In the cells with a normal fatty acid composition (Type C) the energy charge is 0.82, whereas the unsaturated fatty acid-depleted cells (Type E) have an energy charge of 0.48. However, if the unsaturated fatty acid-depleted cells are aerated in glucose (2%, w/v) medium (Type D), their cellular energy charge returns to normal (0.87) owing to the production of ATP by fermentation. Similarly, cells of a high unsaturated fatty acid content have a normal energy charge of 0.82 on glucose medium in the presence of an uncoupler of oxidative phosphorylation (Type B).

Mitochondria were isolated from the various cell types. In these studies the cells were chilled in ice, and oligomycin was added to prevent the breakdown of ATP within the mitochondria. The energy charge of the mitochondria was lower than that of the whole cells, possibly indicating that in spite of the precautions some breakdown of mitochondrial ATP had occurred. As expected, the energy charge of the unsaturated fatty acid-depleted mitochondria (Types D and E) and of the chemically uncoupled mitochondria (Type B) is lower than that of mitochondria from coupled cells with a normal fatty acid composition (Types A and C). The mitochondrial adenine nucleotide translocase can carry ATP from the cytoplasm into the mitochondria of chemically uncoupled

Table 3. *Effects of unsaturated fatty acid depletion on the adenylate energy charge of cells and mitochondria*

Cells were grown, mitochondria were isolated and adenine nucleotides were determined as described in the Experimental section. Cell types and the corresponding mitochondria isolated were as follows. A, Cells grown on glucose (2%, w/v) medium, containing Tween 80 (1%, w/v), whose fatty acids were 80% unsaturated; after harvesting the cells were incubated in glucose (2%, w/v) medium. B, Cells were prepared as in A, but were then incubated in glucose (2%, w/v) medium containing 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (20 μM). C, Cells were grown as in A and B, and contained fatty acids that were 80% unsaturated; after harvesting, cells were incubated in ethanol (1%, w/v) medium. D, Cells were grown on glucose (2%, w/v) medium containing Tween 80 (100 mg/litre), and the harvested cells contained fatty acids that were 25% unsaturated; the cells were then harvested and incubated in glucose (2%, w/v) medium. E, Cells were prepared as in D but were subsequently incubated in ethanol (1%, w/v) medium. Incubations of cells after harvesting were for 10min at 28°C in all experiments. Results are the averages for three separate incubations of cells, and for the corresponding mitochondria ±S.E.

		Types of cells and mitochondria				
		A	B	C	D	E
Cellular	ATP	13.0 ± 1.2	13.5 ± 1.4	15.6 ± 1.4	19.5 ± 1.6	2.3 ± 0.2
Cellular nucleotides	ADP	5.1 ± 0.4	6.6 ± 0.6	7.2 ± 0.6	5.5 ± 0.5	14.2 ± 1.4
(μmol/mg dry wt.)	AMP	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	5.5 ± 0.5
	Total	18.5 ± 1.6	20.5 ± 2.1	23.4 ± 2.3	25.5 ± 2.6	21.0 ± 2.0
	Energy charge	0.84 ± 0.2	0.82 ± 0.2	0.82 ± 0.3	0.87 ± 0.3	0.48 ± 0.3
Mitochondrial nucleotides	ATP	4.38 ± 0.4	2.98 ± 0.3	5.06 ± 0.6	0.93 ± 0.1	1.03 ± 0.1
(μmol/mg of protein)	ADP	4.32 ± 0.4	8.11 ± 0.8	4.84 ± 0.5	5.84 ± 0.7	5.67 ± 0.6
	AMP	1.26 ± 0.1	1.89 ± 0.2	1.42 ± 0.1	1.78 ± 0.1	2.90 ± 0.3
	Total	9.86 ± 1.1	12.98 ± 1.3	11.32 ± 1.2	8.55 ± 0.8	9.60 ± 1.0
	Energy charge	0.66 ± 0.2	0.54 ± 0.2	0.66 ± 0.4	0.45 ± 0.4	0.40 ± 0.3

fermenting cells (Type B), leading to a moderately high mitochondrial adenylate energy charge.

However, the adenylate energy charge of unsaturated fatty acid-depleted mitochondria is significantly lower on glucose medium (Type D) than that of mitochondria from chemically uncoupled cells (Type B), with high unsaturated fatty acid content. The mitochondrial adenine nucleotide translocase would be expected to replenish intramitochondrial ATP in both types of mitochondria, by importing ATP generated by glycolysis in the cytoplasm.

Results of Haslam & Fellows (1975) suggested that unsaturated fatty acid depletion inhibits the activity of the adenine nucleotide translocase *in vitro* in addition to uncoupling oxidative phosphorylation. The present data support the conclusion that *in vivo* the adenine nucleotide translocase is not functioning normally in unsaturated fatty acid-depleted cells, and this results in lower intramitochondrial concentrations of ATP, which could explain previous observations that unsaturated fatty acid depletion inhibits mitochondrial processes such as protein synthesis (Watson *et al.*, 1971; Marzuki *et al.*, 1975) and DNA synthesis (Marzuki *et al.*, 1974) that require intramitochondrial ATP.

Mrs. J. Jones is thanked for her technical assistance. The work was supported by Science Research Council Grant B/RG/45754 to J. M. H.

References

Adam, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–577, Verlag Chemie, Weinheim and Academic Press, New York

Astin, A. M. & Haslam, J. M. (1977) *Biochem J.* **166**, 287–298

Ball, W. J. & Atkinson, D. E. (1975) *J. Bacteriol.* **121**, 975–982

Cobon, G. S. & Haslam, J. M. (1973) *Biochem. Biophys. Res. Commun.* **52**, 320–326

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., Cobon, G. S. & Linnane, A. W. (1973*a*) *Biochem. Soc. Trans.* **2**, 207–209

Haslam, J. M., Spithill, T. W., Linnane, A. W. & Chappell, J. B. (1973*b*) *Biochem. J.* **134**, 949–957

Lamprecht, W. & Trautschold, I. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 538–541, Verlag Chemie, Weinheim and Academic Press, New York

Marzuki, S., Hall, R. & Linnane, A. W. (1974) *Biochem. Biophys. Res. Commun.* **57**, 372–378

Marzuki, S., Cobon, G. S., Crowfoot, P. & Linnane, A. W. (1975) *Arch. Biochem. Biophys.* **169**, 591–600

Perlman, P. S. & Mahler, H. R. (1971) *Nature (London) New Biol.* **231**, 12–16

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

Resnick, M. A. & Mortimer, R. K. (1966) *J. Bacteriol.* **92**, 597–600

Somlo, M. (1970) *Arch. Biochem. Biophys.* **136**, 122–133

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

Watson, K., Haslam, J. M., Veitch, B. & Linnane, A. W. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 162–174, North-Holland, Amsterdam