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The Incorporation of Cytochrome Oxidase into Newly-Forming Yeast Mitochondrial Membranes

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Abstract

Cytochrome c oxidase extracted from a yeast auxotroph grown on different fatty acid supplements, and assayed at various temperatures ($0 \rightarrow 37^{\circ}$), gives transition points (T_t) when the data are expressed in an Arrhenius plot: T_t for linoleic, oleic and elaidic acids were 7.7°, 10.2° and 21.8° respectively. Lipid analysis of isolated mitochondria established that there is a change in lipid profile when yeast is first grown aerobically on linoleic acid and then transferred to elaidic acid under anaerobic conditions. These two observations have been used as a basis for the investigation of the assembly of cytochrome c oxidase when cells are oxygen induced for mitochondriogenesis *via* a linoleic, N_2 elaidic, O_2 transfer experiment. A three-stage assembly process is proposed consisting of (i) $0.0 \rightarrow 0.25$ h, auto-assembly of oxidase from preformed, chloramphenicol-sensitive precursors and newly synthesized cycloheximide-sensitive precursors, (ii) 0.25 h $\rightarrow 0.5$ h, a continuation of (i) plus *de novo* synthesis of new precursors of both kinds and (iii) 0.5 h onwards; normal, synchronised *de novo* synthesis. This model predicts

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one population of oxidase molecules at 0.0 h (linoleic T_t) and two populations of oxidase at 0.5 h (end of stage (ii)); one from the anaerobic precursors (linoleic T_t) and one for the *de novo*, aerobic oxidase (elaidic T_t): these predictions are confirmed by Arrhenius profiles constructed from samples taken at 0.0, 0.5, 1.0 and 3.0 h after oxygen challenge.

Introduction

The facultative yeast *Saccharomyces cerevisiae* is an extremely useful tool for studying the biogenesis of mitochondrial membranes; for a review of this literature, see Mahler, 1973 [1].

The early work of Wilson and Fox [2] demonstrated the value of Arrhenius plots (using the β-galactoside transport system of *E. coli*) for diagnosing the lipid environment of membrane bound enzymes and the large body of work typified by Raison [3], Fox [4], Esfahani *et al* [5] and Overath and Trauble [6] has clearly demonstrated the relationships between temperature, liquid-crystal transitions and the biological properties of membranes. In a preliminary report [7] we subsequently reported similar results for the cytochrome c oxidase of yeast mitochondria: a number of other reports on the temperature sensitivity of mitochondrial enzymes have also appeared in the literature (see 8, 9 and Discussion).

Further work in this laboratory has proven more definitive than that reported earlier [7]. Subsequent experiments, using the terminal enzyme of the respiratory chain, have allowed us to relate the results from Arrhenius profiles, lipid analyses and inhibitor studies to posit a working model for the biogenesis of the cytochrome c oxidase complex.

Methods

Yeast Strains: Mutant yeast strain KD 20, (ol 1-2), isolated by Resnick [10] (generously donated by Dr. S. Fogel, Department of Genetics, University of California, Berkeley, California) and our standard laboratory strain 77 [11] were used in this study. Both strains are respiratory competent (Pρ+) and were maintained as previously described [12].

Growth Conditions: The growth medium used in these experiments was YCB (0.5% casein acid hydrolysate (Oxoid), 0.1% Triton X-100, 1.2% yeast carbon base (Difco), 0.002% ergosterol and 3% glucose). This medium was supplemented with individual fatty acids as indicated in the text. Difficulties in introducing the fatty acids into the medium were avoided by dissolving the lipid along with the ergosterol in absolute ethanol (5 ml/litre of medium) with slight heat. The resulting solution

was mixed into a minimal volume of yeast carbon base and Triton X-100 and filter-sterilized by passage through a bacterial filter (Millipore Filter Corp., Bedfore, Mass.). The sterilized solution was added to the main bulk of the YCB medium which had been previously heat sterilized.

To effect a transfer of yeast from an anaerobic medium (supplemented with linoleic acid) to an aerobic medium (supplemented with oleic or elaidic acid) the following protocol was followed: Anaerobic yeast were cultured at 30° in 5 litre batches of linoleic (0.01%) supplemented YCB contained in a 7 litre commercial fermentor (New Brunswick Scientific, New Brunswick, N.J.). Anaerobiosis was maintained by gassing the medium with a stream of nitrogen, from which residual traces of oxygen were removed by chemical purging [13]. The medium was inoculated and after a suitable time period, when the cell density was approximately 3 mg dry wt/ml culture, the cells were harvested anaerobically by means of continuous flow centrifugation at the rate of 400 ml/min at 15,000 x g (0°). The resulting pellets were washed in cold N_2 bubbled medium supplemented with the new fatty acid. The washed cells were then resuspended in the fermentor vessel in an equivalent volume of fresh aerated medium containing 0.01% elaidic or oleic acid. This transfer procedure was carried out within 20 min and zero time for oxygen challenge was estimated from the time of introduction of the washed cells into the aerated medium.

To determine the standard membrane phase transition points (T_t) for the various fatty acids (ie, linoleic, oleic and elaidic) the fatty acid desaturase mutant KD 20 was used. This organism was cultured aerobically in 1 litre batch cultures containing YCB medium supplemented with 0.02% fatty acid and harvested 1 h prior to the onset of stationary phase (180 mg dry wt/litre). In all instances where cell-free extracts were to be prepared, cells were collected in pre-cooled tubes and after three washings in 100 mM phosphate buffer (pH 7.4) containing D-chloramphenicol (CAP, 4 mg/ml) and cycloheximide (CHI, 50 μg/ml) stored at −20° overnight. Cell extracts were prepared from these frozen cells by using the French Press technique described by Ball and Tustanoff [12].

Isolation of Mitochondria: Harvested cells were once washed with cold distilled water (4°) and twice with cold 0.04 M phosphate buffer, (4°), pH 7.4 containing 0.1% bovine serum albumin, chloramphenicol (4 mg/ml) and cycloheximide (25 μg/ml). The resulting washed cells were suspended in 1.0 M sorbitol containing 0.1 M tris-HCl buffer, pH 8.0, 1 mM EDTA and 0.1% bovine serum albumin. After 30 sec homogenization with glass beads in a Braun shaker a mitochondrial fraction was obtained by differential centrifugation after the procedure of Henson *et al* [14]. The final mitochondrial pellet was suspended in 0.25 M sucrose containing 10 mM tris-HCl buffer (pH 7.4) to yield a suspension containing 5-10 mg protein/ml.

Analytical Procedures: Protein and dry wt estimations were carried out as previously described [12]. The estimation of cyanide-sensitive respiration was carried out polarographically [15]. Cytochrome c oxidase activity was determined spectrophotometrically at various temperatures from 0° to 37° by the method of Smith [16]. The reaction temperatures were monitored by the use of an immersible thermistor (± 0.1°) (Yellowspring Instrument Co., Ohio) placed inside the jacketed reaction cuvette. Removal of condensation from the cuvette chamber at low temperatures was achieved by placing a bag of dried silicia gel inside the chamber and passing a stream of dried air over the optical surface of the cuvette.

Fatty Acid Analysis: Lipids from yeast cells were extracted with chloroform-methanol (2:1) [17]. Methyl esters of the extracted fatty acids were prepared by the boron trifluoride procedure [18] and were resolved in a Beckman GC-45 gas chromatograph using an EGSS-X column and employing standard gas chromatographic techniques.

Results

Since the yeast auxotroph KD 20 is unable to synthesize unsaturated fatty acids [10] it will require these lipids for growth and automony. It is thus possible to manipulate the nature of the fatty acids in the mitochondrial membrane and concomitantly imprint these membranes with different physico-chemical properties by altering the nature of the unsaturated fatty acid supplement in the growth medium. With this in view KD 20 was grown aerobically on a series of minimal media (see Methods) which were supplemented with either oleic, linoleic or elaidic acid (0.02%). Cells were grown to late exponential phase and cytochrome c oxidase activities from each of the three lipid supplemented cell species were assayed spectrophotometrically at different temperatures (0°-37°). The results of these assays are represented as Arrhenius plots (log of the total activity vs the reciprocal of the absolute temperature) and are shown in Fig. 1. It can be seen that for each fatty acid there is a characteristic transition point in the respective plot. When the mutant is grown solely on oleic acid it is 10.2°, on linoleic acid it is 7.4° and on elaidic acid it is 21.8°.

It can furthermore be seen that at temperatures below the transition points the slope of these curves differ whereas above these points they are all parallel. In the latter case, the cytochrome c oxidase is in a gel-liquid phase and the activity of the enzyme is not affected by the nature of the fatty acids constituting the membrane [3]. Below the phase transition point, the diversing slopes of the curves indicate a dissimilar effect of the non-liquid membranes on the activation energy of the enzyme. It is thus possible to use the transition temperatures and

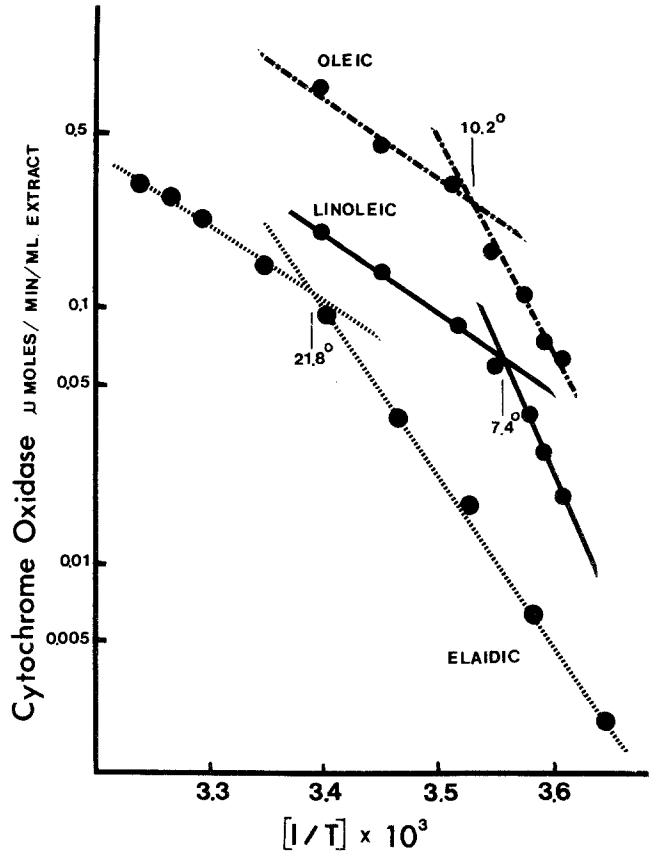

Figure 1: Arrhenius profiles of cytochrome c oxidase activity. Strain KD 20 was grown aerobically as described in the Methods. Total activity in cell-free extracts was measured spectrophotometrically at various temperatures; curves are labelled according to the fatty acid supplement added (0.02%) to YCB medium.

slopes from these Arrhenius plots to identify the fatty acid components of the lipids which encompass the cytochrome oxidase molecule in the inner mitochondrial membrane (cf 1-9).

Under anaerobic conditions wild type yeast have an absolute requirement for unsaturated fatty acids [11]. Growing these cells anaerobically on linoleic acid it was possible to manipulate them to synthesize membranes which, besides having a minimal level of cytochrome c oxidase activity, also contain large amounts of linoleic acid. Thus when these cells are transferred to a fresh aerobic medium containing an excess of oleic or elaidic acid several results are possible:

(1) If oxygen induced the assembly of cytochrome c oxidase into preformed membrane then the Arrhenius plot would show a linoleic-type transition.

(2) If the fatty acids were readily interchangeable in the membrane matrix then there would be no single clear cut transition point.

(3) If the newly synthesized enzyme was only integrated into newly formed membrane one would expect an oleic or elaidic acid type transition (cf, Wilson and Fox) [2].

On implementing the linoleic → oleic transfer in YCB medium samples were taken for cytochrome c oxidase assays at 30 min and 60 min after

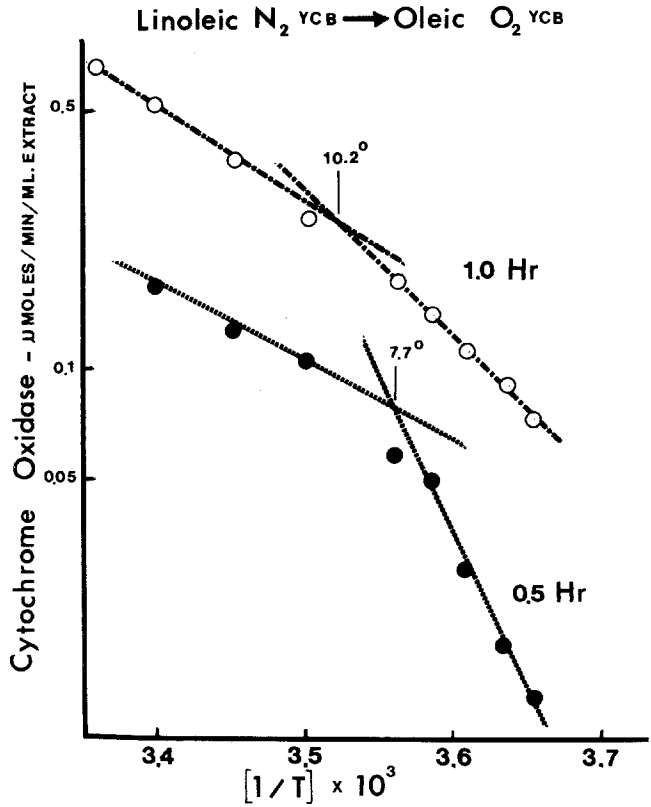

Figure 2: Arrhenius profiles from a linoleic, N_2 → oleic, O_2 transfer experiment. Wild type yeast (strain 77) was grown anaerobically on 0.02% linoleic YCB, harvested, washed and exposed to oxygen in 0.02% oleic YCB. Large samples were taken for cytochrome oxidase assays at 0.5 and 1.0 h after oxygen challenge. Enzyme assays were as described in the Methods and legend to Figure 1.

oxygen challenge. As can be seen from Fig. 2 the Arrhenius plot for 30 min after induction gives a T_t of the linoleic type (7.7°C). At 60 min the transition was oleic in nature (10.2°C). This would imply that immediately after induction (until at least 30 min) either an inactive form of cytochrome c oxidase, present in anaerobically synthesized membranes, was activated, or that newly formed cytochrome c oxidase was integrated into old membranes. Subsequently synthesized cytochrome c oxidase was incorporated into newly formed membranes.

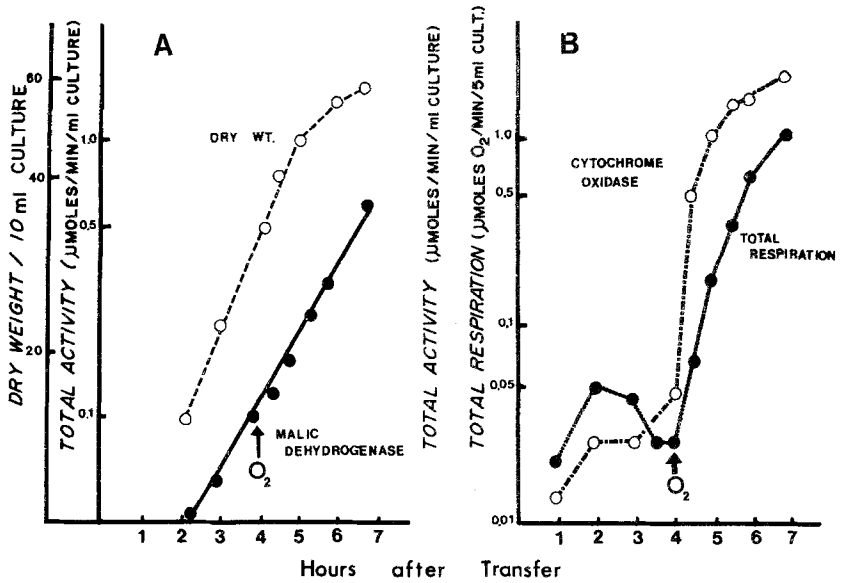

Figure 3: Growth and enzyme synthesis in a linoleic, $N_2 \rightarrow$ oleic, O_2 transfer experiment. Growth conditions were as for Figure 2 with samples taken as indicated:
(A) - - ○ - - - ○ - dry weight; —●—●— total malic dehydrogenase.
(B) - - - ○ - - - ○ - total cytochrome oxidase; - - ● - - ● total respiration.

If one looks at the kinetics of enzyme synthesis during the experiment just described (cf, Fig. 3) it seems clear that the synthesis of cytochrome c oxidase was not a simple induction process. Malic dehydrogenase and dry weight (Fig. 3A) increased exponentially and oxygen had no effect on the kinetics of their synthesis. On the other hand total respiration and cytochrome c oxidase (Fig. 3B) demonstrated non-exponential increases. In the first phase of this increase (up to 30 min after oxygen challenge) there appeared to be a very rapid increase in the rate of synthesis of both of these parameters. In the second phase (from 30 min onwards) the increase in the synthetic rate slowed and finally became parallel to the

dry weight increase. This slowing of the synthetic rate after 30 min appeared to coincide with the association of the bulk of cytochrome c oxidase with newly formed membrane, dominated in this case by oleic acid (Fig. 2).

The difference between a transition at 7.7° and one at 10.2° is not easily discerned: in order to improve our resolution this transfer experiment was repeated using elaidic acid in the aerobic phase (7.7° vs 22°) and samples taken at 0.0, 0.5, 1.0 and 3.0 h after oxygen challenge. Fig. 4A shows the expected exponential growth rate (the dotted line representing the harvesting-washing procedure) and Fig. 4B again shows

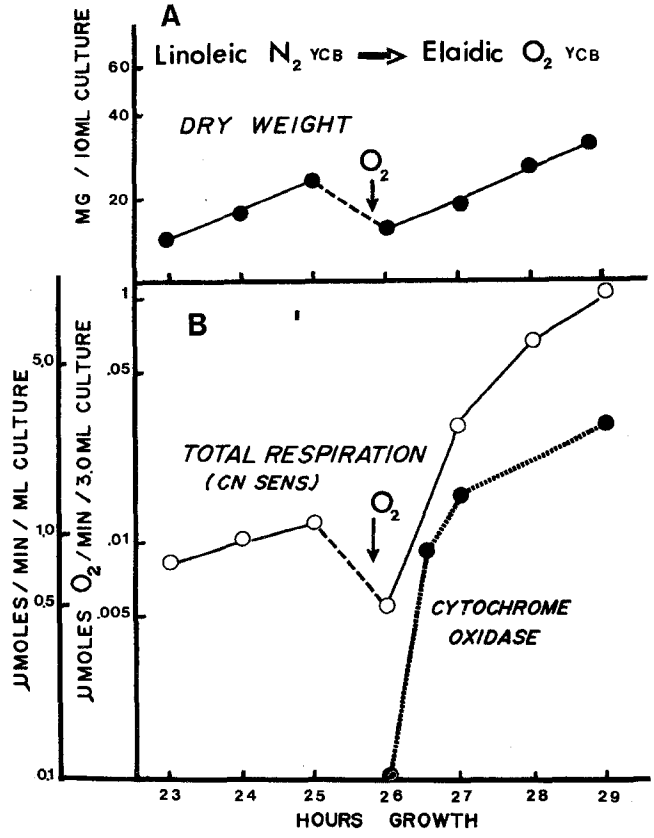

Figure 4: Growth and enzyme synthesis in a linoleic, N_2 → elaidic, O_2 transfer experiment. Growth conditions were the same as for Figures 2 and 3 except that 0.02% elaidic acid was the supplement in the aerobic phase (Strain 77):
(A) dry weight;
(B) —○—○— total respiration; - - ● - - ● - total cytochrome oxidase.

the non-exponential induction of total respiratory ability and total cytochrome c oxidase activity.

The Arrhenius plots of cytochrome c oxidase activity for this experiment are shown in Fig. 5. At zero time the activity was clearly associated with the linoleic acid dominated membrane (T_t = 7.6°). After 0.5 h exposure to oxygen this transition was still present but an elaidic acid-type transition had also appeared. (T_t = 7.7° and 22.5°). By 1 h the activity showed an elaidic transition (T_t = 28°) which was maintained at 3.0 h.

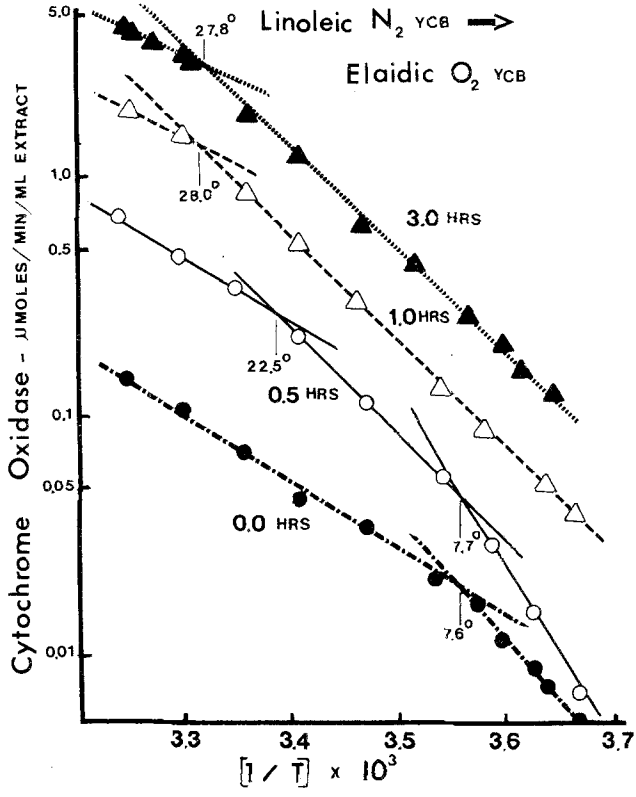

Figure 5: Arrhenius profiles from a linoleic, N_2 → elaidic, O_2 transfer. Growth conditions were as for Figure 4. Samples were taken at 0.0, 0.5, 1.0 and 3.0 h for cytochrome oxidase assays.

In order to substantiate these observations the fatty acid composition of mitochondria isolated from these adapting yeast cells was determined by gas liquid chromatography. At zero time (Table I) the major fatty acid found in these organelles was linoleic (18:2) and it constituted 35%

of the total fatty acids present. After transfer to elaidic medium, the linoleic acid content fell to 20% and the elaidic acid (18:1) increased from 4 to 18% after two hours of adaptation. The reduction of the 18:2 fatty acid and the subsequent increase of the 18:1 fatty acid in this transfer experiment must be due to the incorporation, or exchange, of the second fatty acid into the mitochondrial membrane.

The synthetic curves for total cytochrome oxidase (Figs. 3B and 4B) are similar to the auto-assembly curves obtained by Mason *et al* [19] when they exposed oxygen induced yeast to CHI (inhibits cytoplasmic protein synthesis) and subsequently to CAP (inhibits mitochondrial protein synthesis) in the absence of CHI. The question arises therefore as to whether the rapid increase in total activity seen in Figs. 3b and 4b from 0.0 → 0.5 h is due to (i) *de novo* synthesis, (ii) activation of preformed precursors, or (iii) some combination of these mechanisms. The answer to this question is crucial to an analysis of the Arrhenius profiles of Fig. 5.

We therefore repeated the linoleic, N_2, YCB → elaidic, O_2, YCB transfer in triplicate, adding CAP (4 mg/ml) to one flask and CHI (50 μg/ml) to a second. The results of this experiment are reported in Fig. 6: the inhibitors have the same effect on the synthesis of cytochrome oxidase and on total respiratory ability. The effect of CHI is to inhibit completely the production of both activities from 0.0 to 2.0 h. On the other hand CAP permits a limited fast synthesis from 0.0 to 0.5 h (Phase I) but inhibits the subsequent slow synthetic phase (II). According to Mason *et al* [19] these results would indicate that there is a pool of CAP-sensitive precursors in the anaerobic cells that is activated by CHI-sensitive precursors *via* auto-assembly, and, that anaerobic cells do not contain a complementary pool of CHI sensitive precursors that can be activated by the products of mitochondrial protein synthesis.

Discussion

Before discussing the significance of these results, some small ambiguities should be discussed. The linoleic, N_2 → oleic, O_2 transfer apparently gives one transition point (Fig. 2, 0.5 h). This is probably a function of the close similarity between the expected T_t's of 10.2 and 7.7 for oleic and linoleic acids. It is, however, clear that there are two transitions in the 0.5 h curve from the linoleic N_2 → elaidic O_2 transfer (Fig. 5, 0.5 h). This illustrates the care with which the transfer order (eg, linoleic N_2 → elaidic O_2) and the fatty acids used must be chosen. One other point of interest is the change in T_t for the elaidic-type transition for wild type cells at 1.0 and 3.0 h in Fig. 5. This is in contrast to the control curve obtained for KD 20 (Fig. 1) and can probably be explained

in terms of the overall composition of the mitochondrial membranes (see Overath and Trauble) [6]. The membranes of KD 20 will be absolutely dominated by the fatty acid supplement (in this case elaidic) whereas strain 77 is able to synthesize endogenous fatty acids once it is exposed to oxygen. The change in T_t is most probably due to secondary interactions between exogenous and endogenous fatty acids *via* mixed glycerides. This higher T_t can however still be used as an indicator of "new membrane".

The lipid analyses of Table I show that during a linoleic, $N_2 \rightarrow$ elaidic, O_2 transfer the kinds of lipids found in the mitochondrial membranes change in response to the change in lipid supplement; this could be due to exchange or incorporation. During the first 2.0 h there is a rapid assembly of mitochondria; this is reflected in activity (Figs. 3B, 4B and 6) as well as in the size of the mitochondrial pellets (unpublished results). Thus one would not expect significant amounts of lipid exchange under conditions where lipid precursors were being rapidly incorporated into the "sink" of new mitochondrial membranes. The changes in composition and Arrhenius profiles are therefore due to incorporation rather than to replacement.

The transition profiles (Fig. 5) can only be interpreted in the context of the enzyme used and its mode of synthesis. In Fig. 7 we illustrate the implications of the inhibitor studies reported in Fig. 6. The source of mRNA is deliberately left ambiguous as it is not at all certain as to where the messengers transcribed on the various ribosomes actually originate [1, 20]. Recent work from Schatz's group has indicated that cytochrome oxidase consists of at least six polypeptides and that the various polypeptides are differentially sensitive to CHI, CAP, [21] and genetic lesions [22]. For the sake of simplicity we have represented these various precursors by single symbols; N for nucleo-cytoplasmically synthesized and M for mitochondrially synthesized.

Our interpretation is similar to that of Mason *et al* [19] and Kim and Beattie [33]; the absence of synthesis in the presence of CHI shows that mitochondrial protein synthesis alone (CAP sensitive) cannot produce active oxidase. Conversely, cytoplasmic protein synthesis, in the presence of CAP, *is* able to cause auto-assembly of active oxidase. This leads to the interesting conclusions that (a) components synthesized on mitochondrial ribosomes are synthesized anaerobically and (b) that oxygen induction triggers or accelerates the synthesis of precursors dependent upon cytoplasmic protein synthesis.

Consequently, the control curve for cytochrome oxidase in Figs. 3B, 4B and 6 can be broken down into the following phases: $0.0 \rightarrow 0.25$ h auto-assembly of oxidase on anaerobically produced precursors ($M_{N_2} + N_{O_2} \rightarrow M/N$); $0.25 \rightarrow 0.5$ h, this process continues but *de novo* synthesis has also commenced ($M_{O_2} + N_{O_2} \rightarrow M/N$); $0.5 \rightarrow 3.0$ h most of the activity comes from the *de novo* synthesis (control vs + CAP) (Fig. 6B).

TABLE I. Fatty Acid Composition of Mitochondria Isolated from a Linoleic (N_2) → Elaidic (O_2) Transfer

Yeast were grown anaerobically on 3% glucose minimal medium supplemented with ergosterol and 0.02% linoleic acid to a point 1h prior to stationary phase and then adapted in minimal medium containing 0.02% elaidic acid as described in the Method's Section. Cells were harvested at times indicated and mitochondria were isolated. The fatty acid content of these organelles was determined by gas liquid chromatography.

Time of Growth	Percent Fatty Acid							
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
N_2	6.8	9.7	26.3	10.6	6.0	4.0	35.9	0
O_2 + ½ h	3.0	9.4	20.8	15.4	5.9	13.3	24.9	0
O_2 + 1 h	4.3	7.7	24.4	18.4	6.7	14.3	21.6	0
O_2 + 2 h	4.3	5.1	21.6	24.7	5.8	17.7	20.6	0

The fatty acids are denoted by the convention – number of carbon atoms:number of unsaturated linkages.

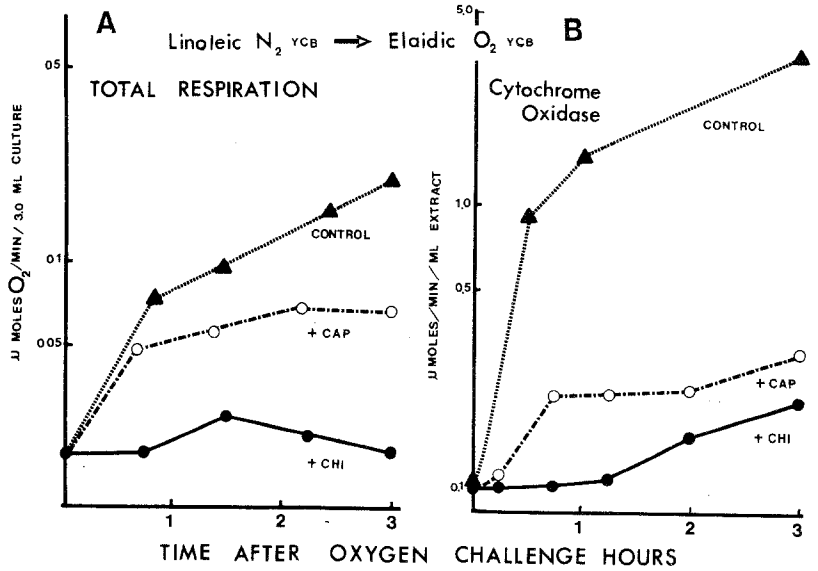

Figure 6: The effects of CAP and CHI on aerobic adaptation. Growth conditions were as for Figures 4 and 5 with CAP and CHI added at 4.0 mg/ml and 50 μg/ml respectively as indicated.
(A) Total respiration (B) Total cytochrome c oxidase.

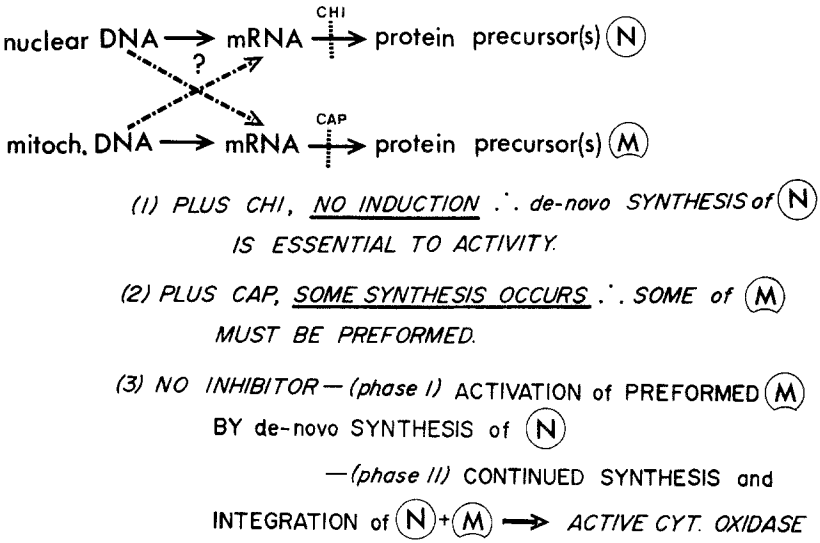

Figure 7: Implications of the inhibitor studies of cytochrome c oxidase assembly. M = precursors synthesized on mitochondrial ribosomes. N = precursors synthesized on cytoplasmic ribosomes.

We can now examine our model for cytochrome oxidase (Fig. 8) and explain it in terms of the synthetic process (Figs. 6 and 7) and the observed lipid environments (T_t's from Fig. 5).

The anaerobic membrane will have traces of oxidase and a pool of mitochondrial precursors (Fig. 8) embedded in a membrane dominated by linoleic acid (cf Figs. 1, 2, 5, (0.0 h) and Methods). Once oxygen is admitted to the culture auto-assembly and *de novo* synthesis of the cytochrome oxidase takes place between 0.0 and 0.5 h after transfer. This should result in two populations of oxidase: (i) assembled from anaerobically synthesized, linoleic associated precursors and (ii) assembled *de novo* in the presence of elaidic acid (Fig. 8—0.5 h). The

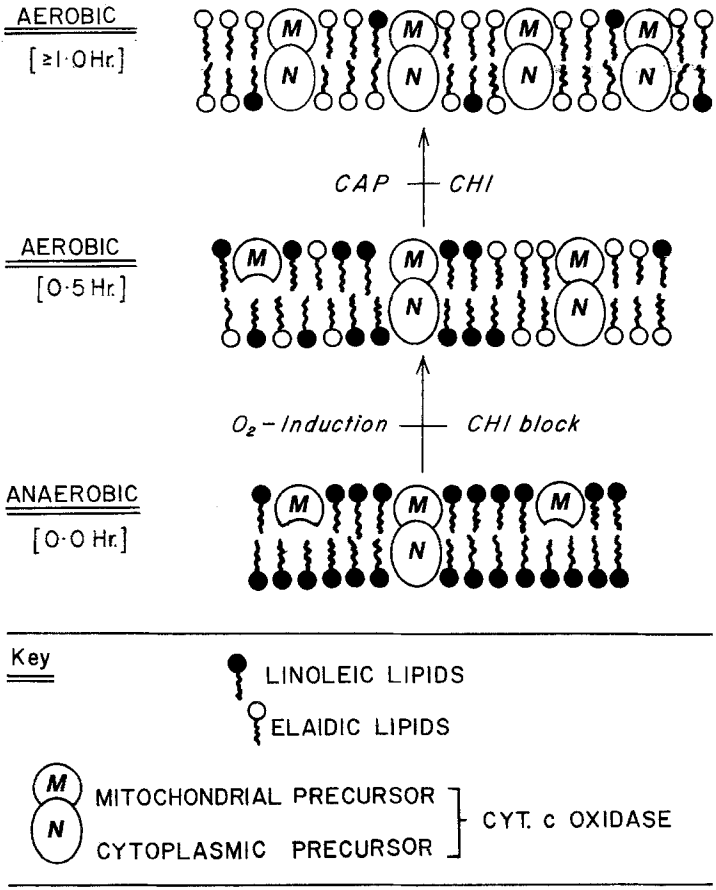

Figure 8: Model for the assembly of cytochrome c oxidase during a linoleic, $N_2 \rightarrow$ elaidic, O_2 transfer experiment.

0.5 h curve of Fig. 5 shows two transitions as predicted by the two population assembly model.

By 1.0 h the preformed oxidase becomes a minor component in the total activity (cf control and + CAP curves for cytochrome oxidase in Fig. 6) and the linoleic transition is probably undetectable in these samples (Fig. 8 $\geqslant$1.0 h and Fig. 5, 1.0, 3.0 h).

A pre-requisite for the correlation of the predicted (two) populations of cytochrome oxidase with the double T_t of the 0.5 h curve (Fig. 5) is the implicit assumption that new enzyme integrates into membrane together with new lipid and remains there: Tsukagoshi and Fox [23, 24] have recently demonstrated the questionability of such assumptions. Cytochrome c oxidase, however, is an enzyme complex which clearly requires lipid for its activity [25, 26]. It seems reasonable to assume that in the case of cytochrome oxidase this tightly bound lipid (essential to activity) will not diffuse or exchange rapidly into the membrane over the time course of the experiment (0.0 → 0.5 h). These tightly bound lipids might in fact act as seed crystals trapping the enzyme in mosaic zones [26, 27] which would show up in the Arrhenius profiles of Figs. 2 and 5. If this is true, then it would seem that the mitochondrially synthesized precursor(s), which gives rise to the linoleic transition (synthesized anaerobically), is responsible for tightly binding the lipid which is essential for catalytic activity.

The effects of varying lipid environment on mitochondrial activities of various kinds is being intensively studied in yeast at the present time [28, 29, 30, 31, 32] and this information, coupled to transition and assembly studies of the kind reported here, should rapidly expand and consolidate our knowledge of the mechanisms involved in mitochondrial biogenesis.

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