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THE RESPIRATORY CHAIN OF *PARAMECIUM TETRAURELIA* IN WILD TYPE AND THE MUTANT Cl_1

II. CYANIDE-INSENSITIVE RESPIRATION. FUNCTION AND REGULATION

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Summary

1. The cyanide-insensitive respiration in *Paramecium tetraurelia* was found to be located in mitochondria.

2. Sensitivity of the mitochondrial respiration to cyanide depended on growth conditions. Under standard conditions of growth, 15—20% of respiration was insensitive to 1 mM cyanide. Full resistance to 1 mM cyanide was observed by growing cells in the presence of erythromycin (100—400 μg/ml) or 0.2 mM cyanide. The mitochondrial respiration of the mutant Cl_1 harvested during the exponential phase of growth was largely insensitive to cyanide (more than 80%).

3. Pyruvate was oxidized at the same rate by wild type mitochondria and mitochondria of the mutant Cl_1. In contrast, succinate oxidation was 2—3 times faster in mitochondria of the mutant Cl_1 than in wild type mitochondria.

4. The cyanide-insensitive respiration was inhibited by 1 mM salicylhydroxamic acid to nearly 100%. Other efficient respiratory inhibitors included amytal and heptylhydroxyquinoline. Antimycin was not inhibitory even at concentrations as high as 5 μg/mg protein, a finding consistent with the lack of antimycin binding sites.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine; FCCP, Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; MOPS, Morpholinopropane sulfonic acid; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

5. The K_m O_2 was less than 2 μM for the cyanide-sensitive respiration and 4—5 μM for the cyanide-insensitive respiration.

6. ADP stimulated the oxidation of pyruvate and succinate by mitochondria of the wild type and the mutant Cl_1 by 2—4-fold. AMP selectively stimulated the cyanide-insensitive respiration. Preincubation of *Paramecium* mitochondria with trace of ADP increased their cyanide sensitivity. This effect of ADP was prevented by uncouplers.

7. P/O ratios in wild type mitochondria were close to 2 for pyruvate oxidation, between 1 and 2 for succinate oxidation and between 0.5 and 1 for ascorbate oxidation. In mitochondria of the mutant Cl_1, P/O ratios for pyruvate and succinate oxidation were lower by about one third in comparison to those of wild type mitochondria. Addition of cyanide decreased P/O ratios with pyruvate and succinate as substrates for both types of mitochondria (30% for the mutant, 40—70% for the wild type). Ascorbate oxidation by mitochondria of the mutant was not coupled to ATP synthesis and did not generate a membrane potential.

8. H^+ efflux dependent on O_2 pulses was measured in the presence of increasing concentrations of cyanide. Using succinate as substrate, the H^+/O ratio was 4 for wild type mitochondria and 1.5 for mitochondria of the mutant. The curve relating H^+ efflux to added cyanide was biphasic in both cases. There was a rapid drop of H^+/O to 30—50% of the original value when cyanide was added to 0.1 mM. Upon further addition of cyanide up to 1 mM, the H^+/O ratio decreased slowly towards zero.

9. Under steady-state conditions of respiration, the percentage of oxidation of the respiratory carriers increased from ubiquinone to cytochrome aa_3, except for the *b*-type cytochromes which remained more oxidized than cytochromes cc_1.

10. The CO-inhibited respiration in mitochondria of the mutant Cl_1 was partially photorestored by white light. As the only CO-reactive pigment in these mitochondria is a *b*-type cytochrome, the photorestoration data suggest that the CO-reactive cytochrome *b* may be autooxidizable.

11. The above data suggest that the respiratory chain of *Paramecium* mitochondria possesses three terminal oxidases, namely cytochrome aa_3 (absent in mitochondria of the mutant Cl_1), a CO-sensitive *b*-type cytochrome and a salicylic hydroxamic acid-sensitive oxidase.

Introduction

Respiration in *Paramecium tetraurelia* is partially insensitive to cyanide [1]. The cyanide-insensitive respiration is inhibited by salicylhydroxamic acid suggesting that *P. tetraurelia*, like a number of other organisms [2], possesses, beside the more common cytochrome *c* oxidase, an alternative pathway to molecular oxygen. The present investigation was undertaken to delineate some physiological aspects of the cyanide-insensitive respiration in wild type *P. tetraurelia* and in the Cl_1 mutant lacking cytochrome aa_3 [3,4]. These aspects include the cellular localization of the cyanide-insensitive oxidative pathway, the branching of this pathway in the respiratory chain and the modulation of its activity by adenine nucleotides.

Materials and Methods

Measurement of respiration

The method of isolating *Paramecium* mitochondria was identical to that described in the preceeding paper [5]. Respiration of mitochondria was measured polarographically with a Clark electrode (Yellow Springs, Cleveland, OH) at 22°C. The medium consisted of 0.5 M mannitol, 0.05% bovine serum albumin, 5 mM $MgCl_2$ and 10 mM phosphate buffer, final pH 7.3, final volume 1.5 ml. Inhibitors were added as small volumes (10 μl or less) of aqueous or ethanolic solutions. Ethanol up to 1% (v/v) had no effect on respiration. KCN was freshly prepared for each experiment; its pH was adjusted to 7.2—7.5. When ascorbate was used as substrated in the presence of TMPD, a control without mitochondria was included to assess its spontaneous oxidation. Pyruvate was always used in the presence of a small amount of malate as indicated in the legends of figures.

Measurement of P/O ratios

The phosphorylation coupled to respiration was determined by the method of Nielsen and Lehninger [6] using orthophosphate labeled with ^{32}P. Orthophosphate labeled with ^{32}P was obtained frm the Commissariat à l'Energie Atomique, Saclay.

Measurement of H^+ efflux

pH changes were recorded using a combination glass electrode connected to a Radiometer PHM64 pH meter. The output of the pH meter was amplified and displayed on a Servotrace recorder at 0.1 pH unit, full scale. The detailed experimental conditions are given in the legend of Fig. 7.

Steady-state reduction of ubiquinone and cytochromes

Mitochondria were incubated for short time periods (15—30 s) at 22°C in the medium, described above, at a concentration of 5 mg protein/ml in the presence of an oxidizable substrate. This ensured that steady-state reduction of the respiratory carriers was reached without anaerobiosis being attained. The suspension was then pipetted into a cuvette of 3 mm optical pathway and immediately frozen in liquid nitrogen [7]. Spectrophotometric measurements were performed with a Cary 15 spectrophotometer. Results were expressed as percentages of the value obtained for dithionite reduced minus H_2O_2 oxidized, which was designated 100% reduction.

Other determinations

Cytochrome aa_3 concentration was obtained from $A_{608}—A_{630}$ (reduced minus oxidized). The molecular extinction coefficient was assumed to be 16.5 $mM^{-1} \cdot cm^{-1}$ as for mammalian cytochrome aa_3 [8]. Ubiquinone concentration was obtained from the difference in absorption $(A_{ox} - A_{red})_{275}$; the molecular extinction coefficient was taken equal to 12.5 $mM^{-1} \cdot cm^{-1}$ [9].

Fluorescence spectra were measured with a Perkin-Elmer spectrofluorimeter MPF-2A with excitation at 350 nm and emission at 470 nm for antimycin titration [10], and excitation at 355 nm and emission at 480 nm for HQNO titra-

tion [11]. Concentrations of antimycin and HQNO were determined spectrophotometrically using absorbance coefficients of 4.8 $mM^{-1} \cdot cm^{-1}$ at 320 nm and 9.45 $mM^{-1} \cdot cm^{-1}$ at 348 nm respectively (Ref. 12). The membrane potential ($\Delta\psi$), positive outside, developed in *Paramecium* mitochondria upon respiration, was monitored with the fluorescent dye 3,3'-dipropylthiodicarbocyanide [12] (excitation and emission wavelengths 620 and 670 nm).

Protein content was estimated by the Biuret method [13] using bovine serum albumin as a standard.

Results

Dependence of cyanide-insensitive respiration of Paramecium

Cyanide-insensitive respiration of *Paramecium* depended on growth conditions and on the time of harvest. It was always assessed on the mitochondrial fraction isolated from a cell homogenate (described in the next section) and was consequently referred to as the percentage respiration insensitive to 1 mM cyanide. Wild type cells harvested during the exponential phase of growth were characterized by a higher cyanide-insensitivity than cells harvested during the stationary phase (40 vs. 15—20%). When erythromycin, an inhibitor of the mitochondrial protein synthesis, was added to the medium, at concentrations between 100 and 400 μg/ml, respiration became totally cyanide-insensitive [14]. When the growth temperature was raised from 28°C to 33—35°C, the percentage of cyanide-insensitive respiration increased to 80—90%. Growth of the wild type strain in the presence of 0.2 mM cyanide at 28°C increased the cyanide-insensitivity to 100%. Respiration of the mutant Cl_1 harvested during the exponential phase of growth was largely insensitive to cyanide (more than 80%). Cyanide-insensitivity markedly decreased (to 40—50%) in mutant cells harvested during the stationary phase of growth.

In the present study, *Paramecium* cells were growth at 28°C. Unless otherwise indicated, the mitochondria were prepared from wild type cells harvested during the stationary phase of growth and from mutant Cl_1 collected during the exponential phase (see preceeding paper [5]).

Subcellular localization of the cyanide-insensitive respiration

The distribution of the cyanide-insensitive respiration in subcellular fractions of an homogenate of cells was compared to that of cytochrome aa_3, a typical marker of mitochondria. Specific activities and protein recoveries obtained by differential centrifugation of the homogenate were plotted according to the method of De Duve et al. [15]. The homogenate was fractionated in five fractions as follows. A first fraction (I) was obtained by centrifugation at $600 \times g$ for 10 min; it consisted of nuclei and unbroken cells. The supernatant centrifuged at $6000 \times g$ for 10 min yielded a sediment made of crude mitochondria (fraction II). Fraction III was obtained by centrifugation of the supernatant of step 2 at $15\,000 \times g$ for 15 min. The resulting supernatant was centrifuged at $100\,000 \times g$ for 60 min to yield the pellet IV and a supernatant (fraction V). The highest specific activity of the cyanide-insensitive and hydroxamic acid-sensitive respiration coincided with that of cytochrome aa_3 (fraction II) indicating its mitochondrial localization.

Rates of respiration, respiratory control and oxidative phosphorylation in mitochondria of the wild-type and the mutant Cl_1

As shown in Table I, rates of respiration on pyruvate were of the same order in mitochondria of the wild type and the mutant. In contrast, the rate of succinate oxidation was 2—3 times higher in mitochondria of the mutant than in wild type mitochondria. The addition of ADP to mitochondria of the wild type and the mutant respiring either on pyruvate or succinate resulted in a two to four fold increase of the respiratory rate. After exhaustion of ADP, mitochondria returned to state 4 respiration. Respiration of mitochondria of the mutant on ascorbate, in spite of the lack of cytochrome aa_3 was found to be nearly as active as that of wild type mitochondria (correction being made for spontaneous oxidation of ascorbate). However, in contrast to wild type mitochondria, oxidation of ascorbate by mitochondria of the mutant was not stimulated by ADP. Mitochondria of the mutant may possess a residual cytochrome oxidase activity despite the absence of optically detectable cytochrome aa_3 (cf. Ref. 16).

Effect of cyanide and salicylhydroxamic acid

Aromatic hydroxamic acids, and especially salicylhydroxamic acid, are considered as rather selective inhibitors of the alternative oxidase in eukaryotic mitochondria [17]. Data in Table I indicate that respiration of wild type mitochondria was partially decreased when either 1 mM salicylhydroxamic acid or 1 mM cyanide was added separately, and virtually completely abolished when the two inhibitors were added together at the same concentrations. This observation is in agreement with the concept [2] that the salicylhydroxamic

TABLE I

RESPIRATORY ACTIVITIES OF MITOCHONDRIA FROM *P. AURELIA*

The medium used is described in Materials and Methods. The oxidizable substrates were added at the following concentrations: pyruvate 10 mM, malate 1 mM, succinate 10 mM and ascorbate 3 mM + TMPD 0.05 mM. When present, ADP was at the final concentration of 180 μM, salicylhydroxamic acid (SHAM) and KCN at 1 mM. The reaction was initiated by addition of 0.8—1 mg of mitochondrial protein in 1.5 ml of medium. Results of seven experiments are given as means ± S.D.

Additions	Wild type (nmol O_2/min per mg)	Mutant Cl_1 (nmol O_2/min per mg)
Pyruvate + malate	18 ± 2	25 ± 4
Pyruvate + malate + ADP	79 ± 2	80 ± 6
Pyruvate + malate + SHAM	12 ± 1	5 ± 1
Pyruvate + malate + KCN	4 ± 1	22 ± 4
Pyruvate + malate + KCN + SHAM	<2	<2
Succinate	41 ± 6	106 ± 22
Succinate + ADP	82 ± 7	138 ± 28
Succinate + SHAM	27 ± 2	12 ± 2
Succinate + KCN	8 ± 1	85 ± 17
Succinate + KCN + SHAM	<2	<5
Ascorbate + TMPD	38 ± 5	32 ± 8
Ascorbate + TMPD + ADP	49 ± 5	32 ± 8
Ascorbate + TMPD + SHAM	29 ± 2	6 ± 2
Ascorbate + TMPD + KCN	13 ± 2	22 ± 5
Ascorbate + TMPD + KCN + SHAM	<2	<2

acid-sensitive, cyanide-insensitive pathway is a branch of the main respiratory chain.

The oxidation of pyruvate, succinate and ascorbate by mitochondria of the mutant was slightly, but significantly inhibited by 1 mM cyanide (10—20%) in spite of spectral evidence for lack of cytochrome aa_3. The cyanide titration curves of succinate oxidation by wild type mitochondria and mitochondria of the mutant were markedly different (Fig. 1). Obviously, a component, which is highly sensitive to low concentrations of cyanide ($K_i < 20$ μM), was present in wild type mitochondria and not in mitochondria of the mutant. This component is cytochrome aa_3. Another component, sensitive to only high concentrations of cyanide, ($K_i > 50$ μM) might be present both in mitochondria of the wild type and the mutant.

It is generally accepted that the branching point of the salicylhydroxamic acid-sensitive oxidase in cyanide-insensitive organisms, at least in plants, is on the substrate side of the *b* cytochromes, probably at the ubiquinone level (for review see Refs. 18, 19). Assuming that the same type of branching holds for *Paramecium* mitochondria, it remained to be explained why ascorbate is oxidized by mitochondria of the mutant which lacks cytochrome aa_3 and why ascorbate oxidation by mitochondria of the mutant is inhibited by salicylhydroxamic acid (Table I). With respect to the latter inhibitor, it must however be noted that ascorbate oxidation by the mitochondria of the mutant is at least four times less sensitive to salicylhydroxamic acid than succinate oxidation. Ascorbate appears to be oxidized in the mutant by a pathway which differs from that used for succinate oxidation by its sensitivity to salicylhydroxamic acid.

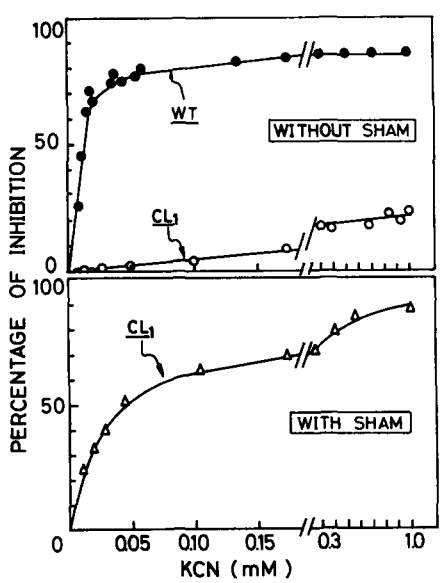
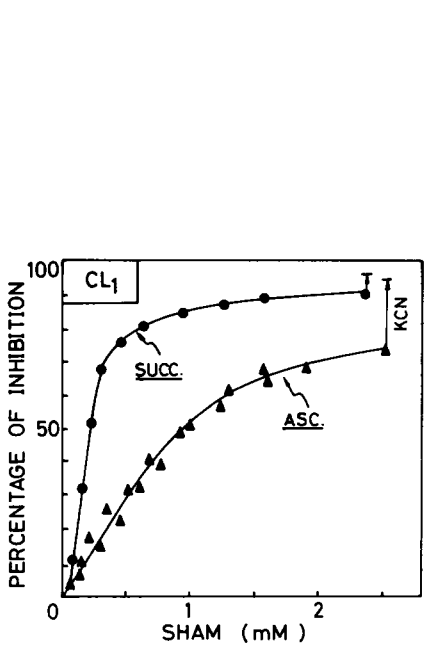


Fig. 1. Titration of the cyanide-sensitive respiration in mitochondria of the wild type and the mutant Cl_1. Conditions as in Table I. Succinate was used as substrate.

Effect of other respiratory inhibitors

Antimycin did not inhibit the oxidation of pyruvate or succinate, even at concentrations as high as 1 μg/mg protein. At concentrations higher than 5 μg/mg protein, it exhibited uncoupling effects [20] which were reflected by an increase in the rate of respiration and by a more oxidized steady state of cytochrome *b* and *c*. The absence of inhibitory effect of antimycin is readily explained by the lack of antimycin binding sites, as demonstrated by the fluorimetric assay illustrated in Fig. 3.

HQNO at saturating concentrations (10 μM) inhibited pyruvate and succinate oxidation to an extent of 40—50% in wild type mitochondria but not at all in mitochondria of the mutant Cl_1. It did not inhibit the cyanide-insensitive, salicylhydroxamic acid-sensitive respiration. The binding of HQNO, studied by fluorimetry (Fig. 3), revealed a number of binding sites at saturation of 0.3 nmol/mg protein and a K_d value of 4 μM (values close to those found in rat liver mitochondria). The occurrence in *Paramecium* mitochondria of binding sites for HQNO, but not for antimycin, is consistent with different binding



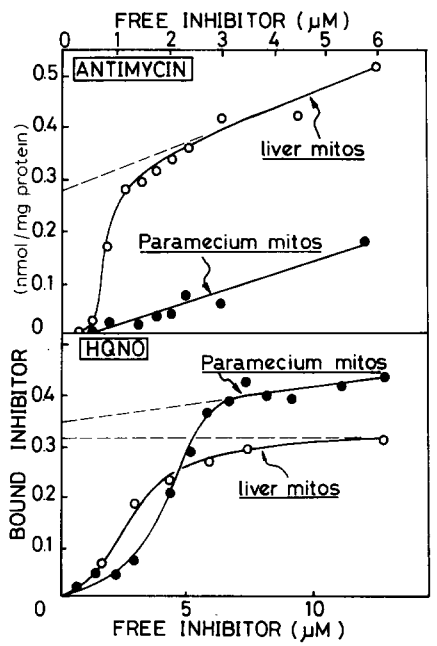


Fig. 2. Titration of the salicylhydroxamic acid (SHAM)-sensitive respiration in mitochondria of the mutant Cl_1. Conditions as in Table I. Succinate and ascorbate (plus TMPD) were used as substrates. At the end of the titration, 1 mM KCN was added, resulting in a supplementary inhibition as indicated by arrows.

Fig. 3. Lack of high affinity antimycin binding sites in *Paramecium* mitochondria. Number of HQNO binding sites. The medium (1 ml) consisted of 0.5 M mannitol, 1 mM EDTA, 1 mM EGTA, 5 mM MOPS, pH 7.3, BSA 6 mg and increasing concentrations of antimycin or HQNO. The fluorescence intensity of antimycin or HQNO was measured as described in Methods, and then wild type *Paramecium* mitochondria (2.5 mg protein in 0.1 ml) were added. After incubation at 22°C for 15 min, the mitochondria were separated from the medium by centrifugation. The fluorescence of the supernatant fluid was again measured. The difference between the first and second value of fluorescence allows calculation of the bound antimycin or HQNO. For comparison, antimycin and HQNO titrations were carried out with rat liver mitochondria (2.2 mg protein) under the same conditions.

sites for the two inhibitors in mammalian mitochondria [21,22].

Paramecium mitochondria were nearly 100 times less sensitive to rotenone, but only 4 times less sensitive to amytal than mammalian mitochondria. At 5 mM, amytal completely stopped pyruvate oxidation, without markedly inhibiting (only 15%) succinate oxidation.

A new type of cyanide- and hydroxamic acid-insensitive, but azide-sensitive respiration has been recently found in mitochondria of *Neurospora crassa* [23], and *Schizosaccharomyces pombe* [24]. In the case of *Paramecium* mitochondria incubated with 1 mM cyanide, there was no further inhibition of respiration by 1 mM azide.

K_m O_2 values for cyanide-sensitive and -insensitive oxidases

The K_m O_2 values were calculated from oxygraphic traces between the point at which the trace deviates from linearity and anaerobiosis. During this non-linear part of the traces, the recorder sensitivity was increased 20-fold and the expanded curves were analyzed by measuring the rates of O_2 uptake, by drawing tangents to the curve at several O_2 concentration. Succinate was used as substrate and oxygen uptake was measured in the presence of either 0.5 mM salicylhydroxamic acid or 0.5 mM cyanide. The K_m values were deduced from Lineweaver-Burk plots. The average measured values of K_m O_2 in wild type mitochondria were 0.7 μM for the respiration insensitive to salicylhydroxamic acid (sensitive to cyanide) and 4—5 μM for the respiration insensitive to cyanide. The K_m O_2 for mitochondria of the mutant Cl_1 was 4 μM, similar to that found for the cyanide-insensitive respiration in wild-type mitochondria. The method of tangential analysis of oxygen depletion curves, which has been employed in this work, has been reported to yield only approximate quantitation of K_m O_2, particularly for low K_m values [25]. On this basis, the experimentally determined value of 0.7 μM for the K_m O_2 related to the cyanide-sensitive respiration could have been underestimated; it would therefore be more correct to state that K_m value is less than 2 μM. This reappraisal, however, does not invalidate our conclusion that the salicylhydroxamic acid and the cyanide-insensitive pathways differ in their affinity for O_2.

Effects of ADP and AMP on the cyanide-sensitive and cyanide-insensitive respiration

Addition of 0.6 mM cyanide to wild type mitochondria inhibited the state 4 respiration by about 80%. AMP added at a concentration of 0.5 mM resulted in a 2-fold stimulated of the residual cyanide-insensitive respiration (Fig. 4 trace a). Comparatively to AMP, ADP had a minor effect. The maximum stimulation obtained with ADP was less than 30% of that obtained with AMP. The K_m for AMP, calculated by plotting the difference in rates of O_2 uptake before and after addition of AMP (Fig. 5) was found to be equal to 120 μM.

After preincubation of mitochondria with ADP (or to a lesser degree with ATP), respiration became much more sensitive to cyanide; it was virtually completely blocked by 1 mM cyanide (Fig. 4 trace b). This effect of ADP was prevented by FCCP (Fig. 4 trace c). In all cases, the residual cyanide-insensitive respiration was markedly stimulated by AMP. Carboxyatractyloside, which inhibits ADP transport in mitochondria [26] and abolishes the stimulation

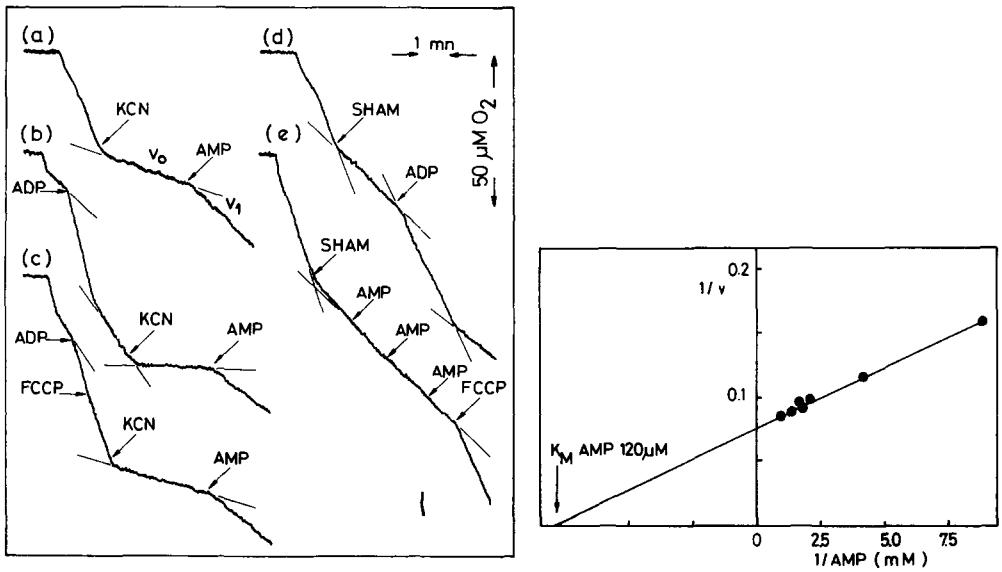


Fig. 4. Effect of AMP on the cyanide-insensitive respiration. Same conditions as in Table I. AMP was used at the final concentration of 0.5 mM, ADP 0.1 mM, salicylhydroxamic acid (SHAM) and cyanide 1 mM. The assays were carried out with wild type *Paramecium* mitochondria.

Fig. 5. Lineweaver Burk plot of the effect of AMP on the cyanide-insensitive respiration. Same conditions as in Fig. 4. v is the difference between v_1 (respiration after AMP) and v_0 (respiration before AMP) (cf. Fig. 5).

of state 4 respiration by ADP [27], had no effect on the AMP-stimulated respiration in *Paramecium* mitochondria.

The salicylhydroxamic acid-insensitive respiration was stimulated by ADP (Fig. 4 trace d), but not by AMP (Fig. 4 trace e).

Phosphorylation efficiency of the cyanide-insensitive pathway

P/O ratios assayed with wild type *Paramecium* mitochondria (in which 20% of respiration was cyanide-insensitive) were slightly higher than 2 for pyruvate

TABLE II

PHOSPHORYLATION EFFICIENCY OF THE CYANIDE-INSENSITIVE RESPIRATION

The medium was similar to that used in Table I except that phosphate was labeled with ^{32}P. Further additions were hexokinase (1 mg) and glucose (10 mM). Cyanide was added at 1 mM and amytal at 5 mM. Results of three experiments are given as means ± S.D.

Additions	Wild type (P/O)	Mutant Cl_1 (P/O)
Pyruvate + malate	2.3 ± 0.1	1.7 ± 0.2
Pyruvate + malate + KCN	1.3 ± 0.1	1.2 ± 0.1
Succinate	1.3 ± 0.1	0.7 ± 0.2
Succinate + KCN	0.4 ± 0.1	0.5 ± 0.2
Succinate + amytal + KCN	0.4 ± 0.2	0.3 ± 0.2
Ascorbate	0.6 ± 0.1	<0.1
Ascorbate + KCN	0.1	<0.1

oxidation, between 1 and 2 for succinate oxidation and between 0.5 and 1 for ascorbate oxidation (Table II). In the presence of 1 mM cyanide, i.e. when the flux of electrons is directed through the cyanide-insensitive pathway, P/O ratios were decreased by one-third in the case of pyruvate oxidation and by two-thirds in the case of succinate oxidation. A significant phosphorylation was still coupled to the cyanide-insensitive oxidation of succinate, even in the presence of amytal added to block oxidation steps of the tricarboxylic acid cycle beyond succinate oxidation.

P/O ratios for pyruvate and succinate oxidation in mitochondria of the mutant Cl_1 were lower than in wild type mitochondria by about one-third and there was no ATP synthesis coupled to ascorbate oxidation due to the lack of the third phosphorylation site (Table II). An unexpected observation concerns the effect of cyanide on P/O ratios obtained with mitochondria of the mutant, using pyruvate or succinate as substrates. One could expect the same P/O in the absence or in the presence of cyanide as cytochrome aa_3 is not present in these mitochondria. However, 1 mM cyanide depressed the P/O ratios by about 30—40%. A plausible explanation is that mitochondria of the mutant possess a cyanide-sensitive pathway from pyruvate or succinate to O_2 which is different from cytochrome aa_3 and is coupled to ATP synthesis. Another possibility is that they possess a residual cytochrome oxidase activity despite the absence of optically detectable cytochrome aa_3 (cf. Ref. 16). This latter alternative, however, is not very likely in view of the finding that ascorbate oxidation in mitochondria of the mutant was not coupled to ADP phosphorylation (Table II) and was not electrogenic, as shown in the next section.

A last comment concerns the effect of salicylhydroxamic acid. In our hand, salicylhydroxamic acid, ever after recrystallization to eliminate any contaminant, exhibited a slight uncoupling effect with mammalian mitochondria. For this reason the effect of salicylhydroxamic acid on P/O ratios with *Paramecium* mitochondria cannot be interpreted on a sound basis and will not be discussed here.

Generation of membrane potential ($\Delta\psi$) by ascorbate and succinate oxidation

Although the fluorescent dye, dipropylthiocarbocyanine, is not accepted to be quantitative, it is considered as a reliable probe which may provide useful information by qualitative monitoring of $\Delta\psi$ [12]. Upon addition of ascorbate plus TMPD to wild type mitochondria, the fluorescent intensity of dipropylthiocarbocyanine abruptly decreased, indicating the generation of a $\Delta\psi$, positive outside (Fig. 6). In contrast, no modification of fluorescence was observed when ascorbate plus TMPD was added to mitochondria from the mutant strain an observation which coincides with the absence of ATP synthesis coupled to ascorbate oxidation (see above). Upon oxidation of succinate, wild type mitochondria generated a larger $\Delta\psi$ than mitochondria of the mutant Cl_1 (Fig. 6), which again is in line with higher P/O ratios for succinate oxidation in wild type mitochondria as compared to mitochondria of the mutant.

Another interesting observation concerns the effect of different concentrations of cyanide on the $\Delta\psi$ generated in wild type mitochondria either by succinate or ascorbate oxidation (Fig. 6). A small concentration of cyanide (50 μM) was sufficient to abolish the $\Delta\psi$ generated by ascorbate oxidation,

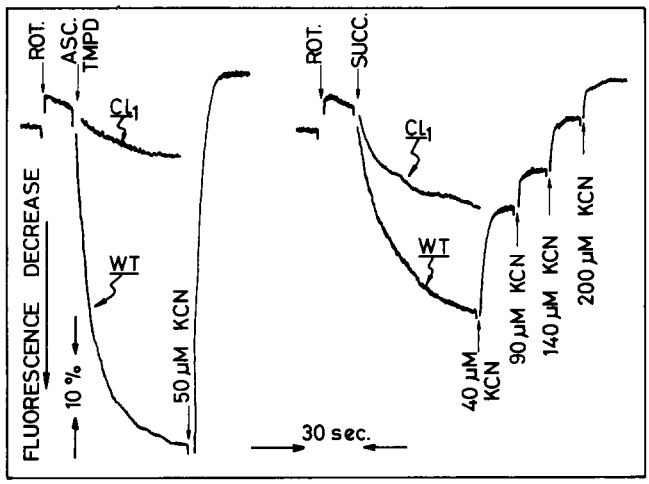


Fig. 6. Generation of a membrane potential by ascorbate and succinate oxidation. The medium (3 ml) consisted of 0.5 M mannitol, 0.1% bovine serum albumin, 1 mM EDTA, 5 mM MOPS, 20 mM KCl, 0.2 mg of mitochondrial protein, 1 µg oligomycin and 3 µM dipropylthiodicarbocyanine. Ascorbate was used at the final concentration of 3 mM, TMPD at 50 µM, succinate 10 mM and cyanide as indicated in the Figure. Final pH 7.10.

consistent with the role played by cytochrome aa_3 as a terminal oxidase under these conditions. At a similar cyanide concentration, only part of the $\Delta\psi$ generated by succinate oxidation was abolished; up to 200 µM cyanide was required to totally inhibit the $\Delta\psi$ generated by succinate oxidation. The most simple explanation is that succinate is oxidized by two electrogenic pathways. One of these pathways is sensitive to low concentrations of cyanide ($\leqslant$50 µM) and involves cytochrome aa_3, while the other one is sensitive to much higher concentrations of cyanide ($\geqslant$200 µM); the latter one could involve the CO-binding cytochrome *b*.

H^+ efflux coupled with the functioning of the different pathways to O_2 in Paramecium mitochondria

Acidification of the medium was observed when pulses of O_2 were injected into an anaerobic suspension of *Paramecium* mitochondria incubated with an oxidizable substrate, oligomycin and valinomycin. In wild type mitochondria, in which the cyanide-insensitive respiration accounts for about 20% of the total oxygen uptake, H^+/O ratios up to 4 were obtained with succinate as substrate. As shown in Fig. 7, addition of increasing concentrations of cyanide decreased the H^+/O ratios to zero. The dose effect curve was biphasic with a rapid decrease of H^+/O ratios from 4 to 2 at concentrations of cyanide lower than 0.1 mM and a slow decrease of H^+/O ratios above 0.1 mM cyanide, the H^+/O value being about 1 at 0.7 mM cyanide. A control experiment carried out with beef heart mitochondria showed, as expected, a drop of the H^+/O from about 4 with succinate as substrate to zero with less than 0.1 mM cyanide. The biphasic curve of inhibition of H^+/O ratios by cyanide in wild-type *Paramecium* mito-

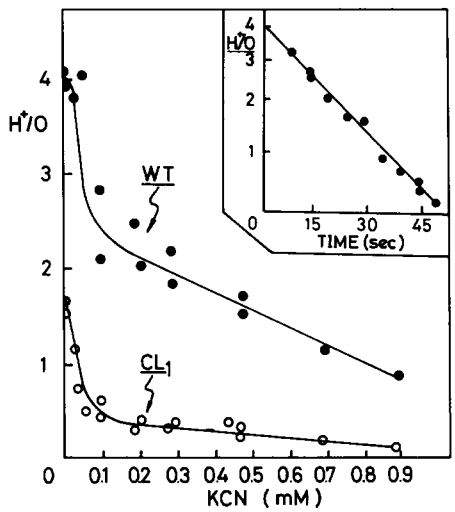


Fig. 7. Effect of increasing concentrations of cyanide on H^+/O ratio with succinate as substrate. Mitochondria of the wild type and the mutant Cl_1 (5 mg protein) were incubated at 22°C in 3 ml of a medium made of 0.5 M mannitol, 50 mM KCl, 0.2 mM MOPS, 1 mM EDTA, 2 mM succinate, 5 μg oligomycin, 2 μg valinomycin and cyanide as indicated. Anaerobic conditions were ensured by sparging with argon gas. The pH was adjusted exactly to 7.10. At zero time, H^+ ejection was initiated by injecting 50 μl of the above medium saturated with air (O_2 concentration was 205 μM at 22°C). The amount of H^+ ejected (and consequently the H^+/O ratio) was calculated, as indicated in the insert, by extrapolating to zero time the first-order decay of acidity.

chondria can be explained by the presence of two terminal cyanide-sensitive oxidases differing by their degree of sensitivity to cyanide (see Discussion).

H^+/O ratio in mitochondria of the mutant with succinate as substrate and in the absence of cyanide was only 1.7 (instead of 4 in wild type mitochondria), which is consistent with the lack of the third phosphorylation site (cf. Table II). It decreased abruptly to a value of about 0.5 upon addition of 0.1 mM cyanide; there was a further slow decrease above 0.1 mM cyanide.

Steady-state reduction of respiratory carriers

The oxidation-reduction state of cytochromes and ubiquinone was examined in steady-state conditions, using succinate as substrate (Table III). In wild type mitochondria, the percentage of reduction was found to decrease from ubiquinone to cytochrome aa_3 in line with the increase in the mid-point potentials of the respiratory carriers (see preceeding paper [5]), except for cytochrome *b*-557 which remained less reduced than cytochrome *b*-553 or cytochrome cc_1. Addition of 0.5 mM cyanide increased the reduction level of ubiquinone, cytochrome cc_1 and cytochrome *b*-553, but did not modify that of cytochrome *b*-557 which remained largely oxidized. The tendancy of cytochrome *b*-553 and *b*-557 to remain oxidized was accentuated in mitochondria of the mutant. It must be added that the *b* cytochromes in both types of mitochondria are reduced to 70—80% of the maximum by succinate in anaerobiosis. The above data therefore suggest that cytochrome *b*-557 interacts with O_2 and is in equilibrium with cytochrome *b*-553.

TABLE III

STEADY-STATE REDUCTION OF MITOCHONDRIAL CARRIERS IN MITOCHONDRIA OF THE WILD TYPE AND THE MUTANT Cl_1. EFFECT OF CYANIDE

Conditions are described in Materials and Methods. The respiratory medium was supplemented with 10 mM succinate. Cyanide was used at a final concentration of 0.5 mM. Results are given in percentages of reduction. The cytochromes are designed by their α band at 77 K.

Strains	Additions	Ubiquinone	Cytochrome			
			b-553	*b*-557	cc_1-549	aa_3-608
Wild type	Succinate	73	45	25	42	15
Wild type	Succinate + KCN	87	70	30	90	—
Mutant Cl_1	Succinate	90	24	12	84	—

Photorestoration of the mitochondrial CO-inhibited respiration in the mutant Cl_1

The photochemical action spectrum for the relief of the CO-inhibited respiration is the best criterion to ascribe an oxidase function to a CO-binding pigment in a complex respiratory chain. For example, in the case of *Paramecium* wild type mitochondria, which possess two CO-binding cytochromes, cytochrome aa_3 and a *b*-type cytochrome, the photochemical action spectrum for restoration of the CO-inhibited respiration would give a definitive answer as to the possible oxidase function of the CO-binding cytochrome *b*. The mutant Cl_1 which is devoid of cytochrome aa_3 offers the possibility of a much simpler photorestoration assay. In fact the only CO-reactive cytochrome *b* present in mitochondria of the mutant Cl_1 is the *b*-type cytochrome; thus no selected

TABLE IV

PHOTORESTORATION OF THE CO-INHIBITED RESPIRATION IN MITOCHONDRIA OF THE MUTANT Cl_1

The medium was that used for respiration assays (cf. Methods). It was partially saturated with CO, the remaining concentration of O_2 being 40 μM. The substrate was succinate (10 mM). The amount of mitochondrial protein was 0.35 mg. The final volume was 1.5 ml. When added, salicylhydroxamic acid was at a final concentration of 0.7 mM and cyanide at 0.14 mM. Respiration was assayed as described in Methods. Photoirradiation was carried out with a 375 W lamp placed at a distance of about 8 cm from the incubation vessel. A screen of water (closed Petri box filled with water) was placed between the light source and the incubation vessel. A control was carried out with the same medium partially saturated with N_2, the remaining O_2 being about 40 μM. The results are given by reference to the rate of respiration in the control in the absence of any inhibitor.

Conditions	Rate of respiration (% of the control)
CO (dark)	66
CO (light)	90
CO + hydroxamic acid (dark)	19
CO + hydroxamic acid (light)	60
CO + KCN (dark)	41
CO + KCN (light)	74

wavelength is required to identify it in a photorestoration assay. A typical experiment of photorestoration of CO-inhibited respiration in mitochondria of the mutant Cl_1 is described in Table IV. Mitochondria were incubated in a medium partially saturated with CO (cf. legend of Table IV), and their ability to oxidize succinate before or after photoirradiation with white light was measured under different conditions. In all conditions, light restored the CO-inhibited respiration. It may be noted that CO (in the dark) inhibits respiration only partially. This may in part be due to the fact that the mutant Cl_1 possesses two functional oxidases, one being the salicylhydroxamic acid-sensitive oxidase and the other the CO-reactive cytochrome *b*. Indeed, salicylhydroxamic acid decreased markedly the rate of the CO-inhibited respiration in the dark. Furthermore, the percentage of photorestoration of the CO-inhibited respiration in the presence of salicylhydroxamic acid was twice higher than in the absence of salicylhydroxamic acid. The inhibitory effect of a small concentration of cyanide (0.14 mM) in the dark is moderate, probably due to the low cyanide reactivity of the CO-binding cytochrome *b*; in the presence of cyanide, photorestoration of the CO-inhibited respiration was less in the presence than in the absence of cyanide, but it was not negligible. This last assay excludes the participation of residual cytochrome aa_3 as terminal oxidase in the mutant Cl_1. If it had been the case, cytochrome aa_3 would have been fully inhibited by the concentration of cyanide used (the cyanide titration curve of succinate oxidation in wild type mitochondria (Fig. 1) shows that the plateau of inhibition for the high affinity region of the curve, due to cytochrome aa_3, is attained at less than 0.10 mM cyanide).

Discussion

In plant mitochondria and in a number of microorganisms, the cyanide-insensitive, hydroxamic acid-sensitive oxidation of NAD-linked substrates exhibits P/O ratios of about 1, and succinate oxidation is not coupled to ATP synthesis. On the basis of these observations, it has been proposed that the branch point of the cyanide-insensitive pathway on the respiratory chain is on the substrate site of coupling site II [17—18], possibly at the level of ubiquinone [28]. In *Paramecium* mitochondria, the situation appears more complex. In fact, beside cytochrome aa_3, there may exist two alternate oxidases, one of which is a CO-reactive *b*-type cytochrome, possibly *b*-557 (cf. accompanying paper [5]). There are different observations which suggest that this *b*-type cytochrome acts as an oxidase. (1) Under steady-state conditions, it is more oxidized than cytochrome cc_1 although its mid-point potential has a lower value. (2) It is present at a concentration 2—3 times higher in mitochondria of the mutant than in mitochondria of the wild type and concomitantly succinate oxidation is 2—3 times faster in the mutant than in the wild type. (3) The CO-inhibited respiration of mitochondria from the mutant Cl_1 can be restored by light. This last point deserves a brief comment. The photochemical action spectrum for the relief of the CO-inhibited respiration is considered to be the best criterion to ascribe an oxidase function to a CO-binding pigment in a respiratory chain which contains several of these pigments. We took advantage of the fact that mitochondria of the mutant Cl_1 possess only one CO-

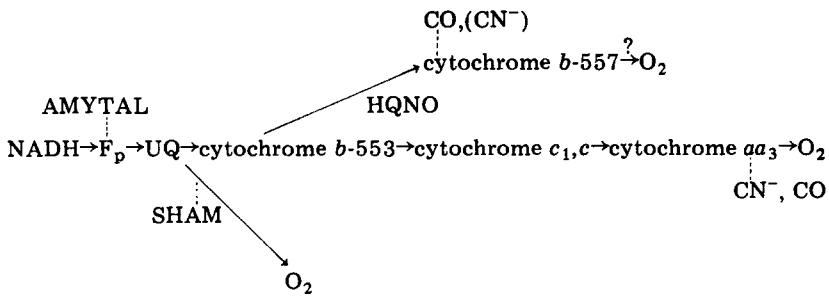
binding cytochrome (a cytochrome *b*) to use a simplified photorespiration assay with white light. Although the photorespiration assay described here is essentially indicative and cannot replace the photochemical action spectrum for a definitive conclusion, it strongly suggests that the CO-binding cytochrome *b* of *Paramecium* mitochondria is autooxidizable.

The cyanide inhibition of the mitochondrial respiration in the mutant Cl_1 is markedly less than in wild-type mitochondria; this is probably due to the low cyanide sensitivity of the CO-binding cytochrome *b* (cf. titration curves of Fig. 1). In wild type mitochondria, the two autooxidizable cytochromes, namely cytochrome aa_3 and the CO-reactive cytochrome *b* would differ by their affinity for cyanide, cytochrome aa_3 exhibiting a higher cyanide affinity than the *b*-type cytochrome. Assuming that succinate oxidation is coupled to the phosphorylation of two ATP when occurring through cytochrome aa_3, and to the phosphorylation of only one ATP when occurring through cytochrome *b*, the cyanide titration of the protein efflux induced by succinate oxidation in wild type mitochondria (Fig. 7) can be easily explained. The H^+/O ratio is indeed considered as an index of the phosphorylation efficiency. The abrupt decrease of H^+/O found with wild type mitochondria treated by low concentrations of cyanide (less than 0.1 mM) is attributable to the predominant inhibition of cytochrome aa_3; the further decrease of H^+/O at higher cyanide concentrations would be due to the inhibition of the autooxidizable cytochrome *b*.

An intriguing observation is that ascorbate is oxidized by mitochondria of the mutant Cl_1 which are devoid of cytochrome aa_3 (all corrections being made for spontaneous oxidation of ascorbate). In contrast to ascorbate oxidation by wild type mitochondria, ascorbate oxidation by mitochondria of the mutant is not electrogenic and is poorly inhibited by cyanide. On the other hand, it is significantly inhibited by salicylhydroxamic acid, but much less than succinate oxidation (4 time less). A plausible explanation is that ascorbate is oxidized in mitochondria of the mutant, both by the CO-reactive *b*-type cytochrome and by the hydroxamic acid-sensitive pathway. The free energy release accompanying the electron transfer from ascorbate to the CO-reactive *b*-type cytochrome would not be high enough to be coupled to ATP synthesis.

The above considerations on the different pathways to O_2 in wild type mitochondria are schematically depicted in Scheme I. Mutant Cl_1 would possess both the hydroxamic acid-sensitive oxidase and the *b*-type cytochrome oxidase (*b*-557), but no cytochrome aa_3. Cytochrome *b*-553 is tentatively included between ubiquinone and cytochrome cc_1. The steady-state reduction experiments indicate that cytochrome *b*-557 is in equilibrium with cytochrome *b*-553 and thus probably branched at the level of cytochrome *b*-553.

Respiration in *P. tetraurelia* was insensitive to antimycin, and concomitantly antimycin binding sites were absent. Antimycin insensitivity is typical of a number of cyanide-insensitive organisms. Correlation between lack of cytochrome *b*-562 (absorbance at 77 K) and the absence of both antimycin-sensitivity and antimycin binding sites has been reported for a mutant of *Schizosaccharomyces pombe* [29]. This correlation also holds for *Paramecium* mitochondria which lack cytochrome *b*-562. The partial inhibition of succinate oxidation by HQNO in wild type mitochondria, but not in mitochondria of the



Scheme I. Tentative model of pathways of electron transport in wild type *Paramecium* mitochondria. Dotted lines show the sites of action of inhibitors.

mutant Cl_1 is consistent with an effect of this inhibitor on electron transfer between the *b* and cc_1 cytochromes (see Fig. 8).

The hydroxamic acid-sensitive pathway of electron transfer to O_2 in *P. tetraurelia* was specifically stimulated by AMP. A similar effect of AMP has been observed in the following hydroxamic acid-sensitive organisms: *Euglena gracilis* [30], *Moniliella tomentosa* [31] and *Acanthamoeba castellanii* [32]. It was of interest that preincubation with ADP increased the cyanide-sensitivity of wild-type *Paramecium* mitochondria. This ADP effect is abolished by uncouplers. In other words, ADP modifies the sharing of electrons by the hydroxamic acid- and cyanide-sensitive pathways, favouring the channeling of electrons into the cyanide-sensitive pathways. Such intricate antagonistic effects suggest an adjustement of the activities of the electrogenic and non-electrogenic pathways, depending on the relative concentrations of AMP and ADP.

A final comment concerns the low H^+/O ratio in mitochondria of the mutant Cl_1 as compared to wild type mitochondria (1.7 vs. 4 with succinate as substrate) and its relevance to the role played by cytochrome aa_3 as a proton pump [33—35]. The decreased ability of mitochondria from the mutant to eject protons adds additional support to the proposal that cytochrome oxidase is a proton pump [33].

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References

1 Doussière, J., Adoutte, A., Sainsard, A., Ruiz, F., Beisson, J. and Vignais, P.V. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th., Neupert, W., Sebald, W. and Werner, S., eds.), pp. 873—880, Elsevier/North-Holland Biomedical Press, Amsterdam
2 Henry, M.F. and Nyns, E.J. (1975) SubCell. Biochem. 4, 1—65
3 Sainsard, A., Claisse, M. and Balmefrezol, M. (1974) Mol. Gen. Genet. 130, 113—125
4 Sainsard-Chanet, A. (1978) Mol. Gen. Genet. 159, 117—123

5 Doussière, J., Sainsard-Chanet, A. and Vignais, P.V. (1979) Biochim. Biophys. Acta 548, 224—235
6 Nielsen, S.O. and Lehninger, A.L. (1955) J. Biol. Chem. 230, 521—530
7 Chance, B. and Williams, G.R. (1956) Adv. Enzymol. Rel. Subj. Biochem. 17, 65—134
8 Yonetani, T. (1961) J. Biol. Chem. 236, 1680—1688
9 Redfearn, E.R. (1967) Methods Enzymol. 10, 381—384
10 Berden, J.A. and Slater, E.C. (1972) Biochim. Biophys. Acta 256, 199—215
11 Van Ark, G. and Berden, J.A. (1977) Biochim. Biophys. Acta 459, 119—137
12 Sims, P.J., Wagoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315—3330
13 Gornall, A.G., Bardawill, C.S. and David, M.M. (1949) J. Biol. Chem. 177, 751—756
14 Addoute, A. and Doussière, J. (1978) Mol. Gen. Genet. 161, 121—134
15 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604—617
16 Edwards, D.L., Klein, J.L., Warden, J.T. (1978) Eur. J. Biochem. 85, 437—443
17 Schonbaum, G.R., Bonner, W.D., Jr., Storey, B.T. and Bahr, J.T. (1971) Plant Physiol. 47, 124—128
18 Rich, P.R. and Bonner, W.D., Jr. (1977) in Functions of Alternative Terminal Oxidases, 11th FEBS-Meeting, Copenhagen, 1977 (Degn, H., Lloyd, D. and Hill, G.C., eds.), Vol. 49, pp. 149—158, Pergamon Press, Oxford
19 Solomons, T. (1977) Annu. Rev. Plant Physiol., 28, 279—297
20 Slater, E.C. (1967) Methods Enzymol., 10, 48—57
21 Izzo, G., Guerrieri, F. and Papa, S. (1978) FEBS Lett. 93, 320—322
22 Eisenbach, M. and Gutman, M. (1977) Eur. J. Biochem. 59, 223—230
23 Edwards, D.L. (1978) in Functions of Alternative Terminal Oxidases, 11th FEBS-Meeting, Copenhagen, 1977 (Degn, H., Lloyd, D. and Hill, G.C., eds.), Vol. 49, pp. 21—29, Pergamon Press, Oxford
24 Goffeau, A. (1978) in Functions of Alternative Terminal Oxidases, 11th FEBS-Meeting, Copenhagen, 1977 (degn, H., Lloyd, D. and Hill, G.D., eds.), Vol. 49, pp. 39—46, Pergamon Press, Oxford
25 Degn, H. and Wohlrab, H. (1971) Biochim. Biophys. Acta 245, 347—355
26 Vignais, P.V., Vignais, P.M. and Defaye, G. (1973) Biochemistry 12, 1508—1519
27 Vignais, P.V., Vignais, P.M. and Defaye, G. (1971) FEBS Lett. 17, 281—288
28 Rich, P.R. and Moore, A.L. (1976) FEBS Lett. 65, 339—344
29 Bandlow, W., Wolf, K., Kaudewitz, F. and Slater, E.C. (1974) Biochim. Biophys. Acta 333, 446—459
30 Sharpless, T.K. and Butow, R.A. (1970) J. Biol. Chem. 245, 58—70
31 Hanssens, L. and Verachtert, H. (1976) J. Bacteriol. 125, 829—836
32 Edwards, S.W. and Lloyd, D. (1978) Biochem. J. 174, 203—211
33 Wikström, M.K.F. (1977) Nature 266, 271—273
34 Moyle, J. and Mitchell, P. (1978) FEBS Lett. 88, 268—272
35 Wikström, M. and Krab, K. (1978) FEBS Lett. 91, 8—14