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Rapid isolation of muscle and heart mitochondria, the lability of oxidative phosphorylation and attempts to stabilize the process *in vitro* by taurine, carnitine and other compounds

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Abstract

We modified the isolation procedure of muscle and heart mitochondria. In human muscle, this resulted in a 3.4 fold higher yield of better coupled mitochondria in half the isolation time. In a preparation from rat muscle we studied factors that affected the stability of oxidative phosphorylation (oxphos) and found that it decreased by shaking the preparation on a Vortex machine, by exposure to light and by an increase in storage temperature. The decay was found to be different for each substrate tested. The oxidation of ascorbate was most stable and less sensitive to the treatments.

When mitochondria were stored in the dark and the cold, the decrease in oxidative phosphorylation followed first order kinetics. In individual preparations of muscle and heart mitochondria, protection of oxidative phosphorylation was found by adding candidate stabilizers, such as desferrioxamine, lazaroids, taurine, carnitine, phosphocreatine, N-acetylcysteine, Trolox-C and ruthenium red, implying a role for reactive oxygen species and calcium-ions in the in vitro damage at low temperature to oxidative phosphorylation.

In heart mitochondria oxphos with pyruvate and palmitoylcarnitine was most labile followed by glutamate, succinate and ascorbate. We studied the effect of taurine, hypotaurine, carnitine, and desferrioxamine on the decay of oxphos with these substrates. 1 mM taurine (n = 6) caused a significant protection of oxphos with pyruvate, glutamate and palmitoylcarnitine, but not with the other substrates. 5 mM L-carnitine (n = 6), 1 mM hypotaurine (n = 3) and 0.1 mM desferrioxamine (n = 3) did not protect oxphos with any of the substrates at a significant level.

These experiments were undertaken in the hope that the *in vitro* stabilizers can be used in future treatment of patients with defects in oxidative phosphorylation. (Mol Cell Biochem **174**: 61–66, 1997)

Key words: heart mitochondria, lability, muscle mitochondria, oxidative phosphorylation, stability, taurine

Introduction

The study of oxidative phosphorylation in intact mitochondria provides important information on the integrity of the mitochondrial transporters, dehydrogenases and the respiratory chain complexes, of the coupling between oxidation and phosphorylation and of the efficiency of the energy transduction.

The aim of the present investigation was to study the stability of oxidative phosphorylation catalyzed by muscle mitochondria obtained with a new isolation procedure. We tried to improve the storage and handling conditions, and studied the effect of the addition of potential stabilizing agents.

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Materials and methods

Human and animal muscle

Human muscle was obtained under local analgesia from the *M. vastus lateralis* of patients and controls. The stability was studied of mitochondrial preparations with a normal oxidation rate of pyruvate plus malate, determined directly after isolation in the range of 52–140 nmol $O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$. To determine the decay, the assay of pyruvate plus malate oxidation was repeated after about 2.5 h. For the calculation of the decay see below. Since there was no statistical difference between the decay of pyruvate oxidation in the patients and the controls, they were taken together.

Masseter muscle and heart muscle were taken from normally fed male Wistar rats of 14 weeks, weighing 180–210 g. The animals were anesthetized with a mixture of ether and air, and killed by decapitation in the morning.

Chemicals

N-Acetyl-L-cysteine, desferrioxamine mesylate, hypotaurine, rotenone and ruthenium red were from Sigma, L-carnitine from Lonza, lazaroids U-74389F and U-74500A from Upjohn, palmitoyl-L-carnitine from Fluka, phosphocreatine and hexokinase from Boehringer Mannheim, taurine from BDH and trolox-C (vitamin E without side chain) from Aldrich. The other chemicals used were of the highest purity available.

Isolation of mitochondria

Rapid isolation of muscle mitochondria in high yield was based upon Bookelman *et al*. [11], which on its turn was based upon Luft *et al*. [21] and Dow [3]. We modified the mincing of the muscle and used shorter centrifugation times at higher forces. This method is suitable for heart and muscle mitochondria, but not for liver mitochondria, which are destroyed by the heparin in the isolation medium. In the past it was found that pretreatment with digitonin stabilized rat liver mitochondria by removing the lysosomes [4]. This treatment was also successful in removing lysosomes from muscle mitochondria, but failed to increase their stability (not shown).

The time required for the isolation was 1 h at a temperature of 0°C. The average yield from human muscle determined by the assay of citrate synthetase in the homogenate and in the mitochondrial preparation was 42%. In our hands the method of Bookelman *et al*. [1] gave rise to an average yield of 12.4% and the isolation took more than 2 h.

The isolation medium was the so-called SETH-medium, containing 250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl, 50 U/ml heparin, pH 7.4. For homogenisation, tightly fitting Potter-Elvehjem glass homogenisers were used, with a Teflon pestle driven by a motor at 800 rpm (homogenate), or by hand power (mitochondrial fraction). One stroke is down- plus upwards.

The centrifuge used was of Beckmann, type J2-21 with the JA 20 rotor containing 10 ml Pyrex tubes with r_{max} of 10.3 cm and r_{min} of 4.4 cm. The brake setting was maximal. The centrifugation of Bookelman *et al*. [1] with minimal brake setting was followed with time, and the sedimentation coefficients in Svedberg units were calculated by the use of the formula of De Duve and Berthet [5] to enable recalculation of reproducible spins at higher speeds in shorter time. In spite of the maximal brake setting, now the acceleration/deceleration phases of the spins gave a much higher contribution to the sedimentation coefficients. The low speed spin was for 1 min at 7000 rpm, corresponding to 4200 g_{av} and the high speed spin for 1.5 min at 18000 rpm (27500 g_{av}).

The isolation procedure, summarized in Fig. 1 is as follows: (1) Biopsy of patients suspected of having a mitochondrial disease from the *M. vastus lateralis* under local analgesia. Male Wistar rats are anaesthetized with a mixture of ether and air, and decapitated. The masseter muscles and heart are used for the experiments; (2) Transport the muscle in icecold SETH-medium; (3) Dry the tissue with paper and weigh it in a glass beaker. Minimal amount required is 80 mg; (4) Add 1 µl SETH-medium per mg of muscle and mince the muscle by cutting it for 90 times with a pair of scissors by hand; (5) Put the minced muscle with 18 µl medium/mg muscle into the homogenizer tube and homogenize by 12 strokes; (6) Sample this 5% homogenate to assay citrate synthetase by the method of Srere [6]. The sample may be frozen, but then the latency of the enzyme, which is a measure for the integrity of the inner mitochondrial membranes cannot be determined; (7) 1st low speed spin. Poor out supernatant in another tube. Transfer pellet with 19 µl medium/mg muscle into the homogenizer tube and homogenize by 12 strokes; (8) 2nd low speed spin. Repeat step 7; (9) 3rd low speed spin. Combine supernatants of steps 7–9 for step 10; (10) lst high speed spin. Remove supernatant with a pipette. Transfer pellet with 19 µl medium/mg of muscle into a smaller homogenizer, homogenize by 5 strokes by hand and transfer the preparation to a centrifuge tube; (11) 2nd high speed spin. Transfer pellet with 19 µl medium/mg muscle into homogenizer and bring the volume to 800 µl. After 5 strokes by hand the mitochondrial fraction is obtained; (12) Sample to assay citrate synthetase (CS) and protein. The total amount of mitochondria in the homogenate in mg mitochondrial protein/g muscle with the same purity as the isolated mitochondria can be calculated by CS homogenate(U/g muscle)/CS mitochondria(U/mg protein).

For another approach to isolate and study pigeon breast muscle mitochondria we refer to the work by HN Rasmusssen and UF Rasmussen in the next paper.

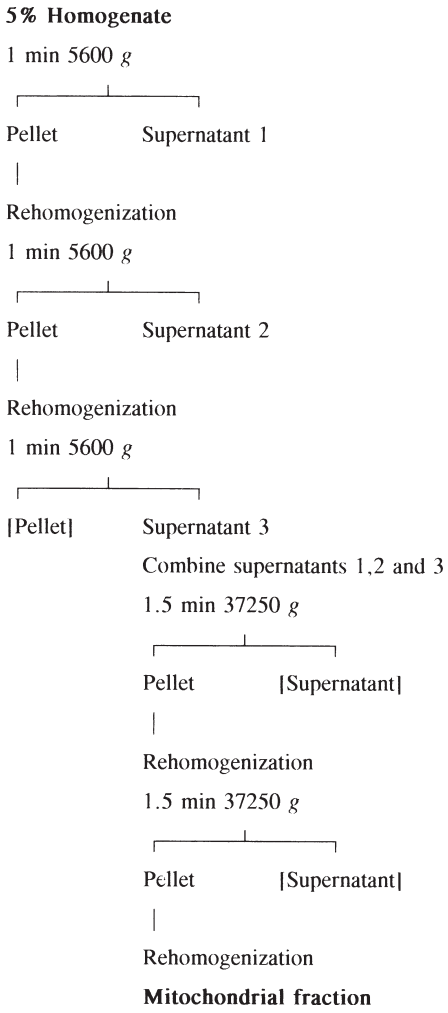

Fig. 1. Isolation scheme of heart and skeletal muscle mitochondria from 5% homogenate to the mitochondrial fraction. [Pellet] and [Supernatant] means that these fractions were discarded.

Assay of oxidative phosphorylation

Oxidative phosphorylation was measured by a Clark electrode with a very small Pt surface, requiring powerful electronics for amplification of the relatively small signal [7]. The advantage of this is that the stirring is not very critical. The double wanded glass oxygraph vessel was of the traditional type, but smaller, with a volume of 0.4 ml. The membrane of the Clark electrode was the thinnest available. The iron stirring rod was surrounded by glass, and driven by a simple magnet stirrer. The oxidative phosphorylation (oxphos) medium contained 5 mM potassium phosphate, 10 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 5 µM EDTA, 75 mM mannitol, 25 mM sucrose, 15 mM glucose, 0.2 mg FA-free bovine serum albumine (pH 7.4). Further additions were 20 µg hexokinase, mitochondria, oxidative substrate, after steady state (5 min) 0.2 mM ADP (pH 7.4). The temperature was 37°C and the volume 400 µl [8, 9]. The mitochondrial substrates tested were 5 mM pyruvate + 2.5 mM L-malate, 5 mM glutamate + 2.5 mM L-malate, 20 µM palmitoylcarnitine + 0.6 mM L-malate, 5 mM succinate + 3 µM rotenone, 5 mM ascorbate + 0.6 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine, TMPD + 0.1 mM desferrioxamine. For the determination of ATP, the produced glucose-6-phosphate was determined in perchloric acid deproteinized samples taken before and 10–20 min after the addition of the ADP [10].

The oxidation of ascorbate is complicated by the iron-catalyzed auto-oxidation of ascorbate. The auto-oxidation was considerably decreased by the addition of the iron-chelator desferrioxamine. There is however still some auto-oxidation, which was experimentally determined at different oxygen concentrations, and subtracted from the oxygen uptake rate.

Calculations

In mitochondria stored in the cold and the dark, the decay of oxidative phosphorylation measured as oxygen uptake with the different substrates measured in the presence of ADP, assayed at different occasions during several days, followed first order kinetics. The decay was expressed in the first order rate constant k in d^{-1}, which is proportional to the rate of the decay. The k was calculated from the oxygen uptake rate J_1 measured at the time t_1 (in d) and J_2 at t_2 according to $k = (\ln J_2 - \ln J_1).(t_2 - t_1)^{-1}$, and from more points by regression analysis between ln J and t. The half life time is equal to ln 2/k. In order to compare the results of the different experiments, we assumed first order kinetics in all experiments.

Statistical analysis was performed by the paired two tailed Students *t*-test. p values of less than 0.05 were considered significant.

Results

Isolated mitochondria are labile. When stored under the same conditions as during our routine experiments, the decay of the oxidation of pyruvate in human mitochondria is about twice as high as in rat masseter muscle mitochondria (Table 1). In human muscle mitochondria the rate of the decay varied 20 fold. The most labile preparation had a half life of 1.21 h, was of a 17 year old boy with low respiratory control, and the most stable one with a half life of 23 h was that of a 90 year old, well-trained lady who had a cardiac septum defect. Her oxidative phosphorylation characteristics were in the range of (younger) controls.

Storage of the rat masseter muscle mitochondria in the dark, with minimal shaking (3 sec) on a Vortex machine, decreased the average decay in pyruvate (plus malate) oxidation

Table 1. Decay of oxidative phosphorylation with pyruvate plus malate by isolated mitochondria

Muscle mitochondria	J_o (nmol $O_2 \cdot mm^{-1} \cdot mg^{-1}$)	$k(d^{-1})$ ± SD (range)	n
A Human	82 ± 22	6.6 ± 4.1 (0.73–13.7)	21
A Rat masseter	258 ± 77	3.5 ± 1.9 (1.45–8.1)	15
B Rat masseter	142 ± 38	0.90 ± 0.39 (0.50–1.38)	5

The *k* was calculated from two assays and we assumed first order kinetics of the decay. (A) Stored in transparent plastic cups on ice and shaken well for a total period of ca 20 sec on a Vortex machine before sampling. Oxidative phosphorylation was measured directly after isolation (J_o) and 2.5 h later. (B) The mitochondria were stored in the dark at 6°C and were shaken for 3 sec. Oxidative phosphorylation was measured directly after isolation (J_o) and after 1 day. Three of the preparations were followed longer for several (3–5) days, and since a straight relation was obtained between ln J and time (r = 0.96–0.98), the decay proved to obey first order kinetics. The decays in the 3 preparations showed a statistically significant difference.

Table 2. Decay of oxidative phosphorylation in by rat masseter muscle mitochondria. Effect of temperature, exposure to light and shaking

Temp	Light	Vortex	Pyruvate + malate	Glutamate + malate	Ascorbate + TMPD
6	–	–	1.08 ; 1.38	0.02; 0.01	0.30 ; 000
23	–	–	2.76	> 4.7	0.34
6	+	–	1.51; 1.64	0.36 ; 0.45	0.37 ; 0.33
23	+	–	> 2.8	> 4.7	0.52
0	++	–	4.42 ; 4.39	0.00 ; 0.00	0.00 ; 040
0	++	+ ; ++	5.45 ; 14.3	3.46 ; 8.90	0.00 ; 6.24

Oxygen uptake rates were measured by a substrate inhibitor method. The oxidation of pyruvate + malate (+ ADP) was inhibited after reaching steady state by 1 mM α-cyano-3-OH-cinnamate, then glutamate was added and complex I inhibited by 2 mM amytal, then ascorbate + TMPD + deferoxamine were added. The results are given of two independent experiments. The preincubation at 23°C was not performed in the second experiment. The assays were performed directly after isolation and after 4 h for the 2 lowest lines and after 22–28 h for the other experiments. Decay was expressed in $k(d^{-1})$, assuming first order kinetics of the decay. In case of *k* >, the oxygen uptake rate became zero. Light: – is in the dark, + is the light in the cold room (regular fluorescence tube 58 W, 55 Hz, Philips with cap, distance 1.1 m), cups in rack, ++ is the light in the laboratory [high frequency(30 kHz) fluorescence tube 58 W, colour 84 without cap, distance nearest tube 2 m, second one 2.5 m] plus daylight from the window (1 × 1.5 m, no direct sunlight, distance 3.2 m), cups on ice. Vortex: – is not shaken, + is shaken for 20 sec, ++ is shaken for 1 min. The concentration of mitochondrial protein in the first experiment was 1.5 mg/ml, and 4.8 mg/ml in the second one. The lowest line, with moderate shaking (+) reflects our previous standard conditions in measuring oxidative phosphorylation (see Table 1).

Table 3. Mode of action of candidate mitochondrial stabilizers

N-Acetylcysteine	FRS, precursor glutathione
L-Carnitine	Acyl carrier, membrane stabilizer (11)
Desferrioxamine	Ferri-chelator (12–15)
Hypotaurine	FRS, decreases low O_2-levels in tissue (16, 17)
Lazaroids	Prevent iron catalyzed lipid peroxidation (18, 19)
Phosphocreatine	ATP regenerator
Ruthenium red	Mitochondrial Ca^{2+}-channel blocker
Taurine	FRS, membrane stabilizer (16, 17)
Trolox-C	FRS

FRS = Free radical scavenger.

by a factor 7 (Table 1). We studied the effects of exposure to light, higher temperature and shaking on the oxidative phosphorylation by rat muscle mitochondria (Table 2) and found that the glutamate oxidation was destroyed by increasing the storage temperature from 6–23°C. That of pyruvate was destroyed by a similar increase of the temperature plus the light in the cold room. Pyruvate oxidation was found to be more affected than glutamate oxidation by the more intense light in the laboratory. Surprisingly, the oxidation of ascorbate was only slightly affected by the exposure to the higher temperature and the light. The conditions routinely used to measure oxidative phosphorylation, including exposure to light and shaking was particularly damaging for the oxidation of pyruvate, but not for the oxidation of glutamate and ascorbate. Prolonged shaking, however, was also found to be harmful for the latter oxidations. The decay of oxidative phosphorylation in the further experiments was studied in mitochondria that had been stored in the dark and in the cold.

Table 3 summarizes the candidate agents to stabilize oxidative phosphorylation used in this work. We studied the decay of the oxidation of pyruvate + malate and of succinate + rotenone by muscle and heart mitochondria, and the effect of the possible stabilizers during several days. Table 4 gives an example of such an experiment. The decays followed first order kinetics. The additions had no acute effect on the oxidative phosphorylation. In this experiment 0.5 mM desferrioxamine proved to be the best stabilizer, followed by 5 µg/ml of the lazaroid U-74389F, 0.5 mM N-acetylcysteine, 50 µM desferrioxamine, 2.5 mM Trolox-C, 2.5 mM L-carnitine, 5 mM Taurine, 1 mM Taurine, 1.25 µM ruthenium red, 7.5 mM phosphocreatine, while 50 µg/ml of the lazaroid U-74500A did not protect. The addition of albumin had no effect on the decay (not shown). In one experiment the most effective protector was found to be 1 mM taurine and in another the lazaroids. These experiments suggested that the phenomenon of stabilization showed a poor reproducibility in different preparations.

To study this in more detail, we measured the decay of oxidative phosphorylation with 5 different substrates in 6 independent preparations of rat heart mitochondria. In addition we studied the effects of taurine and carnitine, and of hypotaurine and desferrioxamine in 3 of the preparations (Table 5). In SETH-medium, the decay of oxidative phosphorylation with the NADH-dependent substrates (pyruvate, glutamate and palmitoylcarnitine) was significantly different from that with succinate and ascorbate. This suggests that these couples of substrates share one vulnerable target. Likely candidates for these targets are complex I for the NAD^+-linked substrates and complex IV for the latter couple. On the average taurine retarded the decay of oxidative phosphorylation with pyruvate from 100 to 66%, glutamate to 83%,

Table 4. Decay of oxidative phosphorylation with pyruvate plus malate by rat masseter mitochondria

	$k(d^{-1})$	RCI after 1 day	r
SETH-medium	1.04	2.2	0.96
1 mM desferrioxamine	0.48	3.7	0.93
0.01 mg/ml U-74389F	0.49	2.6	1.00
1 mM N-acetylcysteine	0.50	3.2	0.99
0.1 mM desferrioxamine	0.51	3.2	0.99
5 mM trolox-C	0.52	2.2	0.93
5 mM L-carnitine	0.53	3.4	0.99
10 mM taurine	0.53	2.4	1.00
2 mM taurine	0.54	2.4	0.99
2.5 µM ruthenium red	0.54	2.8	1.00
15 mM phosphocreatine	0.89	1.6	1.00
0 01 mg/ml U-74500A	1.01	1.3	1.00

Mitochondria in SETH-medium were mixed with equal volume of SETH-medium with the compounds at pH 7.4 and stored in the dark at 6°C in a concentration of 0.36 mg protein/ml. Oxygen uptake rate directly after isolation was 150 nmol $O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$, and the stimulation of respiration by ADP (RCI) was 10.8. The assays were performed 4 times during 5 subsequent days. r = correlation coefficient of ln J vs time.

palmitoylcarnitine to 77%, ($p < 0.05$) succinate to 95% (NS) and ascorbate to 93% (NS). For carnitine these percentages were 89, 94, 88, 110 and 80% (all differences NS). Carnitine exerted a protective effect on the oxidation of pyruvate and palmitoylcarnitine in 5 of the 6 experiments, but did not reach the preset level of significance. Hypotaurine and desferrioxamine did not protect significantly the decay of any of the substrates tested. Desferrioxamine appeared to promote the decay, especially with the substrates pyruvate (from 100–181%) and palmitoylcarnitine (to 170 %).

Discussion

Isolated muscle mitochondria were found to be labile. And our standard conditions for performing oxidative phosphorylation were found to be damaging. Especially mixing the cup with the isolated mitochondria on a Vortex machine and exposure to light, even with the mitochondria on ice, damaged oxidative phosphorylation. Oxidative phosphorylation with the NAD^+-linked substrates was more vulnerable than with ascorbate. Fortunately we always tested oxidative phosphorylation in the order of pyruvate, glutamate, palmitoylcarnitine, succinate, ascorbate. So the most vulnerable substrate oxidations were measured first. Nowadays we protect the isolated mitochondria from light by storage on ice in amber plastic cups (Elkay Élreann, Galway, Fax: 353-91-72309), and shake only for 3 sec before the assay.

Protection of oxidative phosphorylation could be realized by a great variety of compounds with free radical scavenging properties, iron-chelators which inhibits the hydroxyl-radical production by the Haber-Weiss reaction, membrane stabilizers and an inhibitor of the calcium entry in mitochondria. Because of the large variation in the lability and of the improvement in stability by potential stabilizers between individual preparations, large series are needed to confirm the stabilizing action in an average preparation. In such experiments with rat heart mitochondria, taurine was found to be the best stabilizer of the oxidative phosphorylation with the NAD^+-linked substrates, while carnitine, hypotaurine and desferrioxamine did not stabilize significantly. Since taurine has a high concentration in the tissues, one of its functions could be protection of oxidative phosphorylation. In view of experiments by Di Lisa *et al. [11]*, it was a surprise that carnitine did not stabilize at a significant level. In one experiment with pyruvate and in another with ascorbate, it was found to be most effective. In the other experiments it was not harmful for the oxidative phosphorylation. It was of interest that desferrioxamine had a deleterious effect on oxidative phosphorylation, because it was found to have a beneficial effect on the heart during ischemia and reperfusion, by decreasing the iron dependent hydroxyl production, which is harmful to oxidative phosphorylation [12, 13]. It is used in the treatment of iron overload diseases such as porphyria cutanea tarda, thalassemia and other hematological diseases which require regular blood transfusion. Desferoxamine has the disadvantage of many side effects, especially affecting the lens and the retina of the eye [14] and the kidney tubules [15].

Before patients can be treated with drugs that protect oxidative phosphorylation, we need to know the best combination of them in vitro, and then the effect on the realization and the turnover of the components involved in oxidative phosphorylation. Maybe the effect on muscle can be tested in (human) muscle culture, also of patients. In the latter case, the finding of repair of the defect would be of great help. After that, when the drugs are known to be safe (several of them are already tested in other patients), they can be tested on patients with defects in oxidative phosphorylation. Of course together with other treatments that proved valuable in individual patients [20–24].

With appreciation we refer to work by Kraft *et al*. [25] who studied the stability of mitochondria in human muscle fibres. Their muscle fibre model is an interesting one, but requires a high resolution oxygraph.

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Table 5. Decay of oxidative phosphorylation measured with different substrates in rat heart mitochondria

	Pyruvate + malate	Glutamate + malate	Palmitoylcarnitine + malate	Succinate + rotenone	Ascorbate + TMPD	n
SETH-medium	0.73 ± 0.23	0.52 ± 0.06	0.77 ± 0.14	0.21 ± 0.13	0.15 ± 0.09	6
+ Taurine	**0.48 ± 0.24**	**0.43 ± 0.12**	**0.60 ± 0.20**	0.20 ± 0.12	0.14 ± 0.09	6
+ Carnitine	0.65 ± 0.34	0.49 ± 0.11	0.68 ± 0.22	0.23 ± 0.10	0.12 ± 0.08	6
+ Hypotaurine	0.65 ± 0.30	0.45 ± 0.20	0.93 ± 0.46	0.22 ± 0.16	0.20 ± 0.14	3
+ Desferrioxamine	1.32 ± 0.45	0.63 ± 0.18	1.31 ± 0.68	0.30 ± 0.14	0.20 ± 0.07	3

The decays are given in the average k ± SD and n is the number of independent preparations. When the average decay has been given in bold it is significantly lower than in the control (SETH-medium). The drugs were added to the mitochondria in SETH-medium in a similar volume of SETH with 2 mM taurine, 10 mM L-carnitine, 2 mM hypotaurine and 0.2 mM desferrioxamine and stored at 0°C in the dark. Final pH 7.4. The protein concentrations during the incubations were 1.2–2.3 mg per ml. Directly after isolation the average rate of pyruvate + malate oxidation was 164 ± 20 (SD) nmol $O_2 \cdot min^{-1}$ (mg protein)$^{-1}$ and the stimulation of the oxidation by ADP was 8.0 ± 1.3. The oxygen uptake rates with the other substrates were for palmitoylcarnitine + malate 133 ± 29, glutamate + malate 143 ± 57, succinate + rotenone 161 ± 44 and ascorbate + TMPD + deferoxamine 610 ± 202. These measurements were repeated after 1 day of storage for the NAD^+-linked substrates and after 2 days for the other substrates. The assays were done in duplicate.

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