Tetracyano-2,2-bipyridineiron(III), an improved electron acceptor for the spectrophotometric assay of β -oxidation and of succinate dehydrogenase in intact mitochondria

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A recently described direct reading assay for β -oxidation and for succinate oxidation in intact mitochondria using $[\text{Fe}(\text{CN})_6]^{3-}$ as final electron acceptor [Osmundsen & Bremer (1977) *Biochem. J.* **164**, 621–633] has been improved by using instead tetracyano-2,2-bipyridineiron(III) $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, which gives a 2.6 times greater absorbance change on reduction. Some physical and kinetic properties of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ are described. The use of exogenous cytochrome *c*(III) as electron acceptor was also tested; this gives the largest absorbance change, although the absolute rate of reaction is only approx. one-third of that using $[\text{Fe}(\text{CN})_6]^{3-}$ or $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$.

Osmundsen & Bremer (1977) introduced an elegant direct reading spectrophotometric assay for β -oxidation in intact mitochondria by following the reduction of hexacyanoferrate(III), $[\text{Fe}(\text{CN})_6]^{3-}$. The latter complex is able to accept electrons derived from flavoprotein-linked substrate oxidations in the mitochondrial matrix by reacting directly with the respiratory chain at the level of cytochrome *c* located on the outer face of the mitochondrial inner membrane (Klingenberg, 1970). In such experiments free cyanide is required to block transfer of electrons to O_2 via cytochrome oxidase (EC 1.9.3.1) and rotenone to block NADH dehydrogenase (EC 1.6.99.3). Electrons are accepted from flavoprotein acyl-CoA dehydrogenase with acyl-CoA esters as primary substrates, which are in turn generated in the matrix from exogenous acyl(–)-carnitine esters added as substrates by carnitine palmitoyltransferases (EC 2.3.1.21) in the mitochondrial inner membrane. Addition of 10 mM-oxaloacetate is also required as a sink for NADH and acetyl-CoA formed during β -oxidation. Electrons may also be accepted from succinate

dehydrogenase with succinate as substrate (see Osmundsen, 1981). It is also known that $[\text{Fe}(\text{CN})_6]^{3-}$ reacts directly with the flavoprotein L-glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) located on the outer surface of the mitochondrial inner membrane. This can be assayed spectrophotometrically in the presence of antimycin to block the respiratory chain at the level of cytochrome *b* ((Klingenberg, 1970).

The sensitivity of the whole assay is determined by the magnitude of the spectral change which $[\text{Fe}(\text{CN})_6]^{3-}$ undergoes on reduction to $[\text{Fe}(\text{CN})_6]^{4-}$. The sensitivity can be increased by using electron acceptors which undergo larger spectral changes. It is also required that such acceptors should have suitable oxidation–reduction midpoint potentials, should not cross the mitochondrial inner membrane nor react directly with substrates or components of the medium used to suspend the mitochondria. In this paper we compare the use of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ and $[\text{Fe}(\text{CN})_6]^{3-}$ as final electron acceptor for the spectrophotometric assay of β -oxidation and of succinate dehydrogenase activity in intact mitochondria. The oxidant $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ meets all the requirements for a suitable electron acceptor and its use increases the sensitivity by a factor of 2.8. Exogenous cytochrome *c*(III) has also been used as an electron acceptor (Stumpf & Parks, 1981), but, as is shown in this report, its absolute rate of reaction is only about one-third of that of the inorganic acceptors.

Abbreviations used: $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, tetracyano-2,2-bipyridineiron(III); $[\text{Fe}(\text{CN})_4(\text{bpy})]^{2-}$, tetracyano-2,2-bipyridineiron(II); Hepes, 4-(2-hydroxyethyl)piperazine-ethanesulphonic acid.

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Materials and methods

Materials

Hydrogen tetracyano-2,2-bipyridineiron(III), $\text{H}[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 2\text{H}_2\text{O}$ was prepared according to Schilt (1960). All procedures were performed in a fume cupboard. 2,2'-Bipyridine (5.2 g) and $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ (3.9 g) were dissolved in 400 ml of distilled water and heated to just below boiling point, KCN (10 g) in 10 ml of water was added and the solution allowed to cool to 20°C and was kept for a few hours. Dark violet crystals of dicyanobis-(2,2'-bipyridine)iron(II) trihydrate formed and were collected by filtration; 0.9 g was added to a solution of 40 g of KCN in 400 ml of water. The mixture was covered and heated in a steam bath for 24 h. The solution was cooled and extracted with a small volume of CHCl_3 to remove any remaining violet colour and a resulting orange solution was concentrated to 100 ml by rotary evaporation. Orange-brown crystals of $\text{K}_2[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 3\text{H}_2\text{O}$ formed and were recrystallized from a small volume of water. $\text{H}[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 2\text{H}_2\text{O}$ was prepared by passing Cl_2 gas into a solution of 0.82 g of $\text{K}_2[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 3\text{H}_2\text{O}$ in 100 ml of water for a few minutes until the solution changed to a dark colour. Concentrated HCl (5 ml) was then added and the solution left at 0°C overnight. $\text{H}[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 2\text{H}_2\text{O}$ crystallized as red needles which were collected by filtration, washed with ice-cold 0.1 M-HCl saturated with Cl_2 and dried *in vacuo* to give approx. 0.8 g. A summary of its properties is given in Table 1, and the u.v.-visible absorption spectrum (Fig. 1) agrees with that reported by Schilt (1960). Media containing $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ when freshly prepared are stable during a working day. $\text{H}[\text{Fe}(\text{CN})_4(\text{bpy})] \cdot 2\text{H}_2\text{O}$ is stable at room temperature when stored in a desiccator.

Palmitoyl-(−)carnitine was synthesized as described by Holland & Sherratt (1973) and palmitoyl-CoA, cytochrome *c* ($\epsilon_{550} = 29 \text{ mm}^{-1} \cdot \text{cm}^{-1}$) and oxaloacetate were purchased from Sigma. The sources of other biochemicals used are given by Holland & Sherratt (1973). Analar $\text{K}_3\text{Fe}(\text{CN})_6$ was obtained from BDH and 2,2'-bipyridine was purchased from Aldrich.

Preparation of mitochondrial fractions

Mitochondrial fractions were prepared in 300 mM-mannitol / 10 mM-Hepes / 0.1 mM-EGTA (pH 7.2) from the livers of male Wistar rats from a local inbred strain (Gatley & Sherratt, 1976), or in 100 mM-KCl / 1 mM-ATP / 5 mM-MgCl₂ / 1 mM-EDTA / 50 mM-Tris/HCl (pH 7.5) from 0.5 g of human quadriceps skeletal muscle (obtained during a routine diagnostic biopsy by D.M.T.) by the method of Kreiger *et al.* (1980). Protein was measured with the Bio-Rad protein assay kit and Bio-Rad standard protein (freeze-dried γ -globulin).

Spectrophotometric assay of mitochondrial oxidations

Oxidations by mitochondrial fractions (usually 0.05–1.0 mg of protein per ml) were recorded with $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ as electron acceptor by the increase in absorption at 478 nm with 600 nm as the reference wavelength (Fig. 2), with cytochrome *c*(III) by the increase in absorption at 550 nm with 580 nm as the reference wavelength, or with $[\text{Fe}(\text{CN})_6]^{3-}$ by the decrease in absorption at 420 nm with 475 nm as reference wavelength, using an Hitachi 557 spectrophotometer in the dual wavelength mode. A bandpass of 2.0 nm was used. The iso-osmotic reaction medium contained 130 mM-KCl or 300 mM-mannitol, with 10 mM-Hepes, 2.5 mM-phosphate, 0.5 mM-ADP, 0.1 mM-EDTA, 1.5 mg of defatted bovine serum albumin/ml, 1 mM-

Table 1. Absorbance changes on reduction of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, $[\text{Fe}(\text{CN})_6]^{3-}$ and of cytochrome *c*(III), differences in the values of their midpoint oxidation–reduction potentials from cytochrome *c*(III) and second order rate constants for reaction with cytochrome *c*(III)

A value for the midpoint oxidation–reduction potential of cytochrome *c* (E_h) at pH 7.2 of 273 mV is used (N. Osheroff & E. Margoliash, personal communication); other values are from: (a) Stasiw & Wilkins (1969); (b) Butler *et al.* (1981a). Second order rate constants for reaction with cytochrome *c*(III)*, or between cytochrome *c*(II) and cytochrome *c*(III)**, in solution; (c)*, Cassat & Marini (1973); (d)**, Gupta (1973); Gupta *et al.* (1972) (determined by using n.m.r. line broadening).

Electron acceptor	Absorbance change on reduction ($\epsilon_{\text{red.}} - \epsilon_{\text{ox.}}$) (mmol ⁻¹ ·cm ⁻¹) at optimal wavelength	Difference in midpoint oxidation-reduction potential, $E_h - E_h$ (cytochrome <i>c</i>) (mV)	Second order rate constant, k (M ⁻¹ ·s ⁻¹)
$[\text{Fe}(\text{CN})_4(\text{bpy})]^-$	+2.6 (478 nm)	+227 (a)	1.6×10^8 (c)*
$[\text{Fe}(\text{CN})_6]^{3-}$	−1.05 (420 nm)	+147 (b)	0.92×10^7 (b)*
Cytochrome <i>c</i> (III)	+29 (550 nm)	0	1×10^3 (d)**

Fig. 1. Absorption spectra of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ and $[\text{Fe}(\text{CN})_4(\text{bpy})]^{2-}$

Absorption spectrum of: (a) $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, 0.204 mM-H $[\text{Fe}(\text{CN})_4(\text{bpy})]$, 2H $_2\text{O}$ in 10 mM-Tris/HCl, pH 7.6, containing 90 mM-NaCl; (b) $[\text{Fe}(\text{CN})_4(\text{bpy})]^{2-}$ obtained on reduction of (a) by the addition of a few grains of $\text{Na}_2\text{S}_2\text{O}_4$.

KCN (to inhibit cytochrome oxidase), 1 μg of rotenone/ml (to inhibit NADH dehydrogenase), and, as appropriate, 0.1 mM-H $[\text{Fe}(\text{CN})_4(\text{bpy})]$, 2H $_2\text{O}$, 0.5 mM- $\text{K}_3\text{Fe}(\text{CN})_6$ or 0.2 mM-cytochrome *c*(III), pH 7.2, in a final volume of 1.0 or 2.0 ml at 30°C. The hypo-osmotic medium contained 50 mM-phosphate/5 mM-MgCl $_2$ /1.5 mg of defatted bovine serum albumin/ml, 0.5 mM-ADP, 1 mM-KCN, 1 μg of rotenone/ml and 0.2 mM-cytochrome *c*(III), pH 7.2, in a final volume of 1.0 or 2.0 ml.

After recording the slow endogenous rate, oxidations were started by adding 10 mM-succinate or 20 mM-DL-glycerol 3-phosphate. β -Oxidation was measured by the successive addition of (a) 10 mM-oxaloacetate and 20 μM -palmitoyl-carnitine, or (b) 10 mM-oxaloacetate, 1.0 mM-L-carnitine and 20 μM -palmitoyl-CoA. When palmitoyl-CoA was used as substrate the observed rate was corrected by a factor of 7/8 to allow for the direct reduction by $[\text{Fe}(\text{CN})_6]^{3-}$ (Osmundsen, 1981) or by $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ of CoASH released in the bulk phase. The rates were calculated by assuming that β -oxidation suppressed endogenous reactions (see Osmundsen & Sherratt, 1975).

Kinetics of mitochondrial oxidations measured using various electron acceptors

The apparent K_m values for the electron acceptors when 10 mM-succinate was being oxidized by the mitochondria were computed by the method of Eisenthal & Cornish-Bowden (1974) and the experimental and calculated results are displayed in Fig. 3.

Fig. 2. Rates of succinate-, palmitoyl-carnitine- and succinate-dependent reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ and of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ by mitochondrial fractions

Mitochondrial fractions from liver (1.0 mg of protein/ml) were suspended in KCl medium (a-f); or from skeletal muscle (0.5 mg of protein/ml) in mannitol-medium (g-i); with the addition of 0.1 mM-H $[\text{Fe}(\text{CN})_4(\text{bpy})]$, 2H $_2\text{O}$ (a-c, g-i) or of 0.5 mM- $\text{K}_3\text{Fe}(\text{CN})_6$ (d-f). Other additions were made where indicated; succinate (Succ), antimycin (10 μg) (Ant), oxaloacetate (OxAc), palmitoyl-carnitine (PCn), L-carnitine (LCn), palmitoyl-CoA (PCoA). The numbers in parentheses represent the rates of reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ or of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ expressed as nmol reduced/min per mg of protein. Other details are given in the text.

The effect of ionic strength on the rate of reduction of exogenous cytochrome *c*(III) was investigated by using mitochondria in which the outer membrane had been previously damaged by incubating in hypo-osmotic medium at 0 or 30°C before resuspending in iso-osmotic media (Table 2).

Results and discussion

Use of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ as electron acceptor

Reduction of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ to $[\text{Fe}(\text{CN})_4(\text{bpy})]^{2-}$ results in the appearance of an

Table 2. Comparison of the rates of succinate oxidation by rat liver mitochondrial fractions using $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, $[\text{Fe}(\text{CN})_6]^{3-}$ and cytochrome *c*(III) as electron acceptors

Rates are means $\pm$ S.E.M. for six determinations at 30°C and are given as nmol/min per mg of protein; experimental details are given in the text.

Protein concentration (mg/ml)	Rate with:					
	Electron acceptor ... Medium ...	0.1 mM-	0.5 mM-	0.2 mM-	0.2 mM-	0.2 mM-
		$[\text{Fe}(\text{CN})_4(\text{bpy})]^-$	$[\text{Fe}(\text{CN})_6]^{3-}$	cytochrome <i>c</i> (III)	cytochrome <i>c</i> (III)	cytochrome <i>c</i> (III)
		Iso-osmotic KCl			Iso-osmotic mannitol	Hypo-osmotic phosphate
0.02		383 $\pm$ 24	187 $\pm$ 27	12.8 $\pm$ 1.0	14.1 $\pm$ 1.9	99 $\pm$ 6
0.05		382 $\pm$ 21	239 $\pm$ 16	13.6 $\pm$ 1.0	13.1 $\pm$ 1.0	102 $\pm$ 4
0.07		405 $\pm$ 29	325 $\pm$ 28	13.0 $\pm$ 1.0	12.6 $\pm$ 0.6	104 $\pm$ 4
0.55		427 $\pm$ 26	290 $\pm$ 19	11.2 $\pm$ 0.2	11.0 $\pm$ 0.7	111 $\pm$ 5
0.70		365 $\pm$ 16	341 $\pm$ 11	11.0 $\pm$ 0.7	10.7 $\pm$ 0.3	121 $\pm$ 19

Fig. 3. Influence of the concentration of electron acceptor on the rate of mitochondrial oxidation of succinate. The initial rate of reduction of the electron acceptor by the same rat liver mitochondrial fraction (1.0 mg of protein/ml) with 10 mM-succinate as substrate was measured as described in the Materials and methods section: (a) $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, (b) $[\text{Fe}(\text{CN})_6]^{3-}$, (c) cytochrome *c*(III). The experimental points (O) were used to determine the best fit for the relation between velocity and acceptor concentration (●), assuming simple hyperbolic kinetics, and the apparent K_m values by the computer program of Eisenthal & Cornish-Bowden (1974). The apparent K_m values are: (a) $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, 0.021 mM; (b) $[\text{Fe}(\text{CN})_6]^{3-}$, 0.088 mM; (c) cytochrome *c*(III), 0.93 mM.

absorption band at 478 nm (Fig. 1). The change in absorbance (against a water blank) is $2.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 478 nm, or $2.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ using the wavelength pair 478–600 nm, by contrast with a decrease in absorbance of $1.05 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 420 nm on reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ to $[\text{Fe}(\text{CN})_6]^{4-}$ (Table 1) (Osmundsen, 1981). Measured rates of oxidation of several substrates were approx. 15% faster with $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ as final electron acceptor when compared with $[\text{Fe}(\text{CN})_6]^{3-}$, and the absolute rate of change in absorbance was about 2.8 times greater (Fig. 2), both because of a larger change in absorbance of the former (2.5 times) and a small increase in the absolute reaction rate (about

15%). [However, the absorbance change on reduction of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ to $[\text{Fe}(\text{CN})_4(\text{bpy})]^{2-}$ is still much less than those of porphyrin complexes of Fe.] It is possible that some substituted bipyridine compounds may form useful co-ordination compounds of Fe with further increase in sensitivity.

The rate of succinate oxidation with $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ as final electron acceptor was inhibited by 95% by antimycin (Fig. 2), while that of DL-glycerol 3-phosphate was unaffected, indicating that $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ does not cross the mitochondrial inner membrane to a significant extent (see Osmundsen & Bremer, 1977).

Using 0.1 mM- $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ as electron accep-

Table 3. *Effect of pre-incubation of mitochondria in hypo-osmotic medium on the rate of succinate-dependent reduction of exogenous cytochrome c(III) in different media*

Liver mitochondrial fractions were prepared as described in the Materials and methods section up to the stage of the final centrifugation. They were resuspended in 20 vol. of hypo-osmotic medium at either 0 or 37°C for 10 min, centrifuged at 10000 g_{av} for 5 min at 2°C, and finally resuspended in isolation medium with a concentration of 50 mg of mitochondrial protein/ml. The rate of succinate-dependent reduction of exogenous cytochrome c(III) at 30°C, in mannitol, KCl or hypo-osmotic media was measured (1.0 mg of mitochondrial protein/ml); other details are given in the text. Rates are expressed in nmol of cytochrome c(III) reduced/min per mg of protein. Values are means $\pm$ S.E.M. for four experiments.

Treatment of mitochondria	Reaction media		
	KCl	Mannitol	Hypo-osmotic
Untreated	11.7 $\pm$ 1.4	14.3 $\pm$ 2.5	101 $\pm$ 3.6
Pre-incubated in hypo-osmotic medium at 37°C	48.5 $\pm$ 7.9	49.3 $\pm$ 8.0	—
Pre-incubated in hypo-osmotic medium at 0°C	11.9 $\pm$ 1.4	12.9 $\pm$ 1.3	—

tor the initial rate of oxidation of 10 mM-succinate was independent of the concentration of mitochondrial protein over the range 0.02–5.0 mg/ml (Table 2). The initial rate of reduction of 0.5 mM- $[\text{Fe}(\text{CN})_6]^-$ was not linearly related to protein concentration below 0.07 mg/ml; this apparently slower rate reflects practical difficulties in recording accurately the very small initial rates of absorbance changes (Table 2). The rate of reduction of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ with 0.1 mg of mitochondrial protein/ml was independent of the concentration of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ between 0.06 and 5.0 mM (Fig. 3) and of the pH between 6.5 and 8.0. The rates of transfer of electrons to these acceptors are sufficiently unresponsive to acceptor concentrations so as to give reliable assays for mitochondrial oxidations, provided that the concentration of the oxidized form does not fall to less than about three times its respective K_m (Fig. 3). These K_m values are 0.021 mM for $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, 0.088 mM for $[\text{Fe}(\text{CN})_6]^{3-}$ and 0.093 mM for cytochrome c(III). In practice, therefore, an appropriate absorbance range was selected for spectrophotometric recordings.

The complex $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ thus meets all the requirements for an electron acceptor for the assay of β -oxidation, succinate and glycerol 3-phosphate oxidations in intact mitochondria by the method of Osmundsen & Bremer (1977) with an increase in sensitivity of 2.8 times.

Comparison of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$, $[\text{Fe}(\text{CN})_6]^{3-}$ and cytochrome c(III) as electron acceptors for mitochondrial oxidations

The rate of succinate oxidations using exogenous cytochrome c(III) as electron acceptor is about ten times faster in hypo-osmotic medium than in either

iso-osmotic ionic or non-ionic media, since access of cytochrome c(III) to the inner membrane is facilitated by damage to the outer membrane by incubation of mitochondria in hypo-osmotic media. Even with a damaged outer membrane, the maximal attainable rate of electron flux from succinate through endogenous cytochrome *c* to exogenous cytochrome c(III) (in hypo-osmotic medium) is only one-third of the maximal rate to $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ or to $[\text{Fe}(\text{CN})_6]^{3-}$ (in ionic iso-osmotic medium) (Table 2). The lower maximum rate with exogenous cytochrome c(III) as electron acceptor may be partly due to steric constraints for electron transfer between the relatively large exogenous and endogenous cytochrome *c* molecules which have a net positive charge at pH 7.2 (Table 1). Similar constraints might not be expected for reaction with the small negatively charged inorganic complexes $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ or $[\text{Fe}(\text{CN})_6]^{3-}$ (Table 1; Butler *et al.*, 1981b), although the behaviour of endogenous cytochrome *c* may be modified by the proximity of negatively charged phospholipids in the inner membrane. However, charge effects may not be of major importance, since the rate of electron transfer to exogenous cytochrome c(III) by mitochondria with damaged outer membranes is not critically dependent on the ionic strength of the medium (Table 3). Further, the faster rates with $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ or $[\text{Fe}(\text{CN})_6]^{3-}$ may also reflect the greater thermodynamic driving force indicated by the more positive midpoint potentials of these ions compared with that of cytochrome *c* (Table 1). However, even when using cytochrome c(III), the absolute rate of change in absorbance measured as 550–580 nm may be about five times larger than the change at 478–600 nm using $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ or twelve times larger than at 420–475 nm using $[\text{Fe}(\text{CN})_6]^{3-}$.

Application to the measurement of mitochondrial oxidations in biopsy samples of human tissues

The electron acceptor used is important when measurements of succinate oxidation are used to determine the rate of flux of electrons through Complex III of the respiratory chain, for example in investigations of partial deficiencies of cytochrome *b* in human muscle myopathies (DiMauro *et al.*, 1980; Stansbie *et al.*, 1982). An impairment of flux of less than 50% of the normal rate may not be characterized accurately if cytochrome *c*(III) is used. The use of $[\text{Fe}(\text{CN})_4(\text{bpy})]^-$ allows measurements to be made of succinate oxidation with as little as 20 μg of mitochondrial protein and of β -oxidation with 50 μg . This advance is particularly important in diagnostic studies using mitochondrial fractions prepared from the very small samples of human tissue obtained by biopsy.

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