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NITRITE METABOLISM BY SKELETAL MUSCLE MITOCHONDRIA IN RELATION TO HAEM PIGMENTS

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SUMMARY

1. Nitrite is reduced anaerobically by skeletal muscle mitochondria, a product of the action being nitrosylferricytochrome *c* (ref. 1). The anaerobic incubation of nitrite with skeletal muscle minces at pH 6.0 leads to the formation of nitric oxide[2], part of which is located ultimately in combination with endogenous myoglobin.

2. Pig oxymyoglobin is readily oxidised to the met- form by low concentrations of nitrite, whereas nitrosylmyoglobin is relatively stable.

3. Under argon, metmyoglobin is insensitive to reduction by pig muscle mitochondrial enzyme systems and NADH, whilst nitrosylmetmyoglobin is readily reduced to nitrosylmyoglobin.

4. The incubation of nitrosylferricytochrome *c* and metmyoglobin with muscle mitochondria and NADH under N_2 has resulted in the formation of ferrocytochrome *c*, which does not form a nitrosyl- complex, and a stoichiometric equivalent of nitrosylmyoglobin, with nitrosylmetmyoglobin as an intermediate.

5. Thus, nitrosylferricytochrome *c*, formed by anaerobic cytochrome oxidase (EC 1.9.3.1) action in the presence of nitrite[1], is reduced by the reduced NAD dehydrogenase (EC 1.6.99.3) system of muscle, the nitrosyl- group being transferred to metmyoglobin arising from the chemical oxidation of endogenous myoglobin by nitrite. Mitochondrial reduction of nitrosylmetmyoglobin to the stable complex nitrosylmyoglobin follows even in the presence of sufficient nitrite to oxidise oxymyoglobin.

INTRODUCTION

The administration of nitrite *in vivo* causes methaemoglobinaemia or even produces a modified haem–protein complex[3]; *in vitro*, it oxidises oxymyoglobin (MbO_2) readily to the met- form (MetMb). WALTERS AND TAYLOR[2] have found that nitrite is metabolized anaerobically by pig skeletal muscle minces; a product of the metabolism was nitric oxide, part of which was located ultimately in combination with endogenous haem pigment as nitrosylmyoglobin (NOMb).

Abbreviations: Mb, deoxygenated myoglobin; MbO_2, oxymyoglobin; MetMb, metmyoglobin; NOMb, nitrosylmyoglobin; NOMetMb, nitrosylmetmyoglobin; MbCO, carbon monoxide myoglobin.

Using pig skeletal muscle mitochondria, mammalian cytochrome oxidase (EC 1.9.3.1) has been implicated in the anaerobic oxidation of ferrocytochrome *c* by nitrite, a product of the action being nitrosylferricytochrome *c* (ref. 1). A nicotinamide nucleotide dependent reaction has been observed by which pig skeletal muscle mitochondria are able to effect the anaerobic transfer of the nitrosyl- group from nitrosylferricytochrome *c* to MetMb. Unlike uncomplexed MetMb, the resultant nitrosylmetmyoglobin (NOMetMb) is reduced by mitochondria to its ferrous form, NOMb, even in the presence of nitrite at concentrations able to cause rapid oxidation of MbO_2. Thus, it is possible to propose an enzymic pathway of formation of NOMb, the end product of part of the nitrite metabolized anaerobically by skeletal muscle.

METHODS

Skeletal muscle mitochondria

These were prepared from the minced crura of the pig diaphragm within 1–2 h of slaughter by the method outlined by WALTERS AND TAYLOR[1]. The mitochondria were washed twice with Tris–KCl buffer[4] and the lipid adhering to the sides of the centrifuge tubes was removed mechanically to ensure elimination of endogenous substrate. The mitochondria were then suspended in the same medium to a concn. of 0.67 ml per g wet muscle (av. protein content 3–4 mg/ml).

Spectrophotometric determination of haem pigments and NADH

These analyses were made on the basis of the following absorbance coefficients (mM^{-1} cm^{-1}). For reduced *minus* oxidised cytochrome *c*, 21 at 550 mμ (ref. 5); for pig MetMb at pH 6.0–7.4, 3.5 at 630 mμ and 150 at 407 mμ; for pig MbO_2, 15 at 580 mμ; for NADH, 6.2 at 340 mμ.

Anaerobic reduction of NOMetMb to NOMb

NOMetMb was formed anaerobically from NO and 75 μM pig MetMb (ref. 6; $A_{\text{Soret}}/A_{280\text{m}\mu}$ ratio = 4.9) in a Tris–KCl–glucose buffer containing KCl (70 mM), Tris–HCl (pH 7.4, 35 mM), glucose (20 mM), potassium phosphate (pH 7.0, 4.0 mM), $MgSO_4$ (3.5 mM), EDTA (0.7 mM), ADP (0.5 mg sodium salt/ml) and ATP (0.5 mg disodium salt/ml) in 1-cm Thunberg tube type spectrophotometer cells. Under a reduced pressure of argon, additions were made from the side-arm of 0.05 ml mitochondria, NADH sodium salt to 0.2 mM and of $NaNO_2$ to 2.8 mM where appropriate. Spectral changes in the visible region were monitored by the repetitive scanning facility of the Optica CF4R automatic recording spectrophotometer.

Anaerobic reduction of nitrosylferricytochrome c, with transference of the nitrosyl- group to myoglobin

Nitrosylferricytochrome *c* was formed anaerobically from NO and 15 μM ferricytochrome *c* in the Tris–KCl–glucose buffer previously described in 1-cm Thunberg tube type spectrophotometer cells. Excess NO was scrupulously removed by repeated evacuation and entry of O_2-free N_2. The side-arms were replaced by others containing 0.05 ml mitochondria and MetMb to 15–40 μM; NAD^+, NADH or NADPH was present in the main compartment, generally to 40 μM. Atmospheres of N_2 at reduced pressure were restored by repeated evacuation and entry of N_2. After equilibration

at 37° and tipping, haem-pigment changes were determined by scanning spectrophotometrically at frequent intervals.

Fluorimetric measurement of oxidation of NADH in presence of haem pigments

Changes in fluorescence were determined with an Aminco-Bowman spectrofluorimeter using an exciting wavelength of 340 mμ and measuring fluorescence at 460 mμ. The Thunberg tube type fluorimeter cell (1 cm × 1 cm) contained 15 μM nitrosylferricytochrome *c* and 40 μM NADH. The reaction at room temperature under N_2 was started by adding 0.05 ml mitochondria and MetMb to 17.5 μM from the side-arm. The fluorescence of NADH was considerably reduced in the presence of cytochrome *c* and myoglobin; fluorescence was linearly related to NADH concn. over the range 0–40 μM. The combined spectrum of 15 μM nitrosylferricytochrome *c* and 17.5 μM MetMb was almost isosbestic at 340 mμ with that of 15 μM ferrocytochrome *c* and 17.5 μM NOMb; as a consequence, the attenuation of the light incident upon NADH present should have remained virtually unchanged throughout the reaction.

The sulphanilic acid–1-naphthylamine method of SNELL AND SNELL[7] was used with modifications for the determination of nitrite.

Sources of biochemicals

β-NADH disodium salt, NADPH tetrasodium salt, ATP disodium salt and ADP sodium salt from Sigma London Chemical Co ; NAD^+ and cytochrome *c* from Koch Light Laboratories, Colnbrook, Bucks.; amytal from May & Baker Ltd., Dagenham.

RESULTS

Stability of myoglobin derivatives towards nitrite

Both MbO_2, prepared from purified pig MetMb (ref. 6) with $NaBH_4$, and the oxy- pigment contained in fresh aqueous extracts of pig skeletal muscle were very susceptible to oxidation by nitrite. In 0.20 M phosphate buffer (pH 6.0) and at 25°, for example, 50 μM MbO_2 was oxidised rapidly by 0.25 mM $NaNO_2$, the only haem product detected spectrophotometrically being MetMb. The spectrum obtained with 2.5 mM $NaNO_2$ was that ascribed to the nitrite salt of MetMb[8]. NO from concurrent reduction of $NaNO_2$ was not detected in combination with the haem pigment, and the amount of nitrite required to oxidise one mole of myoglobin was consistently less than the one mole needed stoichiometrically for the formation of NO. NOMb, on the other hand, was stable towards $NaNO_2$ concentrations causing rapid oxidation of MbO_2.

Susceptibility of myoglobin derivatives to reduction by muscle mitochondria

The anaerobic reduction of pig MetMb to the ferrous condition by muscle mitochondria with pyruvate + malate did not proceed under circumstances promoting rapid reduction of ferricytochrome *c*; with NADH, a very slow rate of reduction was eventually apparent (*cf.* ref. 9).

However, NOMetMb was readily reduced anaerobically to NOMb by mitochondria and NADH, even in the presence of 2.8 mM $NaNO_2$. The reduction was observed (Fig. 1) by the gradual shift of the α- and β-peaks to higher wavelengths[10,11], by the loss of sharpness of the peaks and by the development of stability of the

pigment to air[6]; in the absence of excess NO, NOMetMb decomposes in air to MetMb[8].

The products of anaerobic incubation of NOMetMb with mitochondria, NADH and $NaNO_2$ were diluted 10-fold in air. The Soret spectrum of the resultant solution (Fig. 2) was consistent with that of NOMb containing a small amount of MetMb arising from the aerobic decomposition of residual NOMetMb. If such diluted incubation mixtures were reduced with $Na_2S_2O_4$ the Soret peak shifted to 419 mμ, the wavelength of maximum absorbance for NOMb. The magnitude of the shift of the Soret peak indicated a mixture of approx. 90% NOMb and 10% NOMetMb before admission of air.

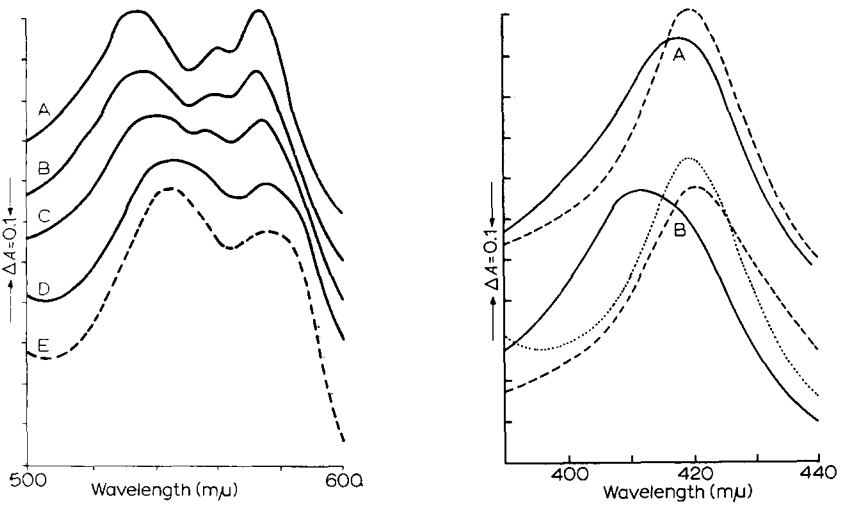


Fig. 1. Absorption spectra of anaerobic incubations at 37° under argon of mitochondria, 68 μM pig NOMetMb, 0.2 mM NADH and 2.8 mM $NaNO_2$ after periods of 0 (A), 10 (B), 25 (C) and 60 (D) min respectively and also the spectrum of 68 μM NOMb (E).

Fig. 2. Absorption spectra of the products, after 10-fold dilution in air, of anaerobic incubation at 37° for 1 h of 68 μM pig NOMetMb (A) with mitochondria, 0.2 mM NADH and 2.8 mM $NaNO_2$ and (B) in buffer only (———); also spectra of the diluted products reduced with $Na_2S_2O_4$ (– – – –) and with subsequent addition of $NaNO_2$ (· · · · · ·).

In the absence of mitochondria and NADH, some autoreduction of NOMetMb occurred during anaerobic incubation at 37° (*cf.* ref. 10); the position of the Soret peak after 1 h (Fig. 2) was consistent with the reduction of about 25% of the pigment present. After reduction with $Na_2S_2O_4$, the products of incubation without $NaNO_2$ always exhibited maximum absorbance displaced slightly above the 419-mμ value associated with NOMb. This displacement is ascribed to a small amount of Mb (peak at 430 mμ), and a small addition of $NaNO_2$ localized the peak precisely at 419 mμ.

Transfer of the nitrosyl- group from ferricytochrome c to myoglobin

The changes in the visible spectrum during the anaerobic incubation of mitochondria, NADH, nitrosylferricytochrome *c* and MetMb are shown in Fig. 3A. An increase of absorbance at the α-peak of ferrocytochrome *c* at 550 mμ was quickly apparent, together with the formation of a substantial shoulder in the 570–590-mμ region close to the original α-peak of nitrosylferricytochrome *c* at 563 mμ. These

developments have been observed in both Tris–KCl–glucose buffer and the ALDRIDGE AND PARKER[12] medium for oxidative phosphorylation modified to pH 6.0. These spectral features were reproduced in model systems containing $NaNO_2$ and reduced chemically with $Na_2S_2O_4$, indicating that the 570–590-mμ shoulder is attributable to

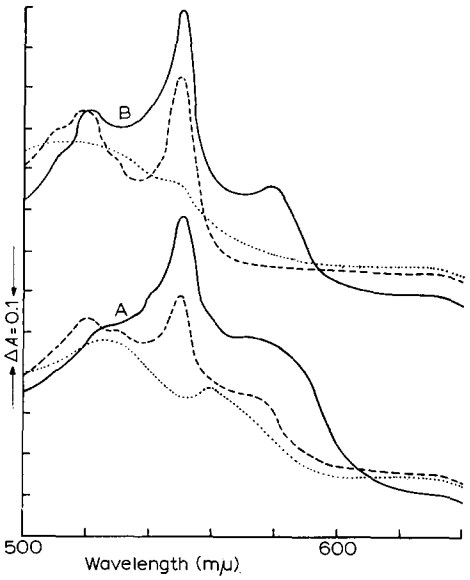


Fig. 3. A. Initial absorption spectrum (· · · · · ·) of an incubation under N_2 of mitochondria, 37 μM pig MetMb, 15 μM nitrosylferricytochrome *c* and 40 μM NADH and spectra after 1 min (– – – – –) and 15 min (———) respectively at 37°. B. Initial absorption spectrum (· · · · · ·) of an incubation under CO of mitochondria, 37 μM pig MetMb, 15 μM ferricytochrome *c* and 40 μM NADH and spectra after 3 min (– – – – –) and 63 min (———) respectively at 37°.

the α-peak of NOMb. On the basis of a millimolar absorbance coefficient of 11 for NOMb at its α-peak, the increase of absorbance in this region represented a molar formation of the nitrosyl- derivative slightly greater than the nitrosylferricytochrome *c* available. The appearance of the β-peak of NOMb was shown by the formation of a shoulder on the α-peak of ferrocytochrome *c* at shorter wavelengths; concurrently a fall of absorbance occurred in the 630-mμ region where MetMb contributes particularly to the absorption of light. Similar incubations with NAD^+ instead of NADH did not result in changes in the visible spectrum, showing that the mitochondrial preparations were virtually substrate free. NOMetMb was not formed directly under these conditions; NO in excess of that required for nitrosylferricytochrome *c* formation must therefore have been removed by the techniques employed. The direct anaerobic reduction of 15 μM nitrosylferricytochrome *c* by 40 μM NADH without the intervention of mitochondria proceeded only very slowly; on increasing the NADH (or NADPH) concentration to the unphysiological level of 0.4 mM, however, the rate approached that mediated by mitochondrial enzymes with 40 μM NADH. The initial rates of formation of ferrocytochrome *c* and of NOMb + NOMetMb were determined by the increases of absorbance at 550 mμ and 575 mμ respectively in repetitive scans at frequent intervals. In the presence of mitochondria and 40 μM NADPH both rates were 25–30 % of those observed with NADH at the same level.

The inclusion of 40 μM NAD^+ with the NADPH increased both rates of formation to approx. 50 % of those for NADH, presumably as a result of transhydrogenase action. The initial rate of formation of ferrocytochrome *c* was reduced to one third by 1.0 mM amytal, but the rate of formation of the nitrosyl- derivatives of myoglobin was inhibited by about 25 % only.

In the absence of NO, the anaerobic incubation of mitochondria, NADH, MetMb and uncomplexed ferricytochrome *c* resulted in rapid reduction to ferrocytochrome *c* without spectral evidence of the concurrent formation of Mb; since the absorbance at 630 mμ remained unchanged there was no loss of MetMb. In similar incubations under CO, rapid reduction of ferricytochrome *c* was followed by the gradual appearance in the visible spectrum of a peak at 580 mμ, corresponding to the α-peak of carbon monoxide myoglobin (MbCO) (Fig. 3B). At the same time, the absorbance was increased at the β-peak of MbCO (542 mμ) and decreased at 630 mμ.

Anaerobic incubations of nitrosylferricytochrome *c*, MetMb, NADH and mitochondria were arrested at intervals by shaking in air. The contribution of ferricytochrome *c*, resulting from aerobic cytochrome oxidase action, to the Soret peaks restricted the shift in wavelength anticipated for the formation of NOMb from MetMb on continued incubation with NADH for 5–40 min (Fig. 4). However, the additions of $Na_2S_2O_4$ and $NaNO_2$ to the diluted incubation products caused a decrease in absorbance at the peak in those of short duration (5–10 min at 37°) and an increase in those from longer incubations (20–40 min). This difference in response is ascribed

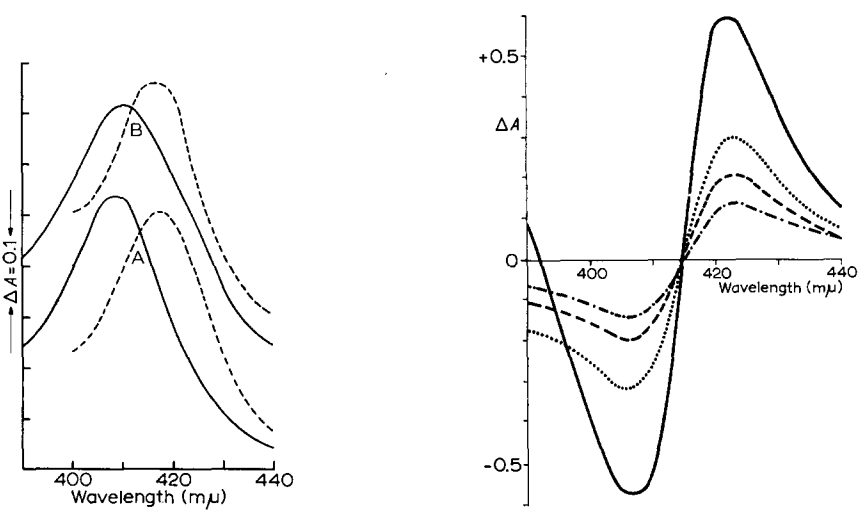


Fig. 4. Absorption spectra of the products, after 10-fold dilution in air, of incubation under N_2 at 37° of mitochondria, 37 μM pig MetMb, 15 μM nitrosylferricytochrome *c* and 40 μM NADH after periods of (A) 5 min and (B) 40 min before (———) and after (– – – –) reduction with $Na_2S_2O_4$ and addition of $NaNO_2$.

Fig. 5. Difference absorption spectra of the products, after 5-fold dilution in air, of incubation under N_2 at 37° of mitochondria, 37 μM pig MetMb, 15 μM nitrosylferricytochrome *c* and 40 μM NADH after periods of 5 min (–·—·–), 10 min (– – – – –) and 40 min (·····) with reference to dilutions of similar incubations containing ferricytochrome *c* uncomplexed with NO. Also shown is the difference spectrum (———) of any one of the above products after complete conversion of the myoglobin present into NOMb with $Na_2S_2O_4$ and $NaNO_2$, the decomposition products of which account for additional absorbance below 400 mμ.

to the continued formation of NOMb throughout the incubation which eventually permitted the observation of the increase in Soret absorbance resulting from the reduction of ferricytochrome *c* with $Na_2S_2O_4$ without interference from the decrease in absorbance on chemical conversion of MetMb to NOMb. A comparison of the time scales involved in Figs. 3 and 4 provides evidence for the enzymic formation of NOMb through the intermediate of NOMetMb, which would appear as MetMb in products after aeration.

Difference spectra in the Soret region have been obtained of the products of anaerobic incubation of mitochondria, NADH, MetMb and nitrosylferricytochrome *c*, after shaking in air and dilution, with reference to similar mixtures incubated with ferricytochrome *c* uncomplexed with NO. The cytochrome oxidase activity of the mitochondria ensured that cytochrome *c* was in the ferric state in both products, and therefore it did not interfere with the spectrophotometric interpretation of myoglobin interactions. Fig. 5 records the increase with time of incubation of the resultant peak and trough, corresponding closely to the difference spectrum of NOMb with reference to MetMb; the peak resulting from subsequent complete chemical conversion of myoglobin to the nitrosyl- form is also shown. No interfering peak at 417 mμ, corresponding to the difference spectrum of nitrosylferricytochrome *c* with reference to ferricytochrome *c*, has been observed in products of incubation with NADH for 5 min or longer.

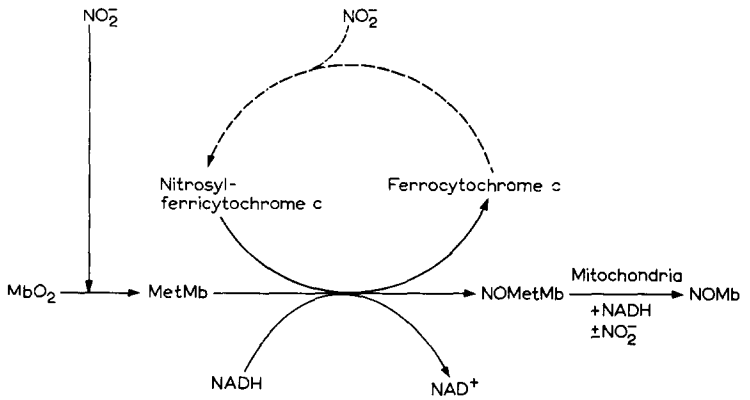
When mitochondria, 40 μM NADH, 17.5 μM MetMb and 15 μM nitrosylferricytochrome *c* were incubated anaerobically the rate of oxidation of NADH, as observed spectrofluorimetrically, was initially fast for 3–4 min and subsequently decayed to a minimum value within approx. 30 min. After this period at room temperature, a small regeneration of NADH was observed on occasions, probably as a result of substrate formation by a degradative change such as lipolysis[13]; subsequent shaking in air caused a resumption of rapid NADH oxidase activity.

DISCUSSION

It was suggested by Dr. E. ANTONINI, of the University of Rome, that the observed difference between MetMb and NOMetMb in susceptibility to reduction by mitochondrial enzyme systems might be dependent upon the relative stabilities of the reduced and oxidised forms in each case. Mb is labile and oxidised by ferricytochrome *c*[14]; NOMb is more stable than NOMetMb and therefore there will be little tendency for re-oxidation to occur. Furthermore, MetMb may be reduced by mitochondria and NADH in the presence of CO which stabilizes the ferrous form by complexing as MbCO. Cyanide prevents this reduction by forming a stable cyanometmyoglobin.

Nitrosylferrocytochrome *c* has not been detected (*cf.* also ref. 11). Therefore, it is probable that reduction of nitrosylferricytochrome *c* will liberate the nitrosyl-group for transfer to myoglobin to form the stable NOMb. The reaction could proceed in two ways: (1) MetMb $\longrightarrow$ Mb $\xrightarrow{NO}$ NOMb or (2) MetMb $\xrightarrow{NO}$ NOMetMb $\longrightarrow$ NOMb. Spectrophotometric studies in the Soret region favour (2). In the presence of amytal, which inhibits NAD^+–flavin interactions[15], the initial rate of reduction of nitrosylferricytochrome *c* is decreased more than the rate of formation of NOMb, although a stoichiometric relation between these reactants is subsequently achieved.

The liberation of the nitrosyl- group does not therefore appear to be the rate limiting factor. Furthermore, the observation of a rapid oxidation of NADH followed by a slower reaction (at a time when nitrosylferricytochrome *c* is completely reduced) indicates a multi-stage process.



Scheme 1. Proposed route of formation of nitrosylmyoglobin (NOMb) from endogenous oxymyoglobin (MbO_2) on anaerobic incubation of $NaNO_2$ with skeletal muscle. (present work ——→; previous researches, ref. 1 – – –→).

Scheme I summarizes the sequence of reactions proposed for the formation of NOMb from nitrite and endogenous myoglobin. Two functions are attributed to nitrite; it oxidises myoglobin to the met- form and it acts anaerobically as a terminal respiratory electron acceptor to ferrocytochrome *c* with the formation of nitrosylferricytochrome *c*. The nitrosyl- group is transferred from ferricytochrome *c* to MetMb by NADH–cytochrome *c* reductase action. Finally, the NOMetMb formed is reduced readily to NOMb by mitochondrial enzyme systems even in the presence of nitrite concentrations causing rapid oxidation of MbO_2. The transfer of the nitrosyl- group from ferricytochrome *c* to other acceptors of biological origin *via* a similar pathway is also a possibility.

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