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The Mechanism of Transmembrane $\Delta\mu_{H+}$ Generation in Mitochondria by Cytochrome *c* Oxidase

By Michele LORUSSO, Ferdinando CAPUANO, Domenico BOFFOLI, Riccardo STEFANELLI and Sergio PAPA
Institute of Biological Chemistry, Faculty of Medicine and Centre for the Study of Mitochondria and Energy Metabolism, CNR, University of Bari, Bari, Italy

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1. In rat liver mitochondria treated with rotenone, *N*-ethylmaleimide or oligomycin the expected alkalinization caused by proton consumption for aerobic oxidation of ferrocyanide was delayed with respect to ferrocyanide oxidation, unless carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present. 2. When valinomycin or valinomycin plus antimycin were also present, ferricyanide, produced by oxidation of ferrocyanide, was re-reduced by hydrogenated endogenous reductants. Under these circumstances the expected net proton consumption caused by ferrocyanide oxidation was preceded by transient acidification. It is shown that re-reduction of formed ferricyanide and proton release derive from rotenone- and antimycin-resistant oxidation of endogenous reductants through the proton-translocating segments of the respiratory chain on the substrate side of cytochrome *c*. The number of protons released per electron flowing to ferricyanide varied, depending on the experimental conditions, from 3.6 to 1.5. 3. The antimycin-insensitive re-reduction of ferricyanide and proton release from mitochondria were strongly depressed by 2-n-heptyl-4-hydroxyquinoline *N*-oxide. This shows that the ferricyanide formed accepts electrons passing through the protonmotive segments of the respiratory chain at the level of cytochrome *c* and/or redox components of the cytochrome b–c_1 complex situated on the oxygen side of the antimycin-inhibition site. Dibromothymoquinone depressed and duroquinol enhanced, in the presence of antimycin, the proton-release process induced by ferrocyanide respiration. Both quinones enhanced the rate of scalar proton production associated with ferrocyanide respiration, but lowered the number of protons released per electron flowing to the ferricyanide formed. 4. Net proton consumption caused by aerobic oxidation of exogenous ferrocytochrome *c* by antimycin-supplemented bovine heart mitochondria was preceded by scalar proton release, which was included in the stoicheiometry of 1 proton consumed per mol of ferrocytochrome *c* oxidized. This scalar proton production was associated with transition of cytochrome *c* from the reduced to the oxidized form and not to electron flow along cytochrome *c* oxidase. 5. It is concluded that cytochrome *c* oxidase only mediates vectorial electron flow from cytochrome *c* at the outer side to protons that enter the oxidase from the matrix side of the membrane. In addition to this consumption of protons the oxidase does not mediate vectorial proton translocation.

Although it is established that cytochrome *c* oxidase (EC 1.9.3.1) directly converts redox energy into transmembrane thermodynamic potential difference of protons ($\Delta\mu_{H+}$) (Hinkle, 1973; Papa, 1976*a*), the mechanism by which this is carried out is a matter of controversy.

Cytochrome oxidase is plugged through the insulating layer of the mitochondrial membrane, being exposed to both aqueous phases (Schneider *et al.*, 1972; Eytan *et al.*, 1975). Mitchell (1966) postulated that the oxidase transfers electrons, accepted from cytochrome *c*, located at the outer side of the membrane (De Pierre & Ernster, 1977), to protons from the matrix space, thus setting up a transmembrane potential and pH difference.

Investigations on the oxidation of endogenous carriers in mitochondria and sonicated submitochondrial particles provided evidence that the protons consumed for reduction of oxygen to water reach the oxidase from the inner or matrix side of the membrane (Mitchell, 1969; Mitchell & Moyle, 1970; Papa, 1976*a*; Papa *et al.*, 1974*a*,*b*, 1978*a*).

It was also shown that the transition of cytochromes *c* and aa_3 from the reduced to the oxidized state did not itself cause transmembrane proton translocation. A rapid proton release at the outer side of the membrane, observed in this transition, was ascribed to antimycin-insensitive oxygen-dependent redox events in the ubiquinone–cytochrome *c* span of the respiratory chain (Papa *et al.*, 1974*a*, 1978*a*).

Investigations based on the use of artificial reductants have, on the other hand, produced contrasting results. Mitchell & Moyle (1967) and Mitchell (1969) observed that aerobic oxidation of ferrocyanide by antimycin-supplemented mitochondria produced no proton translocation other than that arising from consumption of protons from the matrix for reduction of oxygen to water.

Wikstrom (1977) and Wikstrom & Saari (1977) reported that, when specific precautions are taken, an antimycin-insensitive proton ejection from mitochondria can be shown to accompany ferrocyanide oxidation. Similar observations have also been made by Sigel & Carafoli (1978), Azzone *et al.* (1978) and Alexandre *et al.* (1978) using either ferrocyanide or *NNN'N'*-tetramethyl-*p*-phenylenediamine plus ascorbate. Wikstrom & Saari (1977) interpreted these, as well as other observations made with sonicated submitochondrial particles and purified cytochrome oxidase inlaid in liposomes, as evidence that the oxidase functions as a redox proton pump (see also Sorgato & Ferguson, 1978).

This conclusion was challenged by Papa *et al.* (1978*a*,*b*) and Moyle & Mitchell (1978), who obtained independent evidence indicating that the antimycin-insensitive proton release associated with ferrocyanide respiration results from redox reactions on the substrate side of cytochrome *c*.

In the present paper a detailed study is presented of the proton-translocation reactions elicited by pulses of aerobic mitochondria with ferrocyanide or exogenous ferrocytochrome *c*.

It is shown that antimycin-insensitive proton release elicited by reductant pulses results from redox reactions of respiratory carriers situated on the substrate side of cytochrome *c* or from oxidation of the reductant, but not from a proton pump in the oxidase. Information on the nature of these proton-release reactions and their relevance to the function of the respiratory chain is presented. Some of the present results have been communicated in a preliminary form (Papa *et al.*, 1978*c*).

Materials and Methods

Chemicals

Antimycin A, valinomycin, oligomycin, rotenone, 2-n-heptyl-4-hydroxyquinoline *N*-oxide, *N*-ethylmaleimide, bovine serum albumin and bovine heart ferricytochrome *c* (mol.wt. 12327; type IV) were purchased from Sigma Chemical Co. Duroquinone, potassium ferrocyanide and potassium ferricyanide were from BDH Chemicals, Poole, Dorset, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. C. P. Lee, University of Pennsylvania, PA, U.S.A. Dibromothymoquinone was a gift from Professor A. Trebst, University of Bochum, Bochum. All the other reagents were of the highest purity grade available. Stock solutions of 2-n-heptyl-4-hydroxyquinoline *N*-oxide, prepared by dissolving the whole contents of a vial in ethanol, were stored at 0°C and used within 1 month. The exact concentration was determined spectrophotometrically by using an absorption coefficient at 348nm of $9.45\,mM^{-1}\cdot cm^{-1}$ (Van Ark & Berden, 1977). Ferrocytochrome *c* was obtained by reduction with excess ascorbate in 10mM-potassium phosphate buffer, pH7.4, followed by dialysis for 12h against phosphate buffer and for 8h against water. The fresh solution of ferrocytochrome *c* so obtained contained less than 5% ferricytochrome *c* and was free of ascorbate. The exact concentration of ferrocytochrome *c* was measured at 550nm by using an absorption coefficient (reduced minus oxidized) of $21\,mM^{-1}\cdot cm^{-1}$ (Massey, 1959). Duroquinol was obtained by reduction with KBH_4 in methanol, excess KBH_4 being eliminated by acidification with 0.1M-HCl. The methanolic solution, diluted with the reaction mixture at about pH7, was kept at 0°C and used within 1h. Dibromothymoquinone was dissolved in methanol containing a few drops of *NN*-dimethylformamide.

Preparation of mitochondria

Rat liver mitochondria were isolated, as described by Myers & Slater (1957), and suspended in 0.25M-sucrose at a protein concentration of about 60mg/ml. Bovine heart mitochondria were prepared as described by Löw & Vallin (1963) and suspended in 0.25M-sucrose at a protein concentration of about 20mg/ml.

Incubation procedure

Rat liver mitochondria were incubated at 25°C in a basic reaction mixture containing: 150mM-KCl, 1mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, 0.7mM-potassium EDTA, 0.33mM-*N*-ethylmaleimide, 0.5μg of rotenone/mg of mitochondrial protein and 2μg of oligomycin/mg of mitochondrial protein. All other additions are specified in the legends to Figures and Tables.

The pH was adjusted to 7.4 with 0.1M-KOH. Samples from the same mitochondrial suspension were transferred to the measurement cells, thermostatically controlled at 25 ± 0.01°C, for simultaneous determination of pH, ferricyanide production and oxygen consumption. Bovine heart mitochondria were suspended in the reaction mixtures described in the legends to Figures and Tables. Samples were transferred to thermostatically controlled cells for pH measurement and monitoring of redox transitions of cytochrome *c*.

Measurement of proton translocation

pH changes were measured potentiometrically with combination glass electrodes (Beckman no. 39030, Beckman Instruments or Ingold type 104053298, Ingold A.G., Zurich International, Geneva, Switzerland). The electrode was connected to an electrometer amplifier (model 604; Keithley Instruments, Cleveland, OH, U.S.A.). The output of the electrometer was fed into a two-channel potentiometric pen recorder (Speedomax XL 681; Leeds and Northrup Italiana S.p.A., Milano, Italy). The time for overall increase (10–90% change) of the pH recording system was 0.4s, the noise level was less than 5×10^{-4} pH unit, and the precision 10^{-3} pH unit. Potential changes were converted into proton equivalents by double titration with standard solutions of HCl and KOH. pH measurements were made by immersing the electrode in the mitochondrial suspension which was kept vigorously stirred in an open glass cell surrounded by a glass jacket for circulation of water thermostatically controlled at 25°C. Ferrocyanide was added to the aerobic mitochondrial suspension as 1μl portions of a 0.15M freshly prepared solution in 150mM-KCl, containing 1mM-Hepes buffer, pH7.4. It was noted that when ferrocyanide was added to the mitochondrial suspension or to the reaction mixture it caused an immediate small deflection in the potentiometric pH trace in the direction of alkalinity (Wikstrom & Krab, 1978). This deflection, however, does not represent a real pH increase. In fact the magnitude of the deflection of the pH trace was unaffected by changing by one order of magnitude the buffering power of the reaction mixture and varied with the electrode used. This artifact, which probably reflects interference of ferrocyanide with electrodes, was negligible in the experimental conditions we used.

Measurement of O_2 consumption.

O_2 consumption was measured polarographically with a Clark-type membrane-coated oxygen electrode (no. 4004; Yellow Spring Instruments, Yellow Spring, OH, U.S.A.) connected to an electrometer amplifier constructed at the Physics Department of the University of Bari according to the design recommended by the makers of the electrode. The output of the electrometer was fed to the Leeds and Northrup potentiometric pen recorder. The electrode was immersed through an O-ring-sealed side hole into the mitochondrial suspension which was kept vigourously stirred by a magnetic follower in an all-glass reaction cell, sealed at the top with a glass plug with central 3cm-long capillary channel, filled with the mitochondrial suspension for insertion of the micro-syringe needle. This cell excluded detectable O_2 diffusion. The $t_{\frac{1}{2}}$ of the response of the O_2-recording system, which measured O_2 produced from H_2O_2 decomposition with catalase, was 0.5s. This time does not include a lag of 4–5s which elapsed after the addition of catalase or H_2O_2, before the oxygen electrode started to record the change in O_2 concentration. This lag, which was also observed before the oxygen electrode responded to increased respiratory rates elicited by ferrocyanide, succinate and other substrates (see Fig. 1 and Brand *et al.*, 1976; Reynafarje *et al.*, 1976), probably reflects the time required for O_2 or substrate equilibration with the suspension layers sensed by the surface of the oxygen electrode. O_2 consumption was calibrated by setting the null point after dissolving solid sodium dithionite in the reaction mixture and taking the signal displayed after equilibrium of the electrode with water at 25°C and 10^5Pa as corresponding to 263μM-O_2 (250μM-O_2 in 150mM-KCl) (Hodgman, 1961). This calibration was also checked by aerobic oxidation of known amounts of NADH by sonicated mitochondria.

Measurement of ferricyanide production

Ferricyanide production by mitochondrial oxidation of ferrocyanide was directly monitored at the wavelength couple 420–500nm by using a Johnson Foundation dual-wavelength spectrophotometer, in a 1cm cuvette thermostatically controlled at 25°C. The transmittance changes were converted into ferricyanide equivalents by addition to the mitochondrial suspension of small portions of standard ferricyanide solution (final concentration 20μM). Since added ferricyanide underwent partial reduction during the mixing time, to avoid this complication (which could cause substantial overestimation of ferricyanide production) it was added to the mitochondrial suspension after aerobic oxidation of ferrocyanide. In fact, under these conditions, added ferricyanide was not reduced and any optical interference by the mitochondrial suspension is accounted for.

Measurement of ferrocytochrome c oxidation

Oxidation of ferrocytochrome *c* was monitored at the wavelength couple 550–540nm as described for ferricyanide mesurements.

Protein

Mitochondrial protein was determined by the biuret method, with bovine serum albumin as standard and correcting for KCN residual absorbance (Szarkowska & Klingenberg, 1963).

Results

Proton translocation induced by ferrocyanide pulses

Fig. 1 presents a detailed picture of the characteristics of pH changes in the extramitochondrial aqueous

phase, and ferricyanide net production and O_2 consumption caused by ferrocyanide addition to aerobic rat liver mitochondria. Mitochondria were preincubated with rotenone, to inhibit endogenous respiration, oligomycin and *N*-ethylmaleimide to prevent transmembrane proton diffusion via H^+-ATPase and proton/anion symporters respectively (Papa, 1976*b*).

In the absence of valinomycin and antimycin (Fig. 1, Expt. *ai*), ferrocyanide addition caused, after an interval of a few seconds after the commencement of ferrocyanide oxidation, during which there was no detectable pH change, a net alkalinization, which took about 30s to display its maximal rate. In the presence of valinomycin, added to aerobic mitochondria at the beginning of the preincubation period, ferrocyanide oxidation resulted in a small acidification followed by net alkalinization (Fig. 1*b*). This transient acidification induced by ferrocyanide was much faster and larger when valinomycin was added to mitochondria 30s before ferrocyanide (Fig. 1*c*).

It can be noted that valinomycin depressed the rate of net proton consumption when added from the beginning of the preincubation period, but accelerated this reaction when added only 30s before ferrocyanide. Furthermore the addition of valinomycin to aerobic mitochondria itself induced a proton release (Fig. 1*c*), which was inhibited by antimycin (see Fig. 1*d*).

Antimycin had practically no effect on ferrocyanide oxidation and proton movements when valinomycin was added 30s before ferrocyanide (Fig 1*d*). However, it preserved the stimulatory effect exerted by valinomycin on these processes when the two substances were present from the beginning of the preincubation period (Fig. 1*ei*). It is worth noting that these last conditions were those selected by Wikstrom (Wikstrom, 1977; Wikstrom & Saari, 1977) to demonstrate proton release associated with aerobic oxidation of ferrocyanide.

An insight into the nature of the acidification process elicited by ferrocyanide respiration is

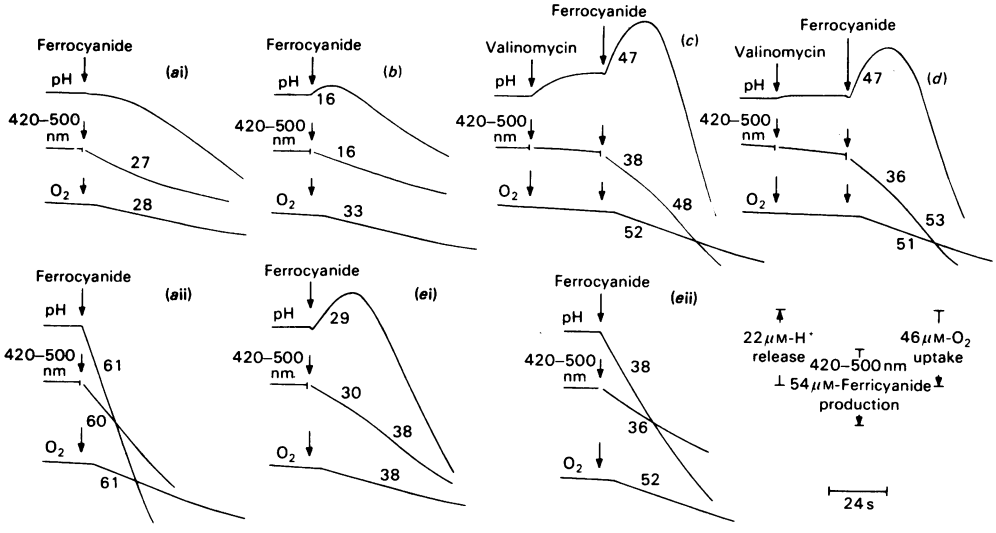


Fig. 1. *Proton movements, ferricyanide production and O_2 consumption elicited by ferrocyanide pulses of aerobic rat liver mitochondria*

Mitochondria (2mg of protein/ml) were preincubated for 5min at 25°C in the basic reaction mixture, pH7.4, described in the Materials and Methods section. Additions: Expt. *ai*, none. Expt. *b*, 0.035μg of valinomycin/mg of protein added at the beginning of the preincubation period. Expt. *c*, valinomycin added 30s before ferrocyanide. Expt. *d*, 0.5μg of antimycin/mg of protein added at the beginning of preincubation, valinomycin added 30s before ferrocyanide pulse. Expt. *ei*, antimycin and valinomycin added at the beginning of the preincubation. In Expts. *aii* and *eii* 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was also present from the beginning of the preincubation. Ferrocyanide was added at the final concentration of 1mM. For other experimental details and procedures see the Materials and Methods section. It should be noted that the delay of 4–6s shown by the O_2 traces before the oxygen electrode responded to the increased respiratory rate elicited by ferrocyanide is a characteristic of the O_2-measuring set-up [i.e. it was also observed with other respiratory substrates or when O_2 was produced from H_2O_2 decomposition with catalase (see the text) and did not affect evaluation of initial rates of O_2 reduction, which were constant for at least 30s]. The numbers on the traces are initial rates expressed as ng-ions of H^+ translocated, nmol of ferricyanide produced and nequiv. of electrons transferred to O_2 (ng-atoms of O×2)/min per mg of protein. In Expts. *c*, *d* and *ei* the rates for the second phase of ferricyanide production are also presented.

provided by the measurements of O_2 consumption and ferricyanide production.

Rotenone-supplemented mitochondria still exhibited, after a 5 min preincubation, a significant endogenous respiration (about 6 ng-atoms of O/min per mg of protein), which was unaffected by antimycin.

In the absence of antimycin and valinomycin, conditions under which ferrocyanide caused no transient acidification, the initial rate of ferricyanide appearance was equal to that of O_2 reduction. On the other hand, under conditions that promoted ferrocyanide-induced acidification, the initial rate of net ferricyanide production was lower than that of O_2 reduction. The larger the transient acidification, the higher the deficit of ferricyanide production with respect to O_2 reduction (Fig. 1, Table 1). It can be noted that under conditions promoting considerable initial acidification (Expts. *c*, *d* and *ei* of Fig. 1) ferricyanide production exhibited, concomitantly with the acidification process, a distinct initial slow phase. As the acidification process reached completion, the rate of ferricyanide production increased and matched the rate of O_2 reduction. The rate of O_2 reduction, on the contrary, did not exhibit comparable changes.

Moyle & Mitchell (1978) have reported that, under conditions where ferrocyanide oxidation resulted in proton release, the net rate of scalar proton consumption, measured in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, was lower than that of O_2 reduction (deficit proton consumption or excess O_2 reduction).

Fig. 1 shows that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone abolished the acidification process associated with ferrocyanide oxidation by mitochondria treated with valinomycin (results not shown) and valinomycin plus antimycin (Expt. *eii* of Fig. 1). In these conditions the initial rate of proton consumption in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was lower than the initial rate of O_2 reduction (cf. Moyle & Mitchell, 1978). The higher the acidification process, the larger the deficit of proton consumption (see Table 1). On the contrary, in the absence of valinomycin and antimycin no deficit of proton consumption was observed (Expt. *aii* of Fig. 1). It can also be noted that in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone the initial rate of ferricyanide production was equal to that of O_2 reduction when ferrocyanide did not cause acidification in the coupled state but was lower under conditions that did lead to transient acidification.

The pattern of ferrocyanide oxidation and proton movements presented in Fig. 1 was constantly observed with several mitochondrial preparations, although the quantitative parameters varied. Table 1 presents the mean values of the quantitative parameters with their statistical evaluation for a series

Table 1. *Statistical analysis of the initial rates and stoicheiometric relationships of proton release, ferricyanide production, O_2 consumption and net proton consumption in ferrocyanide-pulse experiments in rat liver mitochondria*

The values are the means ±S.E.M. for the number of different experiments indicated. The values for the H^+ release/e^- ratio are means ±S.E.M. of the internal H^+ release/e^- ratio measured in different experiments. Experimental conditions: (*a*) see Expts. *ai* and *aii* of Fig. 1; (*b*) see Expt. *b* of Fig. 1; (*c*) see Expt. *c* of Fig. 1; (*d*) see Expt. *d* of Fig. 1; (*e*) see Expts. *ei* and *eii* of Fig. 1; (*f*) see Expt. *b* of Fig. 3; (*g*) see Expt. *b* of Fig. 6; (*h*) see Expt. *c* of Fig. 6. When present 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to the mitochondrial suspension at the beginning of the 5 min preincubation period. For other details see the legend to Fig. 1 and the Materials and Methods section.

							+ FCCP				
Expts.	No. of Expts.	H^+ release (ng-ions/min per mg of protein)	Ferricyanide production (nmol/min per mg of protein)	e^- (ng-atoms of O×2/min per mg of protein)	H^+ release/e^-	Ferricyanide deficit (nmol/min per mg of protein)	H^+ uptake (ng-ions/min per mg of protein)	Ferricyanide production (nmol/min per mg of protein)	e^- (ng-atoms of O×2/min per mg of protein)	H^+ deficit (e^- − H^+ uptake) (ng-ions/min per mg of protein)	H^+release /H^+ deficit (H^+release/e^- flowing to ferricyanide)
(*a*)	6	0	27.2±2	26.2±2	0	0	52.4±4	47.9±4	50.5±5	1.1±0.6	0
(*b*)	6	14.2±4	15.6±2	27.5±4	0.48±0.07	11.6±2	32.4±2	27.7±1	42.7±4	9.5±2	1.50
(*c*)	5	45.5±2	37.4±3	50.8±2	0.9±0.04	13.5±2	42.8±3	49.0±6	55.3±5	12.5±2	3.64
(*d*)	4	44.8±6	38.0±5	50.0±7	0.89±0.09	12.2±3	45.3±7	45.4±7	58.7±10	13.4±3	3.34
(*e*)	8	30.2±4	25.7±2	38.5±4	0.77±0.04	12.5±2	32.5±2	31.3±2	43.6±4	11.2±2	2.70
(*f*)	4	6.7±1	37.0±3	42.4±5	0.17±0.04	5.4±0.5	34.2±2	34.1±5	38.0±5	4.0±3	1.67
(*g*)	3	41.5±4	—	62.7±7	0.73±0.04	—	27.4±4	—	58.7±10	31.3±6	1.33
(*h*)	3	19.9±3	—	44.5±9	0.48±0.08	—	19.7±2	—	40.9±6	21.2±4	0.94

of these experiments. The ferrocyanide-induced acidification process in mitochondria, treated with valinomycin or valinomycin plus antimycin, was accompanied by a significant deficit in ferricyanide production with respect to O_2 reduction, both in the absence and presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and a deficit in scalar proton consumption in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. It can be noted that the deficit in ferricyanide production in the coupled state was practically equal to the deficit in proton consumption measured in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Furthermore in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone the net rates of proton consumption and ferricyanide production were also practically equal. Thus the deficit in proton consumption can be taken as representing the rate of ferricyanide re-reduction by endogenous hydrogenated reductant(s) (cf. Moyle & Mitchell, 1978).

Control experiments showed that KCN abolished ferrocyanide respiration and the associated proton movements. Ferricyanide added to aerobic mitochondria was partially reduced and this resulted in a small but significant acidification. This reaction increased proportionally with the concentration of added ferricyanide and was larger when mitochondria were preincubated with KCN. The rate of proton release induced by added ferricyanide was, however, lower than that elicited by the addition of equivalent amounts of ferrocyanide to respiring mitochondria (see also Fig. 11 and Moyle & Mitchell, 1978). Added ferricyanide (1 mM) produced rates of proton release of 6 and 10 ng-ions/min per mg of protein in the absence and presence of KCN respectively.

The deficit of proton consumption observed in the presence of valinomycin or valinomycin plus antimycin is a measure of the rate of scalar proton production induced by ferrocyanide.

The present measurements provide direct experimental support for the proposal of Moyle & Mitchell (1978) that this deficit of scalar proton consumption is due to oxidation of endogenous hydrogenated reductant(s) by formed ferricyanide. The rate of scalar proton release was considerably lower than the net rate of acidification observed in the coupled state. Thus the latter process represents in large part vectorial proton translocation across the mitochondrial membrane.

It should be mentioned that Wikstrom & Krab (1978) did not observe, in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, a deficit in ferricyanide production with respect to O_2 reduction in rat liver mitochondria supplemented with valinomycin and antimycin, although there was an apparent deficit of proton consumption. Possible explanations for this discrepancy with the present results are: (i) the concentration of mitochondrial proteins used by Wikstrom & Krab (1978) was lower than those used here; (ii) it was not reported whether under the same experimental conditions and with the same mitochondrial preparation there was any transient acidification upon ferrocyanide oxidation in the absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; (iii) processes that were artifacts (see the Materials and Methods section) might have affected their measurements of ferricyanide production and pH changes.

In Fig. 2 a titration curve of the stimulatory effect of antimycin on ferrocyanide-induced proton release from mitochondria preincubated for 5 min with valinomycin is shown. Enhancement of the H^+/e^- ratio for proton release took place in the same concentration range in which inhibition of the respiratory chain between cytochromes *b* and *c* occurs (Estabrook, 1962). Maximal stimulation was observed at about 0.25 nmol of antimycin/mg of protein. Increasing the antimycin concentration above this value had no further effect.

Fig. 3 and Table 1 show the effect of 2-n-heptyl-4-hydroxyquinoline *N*-oxide on ferrocyanide oxidation and proton movements in rat liver mitochondria. 2-n-Heptyl-4-hydroxyquinoline *N*-oxide addition to mitochondria treated with valinomycin and antimycin caused a marked depression of the transient acidification induced by ferrocyanide (see also Papa *et al.*, 1978*a*,*b*). The initial rate of O_2 reduction was practically unaffected, so that the H^+/e^- ratio

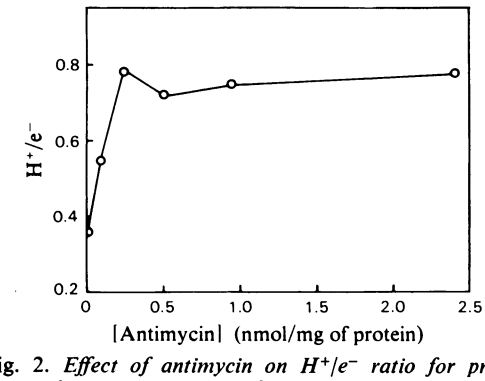


Fig. 2. *Effect of antimycin on H^+/e^- ratio for proton release associated with ferrocyanide respiration*

Rat liver mitochondria (2 mg of protein/ml) were preincubated for 5 min in the basic reaction mixture supplemented with 0.035 μg of valinomycin/mg of protein. Antimycin was added at the concentrations indicated from the beginning of the preincubation period. Ferrocyanide was present at 1 mM. The H^+/e^- ratio was obtained by dividing the initial rate of proton release by the initial rate of O_2 reduction (see the legend to Fig. 1). For other details see the Materials and Methods section.

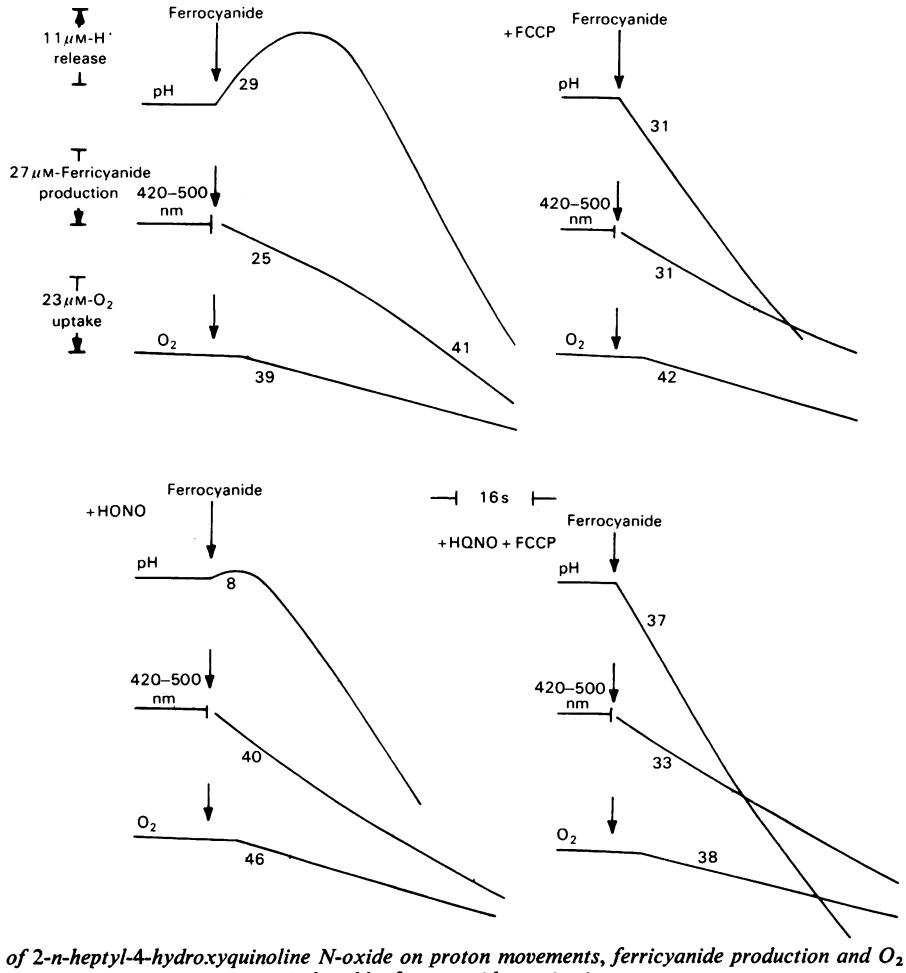


Fig. 3. *Effect of 2-n-heptyl-4-hydroxyquinoline N-oxide on proton movements, ferricyanide production and O_2 consumption induced by ferrocyanide respiration*

Rat liver mitochondria (2mg of protein/ml) were preincubated for 5min in the basic reaction mixture also containing 0.035 μg of valinomycin/mg of protein and 0.5 μg of antimycin/mg of protein. Ferrocyanide was present at 1mM. For other details see the Materials and Methods section. Where indicated 10nmol of 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HQNO)/mg of protein and 1 μM-carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) were added to the mitochondrial suspension from the beginning of the preincubation. The numbers on the traces are described in Fig. 1.

decreased sharply. Interestingly enough, the deficit in net ferricyanide production against O_2 reduction was largely abolished by 2-n-heptyl-4-hydroxyquinoline *N*-oxide. Control experiments in carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-treated mitochondria showed that 2-n-heptyl-4-hydroxyquinoline *N*-oxide also depressed the deficit in proton consumption, i.e. the rate of scalar proton consumption closely approached that of O_2 reduction.

Thus it appears that 2-n-heptyl-4-hydroxyquinoline *N*-oxide suppresses ferrocyanide-dependent acidification by inhibiting antimycin-insensitive oxidation of hydrogenated endogenous reductant(s) by formed ferricyanide. The decrease in the deficit in proton consumption caused by 2-n-heptyl-4-hydroxyquinoline *N*-oxide seems to exclude the possibility that the inhibitory effect exerted on proton release in the absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone results simply from protonophore activity of 2-n-heptyl-4-hydroxyquinoline *N*-oxide (Wikstrom, 1978). This possibility was also ruled out by an independent experimental approach.

The experiments of Fig. 4 show that 2-n-heptyl-4-hydroxyquinoline *N*-oxide caused, at pH7.4, a

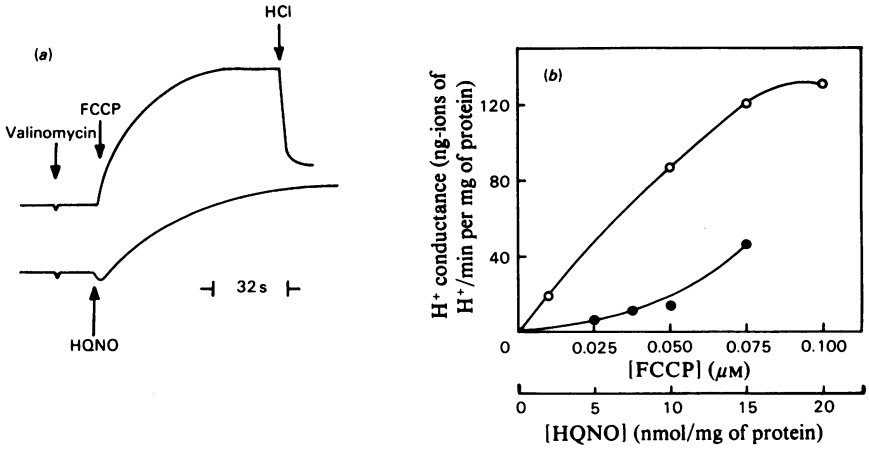


Fig. 4. *Measurement of passive proton conductance induced by 2-n-heptyl-4-hydroxyquinoline N-oxide and carbonyl cyanide p-trifluoromethoxyphenylhydrazone in rat liver mitochondria*

Mitochondria (4mg of protein/ml) were suspended in a reaction mixture containing: 200mM-sucrose, 2mM-KCl, 1mM-Hepes, 0.33mM-*N*-ethylmaleimide, 1mM-EGTA, 2μg of oligomycin/mg of protein, 0.5μg of rotenone/mg of protein. Final pH was 7.4 and the temperature 25°C. (*a*) Where indicated the following additions were made: 0.035μg of valinomycin/mg of protein, 0.05μM-carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP), 10nmol of 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HQNO)/mg of protein and 49μM-HCl. (*b*) Titration of the effect of 2-n-heptyl-4-hydroxyquinoline *N*-oxide and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on passive proton conduction in rat liver mitochondria. ●, 2-n-Heptyl-4-hydroxyquinoline *N*-oxide; ○, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone.

moderate increase in passive proton conductance in mitochondria.

Fig. 5 shows a titration of the depression of the ferrocyanide-induced acidification by 2-n-heptyl-4-hydroxyquinoline *N*-oxide and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. 2-n-Heptyl-4-hydroxyquinoline *N*-oxide was a much more effective inhibitor than carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone. A plot of the H^+/e^- ratio at various concentrations of 2-n-heptyl-4-hydroxy-quinoline *N*-oxide and carbonyl cyanide *p*-tri-fluoromethoxyphenylhydrazone versus the proton conductance, induced by the same concentrations of these substances, shows that, for equal proton-conductance values, 2-n-heptyl-4-hydroxyquinoline *N*-oxide caused a much larger depression in the H^+/e^- quotient than did carbonyl cyanide *p*-tri-fluoromethoxyphenylhydrazone. Thus depression by 2-n-heptyl-4-hydroxyquinoline *N*-oxide of ferro-cyanide-dependent acidification is largely due to its inhibitory action on the respiratory chain.

Fig. 6 and Table 1 show that the initial rate and the extent of proton release induced by ferrocyanide oxidation in the presence of antimycin was enhanced by duroquinol and depressed by dibromothymo-quinone. Duroquinol also caused stimulation of O_2 reduction. The H^+/e^- ratio was slightly lowered. In the presence of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone, duroquinol caused enhancement of the deficit of proton consumption with respect to O_2 reduction (Table 1).

Dibromothymoquinone lowered the H^+/e^- ratio for proton release in the coupled state. The deficit in proton consumption measured in the presence of dibromothymoquinone and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was equal to the rate of proton release in the coupled state (Table 1). This indicates that dibromothymoquinone suppressed vectorial proton translocation caused by ferrocyanide respiration.

Proton translocation induced by ferrocytochrome c pulses

Fig. 7 shows that exogenous ferrocytochrome *c* was rapidly oxidized by aerobic antimycin-supple-mented bovine heart mitochondria. The oxidation of ferrocytochrome *c* was accompanied by rapid transient acidification followed by net proton consumption. It can be noted (see Fig. 7) that the bovine heart mitochondria used in these experiments exhibited a typical pattern of respiration-linked proton translocation with succinate as substrate and rapid proton release upon aerobic oxidation of O_2-terminal respiratory carriers in the presence of antimycin (Papa *et al.*, 1974*a*). The transient acidification reaction caused by oxidation of ferro-cytochrome *c* was included in the stoicheiometry of

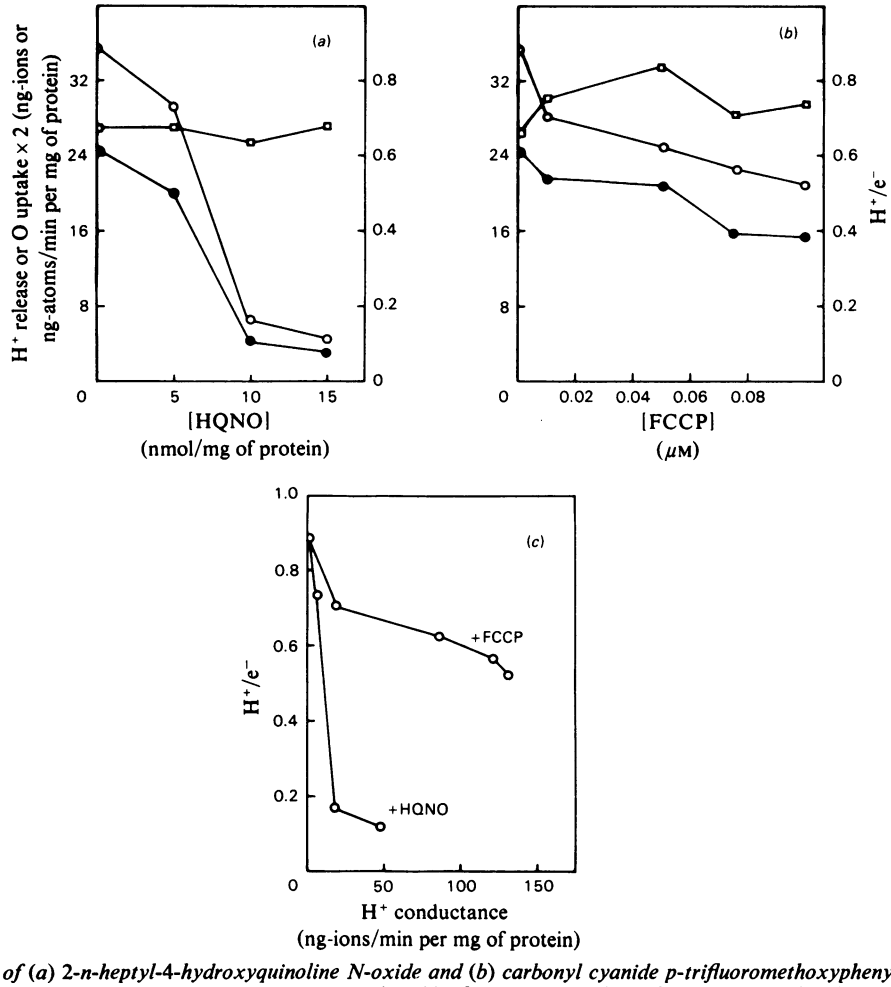


Fig. 5. *Effect of* (*a*) *2-n-heptyl-4-hydroxyquinoline N-oxide and* (*b*) *carbonyl cyanide p-trifluoromethoxyphenylhydrazone on proton release and O_2 consumption induced by ferrocyanide pulses of rat liver mitochondria*

The experimental conditions were those described in the legend to Figs. 3 and 4. 2-n-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added, at the concentrations indicated, from the beginning of the preincubation; ●, Initial rate of H^+ release; □, initial rate of O_2 reduction; ○, H^+/e^- ratios. For other details see the Materials and Methods section and legend to Fig. 1. (*c*) Proton conductance.

1 H^+/e^- for O_2 reduction by ferrocytochrome *c* (Fig. 8). In the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone no net acidification reaction was observed upon ferrocytochrome *c* oxidation; however, net proton consumption was diminished by an amount equivalent to the acidification detected in the control and the net H^+/e^- ratio for proton consumption and cytochrome *c* oxidation decreased from 1 to 0.7 (Fig. 8 and Table 2).

When KCl was omitted from the reaction mixture, the acidification process caused by oxidation of ferrocytochrome *c* was much larger and practically equivalent to proton consumption associated with reduction of O_2 to water so that there was no net alkalinization. In the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone the transient net acidification was lower, but still no net alkalinization occurred. These observations show that proton release associated with oxidation of ferrocytochrome *c* is scalar and not vectorial and that it has to be ascribed to a protolytic reaction other than oxidation of endogenous hydrogenated carrier(s).

In the experiment of Fig. 9 ferricytochrome *c* was added to aerobic bovine heart mitochondria supplemented with antimycin and rotenone and then electrons were fed to cytochrome *c* by exogenous

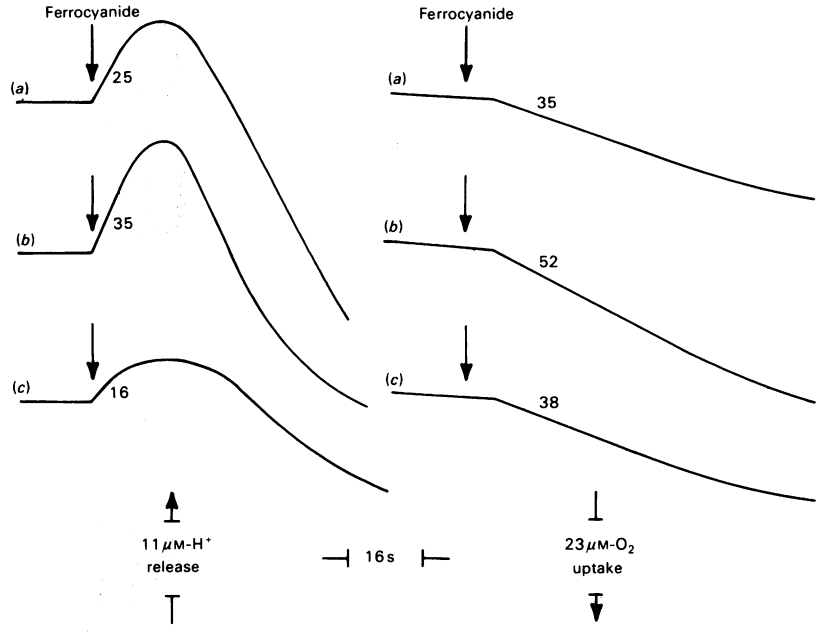


Fig. 6. *Effect of quinones on proton movements and O_2 consumption induced by ferrocyanide pulses of rat liver mitochondria* Mitochondria (3 mg of protein/ml) were preincubated for 5 min in the basic reaction mixture supplemented with 0.5 μg of antimycin/mg of protein and 0.035 μg of valinomycin/mg of protein. (*a*) Control; (*b*) in the presence of 44 μM-duroquinol added 30s before ferrocyanide; (*c*) in the presence of 50 μM-dibromothymoquinone added from beginning of the preincubation. For other experimental details and conditions see legends to Fig. 1 and Table 1, and the Materials and Methods section. Separate controls showed that the solvents used for quinone solutions were without effect on O_2 consumption and proton movements.

NADH via rotenone- and antimycin-insensitive NADH–cytochrome *c* oxidoreductase of the outer mitochondrial membrane (Sottocasa *et al.*, 1967). Addition of both NADH and ferricytochrome *c* produced a respiratory burst, which was accompanied by transient acidification followed by net proton consumption.

Reduction of cytochrome *c* by NADH is itself accompanied by scalar proton production. This proton release was directly measured in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone as a deficit in proton consumption with respect to O_2 reduction. The net proton production measured in the coupled state was quantitatively accounted for by scalar proton production associated with the step NADH→ferricytochrome *c* (Table 3).

Thus it is not electron flow along cytochrome *c* oxidase but only the net transition of cytochrome *c* from the reduced to the oxidized form that causes the acidification reaction observed with pulses of ferrocytochrome *c*. This is confirmed by the experiments of Figs. 10 and 11.

Addition of small amounts of ferricyanide (7.5–80 μM) to bovine heart mitochondria supplemented with antimycin, rotenone and KCN caused proton release, which is attributable to oxidation of endogenous hydrogenated reductants. Added ferrocytochrome *c* was rapidly oxidized upon ferricyanide addition to the mitochondrial suspension and this was accompanied by a fast proton release, which was superimposed on that measured when ferricyanide was added alone (Fig. 10). The enhancement of the initial rate of proton release caused by 10 μM-ferrocytochrome *c* increased with ferricyanide concentration up to 25 μM, then it levelled off (Fig. 11). Furthermore the extent of proton release in the presence of exogenous ferrocytochrome *c* and ferricyanide was slightly smaller than that caused by ferricyanide alone at low concentrations of the oxidant, but larger when the oxidant was added in excess with respect to ferrocytochrome *c*. Thus the rapid proton release induced by the combined addition of ferrocytochrome *c* and ferricyanide was a consequence of direct oxidation of ferrocytochrome *c* by ferricyanide. Rapid ferricyanide reduction by exogenous ferrocytochrome *c* competed for proton-releasing oxidation of endogenous hydrogenated reductants.

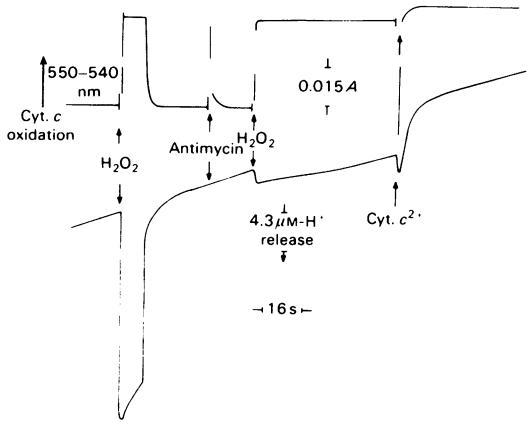


Fig. 7. *Proton translocation induced by O_2 pulses and exogenous ferrocytochrome c pulses of bovine heart mitochondria*

Mitochondria (3.2mg of protein/ml) were incubated in a reaction mixture containing: 200mM-sucrose, 30mM-KCl, 2.5mM-potassium succinate, 0.2mg of purified catalase/ml, 0.5μg of rotenone/mg of protein, 2μg of oligomycin/mg of protein, 0.5μg of valinomycin/mg of protein. The temperature was 25°C and the final pH, 7.0. Where indicated 5μl of 0.3% H_2O_2, 0.5μg of antimycin/mg of protein and 8.5μM-ferrocytochrome *c* (Cyt. c^{2+}) were added. The incubation was carried out in thermostatically controlled cells under a N_2 stream to prevent aeration. For experimental procedure and other details see the Materials and Methods section.

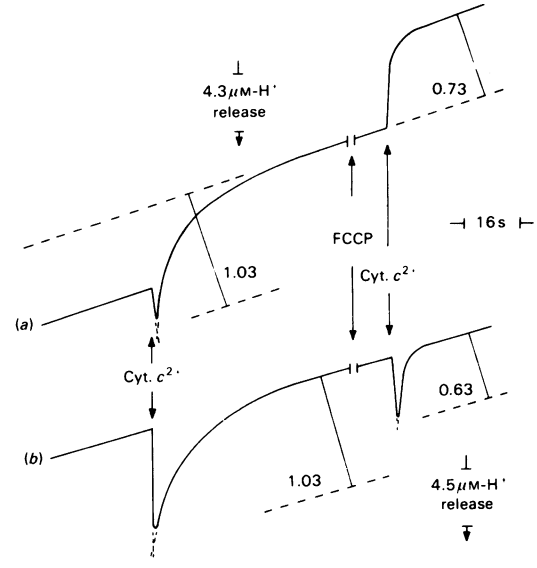


Fig. 8. *Proton translocation induced by oxidation of exogenous ferrocytochrome c in bovine heart mitochondria*

Mitochondria (3.2mg of protein/ml) were incubated in a reaction mixture containing: 200mM-sucrose and 30mM-KCl (Expt. *a*) or 250mM-sucrose (Expt. *b*), 2.5mM-potassium malonate, 0.5μg of rotenone/mg of protein, 2μg of oligomycin/mg of protein, 0.5μg of valinomycin/mg of protein and 0.5μg of antimycin/mg of protein. The temperature was 25°C and the final pH 7.0. Where indicated 8.5μM-ferrocytochrome *c* (Cyt. c^{2+}) and 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added. The values presented are the H^+/e^- ratios for the extent of H^+ uptake and exogenous ferrocytochrome *c* oxidized.

Table 2. *H^+/e^- ratios for proton translocation associated with oxidation of exogenous ferrocytochrome c in bovine heart mitochondria*

For experimental conditions and procedure see the legend to Fig. 8 and the Materials and Methods section. The values are means ± S.E.M. for nine different experiments. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

H^+/e^- (H^+ release)	H^+/e^- (H^+ uptake)	H^+/e^- (+FCCP) (H^+ uptake)
0.28 ± 0.01	1.02 ± 0.04	0.75 ± 0.02

Discussion

The results show that electron flow along cytochrome *c* oxidase, sustained by ferrocyanide or exogenous ferrocytochrome *c*, itself causes no proton translocation in mitochondria other than that arising from consumption of protons for reduction of O_2 to water, which takes place from the matrix side of the membrane (cf. Mitchell & Moyle, 1970; Papa *et al.*, 1974*a*, 1978*a*,*b*). The alkalinization of the extramitochondrial aqueous phase caused by proton consumption was, in all the conditions examined, delayed with respect to O_2 reduction, unless proton conduction through the permeability barrier of the membrane was promoted by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

This situation can, however, be complicated by proton-releasing reactions at the outer side of the membrane, which are unrelated to the function of cytochrome oxidase.

The present data demonstrate that the transient acidification, which can be associated with aerobic oxidation of ferrocyanide by mitochondria and is regarded as evidence of a proton-pumping function of cytochrome oxidase (Wikstrom, 1977, 1978; Wikstrom & Saari, 1977; Wikstrom & Krab, 1978; Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1978), is caused by activation of proton-releasing redox processes on the substrate side of cytochrome *c*.

Rat liver mitochondria contain sufficient endogenous substrates to maintain a significant rotenone-

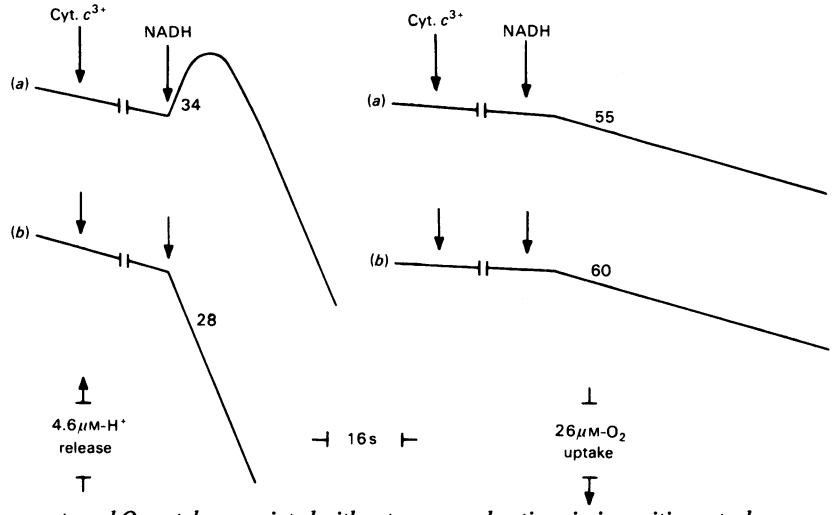
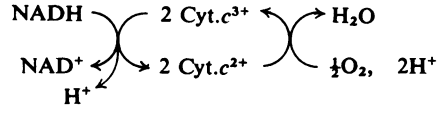


Fig 9. *Proton movements and O_2 uptake associated with rotenone- and antimycin-insensitive cytochrome c-dependent aerobic oxidation of NADH in bovine heart mitochondria*

Mitochondria (1.5mg of protein/ml) were incubated in the reaction mixture described for Expt. (*a*) of Fig. 8. Where indicated 3μM-ferricytochrome *c* (Cyt. c^{3+}) and 200μM-NADH were added. In Expts. (*b*) 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present in the reaction mixture.

Table 3. *Proton-transfer reactions associated with rotenone- and antimycin-insensitive cytochrome c-dependent aerobic oxidation of NADH in bovine heart mitochondria*

Mitochondria (1.5mg of protein/ml) were incubated in the reaction mixture described in the legend to Fig. 8. Final pH was 7.0 and the temperature 25°C. The reactions (see Fig. 9) were started by adding 200μM-NADH in the presence of 3μM-ferricytochrome *c*. The values are means of two experiments. Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Cyt c^{2+}, ferrocytochrome *c*; Cyt. c^{3+}, ferricytochrome *c*.



			+FCCP (1μM)			
H^+ release (ng-ions/ min per mg of protein)	e^- transfer (ng-atoms of O×2/ min per mg of protein)	H^+/e^-	H^+ uptake (ng-ions/ min per mg of protein)	e^- transfer (ng-atoms of O×2/ min per mg of protein)	H^+/e^-	H^+/e^- release in NADH→Cyt. *c*
33.9	56.7	0.6	30	62.6	0.48	0.52

and antimycin-resistant respiratory activity (Fig. 1) (cf. Slater, 1961; Estabrook, 1962). In the presence of valinomycin or valinomycin plus antimycin ferricyanide, produced by aerobic oxidation of ferrocyanide, is re-reduced by endogenous hydrogenated reductants, as shown by a deficit in both ferricyanide production and scalar proton consumption (measured in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) with respect to O_2 reduction. Under these circumstances, and only these, the expected net proton consumption caused by ferrocyanide oxidation is preceded by transient acidification.

Re-reduction of formed ferricyanide and proton release derive from rotenone- and antimycin-resistant oxidation of endogenous reductants through the proton-translocating segments of the respiratory chain situated on the substrate side of cytochrome *c*. The number of protons released per electron flowing to ferricyanide was 3.6 (3.3 in the presence of antimycin) when valinomycin was added to aerobic rotenone-treated mitochondria shortly before ferrocyanide. Under these conditions ferricyanide is probably re-reduced by endogenous NAD(P)H [in the presence of rotenone, mitochondrial NAD and in particular NADP are largely reduced (Klingenberg

& Schollmeyer, 1961; Papa *et al.*, 1967)] with passage of reducing equivalents through the proton-pumping systems of the respiratory chain on the substrate side of cytochrome *c*, whose H^+/e^- value, including the NAD(P) transhydrogenase, is 4 (Mitchell, 1972; Moyle & Mitchell, 1973) or higher than 4 (Brand *et al.*, 1976; Lehninger *et al.*, 1977; Azzone *et al.*, 1977, 1978). The decrease to 1.5 of the number of protons released per electron flowing to ferricyanide, observed upon prolonged aerobic preincubation of rotenone-treated mitochondria with valinomycin, is evidently due to a decrease in the reduction of NAD(P). The detrimental effect exerted by valinomycin preincubation on proton release was partially prevented by the presence of antimycin. The observation that antimycin-insensitive re-reduction of ferricyanide and proton release from mitochondria were both severely depressed by 2-n-heptyl-4-hydroxyquinoline *N*-oxide shows that formed ferricyanide accepts electrons, passing through the protonmotive segments of the respiratory chain, at the level of cytochrome *c* and/or redox component(s) of the cytochrome b–c_1 complex, which are situated on the O_2 side of the antimycin-inhibition site. It should be recalled that 2-n-heptyl-4-hydroxyquinoline *N*-oxide, like antimycin, inhibits electron flow between cytochromes *b* and *c* (Chance & Williams, 1956; Brandon *et al.*, 1972), but also exerts additional inhibitory effects on the cytochrome b–c_1 complex (Izzo *et al.*, 1978).

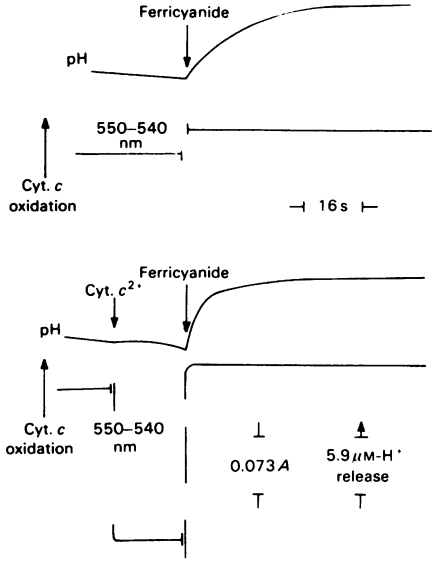


Fig. 10. *Ferricyanide-dependent proton release in KCN-inhibited bovine heart mitochondria*
Mitochondria (3.2mg of protein/ml) were incubated in the reaction mixture described for Fig. 8 Expt. (*a*); 1mM-KCN was also present. Where indicated 50μM-ferricyanide and 10μM-ferrocytochrome *c* (Cyt. c^{2+}) were added.

Dibromothymoquinone, which inhibits ubiquinol oxidation by the cytochrome b–c_1 complex (Melandri *et al.*, 1974), depressed, and duroquinol enhanced, in the presence of antimycin, the proton-release process induced by ferrocyanide respiration. In addition, both analogues enhanced the deficit in proton consumption with respect to O_2 reduction, but lowered the number of protons released per electron flowing to ferricyanide (see Table 1).

Duroquinol, which has a lower affinity for the cytochrome b–c_1 complex than do isoprenic-substituted quinols but is more readily autoxidizable, tends to be directly oxidized by ferricyanide and consequently enhances scalar proton release but not vectorial proton translocation. Dibromothymo-

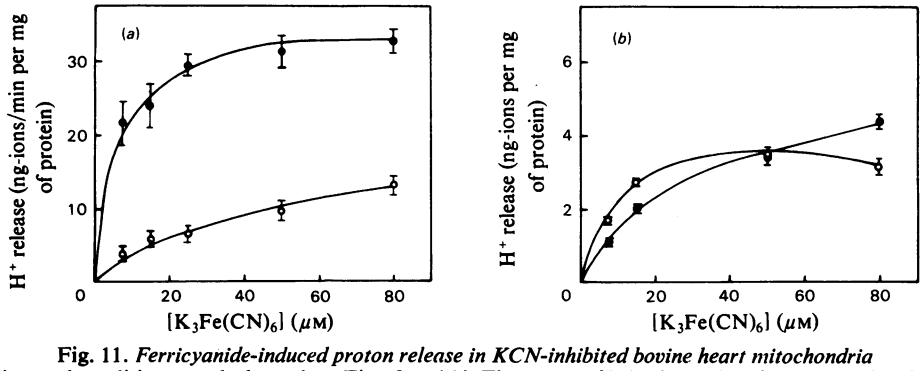


Fig. 11. *Ferricyanide-induced proton release in KCN-inhibited bovine heart mitochondria*
For experimental conditions see the legends to Figs. 8 and 10. The extent of H^+ release (*b*) refers to 60s after ferricyanide addition in the absence (○) and in presence (●) of 10μM-ferrocytochrome *c*. The values represent means ± S.E.M. for five experiments.

quinone suppresses vectorial proton translocation by inhibiting ferricyanide-dependent ubiquinol oxidation through the cytochrome b–c_1 complex as well as by oxidizing endogenous reductants (see Melandri *et al.*, 1974) during the time elapsing between dibromothymoquinone and ferricyanide addition. The enhancement of scalar proton release is probably due to ferricyanide oxidation of dibromothymoquinone, reduced by endogenous hydrogen donors.

It should be noted that conditions favouring re-reduction of formed ferricyanide resulted in a significant increase in the rate of ferrocyanide respiration. It is conceivable that ferricyanide produced by aerobic oxidation of ferrocyanide is accumulated or bound at the surface of the membrane so as to inhibit ferrocyanide oxidation by cytochrome c and be itself effectively reduced by the cytochrome b–c_1 complex. This is not unlikely considering the large ionic shell of the polyvalent ferro(ferri)cyanide complex anion. Independent experiments showed that added ferricyanide inhibits aerobic oxidation of ferrocyanide (M. Lorusso, M. Gutman & S. Papa, unpublished observations). Thus ferrocyanide oxidation by cytochrome c and ferricyanide reduction by the cytochrome b–c_1 complex would both be critically dependent on the local ferrocyanide/ferricyanide concentration ratio. This would explain why ferricyanide produced by oxidation of ferrocyanide is much more effective than added ferricyanide in promoting proton-releasing oxidation of endogenous hydrogenated reductants (Fig. 11; cf. Moyle & Mitchell, 1978).

In the light of the data presented here, it is likely that the proton ejection associated with oxidation of redox mediators such as *NNN'N'*-tetramethyl-*p*-phenylenediamine (Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1978), which by-pass the antimycin inhibition (Lee *et al.*, 1967), results from activation of proton-translocating segments of the respiratory chain on the substrate side of cytochrome c. This would also explain the discrepancies existing for previous measurements of H^+/e^- stoicheiometry for proton release from mitochondria caused by ferrocyanide or *NNN'N'*-tetramethyl-*p*-phenylenediamine, which produced values varying from 1 H^+/e^-(Wikstrom & Saari, 1977; Sigel & Carafoli, 1978) to 2 H^+/e^- (Alexandre *et al.*, 1978).

The net proton consumption caused by aerobic oxidation of exogenous ferrocytochrome c by antimycin-supplemented bovine heart mitochondria was preceded by a rapid scalar proton production, which was included in the predicted stoicheiometry of 1 proton consumed/mol of ferrocytochrome c oxidized. Therefore this scalar proton production, which is different from what is observed with ferrocyanide pulses, cannot be ascribed to oxidation of endogenous hydrogenated carrier(s), and is probably due to displacement of protons from the membrane surface caused by transition of ferrocytochrome c to the oxidized state. This scalar proton displacement could be due to different binding affinities of ferri- and ferrocytochrome c for acidic groups in the mitochondrial membrane (Nicholls, 1974) and might represent the basis (cf. Hinkle, 1978) for the acidification reaction accompanying oxidation of ferrocytochrome c by cytochrome c oxidase inlaid in liposomes (Wikstrom & Saari, 1977; Krab & Wikstrom, 1978).

It is concluded that cytochrome c oxidase, only mediates vectorial electron flow from cytochrome c at the outer side to protons that enter the oxidase from the matrix side of the membrane. In addition to this consumption of protons the oxidase does not mediate vectorial proton translocation. Respiration-linked vectorial proton transport from the mitochondrial matrix to the outer space would derive from proton pumping in the other two segments of the respiratory chain, i.e. NADH–ubiquinone oxidoreductase and ubiquinol–cytochrome c oxidoreductase (Mitchell, 1972; Papa, 1976). The mechanism and H^+/e^- stoicheiometry of proton pumping in these segments of the respiratory chain are still unsettled issues. As things are it seems misleading to draw conclusions on the mechanism of $\Delta\mu_{H^+}$ generation at the third energy-conserving site on the basis of overall H^+/O stoicheiometries measured with succinate and NAD-linked substrates (Lehninger, 1978), a topic that by itself is a matter of controversy (Lehninger *et al.*, 1977; Vercesi *et al.*, 1978; Mitchell & Moyle, 1978; Brand *et al.*, 1978).

It also appears necessary to treat with caution conclusions drawn from measurements of $\Delta\mu_{H^+}$. Sorgato *et al.* (1978) and Sorgato & Ferguson (1978) have shown that the aerobic oxidation of *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate generates, in the presence of antimycin, steady-state membrane potentials and ΔpH in 'inside out' submitochondrial particles, and considered this as evidence in favour of proton pumping by cytochrome oxidase.

It should, however, be pointed out that detection in such a system of a membrane potential is equally consistent with vectorial electron flow or electrogenic proton translocation by cytochrome oxidase. On the other hand the data presented by Sorgato & Ferguson (1978) do not exclude the possibility that the ΔpH generated in the long incubation period used was due to proton production by oxidation of ascorbate and/or $TMPDH^+$ inside the vesicles, or to activation by TMPD of antimycin-insensitive proton-translocating electron flow through other segments of the respiratory chain (Harmon & Crane, 1973).

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