Intramitochondrial Positions of Cytochrome Haem Groups Determined by Dipolar Interactions with Paramagnetic Cations

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E.p.r. (electron-paramagnetic-resonance) spectra of the ferricytochromes were studied in normal and 'nickel-plated' pigeon heart mitochondria and pigeon heart submitochondrial particles. $NiCl_2$ added to either mitochondria or particles was bound completely to the membranes, but none was transported across the vesicles. Hence, any perturbations of the haem e.p.r. spectra by Ni(II) should occur only for those cytochromes in close proximity to the exterior surface. Whenever Ni(II) can approach to within 1 nm of a cytochrome haem, the consequent acceleration of the haem e.p.r. relaxation kinetics should elicit dipolar line broadening. Relaxation acceleration should also increase the incident power level required to saturate the haem e.p.r. signal. In pigeon heart mitochondria, at least three e.p.r. resonances, attributable in part to cytochromes c_1, b_K and b_T, are observed at $g_z = 3.3$, 3.5 and 3.7 respectively. Addition of Ni(II) results in the partial quenching of the $g_z = 3.5$ signal, and increases the saturation power levels for all three cytochrome signals. In submitochondrial particles that are inside-out relative to intact mitochondria, addition of Ni(II) has no measurable effect on the relaxation properties of the $g_z = 3.3$ resonance. In these submitochondrial particles, the peak at $g_z = 3.5$ is missing, and the resonance at $g_z = 3.6$ resolves into two components, neither of which is sensitive to added Ni(II). Addition of free haemin (ferric, a paramagnetic anion) to intact mitochondria elicits the same e.p.r. signal changes as does a preparation of submitochondrial particles. Saturation curves for cytochrome oxidase obtained for e.p.r. spectra of the high-spin form ($g = 6$) and the low-spin form ($g_z = 3.1$) also reveal no effect of Ni(II) on the haem e.p.r. relaxation in either mitochondria or inverted submitochondrial particles. Further, Ni(II) fails to alter the spectra or saturation properties of cytochrome *c* in either mitochondria or submitochondrial particles therefrom. Only with a 50-fold molar excess of Ni(II) can one accelerate the e.p.r. relaxation of cytochrome *c* in aqueous solution, although other more subtle types of magnetic interactions may occur between the cytochrome and either Ni(II) or ferricyanide. Addition of haemin to mitochondria likewise failed to alter the e.p.r. characteristics of either cytochrome *c* or cytochrome oxidase. The present observations strongly suggest that cytochromes b_K, b_T and c_1 reside on the exterior surface of the inner mitochondrial membrane. On the other hand, we find no positive evidence for the location of cytochrome *c* or cytochrome oxidase haem groups within 1 nm of either membrane surface. Because of possible shielding effects from the protein moieties, however, we cannot unequivocally assign the location of the haem groups to the membrane interior. The present results are not inconsistent with the observations of other investigators who used different techniques. However, it is clear that any model of energy coupling in mitochondrial oxidative phosphorylation must account for the positioning of all the $b-c$ cytochrome haem groups on the outside.

The asymmetrical distribution of electron-transfer components within the inner mitochondrial membrane has long been recognized as a crucial factor in

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energy conservation in respiration. Several models of respiratory and photosynthetic energy transduction (Mitchell, 1966, 1975; Boyer, 1967; Slater, 1972; Chance, 1972; Green & Ji, 1972; Green, 1974) incorporate the positions of the electron carriers into their general framework. None of the models necessarily require coincident kinetics through a coupling-site reaction. Nonetheless, the fixed vectoral direction of electron and cation flow is implied,

especially for the chemiosmotic hypotheses (Mitchell, 1966, 1975).

Several investigations in the literature point to the reality of unidirectional proton translocation linked to electron transport. Respiration-linked H^+ extrusion occurs in intact mitochondria (Mitchell & Moyle, 1969), and net proton uptake (Mitchell & Moyle, 1965; Chance & Mela, 1967) in sonicated submitochondrial particles, which are putatively 'inside-out' with respect to intact mitochondria (Ernster & Kuylenstierna, 1969). Analogous work with photosynthetic bacteria (Chance et al., 1970a; Jackson et al., 1968) and higher-plant systems (Jagendorf & Uribe, 1966; Junge & Witt, 1968; Auslander & Junge, 1974) strongly suggests the inward movement of H^+ ions during illumination. In every instance, the polarity of H^+ transport is conserved.

Previous attempts to determine the 'sidedness' of mitochondrial electron-carrier proteins have relied primarily on accessibility studies with probes such as substrates, inhibitors, redox agents or antibodies. In general, the probes must satisfy four conditions in order to be useful and valid in topology studies. The probes must (1) be impermeant, (2) be inert with respect to gross structure, (3) label directly the centre of interest and (4) not interfere in the assays. Also, the ability to isolate mitochondrial preparations which are selectively intact or inverted (Ernster & Kuylenstierna, 1969; Harmon et al., 1974) has rendered mitochondrial membranes fertile ground for all kinds of topology studies. However, the degree of inversion in submitochondrial particles depends on the preparation methods (Astle & Cooper, 1974; Harmon et al., 1974; Tinberg et al., 1974), as does the degree of intactness (Chance et al., 1970b).

The reactivity of cytochrome c in intact mitochondria with $K_3Fe(CN)_6$, for example (Klingenberg & Buchholz, 1970; Tyler, 1970), with cytochrome c peroxidase (Boveris et al., 1972), or with its own antibody (Arion & Wright, 1970; Racker et al., 1971), point to its location on the exterior of the mitochondrial inner membrane. The relative inability of these same agents to interact with cytochrome c in submitochondrial particles (Klingenberg & Buchholz, 1970; Racker et al., 1971) further supports this conclusion.

Extension of these same methods to other electron carriers in mitochondria has also been reported. For instance, a positive antibody reaction with cytochrome c_1 in intact mitochondria has been shown (Schneider et al., 1972). Reaction of cytochrome oxidase with its antibody occurs both in intact mitochondria and in submitochondrial particles (DiJeso et al., 1969; Racker et al., 1970, 1971; Hackenbrock & Hammon, 1975), suggesting that portions of the protein protrude into both aqueous phases. Several investigators (Crane et al., 1956; Lee et al., 1967; Tyler, 1970; Von Jagow & Klingenberg, 1970; Grinius et al., 1971) have observed that utilization of either NADH or succinate by intact mitochondria is not particularly efficient, but that addition of these substrates to submitochondrial particles elicits rapid respiration. The reactive sites of succinate dehydrogenase and NADH dehydrogenase were therefore assumed to lie on the inside surface of the mitochondrial inner membrane. King (1967) confirmed this conclusion on the basis of his isolation procedure for succinate dehydrogenase. However, other work has added some confusion to this picture (Kalina et al., 1972).

Work in several laboratories (Orme-Johnson et al., 1974a; Albracht, 1974; Beinert et al., 1975; Ohnishi, 1973, 1975; Ohnishi et al., 1974a,b) has revealed the existence of several iron–sulphur centres in the NADH dehydrogenase and succinate–cytochrome c reductase regions of the respiratory chain. The proliferation of electron carriers in these large units obviously limits the value of accessibility studies with substrates and oxidants, since the role of iron–sulphur centres in respiration (Gutman et al., 1972; Beinert et al., 1975; Ohnishi, 1975) and energy coupling (Ohnishi, 1976; Case et al., 1976) is only now being learned. Because a detailed investigation of the structural and functional role of the iron–sulphur centres appears in the following paper (Case et al., 1976), it is sufficient to note that studies that concentrate on the localization of the protein moieties of the electron carriers may miss altogether the structural aspects of the various functional groups.

Work in our laboratory with chromatophores from photosynthetic bacteria (Case & Leigh, 1974) has introduced a new approach which should shed light on the positions of the haem groups and the non-haem centres directly. The method, described in the Theoretical section, uses a paramagnetic cation bound to the surface of the membrane as an e.p.r.*-perturbing agent. The criteria for an acceptable e.p.r. probe are similar to those for other probes, except that general binding of the ion to all possible regions of the membrane surface becomes desirable as a means for examining all of the electron carriers. The present report describes the use of two such paramagnetic cations, Ni(II) and Gd(III), and the paramagnetic anion haemin, to locate the positions of the various cytochrome haem groups in the mitochondrial inner membrane. A preliminary report of this work has been given (Case & Leigh, 1975).

Several investigators (Wilson & Leigh, 1972; DerVartanian et al., 1973; Orme-Johnson et al., 1974b; Leigh & Erecinska, 1975) have reported

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* Abbreviations: e.p.r., electron paramagnetic resonance; Mops, 3-(N-morpholino)propanesulphonic acid.

e.p.r. spectra for the various cytochromes in sub-mitochondrial preparations from ox heart and pigeon heart. On the basis of reductive titrations with ascorbate and dithionite, Orme-Johnson et al. (1974b) assigned the e.p.r. resonance at $g_z = 3.8$ to cytochrome b_T*, that at $g_z = 3.5$ to cytochrome b_K, and that at $g_z = 3.3$ to cytochrome c_1. E.p.r. line-shape changes induced by antimycin A were observed for the $g_z = 3.5$ and 3.8 resonances, confirming their identification as b cytochrome signals in ox heart electron-transport particles (DerVartanian et al., 1973).

Potentiometric titrations with submitochondrial particles (Leigh & Erecinska, 1975) have revealed a $g_z = 3.7$ resonance corresponding to cytochrome b_T, and a $g_z = 3.3$ peak reflecting both cytochrome b_K and cytochrome c_1. The observed peak positions were found to be mutually exclusive between the ox heart and pigeon heart systems (Leigh & Erecinska, 1975; Orme-Johnson et al., 1974b). However, none of the original studies was carried out with intact mitochondria, so that any structural alterations which might have resulted from the preparation of the submitochondrial particles were not examined. The present investigation shows that sonication of mitochondria does indeed create structural and functional changes in the membranes. These altera-tions are reflected in the e.p.r. spectra of the b-type cytochromes, which one can mimic by the addition of free haem. Other structural rearrangements which occur during the preparation of submitochondrial particles, such as changes in the environment of certain iron–sulphur centres (Ohnishi, 1975) are examined in the following paper (Case et al., 1976).

Theoretical

The introduction of a paramagnetic species close to an electron carrier will cause magnetic dipole interactions which result in perturbations of the e.p.r. spectrum of the electron carrier. Other types of spin–spin interactions, such as exchange effects, can be superimposed on the dipolar interactions;

* Cytochrome b_T is the designation assigned to the b-type cytochrome in mitochondria with the reduced-minus-oxidized spectral maximum in the α-band at 566nm, and whose midpoint redox potential (E_m) varies with the mitochondrial energy state (Wilson & Dutton, 1970; Chance, 1972; Dutton & Wilson, 1974). It is approximately equivalent to cytochrome b_L as defined by Wikström (1973) and Mitchell (1975). Cytochrome b_K is the designation assigned to b-type cytochrome in mito-chondria with the reduced-minus-oxidized spectral maximum in the α-band at 562nm, and whose E_m is relatively independent of the mitochondrial energization state (Wilson & Dutton, 1970; Chance, 1972; Dutton & Wilson, 1974). It is approximately equivalent to cyto-chrome b_R as defined by Wikstrom (1973) and Mitchell (1975).

however, with membrane proteins dipolar effects should dominate, since exchange interactions are operative only between directly bonded centres. Depending on the position and nature of the relaxa-tion rate of the added probe, the dipolar interaction should appear either as an e.p.r. line 'splitting' (or shift), or as an e.p.r. line broadening and relief from power saturation.

The same kind of magnetic dipole interactions involving paramagnetic nuclei have been discussed extensively by several workers (Eisinger et al., 1962; Mildvan & Cohn, 1970) with regard to effects on the n.m.r. relaxation of biochemically important systems. Although many of the theoretical and experimental treatments now used for n.m.r. relaxation (see Mildvan & Cohn, 1970) can be translated directly to e.p.r. relaxation, several important differences in the experimental conditions (solid versus liquid states, temperature effects, oriented samples etc.) affect both the predicted and observed magnetic dipole effects which are possible.

The presence of a single immobile 'non-relaxing' ion in the vicinity of the membrane-bound electron carrier should elicit dipolar splitting such that:

$$H - H_0 = \pm \mu_{\text{eff}}.(1 - 3\cos^2\theta)/r^3 \qquad (1)$$

where H and H_0 are the peak resonance magnetic fields in the presence and absence of the added probe, μ_{eff}. is the effective magnetic moment of the probe, r is its distance from the electron carrier and θ is the angle subtended by the line connecting the two paramagnetic centres and the applied magnetic field (Slichter, 1963; Carrington & McLachlan, 1967). Dipolar splitting is expected for conditions in which the electron 'sees' the added ion as a permanent magnet, i.e. an ion whose spin-lattice relaxation time, τ_1, is long compared with τ_i for the electron carrier. Fig. 1(a) shows the predicted maximum e.p.r. line splittings as a function of distance, for the electron carrier.

With randomly oriented frozen suspensions at very low temperatures, such that $\cos^2\theta$ is averaged over all θ and $1/\tau_1$ approaches zero, a Pake line-shape should be observed with peak separations of $2\mu_{\text{eff}}./r^3$ (Pake, 1948). If the added probe can bind to numerous membrane sites which contribute appreciably to this effect and which occur over a range of values of r, the averaging of the several Pake lines should give rise to inhomogeneous line broadening (Poole, 1967). This broadening phenomenon differs both theoretically and experimentally from homogeneous (or relaxa-tion) broadening discussed below. Also, higher temperatures, at which $1/\tau_1$ of the probe is fast compared with the resonance (frequency) shift, should elicit diminution of the signal amplitude (Leigh, 1970). All of these effects can, in principle, be exploited to calculate distances between components.
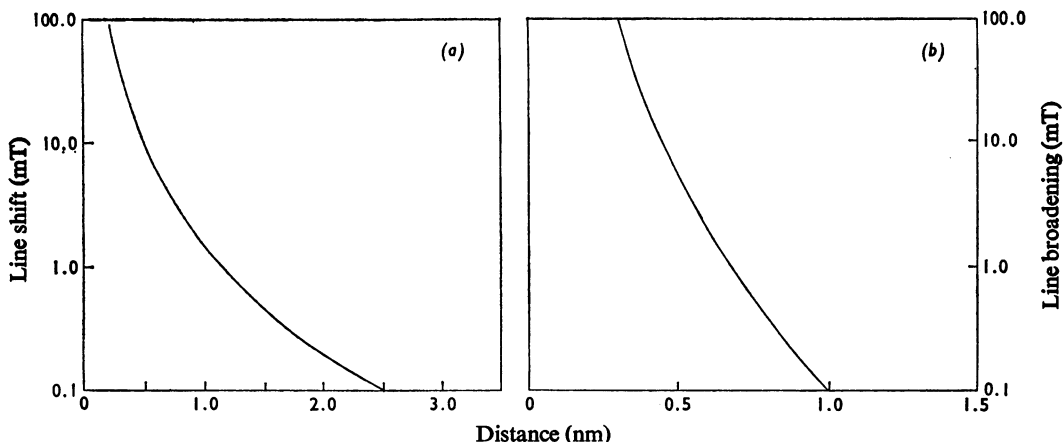
Fig. 1. *Theoretical distance-dependence for e.p.r. dipolar interactions*

(a) Line shift or line splitting from eqn. (1), for $\theta = 0$. (b) Line broadening from eqn. (2), for $\tau_c = 1\,\mathrm{ns}$.

Under conditions in which $1/\tau_1$ of the added ion is rapid $[1/\tau_1 > \gamma(H - H_0)$ where γ is the gyromagnetic constant], or in which spin exchange among the ions is very rapid, the dipolar splittings collapse. Hence the result is then the homogeneous acceleration of both the spin–lattice relaxation rate $(1/\tau_1)$ and the spin–spin relaxation rate $(1/\tau_2)$ for the electron carrier. In this case, the dipole interaction can be described by the general Redfield equations:

$$1/\tau_{2M} \propto \left(\frac{\mu_{\mathrm{eff.}}^2}{\gamma h}\right)\left(\frac{1}{\bar{r}^6}\right)\left(\tau_c + \frac{\tau}{1 + (2\pi\nu)^2\,\tau_c^2}\right) = \Delta H \quad (2)$$

and

$$1/\tau_{1M} \propto \left(\frac{\mu_{\mathrm{eff.}}^2}{\gamma h}\right)\left(\frac{1}{\bar{r}^6}\right)\left(\frac{\tau_c}{1 + (2\pi\nu)^2\,\tau_c^2}\right) \quad (3)$$

Here, ν is the microwave resonance frequency, $\bar{r}$ is the average distance between the ions and the electron carrier, ΔH is the observed e.p.r. line broadening, τ_c is the 'correlation time', which under the present conditions is assumed to be τ_1 of the added ion, and h is Planck's constant (compare with analogous equations for n.m.r. relaxation given by Mildvan & Cohn, 1970). Fig. 1(b) gives the predicted distance (r)-dependence for e.p.r. line broadening (Slichter, 1963).

If the e.p.r. resonance line is sharp, with a Lorentzian shape (therefore homogeneous), one can calculate τ_1, in principle, from the microwave power saturation curve according to the Bloch equation:

$$\text{Signal} \propto H_1\left(\frac{\mathrm{d}\chi''}{\mathrm{d}H}\right) = N_0 \frac{H_1}{1 + \frac{\gamma^2 H_1^2 T_2 T_1}{4}} \quad (4)$$

where $H_1(\mathrm{d}\chi''/\mathrm{d}H)$ is the derivative absorption, H_1^2 is proportional to the incident microwave power, and N_0 is the number of spins in the measuring path (Poole, 1967; Carrington & McLachlan, 1967). At the power level which elicits maximum signal amplitude, eqn. (4) reduces to:

$$\gamma^2 H_1^2 T_2 T_1 = 4 \quad (5)$$

Hence the incident power level at which saturation begins is strictly a function of the relaxation times τ_1 and τ_2. All concentration (redox) terms and associated artifacts which affect the signal amplitude fall out when the signal becomes saturated.

Calculation of τ_1 values from the positions of the saturation maxima (eqns. 4 and 5) requires an accompanying knowledge of both H_1 and τ_2. Whereas τ_2 can be determined readily for a homogeneous line from its linewidth, H_1 must be calculated separately, or else calibrated from the saturation curve of a substance whose τ_1 and τ_2 values are known.

Fig. 2 shows the e.p.r. spectra and saturation curves for methaemoglobin in a 50%-glycerol glass, in the presence and absence of added Gd(III). Stoicheiometric addition of $GdCl_3$ (1:1 with haem) results in the loss of approx. 80% of the e.p.r. signal at $g = 6$. That which remains is broadened from 3.3 mT (33 G) to 5 mT (50 G), and no longer saturates as one increases the microwave power beyond 100 mW. Consequently, dipolar interactions between paramagnetic ions and biological macromolecules appear as line broadening and desaturation, even if τ_1 for the added ion (free solution; Reuben & Cohn, 1970; Poole, 1967) is longer than τ_1 for methaemoglobin.
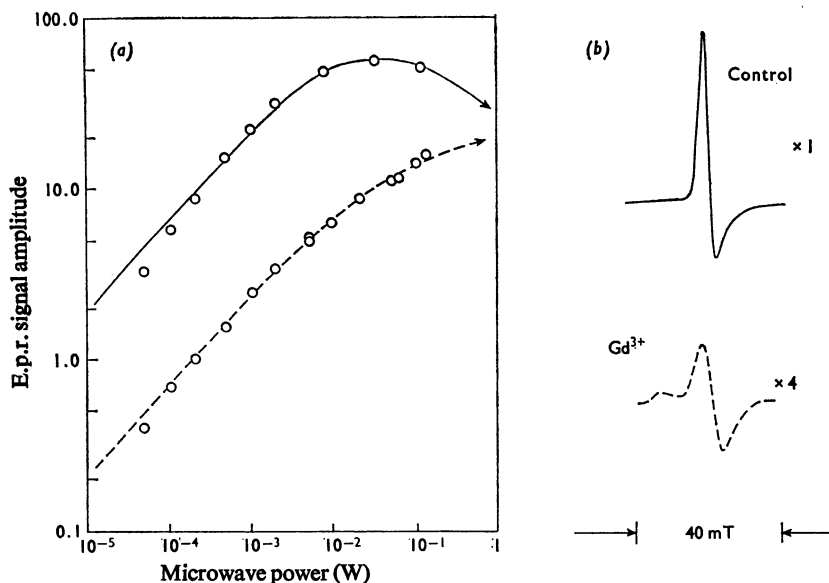
Fig. 2. *Effect of Gd(III) on the e.p.r. spectrum and saturation curve of methaemoglobin*

Methaemoglobin in aq. 50% glycerol containing 0.01M-Tris and 0.01M-Mops, pH7.6, was frozen into a glass. For the spectrum (b), the e.p.r. conditions were: 10mW power, 1mT modulation amplitude, 9.13GHz frequency, 4.2K. For the saturation curve (a), the e.p.r. conditions are the same except for the power, displayed on the abscissa as a logarithmic scale. The signal amplitude is in arbitrary units, shown on the ordinate as a logarithmic scale. ———, No additions. The arrow at the maximum of this curve indicates the power level at which one can solve eqn. (5) for either τ_1 or H_1^2. Given a $\tau_1 = 2\,\mu s$ at 4.2K (Scholes *et al.*, 1971; confirmed in this laboratory by other procedures), 20mW power is equivalent to $H_1^2 = 2.5 \times 10^{-9}$ (T)^2 to within a factor of 2. ----, +100mM-GdCl$_3$. Substitution of 2mM-NiCl$_2$ for GdCl$_3$ gave results which were intermediate between the two sets of curves.

Experimental application of eqns. (2)–(5) to dipole effects in membranes is complicated by several theoretical and experimental drawbacks. If τ_c is not precisely known, for instance, exact distance calculations are not possible. The Gd(III) anomaly in the experiment shown in Fig. 2 suggests that τ_1 of the added ion (τ_c) bound to a macromolecule may be much shorter than τ_1 for the free ion in solution. In addition, there may be a whole range of distances. Further, τ_1 cannot be calculated for an electron carrier whose e.p.r. line-shape is inhomogeneous. Because many of the factors which are built into eqns. (2)–(5) contain large uncertainties when applied to electron carriers, attempts at rigorous calculations of r may give erroneous values. However, errors in the relaxation times as large as two orders of magnitude, which are remotely possible in the calculations or the measurements, would affect r by a factor of less than 2.5. Since the primary purpose of this study is to determine whether an electron carrier is located on one side or the other of a membrane which is some 5–10nm thick (Fleischer *et al.*, 1967; Vanderkooi, 1972), errors in r up to 2nm should therefore have little effect on the final result!

Hence, any observable magnetic effects of Ni(II) or Gd(III) on the electron carriers should definitely demonstrate their exterior location, provided that the perturbing probes remain on the outer surface.

Because the work in the present report concerns itself with e.p.r. relaxation kinetics, all of the e.p.r. spectra are recorded at temperatures colder than those customarily used by other investigators (Wilson & Leigh, 1972; DerVartanian *et al.*, 1973; Orme-Johnson *et al.*, 1974b; Leigh & Erecinska, 1975). Lowering the temperature serves to sharpen a homogeneous e.p.r. line and to increase its amplitude. At the same time, power saturation should occur at lower power levels, and increasing the power level beyond the saturation maximum should result in smaller signals. For an inhomogeneously broadened e.p.r. resonance, lowering the temperature should have no effect on the linewidth or the signal amplitude at low power levels. Power saturation will also occur, at lower power levels as one lowers the temperature but the signal amplitude of an inhomogeneously broadened line does not decrease as one raises the incident power beyond the saturation maximum. Instead, the amplitude remains at the plateau value.

Although all of the cytochrome e.p.r. spectra studied thus far appear to be homogeneous (Case & Leigh, 1974), some of the e.p.r. spectra of ubiquinone and iron–sulphur centres, such as centre N-2 in mitochondria (Case et al., 1976), exhibit line-shapes and saturation profiles characteristic of inhomogeneous broadening.

One additional magnetic effect which may be encountered in studies of this type involves the transmission of energy along a chain of spins. The relaxation process which results is sometimes referred to as 'spin-diffusion'. One can detect the occurrence of spin-diffusion as an identical τ_1 value for large groups of spins (Seiter & Chan, 1973). Its existence apart from other relaxation mechanisms experimentally requires intimate contact (i.e. binding) with a neighbouring spin, and probably does not occur over distances greater than 0.3–0.4nm in the absence of binding. If a long chain of susceptible spins is present, spin-diffusion might be operational over much longer distances. As far as is presently known, spin-diffusion does not exist in solution e.p.r. samples except at very high concentrations. However, in a case such as Ni(II) ions frozen in a suspension of mitochondria or submitochondrial particles, the accumulation of Ni(II) on the membrane surfaces could lead to Ni(II) relaxation by spin-diffusion. This effect is actually beneficial for the purposes of this study, by converting the Ni(II) ions from point sources of relaxation into a planar source. On the other hand, spin-diffusion between Ni(II) and the electron carriers, or among the different electron carriers, could induce long-range changes in the e.p.r. relaxation kinetics of the electron carriers if the phenomenon occurs in the mitochondrion, and thereby obscure our results. In view of the absence of other magnetic interactions among the electron carriers which should exist concurrently, the wide variation in relative τ_1 values (Case et al., 1976), and the relatively long distances between centres, the occurrence of spin-diffusion, although possible, does not appear likely.

Experimental

Pigeon heart mitochondria were prepared by the method of Chance & Hagihara (1963), and sonicated submitochondrial particles by the procedure of Lindsay et al. (1972). EDTA was omitted from the final wash in all cases. For some experiments, both preparations were generously given by Dr. Tomoko Ohnishi (Ohnishi, 1975). Haemin chloride and Mops were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; GdCl$_3$ was from Alfa Inorganics, Danvers, MA, U.S.A., and NiCl$_2$ from B and A, Morristown, NJ, U.S.A. All other organic and inorganic chemicals were reagent grade and were used without further purification.

Binding and transport of both Ni(II) and Gd(III) were followed spectrophotometrically with the indicator murexide (Mela & Chance, 1968; Scarpa, 1972). Because of its very long correlation time and its excessively tight binding to murexide, Gd(III) binding and transport were also monitored by means of water-proton n.m.r. relaxation enhancements (Reuben & Cohn, 1970; Case, 1975). Protein was measured by the biuret method (Gornall et al., 1949).

For the potentiometric titrations, at least 60mg of mitochondrial or submitochondrial protein/ml was suspended in an anaerobic cuvette under Ar gas. No mediator dyes were used. Adjustment of the redox potential (E_h) and the transfer of samples into e.p.r. tubes were performed by established procedures (Wilson & Leigh, 1972; Leigh & Erecinska, 1975; Ohnishi, 1975). In order to assure rapid freezing, the tubes were immersed in a eutectic mixture of methylcyclohexane and isopentane (1:5, v/v) which is liquid at 81K. All samples were frozen within 1min or less after addition of either Ni(II) or Gd(III) in order to preclude any possibility of transport.

E.p.r. spectra were obtained on a Varian E4 spectrometer which was linked to a Nicolet 1074 computer. Scan speeds were typically 2–8min, with instrument time-constants of 0.1–1.0s. The temperature was controlled by a variable-temperature cryostat (Air Products, model LTD-3-110) attached to the liquid-helium transfer line. The sample temperature was monitored continuously with a 56Ω carbon resistor placed between 1cm and 2cm below the e.p.r. cavity. Because the E-4 spectrometer has a linear detector, the output signal is proportional to $H_1(d\chi''/dH)$. At very low power (<0.2mW), deviations from linearity are frequently observed with the E-4 detector such that signal amplitudes may appear lowered. These deviations do not affect the power saturation curves overall.

Results

Absence of paramagnetic cation transport

One of the requirements for the validity of the present e.p.r. relaxation experiments is the lack of uptake of either Gd(III) or Ni(II) under the conditions used for the e.p.r. measurements. Previous work has shown that significant uptake of Gd(III) by intact mitochondria (Case, 1975) or by intact Chromatium chromatophores (Case & Leigh, 1974) occurs regardless of the presence or absence of an energy source. However, the kinetics are sufficiently slow in both cases so that with an addition of ≤1nmol/mg of protein no more than 5% of the Gd(III) is removed from external water within the first 1min. Addition of Gd(III) in amounts exceeding 10nmol/mg of protein accelerates the rate of uptake (Fig. 3a). Similar uptake kinetics are observed for Gd(III) in submitochondrial particles.
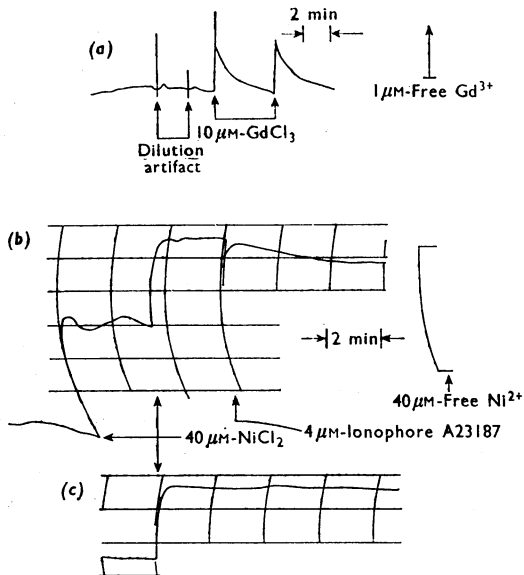
Fig. 3. *Uptake kinetics of Gd(III) and Ni(II) in mitochondria and submitochondrial particles*

Spectrophotometric analysis at 540nm minus 507nm in the presence of murexide (Scarpa, 1972). Calibration was performed by addition of $GdCl_3$ or $NiCl_2$ to murexide solution in the absence of protein. Ordinate is the absorbance difference at the two murexide wavelengths, and is equivalent to cation concentrations according to the calibration curves at the right. The time-scale for curve (a) is given at the top; that for curves (b) and (c) in the centre portion. (a) Pigeon heart mitochondria (1 mg of protein/ml) was present in a solution containing 0.05 mM-murexide, deionized 0.25 M-mannitol, 0.02 M-Tris and 0.02 M-Mops (pH 7.4). (b) Pigeon heart mitochondria (1 mg of protein/ml) were present with 0.1 mM-murexide, 0.1 M-sucrose, 10 mM-sodium succinate and 0.1 M-Mops (pH 7.4). (c) Pigeon heart submitochondrial particles (1 mg of protein/ml) were present with 0.1 mM-murexide, 0.1 M-KCl and 0.01 M-Mops (pH 7.4).

Earlier work (Case, 1975) also suggests that Mn(II) transport in mitochondria is somehow damaged during the isolation (Chance & Hagihara, 1963). No Mn(II) uptake is observed even after massive additions of the ion. Separate measurements of Ni(II) binding and transport (Figs. 3b and 3c) appear to show that Ni(II) behaves like Mn(II) and is not taken up by either mitochondria or particles. The downward drift after addition of ionophore A23187 (Fig. 3b) shows that the amount of Ni(II) transported by the mitochondria is insufficient to oppose the ionophore-mediated diffusion of Ni(II). Nevertheless, transition-metal ions do bind to both mitochondria and particles in copious quantities
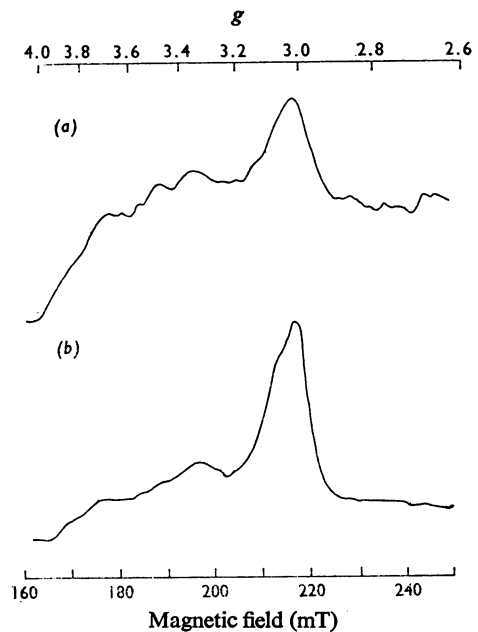
Fig. 4. *E.p.r. spectra of pigeon heart mitochondrial cytochromes*

Pigeon heart mitochondria (90 mg of protein/ml) were incubated at 0°C in the presence of 0.25 M-sucrose, 0.02 M-Mops (pH 7.4) and 40 μM-rotenone. $K_3Fe(CN)_6$ (0.1 mM) was added aerobically to oxidize all of the cytochromes. E.p.r. conditions were: 5 mW power, 2.5 mT modulation amplitude, 9.135 GHz frequency. Spectra are the average of 16 scans at 7.5 K. (a) Control; (b) +2 mM-NiCl_2. Similar spectra were observed for anaerobic samples at controlled redox potential.

(Lehninger et al., 1967; Romslo & Flatmark, 1973; Case, 1975). Both of these observations are fortunate, since they entail the close proximity of an excess of ions to the external surface of the mitochondrial or submitochondrial membranes, without allowing the ions to penetrate into the interior regions of the vesicles. Had the mitochondrial and submitochondrial preparations been fully active with respect to energy-linked bivalent cation transport (Scarpa & Graziotti, 1974), the present study would have been impossible.

Ferricytochrome e.p.r. spectra in mitochondria

Fig. 4 presents typical e.p.r. spectra of the low-spin ferricytochromes in mitochondria in the presence and absence of added $NiCl_2$. The large peak at $g = 3.0$ is due to cytochrome c, with the low-field shoulder ($g_z = 3.1$) arising from a low-spin form of cytochrome oxidase (Wilson & Leigh, 1972). In addition, smaller
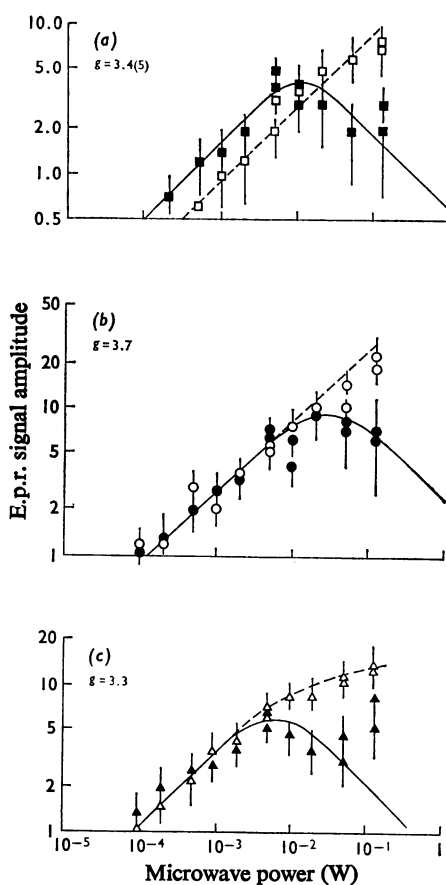
Fig. 5. *E.p.r. saturation curves for cytochromes in intact mitochondria*

Conditions were as in Fig. 4, except that the microwave power was varied. Both scales are logarithmic. Temperature was 7.5K. The determination of signal amplitudes assumed that baselines were straight throughout the region of each resonance peak. This was, in fact, observed for similar spectra in which the cytochromes are reduced. Since the e.p.r. signal at $g = 4.3$ (off scale to the left, Fig. 4) overlaps with some of the cytochrome peaks, and, since its magnitude differs significantly with the cytochrome redox states, spectra obtained under the two sets of conditions are not entirely comparable. The error bars associated with the points represent uncertainties in the assessment of baselines as well as the 'noise' levels in the spectra, at the 95% confidence limit. ●, ▲, ■, No bivalent cations added; ——, fits to eqn. (4) for the control points. ○, △, □, +2mM-NiCl₂; – – – –, fits to eqn. (4) for data in which Ni(II) is present. (a) Cytochrome b_K. (b) Cytochrome b_T. (c) Cytochrome c_1. The curve for cytochrome c_1 (control) is based on data from separate experiments in which the signal amplitude decrease at high microwave power was more clearly observed.

resonances are observed in both cases at $g_z = 3.3$ and 3.7 (cytochromes c_1 and b_T respectively; Orme-Johnson *et al.*, 1974*b*; Leigh & Erecinska, 1975). As Fig. 4 also shows, there is a clearly resolved resonance at $g_z = 3.5$, which is diminished in amplitude in the presence of Ni(II). There may also be present in both spectra a poorly resolved component at $g = 3.8+$ (Fig. 4), which does not saturate even at 100mW power and 4.5K. If previously reported assignments of these e.p.r. spectra are correct (DerVartanian *et al.*, 1973; Orme-Johnson *et al.*, 1974*b*), the $g_z = 3.5$ peak should reflect cytochrome b_K. On the other hand, Leigh & Erecinska (1975) reported that the cytochrome b_K resonance in pigeon heart submitochondrial particles is superimposed on the $g_z = 3.3$ peak of cytochrome c_1, and does not appear at the same g values as does the resonance of b_K in the ox heart systems. The reasons for the apparent discrepancy are resolved below. The origin of the 'bump' at $g = 3.8+$ in intact mitochondria (Fig. 4) is not known, although it could arise from a special state of a b-type cytochrome. It, along with the other three resonances between $g = 3.3$ and 3.9, has also been detected in intact mitochondria from rat liver (C. Saronio & J. Salerno, unpublished work) as well as in intact mitochondria.

The addition of Ni(II) to intact mitochondria elicits no obvious alteration in the e.p.r. spectra, except for the signal decrease at $g_z = 3.5$. However, these resonances are already so broad that linewidth changes as large as 0.001T could easily remain unnoticed. Changes in the microwave power saturation curves, on the other hand, should still reveal magnetic interactions between the haem groups and Ni(II) in a sensitive fashion. Fig. 5 shows the effects of Ni(II) on the power-dependence of the e.p.r. absorptions for cytochrome b_K (a), b_T (b) and c_1 (c). All three haem signals rise, reach a peak and then fall as one increases the incident power, as expected for the saturation of homogeneous e.p.r. lines (Poole, 1967). However, the presence of Ni(II) shifts the positions of the saturation maxima towards higher power levels, indicating an acceleration of τ_1, for all three cytochromes. The effect of Ni(II) on the cytochrome b_K spectrum is so dramatic that one also observes signal quenching at the lower power levels in addition to the shift in the saturation maxima. Had the spectra of Fig. 4 been recorded at 100mW instead of 5mW, the addition of Ni(II) would have enlarged all three cytochrome peaks.

The data of Fig. 5 offer positive evidence of a magnetic interaction between Ni(II) and cytochromes b_K, b_T and c_1. Substitution of Gd(III) for Ni(II) in the experiments of Figs. 4 and 5 gives essentially the same results, suggesting that the magnetic effect is truly dipolar in character. Hence, present data argue that all three of these cytochromes are located on the exterior surface of the mito-
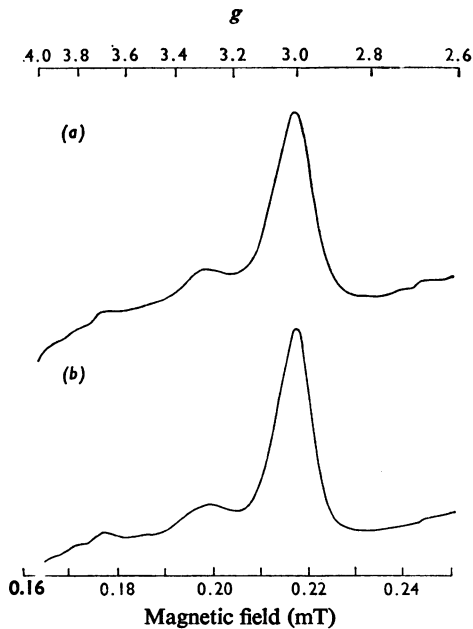
Fig. 6. *E.p.r. spectra of cytochromes in pigeon heart submitochondrial particles*

Pigeon heart submitochondrial particles (80mg of protein/ml) were incubated with 0.25M-sucrose and 0.02M-Mops (pH7.4) under Ar atmosphere. No inhibitors were present. The redox potential (E_h) was adjusted with $K_3Fe(CN)_6$. E.p.r. conditions were as in Fig. 4, except that spectra are averages of four scans at 7.5K. (a) Control, $E_h = +360$mV; (b) +2mM-NiCl$_2$, $E_h = +375$mV.
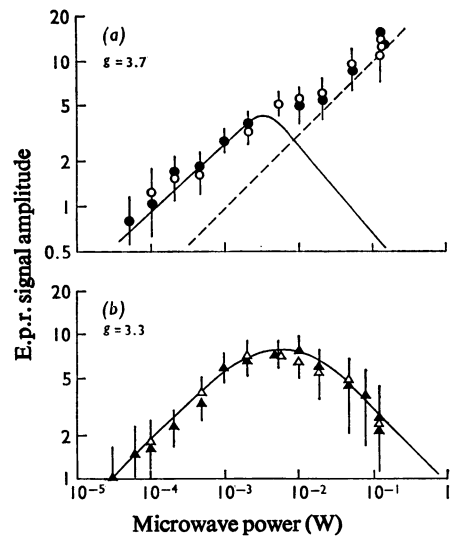

Fig. 7. *E.p.r. saturation curves for cytochromes in submitochondrial particles*

Conditions were as in Fig. 6, except that the microwave power was varied. Both scales are logarithmic. Temperature was 7.5K. Error bars represent uncertainties as in Fig. 5. ●, ▲, No bivalent cations added; ○, △, +2mM-NiCl$_2$. (a) Cytochrome b_T. —— and ----, Theoretical saturation curves from eqn. (4), the sum of which fits the points. (b) Cytochrome c_1. Leigh & Erecinska (1975) have reported that this resonance might also contain a cytochrome b_K component which is not resolved. —— represents a fit to eqn. (4) for both sets of points. No e.p.r. signal attributable to cytochrome b_K was observed at any power level.

chondrial inner membrane, with the haem groups exposed to bulk water.

Ferricytochrome e.p.r. spectra in submitochondrial particles

The converse experiments to those in Figs. 4 and 5 are similar, but use a submitochondrial preparation in which the membranes are turned inside-out (Ernster & Kuylenstierna, 1969; Lindsay et al., 1972). In such a case, Ni(II) should now exert no effect whatsoever on the cytochrome e.p.r. spectra or saturation curves. Fig. 6 gives the e.p.r. spectra of the cytochromes in submitochondrial particles in the presence and absence of Ni(II). Only two resonances, at $g_z = 3.3$ and 3.7, are observed in these particles, in agreement with earlier observations from this laboratory (Leigh & Erecinska, 1975). Note the total absence of the resonance at $g_z = 3.5$. According to Leigh & Erecinska (1975), the $g_z = 3.3$ peak now resolves potentiometrically into two species, one of which has $E_m = +100$mV. The power saturation

curve for this spectrum (Fig. 7b) resembles that for a well-behaved Lorentz line (Poole, 1967). Consequently, if two species give rise to this e.p.r. line, then both must possess identical e.p.r. relaxation times.

If one now examines the power saturation curve of the $g_z = 3.7$ signal, a biphasic curve is obtained, which suggests that at least two different haem species contribute to this resonance. The species which saturates at 1–2mW power, moreover, is not cytochrome b_T, since saturation of the latter requires at least 5–10 times as much power in intact mitochondria (Figs. 5 and 7). Evidently, cytochrome b_T interacts magnetically with some other haem species in submitochondrial particles which is not present in intact mitochondria. Addition of Ni(II) results in no further perturbation of either the $g_z = 3.3$ or 3.7 signal (Figs. 6 and 7). Hence these haem centres appear to be too far away from Ni(II), and therefore water, for any significant magnetic interaction in particles.
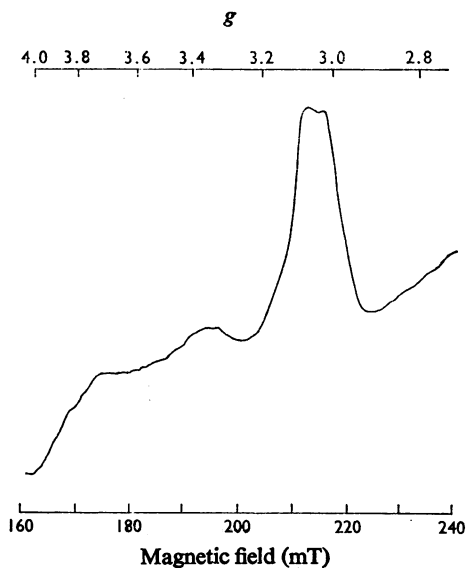
Fig. 8. *E.p.r. spectrum of cytochromes in intact mitochondria in the presence of free haemin*

Conditions were as in Fig. 4, except that $100 \mu M$-haemin chloride was added. Temperature was 7.5 K. The resulting spectrum is the average of eight scans.
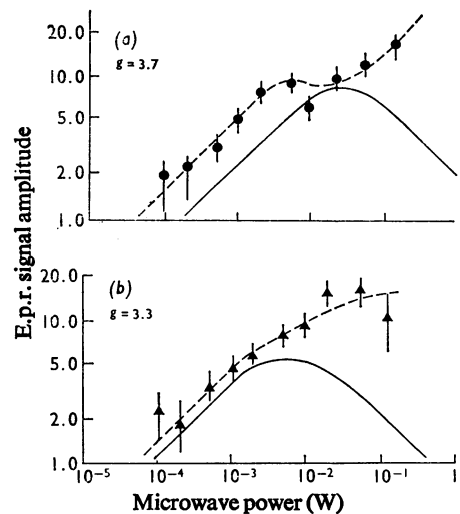

Fig. 9. *E.p.r. saturation curves for cytochromes in intact mitochondria with added haemin*

Conditions were as in Figs. 5 and 8; $100 \mu M$-haemin chloride was present, but no bivalent cations. Temperature was 7.5 K. Both scales were logarithmic. Error bars represent uncertainties as in Fig. 5. (*a*) Cytochrome b_T. ——, Theoretical saturation curve of eqn. (4) fit to the control data points in Fig. 5(*a*) for intact mitochondria. ————, Sum of the theoretical saturation curves fit to data of Fig. 7(*a*) for submitochondrial particles. (*b*) Cytochrome c_1. ——, Theoretical saturation curve fit to the control data points in Fig. 5(*c*) for intact mitochondria. ————, Theoretical saturation curve fit to ▲. No e.p.r. signal attributable to cytochrome b_K could be observed in this preparation at any power level.

What has happened to cytochrome b_K? If it is still present in submitochondrial particles, its e.p.r. spectrum is either buried in the cytochrome c_1 spectrum, or else broadened beyond recognition by whichever species relaxes cytochrome b_T.

The results of the experiments in Figs. 8 and 9 provide some clues to the *b*-cytochrome mystery. Addition of free haem (ferrihaemin chloride) to intact mitochondria results in the e.p.r. spectrum in Fig. 8. The peaks which emerge are identical with those observed in submitochondrial particles to which no free haem has been added (Figs. 6 and 7). Further, the saturation curve for the $g_z = 3.7$ resonance (Fig. 9*a*) now resolves into two components, one of which saturates at 1–2 mW (compare with Fig. 7*a*) and the other of which does not saturate at all. Evidently, addition of free haem to mitochondria perturbs cytochrome b_T, and obliterates the cytochrome b_K spectrum, as does sonication of the mitochondria. Further, addition of free haem to mitochondria accelerates the e.p.r. relaxation of cytochrome c_1, whereas preparation of submitochondrial particles has no effect. Since free haem is a permeant paramagnetic anion (Brault & Rougee, 1974), its magnetic interactions with the b_K, b_T and c_1 cytochrome haem groups is expected and to some extent observed to resemble those induced by Ni(II).

The possible liberation of a free haem group during sonication of mitochondria could explain the similarities between the e.p.r. data of Figs. 6 and 7 and the data in Figs. 8 and 9.

Cytochrome c and cytochrome oxidase

Fig. 10 presents a typical saturation curve for cytochrome *c* in intact mitochondria. As one increases the incident microwave power, the $g_z = 3.0$ resonance amplitude reaches a plateau at approx. 5 mW, and then begins to decrease. The presence of 20–40 nmol of Ni(II)/mg of protein has no detectable effect on the relaxation properties of bound cytochrome *c* (Fig. 10). Similar experiments with submitochondrial particles give essentially identical results, underscoring the inability of Ni(II) to perturb the *c*-haem relaxation in either system.

One explanation which reconciles the absence of a magnetic effect of Ni(II) on the e.p.r. relaxation data for cytochrome *c* (Fig. 10) with previous work on the
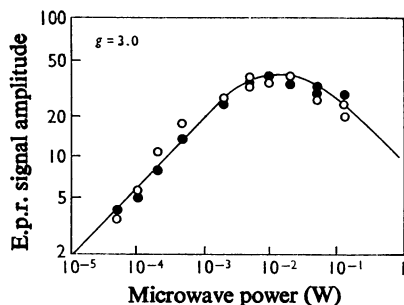
Fig. 10. *E.p.r. saturation curves for cytochrome c in intact mitochondria*

Conditions were as in Figs. 4 and 5. The signal amplitude at $g_z = 3.0$ was determined as a function of microwave power. Both scales are logarithmic. ●, Control; ○, +2 mM-$NiCl_2$. ——, Theoretical saturation curve fit to both sets of data points.

Fig. 11. *Effect of Ni(II) on cytochrome c e.p.r. spectra and relaxation*

Ferricytochrome c (Sigma) was dissolved in 0.01 M-Tris and 0.01 M-Mops (pH 7.6) and frozen. Cytochrome concentration was 3.5 mM. E.p.r. conditions were as in Fig. 2, except that the temperature was 6.5 K. (*a*) Control, no additions; (*b*) 0.16 M-$NiCl_2$ was added before freezing.

positions of the protein in mitochondria (Racker *et al.*, 1970, 1971; Boveris *et al.*, 1972) involves the structure of the cytochrome c molecule. Because the effective dimensions of cytochrome c are 3.0 nm × 3.4 nm × 3.4 nm (Dickerson *et al.*, 1971), giving an 'ion-excluding radius' of 1.8 nm, one should not expect any measurable effect of Ni(II) on its e.p.r. relaxation kinetics (cf. Fig. 1*b*). However, the cytochrome c molecule also possesses a cleft into which water can approach to within 0.4 nm of the haem ring (Dickerson *et al.*, 1971). At a distance of $\bar{r} = 0.4$ nm, the magnetic dipole interactions should give approx. 0.005–0.010 T line broadening, which should be easily seen (Fig. 1*b*).

Several investigations (Dickerson *et al.*, 1971; Vanderkooi *et al.*, 1973) have suggested that the amino acid sequence in this 'cleft' region contains a net positive charge, so that bivalent cations such as Ni(II) might ordinarily be repelled. Separate e.p.r. experiments with soluble cytochrome c suggest that this may indeed by the case. If one freezes cytochrome c solutions in the presence of a 50-fold molar excess of Ni(II), the resulting e.p.r. spectrum is broadened by some 0.063 T and does not saturate at 6.5 K (Fig. 11). Because freezing in water causes ions to migrate into regions of liquid solvent, the Ni(II) ions should aggregate with the cytochrome c, intentionally generating an e.p.r. 'freezing artifact' (Leigh & Reed, 1971). If only one Ni(II) ion is present per cytochrome c molecule, it should preferentially bind to an external site instead of a 'cleft' site because of coulombic forces. However, with 50 Ni(II) ions present per cytochrome molecule, the probability that at least one ion migrates into the cleft becomes substantial.
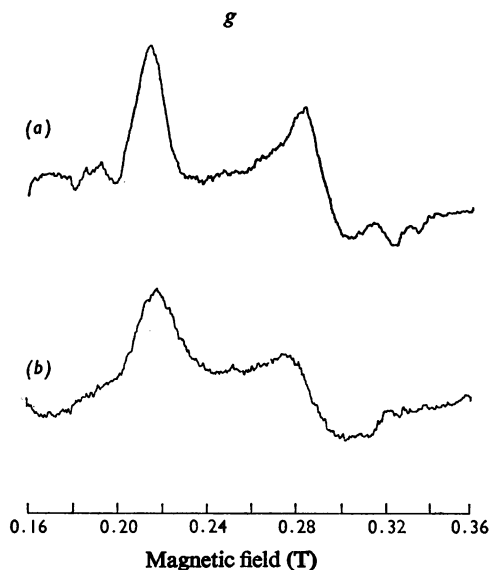
If this interpretation is correct, then the addition of a paramagnetic anion should generate magnetic dipole effects at much lower load levels than does Ni(II). The experiment of Fig. 12 indicates that the addition of equimolar amounts of $NiCl_2$ or $K_3Fe(CN)_6$ elicits changes in the e.p.r. spectra and saturation curves which are very subtle. At 8 K neither agent generates any significant effect. However, the data at 14.5 K show differences in the $g = 3$ amplitudes which are not due to concentration variations. Only the quenching observed with $K_3Fe(CN)_6$ suggests the magnetic origin of the effect. In intact mitochondria, however, we find that a paramagnetic anion such as free haem (which is permeant as well) has no effect on the cytochrome c e.p.r. characteristics. Hence the absence of a magnetic effect appears to be a real indicator of the position of the haem group relative to water.

Under the appropriate conditions, one can observe either a high-spin form of cytochrome oxidase ($g = 6$) or a low-spin form ($g_z = 3.1$) (Wilson & Leigh, 1972). The low-spin oxidase (ferric haem a; Babcock *et al.*, 1976) is customarily observed at E_h above +400 mV, whereas the high-spin form (ferric haem a_3; Babcock *et al.*, 1976) prevails at potentials between +240 mV and +380 mV, characteristic of the
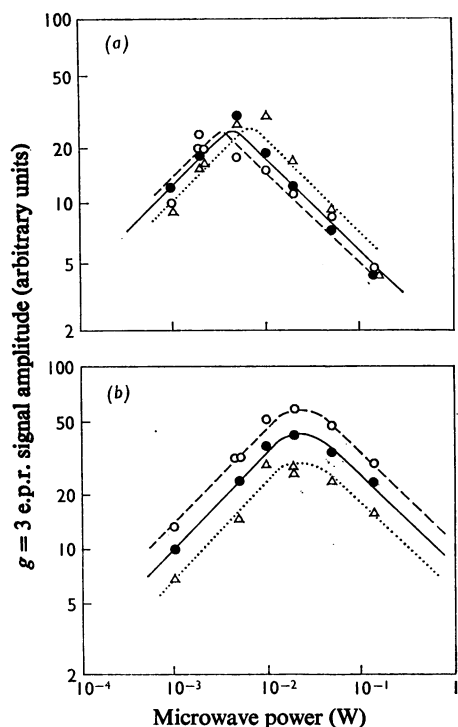
Fig. 12. *Effects of paramagnetic ions on the e.p.r. saturation curves for soluble cytochrome c*

Cytochrome c (Calbiochem, Richmond, CA, U.S.A.) was dissolved in deionized water and frozen. Cytochrome (4.4mM) was present entirely in the ferric form according to spectrophotometric analysis at 550nm minus 540nm. E.p.r. conditions were: scan range, 0.15–0.35T; modulation amplitude, 1mT; frequency, 9.21GHz; power, variable. ●, No paramagnetic ions added; ○, +5mM-NiCl₂; △, +5mM-K₃Fe(CN)₆. ——, ———— and ····· represent theoretical saturation curves fit to control, Ni(II) and ferricyanide data points respectively. (*a*) Temperature 8.0K. (*b*) Temperature 14.5K.

half-reduced oxidase (Wilson & Leigh, 1972). As Fig. 13 shows, the low-spin oxidase e.p.r. signal saturates much more readily than does the e.p.r. signal from the high-spin oxidase. However, the addition of Ni(II) has no effect on the power level at which saturation is attained, for both spin states. Similarly, Fig. 14 shows that, in submitochondrial particles, Ni(II) again fails to perturb either the e.p.r. spectrum or the relaxation kinetics of cytochrome oxidase. Addition of free haem to mitochondria likewise elicits no detectable perturbation in the e.p.r. relaxation kinetics of cytochrome oxidase. One should note that redox (concentration) artifacts
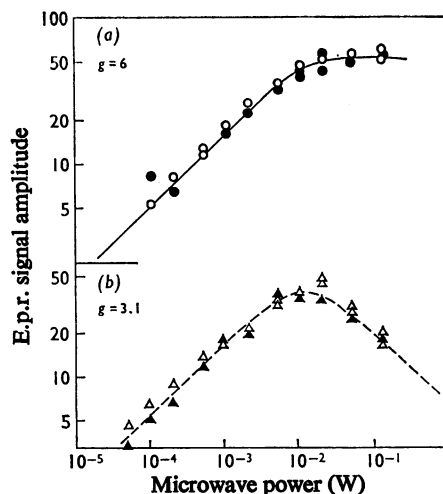

Fig. 13. *E.p.r. saturation curves for cytochrome oxidase in intact mitochondria*

Pigeon heart mitochondria (30mg of protein/ml) were suspended in a solution containing 0.25M-sucrose and 0.02M-Mops (pH7.4) under an Ar atmosphere. No inhibitors were present. E_h was adjusted with K₃Fe(CN)₆. E.p.r. conditions were as in Fig. 4 for the 'low-spin' signal at $g = 3.1$ (*b*; $T = 7.5$K). E.p.r. conditions were as in Fig. 2 for the 'high-spin' signal at $g = 6$ (*a*; $T = 4.8$K). Microwave power was varied; both scales are logarithmic. ●, ▲, $E_h = +400$mV, no additions. ○, △, $E_h = +405$mV, +1.3mM-NiCl₂. —— and ———— represent theoretical saturation curves fit to the data points.

appear in the data as a vertical displacement of the curves, but do not affect the positions of the maxima.

The present observation suggests that the haem groups of cytochrome c and cytochrome oxidase all lie buried in regions which are not accessible to Ni(II). If one assumes that the surfaces of the membrane and the apoproteins can bind Ni(II) near these cytochromes to the same degree as the rest of the mitochondrion, then the data of Figs. 10–14 indicate that the haem groups of cytochrome c and cytochrome oxidase are not close to water from either side. However, one must be careful to note that local size and charge anomalies near these cytochromes may preclude the binding of Ni(II) in these regions only, without excluding water. This is the principal rationale for the use of a paramagnetic anion probe, and the corresponding absence of a magnetic perturbation in the opposite charge situation could argue against specific exclusion of Ni(II).

Discussion

The present results clearly demonstrate a positive magnetic interaction in intact mitochondria of
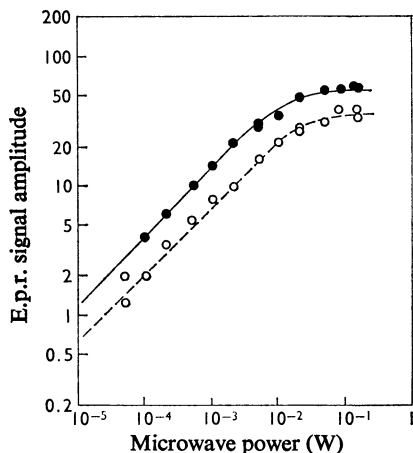
Fig. 14. *E.p.r. saturation curves for cytochrome oxidase in submitochondrial particles*

Conditions were as in Figs. 6 and 7, except that the modulation amplitude was 1 mT, and the temperature was 4.8 K. ●, $E_h = +360$ mV, no additions. ○, $E_h = +375$ mV, +2 mM-NiCl$_2$. —— and ----- represent theoretical saturation curves fit to the control and Ni(II) data points respectively.

exogenous Ni(II) with cytochromes b_K, b_T and c_1. No such interaction of Ni(II) is evident for cytochrome c or the cytochrome oxidase haem groups. If one reconstructs a model for the inner mitochondrial membrane on the basis of the present observations, the b_K, b_T and c_1 haem groups should lie on the outer surface of the membrane, exposed to external solution. The positions of the cytochrome c and cytochrome oxidase haem groups are less certain, although shielded environments for these systems are likely.

One should not consider the evidence in the present paper as being in conflict with earlier observations from other laboratories. Most of the previous studies on the asymmetrical distributions of mitochondrial cytochromes (Arion & Wright, 1970; Racker et al., 1970, 1971; Klingenberg & Buchholz, 1970; Boveris et al., 1972; Hackenbrock & Hammon, 1975) have focused on the proteins or on the substrate/inhibitor binding sites. Ample evidence in the literature (Arion & Wright, 1970; Tyler, 1970; Racker et al., 1970, 1971; Boveris et al., 1972) indicates the exterior location of cytochrome c, yet the e.p.r. relaxation measurements (Fig. 10) fail to confirm this fact.

In mitochondria, there are present a vast number of low-affinity and high-affinity binding sites for bivalent cations, which are presumably non-specific (Lehninger et al., 1967; Romslo & Flatmark, 1973; Case, 1975). Although the addition of large amounts of Ni(II) to the mitochondrial and submitochondrial suspensions is desirable for the e.p.r. relaxation measurements, we were unable to introduce much more than approx. 50 nmol of Ni(II)/mg of protein without incurring damage. Consequently, the likelihood that a Ni(II) ion, or haemin for that matter, could force its way into the cytochrome c cleft in mitochondria under present conditions is small.

Work with specific antibodies for cytochrome oxidase subunits (Eytan et al., 1975) has demonstrated the specific orientation of the cytochrome oxidase complex within the inner mitochondrial membrane. However, one cannot immediately correlate with the subunit structure present information about the positions (Wilson & Leigh, 1972) of the cytochrome oxidase haem groups relative to one another, and their total immobility in the complex (Junge & DeVault, 1975). Because of the difficulty at present encountered by most laboratories in the isolation of a pure cytochrome b protein, antibody experiments have not yet been reported for the location of the cytochrome b protein. At best, any antibody work carried out on this system, once it becomes obtainable in a purified form, would probably give results analogous to those for cytochrome oxidase, showing protrusion of the protein into both aqueous phases.

The implications that the present results pose for the various energy-coupling mechanisms cannot be underestimated. If cytochromes b_K, b_T and c_1 are involved in energy transduction at coupling site II (Wilson & Dutton, 1970; Dutton et al., 1970; Wikström, 1973), at least one of these haem groups should sit on the matrix surface of the mitochondrial membrane for direct H^+ translocation to occur (Mitchell, 1966, 1975; Wikström, 1973). None do. Likewise, if the haems a and a_3 of cytochrome oxidase carry out direct H^+ translocation associated with energy coupling at site III (Dutton et al., 1970), this event would require haem a to reside on the exterior surface, and haem a_3 to sit near the matrix surface of the mitochondrial inner membrane (Mitchell, 1966, 1975; Harmon et al., 1974). Although the present results are probably indeterminate with respect to cytochrome oxidase, there is no positive evidence inherent in these studies to suggest that either haem is exposed to water on either side of the membrane. If the present observations are indicative, none of the energy-transducing systems can function directly in cation transport, within the framework of a solid-state model for electron transport. In the following paper (Case et al., 1976), this conclusion is extended to the electron carriers associated with Site-1 energy conservation.

The sole exception to this conclusion is the possibility that some of the electron carriers do move freely through the membrane. Although such a possibility has been proposed for energy coupling through the *b*-cytochrome region (Green, 1974; J. H. Guth, unpublished work), little direct evidence exists in its support. Because all of the mitochondrial electron carriers are paramagnetic in only one of their two oxidation/reduction states, e.p.r. techniques, such as those used in the present investigation, would be unable to answer such a question. Parallel studies of the cytochromes in their reduced (and diamagnetic) state would have to be carried out to determine whether the haem groups can move through the membrane. Nevertheless, the present investigation has established the positions of several (oxidized) cytochrome haems in mitochondrial respiration, and clarifies some of the conditions which must be satisfied by any models for mitochondrial oxidative phosphorylation.

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