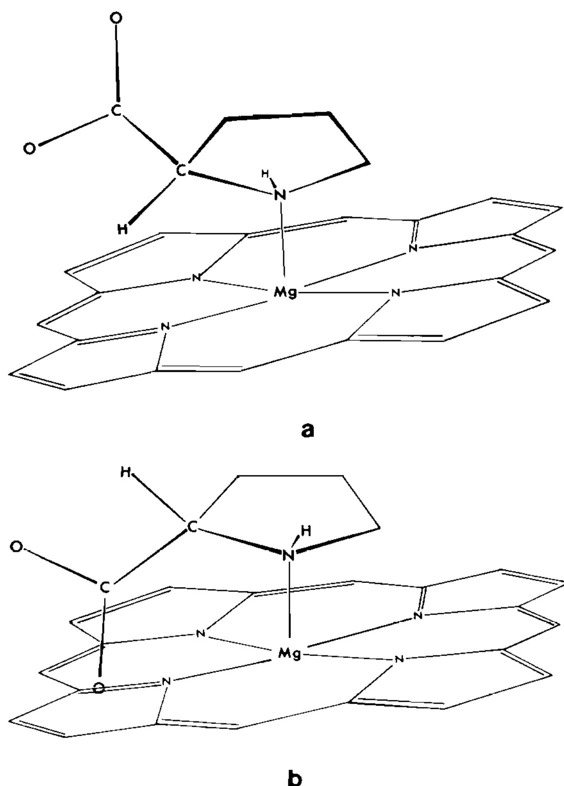

Fig. 1. Diagrams illustrating the formation of an opposite chirality at N to that existing at C for the binding of L-proline to Mg(porphyrin) (as for the optically active Mg-(porphyrin)(L-proline)$_2$ complexes). (a) Sterically acceptable opposite-chirality situation. (b) Sterically unacceptable same-chirality arrangement.

Aqueous solutions of the magnesium porphyrins containing the chiral amino acids, L-histidine, L-serine, L-threonine and L-proline, produce prominent induced Cotton effects [1, 2]. However the CD/ORD spectra of these species differ from those of the magnesium porphyrin Mb and Hb protein complexes. Electronic band positions indicate the complexes producing the Cotton effects to be six-coordinate Mg(porphyrin)(amino acid)$_2$ entities, containing two amino acids bound to the metal rather than only one amino acid residue as for the protein species. The electronic spectra also indicate the presence of five-coordinate Mg(porphyrin)(amino acid) species in the solutions but these do not produce Cotton effects.

Induced Cotton effects of the magnitude observed for the Mg(porphyrin)(amino acid)$_2$ complexes presumably require reasonable firm stereochemical location of the coordinated chiral amino acids. For L-histidine we suggest the ligands are localised primarily by $\pi-\pi$ bonding interactions between the ligand aromatic π system and the d_π orbitals of Mg [2]. For the similar amino acids, L-serine and L-threonine, molecular models indicate that hydrogen bonding between the ligand –OH groups and the porphyrin COO^- side chains fix these ligands in well-defined positions.

L-proline is a special case in that binding through the ring nitrogen atom produces a new chiral centre at that atom. Interestingly, steric interactions between proline and the porphyrin ring determine the chirality of nitrogen to be opposite to that at the asymmetric carbon atom (as illustrated in Fig. 1). Inversion of this type occurs for a similar reason in the case of proline complexes of Cu^{2+} [6].

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Q3

Electron Transfer Mechanism Studies of Cytochrome c_3: pH Dependence of the Redox Equilibria

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Cytochromes c_3 are tetrahaem electron transfer proteins isolated from *Desulfovibrio* spp. which play an important role in the metabolism of sulfur compounds and hydrogen [1]. They have a molecular weight of approx. 13,000 Daltons [1, 2]. The four haems, mesoporphyrins, are covalently bound to the polypeptide chain through thioether linkages provided by cysteinyl residues. The axial ligands are two hystidinyl residues [3, 4]and all the haems have negative, although different mid-point redox potentials [5–8].

Several physico-chemical techniques, mainly Mössbauer [9], EPR [4–7], NMR [3, 10–16], cyclic voltammetry, and differential pulse polarography [6, 7], have been applied to elucidate the mechanism of electron transfer in cytochrome c_3. However, the mechanism by which electrons are transferred between cytochrome c_3 and other electron carriers is only poorly understood. NMR has been shown to be a suitable technique to elucidate the electron exchange mechanism of cytochrome

c_3. The large paramagnetic shifts induced by the low-spin haem ferric ion greatly facilitate the probing of the involvement of each individual center, giving valuable information about the electron exchange mechanism. The general electron distribution equilibrium for a four redox center molecule is rather complicated, since 16 different redox states can be obtained [12]. The complex redox equilibrium of cytochrome c_3 can be described by 32 Nernst equations between these 16 oxidation states. The equilibria between the oxidation states can involve two types of one-electron exchange mechanism: an *intermolecular,* between haems of different molecules, and an *intramolecular,* between haems of the same molecule.

In order to study these redox equilibria, redox titrations of cytochrome c_3, isolated from *D. gigas* [11, 13] and *D. vulgaris* [12], were followed by NMR spectroscopy at 270 and 300 MHz. The redox titration of these cytochromes can be described by grouping the different oxidation states in five steps which, starting with the fully reduced molecules (Step 0), are generated by sequential loss of one electron. Thus, 4 oxidation states with only one haem oxidized (Step I) are first generated; a further loss of one electron generates 6 different oxidation states (Step II); loss of a third electron generates again four different oxidation states (Step III); finally, the loss of a fourth electron leads to the fully oxidized state (Step IV). Indeed, for each haem methyl resonance only one resonance is observed at each intermediate redox Step. Thus, for the concentration and temperature used (2 m*M* and 273 K), the intramolecular electron exchange rates are fast in the NMR time scale ($k_{intra} > 2 \times 10^4$ s^{-1}). The intermolecular electron exchange rates were obtained by saturation transfer experiments, between the resonances due to the same haem methyl group at different intermediate redox Steps ($k_{inter} \cong 10^4$ M^{-1} s^{-1}).

Searching for ionizing groups that might affect the mechanism of electron transfer, several redox titrations were performed at different pH values. The pH dependence of the chemical shifts of the haem methyl resonances in different oxidation stages was followed. A very large chemical shift dependence on pH is observed for the intermediate redox step resonances [15]. This implies that the mid-point redox potential of at least some of the haems changes with pH showing a redox-Bohr effect [16]. Indeed, the pk_a values for the ionizing group depend on the haem oxidation state [12].

The individual potentials obtained from electrochemical studies [6, 7] are macroscopic rather than microscopic parameters and cannot be directly compared with the values obtained from the EPR measurements [5, 6]. Furthermore, the EPR measurements were carried out at 8 K and cannot easily be compared with the room temperature values. When the mid-point redox potential of each haem depends on the oxidation state of the other three, 32 microscopic redox potentials (e_i, e_i^j, e_i^{jk}, and e_i^{jkl}, where i, j, k, l = 1 to 4) must be defined for the equilibria between each pair of the 16 oxidation states. e_i is the microscopic redox potential of haem i when all the other haems in the same molecule remain reduced; e_i^j is the microscopic redox potential of haem i when haem j is oxidized and the other haems (not specified) are reduced, and so far for e_i^{jk} and e_i^{jkl}. The four haems are numbered from 1 to 4 according to increasing mid-point redox potentials e_i (i = 1 to 4) and the interacting potentials between haems i and j are defined as $I_{ij} = e_i - e_i^j = e_j - e_j^i$.

A computer programme which calculates the populations of the 16 different oxidation states for each value of the solution redox potential was used in order to fit the NMR parameters obtained from the NMR redox titrations (*i.e.,* the chemical shifts of the haem methyl resonances due to the intermediate oxidation states in fast exchange, and their maximum intensities).

Extracting these parameters from the NMR redox titrations previously reported [11–13], it was possible to: i) calculate the difference between the microscopic mid-point redox potentials of the haems; and ii) show that the values of the interacting potentials, I_{ij}, are not negligible. For *D. gigas* cytochrome c_3 at pH = 5.6, e_1 is approximately 30 mV more negative than $e_2 \cong e_3$, and e_4 is approximately 15 mV less negative than these last two mid-point redox potentials. The interacting potentials at this pH cover a range between –30 and +45 mV.

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Q4

Infrared Spectra of Carbonyl Lactoperoxidase

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Lactoperoxidase (LP) [1] is a remarkable enzyme, being an efficient scavenger of peroxides, an oxidant of usual peroxidase substrates and halide ions [2] while also catalyzing the disproportionation of dithionite [3]. Like other peroxidases LP has a low redox potential and a protoheme prosthetic group, but a mixed spin type spectrum and a strong heme–protein attachment single out LP as being unique. For this reason we have studied the CO stretch of LP•CO at pH 5–9.

The CO stretch of carbonyl horseradish peroxidases (HRP) A and C is pH dependent in terms of number and positions of ir bands [4]. This was interpreted as indicating the presence of a titratable group near the active center influencing the carbonyl resonator at low pH by H-bonding to the oxygen atom in CO. The ir spectrum of LP•CO exhibits CO stretches at 1940, 1955, and 1961 cm^{-1} (Fig. 1 Spectra at intermediate pH values omitted). The 1940 cm^{-1} band predominates, and its position is independent of pH and buffer species. The 1955 cm^{-1} band is intense at pH 5, gives a shoulder at pH 7, and has vanished at pH 9. The 1961 cm^{-1} band changes in the reverse manner. The low intensity and essentially pH-independent band at ~1913 cm^{-1} may not be related to bound CO.

The ir spectra of LP and HRP A, C have in common *a*) a pH-independent stretch at 1937 ± 3 cm^{-1} with a half-band width of 12 cm^{-1}; *b*) a broader band, sensitive to protons or dipoles. Stretch *a*) is attributed to CO in a narrow, apolar environment, whereas in *b*) CO may dwell in a more open site. The balance between the 1955 and 1961 cm^{-1} stretches may depend upon a change in polarity due to a proton or, more likely, a polar group. The properties *a*) and *b*) seem to be unique to peroxidases, although at present there is no explanation of why stretch *b*) is located on opposite sides of *a*) in HRP and LP. The active site of lactoperoxidase may be different from that of other peroxidases, either lacking the distal base, or the ligand binding site being rigid and preventing carbonyl interaction with the distal base [5].

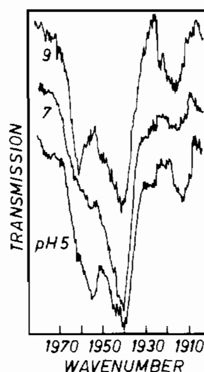

Fig. 1. CO lactoperoxidase (2–2.5 m*M* heme) reduced with dithionite at pH 9 (50 m*M* borate, 1 m*M* EDTA); pH 7 (50 m*M* 3,3-dimethylglutarate, 1 m*M* EDTA); pH 5 (50 m*M* citrate, 1 m*M* EDTA). Digilab Interferometer FT-14C.

The redox potentials of LP and HRP A, C (–180, –212, and –265 mV) and ν_{CO} (1940, 1938, and 1933 cm^{-1}) of the *a*) stretch follow the correlation found for other heme proteins [4].

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