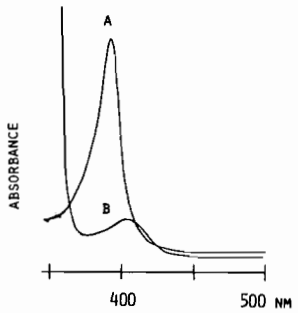

Fig. 1. Ultraviolet–visible spectra in the Soret region of: A, 5.0×10^{-6} *M* urohemin, pH 6.0, in H_2O. This is monomeric urohemin. B, 5.0×10^{-6} *M* urohemin and 4.89×10^{-3} *M* chloroquine, also in H_2O, pH 6.0. The decreased Soret intensity indicates association of chloroquine with urohemin and is characteristic of the decreased intensity observed for urohemin dimer.

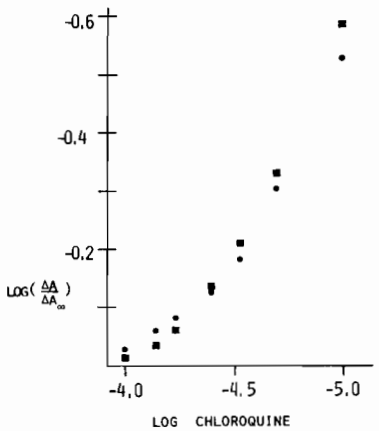

Fig. 2. Analysis of uv–visible data for equilibrium titrations of urohemin with aliquots of chloroquine solution. A standard equation was used from references [6, 7]. Abbreviations: ΔA is the absorbance difference at a wavelength maximum between urohemin alone and urohemin with various aliquots of chloroquine. ΔA_∞ is the absorbance difference between the complex (limiting absorbance) and urohemin with aliquots of chloroquine. This analysis is for a single equilibrium process and nonlinearity in this plot indicates the presence of multiple equilibria. Concentrations: urohemin (initial) 5.0×10^{-6} *M*; chloroquine (maximum) 4.89×10^{-3} *M*.

dimer spectrum chloroquine binding induces a shift in the Soret maximum to longer wavelengths.

Attempts to characterize the chloroquine–urohemin association by standard uv–visible methods [6] revealed that this system cannot be characterized by a single equilibrium process. This is shown by the deviation from linearity of the data in Fig. 2 [6, 7]. This data is the result of successive additions of chloroquine to a solution of monomer urohemin. This process produced standard appearing difference absorption spectra with two isosbestic points between 350 and 500 nm. The fact that linearity in Fig. 2 is not observed suggests that multiple equilibria are present in this system.

Further work on malaria drug interactions with free hemins and heme proteins is in progress in our laboratories.

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Q8

Evidence for Sulphur Ligation in Ferricytochrome *c* at Alkaline pH

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The inactivation of ferricytochrome *c* at alkaline pH proceeds with a pK of 9.3 and it has been shown that the process involves a slow conformational change coupled to a real ionization with a pK of 11 [1]. Important indicators of the alkaline isomerization are the disappearance of the iron–sulfur charge transfer band at 695 nm [2], and the disappearance and formation of signals monitored by NMR [3] and EPR [4]. The prevalent view has been that Met-80, the sixth ligand, is replaced by another strong field ligand [3, 4]. However, the results described below strongly suggest that Met-80 remains ligated to the haem iron.

The ^1H NMR signals (270 MHz) arising from the haem methyl groups of the first alkaline form [3] are found to be composed of overlapping resonances from two slowly exchanging forms of ferricytochrome *c*. Either form can be made dominant over the other by raising the temperature to 330 K or by addition of 0.3 *M* perchlorate at pH 10. In the upfield region of the spectrum two very broad resonances between −8 and 10 ppm are assigned to the Met-80 methyl group in its two environments.

Modification of Met-80 by carboxymethylation [3, 4] or methylation perturbs the equilibrium constant for the conformation change involved in the alkaline isomerization so that it can be observed in the neutral pH range. In the latter case just one 'alkaline' form is observed by NMR and the resonances assigned to Met-80 now show up with twice the intensity observed for the corresponding peaks in the unmodified protein. Also in the carboxymethylated form of ferricytochrome *c* the Met-80 methyl resonances are clearly visible in the same ppm range.

The NMR results were correlated with the signals appearing at alkaline pH in EPR spectra (9.2 GHz and 17 K). The two forms observed by NMR thus correspond to the $g_z = 3.39$ and $g_z = 3.57$ signals. An additional EPR species with $g_z = 3.26$ was correlated with the form of ferricytochrome *c* where the conformation change, but not the deprotonation of the group with pK = 11, has occurred. This form of cytochrome *c* can also be observed at high temperatures [5] or high urea concentrations [6] at neutral pH. As expected both carboxymethylation and methylation of Met-80 yielded the $g_z = 3.26$ species as the major form at neutral pH.

The occurrence of two alkaline forms, as described above, is most likely due to a slow exchange between the two chiral binding modes of Met-80 to the haem iron. This is also strongly supported by the modification experiments on Met-80. The anomalously broadened resonances stemming from the Met-80 methyl group (the width at half height is approximately 350 Hz) are probably due to the slow flipping rate of the nearby Tyr-67. Such an exchange can lead to appreciable broadening since the scalar relaxation of the Met-80 methyl resonance, due to the haem iron, will be modulated by the exchange rate [7]. Considering the positive charge on the sulphur atom of Met-80 in the modified proteins, it is likely that repulsion between the sulphur and the iron will lead to an increased iron–sulphur bond length. This would rapidly explain the disappearance of the 695 nm band, the decreased hyperfine shifts in NMR as well as the EPR signals appearing at alkaline pH.

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Q9

Photoredox Behaviour of Cytochrome *c* in Aqueous Solution

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It is well known that cytochrome *c* has a fundamental importance in biological electron transfer chains. Following the definition of the International Union of Biochemistry its mode of action is electron and/or hydrogen transport by virtue of a reversible valency change of its heme iron. For this reason, a number of authors are attracted by the study of the redox behavior of this compound [1]. Photochemical methods offer a valuable means of obtaining information on the redox properties of iron proteins. In fact, several papers have been recently published on the photoredox behavior of iron porphyrins [2–4], which are known to be the prosthetic groups of heme-proteins.

Here we report some preliminary results obtained in the course of an investigation on the photochemical behavior of cytochrome *c* in aqueous solution.

Results and Discussion

The irradiation in the 330–400 nm range of previously deaerated solutions of cytochrome *c* gave rise to spectral variations (Fig. 1) which clearly indicate that Fe(III) was reduced to Fe(II) without apparent involvement of the porphyrin ring. The plot of

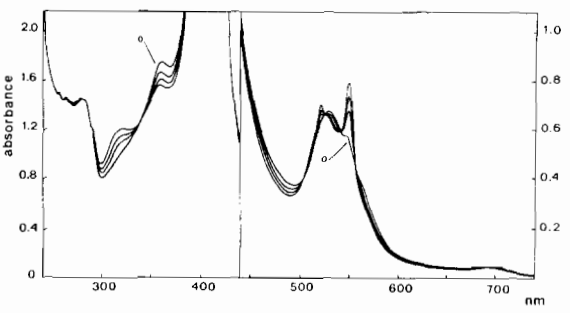

Fig. 1. Spectral variations observed during the irradiation of a 6.5×10^{-5} *M* deaerated aqueous solution of cytochrome *c* at 25 °C, pH = 7.0 initial spectrum; irradiation periods, 10 min.