Inorganica Chimica Acta, 80 (1983) 245–250

Conductometric Studies on Protoporphyrin IX–Iron(III) Alkali Metal Solutions. Evidence for the Alkali Metals Binding to the Protoporphyrin IX–Iron(III) Moiety

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Received May 3, 1983

Conductometric evidence is reported that shows an interaction between alkali metals with protoporphyrin IX–iron(III) in aqueous solutions. Mössbauer spectroscopic and pH titration data are also reported. The results are explained in terms of the alkali metals binding to the propionate groups of the protoporphyrin IX–iron(III) moieties.

Introduction

Protoporphyrin IX iron(II) (or iron(III)) forms the prosthetic group of numerous metalloproteins [1]. Understanding the chemistry of protoporphyrin IX iron in the absence of protein is of interest to both chemists and biochemists. Obviously many differences in the physical and chemical properties of protoporphyrin IX iron in the absence or in association with protein should further the understanding of the role of the moiety in biological molecules. To date, however, the aqueous chemistry of protein-free protoporphyrin IX iron has not been sufficiently well documented and/or explained to render it particularly useful as a model system. One of the main reasons for this is that in aqueous solution protoporphyrin IX iron(III) forms a μ-oxo-oligomer at all pHs above pH 7 (in the absence of competing ligands), [2], while in the same pH range protoporphyrin IX iron(II) forms predominantly a polymer made up of bare protoporphyrin IX iron(II) monomers, where the polymer length depends on concentration and pH [3].

Recently we have shown from studies using Mössbauer spectroscopy that only two protoporphyrin IX iron(III) species occur in aqueous solutions in the pH range 6 to 14 [2]. Below pH 7 a monomeric protoporphyrin IX iron(III) unit containing one hydroxyl ligand is dominant, and above pH 7 a μ-oxo-oligomer containing two protoporphyrin IX iron(III) moieties linked by a μ-oxo-bridge is the major species.

No μ-oxo-oligomers are known to occur in protein molecules. To understand the chemical behaviour of protoporphyrin IX iron(III) over a reasonable pH range *e.g.* 6–12, it is necessary to study the aqueous chemistry in the presence and absence of ligands of sufficient strength to stop the formation of the μ-oxo-oligomer.

One way of overcoming this problem has been to study protoporphyrins in non-aqueous solvents, but such an approach is of limited value if the focal point of the investigation is one of porphyrin reaction kinetics; rather than merely a question of assignment of spectral parameters or purely structural aspects of iron–porphyrin chemistry. A serious drawback to the use of non-aqueous solvents is the very limited solubility of protoporphyrin IX iron(III) (or iron(II)) in such media. Recently protoporphyrin IX iron(III) encapsulated in detergent micelles (of varying surface-charge characteristics) [4–7] has been successful in providing a monomeric system in aqueous media over a wide range of pH. Although micellular porphyrins may yet prove to be a useful and widely applicable model system for haemoproteins the chemistry of the 'naked' protoporphyrin IX moiety in aqueous environments remains of interest. We report here pH and conductometric titration curves of protoporphyrin IX iron(III) solution in the presence of various inorganic salts.

Experimental

Instrumentation

Atomic absorption analyses were performed using a Perkin-Elmer 103 instrument, with a Multisource (Cr, Co, Cu, Fe, Mn, Ni) Intensitron lamp – both

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0020-1693/83/$3.00

TABLE I. Iron Concentration Determinations.

	Atomic Absorption	Spectrophotometric Method (hemochrome 557 nm)
Protoporphyrin IX iron(III) chloride solution	0.36 (±0.04)	0.30 (±0.03) m*M*
Protoporphyrin IX iron(III) hydroxide solution	0.32 (±0.02)	0.28 (±0.02) m*M*

ferric chloride and potassium ferricyanide solutions were employed as calibration standards. Titration experiments were carried out using a Pye-Dynacap pH meter and glass electrode, plus an M.E.L. Equipment Co. type E7566/3 Multard conductivity bridge and pre-calibrated dip-cell. A Pye-Unicam SP8-200 spectrophotometer was used to record electronic absorption spectra.

Materials

Reagents were purchased from the following manufacturers. Bovine protoporphyrin IX iron(III) chloride, protoporphyrin IX iron(III) hydroxide and rubidium chloride (RbCl, 99%) from Sigma Chemicals. Volumetric solutions of sodium hydroxide and nitric acid, plus sodium perchlorate ($NaClO_4$ + H_2O, 75% minimum assay) from British Drug Houses Ltd. Sodium nitrate ($NaNO_3$, AR), sodium chloride (NaCl, AR), potassium chloride (KCl, AR), lithium chloride (LiCl, 98%), ferric chloride ($FeCl_3$, AR), potassium ferricyanide ($K_3Fe(CN)_6$, AR), potassium cyanide (KCN, AR) and potassium thiocyanate (KSCN, AR) from Fisons Ltd. Caesium chloride (CsCl, 99%) from Koch-Light and white spot nitrogen (less than 5 ppm 0) from British Oxygen Ltd.

De-ionised H_2O, prepared in the Department of Chemistry at the University of Essex, was found to have a pH value *ca.* 5.6. Driving off dissolved CO_2 by bubbling nitrogen for twenty minutes resulted in de-ionised H_2O samples of pH 6.6–6.8 and conductivity less than 2.0×10^{-4} mho cm^{-1} (*i.e.* reciprocal ohms/cm).

Methods

Solutions of analar grade reagents were made up assuming 100% purity. Solutions of other reagents were made up using the manufacturer's assay to calculate amounts of solute(s) needed to make up solutions of required concentrations. Solutions of protoporphyrin IX iron(III) salts were made up according to a variation of the procedure outlined by Simplicio [4], as described below. De-ionised H_2O was used throughout.

Forty mg of the protoporphyrin IX iron(III) salt were taken and partially dissolved in 25 ml of 0.01 *M* NaOH by stirring vigorously for two minutes. The resulting solution was then filtered through a Whatman grade I (11.0 cm diameter) filter paper and collected in a 100 ml volumetric flask. After allowing as much of the solution as possible to drain freely into the flask, the filter paper and residual protoporphyrin IX iron(III) salt was removed from the filter funnel and discarded. The solution adhering to the filter funnel was washed into the flask with a small quantity of H_2O. At this stage, the appropriate quantity of a previously prepared salt solution of known concentration (usually 25 ml of a 2.0×10^{-3} *M* solution) was added, if required. Finally, the solution was made up to the 100 ml mark on the volumetric flask with H_2O. Control solutions containing no protoporphyrin IX iron(III) salt were made up in an exactly analogous manner to the procedure described above, except that no protoporphyrin IX iron(III) salt was dissolved in the 25 ml quantities of 0.01 *M* NaOH prior to filtering.

Concentrations of protoporphyrin IX iron(III) solutions were determined by atomic absorption analyses and by bis(pyridine) hemochrome assay, assuming ϵ_{557} = 34 mM^{-1} cm^{-1} after Paul *et al.* [8]. The results of the concentration determinations are given in Table I.

Titrations were performed using a 100 ml three-necked flask with a small depression blown in the bottom, of such a size to accommodate a small (*ca.* 6 mm in length) magnetic stirring bar. The pH electrode and dip-cell were wrapped with Parafilm so that they could be 'push-fitted' into two of the necks of the flask. A pierced Subaseal stopper was fitted to the third neck of the flask and a thin nylon tube (delivering nitrogen gas) inserted through this so that its open end was close to the bottom of the flask, but not close to either the pH electrode or dip-cell. This apparatus was clamped in a retort stand over a magnetic stirrer. All experiments were performed at 20 (±1.0) °C.

Prior to starting each titration, the pH electrode was calibrated against standard buffers. Stirring was then commenced and nitrogen bubbled through the solution for a period of twenty minutes, before the addition of any acid. During this period, the reading

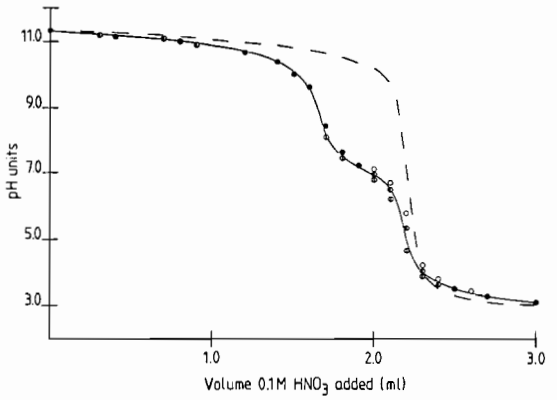


Fig. 1. pH titration curves of protoporphyrin IX iron(III) hydroxide solutions; ○ ◑ ●, triplicate sets of data; •, two or three coincident points; – – –, control NaOH *vs.* HNO_3.

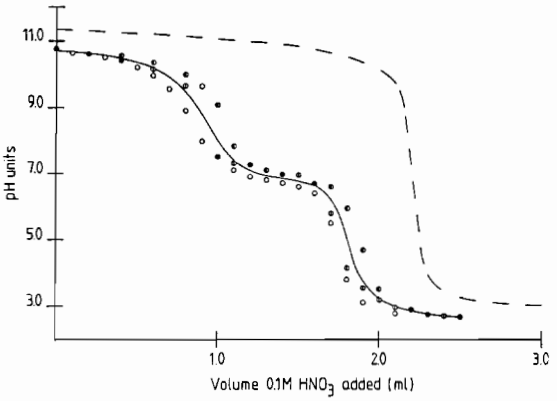


Fig. 2. pH titration curves of protoporphyrin IX iron(III) chloride solutions; ○ ◑ ●, triplicate sets of data; •, two or three coincident points; – – –, control NaOH *vs.* HNO_3.

on the conductivity bridge and pH meter drifted slightly and then became stable. Neither stirring, nor the bubbling of nitrogen, were found to affect the readings on the meters (once CO_2 had been driven off) and were continued throughout; nitrogen gas escaped past the pH electrode and dip-cell. Switching off either of the meter/probe measuring devices did make a small difference to the readings registered by the other; both were left on throughout titrations. 0.1 *M* HNO_3 was added in 0.1 ml quantities by means of a syringe inserted through the Subaseal stopper. One minute was allowed to elapse for every 0.1 ml of acid added, before taking the new readings on the pH meter and conductivity bridge. This was found to be the time required for the systems to equilibrate. The conductivity bridge was always balanced before pH readings were taken. The total volume of 0.1 *M* HNO_3 added never exceeded 3.0 ml, so that volume changes throughout these experiments were small. At the end of each titration, the final readings on the instruments were monitored for at least five minutes to check that they did not change. In addition, the calibration of the pH electrode was checked to ensure that it had not altered by more than 0.05 of a pH unit around pH 7.0. In the event of either of these conditions not being fulfilled, the results were discarded and the experiment was repeated.

Mössbauer Spectroscopy

Preparation of Solutions of Enriched ^{57}Iron(III) Protoporphyrin-IX CN Complexes

5 mg of enriched ^{57}iron–protoporphyrin IX prepared according to the method of Caughey [9] was dissolved in 0.5 ml 1 *N* NaOH solutions and then diluted to 3 ml with distilled water.

This solution was then centrifuged to get rid of the insoluble particles.

To this solution 20 mg KCN was added and stirred until it dissolved.

The pH of the solution was adjusted as required by adding 1 *N* HCl.

The solution was then frozen in a perspex cell and transferred to a liquid N_2 cryostat. The Mössbauer spectrum was then recorded. The Mössbauer spectrometer and curve-curve fitting procedures have been described previously [10].

Results

Upon dissolution in 0.01 *M* NaOH, both the chloride and hydroxide of protoporphyrin IX iron(III) gave green/brown solutions which possessed spectra similar to those previously reported. Apart from KCN the presence of salts did not affect the electronic spectra of protoporphyrin IX iron(III). Upon addition of 0.1 *M* HNO_3 to these solutions, a colour change to red-brown occurred at *ca.* pH 6.0. Below this pH, protoporphyrin IX iron(III) species precipitated from the solution. The resulting red-brown gelatinous solids were readily soluble in acetone. Only high spin Fe(III) was found in these solids (by Mössbauer spectroscopy).

In the pH range *ca.* 6.0 to 8.0, the protoporphyrin IX iron(III) solution became 'soapy' (extensive frothing was introduced by the bubbling of nitrogen). This was never observed outside this pH range or in solution free of the protoporphyrin IX iron(III) moieties.

The results of the pH titrations on 'salt free' protoporphyrin IX iron(III) hydroxide are shown in Fig. 1. These titrations are rather more reproducible than those involving 'salt free' protoporphyrin IX iron(III) chloride solutions (Fig. 2). The difference in the two titration sets reflects the difference

TABLE II. Initial Conductivities of Protoporphyrin IX Iron(III) Chloride Solutions, 0.5 m*M* in Various Salts, Compared to the Protoporphyrin IX Iron(III) Chloride-Free Solutions.

Inorganic Salt	Conductivity (mho $cm^{-1} \times 10^4$)		
	(a) 0.5 m*M* salt and 0.36 m*M* protoporphyrin IX iron(III) chloride	(b) 0.5 m*M* salt solution	b – a
NaCl	3.68	5.35	1.67
$NaNO_3$	3.41	5.23	1.82
$NaClO_4$	3.27	5.14	1.87
KCN	3.20	5.42	2.22

TABLE III. Initial Conductivities of Protoporphyrin IX Iron(III) Hydroxide Alkali Metal Chloride Solutions *cf.* Alkali Metal Chloride Control Solutions.

Inorganic Salt	Conductivity (mho $cm^{-1} \times 10^4$)		
	(a) 0.05 m*M* salt and 0.36 m*M* protoporphyrin IX iron(III) chloride	(b) 0.5 m*M* salt solution	b – a
LiCl	4.06	5.12	1.06
NaCl	3.68	5.35	1.67
RbCl	3.93	5.32	1.39
CsCl	4.06	5.38	1.32

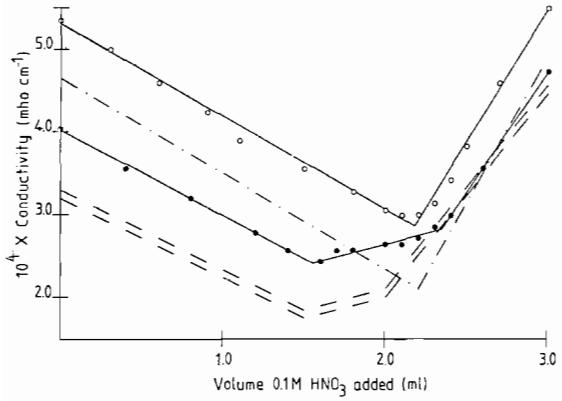


Fig. 3. Conductivity trace – protoporphyrin IX iron(III) chloride/$NaNO_3$; ○, haem free salt solutions; ●, haem + salt; –·–·–, NaOH *vs.* HNO_3 control; – – –, haems, without salt (limiting values).

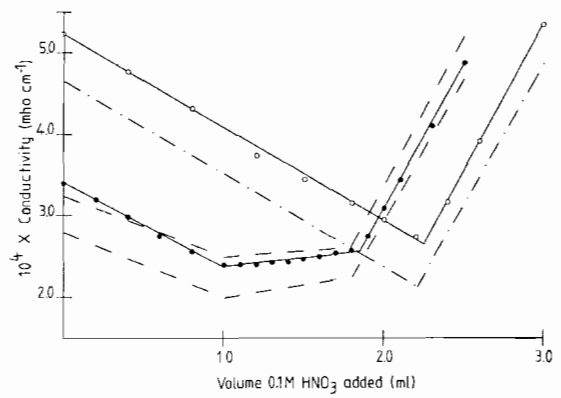


Fig 4. Conductivity trace – protoporphyrin IX iron(III) hydroxide/NaCl; ○, haem free salts solutions; ●, haem + salt; –·–·–, NaOH *vs.* HNO_3 control; – – –, haems without salt (limiting values).

between having chloride or hydroxide present on the initial protoporphyrin IX iron(III) moiety.

The pH titration curves of the protoporphyrin IX iron(III) salt free solutions were equivalent to strong acid *versus* strong base curves as would be expected.

The pH profiles of salt solutions containing either the hydroxide or hydrochloride of protoporphyrin IX iron(III), were the same as those of Figs. 1 and 2 respectively.

In all cases the conductivities of the initially prepared protoporphyrin IX iron(III) salt solutions were less than the conductivities of the protoporphyrin IX iron(III)-free control solutions (Tables II and III). Though there was evidence of deviation from Roult's law, this observation could not be

TABLE IV. ^{57}Fe Mössbauer Parameters for the Protoporphyrin Iron(III) Cyanide Frozen Solutions at 80 K.

	pH	δ/mm s^{-1}	Δ/mm s^{-1}	Γ/mm s^{-1}	% Absorption
site 1	12.5	0.16(2)	1.51(8)	0.31(4)	36.2(7.7)
site 2		0.20(1)	0.73(3)	0.25(2)	63.8(6.8)
site 1	10	0.17(3)	1.73(3)	0.31(8)	24.5(7.2)
site 2		0.19(1)	0.79(3)	0.25(2)	75.5(6.2)

explained by increased ionic interference (in the former solutions relative to the latter) leading to a net decrease in conductivity as in the concentration range studied, addition of further salt to the electrolyte caused a net increase in conductivity.

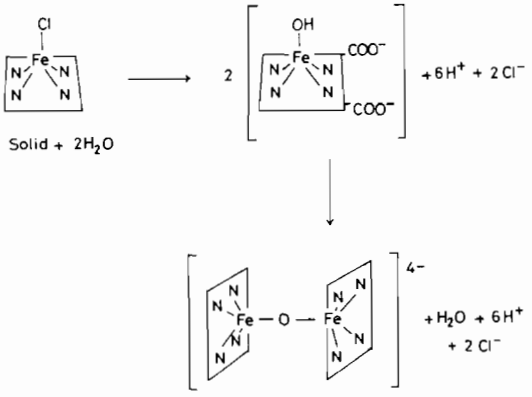
Within the error limits, the conductivity traces for protoporphyrin IX iron(III) chloride in the presence of various salts were all equivalent (*e.g.* Fig. 3), as were those for protoporphyrin IX iron(III) hydroxide salt solutions (Fig. 4). The sole exceptions to this were titrations in the presence of KCN, which deviated from the behaviour displayed by the more ionic salts.

The Mössbauer data for the frozen solutions of protoporphyrin IX iron(III) CN complexes are given in Table IV. As a single site fit is not satisfactory. The spectrum obtained is fitted to a combination of two iron sites.

The isomer shifts of the two sites are more or less the same but their quadrupole splittings are quite different. The smaller quadrupole splitting (0.79(3) mm s^{-1}) is suggested to be from an iron site which contains two CN^- ligands. This value for the quadrupole splitting is smaller than that found for haemoglobin CN which contains one CN^- ligand and one histidine ligand [11]. The larger splitting (1.73 (3) mm s^{-1}) we suggest comes from an iron site where the fifth ligands is CN^- and the sixth ligand is OH^- as this species appears to increase with increase in pH.

Discussion

As seen from Figs. 1 and 2 the pH titration curves for the chloride and hydroxide of protoporphyrin IX iron(III) solutions differ only in the fact that for the chloride, when initially dissolved, Cl^- is replaced first by OH^- so that more OH^- is initially used, as seen in the following scheme:



whereas for the hydroxide of protoporphyrin IX iron(III)

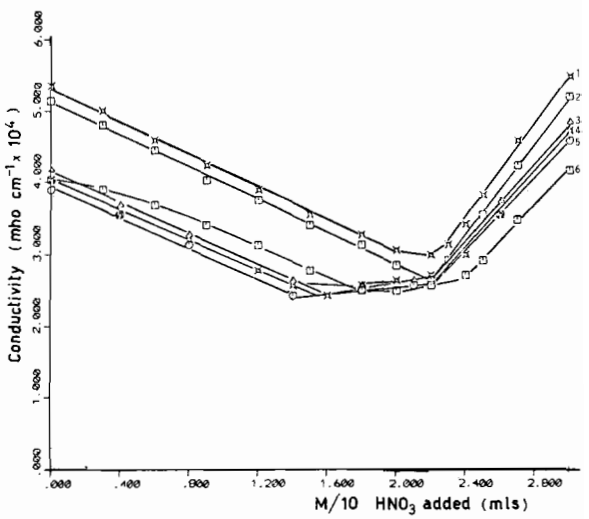
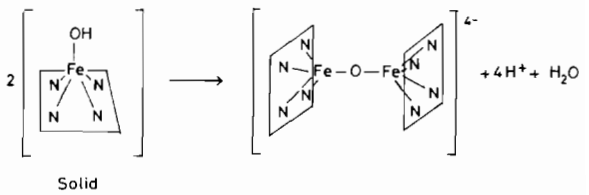


Fig. 5. Conductometric titrations – protoporphyrin IX iron(III) hydroxide solutions with added salts. □, LiCl; △, KCl; ○, RbCl; X, CsCl; 1 and 2 are protoporphyrin IX iron(III)-free controls, 3–6 are protoporphyrin IX iron(III) solutions.



The conductometric data figures show that in all cases where salts are added to alkaline protoporphyrin IX iron(III) solutions, a net drop in the conductivity of the resulting solutions was observed. This represents a reduction in the number of charged species present in the protoporphyrin IX iron(III) salt solution, relative to the control solutions, or a strong interaction between charged species in solution.

At the acid end of the conductivity profiles of solutions made up from the chloride of protoporphyrin IX iron(III) (Fig. 3) there was a net increase in conductivity relative to the salt only control solutions, the 'cross-over' being nonconcomitant with precipitation of the protoporphyrin IX iron(III) species from solutions. This is probably due to the chloride originally present in the solid hydrochloride remaining in solution at acid pH. This is confirmed by the absence of 'cross-over' in the solutions made up from protoporphyrin IX iron(III) hydroxide (Fig. 4).

To understand the nature of the interactions leading to these conductances of the protoporphyrin IX iron(III) salt solutions, Fig. 5 shows the difference observed keeping the anion constant and

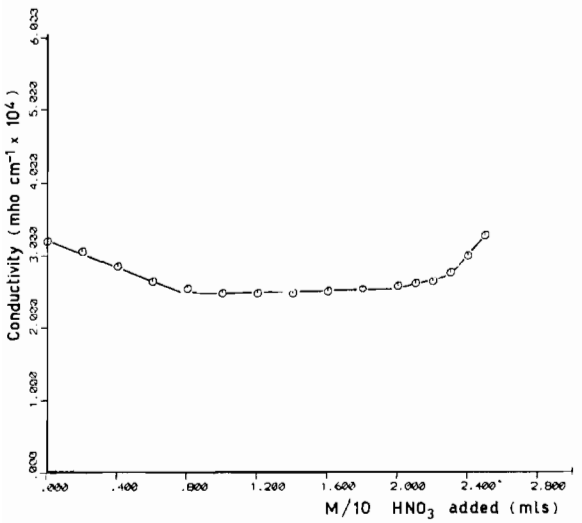


Fig. 6. Conductimetric titration protoporphyrin IX iron(III) chloride/KCN solution.

varying the cation. Clearly some parameter (possibly ionic size) of the cation is influencing the results. From Table II it is clear that for the Na^+ salts the different anions make little difference suggesting the role of the anion is non-coordinating and does not cause interactions. This is confirmed by the potassium cyanide results which are quite different, (Fig. 6), CN^- is known to bind protoporphyrin IX iron(III) at high pH and so this does affect the initial concentration. Indeed, the Mössbauer spectra of the frozen solution containing the CN^- shows that the iron(III) environments are very different to those we have reported previously [2] for protoporphyrin IX iron(III) solutions at high pH. Thus the results show that anions not capable of binding directly to the Fe in the protoporphyrin IX iron(III) moiety do not contribute to the lower conductivity, whereas all the cations do.

Such interactions of metal cations to the protoporphyrin IX iron(III) moieties have been reported previously in the solid state. Hamsik [12] reported a number of salts of protoporphyrin IX iron(III) with the metal cations Ca^{2+}, Ba^{2+}, Ag^+, Pb^{2+} and K^+. We have reported similar materials containing Cu^{2+} and a variety of other metal cations [13]. We presented evidence indicative of these cations binding to the propionic acid groups of the protoporphyrin IX iron(III) moieties [14]. It is therefore apparent that the conductivity data reported here could be explained by complex ion-pair formation, involving the alkali metal cations and the propionic acid groups of the protoporphyrin IX iron(III) moieites of the μ-oxo-oligomers present at alkaline pH. This is then followed by precipitation of the sodium salts of the protoporphyrin IX iron(III) at acid pH. Note that all solutions were made up in 0.01 NaOH and thus Na^+ represents more than 80% of the total alkali metal cation content in each case. Therefore similarity in the results of Table III are not surprising as the differences in the abilities of the different alkali metal cations to interact with the protoporphyrin IX groups may be small.

Acknowledgements

We thank the SERC for support (grant ref. GR/B/95820) to J. S. and M. T. W. and also for a studentship to J. P.

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