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Electrospray and collisionally activated dissociation study of the association of pyocyanin with alkali metal cations and doubly charged alkaline earth and zinc cations

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Abstract

The complexes formed between pyocyanin, an antibiotic $C_{13}H_{10}N_2O$, and alkali, alkaline earth, and zinc cations have been examined by electrospray mass spectrometry. Equimolar (100 μM) aqueous solutions of cation and pyocyanin yielded: $[MP_n]^+$ (M = alkal metal; P = pyocyanin, $n = 1$–3); $[MP_n]^{2+}$ (M = Mg, Ca, $n = 1$–4; M = Ba, $n = 1$–3); and $[ZnP_n]^{2+}$ (n = 2–3). Competition experiments showed that the smallest cation in each group is favoured in complex formation. Collisionally activated dissociation studies showed that pyocyanin is more strongly bound to the smaller cation in each group. $[MP_2]^+$ ions were collisionally decomposed by loss of neutral pyocyanin giving both $[MP]^+$ and M^+ (M = Cs) but only $[MP]^+$ (M = Na, Li) under the same conditions. $[MP_2]^{2+}$ ions decompose to give mainly $[M(\text{P-methyl})]^+$ plus $(\text{pyocyanin})^+$ and $[MP(\text{P-methyl})]^{2+}$. The electrospray spectra from aqueous solutions containing equimolar (100 μM) metal cation, pyocyanin and 2,2′-bipyridine contained both single- and mixed-ligand complexes. There was a preponderance of the bipyridine-containing ions in the spectra. However, for ions containing one or more of both ligands, the collision activated dissociation spectra showed that loss of bipyridine rather than loss of pyocyanin was the preferred decomposition path. The only exception was zinc, for which loss of pyocyanin was preferred. At the lowest collision energies, loss of methyl from $[MP(\text{bpy})]^{2+}$ (M = Mg, Ca, Zn) was the main reaction. (Int J Mass Spectrom 210/211 (2001) 341–359) © 2001 Elsevier Science B.V.

Keywords: Pyocyanin; electrospray; CAD; Alkali metals; Alkaline earth metals; Zinc; Bipyridine

1. Introduction

The biological activities of many antibiotics have been related to their abilities to form metal complexes. Pyocyanin (5-methyl-phenazinone), a substituted phenazine, is a water-soluble blue pigment produced in large quantities by active cultures of the gram-negative bacterium Pseudomonas aeruginosa. Pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells [1], it is a nitric oxide antagonist in a variety of pharmacological preparations [2,3] and its antimicrobial activities are possibly due to interactions with the respiratory chain [4]. Pyocyanin plays an important role as an electron transfer agent and catalyst in the phosphorylation

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Dedicated to Professor Nico Nibbering on the occasion of his retirement.

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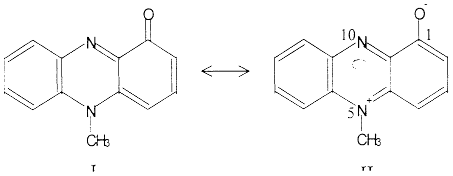


Scheme 1

reactions associated with the photosynthetic processes of bacteria and green plants [5].

Pyocyanin's redox behavior mimics that of riboflavin more closely than do those of other phenazines and as such pyocyanin has been used as a model for flavins which themselves are found to be experimentally difficult to use in electrochemical and spectroscopic studies [6]. The study of metal–flavin interaction is important for understanding some of the properties of metalloflavoproteins and pyocyanin is again a useful model for riboflavin because they both have the ability to form similar divalent metal complexes [7].

There are two contributing resonance structures for pyocyanin, a ketonic form called I, and a zwitterionic form called II (see Scheme 1). In I, the methyl carbon on N(5) is out of the plane of the rings, whereas in II all carbons are coplanar. The geometry of pyocyanin is not known, there being no crystal structure extant, and so the contribution that each structure makes to the overall geometry cannot be assessed. There is a strong C=O stretching band in the infrared spectrum of solid pyocyanin centred at the unusually low frequency of 1622 cm^{-1} but there is no band in the region 1220–1190 characteristic of the C–O stretch [8]. Therefore structure I, with a weakened ketone bond, is the major contributing resonance form. In aqueous solution however, the hydrogen bonding ability of the solvent would favour an enhanced contribution from the dipolar structure II. The association of metal cations with pyocyanin would be expected to favour a bidentate chelation, with interaction between metal cation and N(10) and the oxygen atom, enhancing the contribution of structure II in the complexes.

In this article we report on an investigation by electrospray mass spectrometry of the association of pyocyanin with several metal cations. The work is an extension of previous electrospray, electrochemical and spectroscopic studies of the interactions of pyocyanin with NO [9] and with Mn^{2+} [10]. The metal cations employed were those of the alkali metals, Li, Na, and Cs, the alkaline earth metals, Mg and Ca, and Zn. Zn^{2+}, the metallic components of many enzymes, was chosen for study in order to make comparisons of its complexing properties with those previously reported for Mn^{2+}. Zn^{2+}, although nominally a member of the transition metals, has a filled 3*d* shell and is more similar in many respects to Mg^{2+} than to the transition metals but the d shell is more easily distorted than the noble-gas-like shell of the alkaline earth element [11].

In addition to the study of the metal cation complexes formed by pyocyanin alone, it was instructive to compare the complexing ability of pyocyanin with that of a second ligand for which literature data is available. 2,2′-bipyridine was chosen as the competing ligand because it forms complexes with many metal cations and stability constants for their formation in solution are tabulated [12]. Experiments were carried out to assess the competition between pyocyanin and bipyridine in forming gas-phase metal cation complexes by electrospray. The collisionally activated dissociation (CAD) spectra of some of the mixed complexes containing both pyocyanin and bipyridine were obtained to investigate possible correlations between the intensity of the ions appearing in the electrospray mass spectra and relative metal–ligand bond strengths.

2. Experimental

Electrospray spectra were obtained using a triple sector mass spectrometer (quadrupole/hexapole/quadrupole, Quattro, Fisons, UK) in the manner that has been described previously [13–15]. The metal capillary was at +3.0 kV, the counter electrode was at ~+200 V and the cone was at +15 V. Aqueous solutions at pH values ~5, determined by the solutes present, were infused using a syringe pump at a flow rate of 5 $\mu L\ min^{-1}$. N_2 was used as the drying gas at a temperature of ~350 K. CAD spectra were obtained using argon as the collision gas and laboratory ion energies in the range 20–120 eV.

Most solutions contained 100 μM pyocyanin and, usually, 100 μM of each of one or more metal cations. Some experiments were performed with solutions that also contained 100 μM 2,2′-bipyridine as a competing ligand.

Pyocyanin (Colour Your Enzyme, Bath, Ontario, Canada) and 2,2′-bipyridine (Aldrich) were used as received. All aqueous stock solutions of pyocyanin were stored in a refrigerator in glass flasks under light-free conditions. There was no evidence of sample decomposition over several months when stored in this manner as judged by the electrospray spectrum of the diluted solutions. The metal salts were nitrates, bromides or sulfates and were of the highest purity on hand. The numbers of metal/pyocyanin and metal/pyocyanin/bipyridine complex ions observed in the electrospray spectra were not dependent on the anion of the salt.

3. Results

3.1. Metal complexes containing only pyocyanin

Preliminary experiments were carried out to determine the presence of metal–ligand complexes in the electrospray mass spectra of aqueous solutions containing a variety of metal ions and one of the potential ligands: phenazine, 1-hydroxyphenazine, phenazine-N-oxide, phenazine-N,N-dioxide, and pyocyanin. Of all these phenazines, complex formation was observed only with 1-hydroxyphenazine and pyocyanin, the two molecules having an O atom in the 1-position. All subsequent experiments were carried out with pyocyanin.

The alkali metal ions Li^+, Na^+, and K^+ formed complexes containing one, two and three pyocyanin, $[MP]^+$, $[MP_2]^+$, and $[MP_3]^+$ (M = metal, P = pyocyanin). The mass spectra of solutions of pyocyanin with Cs^+ contained only the complexes $[CsP]^+$ and $[CsP_2]^+$. As seen in Fig. 1(a), the major peak in the spectrum obtained with equimolar concentrations of pyocyanin and Li^+ is protonated pyocyanin PH^+. This was also the major ion in the spectra containing either Na^+ or K^+. In addition, the bare metal ions and/or their monohydrates were present as minor peaks in the spectra of solutions containing Li^+, Na^+ and K^+, but in Cs^+-containing solutions only the bare metal ion was present and it was invariably a major ion. In Fig. 1(a) $[LiP]^+$ (*m/z* 217) is the major complex whereas $[LiP_2]^+$ (*m/z* 427) is 20% as large and $[LiP_3]^+$ (*m/z* 637) is extremely small. No $[MP_4]^+$ was observed in any of the spectra obtained by electrospraying aqueous solutions containing alkali metal cations.

The electrospray spectra of aqueous solutions containing equimolar concentrations of pyocyanin and two alkali metal cations were also examined. A typical example, in Fig. 1(b), shows the electrospray spectrum obtained with a solution containing equimolar (50 μM) Li^+ and Cs^+ and 100 μM pyocyanin. It is apparent that Li^+, the smaller cation, has a greater tendency to form complexes with pyocyanin than does Cs^+. Also in the spectrum are peaks showing the presence of the complexes of pyocyanin with Na^+ and K^+. These cations may have been present as impurities in the reagents but were mainly from the leaching of glassware, the stock pyocyanin solution used in this particular experiment having been stored under light-free conditions in a glass vessel for many months. The degree of complexing of pyocyanin with the alkali metals, taken for each cation as the sum of all $[MP_n]^+$ intensities, was found in competitive experiments to increase in the order, $Cs^+ < K^+ < Na^+ < Li^+$.

CAD experiments were performed on $[MP_2]^+$ (M = Li, Na, Cs) with laboratory ion energies of 40 eV. The major product in all cases was $[MP]^+$, resulting from simple loss of one of the pyocyanin ligands. For $[LiP_2]^+$ and $[NaP_2]^+$, $[LiP]^+$ and $[NaP]^+$ were the respective sole products but for CsP_2^+, in addition to the formation of $[CsP]^+$, loss of both ligands to give Cs^+ accounted for 8% of total decomposition. At 20 eV laboratory energy, the Cs^+ peak was absent, the $[NaP]^+$ peak in the CAD spectrum of $[NaP_2]^+$ was considerably reduced in intensity and no decomposition of $[LiP_2]^+$ was observed. At 40 eV ion energy, with argon as collision gas, the centre of mass

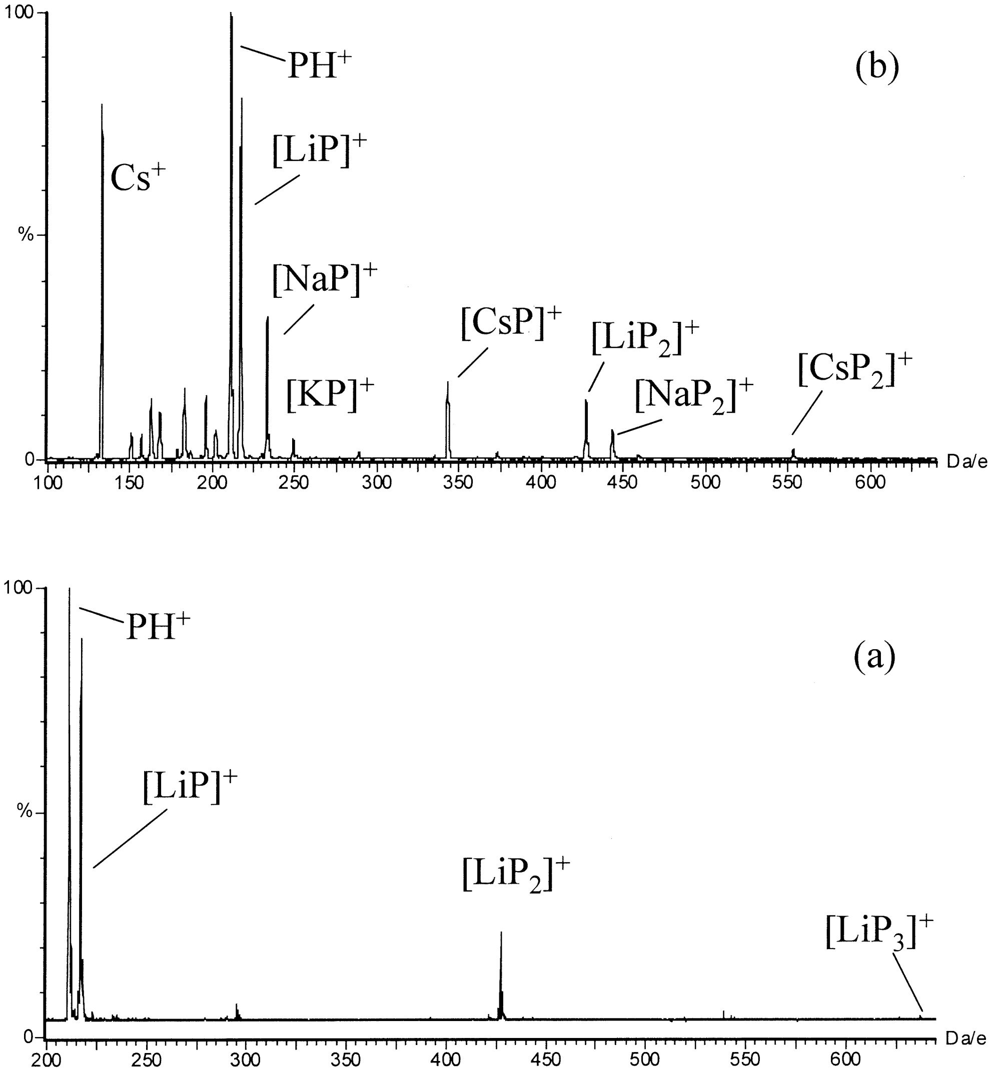


Fig. 1. Electrospray mass spectra from aqueous solutions containing (a) 100 μM Li^+ and 100 μM pyocyanin; (b) 50 μM Cs^+, 50 μM Li^+, and 100 μM pyocyanin. (P = pyocyanin).

collision energy increases in the reverse order of cation mass, viz. M = Cs (2.9 eV), Na (3.6 eV), and Li (3.8 eV). Thus, although $[LiP_2]^+$ is energized to a greater extent than $[CsP_2]^+$ at the same ion translational energy, it still shows less decomposition.

The formation of complexes with pyocyanin by the two alkaline earth divalent ions Mg^{2+} and Ca^{2+} showed some small differences. Each formed $[MP]^{2+}$, $[MP_2]^{2+}$, $[MP_3]^{\ 2+}$, and $[MP_4]^{2+}$. $[MgP]^{2+}$ in the spectrum of the magnesium salt was present almost wholly in the form of the monohydrate $[MgP(H_2O)]^{2+}$ whereas $[CaP]^{2+}$ was present as $[CaP]^{2+}$ (*m/z* 125), $[CaP(H_2O)]^{2+}$ (*m/z* 134), and $[CaP(H_2O)_2]^{2+}$ (*m/z* 143).

The CAD spectra of the pyocyanin complexes of Mg^{2+} and Ca^{2+} were more complicated than those of the corresponding complexes of the alkali metals since both loss of ligand(s) and charge reduction are possible. The CAD spectra of both $[MgP_3]^{2+}$ and $[CaP_3]^{2+}$ obtained with ion energies of 20 eV showed loss of a single pyocyanin to yield $[MP_2]^{2+}$ as the major product. At 40 eV the same product was dominant and a small yield of P^+ was observed together with that of the complementary ion, $[MP_2]^+$. The CAD spectrum for $[MgP_3]^{2+}$ obtained with an ion energy of 60 eV showed, in addition to $[MgP_2]^{2+}$, $[MgP_2]^+$, and P^+, a small peak at *m/z* 429, which corresponds to the loss of 15 u from $[MgP_2]^+$. At the higher energy of 80 eV, as seen in Fig. 2(a), small peaks at *m/z* 214.5 and 207 correspond to losses of 15 u and 2 × 15 u from $[MgP_2]^{2+}$ giving, respectively, $[MgP(P\text{-}15)]^{2+}$ and and $[Mg(P\text{-}15)_2]^{2+}$. The small peak at *m/z* 182 corresponds to the loss of 28 u from the molecular ion of pyocyanin that is present at *m/z* 210. This identification of *m/z* 182 as an ion not containing metal follows from its appearance in the CAD spectra of Zn-containing complexes where the existence of ^{64}Zn and ^{66}Zn isotopes allow the presence or absence of metal in any CAD product to be confirmed. The CAD spectra of $[CaP_3]^{2+}$ were very similar to those of $[MgP_3]^{2+}$.

The CAD spectra of $[MgP_2]^{2+}$, $[CaP_2]^{2+}$, and $[BaP_2]^{2+}$, obtained with ion translational energies of 60 eV are shown respectively in Figs. 2(a), 3(a) and (b). The dependence of the relative yields of the products in the CAD spectrum of $[CaP_2]^+$ on the initial ion translational energy are shown in Fig. 4.

The electrospray spectra obtained with solutions containing Zn^{2+} and pyocyanin showed two doubly charged complex ions, $[ZnP_2]^{2+}$ and $[ZnP_3]^{2+}$. The intensity of $[ZnP_3]^{2+}$ was very small. In the CAD spectra of $[ZnP_2]^{2+}$ with laboratory collision energies up to 80 eV, there were five products, the major ones P^+, *m/z* 182 and *m/z* 167 being derived solely by electron transfer from one of the pyocyanin ligands to the metal. $[ZnP(P\text{-}15)]^{2+}$ and an extremely small contribution from $[Zn(P\text{-}15)]^+$, were the only zinc-containing products. The intensity of $[ZnP_3]^{2+}$ was too low for a CAD study. The ions observed in the CAD spectra of $[MP_2]^{2+}$ (M = Mg, Ca, Ba, Zn) at 80 eV ion energy are listed in Table 1.

3.2. Metal complexes containing both pyocyanin and bipyridine

Electrospray spectra were obtained by infusing aqueous solutions containing equimolar concentrations of an alkali metal cation, pyocyanin and bipyridine. The three electrospray mass spectra for Li^+, Na^+, and Cs^+ shown in Fig. 5 were obtained under identical instrumental conditions. In Fig. 5(c) the base peak is that of Cs^+ and in Fig. 5(b) Na^+ is present, but it is not the base peak. A search was made for Li^+ in the spectra of the lithium-containing solution but neither it, nor any of its hydrates, was observable.

The alkali metals formed complexes with bipyridine alone that, under the experimental conditions, contained no more than two ligands. This was in contrast to pyocyanin that formed $[MP_3]^+$ with all cations except Cs^+ although, as noted, $[MP_3]^+$ was of considerably lower intensity than $[MP]^+$ and $[MP_2]^+$. The relative peak heights of the complexes in Fig. 5 suggested that bipyridine might be the better ligand for Li^+ and Na^+ whereas pyocyanin would be the better ligand for Cs. This idea was tested by obtaining CAD spectra for ions containing both pyocyanin and bipyridine as ligands. The results in Fig. 6, obtained with ion energies of 40 eV, show that loss of neutral bipyridine is favored over loss of pyocyanin. In spectra obtained with ion energies of 20 eV, the products $[Li(bpy)]^+$ and $[Na(bpy)]^+$ had negligible intensities compared with those of $[LiP]^+$ and $[NaP]^+$.

Competition experiments with equimolar concentrations of pyocyanin, bipyridine and one of Mg^{2+} and Ca^{2+} lead to a greater number of permutations and combinations of the ligands than for the alkali metals. Fig. 7(a) shows the formation of MgP_n^{2+} (n = 1–4), $[Mg(bpy)_m]^{2+}$ (m = 1–3) and $[MgP_n(bpy)_m]^{2+}$ (n = 1–3, m = 1 and 2). Fig. 7(b) shows that similar peaks were obtained with Ca^{2+}.

In the CAD spectra of ions containing both pyocyanin and bipyridine as ligands attached to either Ca^{2+} or Mg^{2+}, loss of bipyridine always predominated over loss of pyocyanin. This is illustrated in Fig. 8(a) for the collisional dissociation of $[MgP_2(bpy)]^{2+}$ and Fig. 8(b) for the collisional dissociation of

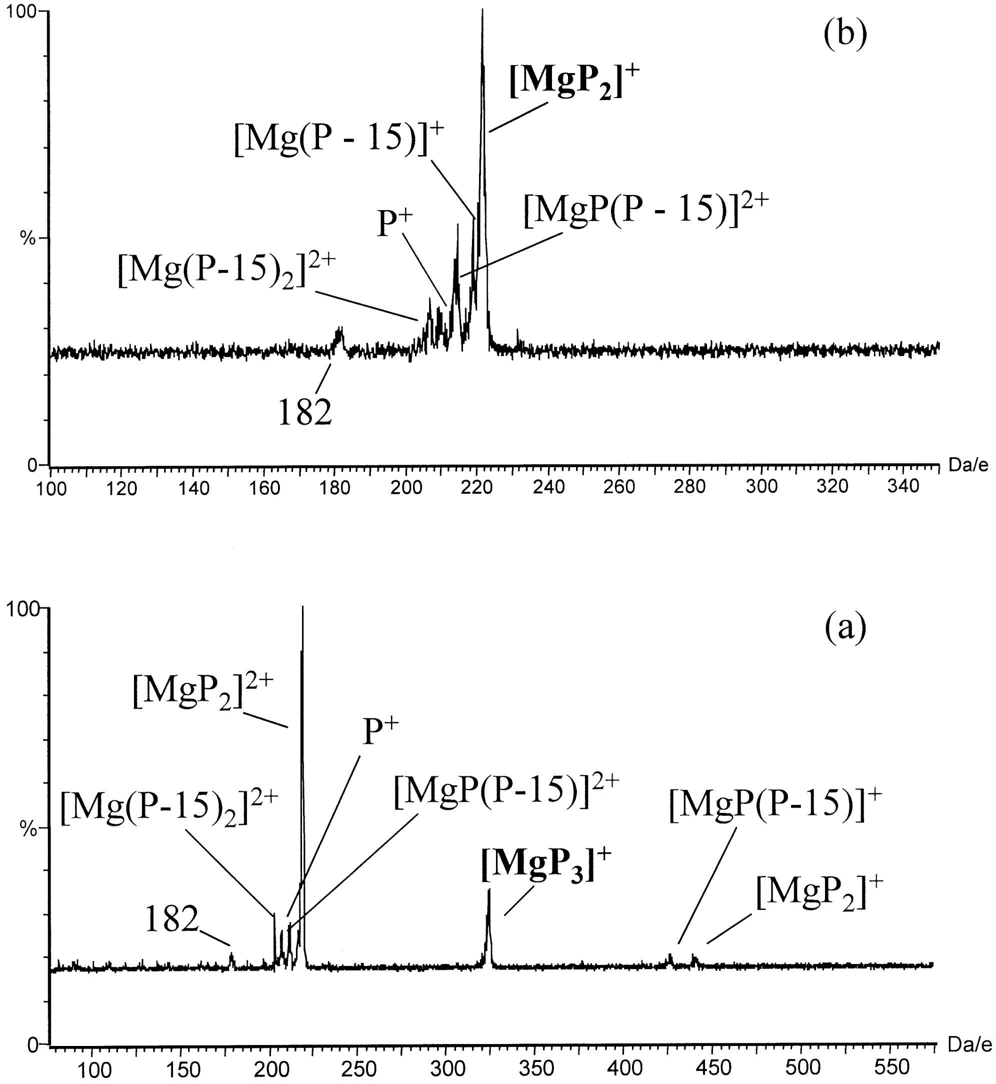


Fig. 2. CAD spectra of (a) $[MgP_3]^{2+}$, ion energy = 80 eV and (b) $[MgP_2]^{2+}$, ion energy = 60 eV. Parent ions are shown in bold type (P = pyocyanin).

$[CaP_2(bpy)]^{2+}$, both at the same ion energy of 40 eV. The dissociation of the 1:2 ions, $[MgP(bpy)]^{2+}$ and $[CaP(bpy)]^{2+}$, was more complex. The major metal-containing product ion at low collision energy was due to the loss of 15 u giving $[M(P–15)(dpy)]^{2+}$ analogous to the $[MP(P–15)]^{2+}$ found for $[MgP_2]^{2+}$ and $[CaP_2]^{2+}$. The fractional product yields from $[CaP(bpy)]^{2+}$ as parent ion as functions of ion energy are shown in Fig. 9.

The electrospray spectra of aqueous solutions containing equimolar concentrations of Zn^{2+}, pyocyanin and bipyridine gave gas-phase complexes in which bipyridine was the predominant ligand. Fig. 7(c) shows that $[Zn(bpy)_2]^{2+}$ has an even higher intensity than PH^+, which is a unique observation amongst all the cation solutions examined. $[ZnP(bpy)]^{2+}$ is also of high intensity, but ions with only pyocyanin as ligand, $[ZnP_2]^{2+}$ and $[ZnP_3]^{2+}$ are, as before, of very low intensity. The CAD spectrum of $[ZnP(bpy)]^{2+}$ showed only two product ions that contained zinc,

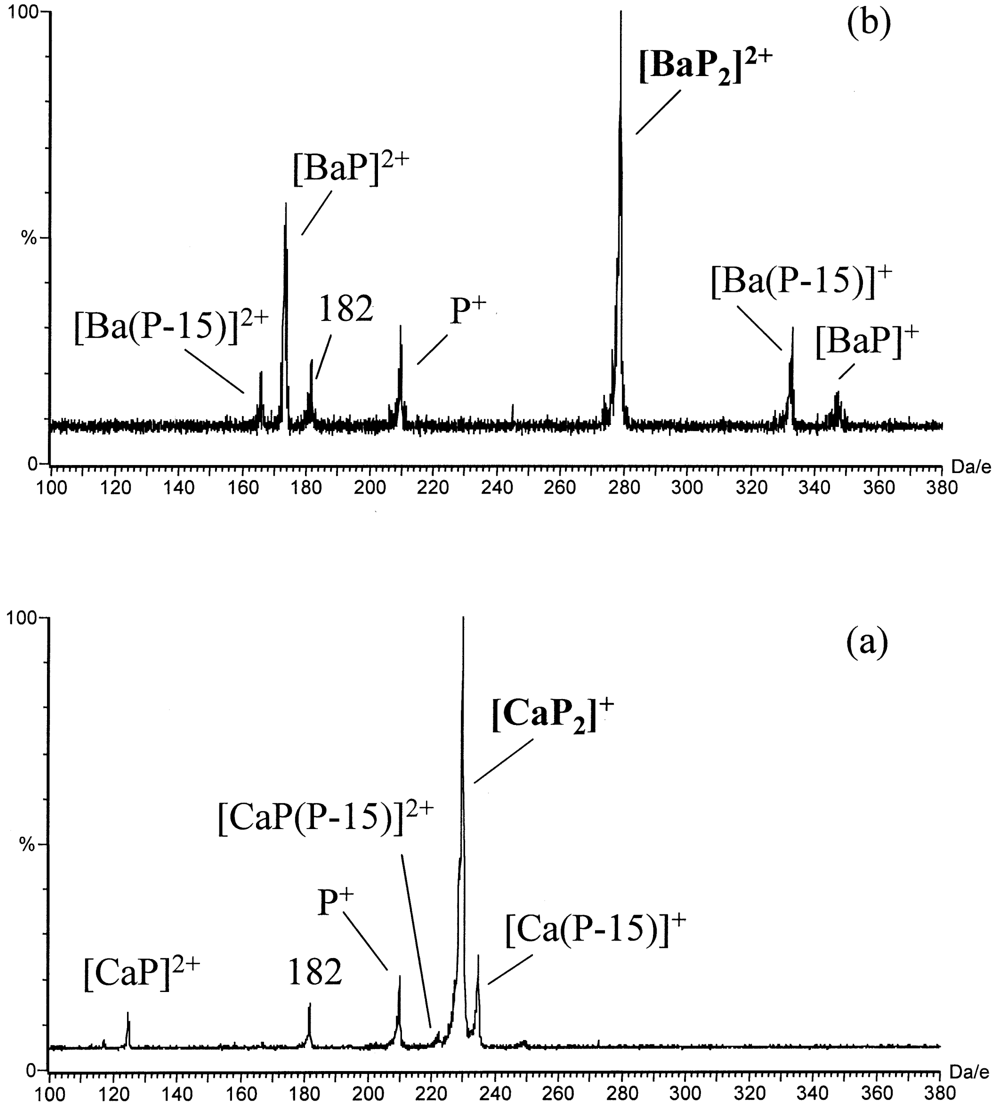


Fig. 3. CAD spectra of: (a) [CaP$_2$]$^{2+}$, ion energy = 60 eV and (b) [BaP$_2$]$^{2+}$, ion energy = 60 eV. Parent ions are shown in bold type (P = pyocyanin).

confirmed by comparing the spectra of complexes containing ^{64}Zn and ^{66}Zn. They were, *m/z* 207.5, which corresponds to [^{64}Zn(P-15) (bpy)]$^{2+}$ and *m/z* 220, which is [Zn(bpy)]$^+$. No [ZnP]$^+$ was observed, although (bpy)$^+$ (*m/z* 156) was a product. Fig. 10 shows how the fractional yields of these products changed with collision energy. The other products were *m/z* 182 and *m/z* 167, resulting from the decomposition of the pyocyanin cation.

The ions observed in the CAD spectra of [MP(bpy)]$^{2+}$, (M = Mg, Ca, Zn) at 80 eV ion energy are listed in Table 2.

4. Discussion

Pyocyanin forms gas-phase complexes by electrospray with all the metal cations that were investigated.

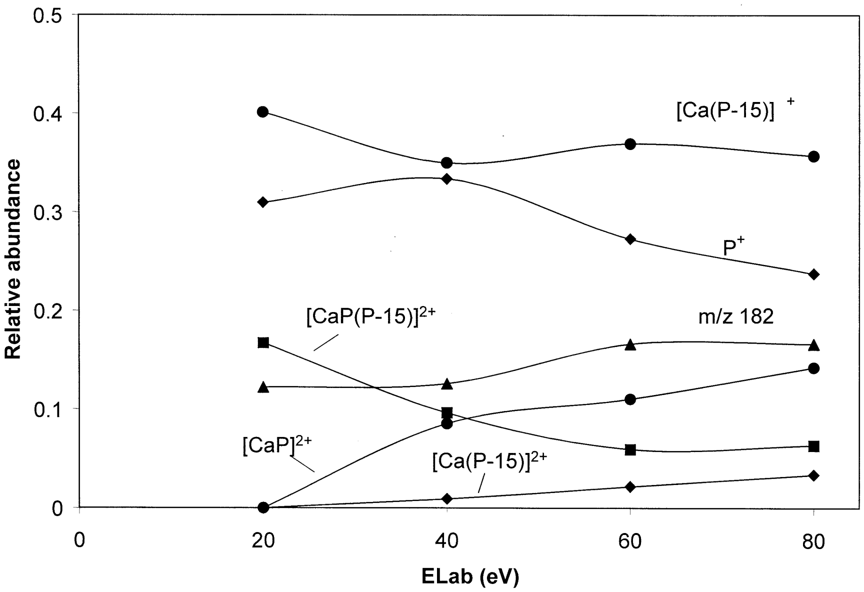


Fig. 4. Product ion yields as functions of ion energy in the CAD spectrum of $[CaP_2]^{2+}$ (P = pyocyanin).

In general the highest intensity in the spectra obtained from equimolar aqueous solutions of pyocyanin with one of the alkali metal cations was that of the 1:1 complex, $[MP]^+$ whereas for the doubly charged alkaline earth and zinc cations it was the 2:1 complex, $[MP_2]^{2+}$. The size of the alkali metal cation plays a major role in the intensities of the ions observed, Li^+ giving the greatest total intensity for all $[MP_n]^+$ ions, either for solutions containing a single cation or from solutions containing equimolar concentrations of two alkali metal cations. The determined order of total intensities was $Li^+ > Na^+ > K^+ > Cs^+$. This is the same order that is usually found in both aqueous and non-aqueous solutions for many organic complexes of the alkali metals.[12] For example the stabilities of the alkali metal cation complexes with 2,2′-bipyridne [16–18], and 1.10-phenanthroline and 8-hydroxyquinoline [17,18] increase with decreasing cation size, consistent with the mainly electrostatic nature of the binding. Also, somewhat surprisingly, the gas-phase binding energies of alkali metal ion–crown ether complexes are determined by the cation size and not by the ion/cavity radius [19]. Size is also obviously important in the gas-phase binding of pyocyanin and the doubly charged cations since in competition experiments for pyocyanin between equimolar concentrations of Ca^{2+} and Zn^{2+} the latter ion, which was strongly favoured, is the smaller of the two cations. The Pauling radii are 0.99Å and 0.74Å, respectively for Ca^{2+} and Zn^{2+}.[11] Surface charge density, which is directly related to ion size, is obviously the controlling factor determining the relative affinities of the cations for pyocyanin.

Table 1
CAD products from the dissociation of $[MP_2]^{2+}$

Product	Mg	Ca	Ba	Zn
$[MP(P\text{-}15)]^{2+}$	√	√	√	√
$[M(P\text{-}15)_2]^{2+}$	√	√		
$[MP]^{2+}$		√	√	
$[MP]^+$		√	√	
$[M(P\text{-}15)]^+$	√		√	√
P^+		√	√	√
m/z 182	√	√	√	√
m/z 167				√

The nature, monodentate or bidentate, of the ligation of pyocyanin to the metal cations is not directly determinable. However, the maximum of three pyocyanin molecules ligated with the alkali metals and Zn^{2+}, and a very low intensity MP_4^+ with Ca^{2+} and Mg^{2+} speaks strongly in favour of a preferred biden-

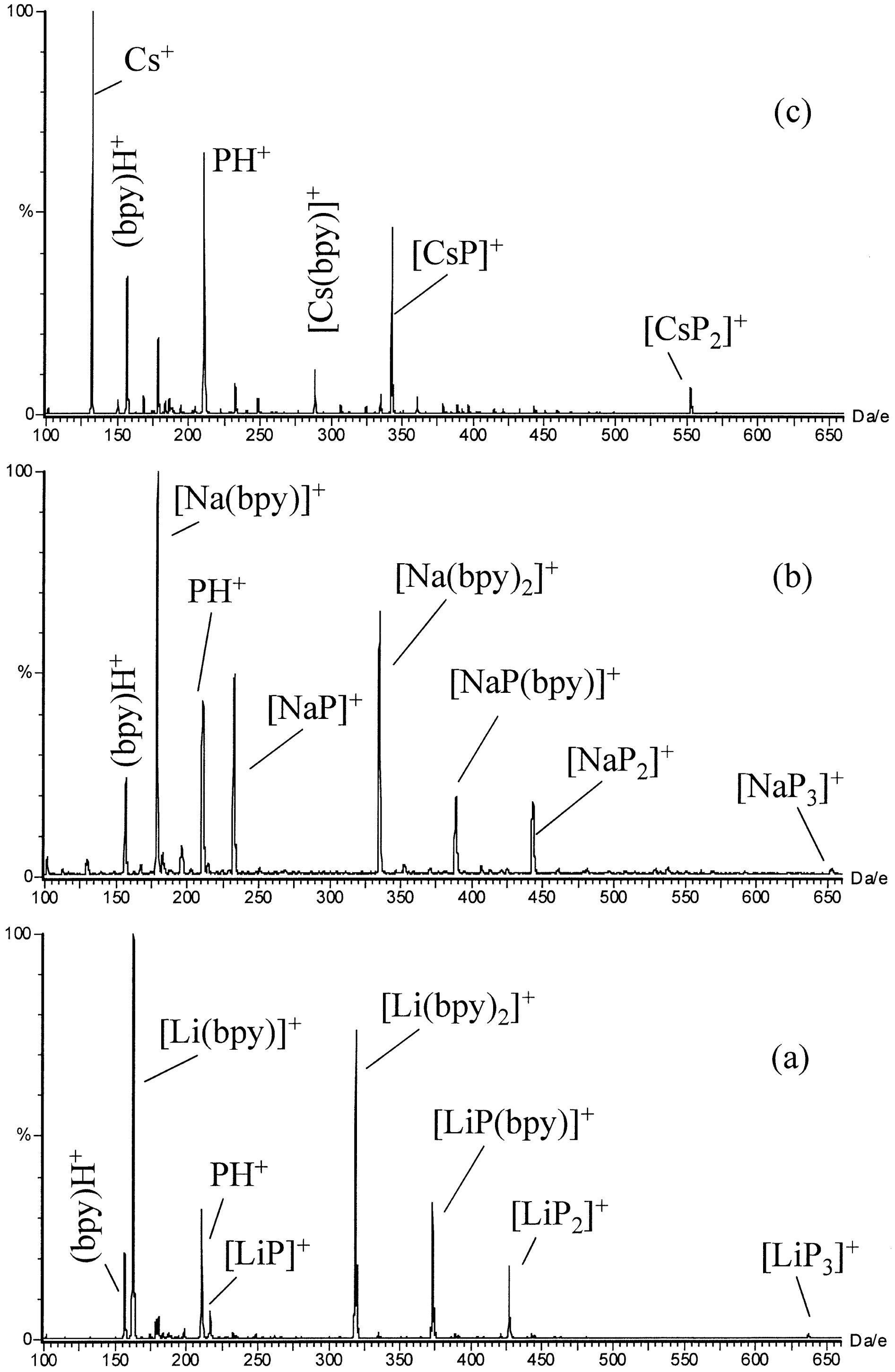


Fig. 5. Electrospray mass spectra from aqueous solutions containing (a) 100 μM Li^+, 100 μM pyocyanin, and 100 μM bipyridine; (b) 100 μM Na^+, 100 μM pyocyanin, and 100 μM bipyridine; and (c) 100 μM Cs^+, 100 μM pyocyanin, and 100 μM bipyridine (P = pyocyanin, bpy = 2,2′-bipyridine).

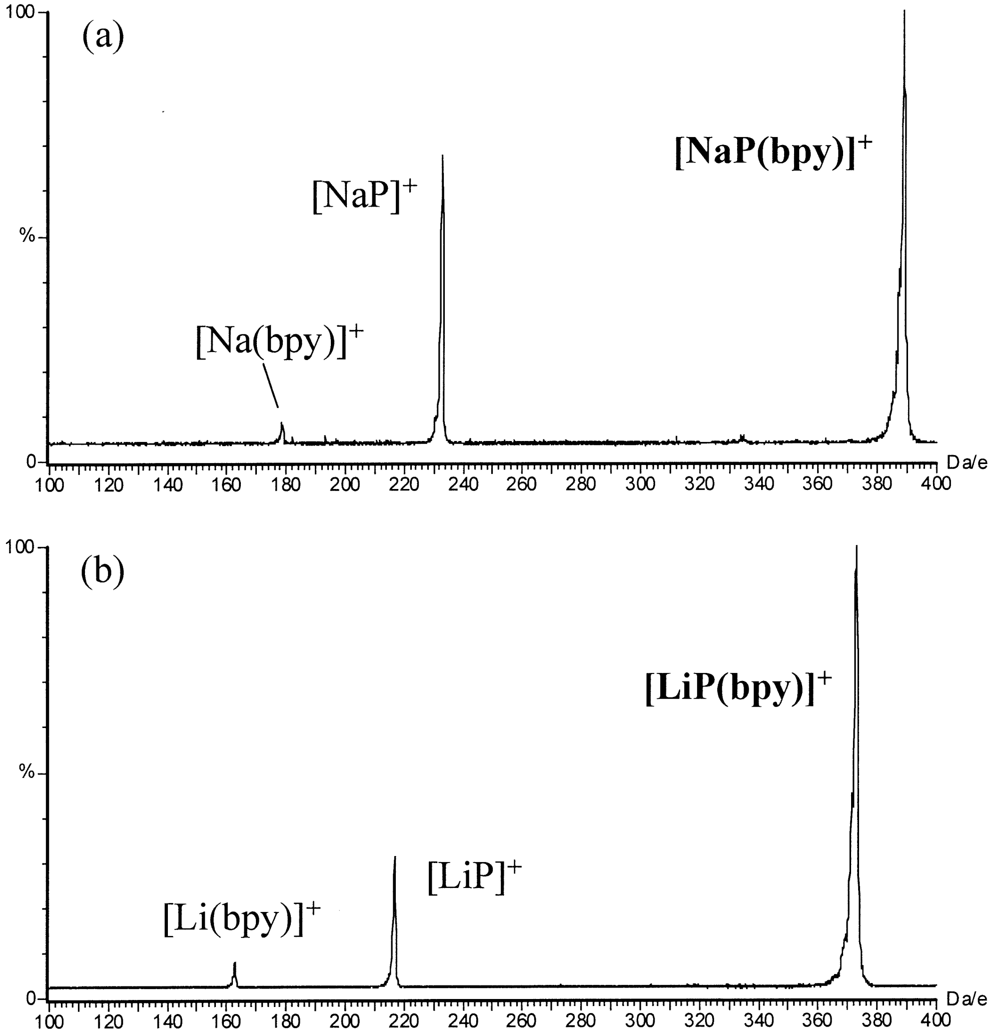


Fig. 6. CAD spectra of (a) $[LiP(bpy)]^+$, ion energy = 40 eV and (b) $[NaP(bpy)]^+$, ion energy = 40 eV. Parent ions are shown in bold type (P = pyocyanin, bpy = 2,2′-bipyridine).

tate attachment with four or six coordination sites. This is supported by a previous electrospray study of the complexing of Mn^{2+} and pyocyanin in which, although the ligand/metal ion concentration ratio was varied from 1:4 to 20:1, no evidence was found for the formation of $[MnP_4]^{2+}$.[10] The $[ZnP]^{2+}$ complex has been observed in nonaqueous solution [7]. Its formation constant in dimethyl sulfoxide as solvent and in the presence of tetrapropyl ammonium perchlorate was found to be $\log K = 3.3 \pm 0.2$, which was essentially the same value determined for the other transition metal cations Fe^{2+}, Ni^{2+}, and Cu^{2+}. The value for Mn^{2+} was slightly lower at 2.2 ± 0.2. No mention was made of higher complexes.

Bidentate attachment would entail interaction between the metal cation and the O and N(10) of each pyocyanin ligand. However, there is no evidence to suggest what the coordination might be. The electrospray spectra and the CAD spectra suggest that the first two pyocyanins are held much more strongly than the third and fourth. The third and fourth pyocyanins would then be present in an

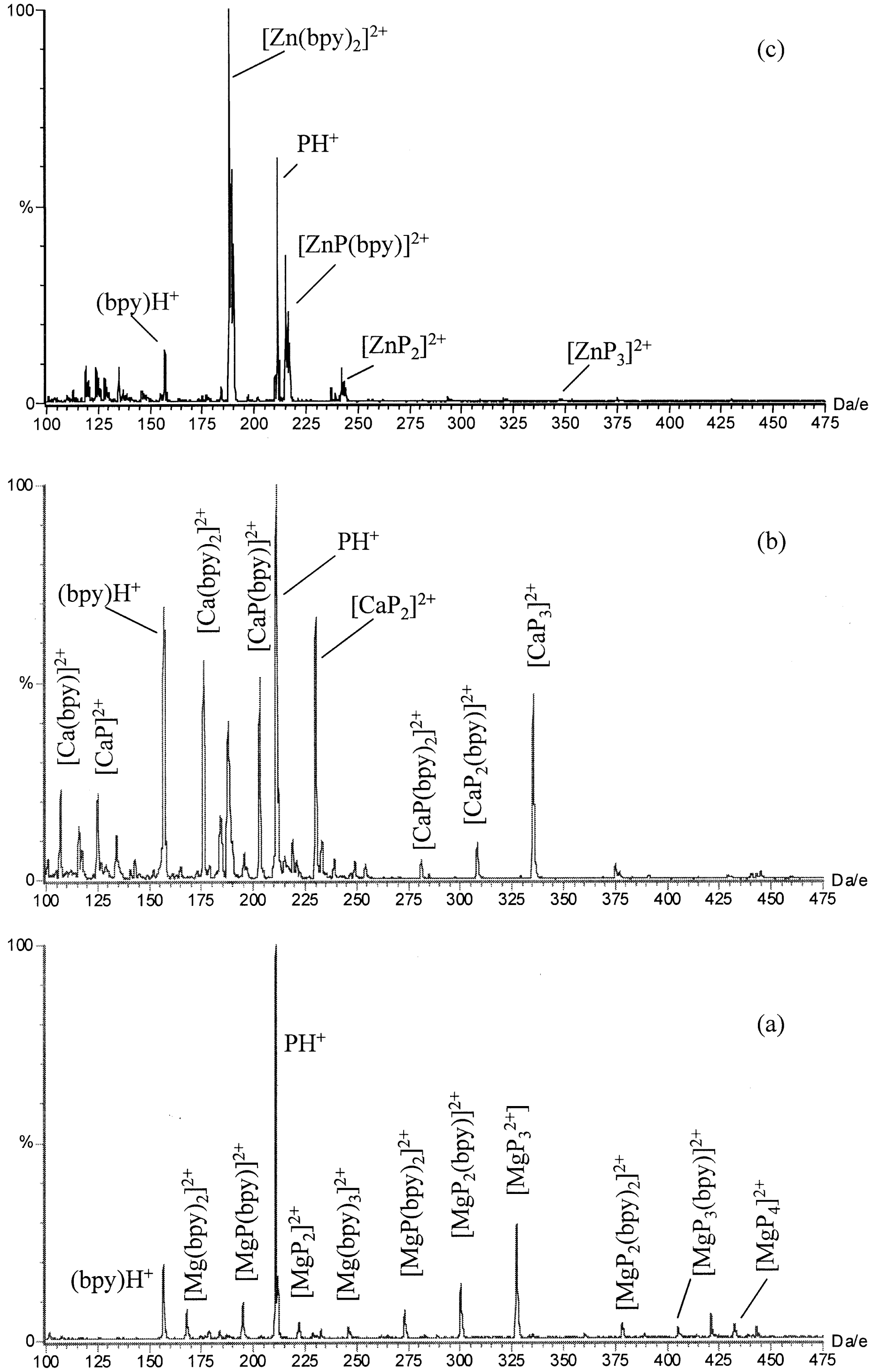


Fig. 7. Electrospray mass spectra from aqueous solutions containing (a) 100 μM Mg^{2+}, 100 μM pyocyanin, and 100 μM bipyridine; (b) 100 μM Ca^{2+}, 100 μM pyocyanin, and 100 μM bipyridine; and (c) 100 μM Zn^{2+}, 100 μM pyocyanin, and 100 μM bipyridine (P = pyocyanin, bpy = 2,2′-bipyridine).

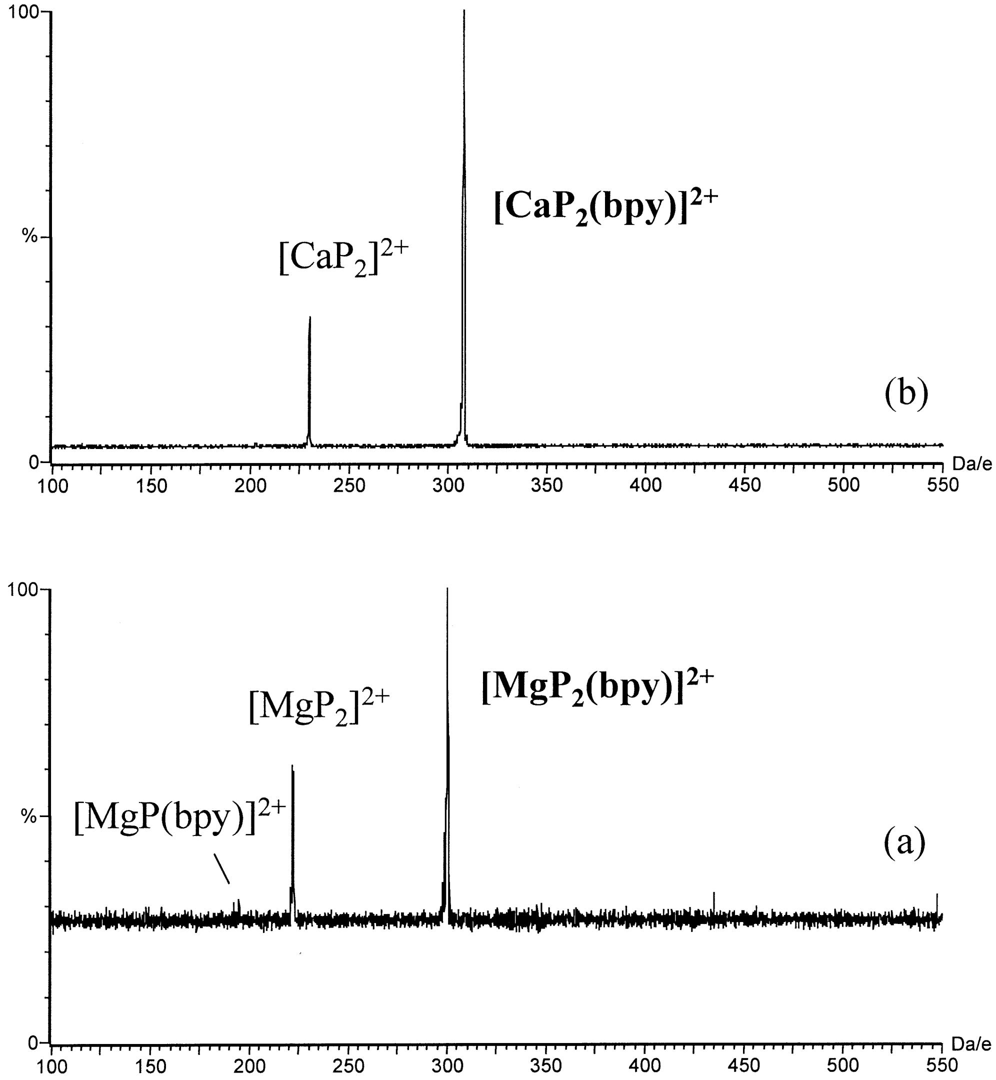


Fig. 8. CAD spectra of (a) $[MgP_2(bpy)]^{2+}$, ion energy = 40 eV and (b) $[CaP_2(bpy)]^{2+}$, ion energy = 40 eV. Parent ions are shown in bold type (P = pyocyanin, bpy = 2,2′-bipyridine).

outer shell if the coordination was tetrahedral, or in axial positions if the coordination were square planar. In the latter case the two extra ligands would be acting in a monodentate manner. If sterically possible, by analogy with the first hydration shells of both Mg^{2+} and Ca^{2+} that are calculated to contain six water molecules, 6-coordination would appear to be preferred [20].

The CAD spectra of the alkali metal complexes $[MP_2]^+$ were unremarkable. The yields of $[MP]^+$ by loss of pyocyanin from $[MP_2]^+$ for all cations at the same laboratory collision energy were in the order $[CsP]^+ > [NaP]^+ > [LiP]^+$, which confirms that the stability of the pyocyanin complexes decrease with increasing cation size. Further confirmation was the presence of a significant intensity of Cs^+, a trace of Na^+, and no Li^+ in the respective CAD spectra.

The CAD spectra of the doubly charged pyocyanin-containing ions were, not unexpectedly, more complex than those of the alkali metals since charge

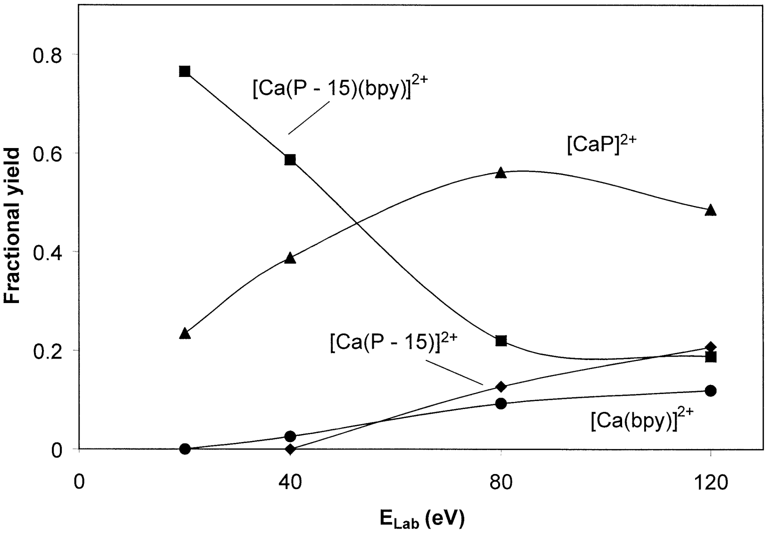


Fig. 9. Product ion yields as functions of ion energy in the CAD spectrum of $[CaP(bpy)]^{2+}$ (P = pyocyanin, bpy = 2,2′-bipyridine).

reduction reactions are now viable. The substantial loss of pyocyanin at 40 eV in the CAD spectrum of $[MgP_3]^{2+}$ [Fig. 2(a)] demonstrates a weakly held third pyocyanin. There are in addition, small peaks at *m/z* 444 and *m/z* 429 corresponding to $[MgP_2]^+$ and $[MgP(P–15)]^+$, formed by charge transfer to, and loss of, one of the ligands. The ions P^+ (*m/z* 210) and $[P-28]^+$ (*m/z* 182) are the complementary ions. Although decomposition of $[MgP_3]^{2+}$ is extensive under the experimental conditions, there is no loss of a second pyocyanin.

Instead, there is loss of 15 u giving *m/z* 214.5, and

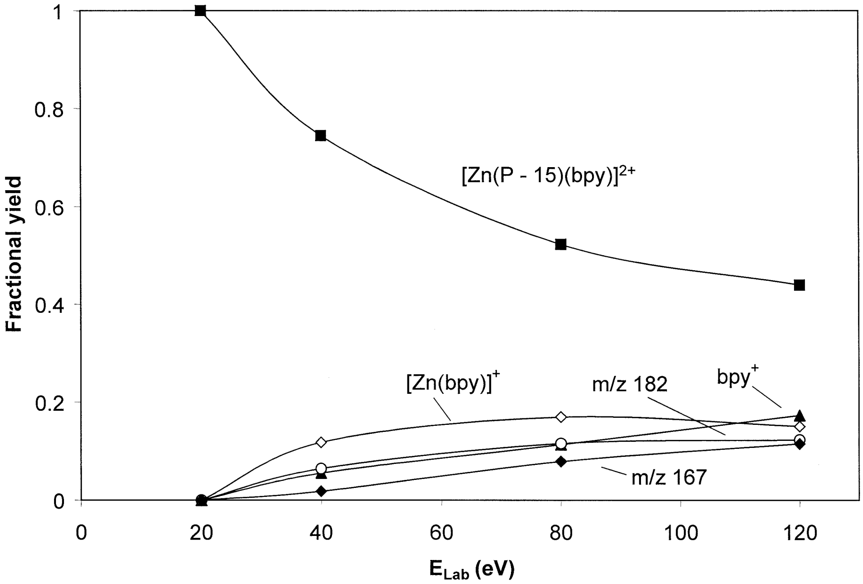


Fig. 10. Product ion yields as functions of ion energy in the CAD spectrum of $[Zn(pyocyanin)(bipyridine)]^{2+}$ (P = pyocyanin, bpy = 2,2′-bipyridine).

Table 2
CAD products from the dissociation of $[MP(bpy)]^{2+}$

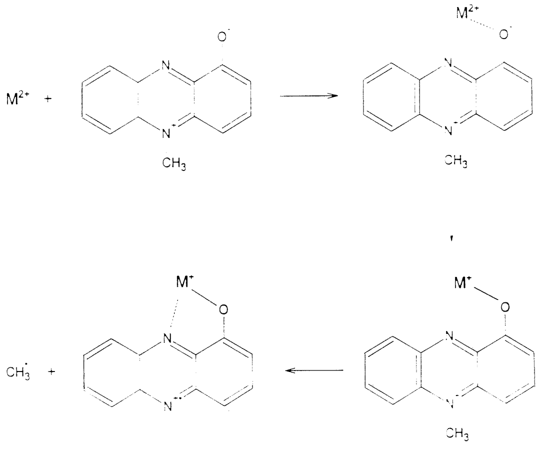
Production	Mg	Ca	Zn
$[M(P\text{-}15)(bpy)]^{2+}$	√	√	√
$[MP]^{2+}$		√	
$[M(P\text{-}15)]^{2+}$		√	
$[M(bpy)]^{2+}$	√	√	
$[M(bpy)]^{+}$			√
m/z 182			√
m/z 167			√
$[bpy]^{+}$			√

loss of 30 u giving *m/z* 207. These ions result from the further decomposition of the product $[MgP_2]^{2+}$. This decomposition is discussed below. At low collision energy the only product ion in the CAD spectra of $[MP_3]^{2+}$ (M = Mg, Ca) is $[MP_2]^{2+}$ consistent with the observation that fully coordinated doubly charged metal cations shed excess energy by “desolvation” rather than in charge reduction reactions [21,22].

The CAD spectra of $[MgP_2]^{2+}$, $[CaP_2]^{2+}$, and $[BaP_2]^{2+}$ in Figs. 2(b), 3(a) and (b), respectively, were obtained at the same ion translational energy of 60 eV, corresponding to centre of mass collision energies of 0.54, 0.52, and 0.40 eV, respectively. The spectra show trends that are attributable to the differences in the sizes of the metal cations. Whereas $[MgP_2]^{2+}$ does not give $[MgP]^{2+}$ as a product, $[CaP_2]^{2+}$ shows a significant peak for $[CaP]^{2+}$ whereas $[BaP]^{2+}$ is the largest product peak in the spectrum of $[BaP_2]^{2+}$. Product ions formed without loss of both a complete ligand and charge predominate in the CAD spectrum of $[MgP_2]^{2+}$ but are of lesser importance in the spectrum of $[CaP_2]^{2+}$ and even less in that of $[BaP_2]^{2+}$. Product ions formed by loss of 15 u constitute the predominant products from $[MgP_2]^{2+}$ but are of lesser importance from $[CaP_2]^{2+}$ and are of even lesser importance from $[BaP_2]^{2+}$. The double loss of 15 u gives rise to $[Mg(P\text{-}15)_2]^{2+}$ from $[MgP_2]^{2+}$, but no analogous ion is present in the spectrum of either $[CaP_2]^{2+}$ or $[BaP_2]^{2+}$. These observations lead to the conclusion that the smallest cation, Mg^{2+}, interacts more strongly with the pyocyanin ligand, resulting in greater retention of charge and ligands.

The ion energy dependence of products in the CAD spectra of $[CaP_2]^{2+}$ (Fig. 4) show that the only metal-containing product ions at low energy are $[CaP(P\text{-}15)]^{2+}$ and $[Ca(P\text{-}15)]^{+}$. Loss of 15 u is therefore the dominant low energy process, whether combined with charge reduction to produce $[Ca(P\text{-}15)]^{+}$, P^{+} and $[P\text{-}28]^{+}$ or by simple formation of $[CaP(P\text{-}15)]^{2+}$. Increasing collision energy does not have much effect on the relative abundance of $[Ca(P\text{-}15)]^{+}$, but the formation of $[CaP(P\text{-}15)]^{2+}$ is superceded mainly by simple loss of one of the ligands to produce $[CaP]^{2+}$ and to a lesser extent by loss of one ligand and 15 u from the remaining ligand to produce $[Ca(P\text{-}15)]^{2+}$.

Ions, both singly and doubly charged, formed by loss of 15 u are important processes in the CAD spectra of the pyocyanin complexes of Mg^{2+}, Ca^{2+}, and Ba^{2+} but decrease in relative importance with increasing cation size. 15 u corresponds to either $\cdot CH_3$ or ·NH. Loss of ·NH would presumably involve N(10), which process would destroy the bidentate character of the pyocyanin ligand. It would also require extensive reorganization and also prior H transfer to N(10) and a reduction in the size of the central ring. This sequence is improbable. In contrast, the contribution of the zwitterionic form (II) of pyocyanin would be enhanced by the association of N(10) and O with a metal cation, resulting in the withdrawal of electron density from N(5). Loss of methyl, as shown in Scheme 2, would then effectively



Scheme 2

leave a formal charge of +1 on N(5) and the odd electron could be delocalized over the whole complex. The delocalization of charge away from the metal would strengthen the association between metal and ligand. The smaller Mg^{2+} is more effective than the larger cations in this methyl elimination, as witnessed by the formation of $[Mg(P\text{-}15)_2]^{2+}$, whereas the analogous ions are not observed in the CAD spectra of $[CaP_2]^{2+}$ and $[BaP_2]^{2+}$. The enhanced covalent character of the binding of simple monodentate ligands to Mg^{2+} compared to Ca^{2+} [23] will lead to the greater propensity to methyl loss in the activated complexes.

The propensity for loss of the methyl radical from one or both pyocyanin ligands is consistent with the electron impact and metastable mass spectra of phenazine and substituted phenazines [24]. Although ionized phenazine lost its nitrogen atoms sequentially as CN and HCN, the substituted phenazines showed a strong tendency for the ionized molecules to lose their substituents before loss of nitrogen occurred A mass spectrum of pyocyanin was not investigated by those workers and indeed the mass spectrum of pyocyanin has not been satisfactorily obtained. Taylor and coworkers found that pyocyanin was readily observed as the protonated molecule when liquid chromatography and a thermospray source were employed [25,26]. However, sample introduction via a gas chromatograph showed that pyocyanin had probably degraded in the heated injection port to yield other phenazines, possibly 1-hydroxyphenazine and 1-methoxyphenazine. Analysis by desorption from a heated wire followed by electron impact gave an ion at *m/z* 210 when high heating wire currents were employed but substantial peaks at *m/z* 211, 212, and 224 showed that significant inter-molecular rearrangements were occurring [25]. There was no confirmation of the identities of these ions. We also have been unable to obtain the molecular ion of pyocyanin by electron impact ionization of the vapour from a solids probe sample. At low sample pressure we saw a spectrum identical to that of 1-hydroxyphenazine and at higher pressure, obtained with the probe at a higher temperature, new ions appeared at *m/z* 211, 212, 213, and 214. Molecular rearrangements were occurring in vaporization from the solids probe. The pyocyanin used in this study was authenticated by both NMR and electrospray mass spectrometry and was judged to have a purity better than 97%. However, its absolute purity was not determined. Protonated pyocyanin, obtained using the electrospray source, was collisionally decomposed at an ion energy of 40 eV to give product ions with almost equal intensities at *m/z* 196 (loss of CH_3), *m/z* 183 (loss of CO or CH_2N), and *m/z* 168 (loss of CO + CH_3).

The competition between pyocyanin and bipyridine in forming complexes with the metal cations is interesting since the electrospray and CAD results present different pictures of the complexation. The electrospray spectra shown in Fig. 5 were obtained with equimolar concentrations of an alkali metal cation, pyocyanin, and bipyridine. Metal complexes containing one or two bipyridine and no pyocyanin are strongly favoured by Li^+ over those containing one or two pyocyanin and no bipyridine. This partiality for bipyridine is less for Na^+, whereas Cs^+ strongly favours those complexes containing only pyocyanin. The mixed complexes, $[MP(bpy)]^+$ (M = Li, Na), are significant ions but $[CsP(bpy)]^+$ (*m/z* 499) is not observed. Similarly, the complexes $[M(bpy)_2]^+$ (M = Li, Na) are major ions but $\{Cs(bpy)_2]^+$ is not observed. $[M(bpy)_3]^+$ is not observed in any of the spectra whereas $[MP_3]^+$ ions (M = Li, Na) are present, as they were in the electrospray spectra of the solutions in the absence of bipyridine. In the electrospray process therefore, bipyridine is favoured as a complexing agent by the smaller cations, Li^+ and Na^+, whereas pyocyanin is favoured by the larger Cs^+. However, this only applies for complexes containing one or two ligands. In complexes containing three ligands only pyocyanin is present.

The influence of cation size in the formation of complexes between the alkali metal cations and bipyridine is in agreement with results from multinuclear NMR studies of complexation in nonaqueous solvents [18]. The estimated stabilities of the alkali metal cation–bipyridine complexes were in the order $K^+ < Na^+ < Li^+$, although the stoichiometry of the complexes could not be established, other than that of $[Li(bpy)_2]^+$ in nitromethane as solvent. No NMR evidence for the formation of Cs^+ complexes was found.

The doubly charged cations, Mg^{2+} and Ca^{2+}, show a preference for forming complexes containing pyocyanin rather than bipyridine, Mg^{2+} more so than Ca^{2+} [(Fig. 7(a) and (b)]. This is in the reverse order of the ionic sizes, contrary to that found for the alkali metals. Zn^{2+} on the other hand strongly favours complexes in which bipyridine is a ligand [Fig. 7(c)]. This figure shows that for the first time in any of the electrospray spectra, PH^+ is not the ion with highest intensity, here $[Zn(bpy)_2]^{2+}$ assumes that role. Zn^{2+} is behaving more like a transition metal ion than like Mg^{2+}, in that it is favouring complexing with the two basic nitrogen sites of bipyridine rather than the oxygen and nitrogen basic sites of pyocyanin. It is also to be noted that, in contrast to complex formation by the alkali metal cations, bipyridine is present in complexes of the general formula $[MP_2(bpy)]^{2+}$ and $[MP\ (bpy)_2]^{2+}$ for all three doubly charged cations, although the peaks for the zinc complexes are of very low intensity.

The CAD spectra of $[MP(bpy)]^+$ of the alkali metals Li and Na shown in Fig. 6, demonstrate an overwhelming preference for loss of bipyridine

$$[MP(bpy)]^+ \rightarrow [MP]^+ + bpy \tag{1}$$

$$\rightarrow [M(bpy)]^+ + P \tag{2}$$

The relative rates of decomposition, k_1/k_2, judged by the relative peak heights of MP^+ and $[M(bpy)]^+$ in the CAD spectra, are 5 for $[LiP(bpy)]^+$ and 14 for $[NaP(bpy)]^+$. Therefore, although complexes containing one or more bipyridine ligands predominate over those containing one or more pyocyanin ligands in the electrospray spectra, the loss of bipyridine is strongly favoured over the loss of pyocyanin in the CAD spectra.

The relative stability constants for the two ligands, bipyridine and pyocyanin, associating with alkali metal cations in aqueous solution are unknown and it is therefore impossible to say how well the electrospray spectra typify ion distribution in solution. Strong correlations between ion abundances in electrospray spectra and known relative stability constants in solution have been reported for 1:1 crown ether-alkali metal ion complexes [27,28]. The suggested reason for the accord is the close correlation of the solvation energies of the complexes [27]. The relative ion intensities of the 2:1 complexes are reported both to show good agreement with expected values [27] and also to show little or no correlation with solution concentrations [28]. The lack of correlation was suggested to be an experimental artifact caused by gas phase reactions, although charged droplet equilibria or kinetics might have been responsible. The CAD results in Fig. 6 showing a weaker bonding of alkali metal cation with bipyridine should rule out gas phase reactions distorting the electrospray spectra. If that were not so then all electrospray spectra should be weighted heavily in favour of pyocyanin, contrary to that which is observed in Fig. 5. If the Iribarne and Thomson [29,30] model for the formation of ions by the electrospray process is correct then, as emphasized by Kebarle and Tang [31], the free energy of solvation of an ion in the liquid phase is the determining factor for its emission to the gas phase. In other words, an ion with a low free energy of solvation is less likely to be ejected into the gas phase than an ion of higher free energy of solvation.

Both phenazine and bipyridine are only slightly soluble in water [32] and we find pyocyanin, with its oxygen substituent, to be considerably more soluble than phenazine. Complexing a metal cation through the nitrogens of bipyridine will eliminate their potential for interacting with liquid water and present only the hydrophobic part of the rings to the surrounding water. When pyocyanin is complexed with a metal cation through oxygen and the adjacent nitrogen then N(5), although somewhat shielded by the methyl group, is still available for interaction with water. A complexed metal cation having as ligands two bipyridines or one bipyridine and one pyocyanin can therefore be more hydrophobic, i.e. have a higher free energy in aqueous solution than the same cation complexed only with pyocyanin. Such complexes will be more surface-active and be preferentially observed in an electrospray spectrum. Also accentuating the difference between the characteristics of the electrospray and the CAD spectra may be a kinetic effect.

Noncomplexed bipyridine has free rotation about the central C·C bond whereas when complexed, a near planar configuration of the two pyridyl rings is adopted [12] while pyocyanin is probably nearly planar in the uncomplexed state in aqueous solution and will certainly be planar in a metal complex. The transitition state for loss of bipyridine will therefore be entropically favoured over that for loss of pyocyanin due to the incipient internal rotation of the bipyridine.

In the electrospray spectra shown in Fig. 7(a) and (b), the total amount of complexed pyocyanin in all ions of Mg^{2+} or Ca^{2+} is greater than that of complexed bipyridine. This preference for complexing with pyocyanin is in accord with the complete absence of $[CaP(bpy)]^{2+}$ as a product in the CAD spectrum of $[CaP_2(bpy)]^{2+}$ and the very small intensity of $[MgP(bpy)]^{2+}$in the CAD spectrum of $[MgP_2(bpy)]^{2+}$ (Fig. 8). Doubly charged metal ion complexes, when collisionally activated, usually have more available pathways for reaction than do the analogous singly charged complexes. Charge reduction, that is electron transfer from the ligand to the metal followed by coulombic repulsion-driven dissociation, can compete with simple ligand loss and interligand proton transfer followed by charge separation may also occur [33]. There is no evidence in this work for interligand reactions, presumably because there are no sufficiently acidic hydrogens in either bipyridine or pyocyanin. The CAD spectra of $[MP(bpy)]^{2+}$ (M = Mg, Ca) show, as do those of $[MP(bpy)]^{+}$ (M = Li, Na, K), a preference for loss of bipyridine but, as seen in Fig. 9, the major product at low collision energy results not from the loss of a whole ligand but from loss of a methyl from pyocyanin with no accompanying charge reduction. This may be compared to the information in Fig. 4 which shows that the major product ion in the CAD spectrum of $[CaP_2]^{2+}$ at all collision energies is $[Ca(P\text{-}15)]^{+}$, i.e. in addition to loss of methyl there is also loss of one ligand and a reduction in charge. It is possible that this comparison is not strictly valid since in Figure 4 the curve for the production of $[CaP(P\text{-}15)]^{2+}$ from $[CaP_2]^{2+}$ is rising more steeply with decreasing collision energy than is $[Ca(P\text{-}15)]^{+}$ and therefore $[CaP(P\text{-}15)]^{2+}$ might be the major product at very low energy. There was insufficient sensitivity in product detection at low collision energy to check this possibility. Aside from this, it is apparent from comparison of Figs. 4 and 9 that, at the same ion energy, $[CaP(bpy)]^{2+}$ is less susceptible to loss of a single ligand and even less susceptible to charge reduction than is $[CaP_2]^{2+}$ and hence that bipyridine confers more stability on $[CaP]^{2+}$ than does pyocyanin. This difference could be associated with the slightly higher ionization energy of bipyridine. The ionization energy of pyocyanin has not been determined but with its additional methyl and oxygen substituents, the value is expected to be lower than that of phenazine (IE = 8.33 eV [34]) which is itself lower than that of 2,2′-bipyridine (IE = 8.6 eV [34]). If charge reduction with associated ligand loss occurs then, on the basis of relative ionization energies alone, the preferred leaving group should be pyocyanin. That it is bipyridine that is lost speaks in favour of pyocyanin being the more strongly bound in the mixed dimers for the alkaline earth cations as well as the alkali metal cations.

Fig. 7(c) shows that ions containing bipyridine are much more strongly favoured in the electrospray spectra of solutions equimolar in bipyridine, pyocyanin and zinc cations than in the spectra obtained from solutions containing the alkali or alkaline earth cations. $[Zn(bpy)_2]^{2+}$ is the base peak and $[ZnP(bpy)]^{2+}$ is only about one-third its size. There are no other peaks of significant intensity. The CAD spectrum of $[ZnP(bpy)]^{2+}$ emphasizes the differences between the alkaline earth metals and zinc. Fig. 10 shows that once again $[M(P\text{-}15)(bpy)]^{2+}$ is the major product at low collision energy and also remains the major product at an ion energies up to 120 meV. There are no other doubly charged ions produced and only one singly charged product ion containing the metal cation. This ion, $[Zn(bpy)]^{+}$, shows the preference of Zn^{2+} for complexing with the two nitrogens of bipyridine rather than with a nitrogen and an oxygen of pyocyanin, the preference of the alkali and alkaline earth cations. Zn^{2+}, even with a completed 3*d* shell, is therefore behaving more like a transition metal in favouring chelation to nitrogen rather than oxygen, and less like Mg^{2+}, with which comparison is often made [11]. No molecular ion of pyocyanin is evident in the CAD spectrum of $[ZnP(bpy)]^{2+}$ but *m/z* 182 and *m/z* 167 from its decomposition are present.

5. Conclusions

Pyocyanin forms gas-phase complexes with both the singly charged alkali metal cations and with the doubly charged alkaline earth and zinc metal cations. The strength of the association between the bidentate ligand and the metal decreases with increasing cation size. Although a maximum of four pyocyanin molecules is observed in the complexes, the strength of the binding of the first two ligands, as judged by the appearance of ions in the electrospray spectra and by CAD studies, is significantly greater than for the third and fourth. Indeed, the CAD spectrum of $[MgP_2]^{2+}$ shows that ligand decomposition by loss of one and two methyl groups is more prevalent than loss of a complete ligand. The relative importance of loss of methyl with retention of charge decreases with increasing size of the alkaline earth metal cation and it is to be noted that methyl loss is not observed for complexes of the alkali metal cations. Loss of methyl leaves the equivalent of 1-hydroxyphenazine minus its hydroxyl H atom coordinated to the metal and it is of interest that 1-hydroxyphenazine is a metabolite of pyocyanin [35–37]. Although the mechanism has not been substantiated it is possible that the metabolism is mediated by a metal cation.

The formation of a metal–oxygen bond and concurrent loss of a carbon–methyl bond in the demethylation process allows an estimate of the minimum energy of the metal–oxygen bond. In terms of changes in the types of Lewis bonds involved, the loss of methyl and formation of a metal–oxygen bond involves the breaking of the $N–CH_3$ bond, the formation of the M–O bond and the change of N–C to N=C and of C=O to C–O. Calculation using average bond enthalpies [11] leads to an estimated M–O bond enthalpy of 360 $kJ\ mol^{-1}$. This estimate cannot allow for any charge delocalization in either the methylated or the demethylated ions and indeed does not consider the ionic nature of the two species. The estimated value does suggest however that the M–O bond energy is not very different to a $C–CH_3$ bond energy the values of which in $(CH_3)_3N$ and $(CH_3)_2NH$ are, respectively, 316 and 347 $kJ\ mol^{-1}$ [34]. The demethylation reaction can therefore have a very low activation energy, as was found.

The competition experiments between pyocyanin and 2,2′-bipyridine in forming complex ions with metal cations emphasize the fact that the intensities of the peaks seen in the electrospray spectrum are not necessarily representative of the strengths of binding of the component ligands in the complexes. Thus, although bipyridine is favoured over pyocyanin in most of the complexes containing two ligands, CAD studies show that pyocyanin is the more strongly bound. The preference for bipyridine probably arises in the ion evaporation stage of the electrospray process.

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