PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Applications of Mass Spectrometry in Studies of Porphyrins and Related Tetrapyrroles

A. H. Jackson

Phil. Trans. R. Soc. Lond. A 1979 **293**, 21-37
doi: 10.1098/rsta.1979.0077

Phil. Trans. R. Soc. Lond. A **293**, 21–37 (1979) [21]
Printed in Great Britain

Applications of mass spectrometry in studies of porphyrins and related tetrapyrroles

By A. H. Jackson
Department of Chemistry, University College, P.O. Box 78, *Cardiff CF*1 1*XL, U.K.*

Electron impact mass spectrometry has played a vital role in the structure determination of a variety of porphyrin and chlorophyll derivatives, especially those occurring in small quantities, and which are related to important intermediates in both normal and abnormal metabolism. More recently, other newer mass spectral techniques have also become of increasing importance, i.e. soft ionization methods and g.c.–m.s. of degradation products.

Field desorption mass spectrometry is particularly useful for porphyrins because of their low volatility and it is now very useful for preliminary analyses of crude mixtures of porphyrins, as well as for direct analysis of individual fractions obtained in high pressure liquid chromatography h.p.l.c. separations. Examples include porphyrins excreted by porphyric patients, petro-porphyrins, chlorophyll derivatives, and synthetic porphyrins. The coupling of a high pressure liquid chromatograph by means of a moving belt system also appears to be a promising technique for the direct mass spectromatic analysis of porphyrins in liquid chromatography fractions, and preliminary results are described.

Chromic acid oxidation, or hydriodic acid reduction, of porphyrins to monopyrrolic fragments (the classical methods used for porphyrin structure determination) have now been refined by use of g.c.–m.s. and can be applied to sub-milligram quantities of porphyrins, or bile pigments; examples include isocoproporphyrin, *d*-urobilin and stercobilin. Biosynthetic studies have also been facilitated by degradative techniques combined with mass spectrometry.

The final section of the paper describes preliminary studies of some complex metastable fragmentation processes occurring with chlorophyll derivatives with the use of the 'Dadi' and linked B/E scan methods, together with field desorption and collisional activation.

Introduction

Haem and chlorophyll (figure 1) occur in abundance and are widely dispersed throughout nature. Many other physiologically important porphyrins and bile pigments are also of widespread occurrence, but as their concentrations are relatively low they have no effect on the pigmentation of the organisms concerned. Visible and ultraviolet spectra were, and still are, extensively used in the characterization of tetrapyrroles as they enable immediate distinction between the different classes (i.e. porphyrin, chlorin, tetrahydroporphyrin and the various types of bile pigment) and often give information about the nature of conjugated side chains. Infrared and n.m.r. spectra provide information about the functional groups and details of the side chain substituents, although a few milligrams of material are usually required for n.m.r. spectrometry, unless a very high field instrument and a very pure sample are available. The intrinsically much higher sensitivity of mass spectrometry, and especially its capacity to give reliable information on molecular mass information, have enabled rapid advances to be made in the structure determination of tetrapyrroles even when insufficient material has been available for n.m.r. spectral studies (Jackson 1977*a*). However, the combination of mass

spectrometry with u.v., n.m.r. (and to a smaller extent i.r.) spectrometry has provided a powerful new dimension in structure determination of tetrapyrroles, just as much, if not more than, with other organic compounds.

Most of the mass spectra of tetrapyrroles obtained so far have involved the use of electron impact (e.i.) mass spectrometry and this has been summarized by Dougherty (1972) and more recently by Smith (1975). However, in recent years other 'softer' ionization techniques have come to the fore, especially chemical ionization and field desorption. The recent developments in high pressure liquid chromatography and its direct combination with mass spectrometry, as well as the degradation of microgram amounts of porphyrins to pyrrolic fragments before analysis by g.c.–m.s., have also become of increasing importance in structural studies of tetrapyrroles. The first part of this paper therefore reviews some of the more important recent applications of e.i. mass spectrometry and the remainder is largely concerned with the newer techniques, and some studies of metastable ionization processes.

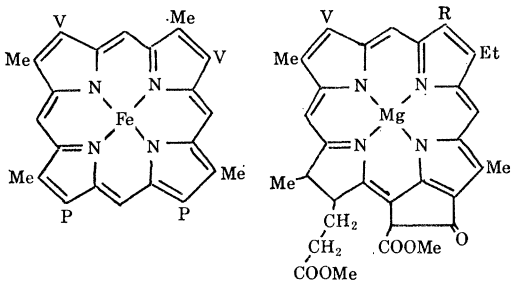

FIGURE 1. Structures of haem and chlorophylls *a* (R=Me) and *b* (R=CHO).

ELECTRON IMPACT MASS SPECTRA

The first systematic studies of porphyrin mass spectra appeared in 1965 (Jackson *et al.* 1965; Hofmann 1965). They included many porphyrins related to haem and chlorophyll and subsequently a further paper described the mass spectral characteristics of a variety of di-, tri- and tetrapyrroles including bile pigments (Jackson *et al.* 1967). The most striking feature of the mass spectra of porphyrins and chlorins is the high intensity of the molecular ion; this is usually the base peak, unless some particularly labile substituent is present such as a β-keto ester side chain (Cox *et al.* 1969). Loss of water also occurs very readily from α-hydroxyalkyl porphyrins, but more useful mass spectra may be obtained with TMS derivatives (Chapman & Elder 1972).

The aromatic nucleus of porphyrins does not fragment and they also exhibit pronounced doubly charged ions (figure 2). The most characteristic fragmentations of porphyrins are 'β-type' cleavages of the side chains (Jackson *et al.* 1965), and similar cleavages are also observed with the open-chain polypyrroles (Jackson *et al.* 1967).

The mass spectra of corroles (which are structurally related to the reduced systems of vitamin B_{12} and the corrins, and like them lack the δ-*meso*-carbon present in porphyrins) are very similar to those of the porphyrins, as expected on account of their aromatic character. The mass spectra of other heterocyclic analogues of the porphyrins and corroles in which one or two of the nitrogen atoms are replaced by oxygen or sulphur, or in which one or more of the *meso*-bridge carbons are replaced by nitrogen (e.g. the phthalocyanins), also exhibit very

similar characteristics to those of the porphyrins. Corrins also show prominent molecular ions, and side chain fragmentations are also analogous to those occurring in the porphyrin series.

Recent examples of the use of mass spectrometry in the structure determination of naturally occurring porphyrins include harderoporphyrin (Kennedy *et al.* 1971), isocoproporphyrin (Elder 1972; Stoll *et al.* 1973), S-411 porphyrin (French *et al.* 1966), chlorophyll *c* (Dougherty *et al.* 1970), degradation products of the *Chlorobium* chlorophylls (Holt *et al.* 1966; Cox *et al.* 1971), bacteriochlorophylls (Brockmann *et al.* 1976), bonellin (Pelter *et al.* 1976) and sirohydrochlorin (Murphy *et al.* 1973; Murphy & Siegel 1973; Deeg *et al.* 1977; Battersby *et al.* 1977). The single most important piece of information obtained in every case was the molecular mass, and side chain fragmentations observed, together with electronic and n.m.r. spectra,

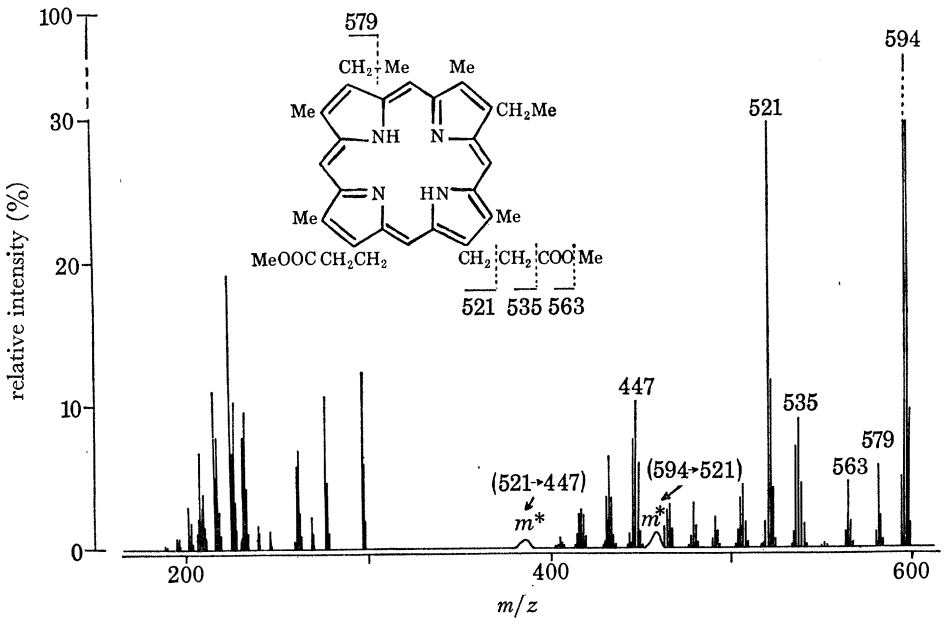

FIGURE 2. Electron impact mass spectrum of mesoporphyrin-IX dimethyl ester.

then allowed formulation of possible structures. As none of the spectral techniques (except n.m.r. in a few special cases) gives information about the order of the side chains around the macrocycle, biosynthetic arguments were applied to limit the number of isomeric possibilities. In most cases total synthesis has been undertaken to provide final proof of structure, as it is not very often that sufficiently pure crystalline material can be obtained for X-ray structure determinations; anhydrobonellin is a notable exception (Pelter *et al.* 1978).

Synthetic and biosynthetic studies in the porphyrin field have also been greatly facilitated by mass spectrometry; relatively little material is usually available in the initial investigations of the multi-stage syntheses required and it is common practice to carry out trial runs at the porphyrin level with milligram quantities of intermediates, the products being characterized by mass and visible spectrometry in combination with thin-layer (and more recently also high-pressure liquid) chromatography.

A common feature of most porphyrin spectra is the presence of a cluster of low intensity ions around M + 60; these are due to metallic complexes (Fe, Cu, Zn, etc.) formed from trace metals in the solvents used, and/or by complexation with the metals in the source itself (cf.

Jackson *et al.* 1965). (The regular use of platinum spatulas in handling free porphyrins is highly recommended, although it does not always obviate the problem.) The mass spectra of the metal complexes are essentially very similar to those of the present porphyrins, although less fragmentation may occur with some of the metalloporphyrins as these may be more volatile than the free bases. Silicon derivatives complex with a variety of additional ligands to form hexacoordinated species which are exceptionally volatile and indeed some petroporphyrins as well as synthetic alkyl porphyrin complexes have been separated by gas chromatography (Boylan & Calvin 1967) and subjected to combined g.c.–m.s. (Games *et al.* 1974*a*). Further g.c.–m.s. investigations of the silicon complexes of petroporphyrins, or of porphyrins with ester side chains were envisaged in Cardiff (Games *et al.* 1974*a*) but these have now been superceded by the advent of h.p.l.c. and f.d. mass spectrometry (see below).

The mass spectra of partly reduced porphyrins exhibit similar features to those already described for porphyrins, i.e. the nucleus remains intact and side chain fragmentations predominate; with the chlorophylls and related dihydroporphyrins, for example, prominent fragmentations are 'benzylic' cleavages of the substituents on the reduced pyrrole rings (Jackson *et al.* 1965). The instability of the fully reduced porphyrinogens, however, leads to practical difficulties in handling, but significant ring fragmentations are observed in their mass spectra; indeed, Budzikiewicz has recently shown that such cleavages may be used to provide some structural information about the order of substituents in the ring system (Budzikiewicz & Pesch 1976). This work has also been extended to *meso*-substituted porphyrinogens and loss of the *meso*-substituent was found to be a dominant fragmentation (Budzikiewicz & Neuenhaus 1977). The ring-opened bile pigments are even more prone to inter-pyrrole cleavages and in a few cases the molecular ion may be very weak (or even absent); they also show a tendency to undergo disproportionation reactions in the mass spectrometer giving rise to M+2 and M−2 ions as well as the expected molecular ion (Jackson *et al.* 1966).

A variety of structural studies of bile pigments have, however, been facilitated by mass spectrometry, including the plant pigments, phycocyanobilin and phycoerythrobilin (cf. Dougherty 1972; Chapman *et al.* 1972; Killilea & O'Carra 1972; Bonnett & McDonagh 1973; Beuhler *et al.* 1976). Unlike the free acids, the bile pigment methyl esters show little tendency to disproportionate, and mono- and diglucuronides have been identified as TMS derivatives (see, for example, Salmon & Fenselau 1974; Gordon *et al.* 1976). Further structural information has also been obtained by diazo coupling to give dipyrrolic azopigments which have also been studied by mass spectrometry (cf. Compernolle *et al.* 1976); oxidative cleavage to maleimides has also proved a useful technique (see below).

The mechanism of the oxidative ring opening of haem to bile pigments and carbon monoxide has also recently been studied by mass spectrometry with the use of mixtures of $^{18}O_2$ and $^{16}O_2$ (Brown & King 1975, 1976, 1978). In model experiments with octaethylhaem, and its *meso*-hydroxy analogue (figure 3), we have now confirmed that the ring-opening process involves *two* separate molecules of oxygen (table 1) and is thus identical with the in-vivo reactions (Jackson *et al.* 1978). In contrast, the apparently similar oxidation of tetracyclone, which also gives rise to carbon monoxide, only involves one molecule of oxygen in the ring opening step (figure 4) (A. H. Jackson & M. G. Lee 1977, unpublished; Chaney & Brown 1978).

As well as the naturally occurring tetrapyrroles, a wide variety of alkyl porphyrins and *meso*-tetraaryl porphyrins have been prepared in recent years; the mass spectra of these compounds and their metal complexes are generally unexceptional. The *N*-alkylated porphyrins, however,

deserve special mention because of their propensity to exhibit pseudomolecular ions at up to $[M+3]^+$, or $[M+4]^+$ (Jackson & Dearden 1973); *N*-alkyl corroles and *N*-oxacorroles also exhibit similar behaviour. This phenomenon has been attributed to hydrogen uptake (probably from water vapour in the spectrometer). However, the chlorophyll *a* degradation product, chlorin e_6, which contains a vinyl group (Budzikiewicz & Drewes 1968), has also been reported to exhibit an $[M+2]^+$ as well as the M^+ ion; porphyrins with acrylic side chains also behave similarly and this has been attributed to intermolecular hydrogen abstraction and reduction of the unsaturated side chains, as their abundances are not affected by the presence of D_2O (Clezy *et al.* 1974).

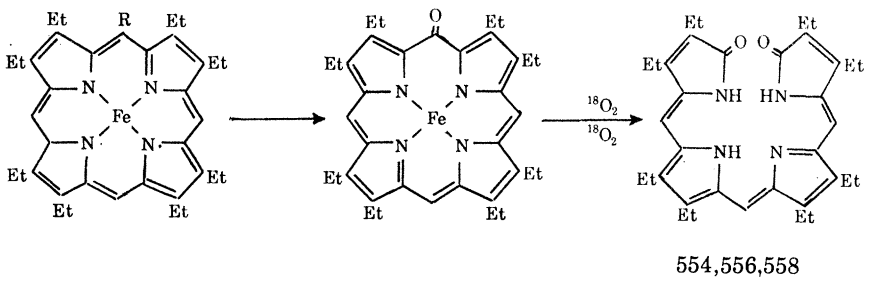

FIGURE 3. Oxidative ring opening of octaethylchlorohaemin (R=H) and *meso*-hydroxyoctaethylchlorohaemin (R=OH) to octaethylbiliverdin by mixtures of $^{18}O_2$ and $^{16}O_2$. (Two-molecule mechanism: see table 1.)

TABLE 1. OXIDATION OF OCTAETHYLCHLOROHAEMIN AND *MESO*-HYDROXYOCTAETHYLCHLOROHAEMIN TO OCTAETHYLBILIVERDIN BY MIXTURES OF $^{18}O_2$ AND $^{16}O_2$

		intensities of the molecular ions of octaethylbiliverdin					
		observed intensities (m/z)			calculated for two-molecule mechanism		
	enrichment in $^{18}O_2$ (%)	554	556	558	554	556	558
octaethylchlorohaemin	27.0	53.5	39.6	7.1	53.2	39.6	7.5
meso-hydroxyoctaethylchlorohaemin	38.5	37.6	47.0	15.4	37.6	47.3	14.8

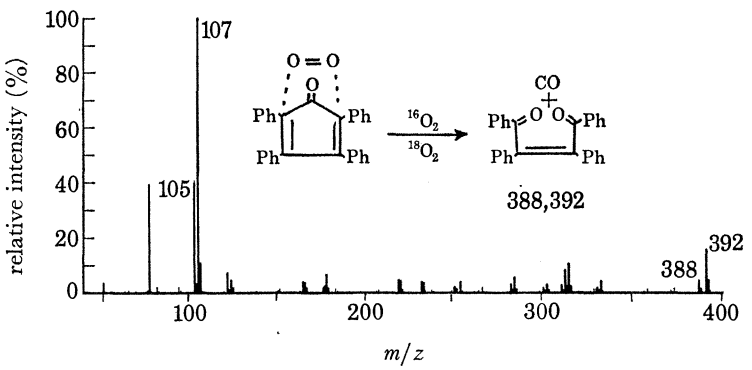

FIGURE 4. Oxidation of tetraphenylcyclopentadienone by singlet oxygen by 'one-molecule' mechanism (with a mixture of $^{18}O_2$ and $^{16}O_2$). The mass spectrum of the product shows molecular ions at m/z 388 and 392 arising from addition of *one molecule* of $^{18}O_2$, or *one molecule* of $^{16}O_2$.

Field desorption mass spectrometry

Field desorption (f.d.) mass spectrometry is becoming of increasing importance in studies of natural products, especially those which are thermally too labile, or too involatile, to be examined by conventional e.i. mass spectrometry. Although it is now over 20 years since the closely related technique of field ionization was first conceived, it is only during the last few years that it has come into widespread use owing largely to the pioneering work of Beckey & Schulten and their colleagues at Bonn (Beckey & Schulten 1975). A specially activated emitter,

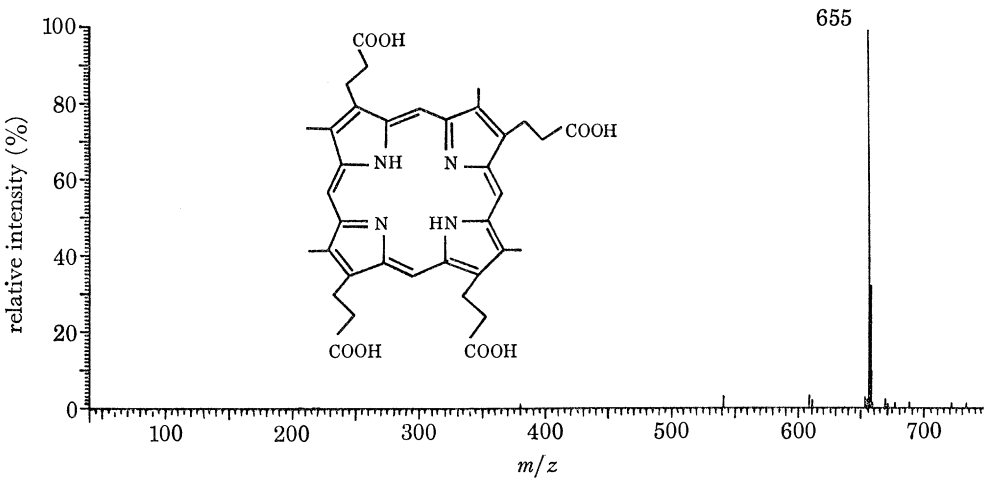

Figure 5. Field desorption mass spectrum of coproporphyrin-III.

consisting of a fine tungsten wire coated with carbon fibre micro-needles (or alternatively with one of a variety of metal needles), is dipped into a solution or suspension of the substance to be examined; after allowing the solvent to evaporate, the coated emitter wire is introduced into the mass spectrometer, and the substance ionized by application of a high potential (10–11 kV) between the emitter and another electrode a few millimetres away. The high field gradient in the neighbourhood of the sample causes ionization and by application of a mild heating current, 'desorption' of the positive ions occurs and these are analysed by the mass spectrometer in the usual manner. In the earlier technique of field ionization, the sample was volatilized into the source and passed over the emitter wire, but field desorption has the advantage that the sample does not need to be heated to volatilize it; consequently the risk of prior thermal fragmentation of labile materials is greatly diminished thereby, and relatively involatile materials can also be handled.

Porphyrin free acids are not very amenable to electron impact mass spectrometry owing to their involatility and most spectra are determined with the much more volatile methyl esters. However, by using the field desorption technique the mass spectra of a variety of porphyrins with up to four free carboxyl groups in the side chain (e.g. coproporphyrin) have now been determined (Evans *et al.* 1975). In most cases the only ions observed are molecular ions; although under some conditions intense $[M+1]^+$ ions may be observed as well, or instead of, M^+ ions, as is the case with many other nitrogenous bases (figure 5). Metalloporphyrins also give excellent f.d. spectra, and, for example, the spectrum of haemin chloride shows only the

molecular ion at m/z 616 (corresponding to the metal complex without the chloride ligand) (cf. Jackson 1977*b*).

The use of f.d. mass spectrometry for preliminary studies of mixtures of related compounds has been a very valuable feature of our recent work; as molecular ions are the predominant or only peaks, qualitative information about the number of components and their molecular masses is readily obtained (Evans *et al.* 1975). In collaboration with our medical colleagues, G. H. Elder and S. G. Smith, we have examined the f.d. mass spectra of mixtures of porphyrin methyl esters obtained by extraction of urinary or faecal samples from patients with disorders

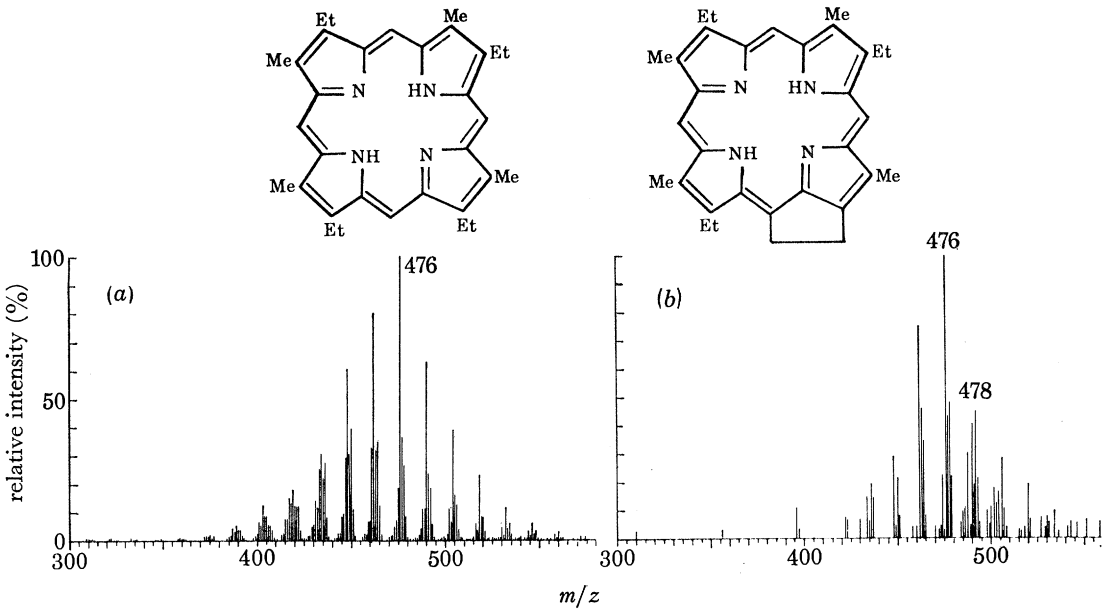

FIGURE 6. Electron impact (*a*) and field desorption (*b*) mass spectra of a mixture of petroporphyrins (La Luna) showing the presence of at least two series of porphyrins differing by 2 u. These are presumably homologues of aetioporphyrin-3 (left) and desoxophylloerythroaetioporphyrin (right).

of porphyrin metabolism (porphyrias), and thereby obtained crude 'fingerprints' of the porphyrins present, which were subsequently confirmed by t.l.c. or h.p.l.c. (the latter now being the technique of choice for quantitative determinations). Preliminary studies have also been carried out with a mixture of petroporphyrins kindly provided by G. Eglinton. At least two series of compounds have been detected corresponding to the aetioporphyrin, and desoxophylloerythroaetioporphyrin series, and the results (figure 6) clearly show the potential of the method, especially as there is no interference in the f.d. spectra from fragment ions.

Accurate mass measurement is more difficult than for e.i. spectra owing to the small amount of material on the emitter wire, but it can be carried out more readily if photo-plate detection is available. With the more common electrical detection systems present on most mass spectrometers it is, however, possible to carry out 'peak-matching' measurements with the use of a known porphyrin as internal standard; in fact in Cardiff we frequently use porphyrin esters as internal standards for mass measurements of all types of unknown compounds.

The phthalocyanines (tetrabenz-tetraazaporphyrin derivatives) have also proved to be very amenable to f.d. mass spectrometry (Games *et al.* 1974*b*) whereas in the electron impact technique source temperatures in the 550° range must be employed to volatilize them (cf. Hill &

Reed 1964). Our recent studies have shown that the f.d. mass spectra of crude polychlorinated phthalocyanine dyestuffs readily give qualitative information about the number of species present and hence the degree of chlorination achieved (Games *et al.* 1974*b*). Attempts to determine the e.i. spectra of chlorophyll derivatives lead to pyrolysis (Jackson *et al.* 1965; see also Dougherty 1972) but the magnesium-free pigment phaeophytin *a*, for example, exhibits an extremely weak molecular ion with extensive fragmentations of the side chain, especially the phytyl ester. In contrast the f.d. mass spectrum of chlorophyll *a* shows the molecular ion at m/z 892 and the base peak is the molecular ion of phaeophytin at m/z 870 (cf. Jackson

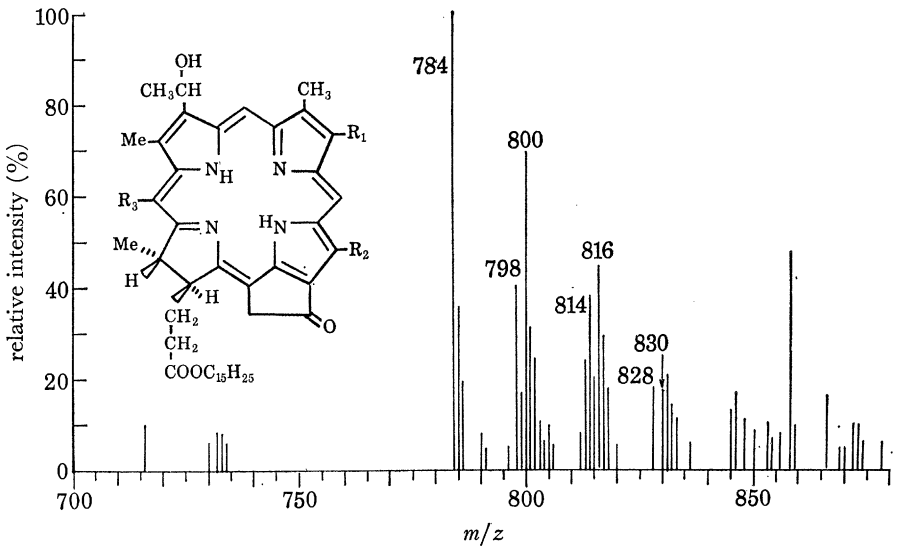

FIGURE 7. Field desorption mass spectra of farnesylphaeophorbide homologues ($R_1 = R_2$ = alkyl; R_3 = Me) obtained from *Chlorobium ethylicum*.

1977*b*). The f.d. mass spectrum of a mixture of phaeophytins *a* and *b* showed intense molecular ions at m/z 870 and 884 respectively, the only significant fragment ions (at m/z 811 and 825) corresponding to cleavage of the isocyclic ring methoxycarbonyl group (Evans *et al.* 1975). A more complex mixture of farnesylphaeophorbides obtained from *Chlorobium ethylicum* has also recently been investigated in Cardiff, and the f.d. mass spectra clearly showed the presence of two series of compounds differing in molecular mass by 2 u, presumably the vinyl and the corresponding ethyl analogues (figure 7).

It is important to note that in all the studies of mixtures described above only qualitative information has been obtained about their composition. The quantification of the components of a mixture by f.d. still presents considerable difficulties owing to the fluctuation in the rates of formation and desorption of the ions. Some preliminary studies on porphyrins have been carried out recently in Cardiff with *meso*-tetraphenylporphyrin, and a deuterated analogue as internal standard; the data shown in figure 8 were obtained by averaging (manually) a series of individual spectra and indicate that the method is feasible; similar conclusions have been reached by D. S. Millington (private communication) in independent experiments.

The use of f.d. mass spectrometry for studies of bile pigments has also proved to be an invaluable technique, and only molecular ions were observed in a series of some 30 synthetic and natural products recently studied in Cardiff (Cooper *et al.* 1976). In contrast, the electron

impact mass spectra often exhibited very weak molecular ions (in some cases these were absent), and thermal disproportionation reactions also occurred with several pigments leading to pseudomolecular ions at [M+2] and/or [M−2], e.g. stercobilin, one of the end-products of bilirubin metabolism by bacterial flora in the intestine; f.d. mass spectra of stercobilin and its dimethylester, however, confirmed that the molecular mass was 594 (not 592 or 596) (figure 9). Similarly, a sample of *d*-urobilin was shown to have a molecular mass of 590 rather than 588, and this was confirmed by degradative experiments (Cooper *et al.* 1976).

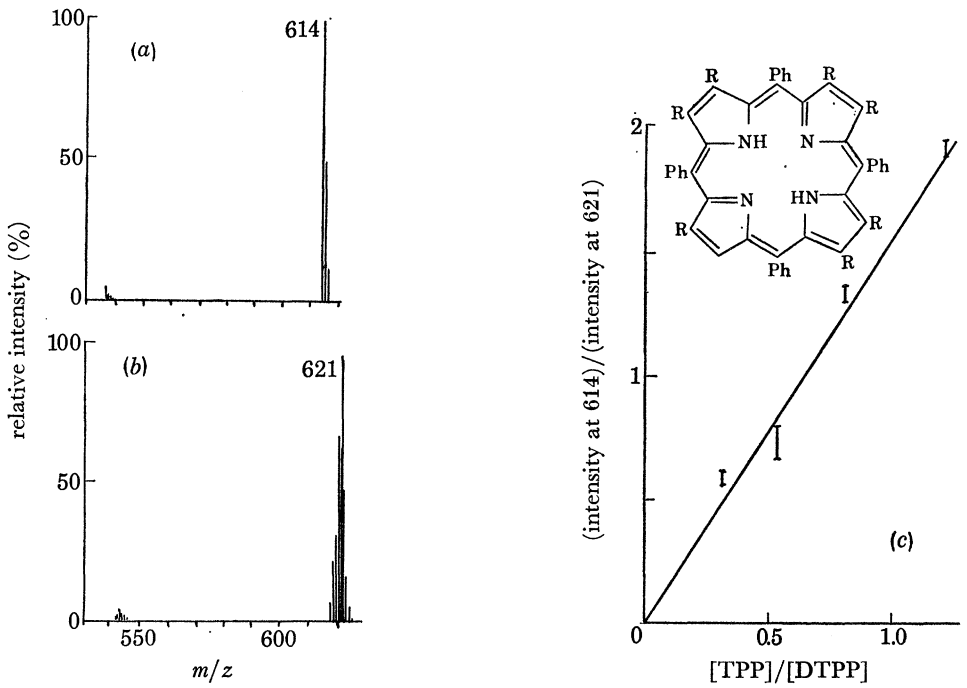

FIGURE 8. Field desorption mass spectra of the molecular ion regions of (*a*) *meso*-tetraphenylporphyrin (TPP; R = H) and (*b*) its deuterated derivative (DTPP; R = D), and (*c*) comparison of the integrated intensities of the molecular ions of mixtures of known composition.

Field desorption mass spectrometry has also proved a very valuable technique in the structural analysis of pterobilin, neopterobilin, sarpedobilin, phorcabilin and other blue-green bile pigments isolated from butterflies (Rüdiger *et al.* 1968; Choussy & Barbier 1975; Bois-Choussy & Barbier 1977); the f.d. mass spectra of some of these compounds were originally determined in Cardiff and helped to confirm their relation to biliverdin γ IX (pterobilin).

HIGH PRESSURE LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

The recent advent of high pressure liquid chromatography (h.p.l.c.) has revolutionized the process of purifying and analysing mixtures of porphyrins, whether from natural sources or of synthetic origin. The main advantages over thin layer chromatography (t.l.c.) are the ease of quantification of each component present, and the potential for preparative use. We have found that the combination of h.p.l.c. with f.d. mass spectrometry is a particularly useful technique for analysis of porphyrin mixtures (Evans *et al.* 1975). Thus in our studies of the sequence of reactions occurring in the biosynthesis of haem from the macrocyclic intermediate

uroporphyrinogen III, preliminary characterizations of the porphyrins isolated and separated (as their methyl esters) by h.p.l.c. were rapidly made in this manner (Jackson *et al.* 1976). A more recent example is the identification of the products of the reaction of picryl azide with protoporphyrin IX dimethyl ester as formyl and diformyl analogues (rather than acetyl derivatives) by f.d. analysis of the crude reaction mixture (figure 10) (Jackson *et al.* 1978); these products were separated by h.p.l.c. and the identity of each fraction confirmed by f.d. mass

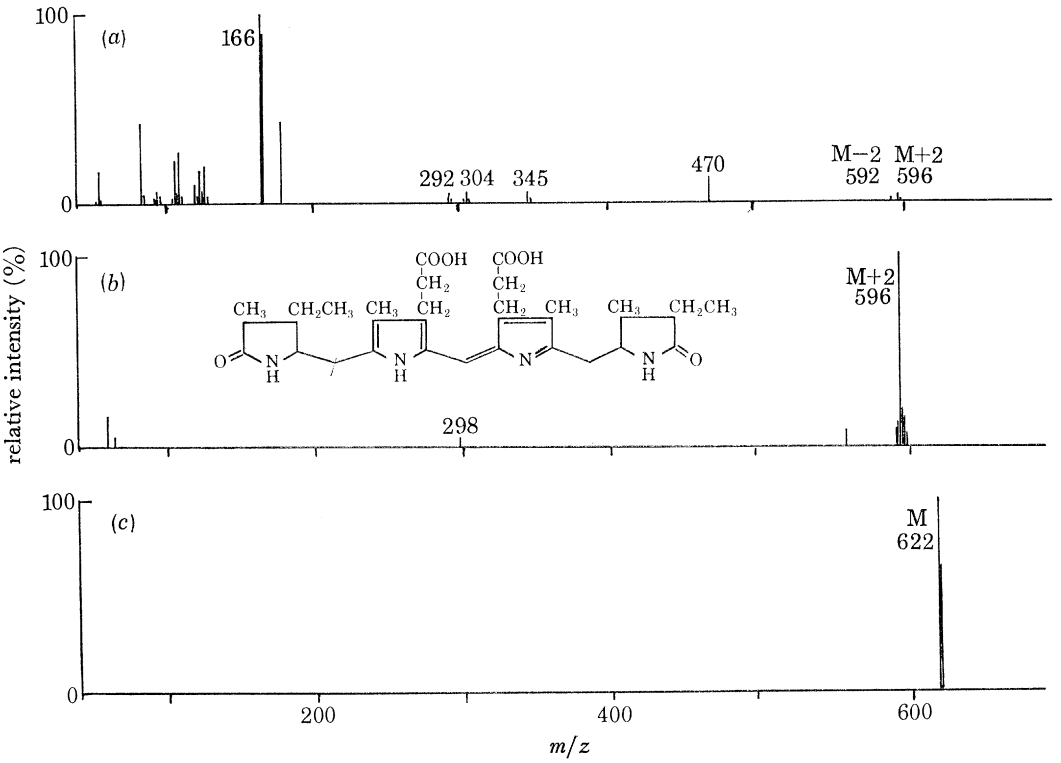

FIGURE 9. Mass spectra of stercobilin (*a*, *b*) and its dimethyl ester (*c*): (*a*) by e.i.; (*b*, *c*) by f.d.

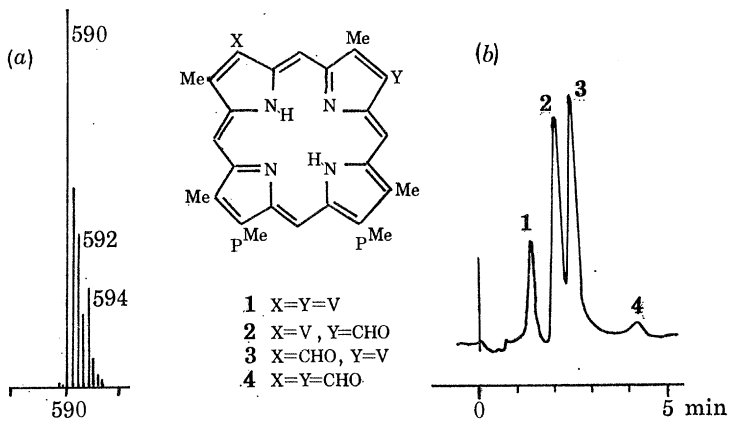

FIGURE 10. (*a*) Field desorption mass spectrum of the crude mixture of products (**1**, **2**, **3** and **4**) obtained by treatment of protoporphyrin-IX dimethyl ester (**1**) with picrylazide. (*b*) H.p.l.c. separation of the products obtained in a subsequent preparative experiment. (The molecular mass of each fraction was confirmed by field desorption mass spectrometry.)

spectrometry and visible spectra, and subsequently the reaction has been extended to the preparative scale.

Apart from our own work, another striking recent example of the use of f.d. mass spectrometry was concerned with haem *a* the prosthetic group of cytochrome *a*; the f.d. spectra of haem *a* dimethyl ester and its acetyl analogue were purified by h.p.l.c. and confirmed the previous structural assignments (DeFilippi & Hultquist 1977).

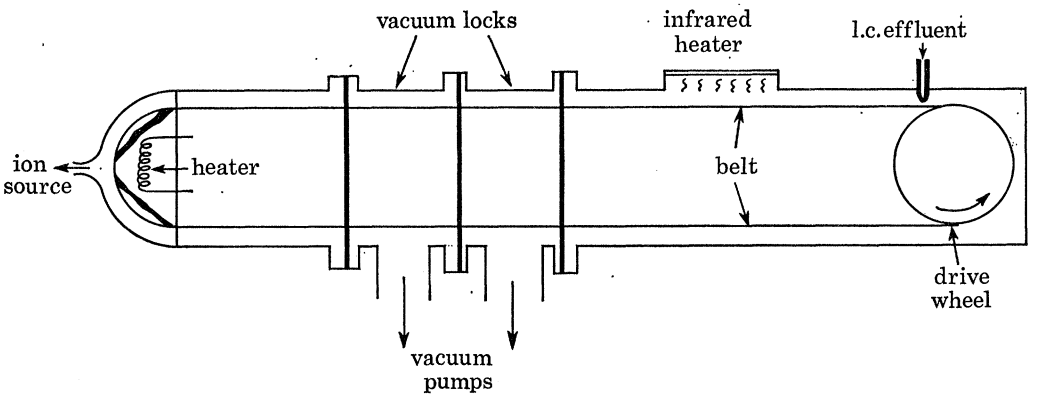

FIGURE 11. Moving-belt interface for direct coupling of a high pressure liquid chromatograph to a Finnigan 4000 mass spectrometer.

Sub-microgram amounts of porphyrins in h.p.l.c. fractions can often be subjected directly to f.d. m.s. by dipping the emitter into the column eluates; if very little material is available the solutions can be concentrated and syringed directly into the emitter wire. In contrast, the mass spectra of porphyrin samples extracted from t.l.c. plates are often more difficult to run owing to contamination, e.g. with plasticizer. This is much more critical with e.i. than with f.d. m.s., and much larger quantities even of pure crystalline material may be required for e.i. than for f.d. m.s., especially as the low volatility of porphyrins usually necessitates the use of source temperatures in excess of 200° for e.i.–m.s. (which may result in partial pyrolysis of the sample).

During the last few months we have also begun to explore the possibilities of using the direct coupling of h.p.l.c. with mass spectrometry (e.i. and c.i.). There are a variety of ways in which this can be carried out on-line, but the most promising at present is a system in which the h.p.l.c. column eluate is allowed to drip onto a moving band, or belt (McFadden *et al.* 1978); the belt then passes through a heater to evaporate the solvent and through a vacuum lock into the mass spectrometer where it is heated to a much higher temperature to volatilize the sample into the source. A schematic diagram of the system installed on our Finnigan mass spectrometer is shown in figure 11. Source temperatures around 250 °C are required for alkyl porphyrins and porphyrin esters, and satisfactory results have been obtained with 100 μg porphyrin. It is hoped that this will be improved by more precise control of such factors as solvent, flow rates and band speed, and perhaps also by single ion monitoring and/or the use of chemical ionization. Some of the more stable bile pigments and chlorophyll derivatives have also proved amenable to direct l.c.–m.s., and we hope to extend the range of compounds investigated and to improve the sensitivity. D. E. Games has already demonstrated the wide potential of direct l.c./m.s. with a variety of other organic compounds, and we also hope to use it in the analysis

of pyrrolic and dipyrrolic intermediates in crude reaction products obtained in the course of porphyrin synthesis. Indeed, this new technique seems likely to become of very widespread use (for less volatile compounds) and will thus complement the now well established g.c.–m.s. method used for more volatile materials.

Gas chromatography–mass spectrometry of tetrapyrrole degradation products

The classical methods of structure determination of porphyrins included oxidative degradation to maleimides and reduction to alkyl pyrroles. The sensitivity of these methods has been greatly improved in recent years firstly by the use of t.l.c. (for maleimides) (Rüdiger 1970) and g.c. (for alkyl pyrroles) (Chapman *et al.* 1971) and latterly by g.c.–m.s. (Stoll *et al.* 1973; Games *et al.* 1974*a*; Jackson *et al.* 1974). Thus great progress has been made in structure determination of algal and other marine bile pigments by degrading them to maleimides and diformyl pyrroles (Rüdiger 1970) while hydrogen iodide–formaldehyde reductions of *Chlorobium* chlorophylls to alkyl pyrroles confirm the presence of a *meso*-methyl group in the '650-series' (Chapman *et al.* 1971). In our own studies the reductive degradation of isocoproporphyrin to a mixture of three alkyl pyrroles, identified by g.c.–m.s., enabled us to distinguish between two isomeric structures deduced from other spectroscopic evidence; this result was also confirmed by oxidative degradation and g.c.–m.s. studies of the three maleimides produced (Stoll *et al.* 1973; Games *et al.* 1974*b*). We also showed that a sample of *d*-urobilin of molecular mass 590 (shown by f.d. m.s.) did not contain a vinyl group in its terminal rings, by oxidizing it to maleimides and showing the absence of methyl vinyl maleimide in the product (Cooper *et al.* 1976).

Biosynthetic studies in the porphyrin field have also been facilitated by mass spectrometry (Battersby *et al.* 1972) and work is now in progress at Cardiff with deuterium labelled intermediates, the products from which are being degraded to maleimides to establish the pattern of labelling. Mass spectral studies of maleimides have also been used in studies of the sequence of porphyrins produced by a series of *Chlorella* mutants (Ellsworth & Aronoff 1968), and partial oxidation of sarpedobiline gave a tripyrrolic pigment which helped to provide structural information (Bois-Choussy & Barbier 1977).

Metastable processes

Studies of the so-called metastable peaks, which arise from ions in the field-free regions of the mass spectrometer, have greatly facilitated structural studies of organic compounds, especially by showing the sequence of fragmentations in the mass spectrometer and in helping to establish the relation between 'daughter' and 'parent' ions (Beynon *et al.*, this symposium). Considerable interest has also been shown in studying the detailed characteristics of metastable ions to establish the thermodynamic parameters of the decomposition processes involved (Cooks *et al.* 1973).

In general, the metastable fragmentations occurring in porphyrin mass spectra are unexceptional, as are those of the bile pigments, although the fragmentations of the latter are much more complex. However, certain chlorophyll derivatives exhibit unusual metastable ions which have been attributed to the *simultaneous* loss of two, or three, side chain substituents

from the reduced pyrrole ring and/or the isocyclic ring (Jackson *et al.* 1965). For example, methyl phaeophorbide *a* (figure 12) undergoes a direct fragmentation of 147 u from the molecular ion due to losses of the isocyclic CO_2Me group, the D ring $CH_2CH_2CO_2Me$ group and one extra hydrogen atom. Similar losses of 147 u occur in other closely related compounds as well as with chlorin e_6 trimethyl ester, and other derivatives in which the isocyclic ring has been opened by base-catalysed methanolysis. In addition, chlorin e_6 trimethyl ester (figure 12) (and its analogues) exhibit pronounced metastable peaks in their mass spectra corresponding to loss of 159 u, which are due to fragmentations of the *meso*-CH_2CO_2Me and the D ring $CH_2CH_2CO_2Me$ groups with transfer of one hydrogen to the macrocyclic ring; these cleavages have been substantiated by deuterium labelling studies.

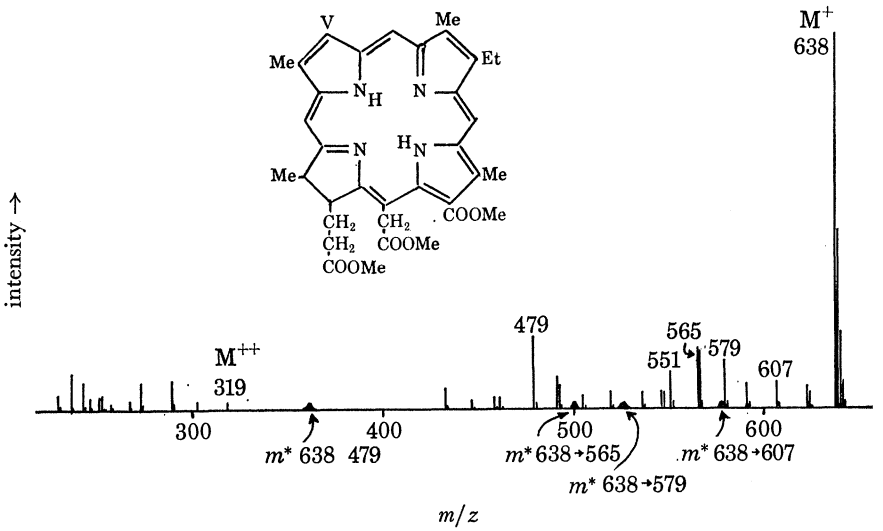

FIGURE 12. Electron impact mass spectrum of chlorin e_6 trimethyl ester.

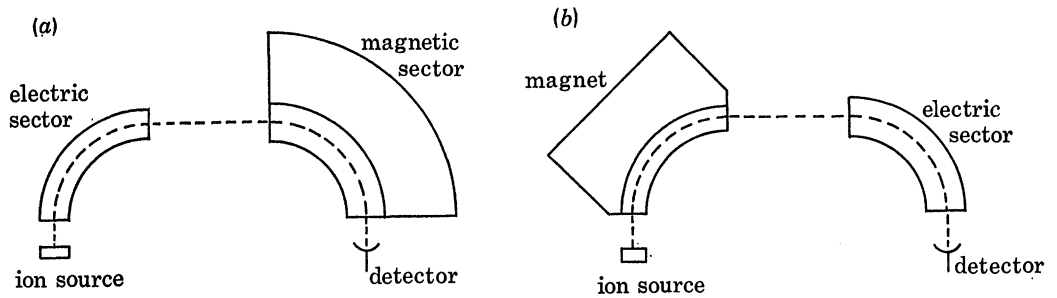

FIGURE 13. Schematic diagram showing the arrangement of the electric and magnetic focusing sections of a high resolution double focusing mass spectrometer: (*a*) 'normal' geometry (Nier–Johnson) and (*b*) 'reversed' geometry.

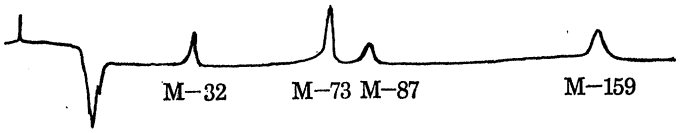

FIGURE 14. Direct analysis of the daughter ions (Dadi) arising from the molecular ion of chlorin e_6 (see figure 12) by metastable transitions (Varian CH5D mass spectrometer with 'reversed' geometry; cf. figure 13*b*).

We have also studied the formation of these novel metastable peaks by the Dadi ('direct analysis of daughter ions') technique by using a mass spectrometer, with a 'reversed' geometry (figure 13), i.e. with the electrostatic focusing sector succeeding the magnetic sector. This enables one to set the magnetic sector to focus on a particular ion, and then to scan the metastable transitions arising from this ion (which occur in the second field-free region) varying the voltage of the electrostatic sector. As shown in figure 14 the Dadi analysis also confirms that the M – 159 arises directly from the molecular ion of chlorin e_6 trimethyl ester.

Metastable transitions of this kind are relatively rarely observed in mass spectrometry, and indeed at one time it was always assumed that each metastable peak corresponded essentially to a *one step decomposition*. However, we have also observed metastable peaks in the spectra of diacyl phloroglucinols involving the simultaneous cleavage of two side chains (A. H. Jackson, G. W. Kenner and D. Ridyard, unpublished 1966). In the spectra of the chlorophyll derivatives it seems likely that the metastable peaks arising from the (M – 147) and (M – 159) fragmentations result from two or three individual cleavages succeeding each other so rapidly as to be almost simultaneous. Separate metastable transitions are also observed for some of the individual cleavages; thus methyl phaeophorbide *a* (M^+, 608) also exhibits metastable ions corresponding to a loss of CO_2Me (608 – 549) and loss of $CH_2CH_2CO_2Me + H$ (549 – 461); *m/z* 549 and *m/z* 461 were also the most intense peaks in the spectrum apart from the molecular ion which was the base peak, while peaks at *m/z* 521 and 520 corresponding to loss of $CH_2CH_2CO_2Me + H$, and $CH_2CH_2CO_2Me$ were very weak, and did not give rise to metastable transitions (Jackson *et al.* 1965). The loss of the isocyclic ring CO_2Me may thus be the primary step in the rapid consecutive cleavages leading to the metastable ions corresponding to M – 147. Similarly metastable peaks have been observed for M – CO_2Me, and M – CH_2CO_2-Me (*not* M – $CH_2CH_2CO_2Me$) in the spectra of chlorin e_6, and thus in these derivatives the loss of CH_2CO_2Me may be the first stage in the M – 159 cleavage process.

In addition to the Dadi technique mentioned briefly above, metastable transitions in the first field-free region of mass spectrometers with conventional Nier–Johnson geometry (figure 11) can also be studied in other ways. There has also recently been an increasing interest in the potential of collisional activation methods for enhancing fragmentations, by allowing the ion beam of the mass to pass through a special cell containing an inert gas at a relatively high pressure and which is inserted in the first field-free region of a conventional mass spectrometer, or in the second field-free region of those with the reversed geometry (McLafferty, this symposium). This technique may be particularly useful with field desorption mass spectrometry as there is usually little or no fragmentation, and hence apart from the mass (and perhaps the elemental composition) of the molecular ion no other structural information can normally be obtained.

It was, therefore, of great interest to discover whether or not collisional activation combined with f.d. mass spectrometry would also show the existence of the unusual metastable transitions in the mass spectra of chlorophyll derivatives. J. R. Chapman of A.E.I./Kratos Ltd kindly investigated the f.d. mass spectra of phaeophytin *a* by using an MS50 instrument with collisional activation in the first field-free region. In addition to the molecular ion (*m/z* 870), phaeophytin *a* exhibited weak fragment ions at *m/z* 812 (loss of CO_2CH_2 from the isocyclic ring) 660 and 592 (M – $C_{20}H_{38}$). Similarly, the f.d. mass spectra of methyl phaeophorbide *a* showed a weak fragmentation at *m/z* 547 (loss of CO_2Me from the isocyclic ring). Studies of the metastable transitions arising in the collisionally activated f.d. mass spectra were also

carried out by linking the magnetic field (B) and the electrostatic sector voltage (E), so that the value of B/E was kept constant throughout the scan. Fragment ions arising from the molecular ion of methyl phaeophorbide *a* were detected in this way by a linked B/E scan and it is very interesting to note that the most intense ion at m/z 459 corresponds to direct loss of 147 u in accord with the metastable transitions observed in the earlier e.i. mass spectra (see figure 15). The next most intense peak in the B/E scan is at m/z 547 (corresponding to a loss of CO_2Me

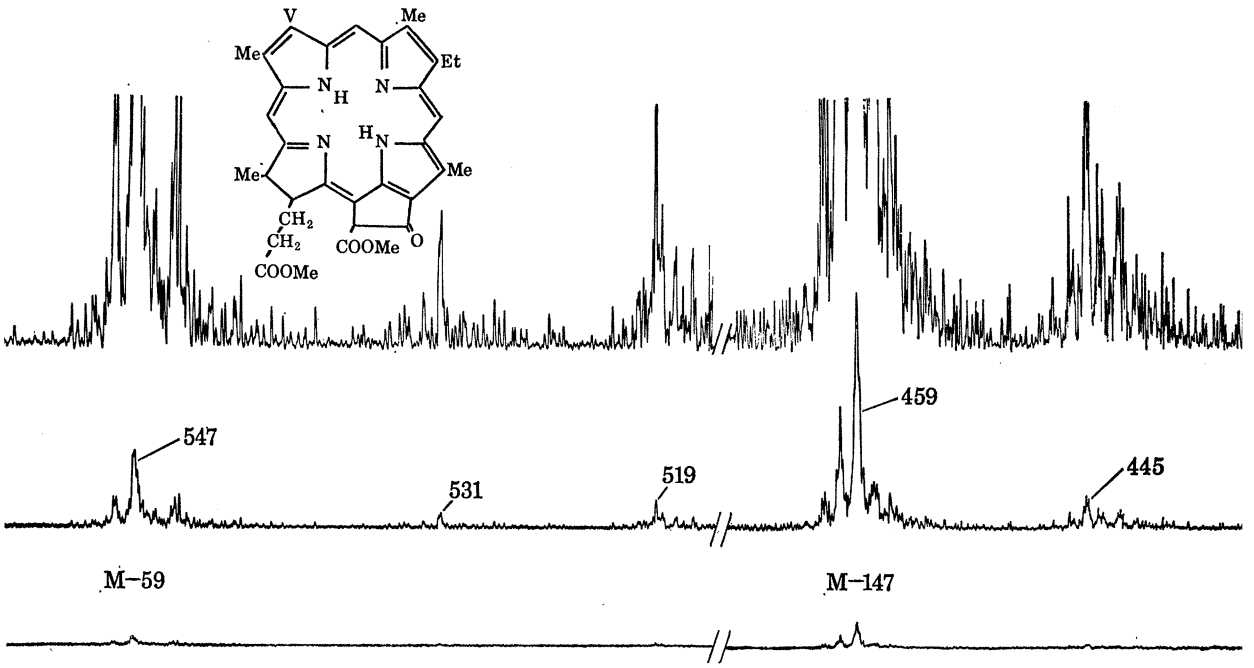

FIGURE 15. Linked B/E scan of the ions arising by metastable transitions from the molecular ion of methyl phaeophorbide *a* (Kratos AEI MS50 mass spectrometer) with the use of field desorption and collisional activation in the first field-free region (see figure 13*a*).

from the parent ion), rather than m/z 548 as observed in the normal f.d. mass spectrum. Similar results were obtained from the spectra of phaeophytin *a* and as the ions observed corresponded to fragmentations of both the phytyl groups and the other side chains, it seems likely that f.d. combined with collisional activation and metastable studies could provide useful structural information with unknown compounds. We hope to investigate this possibility more thoroughly with a wider range of compounds.

I should like to thank the Science Research Council and the Medical Research Council for their support of various aspects of this work, and also the Royal Society for a grant towards the cost of the field desorption equipment. It is a pleasure to acknowledge the assistance of numerous colleagues and former students who were involved with these studies, and whose names are included in the references. I am particularly grateful to Dr D. E. Games and Mr M. Rossiter of Cardiff for advice and assistance, and I should also like especially to thank Dr J. R. Chapman of A. E. I. Kratos for determining the collisional activation spectra. Finally, I should like to dedicate this paper to the memory of my friend and former colleague, Professor George Kenner, F.R.S., who first kindled my interest in the mass spectrometry of tetrapyrroles and whose tragic and untimely death occurred just before the symposium.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

References (Jackson)

Al-Hazimi, H. M. G., Jackson, A. H., Johnson, A. W. & Winter, M. 1977 *J. chem. Soc. Perkin Trans.* 1, p. 98.

Battersby, A. R., Baldas, J., Collins, J., Grayson, D. H., James, K. J. & McDonald, E. 1972 *J. chem. Soc. chem. Commun.* p. 1265.

Battersby, A. R., Jones, K., McDonald, E., Robinson, J. & Morris, H. 1977 *Tetrahedron Lett.* p. 2213.

Beckley, H. D. & Schulten H.-R. 1975 *Angew. Chem. int. Edn* **14**, 403.

Beuhler, R. J., Pierce, R. C. Friedman, L. & Siegelman, H. W. 1976 *J. biol. Chem.* **251**, 2405.

Bois-Choussy, M. & Barbier, M. 1977 *Experientia* **33**, 1407.

Bonnett, R. & McDonagh, A. F. 1973 *J. chem. Soc. Perkin Trans.* I, p. 881.

Boylan, D. B. & Calvin, M. 1967 *J. Am. chem. Soc.* **89**, 5472.

Brockman, H., Gloe, A., Risch, N. & Trowitsch, W. 1976 *Justus Liebigs Annln Chem.*, p. 566.

Brown, S. B. & King, R. F. G. J. 1975 *Biochem. J.* **150**, 565.

Brown, S. B. & King, R. F. G. J. 1976 *Biochem. Soc. Trans.* **4**, 197.

Brown, S. B. & King, R. F. G. J. 1978 *Biochem. J.* **170**, 297.

Budzikiewicz, H. & Drewes, S. E. 1968 *Justus Liebigs Annln Chem.* **716**, 222.

Budzikiewicz, H. & Neuenhaus, W. 1977 *Heterocycles* **7**, 251.

Budzikiewicz, H. & Pesch, R. 1976 *Org. Mass Spectrom.* **11**, 821.

Chaney, B. D. & Brown, S. B. 1978 *Photochem. Photobiol.* **28**, 339.

Chapman, D. J., Budzikiewicz, H. & Siegelman, H. W. 1972 *Experientia* **28**, 876.

Chapman, J. R. & Elder, G. H. 1972 *Org. Mass Spectrom.* **6**, 991.

Chapman, R. A., Rooni, M. W., Morton, T. C., Krajcarski, D. T. & MacDonald, S.F. 1971 *Can. J. Chem.* **49**, 3544.

Choussy, M. & Barbier, M. 1975 *Helv. chim. Acta* **58**, 2651.

Clezy, P. S., Lim, C. L. & Shannon, J. S. 1974 *Aust. J. Chem.* **27**, 2431.

Compernolle, F., Blanckaert, N. & Heirwegh, K. P. M. 1976 *Biomed. Mass Spectrom.* **3**, 155.

Cooks, R. G., Beynon, J. H., Capnoli, R. M. & Lester, G. R. 1973 *Metastable ions*. Amsterdam: Elsevier.

Cooper, G., Games, D. E., Jackson, A. H. & Saxton, R. G. 1976*a* *Biochem. Soc. Trans.* **4**, 214.

Cooper, G., Games, D. E., Jackson, A. H., Saxton, R. G. & Stoll, M. S. 1976*b* In *Advances in mass spectrometry in biochemistry and medicine* (ed. A. Frigerio), vol. 2, p. 257. New York: Spectrum.

Cox, M. T., Howarth, T. T., Jackson, A. H. & Kenner, G. W. 1969 *J. Am. chem. Soc.* **91**, 1232.

Cox, M. T., Jackson, A. H. & Kenner, G. W. 1971 *J. chem. Soc.* C, p. 1974.

Deeg, R., Kriemler, H.-P., Bergman, K.-H. & Müller, E. 1977 *Hoppe-Seyler's Z. physiol. Chem.* **358**, 339.

De Filippi, L. J. & Hultquist, D. E. 1977 *Biochim. biophys. Acta* **498**, 395.

Dougherty, R. C. 1972 In *Biochemical applications of mass spectrometry* (ed. G. R. Waller), p. 591. New York: Wiley-Interscience.

Dougherty, R. C. Strain, H. H., Svec, W. A., Uphaus, R. A. & Katz, J. J. 1970 *J. Am. chem. Soc.* **92**, 2826.

Elder, G. H. 1972 *Biochem. J.* **126**, 877.

Ellsworth, R. K. & Aronoff, S. 1968 *Arch. Biochem. Biophys.* **124**, 358 and **125**, 269.

Evans, N., Games, D. E., Jackson, A. H. & Matlin, S. A. 1975 *J. Chromat.* **115**, 325.

French, J. M., Nicholson, D. C. & Rimington, C. 1966 *Biochem. J.* **120**, 393.

Games, D. E., Jackson, A. H. & Millington, D. S. 1974*a* In *Advances in mass spectrometry in biochemistry and medicine* (ed. A. Frigerio & N. Castagnoli), p. 257. New York: Raven Press.

Games, D. E., Jackson, A. H. & Taylor, K. T. 1974*b* *Org. Mass Spectrom.* **8**, 1245.

Gordon, E. R., Goresky, C. A., Chang, T.-H. & Perlin, A. S. 1976 *Biochem. J.* **155**, 477.

Hill, H. C. & Reed, R. I. 1964 *Tetrahedron* **20**, 1359.

Hofmann, D. R. 1965 *J. org. Chem.* **30**, 3512.

Holt, A. S., Purdie, J. W. & Wasley, J. W. F. 1966 *Can. J. Chem.* **44**, 88.

Jackson, A. H. 1977*a* *Seminars in Haematol.* **14**, 193.

Jackson, A. H. 1977*b* *Endeavour* (N.S.) **1**, 75.

Jackson, A. H. & Dearden, G. R. 1973 *Ann. N.Y. Acad. Sci.* **206**, 151.

Jackson, A. H., Jenkins, R. T., Lee, M. G., Brown, S. B. & Chaney, B. D. 1978 *Tetrahedron Lett.*, p. 5135.

Jackson, A. H., Kenner, G. W., Budzikiewicz, H., Djerassi, C. & Wilson, J. M. 1967 *Tetrahedron* **23**, 603.

Jackson, A. H., Kenner, G. W., Smith, K. M., Aplin, R. J., Budzikiewicz, H. & Djerassi, C. 1965 *Tetrahedron* **21**, 2913.

Jackson, A. H., Millington, D. S. & Games, D. E. 1974 *Adv. Mass Spectrom.* **6**, 215.

Jackson, A. H., Rees, A. H., Matlin, S. A. & Towill, R. 1978 *J. chem. Soc. chem. Commun.* (In the press.)

Jackson, A. H., Sancovich, H. A., Ferramola, A. M., Evans, N., Games, D. E., Matlin, S. A., Smith, S. G. & Elder, G. H. 1976 *Phil. Trans. R. Soc. Lond.* B **273**, 191.

Jackson, A. H., Smith, K. M., Gray, C. H. & Nicholson, D. C. 1966 *Nature, Lond.* **209**, 581.

Kennedy, G. Y., Jackson, A. H., Kenner, G. W. & Suckling, C. J. 1971 *FEBS Lett.* **6**, 9, and **7**, 205.

Killilea, S. D. & O'Carra, P. 1972 *Biochem. J.* **129**, 1179.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

McFadden, W. H., Schwartz, L. & Evans, S. 1976 *J. Chromatogr.* **12**, 389.
Murphy, M. J. & Siegel, L. M. 1973 *J. biol. Chem.* **248**, 6911.
Murphy, M. J., Seigel, L. M., Kamin, H. & Rosenthal, D. 1973 *J. biol. Chem.* **248**, 2801.
Pelter, A., Ballantine, J. A., Ferrito, V., Jaccarini, V., Psaila, A. F. & Schembri, P. J. 1976 *J. chem. Soc. chem. Commun.*, p. 999.
Pelter, A., Ballantine, J. A., Murray-Rust, P., Ferrito, V. & Psaila, A. F. 1978 *Tetrahedron Lett.*, p. 1881.
Rüdiger, W. 1970 *Angew. Chem. int. Edn* **9**, 473.
Rüdiger, W., Klose, W., Vuillaume, M. & Barbier, M. 1968 *Experientia* **24**, 1000.
Salmon, M. & Fenselau, C. 1974 *Biomed. Mass. Spectrom.* **1**, 219.
Smith, K. M. 1975 In *Porphyrins and metalloporphyrins* (ed. K. M. Smith), p. 381. Amsterdam: Elsevier.
Stoll, M. S., Elder, G. H., Games, D. E., O'Hanlon, P., Millington, D. S. & Jackson, A. H. 1973 *Biochem. J.* **131**, 429.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES