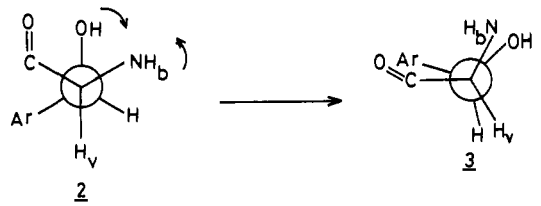
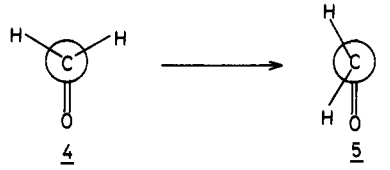
a position where the α C-H_x bond of this amino acid is nearly eclipsed with the N-H_b bond of the neighboring β-hydroxytyrosine unit (Figure 2) to one where this α C-H_x bond is approximately eclipsed with the C=O bond of the *N*-methylleucine (Figure 3). (ii) The -CH(OH)-CHNH- bond of the "right-hand" β-hydroxytyrosine unit is rotated as indicated in **2** until the conformation **3** is attained. This change brings the NH_b of the β-hydroxytyrosine unit very close to proton f. (iii) The rigid $CONH_n$ unit, connecting the CO of the above β-hydroxytyrosine unit to the NH_n of isoasparagine, is rotated about the two bonds connecting it to its α carbons. This rotation, viewed from the isoasparagine CH_2 group, is in a clockwise direction through ~120°; during the rotation of the $CONH_n$ unit, its NH_n proton traverses the face of the chlorine-bearing aromatic ring so that its final position is on the same face as the chlorine atom. (iv) The CH_2-CO bond of the isoasparagine is rotated by rotating the CH_2 group, viewed in the $CH_2 \rightarrow CO$ direction, anticlockwise by ~90°. As a consequence of this motion, the CH_2 group, which is initially orientated relative to the CO group as in **4**, is finally orientated relative to it as in **5**.



2 **3**



4 **5**

Fast Atom Bombardment Mass Spectrometry: A Powerful Technique for the Study of Polar Molecules

Dudley H. Williams,*[1a] Carol Bradley,[1a] Gustav Bojesen,[1a] Sitthivet Santikarn,[1a] and Lester C. E. Taylor[1b]

Contribution from the University Chemical Laboratory, Cambridge, United Kingdom, and Kratos, Manchester, United Kingdom. *Received April 3, 1981*

Abstract: A study of a range of polar organic molelcules by fast atom bombardment (FAB) mass spectrometry is reported. Compounds studied include organic salts, polar antibiotics, nucleoside phosphates, and underivatized peptides. In these classes of compounds, molecular weights in the range 300–2000 daltons have been routinely determined, operating in both positive and negative ion modes. Molecular weights of peptides are readily obtained on less than 1 nmol of material, and sequence information is conveniently deduced from sample sizes in the range 2–50 nmol.

In the past, the application of mass spectrometry to the determination of molecular weight and structure of polar molecules has been severely limited. The limitations have arisen due to our inability to produce efficiently the corresponding ions in the gas phase; thermal decomposition of the solid often occurs in preference to volatilization. The advent of field desorption (FD) mass spectrometry[2] has only slightly alleviated the problem; the technique is difficult in practice and the ions providing molecular weight information, if produced at all, are frequently produced only transiently. Californium plasma desorption mass spectrometry[3] has had some spectacular successes, but is not widely available as a routine method. Additionally, secondary ion mass spectrometry (SIMS) has been used to obtain mass spectra of polar organic molecules, but the mass range has so far proved rather limited.[4-6] We now report the application of the technique of fast atom bombardment (FAB), pioneered by Barber and his colleagues,[7] to the study of organic salts, polar antibiotics, nucleoside phosphates, and underivatized peptides. In these classes of compounds, we have routinely determined molecular weights in the range ca. 300–2000 daltons, operating in both positive and negative ion modes.

Experimental Section Mass spectra were obtained on Kratos MS 50 instruments, equipped either with a conventional magnet (mass range ca. 1000 at 8 kV) or an extended range magnet (mass range ca. 1700 at 8 kV). Higher masses were available by operating at lower accelerating voltages. A Kratos commercial FAB source was employed. In this source, argon ions of kinetic energies 4–6 keV are first produced from argon atoms (by electron bombardment and acceleration of the resulting Ar^+ species). The Ar^+ ions of high translational energy (ion beam current ca 40 μamps) are then converted to Ar atoms of similar energy by charge exchange on passing through argon gas. The argon gun does not employ an electric sector to remove any residual ions which may exist in the beam of Ar atoms. The 4–6 keV beam of atoms is then impacted onto the sample. We have normally dissolved, or dispersed, the sample in glycerol; such a matrix facilitates the production of sample ions in high abundance for relatively long periods. The solution, or suspension, is then introduced into the source on a copper probe tip. The area of the sample matrix on

* Address correspondence to this author at the University Chemical Laboratory.

(1) (a) University Chemical Laboratory, Lensfield Road, Cambridge. (b) Kratos, Ltd., Barton Dock Road, Urmston, Manchester.

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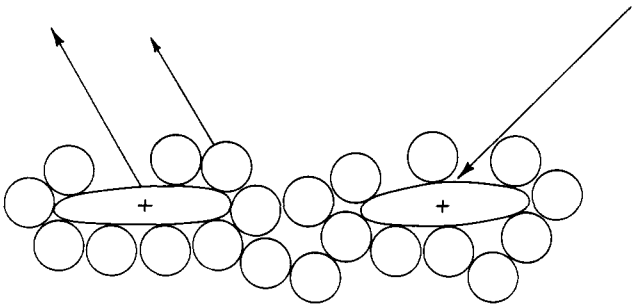


Figure 1. Schematic illustration of argon atom bombardment. A positively charged sample molecule is shown undergoing direct impact, and a second molecule is depicted as leaving the surface due to momentum transfer.

the probe tip was usually in the range 0.04–0.2 cm^2. The probe is inserted into the source through a conventional vacuum lock. The source operating pressure was typically ca. 10^{-5} torr. Spectra were obtained with a magnet scan rate in the range 30–1000 s/decade.

Discussion

Molecular weight information is usually obtained from $(MH)^+$ ions in positive ion spectra, and from $(M-H)^-$ ions in negative ion spectra. These spectra are in general produced with comparable sensitivites; possible exceptions are discussed subsequently. Odd-electron molecular ions are not normally produced in an abundance which gives structural information. We believe that the $(MH)^+$ and $(M-H)^-$ ions (i) are formed by proton transfer reactions which may occur as the molecules are bombarded and pass into the gas phase and/or (ii) are those already existing in the glycerol matrix. We justify the certain operation of (ii) below. In such cases, we propose that the preexisting ions pass into the gas phase due to momentum transfer which they receive from an Ar atom which impacts near, but not upon, the sample ion (Figure 1). A remarkable, and extremely useful, feature of FAB mass spectra is the occurrence of low abundance ions at essentially every mass up to (and usually slightly beyond) $(MH)^+$ or $(M-H)^-$. We propose that this useful background arises from the direct, or nearly direct, impact of Ar atoms on the sample and its supporting matrix (Figure 1). While this overall picture is clearly oversimplified, we feel that it provides an appropriately based physical picture which will prove useful until more detailed mechanistic studies are undertaken.

It follows from the above proposals that a quaternary phosphonium salt, A^+B^-, should produce A^+ in the positive ion spectrum and B^- in the negative ion spectrum; this is indeed the case. The positive ion FAB spectrum of **1** cleanly gives an abundant ion (m/z 349) due to the cation, and the negative ion spectrum shows Br^-. Similarly, the quaternary salt of thiamine (as its hydrochloride **2**) gives an abundant ion m/z 265 corresponding to **3**. Generally we find that if an option exists between the observation of a singly or doubly charged ion (as in **2**), the singly charged ion is observed. This presumably reflects the relatively higher heat of formation of a dication or dianion in the gas phase.

The above experiments define a general strategy for determination of the masses of both cationic and anionic portions of organic salts. Thus the potassium salt of penicillin G (**4**) gives in its negative ion spectrum an intense peak due to the carboxylate anion at m/z 333; this spectrum also shows $(ROO^-K^+ - H)^-$ at m/z 371. Recognition of the molecular ion region in this spectrum is facilitated not only by the characteristic mass separation of m/z 333 and 371, but also by the occurrence of a fragment ion at m/z 289, due to loss of CO_2 from the carboxylate anion m/z 333. Useful structural information is available from the occurrence of the base peak of the spectrum at m/z 192 (formally $C_6H_5CH_2CONHCHCHS^-$). The low internal energies of many of the ions produced, and the potential for operation at high mass, is indicated by the production of dimeric [m/z 705,

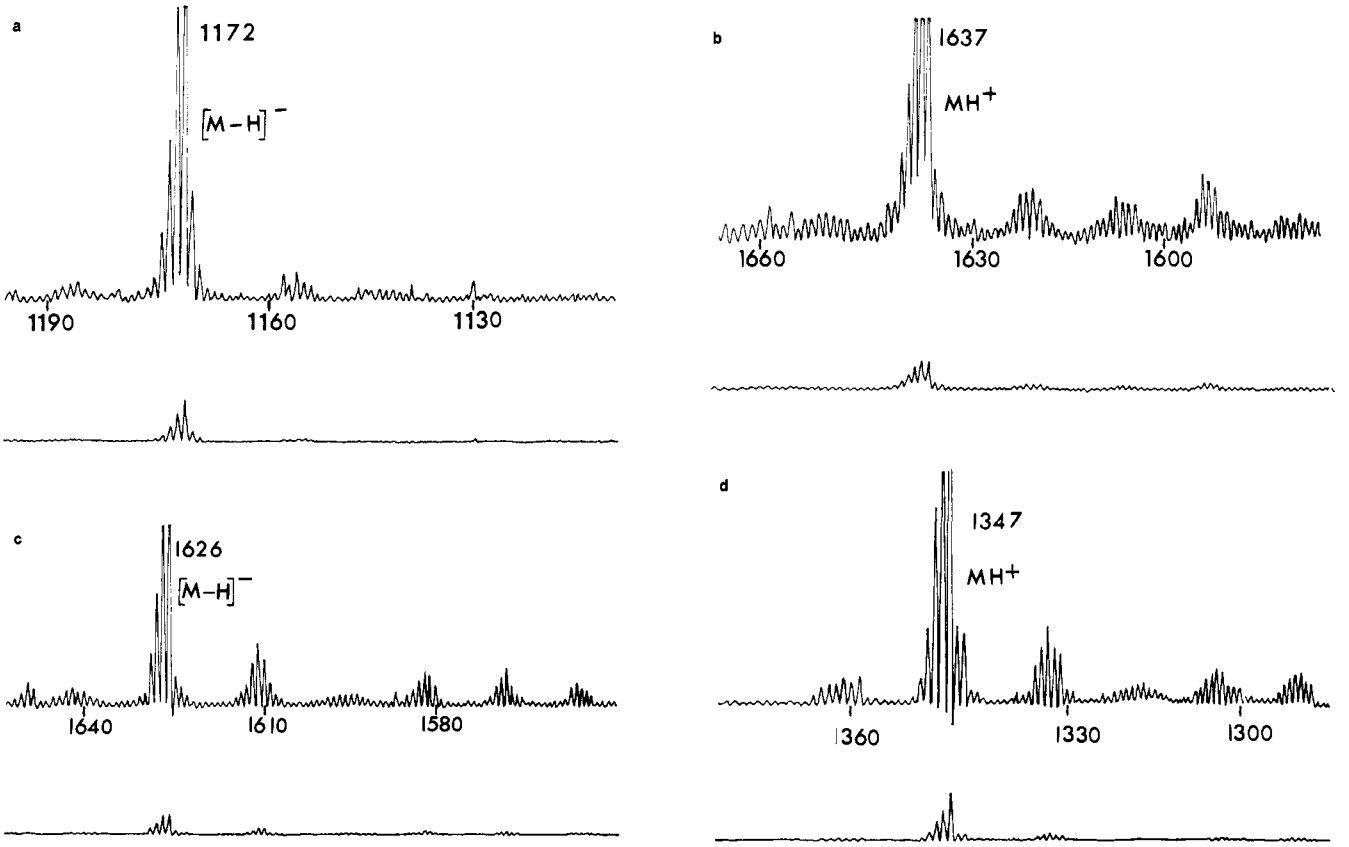


Figure 2. Molecular ion regions in FAB mass spectra of (a) the nucleoside triphosphate **9** (mol wt 1173 daltons) in the negative ion mode, (b) somatostatin (**11**, mol wt 1636 daltons) in the positive mode, (c) gastrin-13 (**12**, mol wt 1627 daltons) in the negative ion mode, and (d) substance P (**13**, mol wt 1346 daltons) in the positive ion mode.

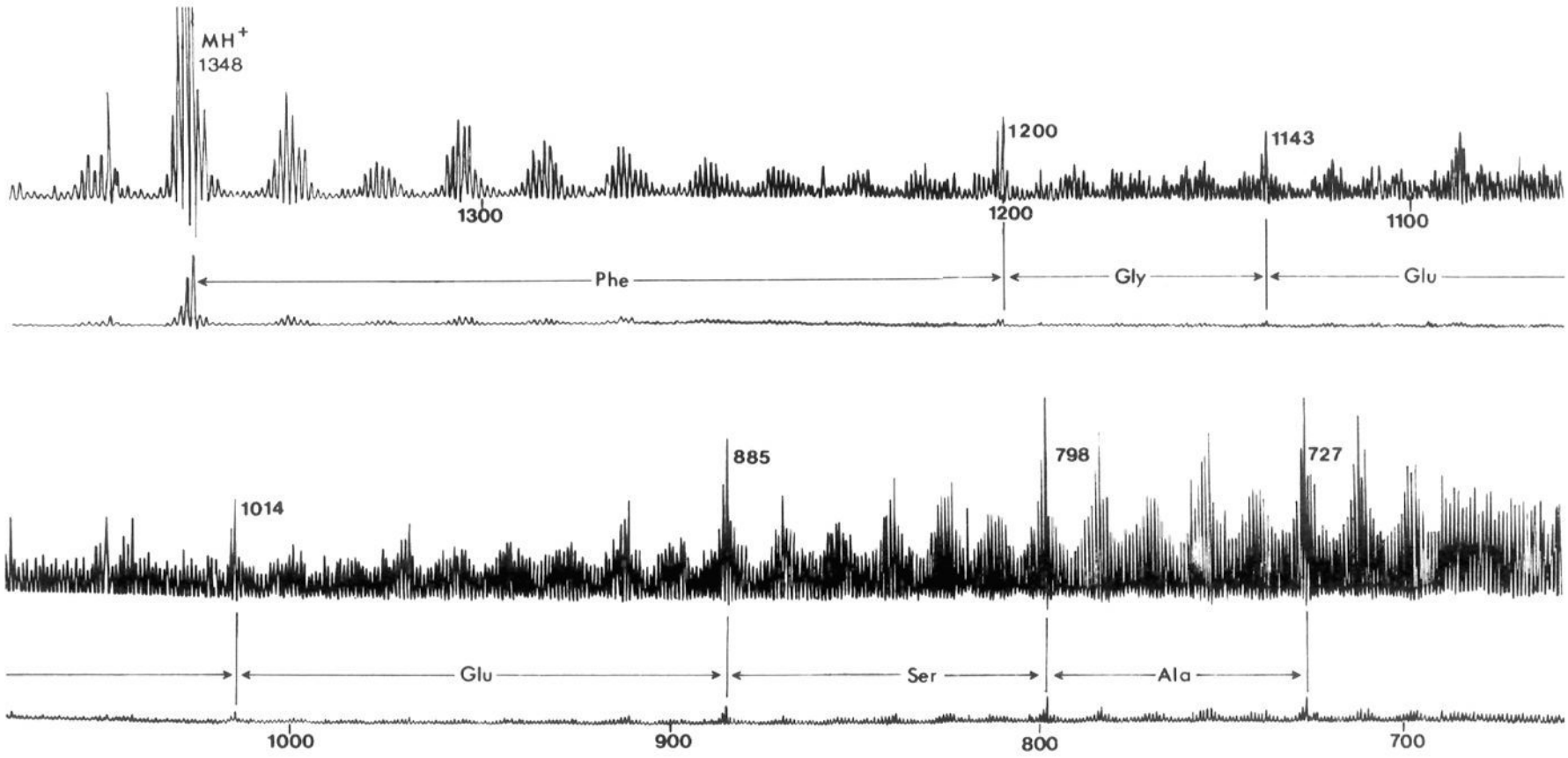
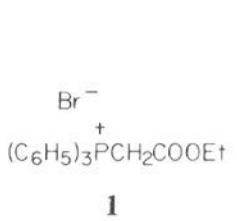


Figure 3. High mass region (m/z 700–1380) of the FAB mass spectrum of Gly-Val-Val-Gly-Arg-Lys-Ile-Ala-Ser-Glu-Glu-Gly-Phe.

(334 + 371)] and trimeric [m/z 1039; (2 × 334 + 371)] ions from **4**; these ions are of an abundance comparable to m/z 333.



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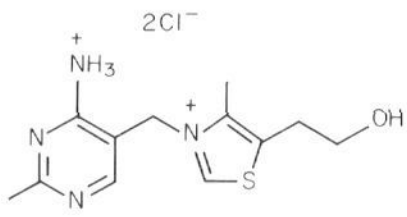

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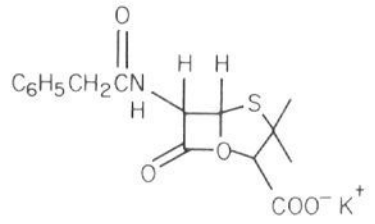

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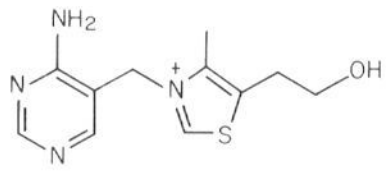


4

We now turn to a discussion of studies of polar compounds of high molecular weight (ca. 900–2000 daltons). In the spectra of such compounds, a mass calibration which relies upon a mass marker (Hall probe device) could in some cases (e.g., calibration mass not close to mass to be measured) be crucially in error by, say, ±1 mass unit. Fortunately, the mass calibration can usually be checked by the presence of an ion at every mass, in conjunction with the use of the characteristic m/z values of ions **5** and **6** derived from glycerol oligomers. In positive ion spectra, the ions corresponding to **5** occur at m/z 93, 185, 277, ..., 1105, 1197. In negative ion spectra, the ions corresponding to **6** occur at m/z 91, 183, 275, ..., 1103, 1195. These negative ions are frequently accompanied by a series m/z 89, 181, 273, ..., 1101, 1193, which are probably due to solvates of anions of monoaldehydes derived by oxidation. In cases where the sample is insufficiently polar to dissolve in glycerol, we have used tetragol (**7**) and, where lack of polarity is an even greater problem, teracol (**8**).

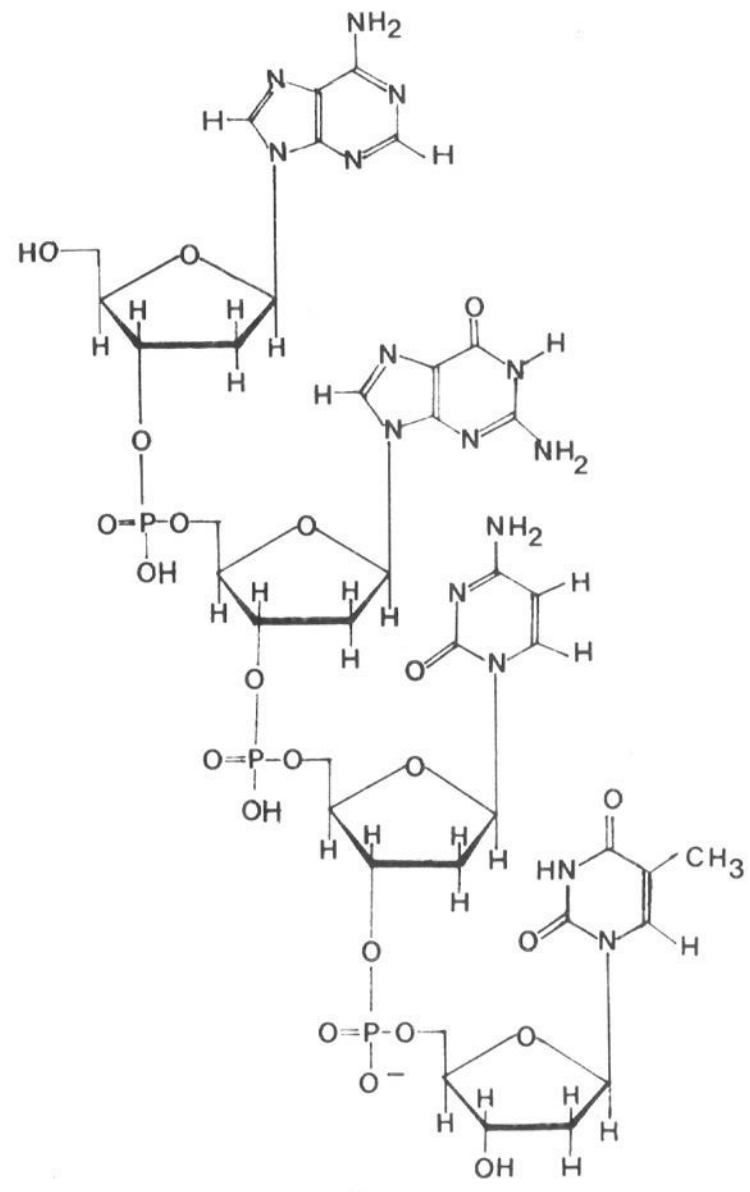
$(C_3H_8O_3)_nC_3H_9O_3^+$ $(C_3H_8O_3)_nC_3H_7O_3^-$ $HO(CH_2CH_2O)_4H$

5 **6** **7**

$HO(CH_2CH_2CH_2CH_2O)_nH$

8

Since it appears that ions which already exist at, or near to, physiological pH may be transferred into the gas phase, we have studied large numbers of compounds which may contain ionic functionalities (e.g., $-N^+H_3$, ArO^-, $-COO^-$, $-OP(O)(OH)O^-$) at, or within a few pH units of, neutral pH. For example, we have obtained an $(M-H)^-$ ion (m/z 1172, Figure 2a) corresponding to the monoanion **9** of a nucleoside triphosphate in its negative



9

ion FAB mass spectrum. The ψ-aglycone **10**, derived from the antibiotic ristocetin A,[8] also produces $(M-H)^-$ in the negative ion mode and produces MH^+ in the positive ion spectrum. These spectra of **10** show an absolute minimum of fragmentation.

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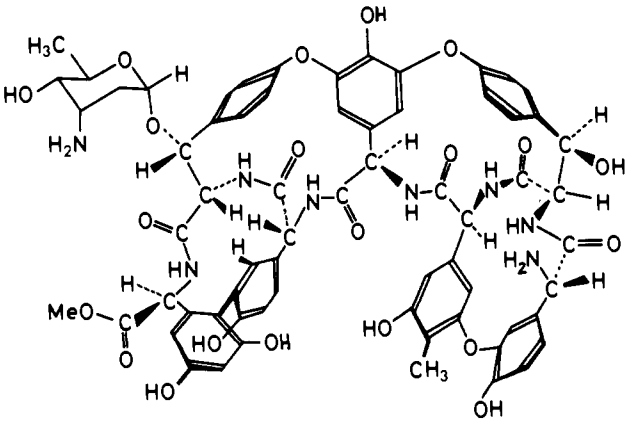
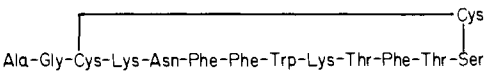


10

Therefore, advantage has been taken of the stabilities of the MH^+ and $(M-H)^-$ ions produced to investigate their binding to cell wall peptide analogues, to which ristocetin A binds in solution.[9] Ristocetin A and the ψ-aglycone **10** bind strongly to the cell wall analogue acetyl-D-Ala-D-Ala, but because of the stereospecificity of the binding show no significant combination with acetyl-L-Ala-L-Ala in solution. Equimolar mixtures of **10** with acetyl-D-Ala-D-Ala, and of **10** with acetyl-L-Ala-L-Ala, were therefore subjected to FAB mass spectrometry in the positive ion mode. In the former case, the intensity ratio of MH^+ from **10** (m/z 1303) to MH^+ from ψ-aglycone/peptide complex (m/z 1505) was ~2:1. In the latter case, the corresponding ratio was ~6:1. In the negative ion mode, the corresponding intensity ratios of m/z 1301:1503 were ~1:1 (association with D,D-peptide) and ~2:1 (association with L,L-peptide). The antibiotic vancomycin, and its aglycon, aglucovancomycin, are also known to show antibiotic properties by virtue of their specific binding to peptides terminating in -D-Ala-D-Ala.[10] The positive ion FAB mass spectrum of an equimolar mixture of aglucovancomycin and acetyl-D-Ala-D-Ala showed MH^+ to (M + peptide + H^+) in the ratio ~2:1; the corresponding data for aglucovancomycin and the L,L-peptide were ~8:1. Although these data appear to suggest stronger binding of the antibiotic ions to the D,D-peptide, we felt that the data should be interpreted with caution since the flux of ions issuing from the matrix may vary during the time of the scan. Neither could complex formation with the L,L-peptide have been predicted. The experiments were therefore repeated with (i) a matrix containing **10**/CH_3CO-D-Ala-D-Ala/CD_3CO-L-Ala-L-Ala in equimolar ratios and (ii) a matrix containing aglucovancomycin/CH_3CO-D-Ala-D-Ala/CD_3CO-L-Ala-L-Ala in equimolar ratios. In these experiments where competitive binding is possible, both antibiotics showed adduct ions of very similar abundances with both CH_3CO-D-Ala-D-Ala and CD_3CO-L-Ala-L-Ala. We conclude that adduct formation is relatively insensitive to the stereochemistry of the peptides (relative to solution studies). It is clear that the contributions (i) of mutual solvation of two components in the gas phase and (ii) of salt bridge formation will be large, and therefore probably less selective than in solution. The experiments show that care must be taken in interpretation of data if a high molecular weight peptide is studied in the presence of a second, low molecular weight component.

Some of the most impressive results with FAB are obtained from underivatized peptides. Molecular weight information has been obtained from MH^+ and $(M-H)^-$ ions up to molecular weights of ca. 2000 daltons. We emphasize that our molecular weight determinations in the m/z 1400–2000 range have been true determinations of m/z values, and not simply observations of ions at or near to the anticipated mass. For example, MH^+ (m/z 1637) is produced from somatostatin (**11**), even in the presence of one disulfide bridge, and (M–H) (m/z 1626) from gastrin-13 (**12**) (Figure 2b and 2c).



Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser

11

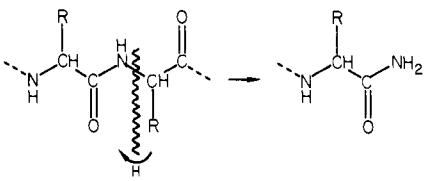
Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-NH_2

12

Underivatized peptides usually produce both excellent positive and negative ion spectra. However, there appears to be an advantage in terms of high sensitivity in running peptides with a net positive charge a pH 6.5 in the positive ion mode, and those with a net negative charge at pH 6.5 in the negative ion mode. Thus, the neuropeptide substance P (**13**) gives MH^+ (m/z 1347, Figure 2d); on a 1 μg (0.74 nmol) sample, a signal-to-noise ratio of 40:1 was obtained. We find that molecular weights up to 1700 can be obtained on less than 0.1 nmol of sample. With larger samples of peptides (2–50 nmol), amino acid sequence information is obtained in both positive and negative ion spectra, from the fragmentations shown in **14**, **15**, and **16**. In **14**, the charge is retained by the N-terminal fragment; the fragments are even-electron ions with either cationic centers in excess of anionic centers by one (positive ion spectra) or anionic centers in excess of cationic centers by one (negative ion spectra). In **15** and **16**, the charge is retained by the C-terminal fragment; again, the fragments are even-electron ions with the charges, positive or negative, determined as for **14**. Ions from **15** and **16** in the same spectrum are frequently recognizable by their characteristic separation of 15 mass units.

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

13



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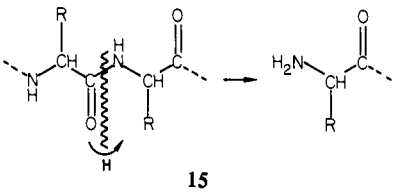

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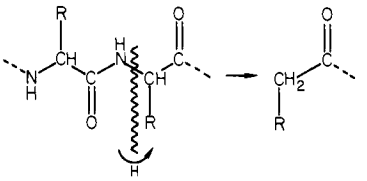

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The sequence ions formed by the above fragmentation processes are generally less abundant than the ions characteristic of molecular weight. As an example of the fragmentation represented in **14**, we reproduce the high mass region (m/z 700–1380) of the peptide Gly-Val-Val-Gly-Arg-Lys-Ile-Ala-Ser-Glu-Glu-Gly-Phe (Figure 3). The sequence ions (m/z 727, 798, 885, 1014, 1143, 1200) of the six amino acids constituting the C-terminal portion are indicated.

Utilizing the fragmentation shown in **14**, a protein-derived mixture of a tetradeca- and a pentadecapeptide (mol wt 1300 and 1371 daltons) has been fully sequenced, aided by the sequence determination of only the first four residues from the N-terminus by electron impact mass spectrometry.[11]

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With use of FAB mass spectrometry, ions characteristic of molecular weight are produced for times ranging from minutes to ca 1 h on samples of 1-50 nmol of highly polar compounds. Thus, conditions suitable for carrying out high-resolution measurements exist. With use of an MS 50 instrument, a resolving power of 80 000 has been obtained. In favorable cases, the intensities of ions characteristic of molecular weight are sufficient to permit precise mass determination (e.g., the antibiotic erythromycin), despite the reduction in the number of ions reaching the collector when operating under high-resolution conditions.

Molecules which do not readily act as proton donors or acceptors are less well suited to examination by FAB mass spectrometry. Thus, we find that structurally informative ions are not produced from nonpolar hydrocarbons with high sensitivity even under a variety of conditions (samples loaded in glycerol, trigol, Nujol, silicone oil, or as a solid). We conclude that FAB mass spectrometry represents a major advance in the study of relatively polar molecules, and appears likely to largely supersede field desorption in this area.

Acknowledgment. We thank SRC and Shell (U.K.) for financial support. We are grateful to Dr. R. C. Sheppard (Laboratory for Molecular Biology, Cambridge) and Dr. R. P. Ambler (Department of Molecular Biology, University of Edinburgh) for samples of peptides and to Dr. S. Salisbury (University Chemical Laboratory, Cambridge) for samples of nucleoside phosphates.

Unified Theory of Aromaticity and London Diamagnetism

Jun-ichi Aihara[1]

Contribution from the Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan. *Received November 18, 1980*

Abstract: The concepts of aromaticity and London diamagnetism were unified into a single theoretical framework. It was then found that a simple relationship exists between the magnitude of the diamagnetic susceptibility due to ring currents and that of the resonance energy due to aromaticity. However, this relationship does not guarantee the proportionality of the susceptibility to the resonance energy. Therefore, it is sometimes dangerous to regard the sign of the susceptibility as an indication of aromaticity or antiaromaticity. The heptalene dianion and bicyclo[6.2.0]decapentaene were predicted to be diatropic but antiaromatic compounds.

In 1961 Elvidge and Jackman suggested that the magnitude of the ring current might be used as a measure of aromaticity of the molecule.[2] An NMR criterion of aromaticity has since been advanced according to which diamagnetic ring currents indicate aromaticity while paramagnetic ring currents indicate antiaromaticity.[3-5] The contribution of ring currents to the magnetic susceptibility is called the London diamagnetism.[6] In 1968 Dauben et al. employed the London diamagnetism as a reliable criterion of aromaticity.[6,7]

However, it was not at all clear that molecules with diamagnetic ring currents also have strong conjugative stabilization.[5,8] In 1966 Abraham and Thomas[9] pointed out that there is little connection between the ring currents and the resonance energy defined by Dewar et al.[10] Labarre and Crasnier[8] and Haddon[11] stressed that it is impossible to link the ring current criterion of aromaticity to the resonance energy or the reactivity of the molecule.

Such a perplexing situation had not changed until the graph theory of aromaticity[12,13] was developed extensively. This theory was found to reproduce analytically the resonance energy of Dewar's type. Furthermore, by the use of the theory, I could prove that both the London diamagnetism and the resonance energy have the same root in the cyclic conjugation of π electrons.[14,15] We are now ready to examine the magnetic criterion of aromaticity in detail. In this paper, I would like to show that the analytic expression of London diamagnetism has a close resemblance in nature to that of the resonance energy. This enables us to clarify the theoretical basis of London diamagnetism as a measure of aromatic stabilization and the limit of its utility. The Hückel molecular orbital model is used throughout this paper.

Theory

Let a characteristic polynomial for the conjugated system G be denoted by $P_G(X)$, and the corresponding reference polynomial $R_G(X)$ can be written formally in this form:[12,13,16]

$$R_G(X) = P_G(X) - \Delta P_G(X) \tag{1}$$

$R_G(X)$ has been interpreted as a characteristic polynomial for the olefinic reference structure of G. Let the jth largest root of the equation $P_G(X) = 0$ be denoted by X_j, and this represents the jth π-electron orbital energy of G. By the use of Newton's method for numerically solving algebraic equations, the corresponding root X_j^0 of the equation $R_G(X) = 0$ can be expressed approximately as

$$X_j^0 \approx X_j + \frac{\Delta P_G(X_j)}{P_G'(X_j)} \tag{2}$$

where $P_G'(X)$ is the first derivative of $P_G(X)$ with respect to X. X_j^0 represents the jth π-electron orbital energy of the reference structure. The validity of eq 2 depends upon the magnitudes of all the $\Delta P_G(X_j)/P_G'(X_j)$ values. Fortunately, they are commonly

(1) Address correspondence to this author at the Department of Chemistry, Faculty of Science, Shizuoka University, Oya, Shizuoka 422, Japan.
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