Principle and Technique of Field-Desorption Mass Spectrometry

Analysis of Corrins and Vitamin B_{12}

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The principle and technique of field-desorption mass spectrometry is described briefly. As is demonstrated for some corrin derivatives and vitamin B_{12} as examples, the method of field desorption extends the range of mass-spectrometric analysis considerably. The molecular weights of substances that are not amenable to conventional mass-spectrometric techniques can be determined. Recent results show that the most important application of field-desorption mass spectrometry will be in the field of biochemical, medical, and environmental analysis.

During the last twenty years, mass spectrometry has developed into a classical procedure for the analysis of organic and organometallic compounds [1–4]. Today, mass spectrometry is a fast-working, highly sensitive, and specific, but also an expensive analytical method for compounds of molecular weight up to 1000 mass units. The range of mass-spectrometric application in biochemistry, medicine, and environmental analysis has increased significantly in recent years as a result of its combination or direct coupling with efficient separation techniques such as gas chromatography or high-pressure liquid chromatography. This combination of modern separation techniques with mass spectrometry enables qualitative and quantitative analysis of extremely complex mixtures. The sensitivity of these methods is in the ppb range, which means that organic compounds present in the original sample in a ratio of $1:10^9$ can be identified and quantitatively estimated; this corresponds to one µg/l.

The principle of mass-spectrometric analysis is the formation of free, gaseous ions from neutral molecules in vacuo and their separation by electric or magnetic fields. The recording of the ion currents produced as a function of their mass:charge ratio (m/e) yields the mass spectrum.

In the case of the classical ionization mode, using electron impact (EI), normally spectra of positively charged ions are measured, since the probability of their formation is about three orders of magnitude greater than the formation of negative ions. Since the electron energy of 70 eV usually employed exceeds the ionization potential of organic molecules, which is approximately 10 eV, a number of fragment ions are formed by consecutive and competitive unimolecular decomposition of the molecular ions. Thus the spectra give not only the molecular weight but also reveal information concerning the molecular structure.

The application of this ionization technique is limited by two factors. First, the necessity of sample evaporation before ionization leads to thermal strain of the substance. Secondly, several eV are transferred to the molecular ion by electron impact. Both factors may result in extensive fragmentation and undetectable molecular ions leading to difficulties in interpretation. To obtain higher volatility and thermal stability of such compounds, various chemical derivatization methods were developed. However, for a series of biologically important substances, even derivatization did not yield satifactory mass-spectrometric results, i.e., the mass spectra did not give unambiguous assignments of molecular weight and structure of the compounds investigated.

In searching for more gentle modes of ionization (with less electronic and thermal excitation), different techniques have been developed of which chemical ionization (CI) [5, 6] and field ionization/field desorption (FI/FD) [7–9] have found a more general application.

Field-Ionization and Field-Desorption Mass Spectrometry

History and Definition

The desorption of positive ions from the surface of a metallic anode under the influence of a high electrostatic field was first observed by E.W. Müller, who used this phenomenon for the construction of the field-ion microscope [10]. A significant advance was made by Inghram and Gomer in 1954, who introduced the mass-spectrometric analysis of field ions by coupling a field-ionization source with a conventional mass spectrometer [11]. The following development of field ionization to an efficient ionization technique supplementing conventional methods in the mass spectrometry of organic compounds was particularly influenced by Beckey [7], who, in 1969, introduced the concept of field-desorption mass spectrometry (FD-MS) for the analysis of large organic molecules [12].

For the use in analytical chemistry, a convenient definition says that field ionization and field desorption of organic molecules can be distinguished by the way in which the sample is supplied to the emitter. If the sample molecules approach the emitter from the gas phase (via the direct or indirect introduction system), one speaks of field ionization. If the sample is adsorbed to the emitter from a solution and is ionized in the adsorbed state, one calls this process field desorption.

Principle

Under the influence of a high electrostatic field of the order of magnitude of 10^7 to 10^8 V/cm, the potential of the outer electron shell of the atom or molecule is changed in such a way that one electron can leave by the so-called tunneling effect. This results in the formation of a positive ion. In the diagram (Fig. 1), interaction of a neutral molecule M and an ionized molecule $[M]^+$ with the emitter surface is demonstrated schematically. If desorption of the neutral molecule M requires an energy A, then the comparatively small energy Q is required for the desorption of the molecular ion $[M]^+$. From this the characteristic feature of the FD-MS to yield molecular ions of high intensity can be explained as follows. While for conventional ionization of a solid sample the complete *sublimation energy* must be applied for evaporation, FD requires only the relatively small *ionic-desorption energy*. In addition, part of the accepted energy is transmitted to the emitter surface (energy relaxation). The average energy transferred during the ionization process is one to two orders of magnitude lower than in the case of EI (note that EI, CI, and FI all require previous evaporation of the sample). Because of the rather small excess energy in the FD ionization process, molecular ions are formed preferentially, so that the FD-MS technique is extremely suitable for the identification of complex mixtures of biological material. Moreover, most of the observed fragments in FD are produced by simple bond cleavage, a fact which considerably simplifies interpretation.

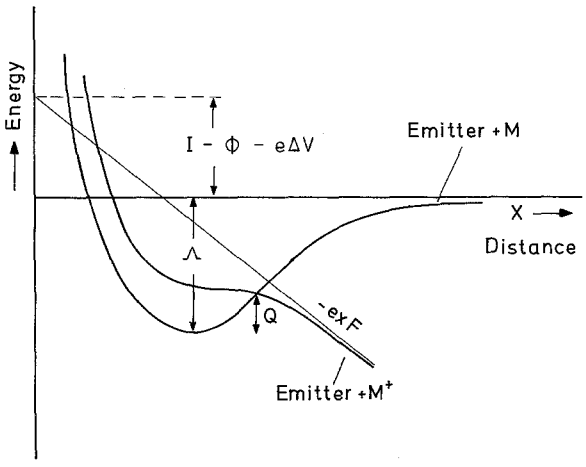

Fig. 1. Interaction curves for a neutral molecule M and an ion $[M]^+$ with the surface of an emitter in the presence of an external electric field F (10^7–10^8 V/cm). ϕ work function of the emitter, I ionization potential for the molecule M, ΔV potential drop between the electron-acceptor level and the surface of the emitter, A desorption energy of the neutral molecule, Q activation energy for desorption in the ionized state

Under the FD conditions, not only radical cations of the molecule $[M]^+$ can be observed, but also protonated molecules $[M+H]^+$ can be formed by field-induced surface reactions. If traces of inorganic salts, e.g., sodium or potassium salts are present, additional signals can be detected as a result of the attachment of the salt cations to the neutral molecules, i.e., $[M+Na]^+$ or $[M+K]^+$, respectively.

Experimental Technique

Field-Ion Emitter

A crucial point in the FD is the production of emitters of uniform quality; 10-μm tungsten wires, activated with benzontrile have proved to be very useful [13].

The result of such an activation process has been shown previously [8, 9]. The tungsten wire is covered with dendrites of partially ordered pyrocarbon

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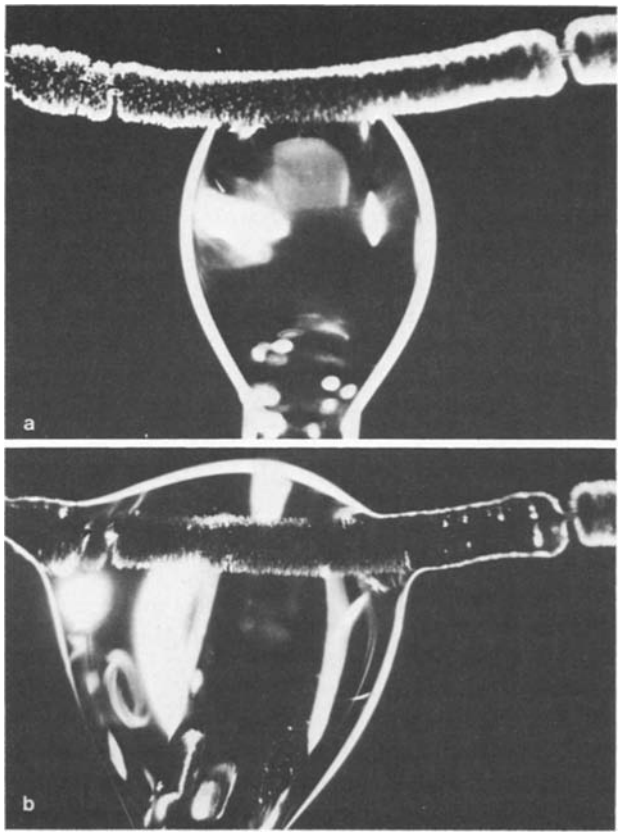


Fig. 2. Syringe technique. Sample supply with a 10-µl syringe. Microscope photographs at a magnification of ca. 1:35 (by courtesy of Dr. H. Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig-Stöckheim)

formed by pyrolysis of benzonitrile in a high electric field in a specially designed activation chamber. Due to the small radii of curvature of the tips of the microneedles, the field strength is enhanced to a level suitable for FD-MS. These emitters are mechanically stable, which is important for repeated use; they can also be chemically and thermally strained, a property which is a prerequisite for pyrolysis mass spectrometry of polymers [14]; and last not least, their surface area is sufficient for sample application.

Sample Application

In the simplest case, the sample can be applied by dipping the activated emitter into a solution [12] or suspension [14] of the substance. The amount of sample deposited should be in the range of 10 µg to a few ng. Diluted solutions can be concentrated most effectively by a µl-syringe under the control with a stereomicroscope (Fig. 2). This method also enables the reproducible deposition of known amounts of sample for quantitative determination [15].

Technique of Measurement

For recording the spectrum, the coated emitter is introduced into the ion source of the mass spectrometer by a pushrod. The exact position in the ion-optical axis of the mass spectrometer is critical for successful operation. The emitter position can be optimized by bleeding acetone into the ion source, using the free points of the coated emitter for field ionization of acetone under conditions that are not sufficient for desorption of the sample itself. For polar substances, the temperature of the emitter has to be increased. This is necessary since a certain amount of thermal energy is required for desorption. For electrical recording of the spectra it is particularly important to know the best anode temperature (BAT) for the desorption of a substance. If the anode is heated too quickly, desorption of the sample occurs so rapidly that the time for recording of a complete spectrum is not sufficient. On the other hand, if the temperature is raised too slowly, the produced ion current is lost in the noise of the detector. The best anode temperature corresponds to maximum intensity of the molecular ion and minimal fragmentation. If the working temperature is slightly above BAT, additional structural information concerning the substance can be obtained by thermally/field-induced formation of fragments.

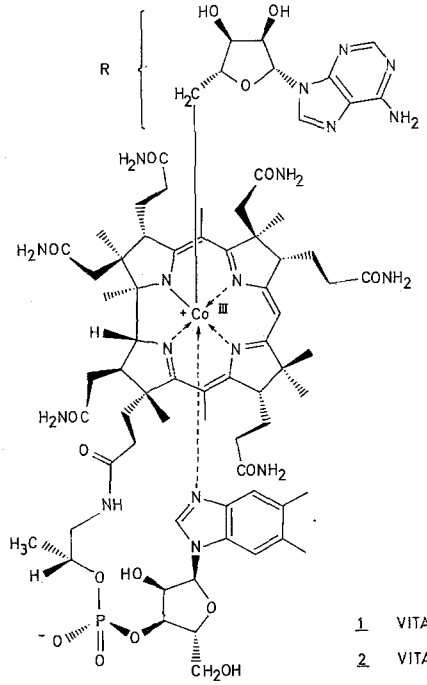
Fluctuating and short-lasting ion currents, which are frequently observed for highly polar substances, are best recorded by integrating techniques such as photographic detection [16]. This facilitates simultaneous and integrating registration of all ions produced during the desorption process. In addition, photoplate detection of the spectra enables determination of the accurate masses of all registered ions. If suitable reference compounds are available, elucidation of the elementary composition is possible. The disadvantage of photographic detection is the low dynamic range of the photoplate and its rather complicated and costly evaluation (using a comparator and computer). The advantages of electrical detection are economy of time, high dynamic range, and high sensitivity. The spectra can be evaluated immediately after registration. In the case of electrical detection, the problem of fluctuating ion currents can be solved by fast accumulation of spectra via multichannel analyzers or computer systems. However, precision measurements of masses of FD ions can only be made if ion currents of sufficient intensity and stability can be produced.

Field-Desorption Mass Spectra of Corrins

Vitamin B_{12} coenzyme (*I*) represents an example of a natural substance of low molecular weight for which the isolation, structural determination, and synthesis

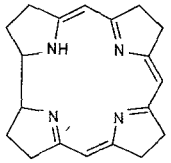
were found to be unusually difficult. In 1926, Minot and Murphy [17] observed that pernicious anemia, which was at that time fatal, could be treated by doses of fresh liver. More than twenty years later, in 1948, Folkers and Smith [18] succeeded in the isolation and cristallization of the coenzymatically inactive vitamin B_{12} (*2*) and in 1958 Barker [19] of the vitamin B_{12} coenzyme (*1*) as well. Attempts to determine the constitution of this biologically important molecule by chemical degradation methods yielded only a partial analysis. The complete structure was solved by Dorothy Hodgkin and co-workers [20, 21] using x-ray analysis.

The total synthesis of this complicated and novel macrocyclic ring system, the skeleton of which was named a corrin (*3*), by the groups of Eschenmoser [22] and Woodward [23], required twelve years and resulted in the synthesis of cobyric acid (*4*) in 1972). From this acid, vitamin B_{12} coenzyme (*1*) and vitamin B_{12} (*2*) may also be prepared [24–26]. These synthetic investigations resulted in a rather complete knowledge of the structure of corrin ring systems and the chemistry of corrins in general.

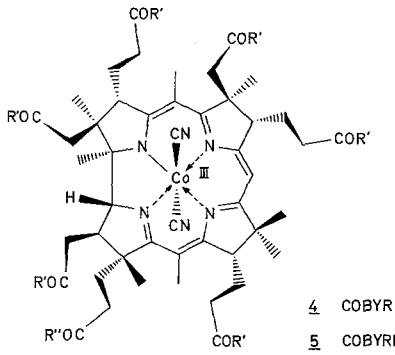


1 VITAMIN B_{12} COENZYME

2 VITAMIN B_{12}, R = CN



3 CORRIN



4 COBYRIC ACID, R' = NH_2 R'' = OH

5 COBYRINIC ACID, R' = R'' = OH

In contrast, a comprehensive understanding of the biosynthesis of vitamin B_{12} coenzyme and its molecular biology, i.e., the molecular principles of its function, is still incomplete. The principal difficulties in all experiments dealing with the biosynthesis and questions of chemical structure and biological activity of the vitamin B_{12} coenzyme are a consequence of the extremely small amounts occurring in living systems. For example, the concentration in human blood is about 10^{-10} g/ml. Hence, in most cases, the necessary amounts are not available for the detection and analysis of vitamin B_{12} coenzyme and its metabolites for the conventional spectroscopic methods.

A basic advantage of mass spectrometry is its high selectivity *and* sensitivity. However, the classical method of electron-impact ionization fails in the case of highly substituted corrins. Neither vitamin B_{12} coenzyme (*1*) nor vitamin B_{12} (*2*) and cobyric acid (*4*) result in analytically useful spectra. The reason for this behaviour is the low vapor pressure and the high thermal instability of these compounds. As mentioned before, in field-desorption mass spectrometry, the problem of sample evaporation is dispensed with. The thermal stress of the sample before ionization is small. Therefore, with a view to making a systematic investigation concerning the enzymatic incorporation of potential synthetic biogenetic precursors of the vitamin B_{12} chromophor and its metabolites, we tested the field-desorption properties of the vitamin B_{12} (*2*) as well as a series of derivatives of cobyrinic acid (*5*).

Cobyrinic Acid Derivatives

The first mass-spectrometric studies on corrins were performed by Seibl on simply substituted synthetic metal complexes [27]. Thermal degradation before ionization is characteristic for these compounds. Thermolysis starts with a cleavage of the axial ligands and results in corrin-metal complexes with a corre-

spondingly lower molecular weight. In the spectra of these compounds, the molecular ions and fragments of the thermally formed complexes are superimposed with those of the axial ligands. While these compounds show rather clear spectra, which make possible a definitive determination of the axial ligands as well as the original metal complexes, the spectra of the cobyrinic acid derivatives are much more complicated (Fig. 3). The signals above m/e 900 are accompanied by intense fragment ions in the lower mass range. All compounds of this structural type show drastic changes of the spectra with increasing temperature as is clearly demonstrated by contrasting the EI mass spectra of *6* obtained at source-temperature

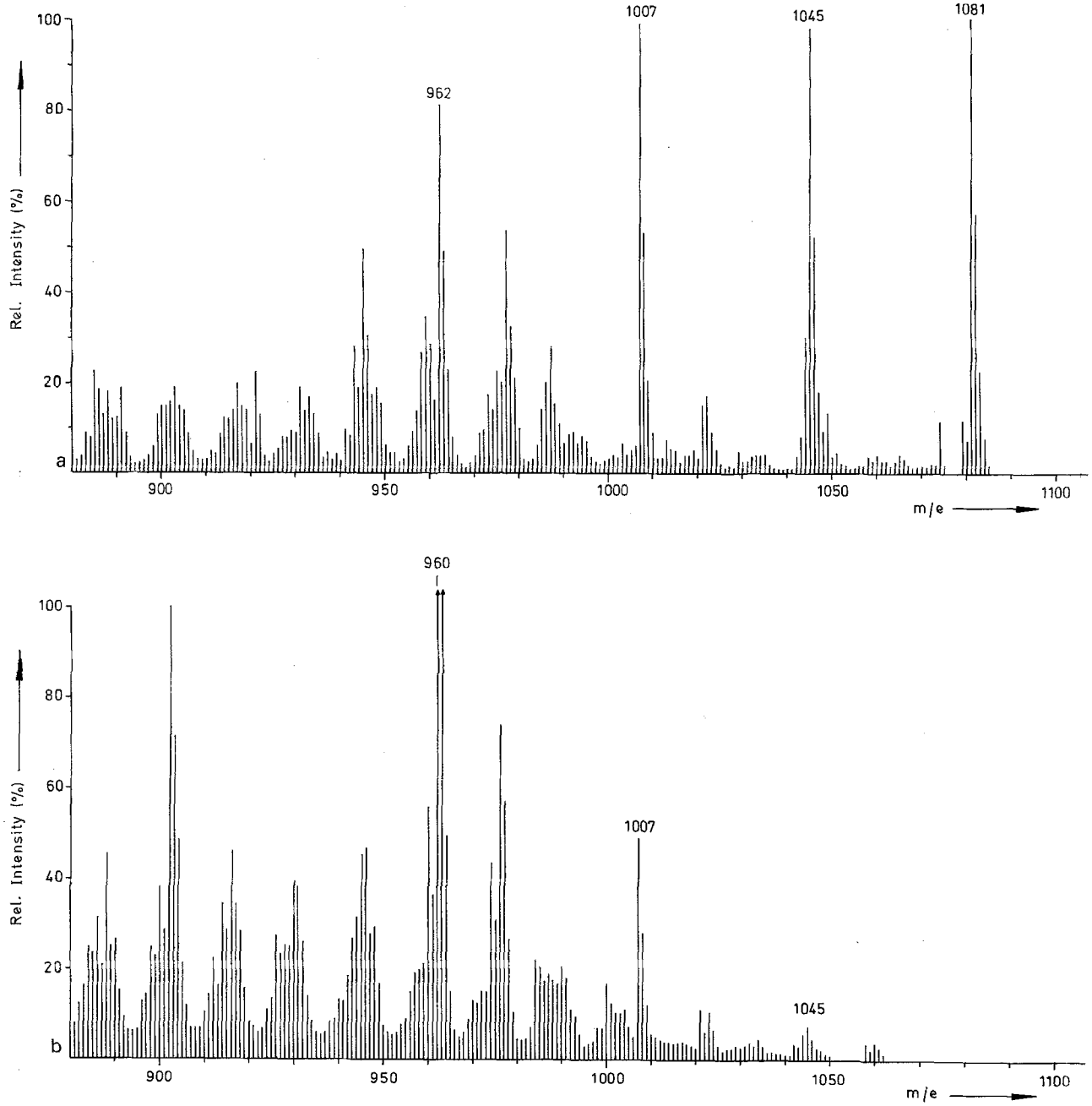


Fig. 3. Electron-impact mass spectra of 10-nitro-cobyrinic acid ester (*6*) recorded at two different temperatures. Experimental conditions: acceleration voltage 8 kV, electron energy 70 eV, electron beam 500 μA. The source temperature was at 200 (a) and 210 °C (b), respectively. The sample was introduced via a direct introduction system into the double-focusing mass spectrometer MS 902, AEI, Manchester

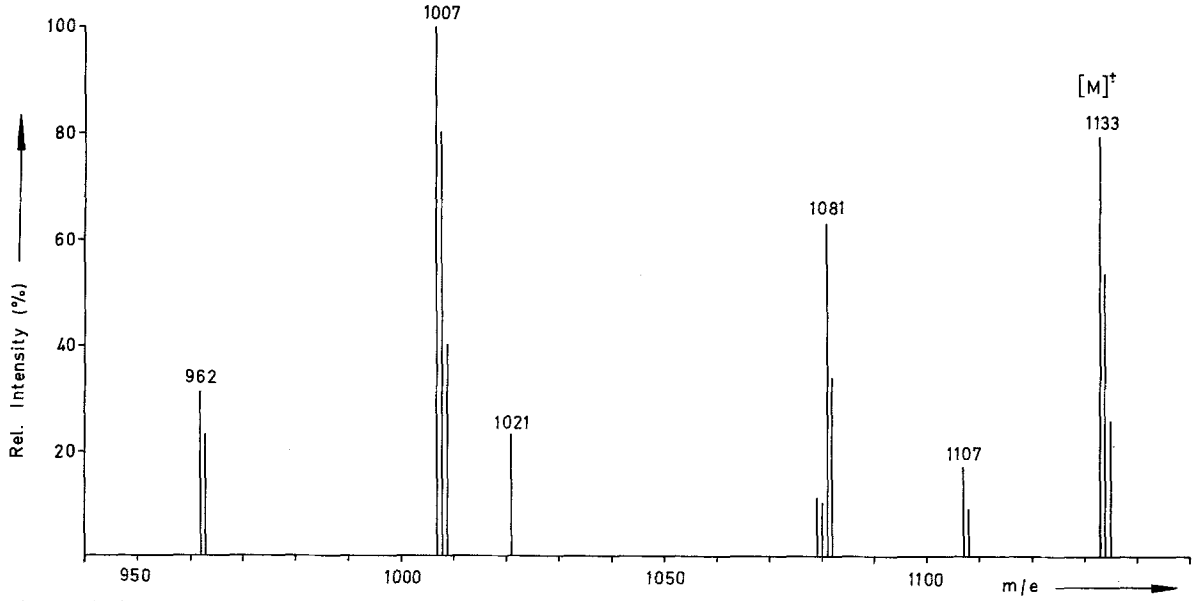
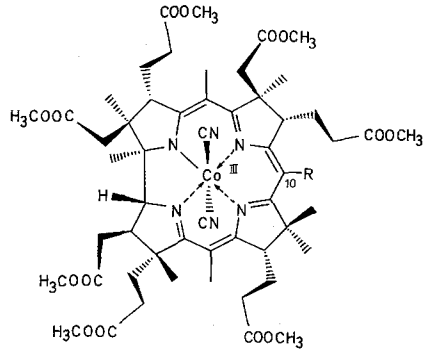


Fig. 4. Field-desorption mass spectrum of 10-nitro-cobyrinic acid ester (*6*). Experimental conditions: photographic registration on vacuum-evaporated AgBr plate (Ionomet, Waban, Massachusetts, USA). Exposure time 10 min, direct heating from 0 to 40 mA emitter-heating current (BAT). Mass resolution 16000 (10% valley definition). The sample (a few micrograms) was transferred to the emitter using the syringe technique (Fig. 2). The solvent was methanol. To simplify a comparison between the EI spectra (electric recording, linear response) and the FD spectra (photographic recording, logarithmic response), the data were normalized and plotted in percent relative intensity



6 10-NITRO-COBYRINIC ACID ESTER, R = NO_2

7 10-BROMO-COBYRINIC ACID ESTER, R = Br

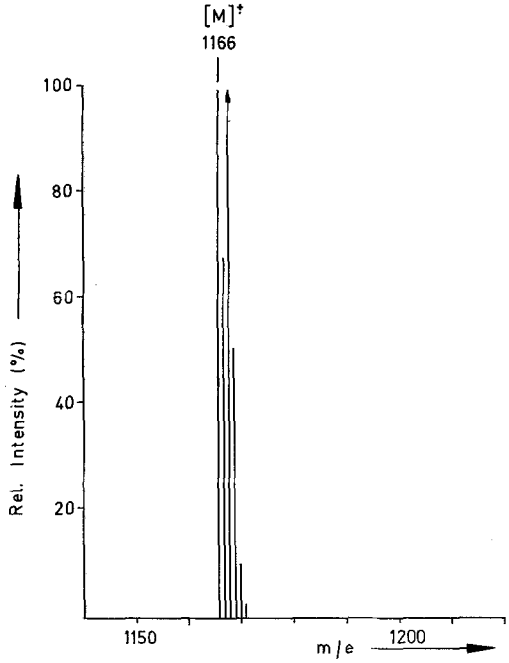


Fig. 5. Field-desorption mass spectrum of 10-bromo-cobyrinic acid ester (*7*). Experimental conditions: photographic registration on vacuum-evaporated AgBr plate. Exposure time 10 min, indirect heating by laser-supported FD-MS [30] (at BAT). Mass resolution 10000 (10% valley definition)

differences of 10 °C only (see Figs. 3a and b). Only under optimal conditions and within a short time interval can fragments be observed that can be interpreted analogously to the simply substituted corrin-metal complexes by cleavage of the axial ligands. However, based on their fragment pattern and some key fragments, e.g., the mass 962, EI spectra of cobyrinic acid derivatives generally yield only an indication of the structure present.

As can be seen by comparison of the mass spectra in Figures 3 and 4, in FD-MS the molecular ion of the 10-nitro-cobyrinic acid ester (*6*) is detected with high relative intensity at m/e 1133.437. At the same time, fragmentation is strongly suppressed and limited to only few ions in the upper mass range. The inten-

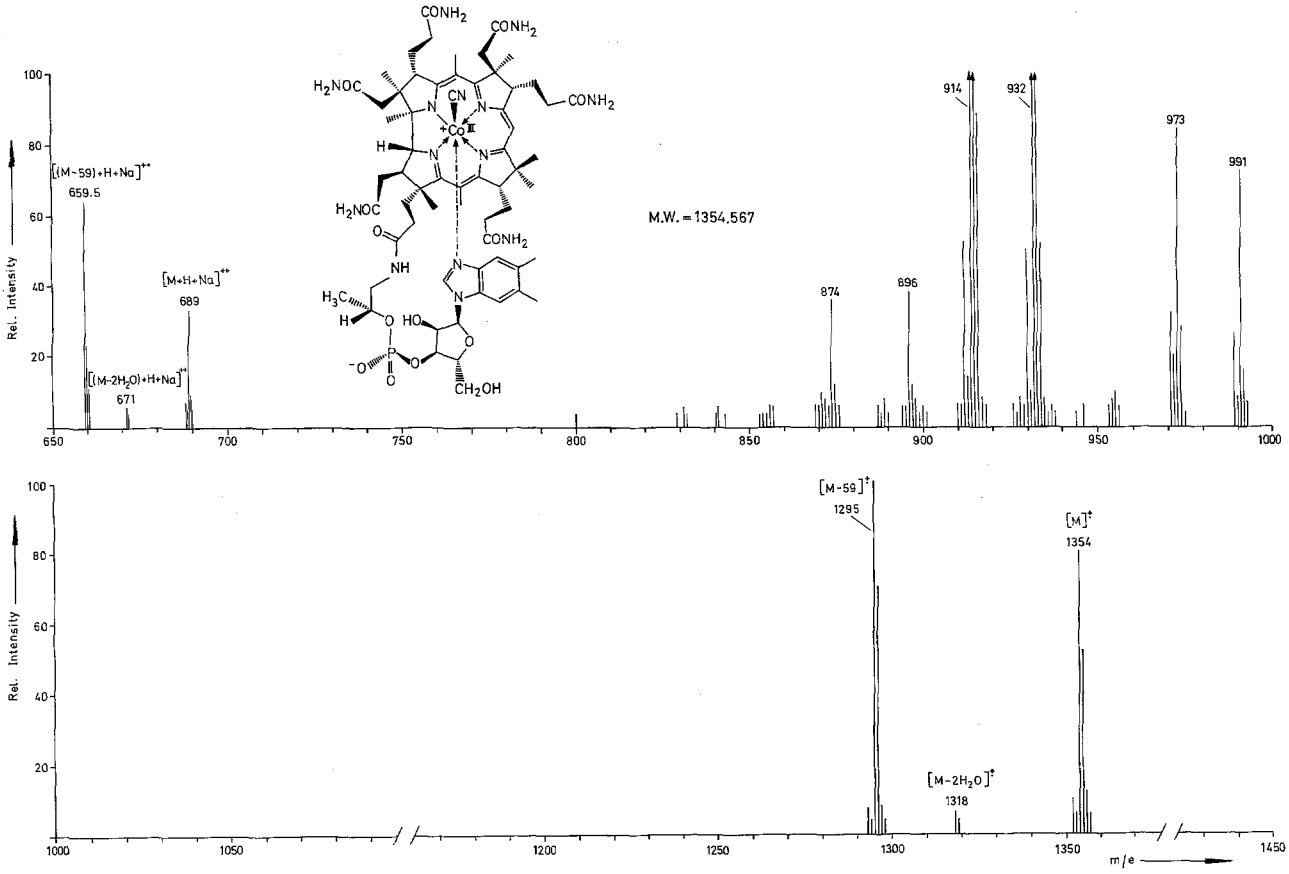


Fig. 6. Field-desorption mass spectrum of vitamin B_{12} (*2*). Experimental conditions: photographic registration as for *6* and *7*. Exposure time 6 min, indirect heating by laser-supported FD-MS [30], 0–200 mW. Mass resolution 8000 (10% valley definition)

sive fragmentation pattern in the lower mass range, which is typical for EI spectra, is completely absent in the FD-spectra. The base peak of the spectrum is an ion at m/e 1007.394 with the elementary composition $C_{49}H_{66}N_5O_{14}Co$. Tentatively, its formation can be explained by the loss of both axial ligands and one acidic acid methylester group. The formation of the ion at 1021.409 results from the loss of both axial ligands and of formic acid methylester. The fragments at m/e 1107.434 and 1081.431 correspond to the cleavage of one or two CN groups, respectively, yielding a corrin-Co(I) complex. As mentioned before, in all EI mass spectra of cobyrinic acid derivatives the ion at m/e 962 appears as a key fragment. From high-resolution FD-MS, its accurate mass was found to be 962.409 and the composition can therefore be specified as $C_{49}H_{67}N_4O_{12}Co$. The appearance of fragments of the same masses in EI and FD spectra of this compound indicates a thermally induced fragmentation prior to desorption, as can be generally observed at temperatures above the BAT. If the BAT is not exceeded, as in the case of the 10-bromo-cobyrinic acid derivative (*7*), only the molecular ions are registered (Fig. 5).

Vitamin B_{12}

Vitamin B_{12} is both a challenge and landmark in mass-spectrometric analysis. In the past, studies were carried out to record vitamin B_{12} mass spectrometrically by the then available ionization procedures. However, records of such studies are scarce [28]. Only recently, Mcfarlane and Torgerson reported investigations that yielded a partial analysis [29]. These workers employed a californium source in connection with a time-of-flight mass spectrometer. While in the spectrum of the positive ions of vitamin B_{12} no masses above m/e 1000 could be obtained, the spectrum of the negative ions show as the highest mass peak a weak signal at m/e 1327, which might be due to the loss of HCN from the original molecular ion $[M-1]^-$, a second signal at m/e 1269, and intense fragments below mass 1000.

Figure 6 shows the upper mass range of the complete high-resolution field-desorption mass spectrum of the vitamin B_{12} (*2*). As expected, thermally/field-induced fragmentation during desorption are more important than in the case of the less polar cobyrinic acid derivatives. Despite these competing degradation reactions,

the molecular ion is detected with high relative intensity at m/e 1354.567. The high abundance of doubly charged ions in the spectrum is also remarkable. The molecular ion as well as the fragments at m/e 1318.546 and m/e 1295.530 are detected because of protonation and cationization at the corresponding half masses 689.282, 671.272, and 659.764, respectively. From the high-resolution data, tentative assignments were made for two relatively intense fragment ions. Thus, for instance, the base peak at m/e 914.445 agrees with an ion of the elementary composition $C_{45}H_{65}N_{11}O_6Co$, the formation of which may result in a nearly complete split of the nucleotide side chain. The loss of acetamide leads to an abundant ion at m/e 1295.530 ($C_{61}H_{83}N_{13}O_{13}CoP$) and a dehydratization process to the ion at m/e 1318.546 ($C_{63}H_{84}N_{14}O_{12}CoP$).

Even though the structures of the fragment ions formed and the associated reactions occurring during field desorption of vitamin B_{12} are still largely unknown, the result of the measurement clearly demonstrates that FD-MS may be of great analytical importance for the investigations concerning the biosynthesis of vitamin B_{12} and related problems.

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