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DETERMINATION OF ACTIVE HYDROGEN CONTENT BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY FOLLOWING HYDROGEN-DEUTERIUM EXCHANGE

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Fast atom bombardment mass spectrometry following hydrogen - deuterium exchange in [hydroxy-$^2H_3$]glycerol and ^2H_2O has been studied as a means of establishing active hydrogen content in molecules of unknown structure. Nucleotides, carbohydrates, one peptide and complex antibiotics, in the mass region to 1500 daltons and 29 exchangeable hydrogens were examined, with a correct hydrogen count unambiguously measured in every case. The method is experimentally simple and applicable on a microgram scale, to salts and a variety of polar compounds of biological origin.

Knowledge of the exact active hydrogen content of a molecule of unknown structure provides an important constraint in the deduction of overall structure. This information is complementary to elemental composition, and to knowledge of the identities of structural subunits or functional groups, and plays a useful role in determining the identity and arrangement of functional groups. The active hydrogen content can often be inferred by chemical derivatization, or measured directly by chemical liberation of H_2 (1), proton nuclear magnetic resonance, or mass spectrometry following hydrogen - deuterium exchange in the ion source or inlet system (2). Unfortunately, these methods become inaccurate and the results ambiguous as the active hydrogen content increases. Mass spectrometric methods are often favorable because of microgram-level sensitivity, but are usually limited by back-exchange, which becomes severe beyond 4-6 hydrogens. Gas-phase exchange under chemical ionization conditions is in principle useful (3-5) but serious errors can result from exchange of non-labile hydrogens (6,7).

Abbreviations used: FAB, fast atom bombardment; M, molecule in which all active hydrogen atoms have been exchanged by ^2H.

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The recent development of fast atom bombardment mass spectrometry is having a major impact on the solution of structural problems in biochemistry and natural products chemistry in the mass region up to several thousand daltons (8-10). The prospect of accurate determination of active hydrogen content for molecules in this region is attractive, and would constitute a major improvement over existing methods. As a consequence, the use of FAB mass spectrometry for materials directly exchanged in [hydroxy-$^2H_3$]glycerol - ^2H_2O has been explored in the molecular weight region up to 1500 daltons, and 29 active hydrogens. The technique was examined from the standpoint of applicability on a microgram-scale, with high accuracy, to a variety of compounds of biological importance.

Sindona et al. have employed the use of deuterated glycerol for labeling of the deoxynucleotide TpT, to study the mechanism of formation of a fragment ion in the negative ion FAB mass spectrum (11). The extent of labeling and related matters were not commented on.

MATERIALS AND METHODS

Materials. [Hydroxy-$^2H_3$]glycerol (97.8 atom % ^2H) C-perdeuterioglycerol, and ^2H_2O (98+ atom % ^2H) were purchased from MSD Isotopes, St. Louis, MO. The following were purchased from commercial sources: uridine 5'-phosphate, Na salt; guanosine 5'-phosphate, Na salt; melibiose; melezitose; streptomycin sulfate; α-cyclodextrin; γ-cyclodextrin. N-[(9-β-D-ribofuranosylpurin-6-yl)-carbamoyl]threonine 5'-phosphate, Na salt was a gift from Dr. M. P. Schweizer, University of Utah; conotoxin MI was provided by Dr. W. R. Gray, University of Utah. A mixture of bleomycins A_2, A_2-demethyl, and B_2 were purchased locally as Blenoxane (Bristol Laboratories).

Sample preparation. Samples were prepared by solution in [hydroxy-$^2H_3$]-glycerol - ^2H_2O (1:1) at a concentration 1-5 μg per 2 μl. Prior to application of the sample, the dry probe tip was rinsed in ^2H_2O. Two μl of the solution were transferred to the probe tip using a 10 μl syringe that had been previously rinsed with ^2D_2O. The probe was immediately transferred to the vacuum lock of the mass spectrometer. These precautions were necessary to avoid reexchange of deuterium by adsorbed H_2O.

Mass spectrometry. Mass spectra were acquired using a Varian MAT 731 instrument, with either magnetic or electric scanning at 8 kV accelerating potential. An Ion Tech FAB 11N ion source was installed, closely following the modifications described by Biemann and co-workers (12). A neutral Xe beam

having 6 KeV energy was used for ion desorption. In some cases ion abundance data were taken from mass spectra accumulated over 2 min. (1024 scans) using a Nicolet 1170 signal averager.

RESULTS AND DISCUSSION

Ion abundance data from the FAB mass spectra of ten compounds following hydrogen-deuterium exchange are given in Table 1. Abundances are normalized to the most intense peak within each isotopic cluster. The notation $(M+^2H)^+$ refers to the fully exchanged molecule, attached to a deuteron, in analogy to the protonated molecule MH^+ (unexchanged) usually produced by FAB (16). The notation $(M+^2H-1)^+$ refers to ion species in which one deuterium has been replaced by protium. Additional ions such as Na^+ adducts, which are characteristic of FAB spectra (17,18), were also observed, but are not listed in Table 1. In every case the extent of exchange in these ions was the same as for those reported.

In every case examined the correct active hydrogen content was established from the mass shift between the spectrum of the unlabeled material (data not

Table 1. Summary of Molecular Weight-Related Ions Following Hydrogen-Deuterium Exchange

Compound	Molecular Weight, Unlabeled	Number of Active Hydrogens	Percent Exchange	m/z (relative intensity)[a] $(M+^2H-3)^+$	$(M+^2H-2)^+$	$(M+^2H-1)^+$	$(M+^2H)^+$
Uridine-5'-phosphate, monosodium salt	346	4	90	349(8)	350(19)	351(55)	352(100)
Guanosine-5'-phosphate,	385	6	92	390(13)	391(26)	392(60)	393(100)
N-[(9-β-D-ribofuranosylpurine-6-yl)carbomyl]threonine 5'-phosphate, monosodium salt	514	7	93	520(11)	521(19)	522(56)	523(100)
Melibiose	342	8	90	349(20)	350(41)	351(94)	352(100)
Melezitose[b]	504	11	93	514(13)	515(34)	516(80)	517(100)
Streptomycin sulfate	581	16	93	596(27)	597(59)	598(100)	599(91)
α-Cyclodextrin	972	18	93	989(45)	990(83)	991(100)	992(85)
γ-Cyclodextrin[b]	1296	24	93	1319(66)	1320(87)	1321(100)	1322(90)
Bleomycins[c]							
A_2, cation[d]	1414	25	93	1436(83)	1437(95)	1438(100)	1439(98)
demethyl-A_2	1399	25	93	1423(71)	1424(88)	1425(100)	1426(96)
B_2	1424	29	93	1452(87)	1453(99)	1454(100)	1455(90)
Conotoxin MI[e]	1492	27	92	1518(79)	1519(95)	1520(100)	1521(96)

[a] Each isotopic cluster is normalized to the largest peak in the cluster.
[b] FAB mass spectrum of unlabeled compound, ref. 13.
[c] FAB mass spectrum of unlabeled compound, ref. 14.
[d] Ion m/z 1439 is fully exchanged M^+; lower mass peaks due to back exchange.
[e] Tetradecapeptide, with 2 disulfide bonds, ref. 15.

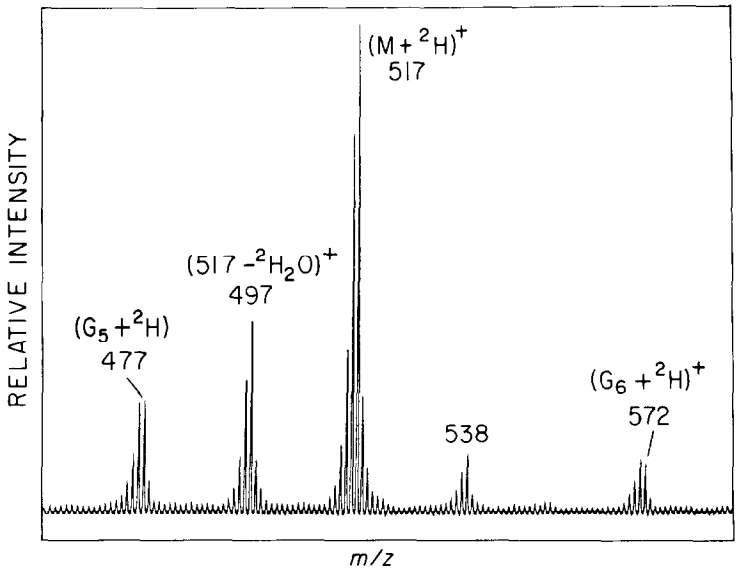

Fig. 1. Fast atom bombardment mass spectrum of melezitose in [hydroxy-$^2H_3$]glycerol (G) and ^2H_2O. The m/z 517 ion contains 12 deuterium atoms.

shown), and that following exchange, up to a maximum of 29 hydrogens. No evidence was found for incorporation of hydrogen from the glycerol skeleton into the protonated molecule or any fragment ions, as judged from spectra determined using C-perdeuterioglycerol matrix.

The extent of exchange from [hydroxy-$^2H_3$]glycerol ranged from 90-93% of the maximum possible. This value was observed to steadily decrease as the labeled sample was allowed to stand for longer times before introduction into the mass spectrometer. In the simpler cases (below m/z 1000 and fewer than 18 exchangeable hydrogens), the deuterium content was unambiguously established by simple visual inspection, as illustrated by the spectrum of [hydroxy-$^2H_{11}$]-melezitose (Fig. 1). The relative intensity of m/z 518 is accounted for by the required contribution from natural heavy isotopes, mainly ^{13}C. In practice, natural heavy isotope contributions can be calculated by standard equations (19) if the elemental composition is known or can be estimated; it can also be established simply from the mass spectrum of the unlabled com-

pound. The lower isotope peaks (m/z 516, 515 in Fig. 1) are due mainly to incomplete exchange, but contributions from M^+ may be possible in some cases (recognized from the mass spectrum of unlabeled material). For ions having many deuterium atoms, the abundances of the first and second lower isotope peaks are sensitive to small changes in overall deuterium content (20). Because the glycerol matrix ions represent the deuterium content of the sample at the time of ionization, the glycerol isotopic clusters can be used to accurately measure the extent of exchange in the sample, regardless of whether glycerol ions appear the mass region of interest. Thus, the levels of exchange of $([\underline{\text{hydroxy}}\text{-}^2H_3]\text{glycerol})_n \cdot {}^2H^+$ cluster ions (n = 3,4,5...) and sample ions, were typically found to be within ±1% of each other. The effect of number of deuteriums on the pattern is illustrated by comparison of m/z 517 ($^2H_{12}$) and 572 ($^2H_{19}$) clusters in Fig. 1. For very large ions (e.g., the bleomycins, Table 1), the isotopic pattern may appear visually confusing. Clarification can be gained by correcting for natural heavy isotopes, and by calculating (20) the lower isotope peak pattern, using deuterium levels measured from glycerol ions.

The three mononucleotides (Table 1) were examined as their sodium salts. If, in a molecule of unknown structure, salt formation from acidic functional groups (phosphate, carboxylate) were suspected, acidification or cation replacement (K^+ or Na^+) experiments (12,18) to establish this fact can be carried out directly on the probe tip.

Several micrograms of sample were used in the present study for convenience. In most cases an intense signal of several minutes duration was obtained. Reduction of sample size to submicrogram levels is therefore possible, particularly for molecular weights over m/z 600, where interference from glycerol cluster ions is minimized. Some fluctuation in ion beam intensity was encountered from scan to scan. For accurate measurement of deuterium content, a signal averager was used in some cases, although multiple scans taken by conventional scanning (Fig. 1) will also suffice.

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