protons if anything fit the former than the latter. The concentration data on β-DPN do not permit a distinction to be made between the (M′)- and (P)-helical forms.

Theoretically computed isoshielding lines[52] for a dihydronicotinamide moiety indicate that this molecule possesses little or no ring current field and, hence, in the case of β-TPNH, one cannot rule out a dihydropyridine–dihydropyridine interaction. Nevertheless, the magnitude and direction of the shifts of the protons of the adenosine moiety (Figure 13) indicate that adenine–adenine interaction exists and these data are compatible with dimerization between two (P)-helices of β-TPNH with adenine–adenine interaction. β-TPNH may exist either as (P)-B-anti-B-anti or as (P)-B-anti-A-anti because the preferred torsional diastereomer constrained to the nicotinamide–ribose glycosidic linkage is anti (*vide supra*) and that in such a conformation the less crowded side is the B side. X-Ray data on lactate dehydrogenase reduced coenzyme binary complex indicate that the dihydronicotinamide is anti in the complex.[54] The data do not allow a distinction between the (M′)- and (P)-helices of β-DPNH.

Acknowledgment. This research was supported in part by grants from the National Cancer Institute of the National Institutes of Health (CA12462-01 and CA12462-02) and the National Science Foundation (GB28015 and GP28061). The authors thank Professor B. Pullman and Dr. C. Giessner-Prettre for providing them with the isoshielding surface for adenine and oxidized and reduced nicotinamides for various z values.

(54) M. J. Adams, A. McPherson, Jr., M. G. Rossman, R. W. Schevitz, I. E. Smiley, and A. J. Wonecott in ref 7, pp 157–174.

Mass Spectrometry of Nucleic Acid Components. *N,O*-Permethyl Derivatives of Nucleosides

D. L. von Minden and James A. McCloskey*

Contribution from the Institute for Lipid Research and Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77025. *Received April 4, 1973*

Abstract: Reaction between nucleosides and methylsulfinyl carbanion–CH_3I or–CD_3I produces permethyl derivatives which are O-methylated in the sugar, and methylated at the following positions in the base: adenosine, N^6,-N^6; guanosine, N^2,N^2,O^6 and N^2,N^2,N-1 (~1:1); uridine, N-3; cytidine, N^4,N^4. The reaction can be carried out on a microgram scale; the products are chemically stable, exhibit relatively low molecular weights, and are sufficiently volatile for gas chromatography–mass spectrometry. Principal fragmentation pathways have been studied, based on D, ^{18}O, and substituent labeling, and measurement of exact mass, using 37 nucleoside and deoxynucleoside models. Major reactions are initiated by transfer of hydrogen from the sugar moiety to the charge-localized base. As an exception, 25% of rearranged hydrogen in the base + H ion is derived specifically from the *O*-2′-methyl group. The influential role of base–2′ interactions is also shown by the effect of methoxyl orientation at C-2′ (ribose *vs.* arabinose) on sugar − H ion abundance. Otherwise, steric features of the sugar have significant effects upon ion abundance, but hydroxyl orientation in the parent nucleoside cannot be assigned directly from the mass spectrum. Characteristic elimination of methylenimine from dimethylamino groups in the base proceeds by a complex mechanism which can include participation of other sterically accessible methyl groups, *e.g.*, the C-5 methyl function in the permethyl derivative of 5-methylcytidine.

Detailed studies of electron impact induced fragmentation of nucleosides and their analogs have led to a clearer understanding of the complex reaction paths which are involved and have provided a powerful tool for the determination of nucleoside structure. However, the dominant experimental problem, particularly for many modified nucleosides which occur in transfer RNA, continues to be low volatility resulting from the presence of multiple hydroxyl and amino groups. Substantial progress in this respect has been made using the field desorption technique in lieu of conventional vaporization,[1] but the question of sensitivity is still unclear and it is evident that the low degree of fragmentation may represent a net loss in structural information compared with electron ionization methods. Chemical derivatization to reduce polarity currently remains as the single most effective approach and in some cases permits the added advantage of gas chromatography. Acetyl,[2,3] phenylboronyl,[3] *O*-isopropylidene,[3] trimethylsilyl,[4] and trifluoroacetyl[5] blocking groups have been proposed for this purpose, but detailed studies have been reported only for the latter derivative.[6,7]

In an effort to explore new approaches to this prob-

(1) H. R. Schulten and H. D. Beckey, *Org. Mass Spectrom.*, 7, 861 (1973). We are indebted to the authors for a copy of their manuscript prior to publication.

(2) S. H. Eggers, S. I. Biedron, and A. O. Hawtrey, *Tetrahedron Lett.*, 3271 (1966).

(3) J. J. Dolhun and J. L. Wiebers, *Org. Mass Spectrom.*, 3, 669 (1970).

(4) J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger, and R. N. Stillwell, *J. Amer. Chem. Soc.*, **90**, 4182 (1968).

(5) W. A. Koenig, L. C. Smith, P. F. Crain, and J. A. McCloskey, *Biochemistry*, **10**, 3968 (1971).

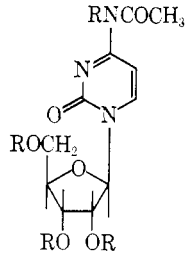
(6) Trimethylsilylation has also been used for mononucleotides, (a), A. M. Lawson, R. N. Stillwell, M. M. Tacker, K. Tsuboyama, and J. A. McCloskey, *J. Amer. Chem. Soc.*, **93**, 1014 (1971); and cyclonucleosides, (b) S. Tsuboyama and J. A. McCloskey, *J. Org. Chem.*, **37**, 166 (1972); J. B. Westmore, D. C. K. Lin, K. K. Ogilvie, H. Wayborn, and J. Berestiansky, *Org. Mass Spectrom.*, **6**, 1243 (1972).

(7) W. A. König, K. Zech, R. Uhmann, and W. Voelter, *Chem. Ber.*, **105**, 262 (1972).

lem, we have examined the mass spectra of *N,O*-permethyl derivatives of nucleosides, prepared by microgram scale reaction using methylsulfinyl carbanion and methyl iodide. This derivative was first surveyed by Dolhun and Wiebers, who employed silver oxide–methyl iodide as reactants.[3] Their report included the mass spectrum of *N,O*-permethyluridine and selected mass values characteristic of the common nucleosides, which did not, however, include ion abundance data.

Choice of the methylsulfinyl carbanion technique[8] was prompted by its success in the permethylation of peptides for sequencing by mass spectrometry.[9,10] The principal alkylating agent used in the present study is CD_3I, which permits clear distinction between native methyl groups and those added in the derivatization procedure. These derivatives possess the general advantages of low molecular weight,[11] high chemical stability over a period of time without special precautions, and sufficient volatility for gas chromatographic introduction of the sample.

This technique has recently been employed in the structure determination of N^4-acetylcytidine (**1**) (as the *N,O*-perethyl derivative **2**) which had been isolated



1, R = H
2, R = C_2H_5

from the first position of the anticodon of *E. coli* $tRNA^{Met}$.[12]

Products of Peralkylation

A typical gas chromatogram derived from permethylation of an equimolar mixture of the common nucleosides is shown in Figure 1. Numbers refer to structures to be discussed. In some cases small amounts of products which are of insufficient volatility to pass through the gas chromatograph may also be formed, but comparative spectra derived from direct probe introduction of the sample show that they are generally absent or of minor importance.

Because of the possibility of keto–enol and amino–imino tautomerism in the base, permethylation of nucleosides can conceivably produce a variety of isomeric derivatives, *e.g.*, the adenosine analogs **3** and **4**. Structures of the products obtained were determined by comparison of their mass spectra with those derived from nucleosides having methyl groups in known positions prior to permethylation. The product from permethylation of adenosine exhibits a spectrum indistinguishable from that derived from N^6,N^6-dimethyladenosine, indicating the structure to be that of the di-

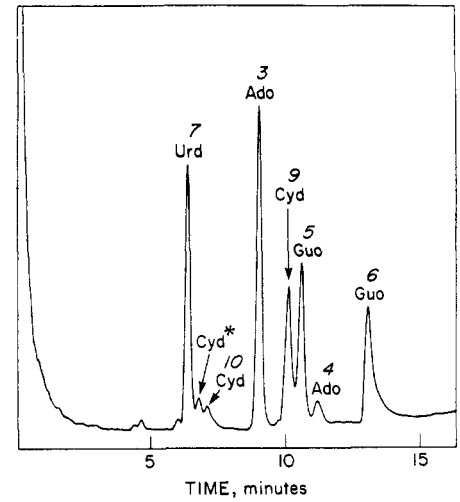
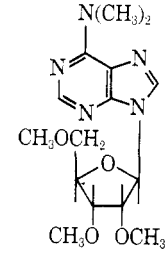
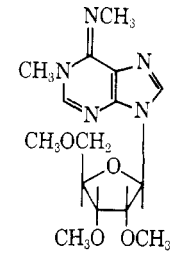


Figure 1. Gas chromatogram of permethylated equimolar mixture of uridine (Urd), adenosine (Ado), cytidine (Cyd), and guanosine (Guo). Cyd* = tetramethyl-Cyd.



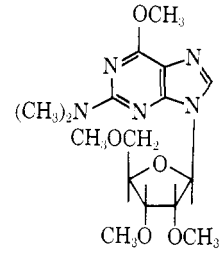
3



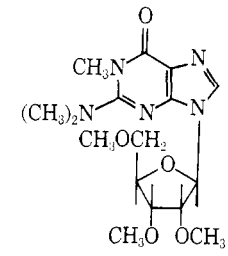
4

methylamino derivative **3**. In practice, the methyl-d_3 derivative is normally prepared (*e.g.*, Figures 2 and 3); therefore derivatives of adenosine and N^6,N^6-dimethyladenosine are readily distinguished. Compound **4**, the product obtained from permethylation of 1-methyladenosine, exhibits an ion intensity pattern which differs significantly from that of **3** (*cf.* Figures 2a and 4) and elutes later gas chromatographically. A small amount of **4** is often formed from permethylation of adenosine, as shown in Figure 1.

As seen in Figure 1, guanosine was found to yield two major products in approximately equal yield. Spectra of the methylation products of N^2,N^2-dimethylguanosine and 1-methylguanosine revealed the earliest eluted product to be **5** (Figure 2b) and the later product, **6** (Table I). Further confirmation of **6** as opposed to



5



6

the isomeric $1,N^2,O^6$-trimethyl structure was established by the loss of methylenimine from the base + H ion in the mass spectrum of **6**, a process highly characteristic of the dimethylamino structure[13,14] as will be discussed.

(8) S. I. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
(9) E. Vilkas and E. Lederer, *Tetrahedron Lett.*, 3089 (1968).
(10) K. Biemann, "Biochemical Applications of Mass Spectrometry," G. R. Waller, Ed., Wiley-Interscience, New York, N. Y., 1972, Chapter 15.
(11) For example, the molecular weight of the trimethylsilyl derivative of guanosine[4] is 643, and the hexa(methyl-d_3) derivative, 385.
(12) Z. Ohashi, K. Murao, T. Yahagi, D. L. von Minden, J. A. McCloskey, and S. Nishimura, *Biochim. Biophys. Acta*, **262**, 209 (1972).
(13) Y. Rahamim, J. Sharvit, A. Mandelbaum, and M. Sprecher, *J. Org. Chem.*, **32**, 3856 (1967).
(14) C. P. Whittle, *Tetrahedron Lett.*, 3689 (1968).

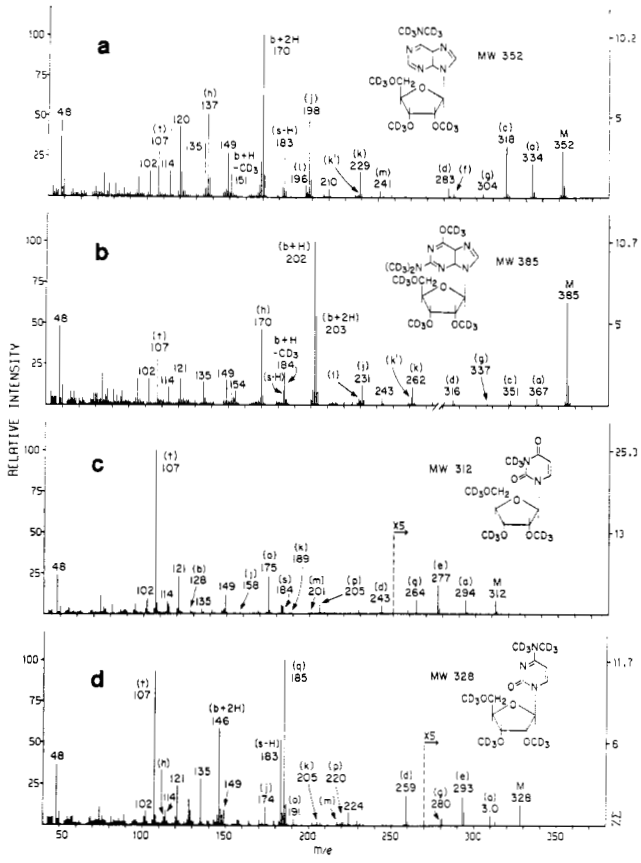


Figure 2. Mass spectrum of (a) N^6,N^6,O-2′,3′,5′-penta(methyl-d_3)adenosine; (b) N^2,N^2,O-6,2′,3′,5′-hexa(methyl-d_3)guanosine; (c) N^3,O-2′,3′,5′-tetra(methyl-d_3)uridine; (d) N^4,N^4-O-2′,3′,5′-penta(methyl-d_3)cytidine.

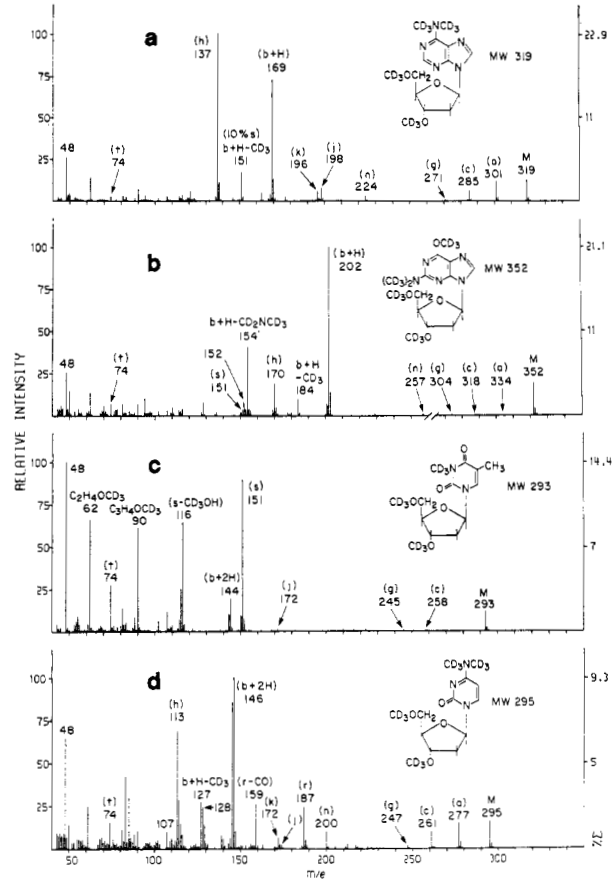
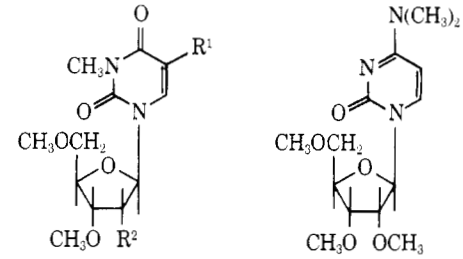


Figure 3. Mass spectrum of (a) N^6,N^6,O-3′,5′-tetra(methyl-d_3)-2′-deoxyadenosine; (b) N^2,N^2,O-6,3′,5′-penta(methyl-d_3)-2-deoxyguanosine; (c) N^3,O-3′,5′-tri(methyl-d_3)thymidine; (d) N^4,N^4-O-3′,5′-tetra(methyl-d_3)-2′-deoxycytidine.

These results suggest that the methylsulfinyl carbanion–methyl iodide reagent may hold promise for synthetic procedures, since conventional alkylation reactions normally involve only ring nitrogen atoms while amino groups are unaffected.[15]

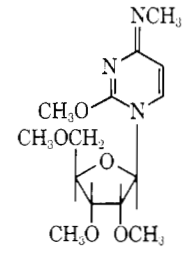
Permethylation of uridine and thymidine produced in each case single, gas chromatographically homogeneous products which were identical with those formed from 3-methyluridine and 3-methylthymidine, respectively. Their structures are therefore **7** and **8** rather than the remaining O^4-methyl possibility. Similar to guanosine, cytidine also yields two isomeric products, the principal and later eluting derivative having the dimethylamino structure **9** as required by the expulsion of methylenimine from the base + H in its mass spectrum (Figure 2d). Two other structural possibilities, **10** and **11**, fit the required molecular weights in which the base bears two methyl groups. The spectra of **9**, **10**, and **11** are substantially different, **11** being indistinguishable from that derived from 3-methylcytidine (Table I, CD_3 derivative). The minor early eluent produced from cytidine therefore has structure **10**, and is characterized by a molecular ion of exceptionally high abundance for a pyrimidine nucleoside (55%, Table I).

As noted in a recent communication,[16] reaction between the 5,6-dihydropyrimidine nucleosides (dihydrouridine, 2′-deoxydihydrouridine, dihydrothymidine) and methylsulfinyl methide–methyl iodide uniquely produces the corresponding 3,5,5-trimethyl analogs, *e.g.*, **12**.

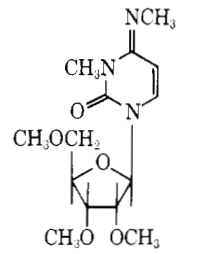


7, R^1 = H; R^2 = OCH_3
8, R^1 = CH_3; R^2 = H

9



10



11

Discussion of Mass Spectra

Fragmentation mechanisms and pathways were studied by deuterium (CH_3 *vs.* CD_3) and substituent labeling (CH_3 *vs.* C_2H_5) for every compound which was

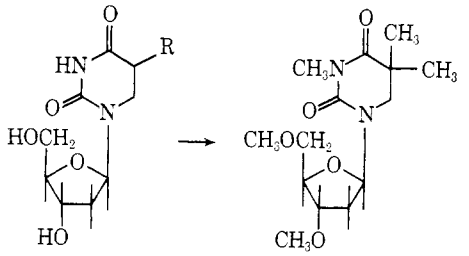
(15) J. H. Lister, "Fused Pyrimidines. Part II. Purines," D. J. Brown, Ed., Wiley-Interscience, New York, N. Y., 1971, p 313. See, however, ref 13, and references therein.

(16) D. L. von Minden, R. Panzica, L. B. Townsend, and J. A. McCloskey, *Biochim. Biophys. Acta*, in press.

Table I. Selected Ions from the Mass Spectra of Nucleoside *N,O*-Per(methyl-d_3) Derivatives

Parent nucleoside	No. of methyl-d_3 groups	*m/e* (Rel abundance[a]) M	k	j	Base + H	Base + 2H	s − H	Other ions
1-Methyladenosine	4	349 (100)	226 (2.5)	195 (1.3)	166 (90)	167 (17)	183 (25)	149 (46), 107 (36)
N^6-Methyladenosine	4	349 (26)	226 (15)	195 (48)	166 (22)	167 (100)	183 (27)	135 (62), 136 (30)
N^6,N^6-Dimethyladenosine	3	346 (26)	223 (15)	192 (46)	163 (20)	164 (100)	183 (27)	134 (58), 312 (29)
9-β-D-Arabinofuranosyladenine	5	352 (26)	229 (16)	198 (100)	169 (31)	170 (86)	183 (11)	137 (65), 318 (20)
2′-*O*-Methyladenosine	4	349 (20)	226 (14)	198 (47)	169 (34)	170 (100)	180 (30)	285 (4.5), 137 (61)
3′-*O*-Methyladenosine	4	349 (19)	229 (14)	198 (48)	169 (20)	170 (100)	180 (29)	286 (2.5), 137 (54)
4′-Thioadenosine	5	368 (24)	229 (4.5)	214 (48)	169 (17)	170 (100)	199 (28)	137 (25), 334 (16)
Guanosine[c]	6	385 (100)	262 (4.7)	231 (5.0)	202 (64)	203 (34)	183 (26)	154 (38), 170 (14)
1-Methylguanosine	5	382 (100)	259 (4.4)	228 (5.2)	199 (63)	200 (34)	183 (27)	107 (72), 135 (42)
N^2-Methylguanosine[d]	5	382 (62)	259 (11)	228 (13)	199 (100)	200 (54)	183 (8.2)	168 (18), 169 (18)
N^2,N^2-Dimethylguanosine	4	379 (62)	256 (11)	225 (11)	196 (100)	197 (54)	183 (7.4)	167 (42), 181 (18)
3-Methyluridine	3	309 (1.7)	186 (0.6)	155 (1.0)	126 (0.8)	127 (1.0)	183 (6.0)	107 (100), 172 (22)
1-β-D-Ribofuranosylthymine	4	326 (9.7)	203 (0.5)	172 (0.4)	143 (2.2)	144 (0.8)	183 (8.2)	107 (100), 189 (5.8)
Cytidine[e]	5	328 (55)	205 (2.0)	174 (2.0)	145 (42)	146 (5.5)	183 (30)	107 (100), 259 (8.0)
3-Methylcytidine	4	325 (5.5)	202 (1.1)	171 (1.0)	142 (1.4)	143 (0.7)	183 (10)	75 (100), 184 (56)
α-Cytidine	5	328 (1.5)	205 (2.5)	174 (3.9)	145 (6.0)	146 (46)	183 (12)	107 (67), 185 (100)
1-β-D-Arabinofuranosylcytosine	5	328 (1.0)	205 (2.5)	174 (7.0)	145 (4.5)	146 (38)	183 (14)	107 (88), 185 (100)
5-Methylcytidine	5	342 (4.0)	219 (2.6)	188 (11)	159 (9.0)	160 (68)	183 (90)	48 (100), 199 (64)
N^6-Methyl-2′-deoxyadenosine	3	316 (21)	193 (8.8)	195 (9.2)	166 (100)	167 (27)	150 (1.1)	135 (71), 136 (64)
1-Methyl-2′-deoxyadenosine	3	316 (18)	193 (1.2)	195 (0.2)	166 (100)	167 (13)	150 (1.2)	48 (37), 62 (27)
3′-Deoxyadenosine	4	319 (15)	229 (7.0)	198 (100)	169 (19)	170 (56)	150 (30)	74 (40), 137 (52)
5′-Deoxyadenosine	4	319 (17)	229 (4.5)	198 (18)	169 (24)	170 (100)	150 (46)	116 (49), 137 (60)
5-Methyl-2′-deoxycytidine	4	309 (14)	186 (4.5)	188 (2.0)	159 (100)	169 (46)	150 (2.0)	48 (64), 201 (16)
Adenosine	5[b]	407 (26)	262 (14)	220 (86)	191 (58)	192 (100)	216 (29)	148 (69), 378 (72)

[a] Uncorrected for isotopic contributions from neighboring peaks. [b] *N,O*-perethyl derivative. [c] Later eluting derivative corresponding to **6**; see Figure 1. [d] Earlier eluting derivative. [e] Structure corressponds to **10**; see Figure 1.



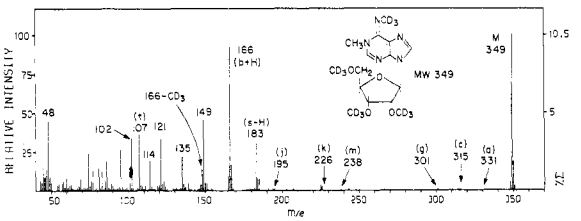
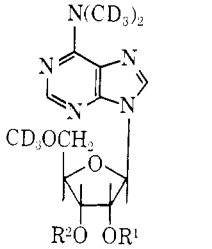
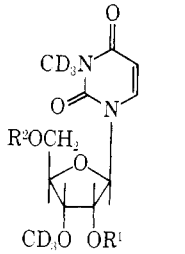


Figure 4. Mass spectrum of N^6,O-2′,3′,5′-tetra(methyl-d_3)-1-methyladenosine.

examined. The latter derivatives were found to follow the same reaction paths as CH_3 or CD_3 derivatives[17] with only minor additional processes superimposed on a similar fragmentation pattern (*e.g.*, the *N,O*-perethyl derivative of adenosine, Table I). Measurement of exact mass, normal and defocussed[18,19] metastable peaks, and ^{18}O-labeling at O-2′ and -4′ in **3** were also employed. Exhaustive labeling of sugar methoxyl groups in adenosine and uridine, **13–16**, prepared from partially labeled intermediates, was used to assess the reactive behavior of individual methoxyl groups in the sugar.





Mass spectra of the *N,O*-permethyl derivatives of the major nucleosides from RNA and DNA are given in Figures 2 and 3.

Ions Closely Related to the Molecular Ion. Molecular ion stabilities were found to vary widely, with the greatest exhibited by the purine nucleosides, in analogy to the behavior of the free compounds and other derivatives.[20] Exceptional stability was observed in spectra of permethylated 1-methyladenosine (Figure 4) and 1-methylguanosine, in which molecular ions were represented as base peaks. Minimum values (<1% rel intensity) were exhibited by derivatives of the 5,6-dihydropyrimidine system, *e.g.*, **12**.

Unambiguous identification of the molecular ion (M) is important because in a system of partially restricted molecular structures the overall structure can be largely defined by molecular mass and composition. This is facilitated in the present case by the usual occurrence of closely related ions resulting from loss of a methyl or methoxyl radical from the sugar moiety (ions a and c), with elimination of methanol from the latter (ion d) or

(17) Unless indicated otherwise the term "methyl" is taken hereafter to include methyl-d_3.

(18) M. Barber and R. M. Elliott, 12th Annual Conference on Mass Spectrometry and Allied Topics, Dallas, Texas, June 1964, p 150.

(19) J. H. Futrell, K. R. Ryan, and L. W. Sieck, *J. Chem. Phys.*, **43**, 1832 (1965).

(20) J. A. McCloskey in "Basic Principles in Nucleic Acid Chemistry," Vol. I, P. O. P. Ts'o, Ed., Academic Press, New York, N. Y., in press.

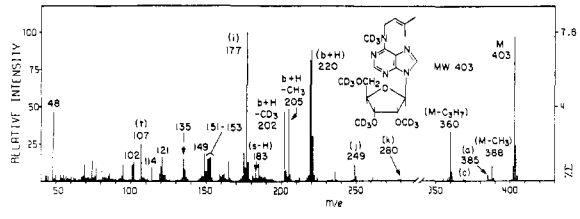
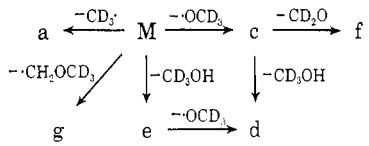
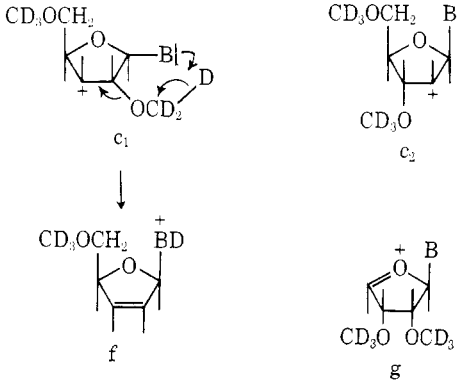


Figure 5. Mass spectrum of N^6,*O*-2′,3′,5′-tetra(methyl-d_3)-N^6-(3-methyl-2-butenyl)adenosine.



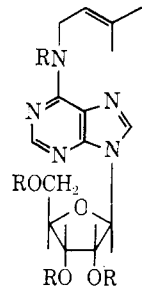
molecular ions (e). The pathway to ion e occurs mainly with pyrimidine nucleosides, while ion c predominates in spectra of purine derivatives. The spectrum of the cytidine (CD_3) derivative **9** shows evidence for expulsion of a second molecule of CD_3OH from d to yield *m/e* 224 (m*, *m/e* 193.8). Comparison of the normal derivatives of adenosine and uridine with the selectively labeled models **13**–**16** shows that production of ions c and e, respectively, involves mainly C-2′ and -3′ rather than C-5′. In permethyladenosine only 9% of ion c is derived from C-5′ in keeping with the reduced stability of primary (5′) *vs.* secondary carbonium ions (C-2′, C-3′).

In the purine series further decomposition of ion c occurs by expulsion of formaldehyde to afford ion f, shown by selective labeling in **13** to occur exclusively from species c_1. This great specificity shown by two otherwise similar precursors is interpreted in terms of direct interaction between the O-2′ alkyl function and electron-rich base, initiated by the charge on C-3′ (c_1 → f). Steric feasibility of O-2′–base interactions can



be readily demonstrated by rotation of the base about the glycosidic bond using space-filling CPK[21] models. Evidence for similar interactions is available in the electron ionization spectra of free nucleosides.[22] Simple cleavage of the C-4′,5′ bond leads to ion g, which gains stability from the unshared electrons of O-4′ and occurs in every case examined with the required exception of permethylated 5′-deoxyadenosine.[23]

Some forms of structural modification lead to reactions which appear in the molecular ion series, although in general such reactions are much more prevalent in decomposition of the base + H ions (following section). The spectrum of the N^6,*O*-2′,3′,5′-tetramethyl derivative (**17**) of N^6-(3-methyl-2-butenyl)adenosine shown in Figure 5 provides an example in which the upper mass range is dominated by ions associated with the isopentenyl group at N^6. Loss of CH_3, *m/e* 388 (clearly distinguished from ion a), and of C_3H_7, *m/e* 360, are produced by rearrangement processes which are highly characteristic of the isopentenyl function.[24,25]



17, R = CD_3
20, R = H

Analogous reactions occur from the base + H ion species, as discussed in the following section.

Fragment Ions of the Base Series. The major and most important decomposition process in nearly all free and derivatized nucleosides which possess a normal glycosidic bond involves rupture of the glycosidic bond with concomitant transfer of one or two hydrogens from the sugar to the base.[26] Of the three ion species which can result, base, base + H, and base + 2H, in the present study the latter two usually predominate as major ions in the spectrum, although in the case of some pyrimidine nucleosides they are of somewhat reduced abundances (see Table I). The base + H species is formally equivalent to the molecular ion of the corresponding free base, and will in general follow the same fragmentation reactions.[20] In the mass spectra of free nucleosides the rearranged hydrogens are derived preferentially from sugar hydroxyl groups,[26] while in trifluoroacetyl[5] and trimethylsilyl nucleoside derivatives[4] they originate in the ribose or deoxyribose skeleton. Results from several representative compounds from the present study are given in Table II.

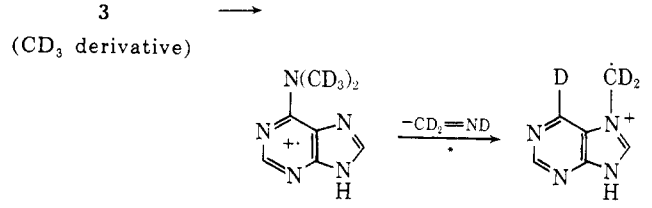
Table II. Protium Contribution to Ions of Type Base + H and Base + 2H from Methyl-d_3 Derivatives

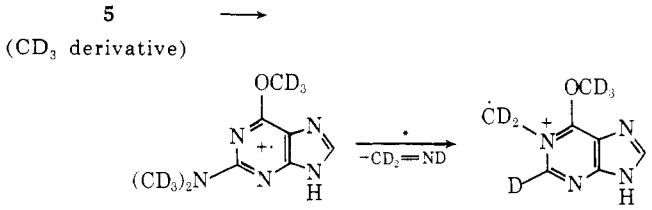
Parent nucleoside	Base + H, %	Base + 2H, %
Adenosine	72	95
2′-*O*-Methyladenosine	100	97
3′-*O*-Methyladenosine	70	93
2′-Deoxyadenosine	97	91
3′-Deoxyadenosine	69	98
5′-Deoxyadenosine	73	98
Guanosine	98	94
Thymidine	100	100

(21) W. L. Koltun, *Biopolymers*, 3, 665 (1965).
(22) S. J. Shaw, D. M. Desiderio, K. Tsuboyama, and J. A. McCloskey, *J. Amer. Chem. Soc.*, 92, 2510 (1970).
(23) Loss of CH_3 from the CD_3 derivative of 5′-deoxyadenosine would produce an ion g of the same structure as from normal ribonucleosides, and is likewise absent.
(24) R. H. Hall, M. J. Robins, L. Stasiuk, and R. Thedford, *J. Amer. Chem. Soc.*, 88, 2614 (1966).
(25) S. M. Hecht, N. J. Leonard, J. Occolowitz, W. J. Burrows, D. J. Armstrong, F. Skoog, R. M. Bock, I. Gillam, and G. M. Tener, *Biochem. Biophys. Res. Commun.*, 35, 383 (1969).
(26) K. Biemann and J. A. McCloskey, *J. Amer. Chem. Soc.*, 84, 2005 (1962).

Assuming the absence of unusually large isotope effects, the ribose or deoxyribose skeleton is shown to be the greatly preferred site for hydrogen isotope abstraction in spite of the numerically greater availability of methyl deuterium atoms. These results must in part reflect the greater ease of abstraction of tertiary *vs.* primary hydrogen atoms. The question as to which skeletal hydrogens are involved, and therefore whether initial opening of the ring is sterically required, is answered with less certainty. However, the similarity of labeling patterns of base + H in Table II for derivatives of adenosine and 3′- and 5′-deoxyadenosine but not for 2′-deoxyadenosine implies involvement of the sterically accessible 2′ region. Further, comparison of base + H data from the two partially labeled (O-2′ *vs.* O-3′) adenosines **13** and **14** shows that the deuterium present in the base + H-type ion from adenosine is derived exclusively from the O-2′ methyl group. This result, in addition to the previous discussion regarding the mechanism of formation of ion f, further confirms the important role played by base–2′ interactions in the mass spectra of nucleosides.

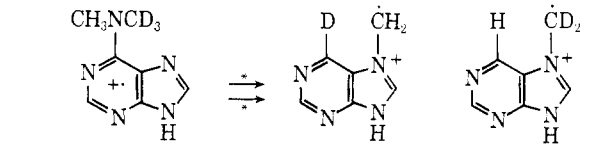
Significant fragmentation of base + H occurs when the derivatized base contains a dialkylamino function. Loss of CD_2NCD_3 or $N(CD_3)_2$ occurs in some instances (*e.g.*, *m/e* 154, 152 in Figures 2a and 3b), but a major process involves expulsion of methylenimine (ion h) usually marked by an appropriate metastable peak. This reaction was first documented by Rahamim and his collaborators[13] and was shown to be generally characteristic of alkylamino heteroaromatic compounds.[14] Mass spectra of permethylated adenosine and guanosine (Figure 2a and b) and their 2′-deoxy counterparts (Figure 3a and b) exhibit major peaks from elimination of methylenimine to form ion h.



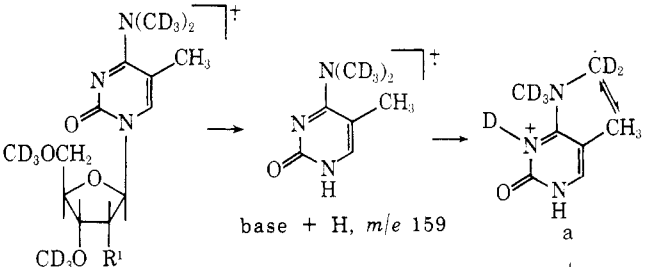


This process is of minor importance in the spectrum of permethylated cytidine (*m/e* 113, Figure 2d) evidently because the base + H precursor is formed in low abundance, while the reverse is true for 2′-deoxycytidine (*m/e* 113, 145, Figure 3d).

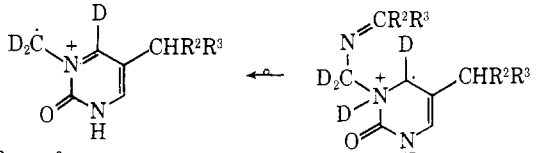
Ion h provides a useful means of detecting amino methylation in the native nucleoside, both by the shifted mass of base + H and the deuterium content of methylenimine which is expelled. Thus base + H from N^6,N^6-dimethyladenosine or N^2,N^2-dimethylguanosine derivatives expels CH_2=NH, 29 mass units (see Table I). However, monomethylation in the starting nucleoside (*e.g.*, N^6-methyladenosine, N^2-methylguanosine) does not produce a split pattern of base + H − CH_2NH and base + H − CD_2ND for ion h. Contrary to previous mechanistic postulates[13,14] recent studies employing deuterium labeling in a number of models have shown that the expulsion reaction involves a net exchange of methyl hydrogens,[27] leading to a characteristic loss of CD_2NH (31 amu) and CH_2ND (30 amu) in a ratio of ~1:1. Models derived from 1- and 7-deazaadenosine showed that N-7 was the preferred but not sole site of rearrangement.[27]



As a consequence of this mechanism, hydrogen from other sterically available methyl groups can in principle participate in the formation of ion h. Derivatives of 5-methylcytidine (**18**) and 5-methyl-2′-deoxycytidine (**19**) were found to produce an analogous split pattern



18, $R^1 = OCD_3$
19, $R^1 = H$



$R^2 = R^3 = H$, *m/e* 127
$R^2 = H$, $R^3 = D$, *m/e* 128
$R^2 = R^3 = D$, *m/e* 129

resulting from losses of CH_2ND, CD_2ND, and CD_2NH from base + H. These observations can be accounted for in terms of our earlier mechanistic rationale,[27] by assuming hydrogen–deuterium exchange in intermediate a. The following abundance ratios were observed for *m/e* 127, 128, 129: **18**, 1:2:2 (with some interference from other unresolved peaks); **19**, 2:2:1. A similar pattern is produced by the methyl-d_3 derivative of 5-methylcytosine, 5:4:1.

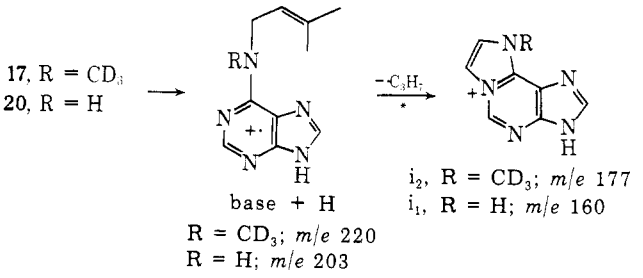
Another common mode of base + H fragmentation involves loss of a CD_3 radical from dimethylamino functions.[13] Protium labeling confirms that *O*-methyl or 1-methyl groups are not preferred.

Some of the most successful applications of mass spectrometry to structural problems of nucleosides have been the work of Leonard and his coworkers[28] in the characterization of adenosine analogs which exhibit cytokinin activity, *e.g.*, the central model **20**.

(27) J. G. Liehr, D. L. von Minden, M. H. Wilson, and J. A. McCloskey, 21st Annual Conference on Mass Spectrometry and Allied Topics, San Francisco, Calif., June 1973; submitted for publication.

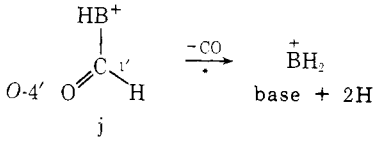
(28) For example, H. J. Vreman, F. Skoog, C. R. Frihart, and N. J. Leonard, *Plant Physiol.*, **49**, 848 (1972); *cf.* ref 16.

The major diagnostic peaks in these spectra represent the expulsion of C_3H_7 from base + H, and to a lesser extent from the molecular ion, usually represented[29] as the cyclized ion i_1. A similar process in the mass spectrum of the permethyl derivative **17** results in the



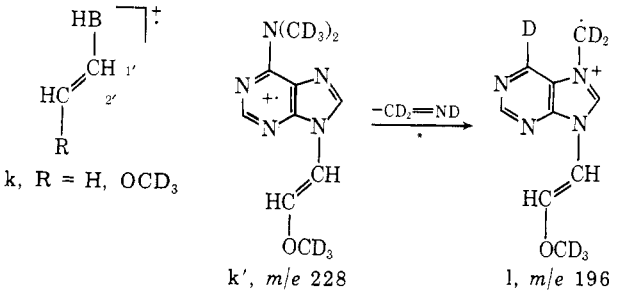
most abundant fragment ion in the spectrum (*m/e* 177), as seen in Figure 5. Loss of a CH_3 radical from base + H (forming *m/e* 205) or M (forming *m/e* 388) also reflects the characteristic behavior of the free compound.[24,25] Loss of the entire side chain (base + H − C_5H_9) also with rearrangement of one hydrogen to the base (base + H − C_5H_8) gives two peaks (*m/e* 148, 149; 2:1) from the CH_3 analog of **17**, but which splits into three peaks in Figure 4 (*m/e* 151–153) showing the existence of a somewhat complex reaction path. Although the mechanistic details of the formation of ion i and other ions related to the isopentenyl side chain have to date not been firmly established by isotopic labeling, their occurrence is clearly useful for purposes of structural characterization.

In general, the stable aromatic character of the base is resistant toward further fragmentation, with the result that most other ions of the base series consist of the intact base plus various portions of the *O*-2′,3′,5′-trimethylribosyl moiety. The principal ions which are formed are in some cases structurally analogous to those formed from free nucleosides or other derivatives, although isotopic labeling reveals that the sugar skeleton is invariably the source of rearranged hydrogen in contrast to free nucleosides, in which hydroxyl hydrogens are preferred. Thus, ion j, the "base + 30" species, was often observed as a major ion in the present study, and in analogy to other systems[4,5,22] decomposes to base + 2H by expulsion of CO.



Its composition was confirmed by ^{18}O labeling in **3** and by the required shift of 16 mass units in the spectrum of **3** (CD_3 derivative) *vs.* the derivative of 4′-thioadenosine (Table I). The structure of j as shown above, in which hydrogen has been transferred to the charge-localized base, is based on previous arguments pertaining to free nucleosides.[22]

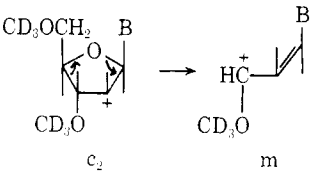
Likewise, inclusion of C-1′ and -2′ plus skeletal hydrogen from the remainder of the molecule is represented by ion k, a useful indicator of 2′-O-methylation in the native nucleoside. Those molecules which contain a dimethylamino function were usually observed to undergo elimination of methylenimine as a minor



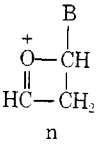
process from a species of ion k having one less skeletal hydrogen (k′ → l). The odd-electron ion k′ therefore behaves similarly to the odd-electron base + H decomposition to ion h, previously discussed. Alternatively, k′ may expel a methyl radical to yield a small peak which can serve to further confirm the presence of a dimethylamino function (*e.g.*, *m/e* 243, Figure 2b).

Metastable defocussing experiments showed both ions j and k to be among the precursors of base + 2H. Since these ions bear rearranged skeletal hydrogens, this accounts in large part for the near-absence of methyl hydrogen (deuterium) in base + 2H ions as reported in Table II.

A new pathway which does not operate in the spectra of free nucleosides or other derivatives produces from most ribonucleoside derivatives a fragment corresponding in composition to base + $C_2H_3OCD_3$ (ion m).



The specifically labeled adenosine derivative **14** reveals the alkoxyl group to be exclusively that from C-3′. A reasonable mechanism can be envisioned using ion c_2 as a precursor, generating a well-stabilized m. This hypothesis is supported by the absence of ion m in the mass spectra of 2′-deoxyribonucleoside derivatives, which do not produce ion c_2. Deoxyribonucleoside derivatives produce an ion of analogous composition, base + C_3H_4O (ion n), observed in Figures 3a, b, and

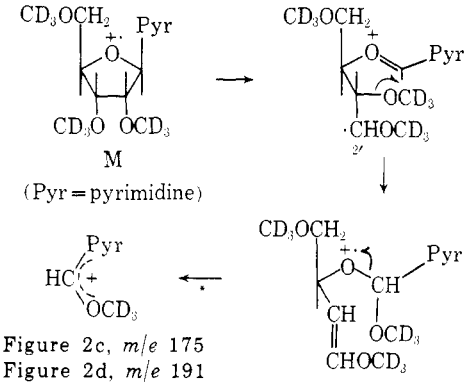


d, but not Figure 3c. This ion undoubtedly contains the first three carbons of the sugar skeleton and is therefore isomeric with the structure shown, but the origin of the heteroatom is presently unknown.

Abundances of base-containing ions from pyrimidine nucleosides are generally lower than their purine counterparts, a fact which has in effect been attributed to relatively lower charge localization in the pyrimidine nucleus,[26] resulting in fewer reactions initiated and controlled by the base. These effects are clearly influential in the present study, one example of which is ion o, unique to the pyrimidine robonucleosides (Figures 2c, d and 6). Measurement of exact mass showed the general composition base + C_2HD_3O, while the

(29) For example: (a) D. S. Letham, J. S. Shannon, and I. R. McDonald, *Proc. Chem. Soc.*, 230 (1964); (b) W. J. Burrows, D. J. Armstrong, F. Skoog, S. M. Hecht, J. T. A. Boyle, N. J. Leonard, and J. Occolowitz, *Science*, **161**, 691 (1968); (c) S. M. Hecht, *Biochim. Biophys. Acta*, **213**, 269 (1970).

spectra of uridine derivatives having ^{18}O labels at O-2′ and O-4′ indicated the absence of the O-2′ and 4′ heteroatoms. Likewise, uridine derivatives selectively deuterated in the methyl groups at O-2′ and O-5′ showed the absence of those groups, with the resulting conclusion that formation of ion o involves the selective migration of a methoxyl group from C-3′.



o, Figure 2c, *m/e* 175
Figure 2d, *m/e* 191

We propose the mechanism shown which is initiated by the positive charge at O-4′ followed by collapse of the 1′,2′ bond. The even-electron species o gains considerable stability through charge delocalization. This mechanism has direct analogy in the electron impact induced methoxyl migrations of dimethoxycyloalkanes studied by Winkler and Grützmacher, in particular 1,3-dimethoxycyclopropane.[30]

Another ion common to pyrimidine ribonucleoside derivatives (ion p) bears the ring oxygen and first two

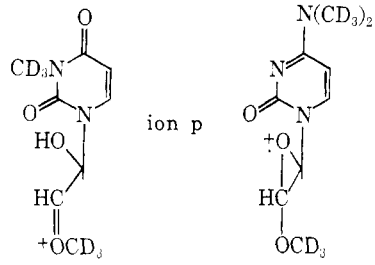


Figure 2c, *m/e* 205 Figure 2d, *m/e* 220

carbons of the sugar, as demonstrated by protium labeling (O-2′), ^{18}O labeling (O-2′ plus O-4′), and measurements of exact mass. Its formation can conveniently be considered in terms of charge localization in the sugar. Ion p generally occurs with rearranged skeletal hydrogen in derivatives of uridine and without rearrangement in derivatives of cytidine. By contrast, an analogous ion containing one additional hydrogen is found in the mass spectra of trimethylsilylated nucleosides[4] but which is not restricted to pyrimidines, evidently due to charge localization in the base.

Nucleosides of cytosine, both free[31,32] and derivatized,[5,33] often exhibit major fragment ions corresponding to base + C_2HO[20] (ion q, ribonucleosides) or base + C_2H_3O[20] (ion r, 2′-deoxyribonucleosides). The latter ion is a precursor for expulsion of CO (*m/e* 159, Figure

(30) J. Winkler and H.-R. Grützmacher, *Org. Mass Spectrom.*, **3**, 1117 (1970).
(31) P. Brown, G. R. Pettit, and R. K. Robins, *ibid.*, **2**, 521 (1969).
(32) H. A. Howlett, M. W. Johnson, A. R. Trim, J. Eagles, and R. Self, *Anal. Biochem.*, **39**, 429 (1971).
(33) D. L. von Minden, J. G. Liehr, S. E. Hattox, and J. A. McCloskey, unpublished results.

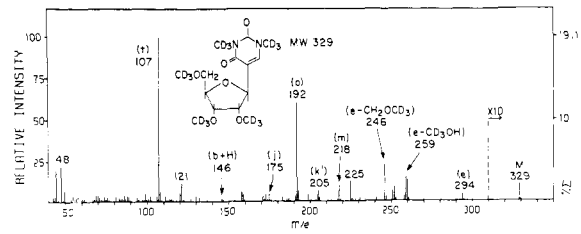
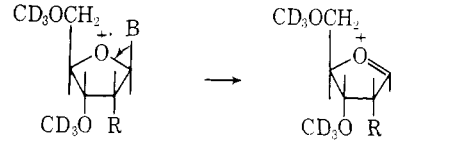


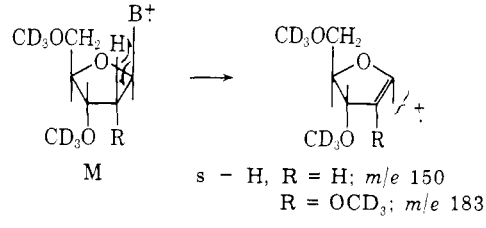
Figure 6. Mass spectrum of1, 3,*O*-2′,3′,5′-penta(methyl-d_3)-pseudouridine.

3d). Metastable defocussing experiments showed that ion q is derived from ions M, e, and d. Further evidence concerning the structures and modes of formation of these highly characteristic ions must await more detailed studies of isotopically labeled models, which are currently in progress in this laboratory.

Fragment Ions of the Sugar Series. The abundances of ions derived exclusively from the ribose or deoxyribose moiety were found to be greater from the pyrimidine nucleosides, in keeping with the previously discussed concept of greater charge localization in the sugar compared with the purine derivatives. Simple cleavage of the glycosidic bond produces the sugar fragment (ion s), which is usually accompanied by a



companion ion having one less hydrogen (s − H). Ion s generally predominates in spectra of pyrimidine nucleosides while s − H is more common in spectra of the purine compounds. These observations are in support of a mechanism of formation of s − H in which the charged base plays an important role, perhaps by inducing elimination of a neutral base molecule.



Participation of H-2′ in this reaction is supported by two factors. (1) Space-filling models show that the base and H-2′ can easily come within bonding distance by rotation of the glycosidic bond in the intact molecular ion, and in some molecules their proximity is so close that it serves to impede free rotation of the base. The abundance of s − H from permethylated adenosine is 27% (2.6%Σ), *vs.* 10% (1%Σ) from the derivative of 9-β-D-arabinofuranosyladenine, reflecting decreased steric accesibility of H-2′. (2) The abundance of s − H is low in the spectra of 2′-deoxynucleoside derivatives, as seen in Table I. In particular, comparison of the substantial differences in s − H abundance between 2′-deoxyadenosine (1% rel intensity) and its 3′- and 5′-deoxy isomers (30% and 46%) shows the marked influence of oxygen at C-2′, an effect best interpreted in

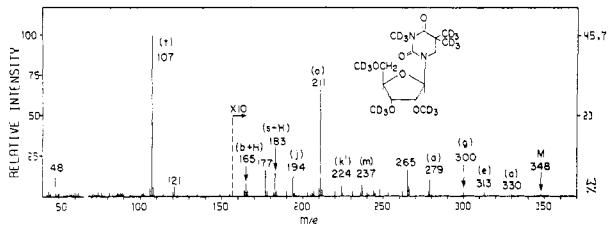
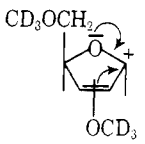


Figure 7. Mass spectrum of 3,5,5,*O*-2′,3′,5′-hexa(methyl-d_3)dihydrouridine.

terms of stabilization of the ionized double bond in s − H following specific removal of H-2′.

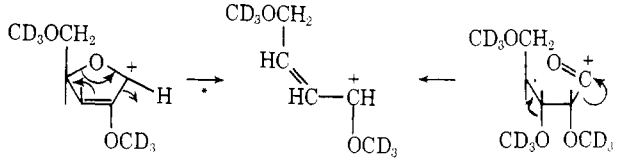
Charge stabilization also appears to play a major role in the formation of *m/e* 149, which corresponds in composition to s − CD_3OH. Comparison of labeling patterns in **3**, **13**, and **14** shows the missing methoxyl group to originate about equally from C-2′ and C-3′: 50% from C-2′, 42% C-3′, and 8% C-5′. Structures



m/e 149

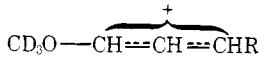
therefore predominate in which a formal charge at C-1′ is delocalized by a 2′,3′ double bond and in addition by an unshared pair from O-4′.

Metastable defocussing experiments show *m/e* 149 to be the precursor by loss of CO to *m/e* 121. The principle species of this ion containing one hydrogen less characteristically occurs at *m/e* 120 in derivatives of adenosine (Figure 2a). Isotopic labeling shows that *m/e* 121 is predominantly missing the 3′-alkoxyl function and is therefore derived from that population of *m/e* 149 in which the 3′-methoxyl is absent. The *m/e* 121 species thus formed gains stability from charge delocalization. However, since the identity of missing hydrogen in s − H is not known with certainty, alternative pathways leading to essentially the same product cannot be completely discounted; for example, the s − H → *m/e* 121 process depicted below.

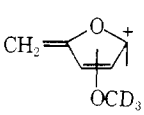


m/e 149 *m/e* 121 s − H

Several other major sugar ion products are often observed in mass spectra of permethylated nucleosides, some in great abundance. Ion t bears three carbons



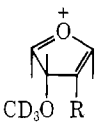
t, R = H; *m/e* 74
R = OCD_3; *m/e* 107



m/e 114

from the sugar skeleton, and is a sensitive indicator of the position of missing oxygen in deoxyribonucleoside derivatives. Its abundance is diminished in 2′-deoxy compounds (*e.g.*, Figure 3a) but is greater in the isomeric 3′- and 5′-deoxyadenosine spectra (see Table I). Specific labeling shows that in the case of adenosine the 3′-alkoxyl function is always present.

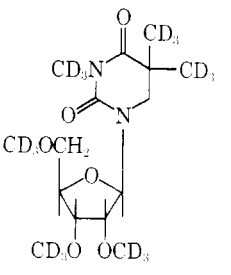
Spectra of ribonucleoside derivatives usually exhibit a peak of *m/e* 114 corresponding to the general structure shown above and is analogous to the abundant *m/e* 169 ion from trimethylsilylated nucleosides[4] and nucleotides.[6a] Isotopically labeled uridine and adenosine derivatives support the well-stabilized structure shown, with a distribution of the OCD_3 moiety at C-2′ being ~35% in uridine and 65% in adenosine. Most spectra of ribonucleoside derivatives exhibit a peak at *m/e* 135 corresponding in structure to a monomethyl ion without C-5′. Metastable peak evidence shows it to be derived from ion s − H, by cleavage of C-4′,5′. In addition, both nucleoside, and to a lesser extent 2′-deoxynucleoside derivatives, produce a peak at *m/e* 102. The composition and labeling pattern in nucleoside spectra indicate that the methoxyl group of *m/e* 102 is derived mainly from the C-3′ position. Simple cleavage of the C-4′,5′ bond produces *m/e* 48 in all compounds which were examined, with the required exception of the derivative of 5′-deoxyadenosine.



m/e 102

Derivatives of Dihydrouridine (21) and Pseudouridine (22). Both dihydrouridine and pseudouridine contain structural modifications at sites which are normally influential in governing fragmentation reactions. Their spectra were therefore examined to determine the precise effects of ring saturation (**21**) and alteration in the strength and nature of the glycosidic bond (**22**).

The previously noted trend toward effects of enhanced charge localization in the sugar, which occurs in pyrimidine nucleosides having less aromaticity and electron density, reaches an extreme in the case of **21**



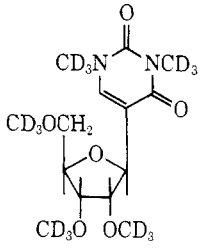
21

(Figure 7). Fragmentation of the base and its lowered ability to initiate reactions by abstraction of hydrogen from the sugar result in the decreased abundance of the principal base-containing ions o and p (*cf.* Figure 2c). Figure 7 is dominated by the sugar ion t more than any other nucleoside which was examined. Similar effects were observed in the spectra of **12** and permethylated dihydrothymidine.[16] A similar trend can be noted in comparing the spectra of free uridine[26] and dihydrouridine[34] and can be attributed to the same factors. These features, and the very low molecular ion abundances, make these spectra less useful for purposes of structural characterization, although as

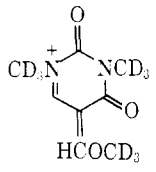
(34) S. M. Hecht, A. S. Gupta, and N. J. Leonard, *Anal. Biochem.*, 30, 249 (1969).

recently demonstrated in the case of **7**, the molecular mass of dihydropyrimidine derivatives can be readily established using chemical ionization techniques.[27]

The permethylated derivative of pseudouridine can be readily distinguished from uridine by differences in molecular mass resulting from differing numbers of methylation sites in the base (2 *vs.* 1). Structure **22** is



22



o, *m/e* 192

assigned in analogy to the *N*-methyl structure of **7**, but O-methylation in the base cannot be excluded with the presently available evidence. Some major features of the spectrum are similar to that of uridine (Figure 2c), such as abundant ions o and t. However, the principal differences arise from the well-known resistance of the C–C glycosidic bond toward electron impact induced cleavage in C-nucleosides.[34-36] The influence of this effect on the abundances of base + H and base + 2H in the present study (**7** *vs.* **22**) is less pronounced than in the mass spectra of the free compounds[26,34-36] because these ions are not prominent in the spectra of permethylated uridine (Figure 2c). Higher glycosidic bond stability is more evident in the decreased abundance or absence of sugar ions which are closely related to or directly derived from ions s or s − H, namely *m/e* 149, 102, and 114 and s or s − H (*m/e* 184, 183), as shown by comparison of Figures 2c and 6. This correlation is not followed by *m/e* 121, possibly reflecting its formation by a different pathway since its precursors (s − H and t, previously discussed) are not present.

Influence of Hydroxyl Orientation in the Sugar. Limited conclusions regarding the effects from changes in sugar hydroxyl orientation can be made from the arabinosyl analogs of adenosine and cytidine, and the α anomer of cytidine. Comparison of ion abundance data in Table I with those from the corresponding normal ribonucleosides shows that significant and sometimes great differences in abundance permit isomers to be readily distinguished, although differences in mass are not observed. Though correlation between abundance of ion s − H and steric accessibility of H-2′ to the base can be made as previously discussed, the direct assignment of hydroxyl orientation cannot in general be established from the mass spectrum. The same conclusion was reached in earlier studies of free nucleosides[22] and trifluoroacetyl derivatives.[7,37]

Comparison with Other Derivatives. Long-term chemical stability of permethylated nucleosides, in particular to traces of moisture, provides a favorable and sharp contrast to the more labile trimethylsilyl derivatives.[4] Although the permethylation procedure is relatively simple and works well on a microgram scale, it is somewhat more cumbersome than silylation[4] or trifluoroacetylation[5] because of the necessity to prepare and store a stock solution of methylsulfinyl carbanion reagent. In our experience the reagent remains reactive for 1–2 months when kept below 0°. The general tendency to form derivative homologs having fewer or greater numbers of added groups is substantially less than in the case of trimethylsilyl derivatives but in some cases (*e.g.*, **5**,**6**) isomeric derivatives of the same molecular weight are formed. In those cases the mass numbers of base-containing ions are unchanged but the gas chromatographic pattern will show additional peaks because the isomers generally elute at different temperatures.

Unlike most other derivatives, the permethylation reaction was found in many cases to produce sufficient trace impurities which result in small peaks at nearly every mass number when sample introduction by direct probe was used. Gas chromatographic sample introduction is therefore desirable in most instances, particularly when upper mass range peaks of low absolute intensity are involved. The chromatographic characteristics exhibited in Figure 1 are reasonably good, but less so than the analogous trimethylsilyl derivatives[39] studied in this laboratory.[40] Minor chromatographic peaks such as those on either side of **7** are of variable intensity and are not always observed. The proportion of **10** was found to increase when very short (2 mins) carbanion reaction times were employed. Overall yield in terms of peak area (flame ionization detector) show the purine nucleosides to be equal (1.0) and greater than the pyrimidine nucleosides (0.66) which were also approximately equal.

An interesting consequence of permethyl derivatives is that nucleosides bearing native methyl groups (CH_3) will elute at essentially the same position as the "unmethylated" nucleoside, *e.g.*, CD_3 derivatives of N^6-methyladenosine (mol wt 349) and adenosine (mol wt 352). When working with hydrolysates of oligonucleotides or RNA, either of which could commonly be expected to contain both the methylated (CH_3) and "unmethylated" nucleoside, clean mass spectra of each individual isomer cannot be obtained. However, as a compensating factor, the exact time of elution of the methyl (CH_3) component in the mixture can be accurately defined since it cochromatographs with the major unmodified nucleoside. A mass spectrum which is acquired from such a gas chromatographic peak will therefore consist of superimposed contributions from each component, with some peaks offset 3 mass units, reflecting the CH_3 − CD_3 structural difference. An example of this effect is given in Table III, which shows the mass shifts of five diagnostic peaks of three methylated analogs relative to adenosine. The number of CH_3 groups in the native nucleoside can be established from M, and their exact location from shifts of fragment ion peaks. Since deuterium isotope effects are relatively small, relative ion intensities in the example shown in Table III can be predicted from the spectrum of adenosine (Figure 2a) by summing the intensity contributions to each mass value.

(35) L. B. Townsend and R. K. Robins, *J. Heterocycl. Chem.*, **6**, 459 (1969).

(36) J. M. Rice and G. O. Dudek, *Biochem. Biophys. Res. Commun.*, **35**, 383 (1969).

(37) However, in the mass spectra of free nucleosides the orientation of C-5′ with respect to the base produces a systematic and predictable influence on the relative abundance of the M − CH_2O ion.[22,38]

(38) K. L. Nagpal and J. P. Horwitz, *J. Org. Chem.*, **36**, 3743 (1971).

(39) For example: (a) Y. Sasaki and T. Hashizume, *Anal. Biochem.*, **16**, 1 (1966); (b) W. C. Butts, *ibid.*, **46**, 187 (1972).

(40) S. E. Hattox and J. A. McCloskey, unpublished results.

Table III. Mass Shifts Relative to *N*,*O*-Per(methyl-d_3)adenosine

Parent nucleoside	Ion M	c	k	Base + H	s − H
N^6-Methyladenosine	−3	−3	−3	−3	0
N^6,N^6-Dimethyladenosine	−6	−6	−6	−6	0
2′-*O*-Methyladenosine	−3	0, −3	−3	0	−3

Table IV. Comparison of Nucleoside Derivatives

Parent nucleoside	Derivative —$SiMe_3$[a,b]— % Σ	Mol wt	—$OCOCF_3$[c]— % Σ	Mol wt	—CD_3— % Σ	Mol wt
Adenosine	22	555	38	651	34	352
Guanosine	16	643	30	667	41	385
Uridine	10	460	12	532	12	312
Pseudouridine	12	604	27	628	27	329
Cytidine	23	531	32	628	25	328
2′-Deoxyadenosine	36	467	31	515	45	319
2′-Deoxyguanosine	16	555	18	555	42	352
Thymidine	4.3	386	36	434	6.3	293
2′-Deoxycytidine	44	443	23	515	36	295

[a] Reference 4. [b] Reference 41. [c] Reference 5.

For purposes of structural characterization, spectra of permethyl derivatives compare well with other alternatives.[3-5,7] Ions of the molecular ion series (a, c–f) provide a number of reference points for identification of M, a favorable characteristic also shown by trifluoroacetyl derivatives.[5,7] Also important, the diagnostic base-containing ions carry a relatively large fraction of the total ion current, as shown by the data in Table IV. With the exception of thymidine, which is dominated by ions from the sugar moiety (Figure 3c), no advantages are offered in this respect by either trimethylsilyl[41] or trifluoroacetyl derivatives. Lastly, the simpler methyl groups produce the usual experimental advantages of lower molecular weight (see Table IV), and less ambiguity in conversion of exact mass to elemental composition, which is primarily governed by the number of elements present. For example, the number of possible compositions for base + H from derivatives of adenosine which fall within ±10 ppm of the theoretical value using composition limits of the molecular ion are: permethyl, 7; trimethylsilyl, 9; trifluoroacetyl, 15.

Experimental Section

Sources of Nucleosides. Purity of all underivatized nucleosides was checked by gas chromatography[39] or mass spectrometry[4] of their trimethylsilyl derivatives. Most nucleosides were commercial products; less common compounds were obtained from the following sources: Terramarine Bioresearch, La Jolla, Calif., 1-α-D-ribofuranosylcytosine (α-cytidine); Cancer Chemotherapy National Service Center, National Institutes of Health, 4′-thioadenosine and N^6-(3-methyl-2-butenyl)adenosine; Dr. M. Honjo, Takeda Chemical Co., Osaka, Japan, 2′-*O*-methyluridine. 5′-Deoxyadenosine and 2′- and 3′-*O*-methyladenosine were previously prepared in this laboratory.[22]

Nucleosides specifically labeled with ^{18}O (*ca.* 4% ^{18}O in O-2′, 9–10% ^{18}O in O-4′) were obtained from ^{18}O-labeled RNA provided by Dr. R. Caprioli, from which the location and distribution of ^{16}O had been previously established.[42] RNA (2 mg) was incubated overnight at 45° with snake venom phosphodiesterase (0.2 mg) and alkaline phosphatase (0.16 unit) at pH 8.6. The hydrolysate was heated at 60° for 30 min and then refrigerated and centrifuged, and the supernatant evaporated to dryness and permethylated. The mixture was submitted to gas chromatography–mass spectrometry, from which spectra of individual components were recorded.

N^3,*O*-2′,3′-Tris(methyl-d_3)-5′-*O*-methyluridine (15) was prepared by permethylation (CD_3) of 5′-*O*-trityluridine (2 mg), followed by removal of the trityl group by heating in presence of 80% acetic acid (2 ml) at 80° for 1 hr. After evaporation of solvent the residue was dissolved in chloroform and separated into two bands by preparative tlc (chloroform–2-propanol, 9:1). Elution of the slower band gave 0.85 mg (70%) of N^3,*O*-2′,3′-tris(methyl-d_3)-uridine; exact molecular mass 295.1721 (required for $C_{12}H_9D_9N_2O_6$, 295.1730). The solution was remethylated using CH_3I. **15** was separated from methyl trityl ether by preparative gas chromatography (6 ft × 0.5 in., 1% OV-17). Insufficient material was recovered for recrystallization; the collected material was shown to be homogeneous by gas chromatography–mass spectrometry and exhibited the required mass spectrum: M, *m/e* 309 (1.7 rel intensity); ion g, *m/e* 264 (1.0); ion p, *m/e* 205 (5.2).

Preparation of *N*,*O*-Permethyl Nucleoside Derivatives. Methylsulfinyl carbanion solution was prepared by the procedure of Lande and coworkers by addition of sodium hydride to dimethyl sulfoxide.[43] Particular care was taken in the initial step[43] involving removal of oil from sodium hydride suspension by washing with anhydrous ether in order to avoid contamination of the final product by traces of oil. When not in use the anion solution was stored below 0°.

Conditions for permethylation were similar to those previously reported for peptide work, which employed equimolar amounts of anion and alkyl iodide[43] in tenfold excess[44] over the number of "reactive equivalents" required (*i.e.*, the product of millimoles of nucleoside times the approximate number of available permethylation sites[43]). From 2 to 100 μg of nucleoside was dissolved in DMSO (80 μl), followed by addition of tenfold reactive equivalent excess of anion solution. After 30 min an equimolar amount of CD_3I (or other alkyl iodide) was added and allowed to stand for additional 90 min. The reaction was terminated by addition of H_2O (1 ml), then the nucleoside was extracted by shaking with $CHCl_3$ (1 ml). The $CHCl_3$ layer was washed successively with three 1-ml portions of H_2O and evaporated to dryness under vacuum. The permethylated nucleoside was redissolved in $CHCl_3$ for transfer to the gas chromatographic inlet system of the mass spectrometer.

Gas Chromatography–Mass Spectrometry. Low-resolution mass spectra were recorded on a LKB 9000 instrument, with sample introduction through the gas chromatographic inlet system (0.25 in. × 6 in. or 0.25 in. × 3 ft, 1% OV-1, temperature programmed at 10°/min from 100°); ionizing energy 70 eV; ion source and carrier gas separator temperatures 250°.

High-resolution mass spectra of the following compounds were photographically recorded at R 15,000–18,000 using a CEC 21-110B instrument, with sample introduction by direct probe: **3**, **21**, **22**; CH_3 derivative of 2′-deoxyadenosine; CD_3 derivatives of 1-methyladenosine, 3′-deoxyadenosine, N^6-methyl-2′-deoxyadenosine, guanosine, N^2,N^2-dimethylguanosine, 2′-deoxyguanosine, 1-ribosylthymine, 2′-*O*-methyluridine, thymidine, dihydrothymidine, cytidine, 2′-deoxycytidine. Elemental compositions were computer derived from exact mass measurements, with maximum errors of ±3 millimass units.

Gas chromatographic data in Figure 1 were recorded with a Barber Colman 5000 instrument and a Keithley 417 electrometer; 0.25 in. × 6 ft, 1% OV-1; carrier gas flow 40 cc/min; temperature programmed at 10°/min from 100°.

Acknowledgments. This work was supported by the National Institutes of Health (GM-13901, GM-02055) and the Robert A. Welch Foundation (Q-125). We are grateful to Misses S. E. Hattox and P. F. Crain for their assistance, to Dr. K. D. Haegele for his advice and help in preparing methylation reagents, Drs. R. Caprioli and M. Honjo for generous gifts of samples, and to Dr. Harry B. Wood, Jr., of the Cancer Chemotherapy National Service Center for his aid in obtaining nucleoside samples.

(41) R. N. Stillwell, K. J. Lyman, and J. A. McCloskey, unpublished data.

(42) R. Caprioli and D. Rittenberg, *Biochemistry*, **8**, 3375 (1969).

(43) M. L. Polan, W. J. McMurray, S. R. Lipsky, and S. Lande, *Biochem. Biophys. Res. Commun.*, **38**, 1127 (1970).

(44) P. A. Leclercq and D. M. Desiderio, Jr., *Anal. Lett.*, **4**, 305 (1971).