carbonates (VI, X = H, CN, OCH_3) were made by converting methanol-^{18}O (55.5% enriched)[45] to the chloroformate, which was then treated with the appropriate phenol in pyridine.[8] ^{18}O-Labeled methyl phenyl carbonate (V) and all ^{18}O-labeled aryl phenyl carbonates XII were prepared by transforming ^{18}O-phenol[45] (diluted to 37.5% enrichment) to the chloroformate, and then adding methanol or the appropriate substituted phenol, respectively.[8] The carbonate esters were purified as before,[8] using gas–liquid chromatography and several injection–collection cycles for liquids, and recrystallization to constant melting point for solids.

Identity and purity of all unlabled carbonates were determined by elemental combustion analysis, gas–liquid or thin layer chromatography, and infrared and low-resolution mass spectra. The retention times or R_f values, infrared and mass spectra, and melting points (Table III) of the labeled carbonates were critically compared with those of the corresponding unlabeled materials.

(45) Supplied by Yeda Research and Development Co. Ltd., Rehovoth, Israel.

Mass Spectra of Nucleic Acid Derivatives. II. Guanine, Adenine, and Related Compounds[1a]

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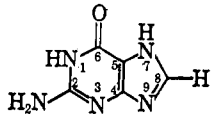
Abstract: The mass spectra of guanine, its 1-, 3-, and 7-methyl derivatives, and of isocytosine, hypoxanthine, adenine, and 6-methylaminopurine have been analyzed with the aid of high-resolution mass measurements, metastable peaks, and deuterium labeling. The major electron-impact fragmentation pattern exhibited by guanine and its mono-N-methyl derivatives consists of initial expulsion of a cyanamide fragment, which contains N-1, C-2, and the amino nitrogen atom, followed by decarbonylation. This sequence thus involves all the guanine atoms which take part in the Watson–Crick pattern of hydrogen bonding in polynucleotides. In contrast, the initial fragment expelled from the adenine molecular ion originates uniquely from a single site only when the amino group is methylated.

Guanine (2-amino-6-oxypurine; the prevailing 1H tautomer is shown as **1**) and adenine (6-aminopurine, **2**) comprise the major purine components of



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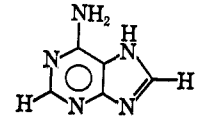

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both ribo- and deoxyribonucleic acids, and of a variety of coenzymes and biosynthetic intermediates as well.[2] Most of the minor purine constituents of both transfer RNA[3,4] and of DNA[5] consist of mono- and dimethyl derivatives of **1** and **2**.

It has recently been demonstrated that adenine also is the principal component of a number of alkaloids, including the cytokinins, which under certain conditions are powerful stimulators of plant cell division. The naturally occurring compounds in this series include zeatin, 6-(4-hydroxy-3-methyl-2-butenyl)aminopurine, isolated as the free base[6] and possibly as a nucleoside and nucleotide[7] from maize, and its deoxy derivative, 6-(3-methyl-2-butenyl)aminopurine.[8,9] The latter compound has been isolated both from a bacterium[10] and, in the form of its ribonucleoside, as a minor component of transfer RNA in yeast[8,11] and in calf liver.[8] The isomeric 6-amino-3-(3-methyl-2-butenyl)purine, which is devoid of cytokinin activity, has been identified as the alkaloid triacanthine.[12]

Mass spectrometry played a significant but limited role in the elucidation of the structures of these substituted adenine derivatives, and has also been applied in studies of the mechanism of biological methylation of transfer RNA,[13] but to date no systematic study of the mass spectra of the parent compounds has been published. The mass spectra of purine nucleosides, in which the fragmentation patterns of the purine moieties have received relatively little attention, have been used in studies both of nucleic acid components[13,14] and of certain antibiotics.[15,16] We present here the mass spectra of guanine, adenine, and some of

(1) (a) Part I of this series: J. M. Rice, G. O. Dudek, and M. Barber, *J. Am. Chem. Soc.*, **87**, 4569 (1965). (b) National Cancer Institute. (c) To whom inquiries should be addressed at Harvard University.

(2) The 7(9) protropic tautomerism of purines unsubstituted in the imidazole ring is ignored in these studies; the imidazole proton is generally represented arbitrarily as residing on N-7.

(3) Abbreviations and notation used in this paper: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; M, molecular (parent) ion; M − *n*, the *m/e* value of a fragment ion formed by loss of *n* mass units from the molecular ion.

(4) L. R. Mandel and E. Borek, *Biochemistry*, **2**, 555 (1963).

(5) D. B. Dunn and J. D. Smith, *Nature*, **175**, 336 (1955).

(6) D. S. Letham, J. S. Shannon, and I. R. McDonald, *Proc. Chem. Soc.*, 230 (1964).

(7) C. O. Miller, *Proc. Natl. Acad. Sci. U. S.*, **54**, 1052 (1965).

(8) R. H. Hall, M. J. Robins, L. Stasiuk, and R. Thedford, *J. Am. Chem. Soc.*, **88**, 2614 (1966).

(9) K. Biemann, *et al.*, *Angew. Chem.*, **78**, 600 (1966).

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(11) H. G. Zachau, D. Dutting, and H. Feldmann, *Angew. Chem.*, **78**, 392 (1966).

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(13) B. E. Tropp, J. H. Law, and J. M. Hayes, *Biochemistry*, **3**, 1837 (1964); B. E. Tropp, Doctoral Dissertation, Harvard University, July 1965.

(14) K. Biemann and J. A. McCloskey, *J. Am. Chem. Soc.*, **84**, 2005 (1962).

(15) S. Hanessian, D. C. DeJongh, J. A. McCloskey, *Biochim. Biophys. Acta*, **117**, 480 (1966).

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

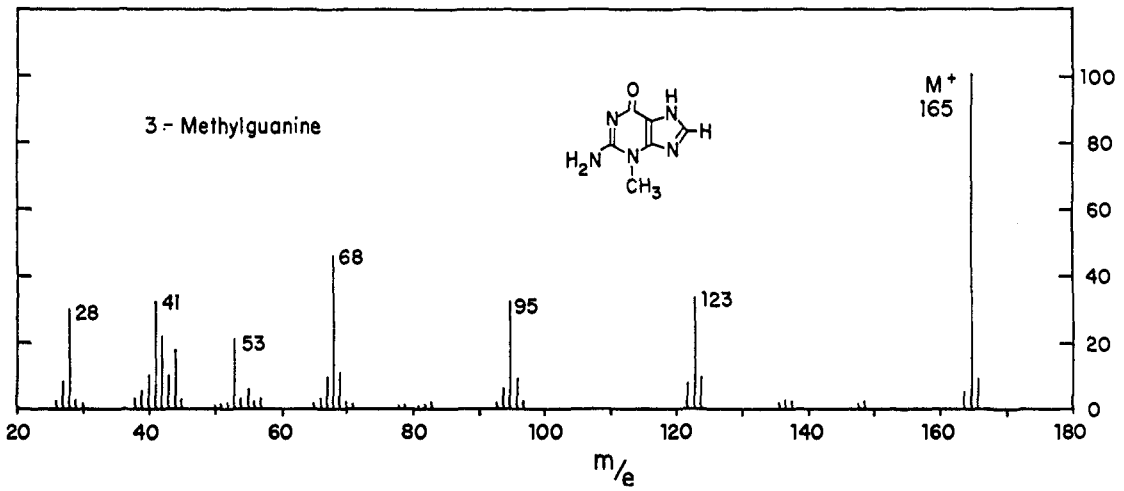


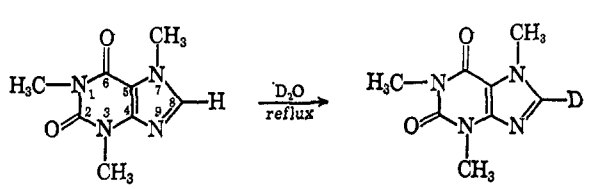
Figure 1. Mass spectrum of 3-methylguanine at 70 ev.

their derivatives, in which the fragmentation patterns have been characterized by metastable peaks and by high-resolution mass measurements of fragment ions. We have sought especially to identify fragments which originate characteristically and uniquely from specific atomic groupings within these molecules, and, where possible, to suggest reasonable structures and mechanisms for the formation of fragment ions.

Experimental Section

The compounds studied were commercial preparations of the highest purity available, and were obtained from the California Corporation for Biochemical Research, Schwarz Bioresearch Inc., Cyclo Chemical Corp., K and K Laboratories Inc., or Aldrich Chemical Co. All preparations were checked for chromatographic homogeneity on paper prior to use. 3-Methylguanine (Cyclo) contained a trace of yellow contaminant, apparently quite polar from its chromatographic behavior, which could be removed by recrystallization from water and which did not contribute significant "noise" to the spectrum of the much more volatile purine. A more polar, ultraviolet-absorbing impurity, identified by R_f values and by infrared spectroscopy as guanine, invariably contaminated samples of 1-methylguanine, and was found in amounts ranging from a trace to more than 90% of the mixture. Preparations containing only traces of guanine provided intense spectra of the more volatile 1-methyl derivative which displayed no significant peak at *m/e* 151, the molecular ion and base peak in the spectrum of guanine, and were used without purification.

Readily exchangeable protons (amino, imidazole, "hydroxy") were replaced with deuterium by warming briefly (*ca.* 15 min) in neutral or basic D_2O and then rotary evaporating the neutralized solution to dryness. If necessary, the procedure was repeated until the expected number of deuterium atoms had been incorporated. Prolonged refluxing (*ca.* 1 hr) in D_2O effects replacement of an additional proton by deuterium. This has been shown to occur at C-8 in purine,[17] *and is presumed to occur at this position in substituted purines as well.* The reaction seems to be general for imidazole derivatives,[18,19] and occurs readily in caffeine (**3**), where the reaction



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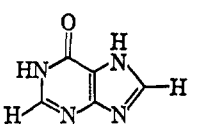
can be followed unambiguously by pmr spectroscopy.[19] In the cases of adenine and hypoxanthine (6-oxypurine; the 1H tautomer is illustrated as **4**), where the magnetic resonance signals of the C-2 and C-8 protons are very close together and are both pH and concentration dependent, only one peak decreases steadily in intensity and finally disappears as deuteration proceeds. Thus hypoxanthine (mol wt 136) readily exchanges two protons for deuterium, forming 1,7(9)-dideuteriohypoxanthine (hypoxanthine-d_2,



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mol wt 138), which on prolonged refluxing slowly exchanges a third hydrogen atom to yield 1,7(9),8-trideuteriohypoxanthine (hypoxanthine-d_3, mol wt 139). Adenine behaves similarly.

Mass spectra were obtained with an Associated Electrical Industries (Ltd.) MS-9 double-focussing mass spectrometer, using a direct-insertion probe. The voltage of the electron beam was occasionally lowered from the standard 70 ev to 30, 20, or 16 ev to suppress high-energy fragmentation paths and to increase the intensity of metastable peaks. Standards for high-resolution mass measurements included argon, nitrogen, and fragments of perfluorotri-*n*-butylamine.

Results and Discussion

The most intense peak in the mass spectra of guanine and its 1-, 3-, and 7-methyl derivatives is that of the molecular ion, from which a variety of fragments are expelled to produce three groups of primary fragment peaks of weak (less than 10%) to moderate (less than 40%) relative intensity. These occur at M − (16–17), due to loss of the amino group or ammonia; at M − (41–42) or, in the case of 1-methylguanine, at M − (55–56), resulting from expulsion of cyanamide fragments; and in some cases at M − (27–30) due to ejection of carbonyl and methylamine fragments. The initial expulsion of a neutral cyanamide fragment is the predominant and most characteristic mode of decomposition of a guanine molecular ion, and is followed by a well-defined sequence of subsequent fragmentation steps which give rise to the major peaks in the mass spectra of these compounds. The cyanamide fragments originate uniquely from N-1, C-2, and the amino group in the pyrimidine ring of guanine, a conclusion which follows from comparison of the mass spectra of its N-methyl derivatives.

The mass spectrum of 3-methylguanine (Figure 1) is characterized by fragment ion peaks at *m/e* 123, 95,

(17) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 86, 696 (1964).
(18) T. M. Harris and J. C. Randall, *Chem. Ind.* (London), 1728 (1965).
(19) G. O. Dudek and J. M. Rice, unpublished observations.

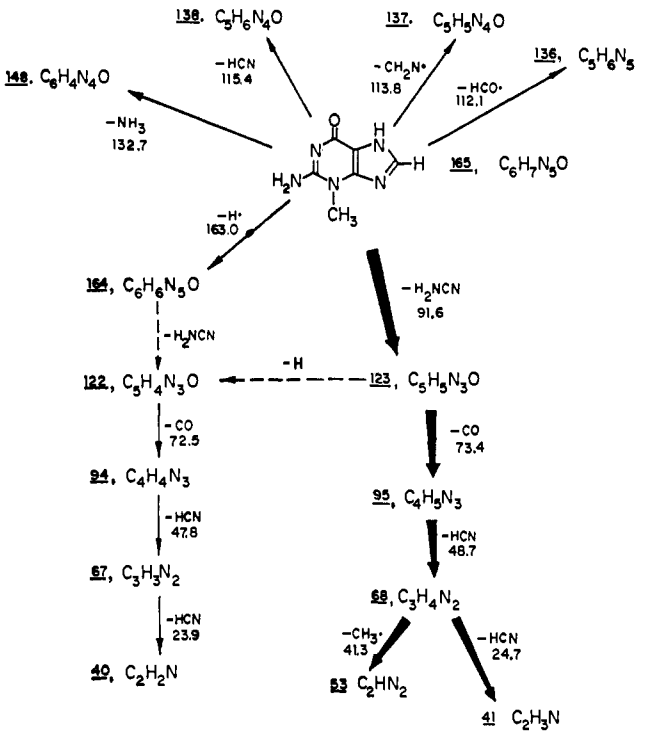
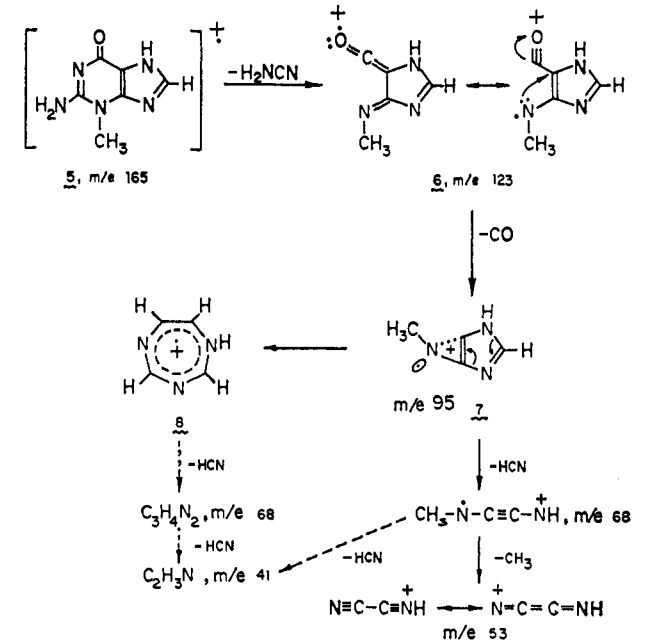


Figure 2. Fragmentation paths of 3-methylguanine. The *m/e* values of observed metastable peaks are given to 0.1 mass unit, together with the transitions to which they have been assigned. Dashed arrows designate transitions for which metastable peaks were not observed, but which reasonably account for the presence in the spectrum of the indicated peaks. Heavy arrows indicate the principal fragmentation processes. The elemental compositions of all ions indicated in this and other fragmentation diagrams were established by high-resolution mass measurements.

68, 53, and 41, whose sequential formation from the molecular ion at *m/e* 165 is well documented by the presence of metastable peaks (Figure 2). Mechanisms for some of these fragmentation processes are proposed in Scheme I.[20] Expulsion of cyanamide from the molecular ion, **5**, is readily explained by a retro Diels–Alder mechanism which results in the formation of ion **6** at *m/e* 123. Decarbonylation is the only significant fragmentation reaction of ions having the elemental composition of **6**; a metastable peak at *m/e* 75.0 (123 → 96?) for the loss of HCN is too weak to be considered unequivocal. The decarbonylation product at *m/e* 95, ion **7**, loses HCN followed by either the methyl group or another molecule of HCN. While **7** is a likely candidate for rearrangement, and could give rise to highly stabilized heteroaromatic ions such as **8** by redistributing its methyl hydrogen atoms within an expanded ring, the methyl group remains intact in a substantial fraction of these ions. The fragmentation of **7**, therefore, probably proceeds by several paths, among them the sequence indicated.

Scheme I



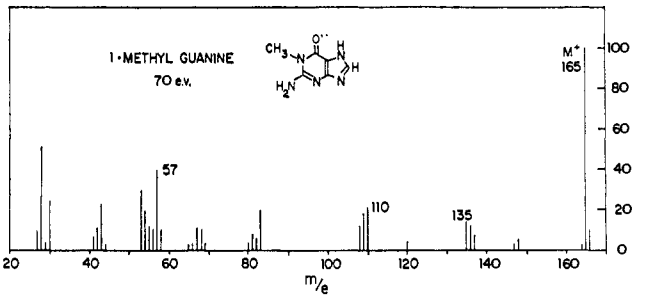


Figure 3. Mass spectrum of 1-methylguanine at 70 ev.

The ions at *m/e* 122 and 124 may derive partly from the molecular ion, but this interpretation is not supported by the presence of metastable peaks. The *m/e* 122 ion is probably formed primarily by expulsion of a hydrogen atom from **6**, chiefly from the methyl group. Except for the ^{13}C-containing fragment at *m/e* 124, these are even-electron ions, and although they follow basically the fragmentation sequence outlined in Scheme I, their fragments at *m/e* 67 and 69 show no tendency to eject a methyl radical: the peak at *m/e* 54 is small and that at *m/e* 52 is negligible. The molecular ion can also lose HCN and, by processes involving hydrogen rearrangements, H_2CN (not CO!), HCO, or NH_3. However, the resulting peaks at *m/e* 136–138 and at 148 are very small, as are the secondary fragmentation peaks which appear in clusters at intervals of approximately 27 mass units below the group at *m/e* 136–138.

The location of the methyl group in 1-methylguanine has several important consequences for the electron-impact fragmentation patterns of this molecule. First, the methyl group is always lost with the cyanamide fragments, as a result of which peaks are observed at *m/e* 110 and 109 (*i.e.*, at M − 55 and M − 56 in Figure 3). Neutral methylcyanamide fragments expelled from the molecular ion retain three or four of the five hydrogen atoms originally associated with the amino and methyl groups. When all five are retained, *i.e.*, when hydrogen rearrangement does not accompany frag-

(20) The conjugated π-electron systems of the molecular ion and unrearranged fragment ions can accommodate delocalization of the positive charge (and the unpaired electron in ion radicals) over all the atoms of second-row elements present except (perhaps) methyl carbon atoms. It is often extremely difficult adequately to represent the structures of such ions, especially when they contain many heteroatoms whose "lone pair" electrons in fact contribute to the π-electron population. In attempting to understand fragmentation patterns it is frequently more instructive to consider a single valence-bond structure for a given ion, rather than the resonance hybrid to which it contributes. We have freely adopted this approach in Scheme I and elsewhere in this paper.

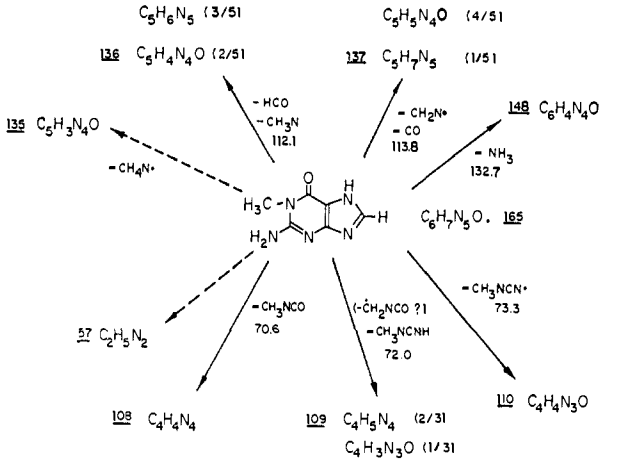


Figure 4. Electron-impact fragmentation reactions of the 1-methylguanine molecular ion.

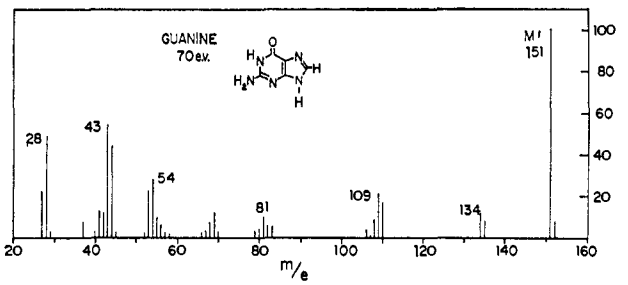


Figure 5. Mass spectrum of guanine at 70 ev.

mentation, the methylcyanamide fragment also retains the charge, and gives rise to the major component of the peak at *m/e* 57. As expected, loss of neutral cyanamide fragments is followed by decarbonylation and subsequent loss of HCN. There are no significant peaks at M − 41 and M − 42, which indicates that N-1 is always the sole nuclear nitrogen atom lost when cyanamide fragments are expelled from guanine derivatives. This generalization applies even when, as in the present case, the arrangement of double bonds in the pyrimidine ring interferes with the mechanism presented in Scheme I for this reaction. This interference is a second consequence of the location of the methyl group at N-1, and results in lower relative intensities for the ions produced in the series of reactions initiated by expulsion of a cyanamide fragment (compare peaks at *m/e* 123, 95, and 68 in Figure 1 with corresponding peaks at *m/e* 109–110, 81–82, and 54–55 in Figure 3). The presence of the methyl group at N-1 also tends to stabilize the positive charge in that region of the guanine molecule. The stabilization of charge, together with the decreased occurrence of competing processes, results in an increased tendency for cleavage of the "single" bonds in the molecular ion in the vicinity of N-1, as indicated by the increased intensity of peaks at *m/e* 135–137 (loss of carbonyl or methylamine fragments). Some of these fragments can also be lost simultaneously, producing the major component of the *m/e* 109 peak and all of the *m/e* 108 ions (Figure 4).

The mass spectrum of guanine (Figure 5) is essentially identical with that of 1-methylguanine below *m/e* 120, with the exception that peaks at *m/e* 43 and 44, due to $CH_3N_2^+$ and $CH_4N_2^+$ (cyanamide) ions originating at

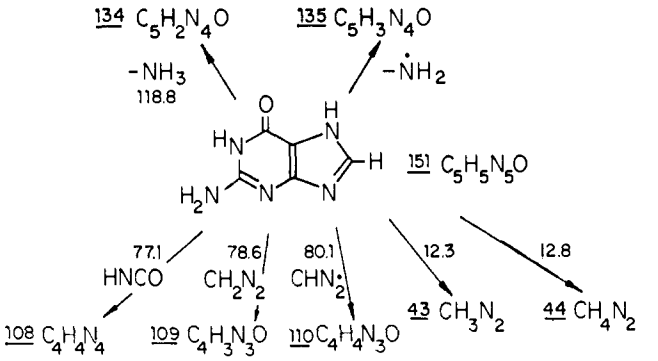


Figure 6. Major electron-impact fragmentation reactions of the guanine molecular ion.

least in part from the molecular ion, replace the corresponding *m/e* 57 peak in the spectum of 1-methylguanine. Decarbonylation is not a significant decomposition reaction for the guanine molecular ion in the absence of a methyl group at N-1. Deamination (followed by decarbonylation) and expulsion of HNCO are the only major fragmentation processes which compete with loss of cyanamide (Figure 6). The ions at *m/e* 109–110 produced by the latter reaction eject CO and HCN, preferably but not exclusively in the order given, producing the peaks at *m/e* 81–83 and 54–55; these processes are not completely defined by metastable peaks. The ability of guanine to lose cyanamide fragments containing one or two hydrogen atoms, the loss of fragments other than cyanamide from the molecular ion, and the over-all low intensity of fragment ion peaks resemble the characteristics of 1-methylguanine, rather than those of 3-methylguanine. This suggests that the prevailing tautomer of guanine in the vapor phase is the linearly conjugated structure **1** in which the pyrimidine ring proton resides on N-1; this is, of course, the familiar tautomer encountered in aqueous solutions and in polynucleotides.

The mass spectrum of 7-methylguanine (Figure 7) is more plentifully endowed with metastable peaks than the spectrum of guanine. A more complete analysis of the fragmentation paths is therefore possible (Figure 8), but the major features of the spectra of the two compounds are quite similar. The 7-methylguanine molecular ion undergoes deamination and loss of cyanamide fragments, but reactions involving expulsion of CO, HCO, or methylamine fragments are extremely minor processes in sharp contrast to the behavior of the 1-methyl isomer. Ejection of an isocyanate fragment from the molecular ion also does not occur to any significant extent, although this reaction is undergone both by the 1-methyl isomer and by guanine itself. The major fragmentation path consists of ejection of cyanamide fragments, giving rise to the peaks at *m/e* 123 and 124, after which much of the fragmentation pattern is analogous to that of 3-methylguanine with respect to the *m/e* values, but not the relative intensities, of successive fragmentation peaks (compare Figures 2 and 8). It is noteworthy that ion **9** at *m/e* 95, the unrearranged product of successive loss of cyanamide and CO from the 7-methylguanine molecular ion, differs from the corresponding daughter ion of 3-methylguanine (**7**) only in the position of the methyl group, which in **9** is poorly situated to participate in

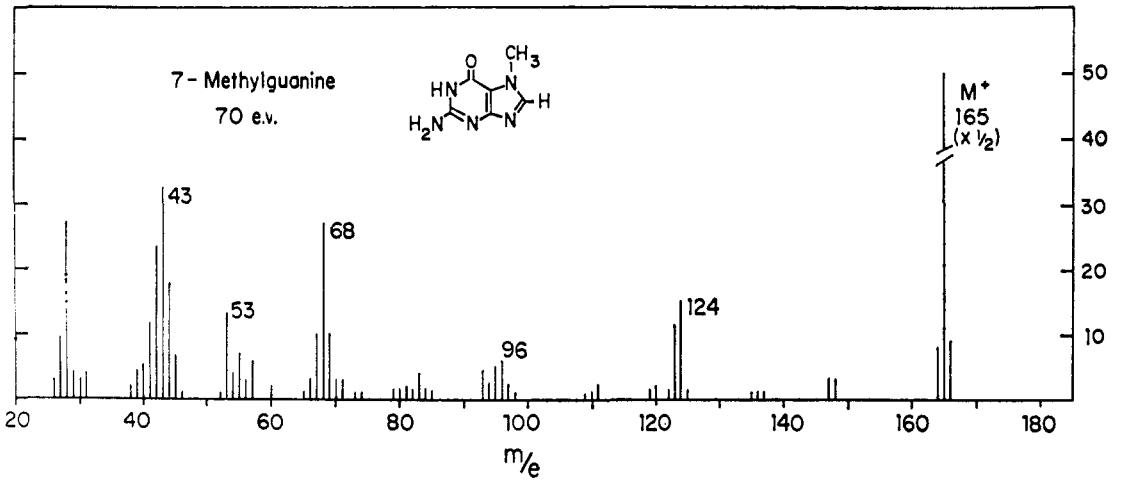


Figure 7. Mass spectrum of 7-methylguanine at 70 ev.

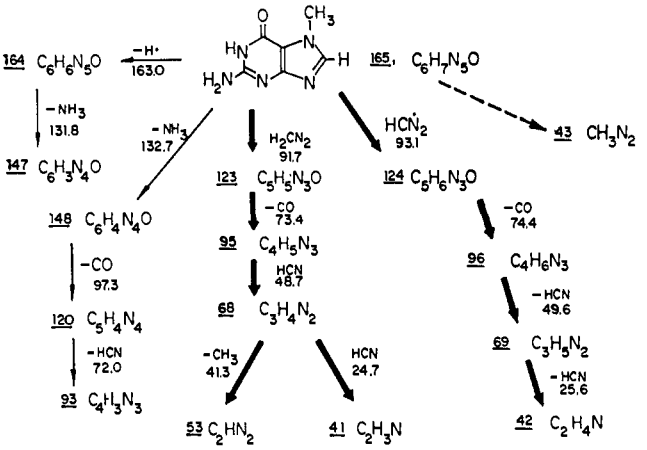


Figure 8. Fragmentation paths of 7-methylguanine.

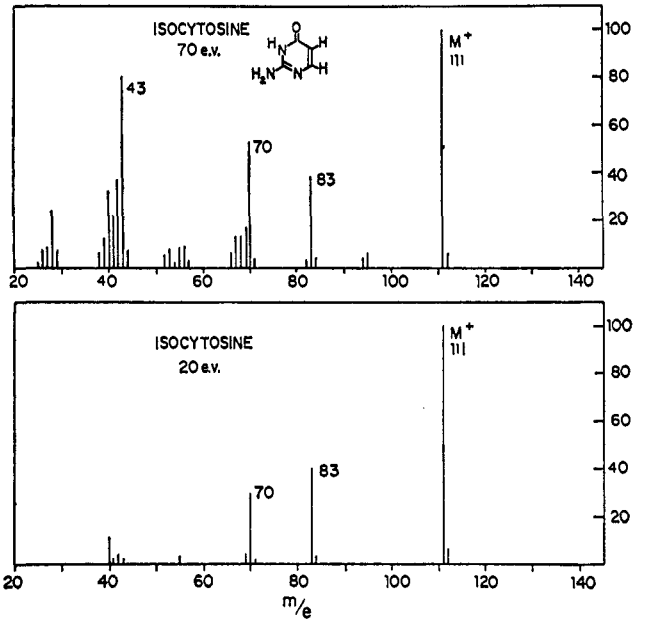
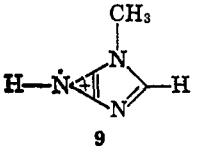


Figure 9. Mass spectra of isocytosine at 70 and 20 ev.

ring expansion to structures such as **8**. This may explain why the *m/e* 95 peak is prominent in Figure 1 but very weak in Figure 7, while the *m/e* 68 peak which results from loss of HCN from the *m/e* 95 ions is prominent in both.



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The combined presence of the amino group at C-2 and the keto function at C-6 in guanine and its monomethyl derivatives serves to limit the fragmentation reactions of the molecular ion to the pyrimidine ring. The mass spectrum (Figure 9) of 2-amino-4-oxypyrimidine (isocytosine), whose arrangement of functional groups corresponds to that of guanine, is qualitatively quite similar to the high-mass region of the spectrum of the latter. Analysis of metastable peaks, together with high-resolution mass measurement of fragment ions (Figure 10), confirms that the same neutral fragments and the $CH_3NH_2^+$ ion are expelled from the molecular ions of both compounds. However, it is apparent from Figure 9 that loss of CO from the molecular ion is a far more important process for isocytosine than it is for guanine or any of the mono-N-methylguanines, and that fragmentation in general is much more facile for the pyrimidine ion. It follows that the aromatic imidazole ring fused to the isocytosine molecule in guanine derivatives stabilizes the molecular ion by delocalizing the positive charge and thus depresses the tendency toward fragmentation, but does not otherwise alter the modes of decomposition of the molecular ion. This stabilization is least effective when the arrangement of bonds in the pyrimidine ring allows the molecular ion to undergo a facile retro Diels–Alder reaction, as in the case of 3-methylguanine, or of 2,6-dioxypurines (xanthines).[21]

Purines which contain only one functional group in the pyrimidine ring show a much less pronounced tendency for one fragmentation reaction of the molecular ion to predominate over all the others. Omission of the amino group from the guanine molecule yields hypoxanthine (**4**), whose mass spectrum (Figure 11) is notable for the extremely low intensities of its fragment

(21) G. Spiteller and M. Spiteller-Friedmann, *Monatsh. Chem.*, 93, 632 (1962); J. M. Rice and G. O. Dudek, in preparation.

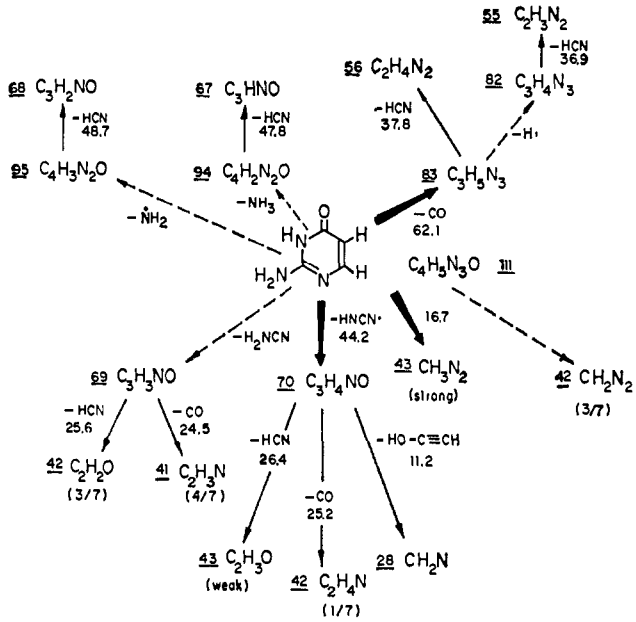

Figure 10. Major fragmentation paths of isocytosine. The distribution of fragment ions suggests that the 3H tautomer of isocytosine (shown) predominates in the vapor phase.

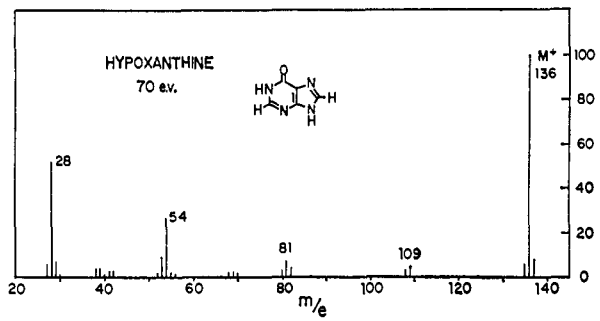

Figure 11. Mass spectrum of hypoxanthine at 70 ev.

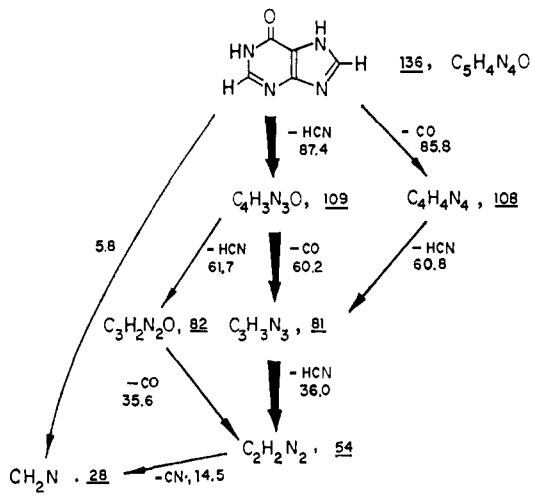

Figure 12. Fragmentation paths of hypoxanthine. The existence of multiple fragmentation reactions for the molecular ion suggests that the 1H tautomer predominates in the vapor phase.

ion peaks. This spectrum suggests, however, that the major determinant of the guanine fragmentation pattern is the carbonyl function and not the amino groups, for to a large extent hypoxanthine expels neutral fragments

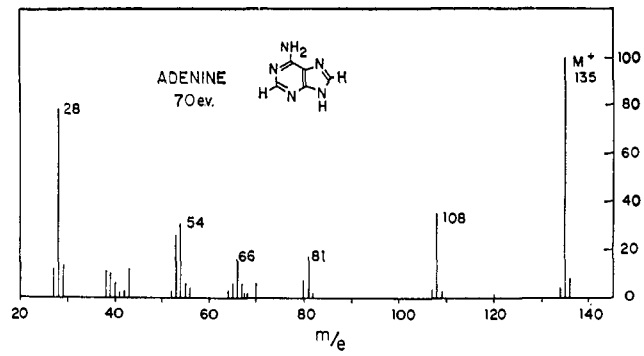

Figure 13. Mass spectrum of adenine at 70 ev.

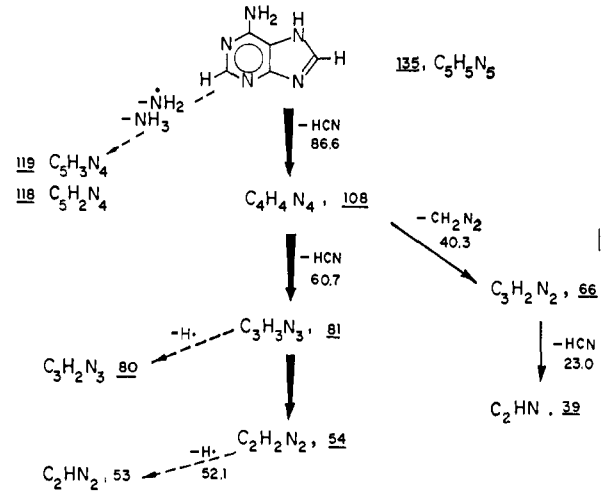

Figure 14. Principal fragmentation paths of adenine.

from the same sites and in the same order as guanine. The molecular ion can eject a hydrogen atom, CO, or HCN, but the preferred process is expulsion of HCN, followed by CO and another molecule of HCN (Figure 12). The M − 27 peak persists *with undiminished relative intensity* in the mass spectra of hypoxanthine-d_2 and hypoxanthine-d_3, and loss of HCN from the latter *must* include C-2. The initial loss of HCN from the hypoxanthine molecular ion thus corresponds principally to the expulsion of cyanamide fragments by guanine, and is inferred to involve N-1 as well as C-2. The subsequent decarbonylation and loss of HCN represent a continuation of the guanine fragmentation pattern. Expulsion of a charged cyanamide fragment from guanine also finds an analogy in the formation of H_2CN^+ from hypoxanthine, but not all of the protonated HCN fragments which originate from the molecular ion do so from the same site as the neutral HCN discussed above. The *m/e* 28 peak due to H_2CN^+ shifts cleanly to *m/e* 29 ($HDCN^+$) in the spectrum of hypoxanthine-d_2, but its total intensity is divided nearly equally between peaks at *m/e* 29 and 30 (D_2CN^+) in the spectrum of hypoxanthine-d_3. A metastable peak in the latter spectrum at *m/e* 6.5 (139 → 30) confirms the origin of some of the D_2CN^+ ions from the molecular ion, and therefore from the imidazole ring.

If the carbonyl group of hypoxanthine and its associated proton are replaced by an amino group, the neutral fragments expelled from the resulting adenine

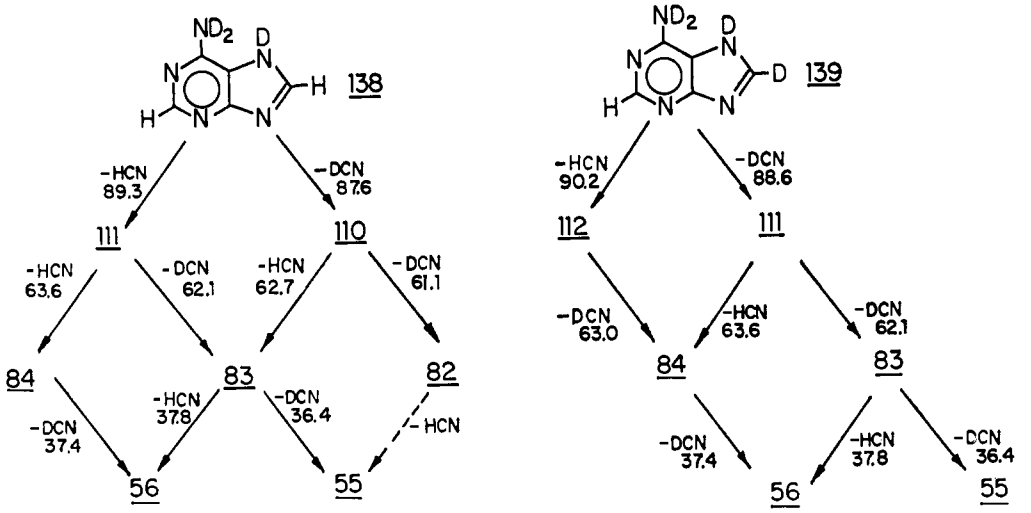

Figure 15. The multiple origins of cyanide molecules produced at each stage of fragmentation in adenine-d_3 and adenine-d_4.

molecular ion no longer show any tendency to originate uniquely from a single site. The mass spectrum of adenine (**2**; Figure 13) is characterized chiefly by fragment ion peaks at *m/e* 108, 81, and 54 due to successive expulsion of three molecules of HCN, although other fragments are occasionally lost along the way (Figure 14). HCN and DCN can be lost in all possible sequences from the adenine-d_3 and adenine-d_4 molecular ions, as indicated in Figure 15, proving that many fragmentation paths contribute to the mass spectrum. The similar behavior of the unsubstituted purine molecule has recently been analyzed in detail.[22]

Loss of the amino group from the molecular ion is an extremely minor process for adenine, but if the amino group is methyl substituted, the initial fragments lost from the molecular ion originate predominately from the alkylamino group. 6-Methylaminopurine (Figure 16) preferentially expels 28 or 29 mass units (CH_2N or CH_3N) from the molecular ion. This process consists of loss of the methylamino group with concomitant transfer of one or two hydrogen atoms to the purine nucleus, followed in each case by successive loss of three molecules of HCN. A high-energy variant, not seen at 16 ev, involves initial loss of a hydrogen atom followed by the remaining 29 mass units of the methylamino group. When the entire methylamino group is lost as a single unit, it retains the positive charge, giving rise to the CH_4N^+ ion at *m/e* 30. Methylation of the amino group thus introduces a powerful and unifying directive influence into the chaotic array of competing fragmentation patterns which otherwise characterize the adenine molecule.

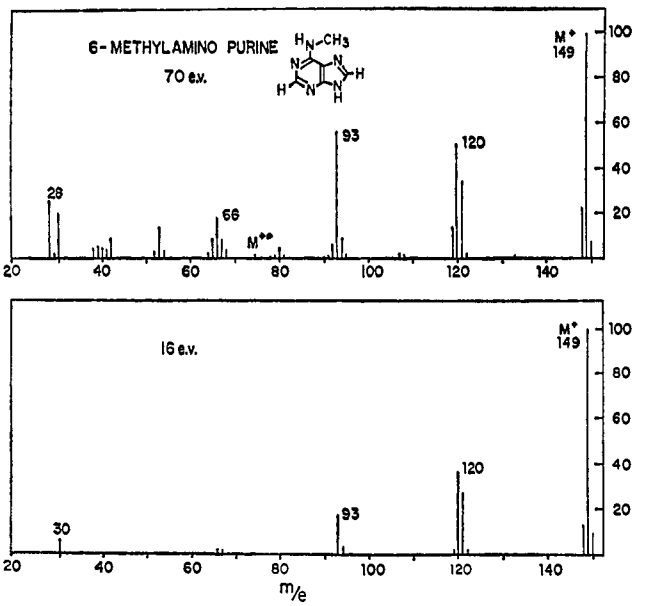

Figure 16. Mass spectra of 6-methylaminopurine at 70 and 16 ev.

Conclusions

The characteristics of guanine appear to be much more favorable than those of adenine or hypoxanthine for obtaining unambiguous structural information from the mass spectra of derivatives of these compounds. It is noteworthy that guanine in nucleic acids is much more reactive than adenine toward both mutagens of the mustard type[23] and certain carcinogenic compounds,[24] and the application of mass spectrometry to the study of such reactions should prove fruitful.

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