# Mass Spectrometry and Carbon-13 Labeling

## Carbon-13 helps to define the chemistry underlying the mass spectra of organic compounds.

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Against a backdrop of traditional methods for the determination of molecular structures, mass spectrometry may be viewed as an idealized example of automation (*1*). The principle is unchanged. The sample undergoes chemical reactions; if these reactions proceed in accord with the chemist's expectations and he is successful in identifying the products, he can reconstruct the original molecule on paper. The mass spectrometer, in the course of measuring the spectrum, initiates reactions and separates and characterizes the products, all in a matter of several seconds to several minutes. All that remains is for the chemist to interpret the results, that is, to reconstruct the underlying chemistry and from that the original molecule.

There are a few hitches in this neat picture. First, both the means employed to activate the molecules—ionization-excitation by electron impact—and the environment in which the action takes place—the gas phase at a pressure low enough to allow only unimolecular reactions—differ considerably from those encountered by organic chemists in most other contexts. Hence, much of the chemistry also differs from that in more familiar systems; even where similarities in behavior are observed, they may be more apparent than real and must be regarded with skepticism until proved. Thus, chemists wishing to realize the potential of mass spectrometry for the determination of molecular structures have been faced with the necessity of formulating a systematic organic chemistry peculiar to this context (*2*). Second, the only direct information available for the characterization of a reaction product is its mass, or, more precisely, its mass-to-charge ratio. Ion structures must be inferred from other considerations.

Four different kinds of data—in effect, four different tools or experimental approaches—have proved generally useful in the definition of reaction paths (*1*). These are (i) correlations of spectra with molecular structures, (ii) appearance potentials, (iii) metastable peaks, and (iv) the spectra of labeled compounds. By and large, the newer techniques that have been developed during the past few years may be viewed as elaborations on or combinations of these four (*3*).

Empirical correlations of spectra with structures furnish at least starting points for a study of the underlying chemistry. Appearance potentials and measurements of related energetics permit calculation of bond strengths, heats of reaction for ionization and decomposition processes, and heats of formation of ions, radicals, and molecules. Such thermochemical values can in turn be used to test postulated mechanisms. Metastable peaks differ from normal peaks in a mass spectrum in that they are more diffuse and appear at nonintegral mass numbers, and they are thus readily identified. Whereas normal peaks are produced by ions that undergo no further decomposition after leaving the ionization chamber, metastable peaks originate from decompositions occurring in a specified portion—or two such portions in double-focusing instruments—of the subsequent flight path. Most importantly, the apparent mass of a metastable peak nearly always determines uniquely the ionic masses before and after decomposition and to this extent defines one step in a contributing decomposition path.

The use of isotopic tracers has been almost certainly the most fruitful single approach. In principle, and to a large extent in actual practice, labeling can establish unequivocally what parts of the original molecule have been incorporated into the various decomposition products. The spectrometer detects and measures the resultant mass differences and hence yields information on the extent of label retention in every ion in the spectrum.

The logical path from specifically labeled compounds by way of mass spectra to the underlying chemistry is reversible. In a system for which the chemist has done his homework, that is, in which he has established the pertinent reaction paths, mass spectra can usually be used to locate a label within a labeled molecule, as well as to assay the isotopic purity of the material. Depending on the nature of the reactions involved, the extent of localization may vary from a single precisely defined point to a specified portion of the molecular structure.

The great bulk of work in this area has, of course, been done with deuterium, but heavier nuclei—principally $^{13}C$, $^{15}N$, and $^{18}O$—have also been used. Deuterium-labeling can sometimes fully define the aspects of a reaction that are of interest; even in these cases independent confirmation by $^{13}C$-labeling or other labeling is reassuring. Very often, $^{13}C$-labeling can furnish information complementing that gained by means of deuterium, and the two together can be used to detect and clarify fine detail of reaction mechanisms. Let us illustrate.

### Illustrative Examples

The spectra of several neopentyl esters of carboxylic acids show small peaks indicating the loss of 30 mass units; the corresponding neopentyl-1,1-$d_2$ esters lose 32 mass units, but deuterium incorporated into the acid portion of the molecule is retained (*4*). Evidently the neutral product is $CH_2O$ comprising the $\alpha$-methylene group and the attached oxygen atom. Three neopentyl-1-$^{13}C$ esters all lose the label in this process, as expected (*4*). In contrast, consider the scrambling of atoms that takes place during decomposition of toluene under electron impact (*5*). Toluene loses a hydrogen atom and

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Table 1. Partial spectra of tertiary butyltoluenes.* Entries in columns 2 through 6 are relative intensities defined by assigning a value of 100.0 to the most abundant ion in each spectrum.

| Mass | Reaction products of neopentane with | | | Reference spectra | |
|---|---|---|---|---|---|
| | Toluene-$d_0$ | Toluene-$\alpha$-$d_3$ | Toluene-$d_8$ | *m-tert*-Butyltoluene† | *p-tert*-Butyltoluene† |
| 155 | | | 23 | | |
| 151 | | 22.4 | | | |
| 148 | 23.2 | | | 24.7 | 22.6 |
| 140 | | | 100 | | |
| 136 | | 100.0 | | | |
| 133 | 100.0 | | | 100.0 | 100.0 |
| 126 | | | 13 | | |
| 122 | | 6.7 | | | |
| 119 | 7.1 | | | 0.5 | 0.4 |
| 112 | | | 23 | | |
| 108 | | 32.7 | | | |
| 105 | 35.6 | | | 37.4 | 31.4 |

* Measured with 70-volt electrons on a Consolidated model 21-103 instrument. † American Petroleum Institute standard samples.

acetylene in succession. The spectra of deuterium-labeled toluenes show almost complete scrambling before the loss of hydrogen and complete scrambling before the loss of acetylene (*5, 6*). The inferred high symmetry of the intermediate $C_7H_7^+$ ion, pictured as the seven-sided tropylium ion rather than the previously assumed benzyl ion, received striking confirmation from $^{13}C$-labeling. The degree of label retention in $C_5H_5^+$ derived from toluene-$\alpha$-$^{13}C$, 70 percent, agreed almost exactly with the value predicted for the random loss of two out of seven carbons, 71.4 percent (*5, 7*). The precise route by which the ring expands and the component atoms all lose position identity remained obscure, however, and the hope of gaining further clarification prompted a study of toluene-$\alpha$,1-$^{13}C_2$ (*8*). The distribution of $C_5H_5^+$-$^{13}C_0$, $C_5H_5^+$-$^{13}C_1$, and $C_5H_5^+$-$^{13}C_2$ indicates complete randomization of the seven carbons in the path leading to $C_5H_5^+$. The observed distribution and that calculated for such randomization are, respectively: 6.0, 45.7, and 48.3 percent; and 4.8, 47.6, and 47.6 percent. Similarly, the approximately random loss of three out of the original eight hydrogens of 1-butene as methyl groups in the decomposition to $C_3H_5^+$ (*9, 10*) involves the loss of a carbon also taken at random from the entire molecule (*10*); in the spectrum of 1-butene-4-$^{13}C$, the label is retained in 75 percent of the $C_3H_5^+$ ions. In both cases, hydrogen scrambling does not imply, as has sometimes been vaguely hinted, that the hydrogen atoms wander freely over the carbon skeleton. Rather, even though the exact sequence of events is still not certain, hydrogen scrambling is clearly a reflection of a drastic skeletal reorganization.

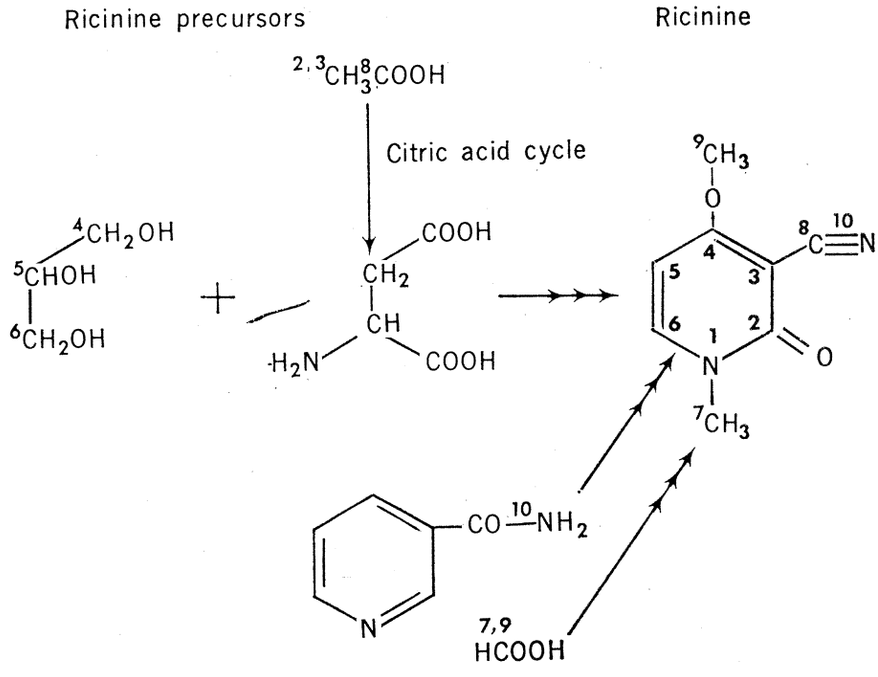
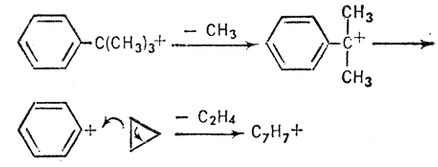



Fig. 1. Labeling pattern of ricinine formed by *Ricinus communis* L. plants from isotopically labeled precursors.

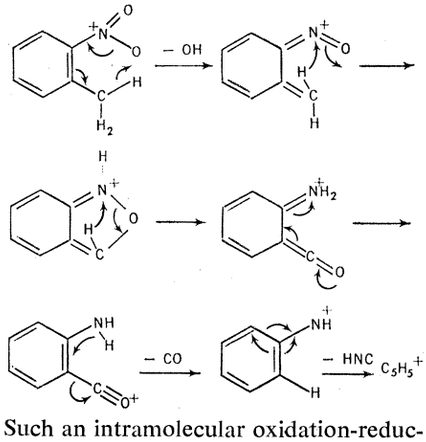
Lest the last two examples paint too bleak a picture, let us look at a few examples of molecules that are better behaved, at least from our point of view. Under electron impact *tert*-butylbenzene breaks down with the loss of $CH_3$ and $C_2H_4$ in succession. Labeling with $^{13}C$ in the $\alpha$-position confirmed that the methyl group lost is one of the original methyl groups and suggested that the ethylene is derived from the remainder of the side chain after a reorganization in which the three remaining side-chain carbons lose identity (*5, 11*). All the ions derived from the parent molecule by the loss of a methyl group and about one-third of the $C_7H_7^+$ ions retain the label. The intermediate is pictured as a phenyl cation coordinated with cyclopropane:




Deuterium-labeling points to the same intermediate in the decomposition under electron impact of 3-phenylpentane (*12*), 1-nitro-1-phenylpropane (*13*), and 2-nitro-1-phenylpropane (*13*), and to a similar species with two methyl side chains on the cyclopropane ring in the decomposition of 3-ethyl-3-phenylpentane (*5*). The spectra of the tertiary butyltoluenes closely resemble that of *tert*-butylbenzene with all the major peaks displaced 14 mass units; this suggests that the reaction paths for the decomposition of tertiary butyltoluenes and *tert*-butylbenzene are the same, with the added methyl group simply going along for the ride. Toluene has been alkylated with neopentane by gamma irradiation in the gas phase to yield a mixture of isomeric tertiary butyltoluenes plus about 10 percent *sec*-butyltoluene (*14*). Table 1 shows partial mass spectra of the products so obtained from toluene-$d_0$, toluene-$\alpha$-$d_3$, and toluene-$d_8$, as well as reference spectra of *m-tert*-butyltoluene and *p-tert*-butyltoluene (*15*). The parent peaks of the butyltoluenes derived from toluene-$\alpha$-$d_3$ and toluene-$d_8$ contain three and seven deuterium atoms, respectively, and the deuterium is retained quan-

titatively in the fragment ions arising by successive loss of $CH_3$ and $C_2H_4$. These findings support the conclusions drawn from $^{13}C$-labeling and demonstrate that labels in the ring and side chain of tertiary butylarenes are cleanly distinguished by mass spectrometry. The peaks at masses 119, 122, and 126 are attributed to primary loss of $C_2H_5$ from *sec*-butyltoluene.

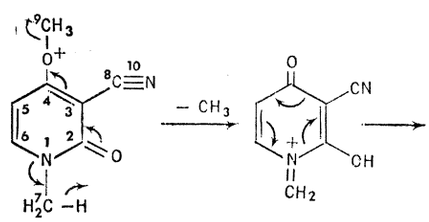
The spectrum of *o*-nitrotoluene furnishes a striking example of high specificity, as shown by deuterium- and $^{13}C$-labeling, through a rather complex reaction sequence. In a major decomposition path, this molecule loses OH, CO, and HCN in succession. The fact that this path is absent in the spectra of the isomeric nitrotoluenes suggested that the hydroxyl hydrogen is derived from the methyl group; this inference was confirmed by deuterium-labeling of the methyl group (*16*). Labeling with $^{13}C$ showed that the methyl carbon is lost as CO in the second step (*17*), and deuterium-labeling showed that the hydrogen lost as HCN in the third step is derived mainly if not solely from the methyl group (*16*). The sequence thus clearly involves an interchange of hydrogen and oxygen atoms between the nitro and methyl groups:




Such an intramolecular oxidation-reduction is apparently also involved in the pyrolytic conversion of *o*-nitrotoluene to anthranilic acid (*18*), as well as in numerous other thermal and photochemical rearrangements of *o*-nitroarenes. The parallel here is of particular interest. In seeking such parallels between reactions in the mass spectrometer and those in more conventional systems (*19*), attention has been focused chiefly on readily recognizable instances in which products or well-defined intermediates in the two contexts can be represented by a single chemical species, differing at most by the presence or absence of a net charge. In the case of *o*-nitrotoluene, the identifiable products resulting from electron impact and from pyrolysis are altogether different species, but labeling and other evidence establishes a common series of events at the molecular level incorporated into the two reaction sequences.

## A Biosynthetic Application

Lastly, we would like to call attention to a somewhat unusual example of the use of mass spectrometry in a biosynthetic study (*20*). The compound under study was ricinine, extracted from the castor plant. Prior work with $^{14}C$ and $^{15}N$ had established the positions of isotopic labels in ricinine formed from certain precursors labeled in specified positions, as shown in Fig. 1: (i) $^{15}N$ in the amide group of nicotinamide appears solely in the nitrile group of the derived ricinine; (ii) the tagged carbon atoms introduced as formate are distributed equally between the *O*- and *N*-methyl groups of ricinine; and (iii) tagged carbon atoms in the 2-position of acetate emerge chiefly as C-2 and C-3 of ricinine, with no contribution to C-4, C-5, and C-6. The nitrile group of ricinine is derived from the carboxamide group of nicotinamide, presumably through an intramolecular dehydration, and the pyridine ring of nicotinamide becomes the pyridine ring of ricinine. The spectrum of ricinine contains a prominent peak at mass 82, shown by high-resolution mass measurement to be due to a $C_4H_4NO^+$ ion, which arises by sequential loss of $CH_3$ and $C_3HNO$. Mass spectra of ricinine samples prepared biosynthetically from nicotinamide-$^{15}N$, formate-$^{13}C$, and acetate-2-$^{13}C$ were measured. None of the label from the nicotinamide or acetate, but about half that from the formate, was retained in the fragment ion. The ion thus contains N-1, C-4, C-5, C-6, either C-7 or C-9, and presumably the methoxyl oxygen and all but one of the hydrogens bound to these atoms in the original molecule. The results can be represented mechanistically by the path:




$CH_2{=}N^+{=}CHCH{=}C{=}O$ + $HOC{\equiv}CC{\equiv}N$
mass 82 mass 67

Once this path was known, mass spectrometry could be used to extend biosynthetic studies of ricinine to other precursors, the spectrum now serving to locate the labeled precursor atoms in the ricinine structure. Thus, in the spectrum of ricinine derived from formamide-$^{15}N$, the parent ion was labeled but the $C_4H_4NO^+$ ion was devoid of label, thus showing that nitrogen from this precursor was incorporated solely in the cyano group. Thus, each new bit of knowledge derived from either the biosynthesis or the electron impact–induced decomposition can be fed back into the loop consisting of the two reaction systems and can help to advance knowledge of the other system as well.

In retrospect, the substantial existing body of knowledge of reactions of organic molecules induced by electron impact furnishes a frame of reference and a good foundation for the use of mass spectra as a way of following $^{13}C$-labels in studies of reaction mechanisms. Conversely, the more widespread use of $^{13}C$-labeling can be confidently expected to contribute a great deal to the further growth of that body of knowledge (*21*).

## Summary

The use of stable isotopic tracers has been probably the most fruitful single technique used in accumulating the substantial existing body of knowledge of the chemistry induced by electron impact in the mass spectrometer. Most such labeling has been done with deuterium, but considerable experience has been gained with $^{13}C$ as well. This experience clearly establishes both the utility of $^{13}C$-labeling in extending knowledge of the reactions underlying mass spectra and the reciprocal utility of mass spectra in the investigation of reaction mechanisms, not only by making possible the measurement of total $^{13}C$-incorporation into a molecule, but also by the location of the label within the molecular structure.

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# Spectre II: General-Purpose Microscope Input for a Computer

## Modular design and digital control facilitate optical measurements in biology.

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A connection between a digital computer and an optical microscope is considered to be essential for the solution of several classes of problems in biology and medicine. Automatic control facilitates quantitative microspectrophotometric histochemical studies (*1*) for which the limitations of the human eye as a colorimeter have necessitated various electronic additions to the microscope itself. Also, the difficulty of training human observers in diagnostic cytology and hematology, and the amount of material to be observed, provide motivation for the development of automated image-processing systems to be used in clinical screening. Finally, techniques for automatic analysis of microscopic images and storage and retrieval of such images as data would significantly advance the study of the three-dimensional microarchitecture of tissues.

Several systems incorporating an interface between microscope and computer have been built and described (*2–5*). The design of most of these has been restricted by the particular application for which the system was intended. The apparatus described in this article is designed to be as flexible as possible so that, in addition to being employed as a research instrument in the above-mentioned areas, it can be used to evaluate optical, electronic, and computer system requirements for the solution of specific problems.

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### Microspectrophotometry

The microspectrophotometric analysis of biological specimens (*6, 7*) involves two dissimilar procedures. First, the area of the specimen to be analyzed must be placed in the light path of the photometer. This requires a complex and ill-defined series of decisions on the part of a trained biologist. The process becomes intuitive, and is rapidly performed. The second procedure, the actual measurement of light absorption, fluorescence, and so forth, at one or more wavelengths is operationally well defined, but it is time-consuming even for skilled experimenters. It therefore seemed logical to separate the two phases of microspectrophotometry in an initial attempt at automation. The first step in this direction was the development of a microscope stage with a "memory." This permits the biologist to control stage motion and focus controls, and to record the position coordinates of all areas of interest on a given specimen, for example, a stained slide. A list of objects to be measured and the measurements to be made on each is transmitted to the photometer controller. The controller, now unattended, makes a second pass over the slide. At this time the measurements requested are made automatically, with any change of wavelength accomplished by a motor-driven monochromator, on all areas of interest on the slide. A list of the measured values is returned to the experimenter. To our knowledge, no other apparatus currently used for biological microspectrophotometry is automated to even this extent.

### Scanning Optical Microscopy

Two biological research programs led us to develop an instrument to scan microscopic fields and produce digitized images for analysis by computer. The first of these required a comparison of reflectance photometry (*8*), special-purpose image-analyzing devices (*9, 10*), and more general computer techniques for counting grains in blood cell autoradiographs. The second involved the creation of a formal system for the description and analysis of the structure of nervous tissues (*11*). Both of these projects, and particularly the latter, give rise to an unmanageable amount of stored data when handled by conventional scanning techniques.

Consider that the practical limit of lateral resolution in light microscopy is of the order of 0.25 micrometer. Although the limit of vertical resolution is less simply expressed, we can approximate a three-dimensional resolution element in the object plane by a cube 0.25 micrometer on a side. A