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Carbon-13 as a Label in Biosynthetic Studies

Carbon-13 nuclear magnetic resonance spectroscopy is useful for the elucidation of biosynthetic pathways.

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In the elucidation of the biosynthetic pathways leading to secondary metabolites, one of the classical approaches consists of the feeding of precursors labeled in specific positions with carbon-14 to the appropriate culture. The metabolite in question is then isolated and the labeling pattern of the carbon skeleton is established by careful and often tedious degradation procedures which should proceed with reasonable yield and lead to unambiguous products. In the ideal case, the level of radioactivity can be established for each carbon atom of the metabolite. However, in most cases this is not possible, and only a few locations are usually checked for labeling.

When carbon-13 labeled precursors are fed, labeling of the molecule may be detected by mass spectrometry (*1*) without the necessity of degradation. However, the exact location of the enriched sites is problematic, since only the total enrichment of each fragment ion can be measured, and therefore the exact scheme of mass fragmentation must be established.

Carbon-13, having a nuclear spin $I = ½$, lends itself to nuclear magnetic resonance (NMR) experiments (*2*, *3*). Thus the advent of highly sensitive NMR spectrometers either equipped with a computer of average transients (CAT) or operating in the pulse Fourier transform mode (PFT) (*4*), as well as the ever growing amount of data available on ^{13}C chemical shifts, ^{1}H-^{13}C and ^{13}C-^{13}C coupling constants, have provided the chemist with two new and elegant possibilities for the detection of ^{13}C-labeled carbon atoms in biosynthetic studies: the observation of ^{13}CH satellite signals and the direct measurement of carbon magnetic resonance (CMR) spectra. The ease with which labeling patterns can be established with the use of these methods is reflected by the exponential increase in the number of publications dealing with this subject. Whereas Lukacs' review of the topic (*5*) listed only 12 papers that had appeared before mid-1971, and Grutzner's (*6*) about 20, papers that covered the literature through mid-1972, the number has now grown to more than 60.

Many common biogenetic precursors like acetate, propionate, methionine, and related compounds are now commercially available and contain as much as 90 percent ^{13}C in specific positions. Yet the selection is still limited by comparison with ^{14}C-labeled substrates. Appropriate precursors usually have to be synthesized by standard procedures for the synthesis of isotopically labeled compounds, the advantage being the stability of the ^{13}C nucleus. In the past 8 years since the classical publication of Tanabe and Detre on the biosynthesis of griseofulvin with ^{13}C-labeled acetate (*7*), some larger precursors have been prepared and fed, including butyrate (*8*), mevalonate (*9–11*), serine, proline (*12*), tryptophan (*13*), phenylalanine (*14*), and four different valines (*15*), two of which contain a chiral ^{13}C labeling at the isopropyl group (*16*, *17*). For the elucidation of the biosynthesis of corrins and porphyrins even more complicated substrates have proved necessary—for example, δ-aminolevulinic acids (*18–21*), porphobilinogens (*21–23*), pyrromethanes (*24*), and uroporphyrinogens (*22*) with labels in different positions. Very recently [6-^{13}C]glucose and [1-^{13}C]-glucosamine were synthesized and successfully used in a study of the antibiotic neomycin (*25*).

Satellite Method

Since ^{13}C has a nuclear spin $I = ½$, hydrogen atoms attached to a ^{13}C atom show spin coupling, the coupling constant usually being in the range of 100 to 250 hertz (*3*). Therefore in a regular ^{1}H NMR spectrum each proton resonance is accompanied by two satellite bands (*26*), one at higher field and one at lower field than the main signal, their spacing being equal to the coupling constant J_{CH}. Since the natural abundance of the nuclide ^{13}C is 1.1 percent, the satellite signals in nonenriched molecules have very low intensity, each about 0.55 percent of the parent signal. They usually cannot be observed in a single sweep in the NMR experiment, and time averaging techniques must be used.

The feeding and subsequent incorporation of ^{13}C-labeled precursors into a metabolite will cause certain locations in the carbon skeleton of the molecule to contain more ^{13}C than the 1.1 percent corresponding to the natural abundance of this isotope. Accordingly the ^{13}CH satellite signals corresponding to labeled sites will have increased intensities as compared with those corresponding to unlabeled sites. Thus it

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is possible to deduce from a ^1H NMR spectrum which carbon atoms in the molecule have been labeled by the precursor fed.

However, several limitations of this method are as follows:

1) Only carbon atoms that have hydrogens attached can be checked for enrichment with ^{13}C. It is sometimes necessary to convert the metabolite into a more suitable form; for example, the reduction of a carbonyl group will convert this carbon atom into one with a hydrogen attached.

2) An unambiguous assignment of the proton NMR spectrum is mandatory. In large molecules at least those segments where ^{13}C labeling is to be detected must be rigorously assigned.

3) In order to detect any enrichment at a specific site, one must be able to observe at least one of the two ^{13}CH satellite bands. This requirement is often hard to fulfill. The satellites may overlap with the strong main signals of other protons, with signals of impurities (*27*), or even with other satellite bands. Such an overlap interferes, of course, with the determination of the intensity of the satellites. Some advantage may be gained by using paramagnetic shift reagents to separate the overlapping bands (*5*).

4) The incorporation of the precursors fed must be high enough to give a distinct enhancement of satellite intensity in order to decide whether a given carbon atom has been labeled, and to what extent.

The different satellite bands are identified by their shape and positions relative to the respective main signals. In an ideal case both satellites can be observed. This, of course, helps their identification and allows a more accurate determination of their intensity by taking the average of the two.

To investigate whether a satellite signal has increased intensity as compared with natural abundance, two methods may be used. The peak area of the satellite in question may be compared with the area of a satellite corresponding to protons attached to a carbon atom which has not been labeled, thus serving as an internal standard [see, for example, the study on piericidin A (*28*)]. A second possibility is to compare peak areas of the same satellite in the spectrum of the labeled metabolite with those in the spectrum of the substance containing ^{13}C only in natural abundance. In this case the two NMR spectra must be recorded under rigorously identical conditions. This technique was used, for example, to determine the enrichment of some of the carbon atoms in labeled variotin (*29*). It is obvious, that the results from this second method will not be as accurate as those obtained from comparison with an internal standard.

The satellite method was mainly used in the years before 1970. Seven out of eight papers reporting labeling patterns established in this manner, together with some of the early results with the direct CMR approach, were reviewed by Lukacs in early 1972 (*5*). An additional application of the satellite method dealt with the biosynthesis of mollisin (see Fig. 1), a yellow naphthoquinone pigment isolated from cultures of the mold *Mollisia caesia* (*30*). Earlier radiocarbon experiments had shown that the methyl groups did not originate from methionine, but that acetate and malonate were good precursors. As a result of feeding of [1-^{14}C]acetate, a Kuhn Roth degradation of mollisin showed that carbon atoms 2 or 7 contain one-third of the total radioactivity; however, the distribution of the label between these two could not be determined. A biosynthetic pathway was proposed featuring two tetraacetate chains (pathway A in Fig. 1). In order to determine the relative extent of labeling of the two acetate chains, Tanabe and Seto (*30*) fed [2-^{13}C]acetate. From Fig. 1 it can be seen that, assuming pathway A to be true, the methyl carbon of acetate should label eight carbon atoms, that is, those at positions 1, 3, 6, 8, 10, 11, 12, and 14. Three of these (carbons 1, 8, and 10), however, cannot be checked for enrichment, since they have no hydrogens attached. The proton NMR spectrum of mollisin showed clearly separated signals, and both the satellites could be observed for the protons at-

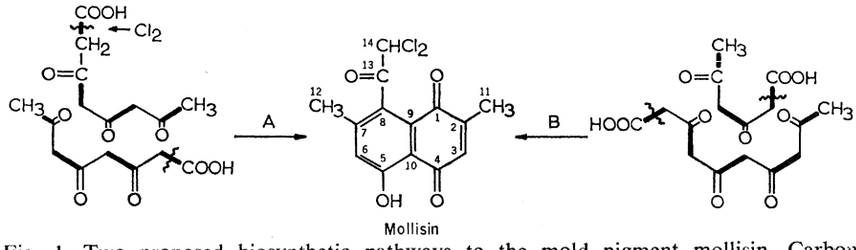


Fig. 1. Two proposed biosynthetic pathways to the mold pigment mollisin. Carbon atoms originating from the same acetate unit are linked by heavy lines.

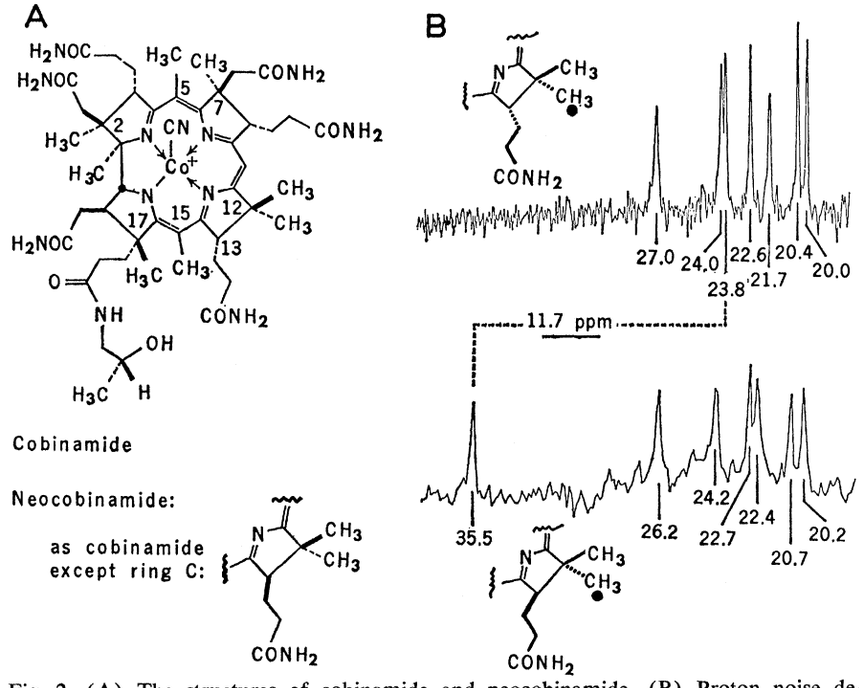


Fig. 2. (A) The structures of cobinamide and neocobinamide. (B) Proton noise decoupled CMR spectra of [$^{13}CH_3$]methionine (●), enriched dicyano cobinamide (top), and dicyano neocobinamide (bottom). [Redrawn from the *Journal of the American Chemical Society* (*37*)]

tached to carbon atoms 3, 6, and 14, whereas only the downfield satellites belonging to the C(11) and C(12) protons were visible. The extent of labeling in mollisin was determined by comparison of the satellite peak areas with the corresponding peak areas in the spectrum of unlabeled material. All five carbon atoms examined proved to be labeled by [2-^{13}C]acetate, and labeling occurred in all positions to the same extent within experimental error. The overall ^{13}C enrichment of the metabolite was determined by mass spectrometry and found to be consistent with the NMR experiments. All these findings were in agreement with the proposed pathway A and show that the two acetate chains are labeled to the same extent. A more recent study by Seto, Cary, and Tanabe (*31*), however, with ^{13}C NMR showed that probably a different pathway, pathway B in Fig. 1, featuring a triketide and a pentaketide is involved.

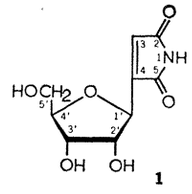
Although used rather infrequently today, the satellite method is by no means obsolete. It is still very valuable in those cases where only a minute amount of labeled metabolite is available for the NMR measurement. Here the more sensitive ^{1}H NMR technique may be advantageous. This is shown in a study by Battersby, which resulted in an unambiguous assignment of the "biosynthetic stereochemistry" at C(12) of vitamin B_{12} (*20*), a result which was independently derived by the "direct" ^{13}C method.

Direct CMR Method

The first paper reporting the direct observation of biosynthetically labeled carbon atoms with the use of CMR spectroscopy was published by Tanabe and his co-workers (*32*). At that time (early 1970) most of the CMR spectra were still measured with the continuous wave technique. As a result of the low sensitivity of the ^{13}C nucleus in an NMR experiment, the recording of natural abundance CMR spectra was not easy. Tanabe's study of radicinin (*32*), a metabolite isolated from the fungus *Stemphyllium radicinum*, elegantly avoided this difficulty. It had been suggested earlier that all the radicinin carbon atoms originated from acetate units. The ^{13}C-labeled acetates fed by Tanabe *et al.* were so well incorporated that the CMR spectra of the now highly enriched samples could easily be measured. However, only the enriched carbon atoms could be detected in the spectrum, the resonances of those containing ^{13}C at natural abundance were too weak to be observed. Yet the feeding of [1-^{13}C]- and [2-^{13}C]acetate in different experiments gave two complementary spectra representing all the carbon atoms.

Today the measurement of ^{13}C resonances at natural abundance has become easier; highly sensitive NMR instruments operating in the PFT rather than in the continuous wave mode are widely used. But the low sensitivity of the ^{13}C nucleus relative to that of the proton still poses problems. In biosynthetic studies very small samples of metabolite are usually obtained; in order to reach a reasonable concentration (preferably 0.3*M* or more), the sample volume is kept small and therefore has to be measured in a microcell or in a regular sample tube using vortex plugs. In some cases, however, the problem is not the absolute amount of material available but rather its low solubility. Here a good signal-to-noise ratio can be obtained by measuring the spectrum in sample tubes with much larger diameters (*33*) than the 8- to 13-mm tubes commonly used.

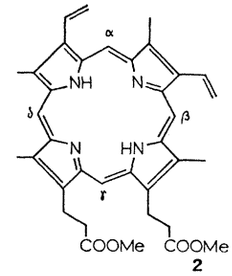
In more recent biosynthetic studies it has become customary to record the natural abundance CMR spectrum of the unlabeled metabolite. In order to be able to locate the labeled sites, unambiguous assignment of all the carbon resonances has been attempted, if possible on a one-to-one basis. Assignments are made by means of the well-established procedures common in ^{13}C NMR spectroscopy (*2*, *3*): comparison with derivatives, comparison with model compounds (*34*), and off-resonance and specific proton decoupling. However, assignments may also be made from biosynthetic evidence obtained from earlier studies. Thus the two carbonyl groups of the maleimide moiety in showdomycin (**1**) could easily be distinguished in the CMR spectrum after feeding [1-^{13}C]acetate:



1

Showdomycin

it was known from a previous study that ^{14}C from [1-^{14}C]acetate was only incorporated into C(5) (*35*). Another method for the assignment of carbon resonances consists of the chemical synthesis of model compounds where the sites of interest have been specifically enriched with ^{13}C. Thus Battersby and co-workers undertook the synthesis of three protoporphyrin-IX dimethyl

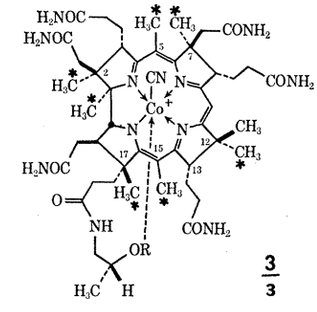


2

Protoporphyrin-IX dimethyl ester

esters (**2**), each labeled at a different meso carbon (*36*). From the CMR spectra of these compounds, the chemical shift of each of the four meso carbons (designated in the formula by α, β, γ, δ) could easily be determined. These assignments were the basis for a study concerning the incorporation of porphobilinogen (*23*) and pyrromethanes (*24*) into the porphyrin nucleus.

A one-to-one assignment of all the carbon atoms in complex molecules is, however, not always possible. Yet in most of these cases valuable information about biosynthetic pathways can be obtained even if only a partial assignment of the natural abundance spectrum is possible. This approach is illustrated in recent studies of vitamin B_{12} (**3**) (*19*, *37*). The natural abundance spectrum of vitamin B_{12}



3

Vitamin B_{12}

R = phosphate · ribose · dimethylbenzimidazole → Co

was recorded by Doddrell and Allerhand (*38*), and some assignments were made. However, in view of the complexity of the molecule (vitamin B_{12} has the molecular formula $C_{63}H_{88}CoN_{14}O_{14}P$), a one-to-one assignment within certain groups of resonances was not possible. Yet, the question of whether six or seven of the "extra" methyl groups of vitamin B_{12} (designated in the formula with *) origi-

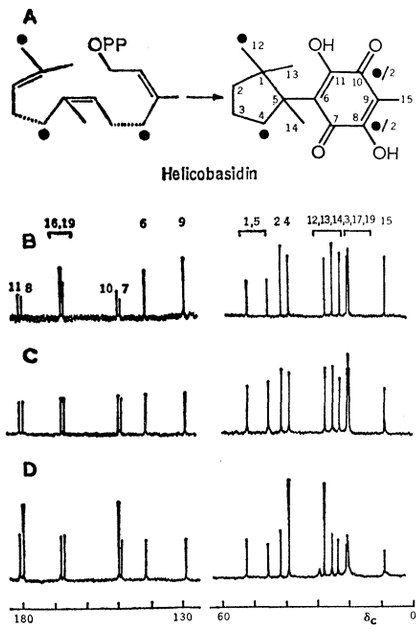


Fig. 3. (A) Helicobasidin and its formation from three isoprene units. (B, C, and D) Proton noise decoupled CMR spectra of helicobasidin diacetate. (B) Natural abundance; (C) natural abundance, 0.1*M* tris(acetylacetonato)chromium (III) added; (D) [2-^{13}C]mevalonate enriched, 0.1*M* tris(acetylacetonato)chromium (III) added. [Redrawn from *Tetrahedron Letters* (*9*)]

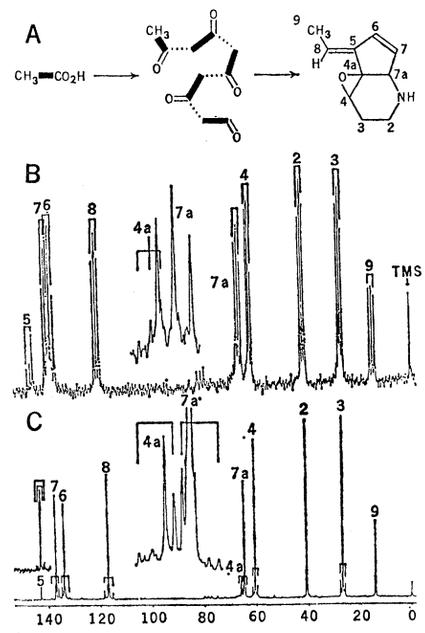


Fig. 4. (A) Dihydrolatumcidin and its formation from acetate. (B) Proton noise decoupled CMR spectrum of dihydrolatumcidin obtained after feeding of [1,2-$^{13}C_2$]acetate. (C) Proton noise decoupled CMR spectrum of dihydrolatumcidin obtained after feeding of a 1 : 1 mixture of [1-^{13}C]acetate and [2-^{13}C]acetate. [From (*49*); courtesy of the American Chemical Society]

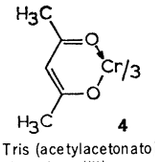
nated from methionine could easily be answered by feeding [$^{13}CH_3$]methionine to a culture of *Propionibacterium shermanii*. The CMR spectrum of the resulting vitamin B_{12} (measured in the dicyano form) showed *seven* strongly enhanced signals (*19*). Another question still remained: which of the two geminal methyl groups at C(12) originated from the decarboxylation of an acetate side chain and which from methionine. The specimen of vitamin B_{12} obtained from the feeding experiment with [$^{13}CH_3$]methionine was degraded with trifluoroacetic acid to give cobinamide together with neocobinamide, the two differing only in the stereochemistry at C(13) (Fig. 2). In the CMR spectrum of the neocobinamide (measured in the dicyano form) one of the seven resonances corresponding to the labeled methyl groups was shifted 12 parts per million downfield as compared with cobinamide. This effect could be accounted for, if one assumes that the labeled (and thus methionine-derived) methyl group has the α configuration. Its resonance in the neocobinamide, where this methyl group is not in a synperiplanar relation to the propionamide at C(13), would be expected to be at lower field than in the cobinamide, because of the absence of the γ effect (*39*). This is indeed observed, and the stereochemistry of the methionine-derived methyl at C(12) is thus truly established as being α (*37*).

To determine whether a position of the carbon skeleton contains more ^{13}C than that corresponding to natural abundance, the spectra of the unlabeled and labeled metabolites are compared. Enhanced signals in the spectrum of the labeled metabolite correspond to labeled carbon atoms.

However, the comparison of spectra, even when recorded under identical conditions, or the comparison of peak intensities within one spectrum can present problems. Often the peak integral is not proportional to the ^{13}C content of the site in question (*2*, *6*) because of differences in nuclear Overhauser enhancement from proton noise decoupling, and in PFT experiments because of short recycling times as compared with the different relaxation times of the different carbon atoms involved. Uncertainties in peak intensities may, however, also arise from poor digitalization of data during accumulation or transformation.

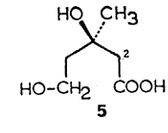
Fortunately small precursors are usually well incorporated by microorganisms, and in many of the cases studied the enriched sites contained 4 percent or more ^{13}C (including the 1.1 percent arising from natural abundance), thus facilitating the detection of the labels in the carbon skeleton. Where the extent of enrichment could not be determined with reliability, it was usually confirmed by mass spectrometry or by measuring ^{13}CH satellite peak areas.

Another method for obtaining more accurate intensity measurements is to equalize the differences in peak intensities arising from various nuclear Overhauser effects and relaxation times by adding a paramagnetic relaxation reagent such as tris(acetylacetonato)chromium (III) (**4**) (*40*).



4

Tris (acetylacetonato) chromium (III)



5

Mevalonic acid

This paramagnetic complex will not induce shifts in the carbon resonances, but will quench the nuclear Overhauser effect and shorten the relaxation times. With careful selection of the pulse interval in the NMR experiment it is then possible to obtain natural abundance CMR spectra with nearly equalized peak intensities. It is obvious that with this technique, even relatively small enrichments can be determined with certainty (*41*). Thus, when [2-^{13}C]mevalonate (**5**) was fed to *Helicobasidium mompa* (*9*), the isolated sesquiterpene helicobasidin was (compare Fig. 3) supposed to carry a label in positions 4 and 12 and half a label at C(8) and C(10), the label arising from the third isoprene unit being equilibrated between the two positions because of tautomerism of the molecule. However, the spectrum of the enriched sample of helicobasidin showed very large variations in intensities and no firm decision could be made whether C(8) and C(10) had been labeled. After bringing the sample solution to 0.1*M* in **4**, however, an equal enrichment of C(8) and C(10) could easily be detected (*9*) (Fig. 3).

Yet a further difficulty may occur when trying to assign the sites of enrichment from the CMR spectrum: two carbon resonances may overlap. It is of course not possible to determine merely from an increase of intensity of such a signal which of the two carbon atoms actually carries the label. However, it may be possible to separate the resonances by the addition of a paramagnetic shift reagent such as Eu(fod)$_3$ (*42*).

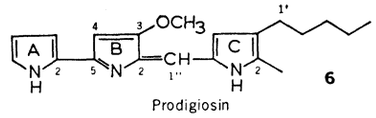
This procedure was successfully applied to the biosynthetic study of the fungal metabolites hirsutic acid C and complicatic acid (*43*).

Carbon-Carbon Coupling

Two ^{13}C nuclei may show coupled resonances (as is familiar from the proton NMR spectra), the C-C coupling through one bond being the largest and most important one. In natural abundance CMR spectra a coupling between two ^{13}C nuclei is usually not observed, since the probability of having two ^{13}C atoms in adjacent positions is equal to the square of the natural abundance: the two signals corresponding to a ^{13}C resonance split by coupling with a second ^{13}C nucleus therefore have an intensity of only 0.55 percent of that of the main signal. However, in the spectra of some biosynthetically enriched metabolites C-C coupling has been detected because of the higher level of ^{13}C in the labeled positions.

Thus the detection of resonances corresponding to coupled ^{13}C nuclei is usually proof that two labels have been incorporated into adjacent positions of the carbon skeleton of the metabolite under investigation. This can occur when an acetate derived chain is cyclized to give a five-membered carbocyclic ring, as in dihydrolatumcidin (*44*) or helicobasidin (*9*). Other metabolites, where C-C coupling was detected after a feeding experiment are sterigmatocystin (*45*), protoporphyrin-IX (*23*), vitamin B_{12} (*18, 19*), and fusidic acid (*46*).

Labeling of adjacent positions may also occur when the precursor is metabolically transformed in a primary sequence before incorporation into the secondary metabolite in question. This was observed when feeding [2-^{13}C]glycine to *Serratia marcescens* (*12*) in the presence of serine and recording the CMR spectrum of the prodigiosin (**6**)



Prodigiosin **6**

produced. The carbon in the 2 position in the B ring and the carbon at 1″ were strongly enriched and showed coupling. Glycine was obviously transformed to doubly labeled [2,3-$^{13}C_2$]serine by the known glycine-serine interconversion [all the acetate derived carbons (*47*) were also labeled]. In the case of avenaciolide, a metabolite of *Aspergillus*

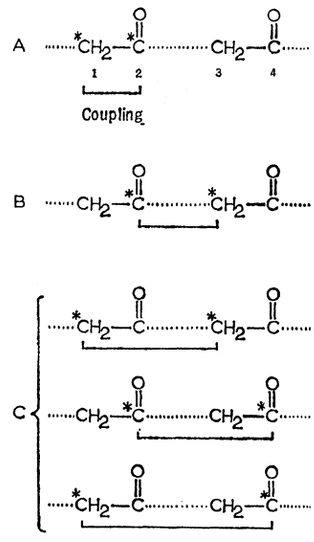


Fig. 5. Double labeling of acetate derived chains. (A) Pattern obtained after feeding [1,2-$^{13}C_2$]acetate [note that coupling between C(2) and C(3) will only occur when two labeled acetate units are incorporated simultaneously]. (B and C) Patterns obtained from feeding a 1 : 1 mixture of [1-^{13}C]- and [2-^{13}C]acetate, pattern B giving rise to 1,2-coupling and pattern C to 1,3- or 1,4-coupling.

avenaceus, acetate was transformed in the citric acid cycle to doubly labeled succinate, which was then incorporated. The corresponding C-C coupling could be observed (*48*).

Double Labeling Experiments

A very useful method for the investigation of acetate-derived compounds was introduced in late 1973 by Seto and co-workers (*49*), using dihydrolatumcidin as an example (compare Fig. 4). The experiment consists of either feeding [1,2-$^{13}C_2$]acetate or a 1 : 1 mixture of [1-^{13}C]acetate and [2-^{13}C]acetate. When the doubly labeled precursor is used, all the carbon atoms derived from acetate will become labeled. Furthermore each acetate *incorporated as a whole unit* will give rise to two doublets due to C-C coupling, both doublets showing the same splitting corresponding to the coupling constant J_{CC} (*50*). Thus from the CMR spectrum, conclusions can be drawn whether acetate units were broken up prior to incorporation or during subsequent transformations leading to the isolated metabolite. A nice example, where the breaking of an acetate unit could be observed, is the study of mollisin (*31*), which suggested that this metabolite was formed through pathway B in Fig. 1. Tanabe and Suzuki recently reported on the biosynthesis of ascochlorin (*51*). This is an additional example where the loss of C-C coupling suggested that an acetate unit was broken up during biosynthetic transformation.

When administering [1,2-$^{13}C_2$]acetate, the feeding conditions have to be carefully adjusted by eventually diluting the precursor with unlabeled material. The goal is to get enough incorporation to see the two doublets arising from each acetate unit but to avoid a too high level of incorporation, which would lead to an appreciable amount of metabolite molecules containing more than one labeled acetate unit simultaneously (see Fig. 5A). This of course would give rise to coupling between carbon atoms originating from different acetate units and would unnecessarily complicate the spectrum (*52*).

In the reverse experiment, however, where a 1 : 1 mixture of [1-^{13}C]- and [2-^{13}C]acetates is fed, the highest possible incorporation is required. Then the probability of having simultaneous labeling of the metabolites by two labeled acetate units will be reasonably high, and the resulting coupling can be observed. The coupling through one bond (Fig. 5B) being large (about 30 to 80 hertz), it is easily distinguished from coupling through two or three bonds (1 to 10 hertz) arising from incorporation patterns of the labeled acetates according to Fig. 5C, which are equally probable. This experiment thus enables the investigator to assign pairs of adjacent carbon atoms derived from the linkage of two acetate units. This set of pairs is complementary to the one obtained from the incorporation of doubly labeled acetate (Fig. 4).

Double labeling experiments also prove useful in the structural elucidation of natural products since they provide information on the sequence of the atoms in the carbon skeleton of the molecule under investigation. There are two recent reports on the use of this technique in combined studies of structure and biosynthesis. The compounds investigated were the fungal metabolite ochrephilone (*53*) and the pigments tenellin and bassianin (*14, 54*).

Conclusion and Outlook

Without any doubt, the use of ^{13}C-labeled precursors makes an excellent addition to the tools already used in the elucidation of biosynthetic pathways. Besides the examples cited so far, a wide variety of secondary metab-

olites have already been investigated with the use of ^{13}C-labeled precursors, the antibiotics being by far the most numerous. Molecules studied include penicillins (*16, 17*) and cephalosporins (*15–17, 55*), ansamycins (*56*), the nucleoside antibiotic maleimycin (*57*), the lactones asperlin (*58*) and ochratoxin A (*59*), the antibiotics nybomycin (*60*), neomycin (*25*), thermozymocidin (*61*), and lasalocid A (formerly X-537A) (*8*), the antitumor metabolite epoxydon (*62*) and even two nitro-containing metabolites: pyrrolnitrin (*13*) and auerothin (*63*). Besides the examples already mentioned, that is, helicobasidin, fusidic acid, hirsutic acid, and ascochlorin, four other groups of terpenoids were investigated: the virescenosides (*64*), growth regulators from an *Acrostalagmus* species (*65*), trichothecolone (*11*), and the coriolins (*10*), the latter by the double labeling technique. Furthermore, reports can be found on the fungal tropolone, sepedonin (*66*); the benzoquinone pigment, shanorellin (*67*); and methyl palmitoleate (*68*) obtained after saponification of yeast lipids.

We believe that in the future novel metabolites will be studied more and more with ^{13}C as a biosynthetic label. The double labeling experiments are especially promising since they give simultaneously information on the biosynthesis as well as on structural properties of the molecule investigated. Thus, the era, where the structure of a secondary metabolite is first determined and only then its biogenetic origin, might well be over.

In contrast to the signal success of ^{13}C enrichment experiments with microorganisms, the application of this method to a study of higher plant biosynthesis is fraught with the difficulty of achieving greater incorporation than 0.5 to 1.0 percent (*69*), which precludes observation of enrichment via labeled precursor above the natural abundance of ^{13}C. A logical solution to this dilemma has, however, been discovered in our laboratory, and although the experimental work is somewhat tedious and as yet incomplete, the methodology merits description as a technique which holds great promise.

Thus, by using biological material whose carbon isotope composition is almost entirely (99.9 percent) ^{12}C, enrichments (from 90 to 95 percent ^{13}C precursors) of 0.3 percent or more can, in principle, be observed. The simple expedient of growing tissue cultures, or germinating seeds in an environment containing only $^{12}CO_2$ as the carbon source for several weeks ensures a very low (0.1 to 0.2 percent) ^{13}C content of the various pools of organic intermediates, cofactors, and protein. At the end of the $^{12}CO_2$ feeding experiments, the ^{13}C precursor is added; and after the appropriate time, usually 5 to 7 days, the metabolite is isolated and examined by ^{13}C FT NMR. In this way the "background" of ^{13}C natural abundance is lowered by an order of magnitude and for the first time it becomes possible to enter the domain of higher plant biochemistry with this powerful technique.

The use of ^{12}C as a "negative" label—that is, one which produces a spectrum lacking a particular signal—has already been used to study mechanisms of reactions in organic chemistry (*70*), and it has been pointed out by Roeder (*71*) that labeling with ^{12}C (> 99.9 percent) could be used in the same biosynthetic work. However, the requirement for relatively high incorporations (> 2 percent) would still have to be met, and this methodology, although important for microbial metabolism studies, cannot be used at present for biotransformation in higher plants.

A final and very important application which is now on the horizon is the utilization of ^{13}C enriched precursors for the study of human metabolism. The complication of radiation damage by ^{14}C and ^{3}H is of course absent in work with ^{13}C and we can look forward to a vigorous program of metabolic studies with mammalian and especially human biochemistry both in vitro and in vivo.

To summarize, in the few years since the introduction of ^{13}C NMR a revolution in biosynthetic methodology has taken place, and the ramifications will no doubt extend to the study of all types of biological material as well as the obvious applications with labeled drugs in medical and pharmacological research, first in animals and very soon in man.

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Avian Incubation

Interactions among behavior, environment, nest, and eggs result in regulation of egg temperature.

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"The stable, warm temperature for early avian development is common to all climates, and is maintained through an equally common prescription for temperature regulation by parental behavior" (*1*). We will consider herein the nature of some major ingredients which are formulated into this "common prescription" for incubation of the avian egg.

The importance of regulating the thermal environment of the developing embryo has been emphasized by studies on the domestic fowl by Lundy (*2*). He found that the optimal temperature for embryonic development in temperature-controlled cabinets was between 37° and 38°C, and that no embryos survived continuous incubation above 40.5°C or below 35°C. Similar results for domestic fowl, pheasant, duck, and quail were reported by Romanoff and Romanoff (*3*). It is generally recognized that 25° to 27°C is a temperature range below which no development occurs, this being the so-called physiological zero temperature. Incubation for any protracted period between physiological zero and the optimum range results in various developmental anomalies. However, maintenance in suspended development below physiological zero for moderate periods is compatible with later development at appropriate temperatures. This correlates with the observed delay in incubation which may occur between the laying of the first egg and the completion of the full clutch. These results emphasize the scope of the task faced by incubating parents in providing a thermal environment that will ensure successful embryonic development and hatching.

With rare exceptions (*4, 5*), the avian embryo is incubated by an attending adult transferring its body heat to the egg. The mean egg temperature is an approximation of the proper developmental temperature. Huggins (*6*) reported a mean egg temperature of 34.0°C ± 2.38° standard deviation (S.D.) for 37 species representing 11 orders. An extensive study of the house wren, *Troglodytes aedon*, revealed mean egg temperatures well within this range (*7*) while other reports have indicated a range of 34° to 36°C for various species (*8, 9*). The narrow range of mean egg temperatures achieved by diverse species suggests that the thermal requirements for successful development are similar for most if not all birds. This requirement must be met through incubation strategies that compensate for fluctuations in environmental temperature and that allow the attending parents to acquire sufficient food to support metabolism.

The relative responsibilities of parent birds varies from mutualistic sharing of the task to total involvement by one parent for the entire incubation

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