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The Quantitative Analysis of Carbon–Carbon Coupling in the ^{13}C Nuclear Magnetic Resonance Spectra of Molecules Biosynthesized from ^{13}C Enriched Precursors[1]

Robert E. London, Victor H. Kollman, and Nicholas A. Matwiyoff*

Contribution from the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received November 25, 1974*

Abstract: The quantitative dependence of the intensities of the various multiplet lines arising from ^{13}C–^{13}C nuclear spin coupling as a function of ^{13}C enrichment is considered. Two cases are distinguished, depending on whether or not the enrichment of the interacting carbons is statistically independent. For statistically independent labeling, the splitting is simply related to the enrichment of the various carbons. For cases in which this condition does not hold, the splitting provides a measure of the correlation in the enrichment of interacting carbons. The quantitative analysis of ^{13}C–^{13}C coupling is shown to provide additional information in biosynthetic experiments in which a correlation in the labeling of the substrates is introduced. In contrast to the information which is obtained by looking for the incorporation of a label into a specific position of a biosynthesized molecule, a quantitative analysis of the correlation in the labeling of the product can give information about the direct incorporation of more complex structural units. Three examples are discussed: the glycolysis of glucose to lactate, the biosynthesis of galactosylglycerol by species of red algae, and the use of doubly labeled acetate to study the biosynthetic incorporation of acetate units into more complex molecules.

I. Introduction

The interpretation of natural abundance ^{13}C NMR spectra is simplified by the negligible amount of carbon–carbon coupling due to the low frequency of occurrence of adjacent ^{13}C nuclei. Although the carbon–carbon splitting which arises in studies of enriched compounds is usually considered to be an unwanted complicating factor,[2] analysis of the multiplet structures of ^{13}C resonances which arise from this coupling can furnish useful quantitative information on the distribution of the label in the compound being studied. This has been noted by several authors;[3-11] however, quantitative analyses have usually not been attempted and the dependence of the splitting pattern on the level of enrichment has been considered only for specific cases. In particular, it is not generally appreciated that the ^{13}C–^{13}C coupling pattern for a multiply bonded carbon atom in a ^{13}C enriched system is a more complex function of the level of enrichment and of the number of carbon atoms bonded than a consideration of pair-wise ^{13}C–^{13}C interactions alone would suggest. In this work we present a general analysis of the problem in the context of the use of ^{13}C and NMR spectroscopy in the study of biosynthetic pathways and consider two cases of importance in tracer studies. First, we show that for compounds in which the labeling of each carbon is statistically independent, the ^{13}C enrichment can be readily determined for each carbon atom. Specifically, the splitting patterns expected for uniformly labeled compounds are analyzed. Alternatively, if the statistically independent labeling condition is not met, the line splitting gives imformation about how the ^{13}C nuclei are distributed. Since coupling arises only when nearby atoms in the same molecule are labeled, it can be used to determine the correlation in the labeling of interacting nuclei.

The use of ^{13}C–^{13}C coupling as a diagnostic tool extends the value of ^{13}C as a biosynthetic tracer in several ways.

(1) The ^{13}C–^{13}C splitting patterns of enriched products can be used to assign the carbon resonances by the multiplet pattern produced.[3]

(2) Quantitative analysis of the multiplet pattern indicates any dilution of the labeled substrate by unlabeled material which may be present in the medium or be derivable from other nonlabeled sources.[4,10,11]

(3) By introducing a correlation in the enrichment of the interacting nuclei in the substrate and by examining the correlation in the ^{13}C enrichment of the product as reflected in the ^{13}C spin–spin multiplet pattern observed in the ^{13}C NMR spectrum, information can be obtained about the intermediate steps in the biosynthetic pathway. Thus far, this method has been exploited with mixtures of C-1 and C-2 labeled acetate in which there is a low correlation in the labeling of the substrate,[6] and doubly labeled acetate diluted with unlabeled acetate, in which the enrichment correlation is high.[6-8,10,11] The use of the technique ob-

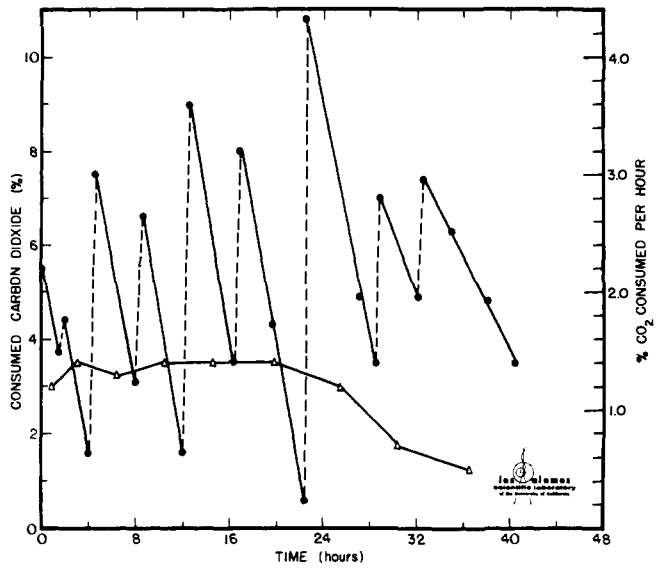


Figure 1. Rate of consumption of carbon dioxide by thalli: (—) carbon dioxide consumed; (- - -) carbon dioxide added to chamber; and (Δ) rate of carbon dioxide consumed per time interval.

viously is not restricted to acetate. A study employing a correlation in the labeling of porphobilinogen was used to study the biosynthesis of the porphyrin ring system.[5] A further illustration of this technique with labeled carbohydrates as the substrate in a study of glycolysis is given here.

(4) Carbons which are equivalent and give rise to a single resonance may not be labeled equivalently by a biological system. The differential labeling of such carbons can be studied by observing how the resonances of adjacent carbons are split.

II. Materials and Methods

The ^{13}C labeled materials described herein were obtained from this laboratory's program of synthesis with stable isotopes which is directed toward the development of methods for the efficient bio- and organosynthetic incorporation of ^{13}C and ^{15}N into a variety of substrates for use in biomedical research.[12,13]

Fermentation of Glucose-^{13}C to Lactate. Glucose-$^{13}C_6$ was fermented to L-lactate-$^{13}C_3$ acid by *Lactobacillus delbrueckii* (ATCC No. 11443). Lactic acid was removed from the concentrated acidified fermentation medium by extraction with ether and isolated as crystalline zinc lactate which could be converted subsequently to sodium lactate solution by ion exchange methods. This technique is a large scale modification of that described elsewhere.[14]

The lactic-$^{13}C_3$ acid used in these experiments was prepared from a mixture of 15 g of glucose-$^{13}C_6$ uniformly enriched to the 73 atom % carbon-13 level and 15 g of glucose having natural abundance levels of the carbon isotopes. The fermentation medium was prepared by dissolving trypton (5 g), yeast extract (5 g), Tween-80 (2 cm^3), L-(+)-cysteine hydrochloride (100 mg), sodium thioglycolate (50 mg), vitamin B_{12} (10 μg), sodium acetate (10 g), Brin's inorganic salts solution (5 cm^3), and the two sugars in about 800 cm^3 of water. After the pH of the fermentation medium was adjusted to 6.8, the volume was increased to 1 dm^3 with water and the solution was autoclaved. The inoculum of *L. delbrueckii* was from 10 cm^3 of inoculated medium which had been kept at 37° for 16 hr. The large scale fermentation was done in an automatic Micro Ferm Laboratory Fermentor (New Brunswick Scientific Co.) and was continued until production of lactic acid ceased (~20 hr) as indicated by stable pH and glucose depletion.

After fermentation, the mixture was rapidly cooled to 10° and acidified with cold 4 *N* sulfuric acid. The cells were then removed by centrifugation and the cell-free medium was concentrated to a semisolid mass by evaporation at 5° under reduced pressure. This material was subsequently extracted with diethyl ether and the combined extracts were concentrated to an oil and taken up in water. The aqueous lactic acid solution was shaken several times with ether and the combined ether layers were back-washed with water. Solid zinc carbonate was added to the combined aqueous phases until a constant pH of 6.5 was obtained. This suspension was treated with decolorizing carbon and filtered, and the resulting clear solution was concentrated, affording crystalline zinc lactate dihydrate which was filtered and washed. Anhydrous material was obtained by heating at 110° in a vacuum desiccator for 24 hr. Product purity was determined by optical rotation and enzymatic assay using Calbiochem's Rapid Lactate Stat-Pack. Carbon-13 enrichment of the lactic acid was determined by oxidation to carbon dioxide followed by mass spectrometric analysis.

The carbon-13 enriched glucose was obtained from starch produced by the photosynthetic incubation of mature excised tobacco leaves in the presence of carbon-13 dioxide using published methods.[12]

Preparation of Galactose-$^{13}C_6$ and Glycerol-$^{13}C_3$. The carbohydrate 2-hydroxy-1-(hydroxymethylethyl) α-D-galactopyranoside [galactosylglycerol] was prepared from the photosynthetic incubation of the marine red algae *Gigartina* in the presence of carbon-13 dioxide enriched to the 91.5 atom % level. The carbon-13 enriched galactoside was extracted from the incubated thalli and acid hydrolyzed to galactose and glycerol. Pure compounds were obtained by column chromatographic separation.

The three species of algae used in these experiments were *Gigartina corymbifera*, *Gigartina harveyana*, and *Gigartina californica*. The algae were obtained fresh in 5-kg quantities within 36 hr of harvest. During shipment the algae were kept in the dark at 18–20°. Photosynthetic incubation of about 4.5 kg of thalli was carried out in three sealed polycarbonate chambers joined together on a common oscillating mechanism and illuminated from the upper and lower surfaces.[13] Carbon-13 dioxide was added to the chambers until the desired concentration was reached (Figure 1). As needed, carbon dioxide was added during the incubation period. Photosynthesis was continued for varying time intervals to 40 hr. The decline in CO_2 uptake after 30 hr was accompanied by a noticeable bleaching of the thalli with a consequent decrease in photosynthetic activity.

At the conclusion of the illumination period, the thalli were removed from the chambers, washed free of salts, and extracted with 80% ethanol. The ethanolic extract was concentrated to a thick syrup and the crude galactoside was purified by acetylation, deacetylation and crystallization from absolute ethanol. Purity of the crystalline galactosylglycerol-$^{13}C_9$ was determined by melting point (128–129°) and by its optical rotation in water $[\alpha]_D$ +161°.

Galactosylglycerol-$^{13}C_9$ was hydrolyzed to a mixture of galactose-$^{13}C_6$ and glycerol-$^{13}C_3$ using 0.1 *N* trifluoroacetic acid. Acid removal was by anion exchange followed by concentration of the sugar mixture under reduced pressure to a thick syrup. The sugars were separated on a 5 × 100 cm column of Dowex 50WX8 (200–400 mesh, Ba^{2+}) resin.[15] Solid galactose-$^{13}C_6$ was obtained by crystallization from alcohol; the glycerol-$^{13}C_3$ was concentrated and stored at −20°. Purity of the galactose was determined by optical rotation measurements, melting point, and enzymatic assay. Glycerol purity was established by enzymatic assay using Calbiochem's Glycerol Stat-Pack and by ^{13}C NMR spectroscopy. Average carbon-13 enrichment was determined by oxidation to CO_2 followed by mass spectrometric analysis.

NMR Experiments. Proton decoupled ^{13}C NMR Fourier Transform spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Supernova using 4K time domain data points. Glycerol samples were dissolved in H_2O and an external D_2O capillary was used for the lock; acetylated galactosylglycerol samples were dissolved in $CDCl_3$, which also served as an internal lock. Peak intensities were determined by digital integration.

Sensitivity of the ^{13}C NMR experiments depends on several factors, including: (1) the spin-lattice relaxation time (T_1) for the particular resonance; (2) the nuclear Overhauser enhancement for the particular resonance; (3) the number of labeled carbon positions in the molecule contributing to the observed resonance; and (4) the amount of ^{13}C–^{13}C coupling. The first effect reflects the fact that for resonances with longer values of T_1 it is necessary to have a longer delay between pulses and, thus, less data can be obtained in a given time period. The last effect listed above will cause the total intensity of a particular labeled carbon position to be split

into several peaks. This will decrease the sensitivity of the experiment since the ^{13}C concentration requirement for the individual peaks must be considered. Using the apparatus described above, the minimum quantity of ^{13}C nuclei required to produce an observable peak for carbons in small molecules with directly bonded protons is $\sim 10^{-6}$ mol. In order to obtain reasonably accurate values for the peak areas, the quantity of ^{13}C nuclei contributing to each peak should exceed this minimum value by at least a factor of 10. Estimates of the ^{13}C requirement for specific biosynthetic experiments must therefore be based on the above factors, as well as on the fraction of ^{13}C in the precursor which will actually be incorporated into the product.

III. Theory

The results obtained below apply to systems in which the following conditions hold: (1) proton decoupling is complete; (2) contributions to T_1 by directly bonded ^{13}C nuclei are negligible (as will be the case for carbons with directly bonded protons);[16,23] (3) coupling with other nuclei is absent or of negligible magnitude; (4) the $J/\Delta\nu$ values (ratio of coupling constants to chemical shifts) are sufficiently small so that the resonance multiplets can be assigned to separate nuclei. For systems of interacting nuclei in which $J/\Delta\nu$ is not small, the assignment of the various peaks to specific nuclei is only an approximation.[17] Due to the large range of chemical shifts for ^{13}C nuclei, condition 4 will usually be met in practice.

An essential point in the analysis of the spin–spin splitting data is that knowledge of the intensity distribution within the multiplet pattern alone is insufficient to determine the labeling probabilities of the carbons unless additional information is known or can be obtained from other data, such as the intensity ratios of the resonances of different nuclei. In particular, the simplest case to treat theoretically, and the one which is usually assumed, is that the labeling of interacting carbons is known or assumed to be statistically independent. In this case, the probablity that several interacting carbons are labeled is just the product of the individual carbon labeling probabilities. Considering a total of N interacting carbons, C-i (usually only adjacent carbons need be considered), denoting the labeling probability of the C-i carbon by α_i, and assuming in each case that the C-1 resonance is being observed, the probability of observing a singlet for the C-1 resonance, P_s, is given by

$$P_s = \alpha_1(1 - \alpha_2)(1 - \alpha_3) \ldots = \alpha_1 \prod_{i=2}^{N} (1 - \alpha_i) \quad (1)$$

where $(N - 1)$ is the total number of carbons which can split C-1. The probability of obtaining a doublet from C-1 due to the labeling of the jth interacting carbon, C-j, P_d^j, is given by

$$P_d^j = \alpha_1\alpha_j(1 - \alpha_k)(1 - \alpha_l) \ldots = \alpha_1\alpha_j \prod_{i\neq 1,j}^{N} (1 - \alpha_i) \quad (2)$$

For a carbon with more than one interacting neighbor, the total probability of obtaining a doublet due to the labeling of any one of its neighbors is a sum of terms in the form of eq 2.

$$P_d = \alpha_1\left(\alpha_j \prod_{i\neq 1,j}^{N} (1 - \alpha_i) + \alpha_k \prod_{i\neq 1,k}^{N} (1 - \alpha_i) + \ldots\right) \quad (3)$$

For a carbon with $(N - 1)$ neighbors which are coupled, there will be $(N - 1)$ terms in the sum. The labeling of C-1 and two interacting nuclei can produce a triplet (in the case of an AX_2 spectrum) or, more generally, a quartet with probability

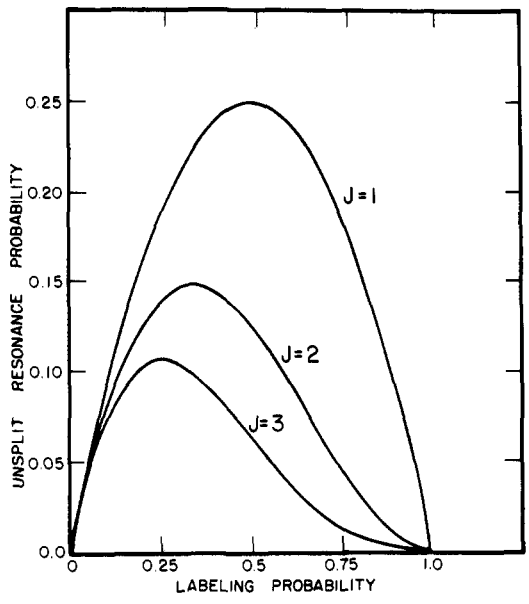


Figure 2. The probability of obtaining a singlet as a function of the individual carbon labeling probability for a uniformly labeled compound. Results are shown for carbons with $j = 1$, 2, or 3 nearest neighbors.

$$P_Q = \alpha_1\alpha_j\alpha_k \prod_{i\neq 1,j,k}^{N} (1 - \alpha_i) \quad (4)$$

As above, the total intensity of the observed quartet (or triplet) may represent a sum of terms, each corresponding to the labeling of C-1 and a pair of neighboring nuclei.

Since the total probability of obtaining a split or unsplit resonance from C_1 is given by α_1, the fractional intensity of a given multiplet can be obtained by dividing the probabilities given above by α_1. Thus, for example, the fractional intensity of the unsplit resonance is given by P_s/α_1. Similarly, the intensity ratio of singlet to remaining multiplet will be given by $P_s/(\alpha_1 - P_s)$.

A particularly simple case to treat is a uniformly labeled compound, in which the labeling probabilities for all carbons are identical. In this case, the various multiplet intensities are proportional to the terms in a binomial expansion. Thus, for example, for a carbon having three nearest neighbors, the singlet:doublet:quartet (triplet):octet ratio is given by

$$S:D:Q:O = (1 - \alpha)^3 : 3\alpha(1 - \alpha)^2 : 3\alpha^2(1 - \alpha) : \alpha^3$$

where α is the labeling probability of each carbon. For a carbon with j nearest neighbors, the probability of obtaining an unsplit resonance, P_s, is then a function of the number of nearest neighbors which are coupled to the observed carbon

$$P_s^j = \alpha(1 - \alpha)^j \quad (5)$$

where the superscripts j indicate the number of coupled neighbors. The probabilities have the property of passing through a maximum which occurs at $\alpha = 1/(j + 1)$. Thus, the probability of obtaining an unsplit resonance increases as the labeling probability α, until α is high enough to reduce the unsplit intensity. P_s^j is shown in Figure 2 for $j = 1$, 2, and 3. It is interesting to note that at natural abundance all of the $P_s^j \simeq \alpha$. However, even at relatively low labeling percentages the splitting increases rapidly and is strongly dependent on the number of nearest neighbors. This result is not an obvious one and frequently has been overlooked.[3,4]

Thus, for 20% enrichment, the ratios of unsplit to split resonances are 4:1, 1.8:1, and 1.05:1 for carbons with one, two, and three nearest neighbors. Most of the differences arise from the increased probability of having a single neighbor labeled, since the probability of having two neighbors labeled at 20% is relatively small, as is indicated by the relative sizes of α, α^2, and α^3.

Statistically Dependent Labeling. In many cases of interest, including compounds which are labeled biosynthetically, the labeling of the various carbons cannot be assumed to be statistically independent. That is, the labeling of the carbons may be a function of time, or a mixture of several samples of identical molecules with different labeling percentages may be present. If the time course of labeling or the composition of the mixture is known, the observed values of the fractional intensities of the various multiplets can be calculated by taking the appropriate averages. Thus, for example, the fractional intensity of the singlet resonance, F_s, will be given by

$$F_s = \alpha_1 \prod_{i=2}^{N} (1 - \alpha_i)/\alpha_1 \qquad (6)$$

The simplest case to treat is a mixture of N components of identical species, each of which is characterized by statistically independent labeling. If each component is present at fractional concentration f_i, the observed fraction of the resonance intensity of C_1 which will be in the singlet is given by

$$F_s = \sum_i f_i \alpha_1{}^i \prod_{l=2}^{N} (1 - \alpha_l{}^i)/\sum_i f_i \alpha_1{}^i \qquad (7)$$

where all i subscripts and superscripts refer to the ith component of the mixture. In the simplest case, for a two-component mixture and a carbon split by only one neighbor, eq 7 becomes

$$F_s = \frac{f\alpha_1(1 - \alpha_2) + f'\alpha_1'(1 - \alpha_2')}{f\alpha_1 + f'\alpha_1'} \qquad (8)$$

where, in order to simplify the notation, the concentrations and labeling probabilities of the two species are denoted by primed and unprimed letters. If the nature of the various components is unknown, the line splitting data alone are insufficient to determine the average labeling percentages of the different carbons.

A more complicated situation arises if there is a mixture of multiply labeled compounds, as in the case of doubly enriched acetate (or porphobilinogen) and natural abundance acetate. In this case, the probabilities of each multiplet which arise from carbon–carbon coupling can be calculated with a formula analogous to eq 7. However, the number of components which must be considered depends on the number of carbons coupled to the one being observed. This analysis is discussed further in the following section.

There are a number of possible labeling experiments in which the labeling probabilities of a given compound will vary with time. For example, the incorporation of a label in a biosynthetic experiment may be retarded if initially a number of unlabeled intermediate metabolites in the synthesis are present. Any realistic model for such effects becomes extremely complex and a description requires more parameters than can reasonably be fit from the splitting data unless a large number of samples extracted at different times are analyzed. One general conclusion which can be drawn is that if the labeling probabilities of the interacting carbons increase with time, there will be a greater correlation in the labeling than if it were statistically independent. Thus, the same splitting can be obtained with a lower average enrichment. As a simple illustrative example of this point, and one which may be a reasonable approximation if very short times are considered, assuming a concerted synthesis of an N carbon product which is only created, but not used as an intermediate for further synthesis, so that the product concentration monotonically increases, the singlet fraction will be given by

$$F_s = \int_0^{t_F} \frac{\partial c(t)}{\partial t}\, \alpha_1(t) \prod_{j=2}^{N} [1 - \alpha_j(t)]\, dt \Big/ \int_0^{t_F} \frac{\partial c(t)}{\partial t}\, \alpha_1(t)\, dt \qquad (9)$$

where $c(t)$ is the product concentration and $\partial c(t)/\partial t$ is the rate of product formation. Then further assuming a constant rate of product formation, and assuming that the $\alpha_j(t)$ increase linearly from 0 to $\alpha_j{}^f$ as t increases from 0 to t_F, for a carbon with one nearest neighbor, F_s is given by

$$F_s = 1 - \tfrac{2}{3}\alpha_2{}^f \qquad (10)$$

where $\alpha_2{}^f$ is the final labeling probability of the nearest neighbor. Thus, the singlet to doublet ratio will be the same as that which would be obtained for a uniformly labeled compound if the nearest neighbor carbon had a labeling probability equal to $\frac{2}{3}$ of the final value in the linear case. The two cases can be distinguished, however, by noting that the average labeling probability of the nearest neighbor is α in the uniformly labeled case, but $\alpha_2{}^f/2 = \frac{3}{4}\alpha$ in the case where the labeling probability is a linear function of time.

Intensities of ^{13}C Resonances Within a Multiplet. From the above discussion, it can be seen that the quantities of experimental interest are the total intensities of each ^{13}C–^{13}C multiplet. In practice, direct measurements of some of the intensities may be impossible due to overlapping peaks. Thus, for example, the center line of a triplet may overlap the singlet peak, or one of the doublet lines may overlap a peak from another carbon resonance. These difficulties can be circumvented if: (a) one peak of the multiplet is clearly resolved and, (b) the intensity ratios of the peaks within the multiplet are known. Thus, for example, for the A triplet in an AX_2 spectrum, measurement of one of the outer lines of the triplet is sufficient to determine the intensities of the other outer line and of the center line if J and $\Delta\nu$ are known. It has recently been pointed out[18] that the relative intensities of the multiplet lines may be altered in Fourier transform experiments on nonequilibrium systems. In particular, an insufficient time delay between pulses will lead to a nonequilibrium situation. Experimentally, we have found that the slow passage intensity ratios within a multiplet appear to be maintained for cases in which the pulse spacing is insufficient for the system to completely relax. This may reflect the fact that for systems enriched in ^{13}C all of the lines within a multiplet and, in fact, all of the lines corresponding to a particular labeled carbon with a directly bonded proton, relax at approximately the same rate due to the dominance of ^{13}C–^{1}H dipolar coupling over ^{13}C–^{13}C dipolar coupling. Thus, the population differences between the various energy levels which correspond to all of the resonances of a particular carbon will be proportional to the equilibrium values. Alternatively, for strongly coupled systems the Fourier transform experiment introduces mixing of the intensities of the lines in different multiplets corresponding to different carbons which may have different relaxation times. In this case, deviations from the slow passage intensity pattern may result for an incompletely relaxed system. We have, however, observed no significant deviations from the slow passage intensity patterns for the systems under study.

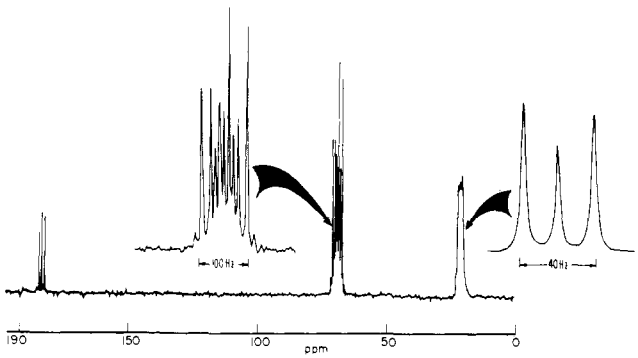


Figure 3. Proton noise decoupled ^{13}C NMR spectrum of zinc lactate (23.7 mg/ml). The spectrum represents 1200 pulses accumulated over a period of 8 min. The methyl (20.9 ppm) and carboxyl (182.6 ppm) resonances are a combination of a singlet, corresponding to species in which C-2 (68.5 ppm) is unlabeled, and a doublet, corresponding to species in which C-2 is labeled. The C-2 resonance is a combination of a singlet, two pairs of doublets, and a quartet for a total of 9 peaks. Due to the differences between $J_{1,2}$ = 37.6 Hz and $J_{2,3}$ = 55.8 Hz, the doublets arising from the labeling of C-2 and either the carboxyl or the methyl carbon can be distinguished.

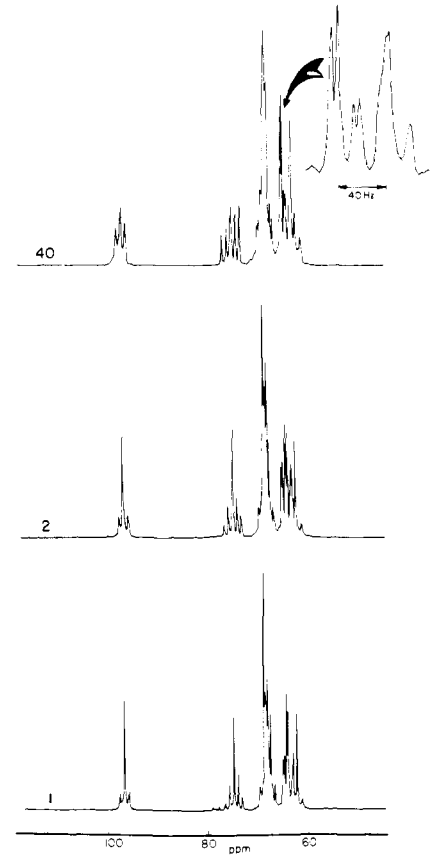


Figure 4. Proton noise decoupled spectra of organically acetylated α-D-galactosyl-2-glycerol dissolved in $CDCl_3$ (the acetyl resonances are not shown). The singlet resonances correspond to galactose C-1 (96.6 ppm), galactose C-2–C-5 (67.2–68.7 ppm), and galactose C-6 (62.3 ppm) and to glycerol C-2 (74.8 ppm) and glycerol C-1 and C-3 (64.0 and 64.2 ppm). The insert illustrates the region of the spectrum containing the glycerol C-1 and C-3 resonances. In the glycerol spectrum (Figure 5) these carbons are equivalent so that the pattern consists of three lines: a singlet plus a doublet due to coupling with C-2. In the galactosylglycerol spectrum, the C-1 and C-3 carbons are not equivalent so that six lines are observed. The C-1 and C-3 lines of the upfield doublet overlap one of the galactose C-6 peaks. The intensities for these peaks were calculated from the intensities of the two lines of the downfield doublet as described in the text. The spectra are for samples taken after 1, 2, and 40 hr of photosynthetic activity. A noticeable change is apparent in the singlet/doublet ratio of the C-1 and C-3 resonances between 1 and 2 hr. The 1, 2, and 40-hr spectra were taken using 90, 170, and 20 mg/ml concentrations using 11,000, 12,000, and 1500 pulses, respectively. The pulse repetition rate was 1.2 pulses/sec.

IV. Biosynthetic Applications

We have employed the theory developed in the previous sections to study several systems about which varying amounts of information are known. These applications are discussed below.

The Formation of Lactate by Glycolysis. The technique of using correlated enrichment to study a biosynthetic pathway is illustrated by the fermentation of a mixture of ^{13}C enriched and natural abundance sugars to lactate by *Lactobacillus delbrueckii*, a particularly simple example. Using an equimolar mixture of glucose uniformly labled with carbon-13 to the 71.1% level and natural abundance glucose, lactate was isolated and examined by mass spectrometry and NMR. Mass spectrometry indicated that 36.2% of the carbon atoms are ^{13}C; however, the NMR spectrum (Figure 3), which exhibits small ratios of singlet to multiplet resonances, can be fit well by assuming that the lactate is uniformly enriched to 73% ^{13}C. This disparity in the determinations of the ^{13}C enrichment indicates that the distribution of ^{13}C is nonuniform. The results can be accounted for by assuming that the lactate is an equimolar mixture of natural abundance and ~71% uniformly enriched components. The application of eq 7 and 8 then indicates that the natural abundance material will make a negligible contribution to the NMR spectrum; however, the average enrichment will be only about half that of the lactate derived from enriched glucose. This result is consistent with the glycolysis pathway in which lactate is formed by the direct breakdown of the six-carbon sugars into two three-carbon units. If, for example, the sugars were broken down into one-carbon units and then randomly reassembled to form lactate, both the NMR spectrum and the mass spectrometric analysis would be consistent with 36% uniform enrichment. Alternatively, if the sugars were broken down into one- and two-carbon units and then reassembled to form lactate, the NMR spectrum would reflect this, and would indicate whether the one-carbon unit formed the carboxyl or the methyl carbon.

Photosynthetic Incorporation of CO_2 into Galactosylglycerol by Red Algae. The use of red algae for obtaining large quantities of ^{14}C labeled α-D-galactosyl-2-glycerol was first studied by Bean et al.[19] and by Bean and Hassid.[20] They found that galactosylglycerol become radioactive very early in the photosynthetic process, and that after 4 min of photosynthesis the galactose:glycerol activity ratio is approximately 2. Although, as was pointed out by Bean and Hassid, this result is consistent with the equivalent labeling of all carbons, a number of inequivalent labeling schemes can lead to the same ratio. We have applied this biosynthesis to obtain ^{13}C labeled galactosylglycerol, and the resulting product can be analyzed using ^{13}C NMR.

The biosynthesized galactosylglycerol was extracted at 1, 2, and 40 hr as described in the methods section. NMR spectra of the organically acetylated species, dissolved in $CDCl_3$, are shown in Figure 4. Glycerol samples extracted from galactosylglycerol samples obtained from different runs after 12 and 24 hr of photosynthetic activity were also analyzed (Figure 5). Peaks in the galactosylglycerol spectrum may be assigned to the galactose C-1 (96.6 ppm), the glycerol C-2 (74.8 ppm), the galactose C-2–C-5 (67.2 to 68.7 ppm), glycerol C-1 and C-3 (64.0 and 64.2 ppm), and galactose C-6 (62.3 ppm), where the peak positions are given relative to external TMS. The fact that the glycerol C-1 and C-3 resonances appear as distinct peaks reflects nonequivalence of these carbon atoms due to the asymmetry

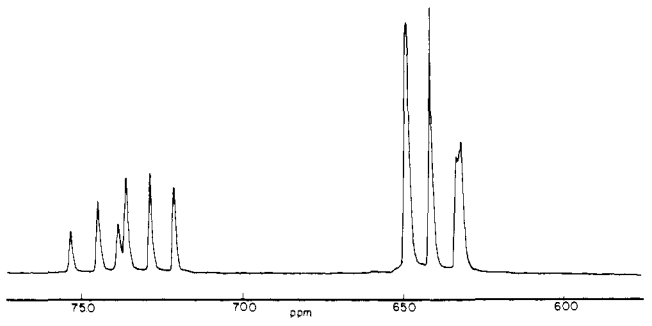


Figure 5. Proton noise decoupled ^{13}C spectrum of glycerol (600 pulses) taken with a pulse rate of 1 pulse/6 sec on a sample containing 50 mg/ml of glycerol prepared from galactosylglycerol extracted after 12 hr of photosynthesis. The flip angle was $\pi/4$ radians. The C-2 resonance (73.7 ppm) consists of a combination of a singlet, doublet, and quartet, where the quartet structure is that calculated for an AB_2 spectrum. The central quartet peaks overlap the singlet resonance, but the relative intensities can be separated, as described in the text. The C-1 and C-3 resonances (64.3 ppm) also consist of a combination of singlet, doublet, and quartet peaks. The quartet structure is, however, not resolvable from the doublet structure, with the two downfield quartet lines overlapping the downfield doublet, and the two upfield quartet lines overlapping the upfield doublet. The intensity ratio of the downfield doublet to the upfield doublet is approximately equal to the ratio of the two downfield quartet lines to the two upfield quartet lines. (See Appendix A.)

of the nearby galactose. In free glycerol (Figure 5) only a single resonance is observed for carbons C-1 and C-3. In addition to the singlet peaks, the multiplets arising from ^{13}C–^{13}C coupling in multiply labeled compounds are also present. A complete analysis of the galactose spectrum cannot be performed due to the overlapping of the C-2–C-5 resonances; however, the multiplet structure of the glycerol spectrum can be considered quantitatively. The C-1–C-3 resonance is a singlet if C-2 is unlabeled (neglecting the small splitting in galactosylglycerol) or a doublet if C-2 is labeled. The C-2 resonance can be either a singlet, doublet, or quartet, as is characteristic of an AB_2 spectrum. The two center lines of the quartet, which are separated by ~6.8 Hz, overlap the singlet. Using values of J = 41.1 and $\Delta\nu$ = 242 Hz for the glycerol carbon–carbon coupling constant and peak separation between C-2 and C-1–C-3, the relative intensities of the spectral lines can be calculated if the slow passage relationships between peak intensities are assumed to hold. Thus, the intensity ratio of the upfield to the downfield doublet for C-2 should be 1.4, the intensity ratio of the upfield quartet line (line 4) to the downfield quartet line (line 1) for the C-2 quartet should be 1.95, the ratio of the downfield doublet to the upfield doublet for the C-1–C-3 peaks should be 1.40, and the ratio of lines 5 + 6 to lines 7 + 8 in the C-1–C-3 signal which arise when all carbons are labeled to produce an AB_2 spectrum is also 1.4 (see Appendix A). These values can be measured directly from the glycerol spectrum and are in complete agreement with the calculated results indicating that the AB and AB_2 intensity pattern is valid in the present Fourier transform experiments. Similar agreement was found for the peaks in the acetylated galactosylglycerol spectrum. The intensity of the center two lines of the C-2 quartet is calculated to be 0.917 of the intensity of the outer two lines. Thus, the intensity of the center two lines of the quartet can be calculated and subtracted from the intensity of the observed center resonances to obtain the singlet intensity. An additional problem in the galactosylglycerol spectrum is the overlapping of the upfield side bands of the glycerol C-1, C-3 and the downfield side band of the galactose C-6. Using parameters measured in the galactosyl glycerol spectrum, J = 43.5 Hz, and $\Delta\nu$ = 268.3 Hz, the intensity of the upfield side band is calculated to be 0.724 times the intensity of the downfield glycerol C-1–C-3 side band (see Appendix A). Three independent ratios can be measured for the glycerol spectrum. These have been arbitrarily chosen as the doublet/singlet ratio (d/s) for C-2 and C-1–C-3 and the quartet/doublet ratio (q/d) for C-2. Measured values, along with some values obtained for C-1 and C-6 for galactose, are summarized in Table I. In addition, the average enrichment of the various samples, as determined by mass spectrometric analysis of samples burned to CO_2, is also shown.

Table I. Multiplet Intensity Ratios for Glycerol and Acetylated Galactosylglycerol Samples

Sample	Photosynthetic period, hr	C-1 d/s	C-6 d/s	C-2 d/s	C-2 q/d	C-1 and C-3 d/s	Av % ^{13}C (mass spec)
Galactosyl-glycerol	1	0.469	0.478	0.883	0.650	0.652	5.0
Galactosyl-glycerol	2	0.640	0.615	1.101	0.938	1.090	5.1
Glycerol	12			3.909	1.366	2.436	24.1
Glycerol	24			3.320	1.248	2.301	28.0
Galactosyl-glycerol	40	1.408	1.652	6.035	1.859	3.810	43.8

A comparison of the average enrichment with the observed multiplet splitting indicates that the sample is not uniformly enriched. The data can most readily be explained by assuming that a large concentration of natural abundance galactosylglycerol is present initially, and to this pool newly synthesized and highly labeled material is added as photosynthesis progresses. Thus, the gradual increase in the doublet/singlet ratios indicated in Table I corresponds to a decreasing fraction of natural abundance galactosylglycerol which contributes, to a good approximation, only to the observed singlet. Bean and Hassid have characterized the galactosylglycerol of red algae as the main reserve carbohydrate, analogous to sucrose in the higher plants.[20] The presence of a large pool even after 36 hr in the dark indicates that it is not rapidly converted into energy and CO_2, consistent with the fact that galactose, unlike glucose, is not immediately fermentable. The function of the galactosylglycerol in the red algae may therefore be primarily as a reserve for structural material, particularly since a galactose polymer is known to be a main structural component of the organism.[20]

Another interesting feature of the spin splitting ratios is the gradual increase in the quartet/doublet ratio of C-2. Since the natural abundance galactosylglycerol does not contribute appreciably to these multiplets, this increase indicates a slow change which is occurring in the labeling probabilities of the newly synthesized material. There are several possible causes for this. (1) At the time when the labeling begins, all of the photosynthetic metabolites, e.g., ribulose diphosphate, seduheptulose 7-phosphate, etc., are not labeled. Therefore, the metabolic products of the photosynthetic process will not be labeled with the probability of the input CO_2 until all of the intermediates have been labeled to this level. Although the rate at which this occurs will be a function of the experimental conditions, most experiments reported the labeling is nearly complete after several minutes.[21] (2) The respiration process will produce unlabeled CO_2, thus decreasing the labeling probability of the CO_2 which is being fixed relative to that supplied externally. As time progresses, newly synthesized metabolites will increasingly contribute to the respired CO_2 and the dilution effect will decrease. (3) As a result of the long period in the

Table II. Results of Uniform Labeling Model

Sample	Photosynthetic period, hr	Fraction labeled, %	% enrichment of labeled material	Calculated ratios C-2 d/s	C-2 q/d	C-1,C-3 d/s
Galactosyl-glycerol	1	4.9	56%	0.881	0.647	0.660
Galactosyl-glycerol	2	5.4	66%	1.098	0.945	1.081
Glycerol	12	34.5	73%	3.908	1.352	2.448
Glycerol	24	26.0	72%	3.313	1.253	2.328
Galactosyl-glycerol	40	51.0	80%	6.047	2.000	3.755

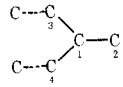
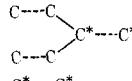
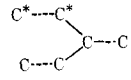
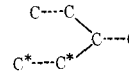
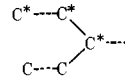
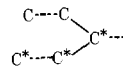
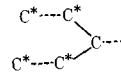
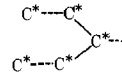
dark, the algae probably develop catabolic enzymes which may continue to function for varying lengths of time after the light is turned on. Therefore, catabolic processes may be producing unlabeled CO_2 at the beginning of the experiment.

As discussed in the previous section, the carbon–carbon coupling data alone are insufficient to completely determine the labeling probabilities of the individual carbon atoms. However, information can be obtained by considering various reasonable models and fitting the observed data. The simplest model is that the sample is a mixture of natural abundance and newly synthesized, uniformly labeled galactosylglycerol. The results obtained with this model, summarized in Table II, indicate that a very good fit to the observed splitting ratios (Table I) can be obtained, most of the errors being <5%. Of the three possibilities suggested above for the gradual increase in the labeling of the newly synthesized material, only the first would be expected to result in substantial differences in the labeling probabilities of the different carbons. Since a uniform labeling model provides such a good fit to the data, it is unlikely that this effect is important. Nevertheless, the labeling probabilities calculated for the 1- and 2-hr samples probably represent an average of gradually increasing probabilities. For example, for the C-1 and C-3 carbons, the same splitting ratios for the 1-hr sample would be obtained if the labeling probability of C-2 increased linearly to $\frac{3}{2}(56\%) = 84\%$. In fact, the entire 1-hr period cannot reasonably be approximated by a linear increase, since the labeling probabilities appear to level off at ~73%. The model also gives rough agreement with the average labeling data, as determined by mass spectrometry, although the NMR values obtained, assuming that the galactose and glycerol are labeled equivalently, are somewhat lower. Determinations of the fraction of natural abundance product become more inaccurate as the fraction of highly labeled product increases; thus estimations of the average labeling based on these fractions are worse for the later samples.

The labeling of the galactosyl glycerol appears to follow three stages. (1) Initially, the labeling percentages of the glycerol carbons increase, eventually leveling off at about 73%. (2) Between 12 and 24 hr there is relatively little change in the net ^{13}C content and ^{13}C NMR spectrum of the galactosylglycerol pool. During this period, the organism may be drawing on the pool to some extent but not synthesizing much highly labeled new material thus leaving the labeling percentages relatively stable. (3) Between 24 and 40 hr there is a further apparent increase in the labeling probability of the newly synthesized material (i.e., not considering the natural abundance pool) to 80%. It is interesting to note that this increase occurs despite a marked decrease in the uptake of CO_2 after 24 hr (Figure 1). The decrease in CO_2 uptake is accompanied by a bleaching of the

Table III. Labeling Probabilities of Compounds Synthesized from Doubly Labeled Acetate and Natural Abundance Acetate

Species	Fractional concn	Labeling probabilities C-1	C-2	C-3
(a) Carbons with Two Nearest Neighbors				
C---C---C---C (3 1 2)	$(1-f)^2$	0.01	0.01	0.01
C---C---C*---C*	$f(1-f)$	α	α	0.01
C*---C*---C---C	$f(1-f)$	0.01	0.01	α
C*---C*---C*---C*	f^2	α	α	α

	Fractional concn	Labeling probabilities C-1	C-2	C-3	C-4
(b) Carbons with Three Nearest Neighbors					
	$(1-f)^3$	0.01	0.01	0.01	0.01
	$f(1-f)^2$	α	α	0.01	0.01
	$f(1-f)^2$	0.01	0.01	α	0.01
	$f(1-f)^2$	0.01	0.01	0.01	α
	$f^2(1-f)$	α	α	α	0.01
	$f^2(1-f)$	α	α	0.01	α
	$f^2(1-f)$	0.01	0.01	α	α
	f^3	α	α	α	α

cells indicating a decline in photosynthetic activity which may reflect a loss in viability of the cells. The quantity of galactosylglycerol extractable per unit cell mass also drops from 3.8 to 2.7 gm/kg between 24 and 40 hr, indicating that the organism may be drawing on its galactosylglycerol reserve, perhaps for the synthesis of the structural galactose polymer. Since any output from the galactosylglycerol pool reflects the average labeling of the pool while the input is of highly labeled material, these effects will increase the labeling percentages as observed, but at the expense of galactosylglycerol labeled to lower levels.

Finally, we note that a comparison of the doublet/singlet ratios of the glycerol and galactose carbons given in Table I indicates that there may be a lag in the labeling of the C-2 and C-5 carbons. These carbons are among the last to become labeled as photosynthesis progresses. The data for galactose are subject to some inaccuracy, however, since the labeling of C-1 and C-6 cannot be determined from the C-2 and C-5 splitting, and since there is a considerable overlap between the doublet and singlet resonances, especially for C-1.

Analysis of Biosynthetic Experiments with Doubly Labeled Acetate. A number of experiments have recently been reported in which doubly labeled acetate was used to study the biosynthesis of products derived from acetate.[6-8,10,11] With the exception of one study,[11] the results have not been analyzed quantitatively. An approximate expression for the percent enrichment at each carbon position of a compound synthesized from a mixture of doubly labeled and natural

abundance acetate was given by McInnes *et al.*[11] A similar, but not identical, expression can be derived using the entries in Table III, assuming that $f \ll 1$ (equivalent to the McInnes et al. assumption that the probability of doubly labeled acetate units being adjacent is small) and $\alpha \gg 0.01$, so that only the first two entries in either table need be considered. The result for the percent enrichment of each carbon, given by 100 times the fraction of doubly labeled acetate units in the product times α differs from the McInnes *et al.* result by a factor of $1/\alpha$. The spectra can, however, be described using the mixture equations such as eq 7 for the singlet fraction of a multiplet. The number of components of the mixture which must be considered depends on the number of nearest neighbors of the carbon under observation; for a carbon with n nearest neighbors, 2^n components of the mixture must be considered. Thus, for a carbon in the product with only one nearest neighbor, only two components, corresponding to the labeled and unlabeled acetate species from which it might have been derived, must be used in the mixture equation. For carbons in the product with two or three nearest neighbors, the four and eight species which must be considered, respectively, are listed in Table III. The concentration of each species is given as a function of f, the fraction of the acetate in the medium which was doubly labeled from which the product was synthesized; α is the uniform labeling probability of the doubly labeled acetate. Of course, each of the components given in these tables can make more than one contribution to a given multiplet. Thus, for example, the total doublet probability for a carbon with two nearest neighbors (Table IIIa) is given by

$$P_d = (1 - f)^2(0.01)[(0.01)(0.99) + (0.99)(0.01)] + f(1 - f)(\alpha)[(\alpha)(0.99) + (1 - \alpha)(0.01)] + f(1 - f)(0.01)[(0.01)(1 - \alpha) + (0.99)(\alpha)] + f^2(\alpha)[(\alpha)(1 - \alpha) + (1 - \alpha)(\alpha)]$$

The additional information which can be obtained from a quantitative analysis can be illustrated by considering the spectra obtained for the antibiotic dihydrolatumcidin in ref 6. The antibiotic was synthesized by *Streptomyces reticuli* var. *latumcidicus* from an exogenous acetate pool consisting of a mixture of 90% uniformly enriched acetate, $^{13}CH_3{}^{13}CO_2Na$, combined with 2.5 times the concentration of natural abundance acetate. The latter was added to reduce the amount of ^{13}C–^{13}C spin–spin coupling and simplify the NMR spectrum of the natural product.

Using $\alpha = 0.90$, $f = 1/3.5$, and $1 - f = 2.5/3.5$, the various multiplet intensities can be calculated. For carbons C-2 and C-9, which have only one nearest neighbor, the singlet to doublet ratio expected is 0.142. For carbons C-3, C-4, C-6, C-7, C-7a, and C-8, which have two nearest neighbors, the singlet/doublet/quartet ratios would be 9%/68%/23%. For carbons C-4a and C-5 which have three nearest neighbors, the singlet/doublet/quartet/octet ratios calculated using the eight components in Table IIIb are 6.7%/52.2%/34.9%/6.1%. Assuming the signal heights of the completely resolved resonances to be roughly proportional to the peak areas, as we have found to be generally true, the spectra given in ref 6 are inconsistent with the above calculations. The apparent singlet/doublet ratios for C-2 and C-9 are about 0.57, and there is no visible higher order splitting for the carbons with more than one nearest neighbor although, for example, for carbons with two nearest neighbors 23% of the resonance intensity should be in a quartet for $\alpha = 0.90$ and $f = 1/3.5$. The observed singlet/doublet ratios of C-2 and C-9 are consistent with the presence of an acetate pool having a ratio of unlabeled to labeled acetate of about 45:1. If, as in the case of galactosylglycerol, there were a combination of highly labeled and natural abundance dihydrolatumicidin present, the quartet/doublet ratios for the carbons with two nearest neighbors would be approximately 1:3 as calculated above. However, this is not observed, so the large dilution of the acetate is probably a steady state process. This discrepancy can be explained by assuming that there is a relatively large pool of derivable acetate units present in the growth medium. Thus, acetate must be charged as acetyl CoA in an energy consuming process, prior to incorporation, whereas glucose present in the medium and containing natural abundance ^{13}C, for example, can be metabolized directly into charged acetate without requiring the additional expenditure of energy. In particular, acetate units derived from glucose have been found to be much more effectively incorporated into the fatty acids of mammalian cells than exogenous acetate.[22] We conclude, therefore, that the dilution of the labeled acetate by 2.5 times with exogenous, natural abundance acetate had relatively little effect on the spectrum since a much larger dilution apparently occurred as the antibiotic was biosynthesized.

The technique of diluting a multiply labeled substrate with natural abundance substrate introduces a correlation in the labeling which can be used to determine whether the substrate is incorporated as a unit into the isolated product. In systems where such a dilution occurs *in vivo* due to the availability of a derivable natural abundance substrate, additional dilution is unnecessary.

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Appendix A

In the analysis of the glycerol and galactosylglycerol spectra, use was made of the expected intensity ratio of the downfield to the upfield doublet of C-1 and C-3. These resonances are actually a superposition of the B parts of an AB and an AB_2 spectrum. In the latter, the B resonances are actually a quartet which appears, however, as a doublet

AB spectrum

$$\frac{3}{4} = \frac{C + (J/2)}{C - (J/2)} = \frac{1 + [x/(1 + x^2)^{1/2}]}{1 - [x/(1 + x^2)^{1/2}]} \simeq 1 + 2x + 2x^2 + \ldots \quad (12)$$

AB_2 spectrum

$$\frac{5 + 6}{7 + 8} = \frac{[2^{1/2}\cos\Delta\theta + \cos\theta_+\sin\theta_-]^2 + [2^{1/2}\cos\theta_+ + \sin\theta_+]^2}{[2^{1/2}\cos\Delta\theta - \sin\theta_+\cos\theta_-]^2 + [2^{1/2}\cos\theta_- - \sin\theta_-]^2} =$$

$$\frac{[1 + 2\cos^2\Delta\theta + \cos^2\theta_+(1 + \sin^2\theta_-) + 2\,2^{1/2}\cos\theta_+(\cos\Delta\theta\sin\theta_- + \sin\theta_+)]}{[1 + 2\cos^2\Delta\theta + \cos^2\theta_-(1 + \sin^2\theta_+) - 2\,2^{1/2}\cos\theta_-(\cos\Delta\theta\sin\theta_+ + \sin\theta_-)]} =$$

$$\simeq \frac{4 + 4x + O(x^3)}{4 - 4x + O(x^3)} \simeq 1 + 2x + 2x^2 + \ldots \quad (13)$$

with the two downfield peaks and the two upfield peaks coinciding with the downfield and upfield doublet lines of the AB spectrum, respectively (see Figure 5). Using the notation of ref 17, the intensity ratio of line 3 to line 4 for the AB spectrum and the ratio of lines 5 + 6 to lines 7 + 8 for the AB_2 spectrum are identical to order $(J/\Delta\nu)^2$ (eq 12 and 13).

For the spectra considered, $J/\Delta\nu = 0.16$ so that the error is $\lesssim 0.4\%$ and we can treat the doublet as arising from nearest neighbor coupling only with very little error. (Note: these ratios are the inverse of the ratios given in the text.)

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(23) For ^{13}C nuclei not directly bonded to hydrogens, ^{13}C–^{13}C dipolar relaxation can contribute significantly to T_1. As a result, the Overhauser enhancements for the singlet will be greater than that for the multiplets due to three spin effects. The relative intensities of the singlet and multiplet lines will then not reflect the labeling probabilities in the decoupled spectrum. However, accurate values can be obtained by examining the coupled spectrum for these carbons. A preliminary study of these effects has been submitted to *J. Magn. Reson.*

Carbon-13 Nuclear Magnetic Resonance. ^{13}C Shieldings and Spin–Lattice Relaxation Times in Chlorinated Biphenyls

Nancy K. Wilson

Contribution from the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709.

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Abstract: Carbon-13 shieldings were measured for 25 chlorinated biphenyls. For 13 of these, the ^{13}C assignments of the protonated carbons were confirmed by selective proton decoupling. The effects of chlorine substitution on the ^{13}C shieldings could be approximately predicted using additive parameters obtained from chlorobenzene and 2-, 3- and 4-chlorobiphenyl data. Marked deviations from the predicted substituent effects occur when chlorines are ortho on the same ring or in 2 and/or 6 positions on both rings. The effects of a chlorine substituent are significantly transmitted through eight covalent bonds. Spin–lattice relaxation times were measured for the protonated carbons in several biphenyls. The relaxation is dominated by the carbon–hydrogen dipole–dipole interaction, which results in maximum nuclear Overhauser enhancements. The relaxation times are related to the rotational barriers about the interring bond.

Polychlorinated biphenyls (PCBs) are of considerable current interest because of their ubiquitous distribution and their remarkable persistence in the natural environment. Investigations centering about their identification in complex mixtures and their biological interactions have been numerous.[1] Several biological studies have indicated that certain PCB isomers are preferentially concentrated in living organisms. Evaluation of the potential health hazards associated with this class of compounds therefore clearly requires knowledge of the characteristics of specific PCB isomers.

Nuclear magnetic resonance, as an analytical tool sensitive to molecular structure, electronic charge distributions, and molecular interactions, is useful for discrimination between the many PCB isomers[2-4] and for study of the different potentials of these isomers for interactions in biological systems. Certain NMR parameters such as ^{13}C spin–lattice relaxation times can be interpreted in terms of molecular motion[5,6] and stereochemistry. Knowledge of the NMR parameters may thus allow more meaningful examinations of the biological effects of PCBs.

Proton NMR data at 220 MHz for a large number of PCBs have been reported,[3] as well as some data at 60 MHz.[4] Detailed analyses of the ^1H spectra of symmetrically substituted dihalobiphenyls[7] as well as several earlier studies of proton chemical shifts and substituent effects on these in halobiphenyls[8] are in the literature. Proton NMR in nematic liquid crystalline solvents has been used to investigate the conformation of 3,5,3′,5′-tetrachlorobiphenyl[9] and of 4,4′-dichlorobiphenyl,[10] but the first of these studies unrealistically predicts a single rigid conformation at room temperature. Fewer ^{13}C NMR studies of biphenyls have been published. Carbon-13 data for ten symmetrically substituted chlorinated biphenyls have been reported and related to trends in charge density distributions calculated by the CNDO/2 method.[2]

Recently, ^{13}C NMR data for some halobiphenyls includ-