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DIRECT ANALYSIS OF ^{13}C ABUNDANCE IN PLANT CARBOHYDRATES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method for the direct analysis of ^{13}C abundance in several kinds of plant carbohydrates by gas chromatography–mass spectrometry (GC–MS) has been developed. The analysis was conducted with the quasi-molecular ion peak and its isotopic species obtained by chemical ionization with isobutane as a reagent gas, and the results are given both as ^{13}C content (atom %) and as the distribution of labelled molecules (mole %). The precision of ^{13}C measurements was within $\pm 2\%$ for ^{13}C abundance from 2 to 10 atom % and less than $\pm 1\%$ for ^{13}C abundance above 10 atom %. Contents of ^{13}C obtained by this method for soluble carbohydrates in corn leaves fed $^{13}CO_2$ were in good agreement with the results obtained with an infrared ^{13}C analyser.

Using this method, the incorporation of ^{13}C assimilated photosynthetically into several carbohydrates in soybean plants and their translocation into roots and nodules were investigated and it was shown that the GC–MS method was applicable as a routine method for the ^{13}C analysis of many plant metabolites in ^{13}C tracer experiments.

INTRODUCTION

The use of ^{13}C as a tracer in the study of plant physiology has developed in recent years. In spite of the disadvantage that the detection of ^{13}C as a stable isotope is much less sensitive than that of the radioactivity of ^{14}C, the application of ^{13}C in numerous branches of plant science should be developed increasingly in the future because first, the use of ^{13}C avoids the biological hazards of and the complicated restrictions accompanying the use of ^{14}C compounds, and second, the results are obtained directly in the form of an isotope ratio, which is more useful for the investigation of metabolic processes, especially the rate of turnover or translocation of metabolites.

The abundance of ^{13}C in biological materials has generally been measured with a mass spectrometer designed for isotope analysis or, more recently, by infrared absorption spectrometry[1]. In these methods, the sample is combusted by appropriate methods and ^{13}C content is determined by measuring the $^{13}CO_2$ to $^{12}CO_2$ ratio, so

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that the sample must be purified carefully prior to analysis in order to prevent the simultaneous combustion of contaminants, and also relatively large amounts of sample are required. Hence, these methods have not necessarily been adequate for the ^{13}C analysis of small amounts of biological compounds such as carbohydrates, organic acids and amino acids.

Gas chromatography–mass spectrometry (GC–MS) is considered to be the best method for the analysis of stable isotopes in trace amounts of biological compounds. In particular, the direct analysis of isotopically labelled compounds without any combustion or digestion process has the great advantage that it can determine the isotope ratio and also provide structural information on molecules and the intermolecular distribution of isotopes. The application of GC–MS to stable isotope analysis has been investigated since the early days of the development of GC–MS[2,3], and several workers reported its application in biochemistry and medicine[4,5]. However, most of this work was aimed at the quantification of isotopically labelled or non-labelled compounds with the aid of tracer molecules in which the position and abundance of particular isotopes was known previously, or was aimed at obtaining qualitative information on the positions of tracer atoms in molecules by analysis of appropriate fragment ion peaks. Thus, little work on either theoretical or methodological aspects of the routine analysis of stable isotope in compounds separated gas chromatographically has been done with the purpose to chase the fate of tracer atoms in metabolic process.

The direct analysis of stable isotopes in molecules by GC–MS involves the measurement of the intensities of several isotopic species of molecular, quasi-molecular or fragment ion peaks. In GC–MS, a relatively wide mass range is scanned rapidly at a rate of a few milliseconds per mass unit for small gas chromatographic peaks with a continuously varying intensity, so that the precision and accuracy of the intensities measured for each ion peak are much poorer than those in other methods for stable isotope analysis. Because of this disadvantage, GC–MS has not been adopted for the routine analysis of isotope ratios in biological materials generally containing isotopes in low abundance. In this regard, as suggested by Caprioli *et al.*[6], a quadrupole mass analyser has some advantages with respect to precision and accuracy over the magnetic deflection type mass spectrometer, mainly because of its extremely high switching rate of mass dispersion. However, the quadrupole instrument has the disadvantage that the practical sensitivity decreases rapidly in high mass regions. Most biological compounds, for analysis by gas chromatography, must be converted into volatile forms such as trimethylsilyl derivatives, which have relatively high molecular weights, so that for the direct analysis of these compounds it seems to be preferable today to use a magnetic deflection type mass spectrometer.

On the other hand, Sano *et al.*[7] developed a technique of mass fragmentography for monitoring continuously $^{12}CO_2$ and $^{13}CO_2$ from a pyrolyser for the continuous combustion of effluents from a gas chromatographic column. This method is expected to provide a better precision of ^{13}C measurement and also it is very attractive as it allows possible improvements to the simultaneous measurement of ^{13}C and ^{15}N abundance in doubly labelled compounds by an appropriate combustion method[8]. However, this method is not released from the apprehensions to reduce the accuracy of ^{13}C abundance owing to contamination and/or incomplete combustion, and also provides no structural information.

The precision and accuracy of the peak intensities in mass spectra have been greatly improved in recent years with a combination of GC–MS and computer systems that allow rapid repeat scanning and data acquisition with high performance. Therefore, although the limit of precision inherent in the direct analysis of isotope ratios by GC–MS should be borne in mind, it would seem to be valuable to investigate the possibility of using GC–MS for the routine measurement of isotope contents in various biological compounds with regard to the level of precision required in tracer experiments.

We have therefore investigated the direct GC–MS analysis of the ^{13}C contents of some plant metabolites labelled with ^{13}C assimilated photosynthetically and in this paper we discuss its application to the study of the fate of photosynthetic products in higher plants.

CALCULATION OF ^{13}C CONTENT FROM MASS SPECTRAL DATA

Biological molecules in tracer experiments are generally considered to be mixtures of molecules containing a varying number of isotopes. For example, ^{13}C-labelled glucose obtained by photosynthesis in $^{13}CO_2$ is a mixture of molecular species in which 0–6 carbon atoms are substituted by ^{13}C. In such mixtures, data on the distribution of isotopic molecular species are required in order to evaluate the isotope abundance. Thus, isotope contents of molecules are determined with complex arithmetic manupulations from data on the intensities of all possible isotopic species of molecular or fragment ion peaks. In a particular instance in which it is assumed that the isotopes are uniformally, *i.e.*, randomly, distributed in molecules, the equation proposed by VandenHeuvel *et al.*[9] could be used, which requires the intensities of only two ion peaks, *viz.*, M and M + 1 corresponding to the non-labelled molecule and the molecule containing only one heavy isotope, respectively. However, in a short-time labelling experiment, it is evident that this assumption of random distribution is by no means justified. On the other hand, Biemann[2] discussed in detail an approximate method using all of the peak intensities corresponding to each isotopic species, based on the assumption that the ratios of the intensity of the isotope peak at M + 1, M + 2, ..., etc., to the intensity of peak at M are same in both unenriched and enriched materials. This assumption is approximately justified in most instances but the resulting error is not small if the contribution of particular isotopes to the natural abundance of each isotopic molecular species is not negligible in comparison with that of other isotopes in the molecule.

The method for the evaluation of the ^{13}C content (in atom %) and the distribution of isotopic molecular species (in mole %) devised in this work can be outlined as follows.

An empirical expression C_nT was initially introduced, corresponding to the molecular or fragment ion peaks concerned, where C_n corresponds to n carbon atoms expected to incorporate ^{13}C as a tracer and T contains all of the other elements in molecule. For example, the quasi-molecular (MH^+) ion peak (m/e = 628) of the trimethylsilyl (TMS) derivative of glucose oxime, which is obtained by chemical ionization with isobutane as a reagent gas, is expressed as C_6T, where T is $C_{18}H_{63}O_6NSi_6$. Various isotopic species of T are denoted by T^0, T^1, T^2, ..., etc., each with one mass unit difference, where T^0 is one containing no heavy isotope. The

abundance of T^i ($i = 0, 1, 2, \ldots$) depends on the natural abundance of the isotopes of each element constituting T and is considered to be identical in unenriched and enriched molecules.

Let M be the mass number of C_nT containing no heavy isotope, then the molecular species corresponding to each isotopic ion peak with mass number at M, M + 1, M + 2, ..., M + n are as follows:

$$\begin{aligned}
&\text{M:} && {}^{12}C_nT^0\\
&\text{M + 1:} && {}^{13}C^{12}C_{n-1}T^0,\ {}^{12}C_nT^1\\
&\text{M + 2:} && {}^{13}C_2{}^{12}C_{n-2}T^0,\ {}^{13}C^{12}C_{n-1}T^1,\ {}^{12}C_nT^2\\
&\vdots\\
&\text{M + } n\text{:} && {}^{13}C_nT^0,\ {}^{13}C_{n-1}{}^{12}CT^1, \ldots,\ {}^{12}C_nT^n
\end{aligned}$$

Let A_i ($i = 0, 1, 2, \ldots, n$) be the abundance of T^i and B_i the abundance of isotopic species of the molecule to be analysed in which i carbon atoms in n are substituted by ^{13}C. Then, the intensity R_i ($i = 0, 1, 2, \ldots, n$) of the ion peak at M, M + 1, M + 2, ..., M + n is related to A_i and B_i by the following equations:

$$\left.\begin{aligned}
R_0 &= k(A_0B_0)\\
R_1 &= k(A_0B_1 + A_1B_0)\\
R_2 &= k(A_0B_2 + A_1B_1 + A_2B_0)\\
&\vdots\\
R_n &= k(A_0B_n + A_1B_{n-1} + \ldots + A_nB_0)
\end{aligned}\right\} \qquad (1)$$

where k is a proportionality constant depending on the amounts of sample injected and the ionization efficiency. It should be noted that the ionization efficiencies of the ion peaks concerned are assumed to be identical for all labelled species of the same molecule.

Dividing each equation in eqn. 1 by the first equation in the series, $R_0 = k(A_0B_0)$, gives nth-order simultaneous equations:

$$\left.\begin{aligned}
R_1' &= A_1' + B_1'\\
R_2' &= A_2' + A_1'B_1' + B_2'\\
R_3' &= A_3' + A_2'B_1' + A_1'B_2' + B_3'\\
&\vdots\\
R_n' &= A_n' + A_{n-1}'B_1' + \ldots + B_n'
\end{aligned}\right\} \qquad (2)$$

where

$$\left.\begin{aligned} R_i' &= R_i/R_0 \\ B_i' &= B_i/B_0 \\ A_i' &= A_i/A_0 \qquad (i = 1, 2, \ldots, n) \end{aligned}\right\} \tag{3}$$

R_i' are the relative intensities of each of the ion peaks of isotopic species of C_nT based on $R_0 = 1$.

Eqns. 2 are solved for B_i' and the abundance of labelled molecules, $B_0, B_1, \ldots, B_n$ are obtained with

$$\left.\begin{aligned} B_0 &= 1\Big/\left(\sum_{i=1}^{n} B_i' + 1\right) \\ B_i &= B_0 B_i' \qquad (i = 1, 2, \ldots, n) \end{aligned}\right\} \tag{4}$$

as the sum of molecular abundance, $\sum_{i=0}^{n} B_i$ must be equal to unity. Then, the ^{13}C content, X (atom %), is calculated with

$$X = \frac{1}{n} \sum_{i=1}^{n} iB_i \cdot 100 \tag{5}$$

The abundances of $T^0, T^1, \ldots, T^n$ can be calculated theoretically from the natural abundances of the isotopes of each element in T. However, as discussed later, the peak intensities measured by a mass spectrometer contain some systematic errors considered probably to be due to contamination of small peaks by electrical noise and/or background noise (see Tables I and II). Therefore, it is preferable to use the A_i' values determined experimentally by the analysis of unenriched molecules.

It is assumed initially that ^{13}C is distributed randomly in unenriched molecules. This assumption is entirely reasonable as isotope discrimination occurring naturally is negligible for the level of precision achieved with GC–MS analysis. Let a and b be the natural abundance of ^{12}C and ^{13}C, respectively, $(a + b = 1)$ and the probability of finding any molecule with a certain number of atoms of ^{12}C and ^{13}C will be the appropriate term of the binomial expansion

$$(a + b)^n = a^n + {}_nC_1 a^{n-1} b + {}_nC_2 a^{n-2} b^2 + \ldots + b^n \tag{6}$$

where

$${}_nC_i = \frac{n(n-1)\ldots(n-i+1)}{i!}$$

Therefore, the B_is in eqns. 1 are substituted by

$$B_i \equiv {}_nC_i a^{n-i} b^i \qquad (i = 0, 1, 2, \ldots, n) \tag{7}$$

Let K_i ($i = 1, 2, \ldots, n$) be the intensities of the M + 1, M + 2, ..., M + n peaks relative to the intensity of the M peak in the unenriched molecule; the resulting equations corresponding to eqns. 2 are

$$\left.\begin{aligned} K_1 &= A'_1 + {}_nC_1 e \\ K_2 &= A'_2 + {}_nC_1 e A'_1 + {}_nC_2 e^2 \\ K_3 &= A'_3 + {}_nC_1 e A'_2 + {}_nC_2 e^2 A'_1 + {}_nC_3 e^3 \\ &\vdots \\ K_n &= A'_n + {}_nC_1 e A'_{n-1} + {}_nC_2 e^2 A'_{n-2} + \ldots + e^n \end{aligned}\right\} \tag{8}$$

where $e = b/a$. If the official value of ^{13}C natural abundance, $b = 0.01107$, is adopted, then e is calculated to be 0.011194.

Eqns. 8 are solved for A'_i and the resulting values are used for eqns. 2 for an enriched sample.

The abundances A_i ($i = 0, 1, 2, \ldots, n$) of T^0, T^1, T^2, ..., T^n are determined in the same manner as shown in eqns. 4. Here, the assumption that $\sum_{i=0}^{n} A_i = 1$ is not entirely justified because A_{n-1}, A_{n-2}, ..., etc., are very small but not zero. However, the resulting error is not large and also this approximation has no effect on the calculation of ^{13}C abundance in enriched materials because eqns. 2 require values of A'_i but not of A_i.

EXPERIMENTAL

Preparation of trimethylsilyl derivatives

A solution containing 1–5 mg of carbohydrates was evaporated *in vacuo* completely to dryness over phosphorus pentoxide. Aliquots (about 200 µl) of anhydrous pyridine containing hydroxylamine hydrochloride (25 mg/ml) were added to the residue and reaction was carried out at 75°C for 1 h to give oximes of reducing sugars. After cooling to room temperature, 1 ml of TMS-HT (Tokyo Kasei Kogyo, Tokyo, Japan) was added and the mixture was allowed to stand overnight at room temperature to give trimethylsilyl (TMS) derivatives. A few microlitres of the clear supernatant solution were injected into the gas chromatograph.

Preparation of ^{13}C-labelled plant carbohydrates

Corn plants were grown in phytotron (25°C, natural light) for 20 days after sowing in plastic pots filled with a mixture of vermiculite, perlite, peat moss and fine gravel in the proportions 2:2:1:1 (v/v). The pots were fertilized with 5 g of Magamp-K and 15 g of magnesia lime.

Soybean plants were grown for 35 days with complete nutrient solution aerated continuously in a growth chamber controlled at 25°C (day, 14 h) and 19°C (night, 10 h) with artificial light at about 40 klux from a metal halide lamp. The plants were well nodulated by inoculation of a commercial strain of *Rhizobium japonicum*. The composition of the nutrient solution was as follows (mg/l in deionized water): KNO_3, 18.1; $MgSO_4 \cdot 7H_2O$, 202.8; KH_2PO_4, 87.9; K_2SO_4, 16.6; $CaCl_2 \cdot 2H_2O$, 73.4; H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.08; $ZnSO_4 \cdot 7H_2O$, 0.44; $CuSO_4 \cdot 5H_2O$, 0.04; $Na_2MoO_4 \cdot 2H_2O$, 0.10; $CoCl_2 \cdot 6H_2O$, 0.04; Fe-EDTA, 22.5; and pH, 5.8–6.2.

Whole plants of soybean and attached leaf (the fourth from lowest leaf) of corn were used for $^{13}CO_2$ assimilation. Assimilation chambers made of acrylic sheet designed for whole plants or attached leaves were set up in the growth cabinet with artifical light at 25 klux (for corn leaves) or 40 klux (for soybean plants). The temperature in the assimilation chamber was controlled at 25°C. After the plants or attached leaves had been introduced, the air in the chamber was purged with carbon dioxide-free air and then closed air-tight. $^{13}CO_2$ was generated from $Ba^{13}CO_3$ by reaction with 1 *N* hydrochloric acid. The concentration of carbon dioxide in the chamber was controlled at about 300–400 ppm by regulating manually the generation of $^{13}CO_2$, monitoring with an infrared carbon dioxide analyser. The abundance of ^{13}C in the air was denoted as that of the $Ba^{13}CO_3$ used.

After feeding of $^{13}CO_2$ for a certain period, the plants were immediately separated into the component parts and killed in liquid nitrogen or in a deep-freeze at −80°C. The frozen plant tissues were macerated with 80% (v/v) ethanol using a Waring blender or a mortar and pestle, followed by centrifugation at 3000 *g* for 30 min, then the pellet was washed twice with 80% ethanol. The supernatant and washings were combined and evaporated to remove ethanol. The resulting aqueous solution was passed through columns of Dowex 50 (H^+) and Dowex 1 ($HCOO^-$), and the neutral fraction eluted (containing carbohydrates) was evaporated and dried completely over phosphorus pentoxide, followed by the preparation of TMS derivatives.

Uniformally labelled [^{13}C]glucose

[U-^{13}C]Glucose containing 62.3 atom% of ^{13}C as an authentic value (Merck, Rahway, NJ, U.S.A.) was kindly supplied by Dr. S. Murayama of the Department of Soil Science, National Institute of Agricultural Sciences, Ibaraki, Japan.

Gas chromatograph–mass spectrometry

A Model JMS-D300 double-focusing mass spectrometer (JEOL, Tokyo, Japan) was equipped with a Model 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.). For the separation of plant carbohydrates, a 1.8 m × 2 mm I.D. glass column packed with 3% OV-17 on 100–120-mesh Chromosorb W HP was used. The flow-rate of the carrier gas (helium) was 40 ml/min, the injector temperature was 250°C and the column temperature was programmed from 140 to 260°C at 2°C/min.

The chemical ionization (CI) method with isobutane as the reagent gas was used to obtain quasi-molecular ion peaks. The pressure of the reagent gas in the ionization chamber was kept at about 1 Torr, the ionization potential was 70 eV, the ion source temperature was 240°C and the ionization current was 300 μA. By using these conditions, the MH^+ ion peak was obtained as a base peak for the TMS

derivatives of any carbohydrate analysed in this experiment except sucrose. Mass spectra were obtained with repeat scanning over the mass range *m/e* 440–640 with a scanning speed of 0.6 sec and a scanning interval of 1.0 sec. The data for the intensity of each ion peak were stored for each scan in a JMA-2000 computer system and each mass number was integrated from the start to the end of one chromatographic peak. These integrated intensities of each mass number were recorded and used for calculation.

Liquid chromatography and ^{13}C analysis with an infrared absorption spectrometer

Soluble carbohydrates in corn leaves fed $^{13}CO_2$ were also separated by ion-exchange chromatography to measure the ^{13}C abundance by infrared absorption spectrometry. A Model JLC-6AH automatic liquid chromatograph (JEOL) was equipped with a water-jacketed glass column (280 × 8 mm I.D.) packed with LCR-3 anion-exchange resin. Borate–sodium hydroxide eluting buffers with the following concentrations were used: first, 0.11 *M* (pH 7.5); second, 0.25 *M* (pH 9.0); and third, 0.35 *M* (pH 9.5). The first buffer was changed after 130 min to the second buffer and after 310 min to the third buffer. The buffer flow-rate was 0.51 ml/min and the column temperature was constant at 55°C.

Carbohydrates (10–15 mg) were dissolved in 1 ml of the first buffer and loaded on the column with the automatic sample injector. The eluent was collected in 2-ml fraction tubes and aliquots of each fraction were heated with 1% orcinol in 96% sulphuric acid at 95°C followed by monitoring of the absorbance at 470 nm. The fractions corresponding to each carbohydrate were combined, evaporated, passed through a column of Dowex 50 (H^+) to remove sodium ions and concentrated so as to contain about 1 mg of carbohydrate in 0.1 ml of solution. A Model EX-130 infrared ^{13}C analyser (Japan Spectroscopic, Tokyo, Japan) was employed for the measurement of the ^{13}C abundance in carbohydrates. Borate saturating in the carbohydrate solution did not interfere in the ^{13}C analysis.

RESULTS

Analysis of authentic [^{13}C]glucose

Mass spectra of the TMS-oxime of glucose are shown in Fig. 1. The MH^+ (*m/e* 628) ion peak was a base peak in non-labelled glucose, whereas in authentic [U-^{13}C]glucose (62.3 atom%), an isotopic ion peak at *m/e* 634 exhibited the maximum intensity. Occasionally, a molecular ion peak (M^+, *m/e* 627) or another quasi-molecular ion peak ($M - H^+$, *m/e* 626) was detected with trace intensity. If the intensities of these peaks are relatively large, the effects of the isotopic species of these ion peaks on the peaks at *m/e* 628, 629, ..., etc., must be corrected, as discussed by Biemann[2]. However, in the experiment reported here, these peaks were usually very small or not detectable, and can then be neglected in the calculation.

Table I shows the relative intensities of the MH^+ ion and its isotopic species for the TMS-oxime of authentic glucose with ^{13}C in natural abundance. The ion multiplier corresponds to the gain control of the amplifier of the ion detector. Theoretical values were obtained by calculation from the natural abundance of isotopes of each element constituting the MH^+ ion (empirical formula $C_{24}H_{63}O_6NSi_6$) based on the assumption of random distribution of each isotope. The observed intensities above

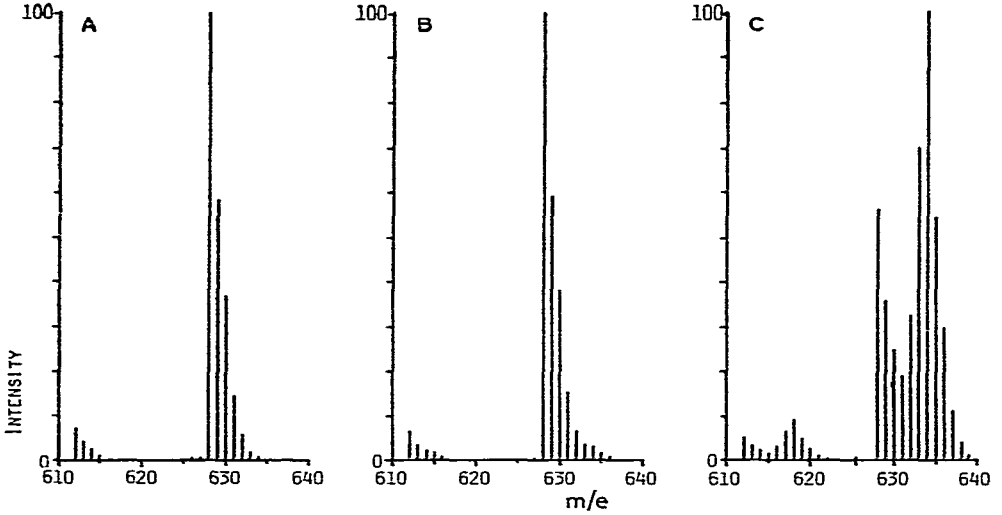


Fig. 1. Mass spectra (*m/e* 610–640) of TMS-oxime of authentic glucose containing ^{13}C in natural abundance (A), 3.84 atom% (B) and 62.3 atom% (C).

m/e 632 were higher to some extent than the theoretically expected values and the differences were much larger with high ion multiplying. This is considered to be caused by the fact that the small peaks suffered from a relatively large influence of electrical noise. The level of ion multiplying also affected to the deviation of the intensity of each ion peak, and the level required to detect small isotopic peaks depended on the amount of sample injected and also on the scanning speed per mass

TABLE I

RELATIVE INTENSITIES OF MH^+ ION PEAK AND ITS ISOTOPIC SPECIES OF TMS-OXIME OF GLUCOSE CONTAINING ^{13}C IN NATURAL ABUNDANCE

Data are expressed as mean ± standard deviation. $\sqrt{\Sigma (x - \bar{x})^2/n}$ ($n = 6$). Theoretical values were calculated by assumption of random distribution of ^{13}C, ^{2}H, ^{17}O, ^{18}O, ^{15}N, ^{29}Si and ^{30}Si each in natural abundance.

m/e	*Sample injected (μg)*			*Theoretical*
	5	*1*	*0.2*	
	Ion multiplier			
	1.10	*1.40*	*1.65*	
628	100.00	100.00	100.00	100.00
629	58.79 ± 0.36	58.43 ± 0.51	59.28 ± 0.57	58.98
630	38.04 ± 0.36	38.06 ± 0.37	38.43 ± 0.83	37.80
631	14.82 ± 0.09	14.73 ± 0.33	15.08 ± 0.41	14.46
632	5.69 ± 0.06	5.80 ± 0.05	5.98 ± 0.18	5.26
633	1.80 ± 0.02	1.85 ± 0.05	2.02 ± 0.09	1.47
634	0.63 ± 0.01	0.70 ± 0.03	0.75 ± 0.05	0.38

TABLE II

ABUNDANCE OF ISOTOPIC SPECIES OF T FOR MH^+ IONS OF TMS DERIVATIVES OF GLUCOSE AND FRUCTOSE OXIMES

T = $C_{18}H_{63}O_6NSi_6$. Data are expressed as mean ± standard error (n = 6).

Species	*Theoretial*	*Authentic*	*Corn leaves*	
		Glucose	*Glucose*	*Fructose*
T	0.4894	0.4855 ± 0.00083	0.4851 ± 0.00063	0.4858 ± 0.00059
T + 1	0.2558	0.2534 ± 0.00037	0.2508 ± 0.00063	0.2517 ± 0.00056
T + 2	0.1669	0.1668 ± 0.00058	0.1675 ± 0.00041	0.1660 ± 0.00067
T + 3	0.0590	0.0608 ± 0.00018	0.0617 ± 0.00033	0.0613 ± 0.00049
T + 4	0.0215	0.0237 ± 0.00011	0.0247 ± 0.00031	0.0245 ± 0.00037
T + 5	0.0056	0.0073 ± 0.00053	0.0076 ± 0.00018	0.0081 ± 0.00017
T + 6	0.0014	0.0025 ± 0.00004	0.0026 ± 0.00007	0.0025 ± 0.00004

unit. Under the analytical conditions adopted in this experiment, a level of 1.10 in the ion multiplier gave deviations within ±1.0% for the most of the peak intensities and then the amount of sample required was 5 μg or more.

Table II shows the abundance of isotopic species of T ($C_{18}H_{63}O_6NSi_6$) for MH^+ ions of TMS derivatives of glucose and fructose oximes. The measured abundances for authentic glucose and for glucose and fructose from corn leaves generally agreed well with theoretical values. However, the measured abundances for T + 4, T + 5 and T + 6 were significantly higher than the calculated values, reflecting the overestimation of small peak intensities in the mass spectra as shown in Table I. Thus, the use of theoretical values of the abundance of T for A_i in eqns. 1 resulted in some overestimation of the ^{13}C abundances. However, the atom excess percentage obtained by substracting the ^{13}C abundance in unenriched material determined by using

TABLE III

^{13}C ATOM% AND ISOTOPIC MOLECULAR ABUNDANCE OF AUTHENTIC [U-^{13}C]GLUCOSE

Original and diluted samples were expected to have 62.3 and 3.84 atom% as theoretical values, respectively.

Species	*Isotopic molecular abundance*		*Atom%*	
	Original	*Diluted*	*Original*	*Diluted*
M	0.2647 ± 0.0002	0.9058 ± 0.0004	60.77	3.72
M + 1	0.0227 ± 0.0004	0.0624 ± 0.0006	60.76	3.75
M + 2	0.0133 ± 0.0001	0.0014 ± 0.0009	60.89	3.67
M + 3	0.0397 ± 0.0001	0.0017 ± 0.0003	60.82	3.70
M + 4	0.1115 ± 0.0002	0.0052 ± 0.0003	60.94	3.65
M + 5	0.2529 ± 0.0003	0.0108 ± 0.0002	60.83	3.66
M + 6	0.2952 ± 0.0008	0.0127 ± 0.0001		
			Mean: 60.83 ± 0.045	3.69 ± 0.017

TABLE IV

ANALYSIS OF AUTHENTIC [^{13}C]GLUCOSE

Samples were obtained by dilution of [U-^{13}C]glucose (62.3 atom%). Data are expressed as mean ± standard error (n = 4).

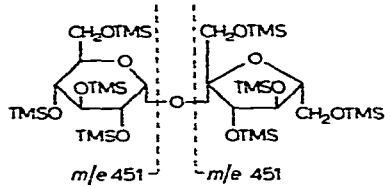
Sample No.	*Atom%*	
	Expected	*Observed*
1	13.25	12.94 ± 0.062
2	7.87	7.82 ± 0.035
3	4.69	4.53 ± 0.059
4	2.95	2.92 ± 0.034
5	1.86	1.84 ± 0.020
6	1.49	1.51 ± 0.026

the same theoretical abundance of T was identical with the results obtained by using the measured abundance of T as the natural abundance of ^{13}C was 1.107 atom%. Therefore, it was considered to be preferable to use the measured abundance of T obtained from the analysis of unenriched material with the same conditions of measurement as for the analysis of enriched material.

The abundances of isotopic molecular species and ^{13}C (atom%) are shown in Table III for [^{13}C]glucose with 62.3 and 3.84 atom% of ^{13}C as authentic values. The measured ^{13}C contents were lower in both samples than the authentic values. A similar tendency was observed in the analysis of [^{13}C]glucose with a varying ^{13}C content, as shown in Table IV. The standard deviations varied from 0.2% of 60.83 atom% to 3.4% of 1.51 atom% [^{13}C]glucose. The isotopic molecular abundance of ^{13}C-enriched molecules provides information on the distribution of species of molecules containing a varying number of ^{13}C atoms. The results obtained for the original [U-^{13}C]glucose used in this experiment indicated that most of this sample consisted of non-labelled and labelled molecules with five or six ^{13}C atoms. The uniformity of labelling of this authentic [U-^{13}C]glucose will be discussed later.

Analysis of soluble carbohydrates in corn leaves fed $^{13}CO_2$

The total ion monitoring (TIM) mass chromatogram of the neutral fraction of corn leaves is shown in Fig. 2. Fructose, glucose and sucrose were the major components of this fraction. The abundances of ^{13}C obtained by GC–MS were compared with those obtained with the infrared ^{13}C analyser (Table V). The data from the two methods were nearly identical except for sucrose, which showed relatively high ^{13}C contents. For the analysis of sucrose, the fragment ion peak at *m/e* 451 was used because the MH^+ ion peak of the TMS derivative of sucrose (expected to show *m/e* 919) could not be obtained. This fragment ion was considered to contain two components from glucose and fructose residues of sucrose with the following mass fragmentation:



This interpretation was supported by a comparison of measured and theoretically calculated abundances of T for the empirical formula of this fragment ion (Table VI). Thus, if the ^{13}C abundances in glucose and fructose residues are not identical and also the contributions of these two components to this fragment are different, the resulting data on ^{13}C abundance would differ significantly from the ^{13}C abundance in sucrose. This is inevitable for the compound, which has a large molecular weight and for which molecular, quasi-molecular or fragment ion peaks containing all of the carbon atoms in original molecule cannot be obtained. Therefore, it should be noted that the data given for the ^{13}C abundance of sucrose in this paper possibly contain significant errors, probably giving lower values than the true ones.

Fig. 3 shows time course of the incorporation of ^{13}C into carbohydrates in corn leaves. The two experiments indicated that fructose and glucose were labelled more rapidly than sucrose in the short labelling period. However, the incorporation of ^{13}C into sucrose exceeded increasingly those of other two carbohydrates with increasing labelling period.

Labelling of carbohydrates with ^{13}C and their translocation in soybean plants

The incorporation of ^{13}C assimilated photosynthetically into soluble carbohy-

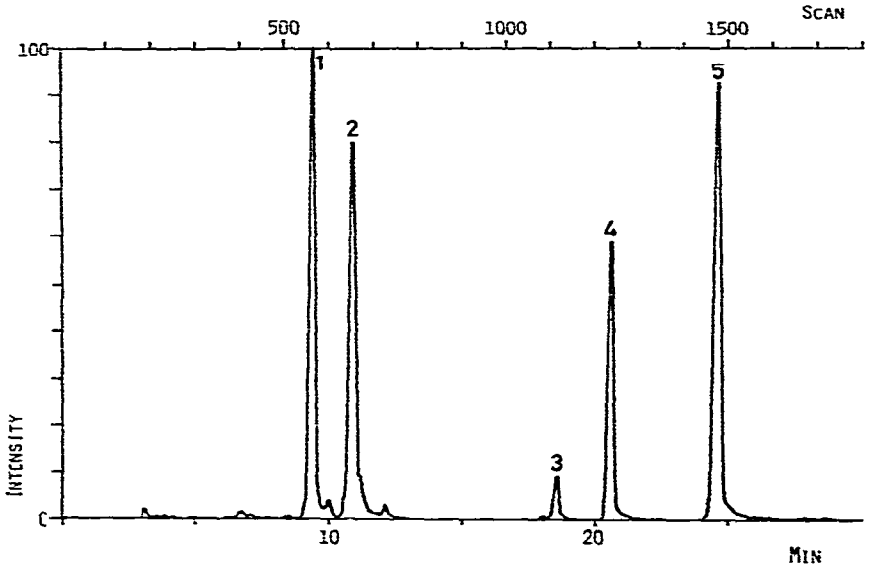


Fig. 2. TIM mass chromatogram between *m/e* 440 and 640 of TMS-oximes of soluble carbohydrates in corn leaves. 1 = Fructose; 2 = glucose; 3 = unknown; 4 = β-phenyl glucoside (internal standard); 5 = sucrose.

TABLE V

^{13}C ABUNDANCE OBTAINED BY GC–MS ANALYSIS AND INFRARED ABSORPTION SPECTROMETRY FOR SOLUBLE CARBOHYDRATES IN CORN LEAVES FED $^{13}CO_2$ UNDER VARIOUS CONDITONS

Carbohydrates were separated by ion-exchange chromatography (see text) followed by infrared analysis. Data are given as averages of three and four independent measurements for infrared and GC–MS analysis, respectively.

Sample	*Sample No.*	*Atom%*	
		IR	*GC–MS*
Sucrose	1	7.85 ± 0.069	7.09 ± 0.082
	2	3.58 ± 0.021	2.89 ± 0.038
	3	1.27 ± 0.010	1.24 ± 0.038
	4	1.16 ± 0.010	1.17 ± 0.033
Glucose	2	2.62 ± 0.115	2.56 ± 0.032
	3	1.61 ± 0.023	1.50 ± 0.047
	4	1.26 ± 0.006	1.27 ± 0.062
Fructose	3	1.50 ± 0.015	1.51 ± 0.062
	4	1.38 ± 0.015	1.37 ± 0.007

drates in soybean plants and their translocation to roots and nodules were investigated as an example of the application of the method presented here.

Soybean nodules contain large amounts of cyclitols, such as pinitol (5-O-methyl-D-inositol), (+)*chiro*-inositol, sequoyitol (2-O-methyl-*myo*-inositol) and *myo*-inositol[10]. These cyclitols form 60% or more of soluble carbohydrates in nodules and also are present in leaves, stems and roots at relatively low concentrations. The metabolism and physiological role of these cyclitols, especially in relation to the dinitrogen-fixing activity of soybean nodules, have not yet been elucidated.

Soybean plants were pre-treated in the dark for 48 h to reduce the endogenous pool of carbohydrates and then exposed to $^{13}CO_2$ (90 atom%) for 3 h under irradiance at about 40 klux. Plants were sampled 3, 9 and 23 h after the beginning of $^{13}CO_2$ exposure and the ^{13}C abundance of carbohydrates soluble in 80% ethanol was determined.

TABLE VI

ABUNDANCE OF ISOTOPIC SPECIES OF T FOR FRAGMENT ION (m/e = 451) OF TMS DERIVATIVE OF SUCROSE

T = $C_{12}H_{43}O_5Si_4$. Data are expressed as mean ± standard error (n = 6).

Species	*Theoretical*	*Observed*
T	0.6210	0.6135 ± 0.00189
T + 1	0.2152	0.2156 ± 0.00043
T + 2	0.1232	0.1287 ± 0.00047
T + 3	0.0299	0.0322 ± 0.00017
T + 4	0.0089	0.0065 ± 0.00176
T + 5	0.0016	0.0023 ± 0.00029
T + 6	0.0003	0.0010 ± 0.00017

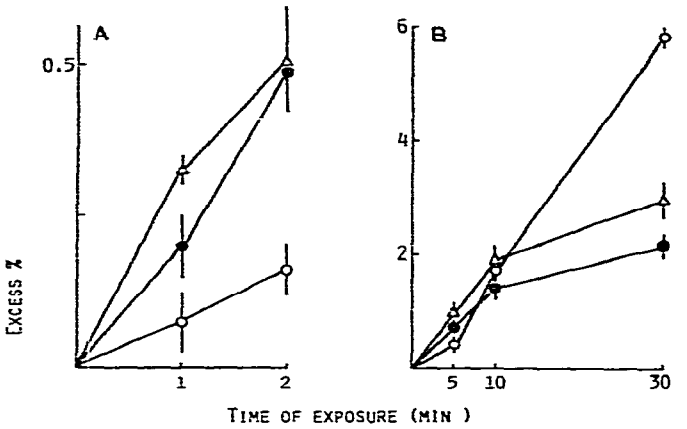


Fig. 3. Incorporation of ^{13}C into soluble carbohydrates in corn leaves fed $^{13}CO_2$ of 90 atom % (A) and 30 atom % (B). Data are given as averages of four or six independent measurements. Vertical lines = 2 × standard error. ○, Sucrose; ●, glucose; △, fructose.

The TIM mass chromatogram is shown in Fig. 4 for the nodule extract. Pinitol, (+)*chiro*-inositol, sequoyitol, glucose, *myo*-inositol and sucrose were the major components of this extract. Fructose was not completely separated from (+)*chiro*-inositol. Penta-TMS derivatives of pinitol and sequoyitol (mol.wt. 554) showed the MH^+ ion peak at *m/e* 555, and hexa-TMS derivatives of (+)*chiro*-inositol and *myo*-inositol (mol.wt. 612) showed the MH^+ ion peak at *m/e* 613, both as the base peak. Thus, these MH^+ ion peaks and their isotopic species were employed for ^{13}C analysis.

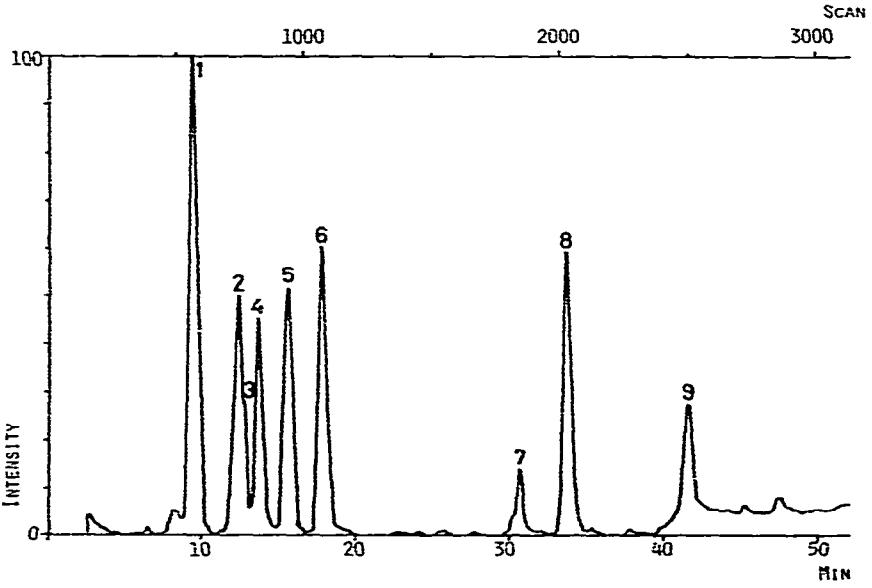


Fig. 4. TIM mass chromatogram of TMS-oximes of carbohydrates in 80% ethanol extract of soybean nodule. 1 = Pinitol; 2 = (+)*chiro*-inositol; 3 = fructose; 4 = sequoyitol; 5 = glucose; 6 = *myo*-inositol; 7 = unknown; 8 = β-phenyl glucoside (internal standard); 9 = sucrose.

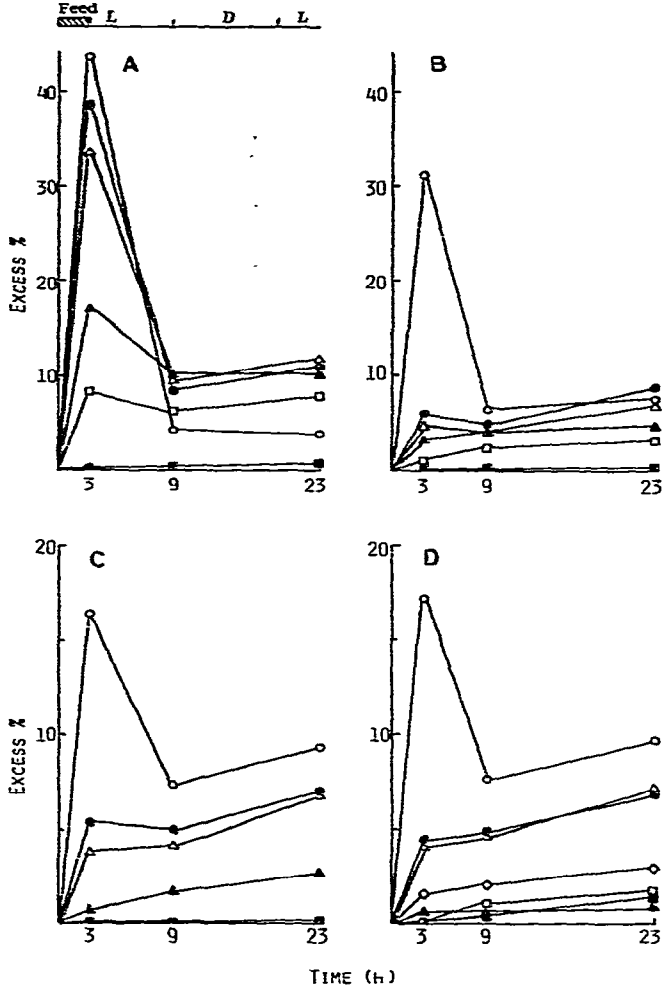


Fig. 5. Incorporation of ^{13}C into soluble carbohydrates in leaves (A), stems and petioles (B), roots (C) and nodules (D) of soybean plants following photosynthetic assimilation of $^{13}CO_2$ for 3 h. Data are given as averages of four independent measurements. Experimental conditions are briefly illustrated on the upper part of (A); L and D are light (40 klux) and dark, respectively. ○, Sucrose; ●, glucose; △, fructose; ▲, *myo*-inositol; □, sequoyitol; ■, pinitol; ◇, (+)*chiro*-inositol.

The results are shown in Fig. 5. The incorporation of ^{13}C into cyclitols other than *myo*-inositol and sequoyitol in leaves was much less than that into saccharides. In particular, pinitol, which occupied the largest part of cyclitols not only in nodule but also in other parts of the plant, exhibited little incorporation of ^{13}C. This result is contrary to the suggestion by Phillips and Smith[11] that pinitol may be one of the photosynthates that are translocated into the nodules. The results obtained in this experiment may indicate that sucrose is the major compound translocating carbon assimilated by photosynthesis into the root and also the nodules. The large amounts of cyclitols in the nodules are considered to be metabolically inactive as a whole. This suggests that these cyclitols may have a certain physiological role, different from the

intermediate metabolites supplying the energy fixed by photosynthesis to nodules for dinitrogen fixation.

It should be noted that the incomplete separation of fructose and (+)*chiro*-inositol may result in some errors in the measurement of ^{13}C abundance in (+)*chiro*-inositol. As shown in Fig. 1, the TMS-oxime of glucose (and also fructose) exhibited a small fragment ion peak at *m/e* 612 caused by the demethylation of the molecular ion, and isotopic species of this fragment ion are considered to overlap with MH^+ ion peaks of (+)*chiro*-inositol. For this reason, the ^{13}C abundance of (+)*chiro*-inositol was not measured in leaves, stems and roots. In nodule extracts the fructose content was very low and it seemed to be reasonable to neglect the contribution of fructose to the MH^- ion peaks of (+)*chiro*-inositol. However, the possibility of overestimation of the ^{13}C abundance in (+)*chiro*-inositol was not removed completely because fructose showed a much higher ^{13}C abundance than (+)*chiro*-inositol.

DISCUSSION

For the calculation of the ^{13}C abundance in labelled compounds, it is essential that there is no contamination of the background or other impurities on the ion peaks to be measured. This assumption is considered to be satisfied completely for the quasi-molecular (MH^+) ion peak and its isotopic peaks obtained by the CI method. Some other inherent assumptions for the calculation of isotope abundance from GC–MS data together with the sources of error were discussed in detail by Biemann[2]. However, in ^{13}C-labelled compounds, the error caused by factors such as fractionation or loss of labelled molecules in the mass spectrometer is considered to be negligibly less than that in the deuterium-labelled compounds which Biemann mainly discussed.

In the analysis of authentic [^{13}C]glucose with varying abundance, the ^{13}C contents obtained by GC–MS were lower than the expected values (Tables III and IV). A satisfactory interpretation of these systematic differences is not given here, but

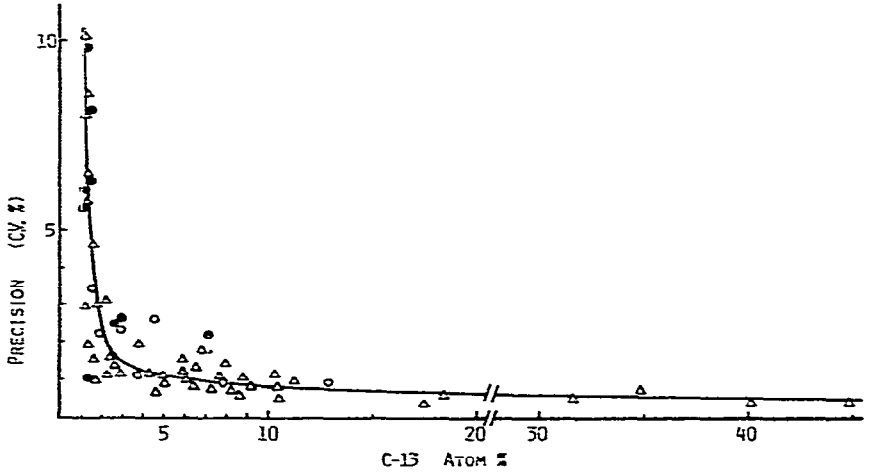


Fig. 6. Precision (coefficient of variation, %) as a function of ^{13}C abundance (atom%) for authentic [^{13}C]glucose (○) and ^{13}C-labelled carbohydrates in corn leaves (●) and in soybean plants (△).

the differences were not large in most instances and also were sufficiently reproducible.

The results of the analysis of authentic glucose (Tables I–IV) give an indication of the precision of the method. The variance of peak intensity (Table I) depends on the level of ion multiplying, and therefore on the amount of ions introduced into the detector. On the other hand, the scanning speed per mass unit and the number of repeat scans for one chromatographic peak were also important factors influencing the precision of measurement. The upper limit of the scanning speed permitted depends on the mass resolving power required for the measurement of the ion peaks concerned. In this experiment, the scanning speed was established at 0.6 sec for the mass range *m/e* 440–640, which included all of the MH^+ or fragment (for sucrose) ion peaks of carbohydrates analysed in this experiment. In addition, at least 0.4 sec was required for the dead time of data acquisition and for the reversion time of the magnetic field. Therefore, repeat scanning was conducted every 1.0 sec and then 40–80 scans were made from the start to the end of each chromatographic peak. If a very narrow mass range can be adopted, the scanning interval is less than 1.0 sec, and then the increase in the number of repeat scans may result in some improvement in precision, but a great improvement cannot be expected because the scanning interval cannot be less than 0.5 sec.

Fig. 6 shows the precision (coefficient of variation) of measurement as a function of ^{13}C atom%. The results indicate that a precision within about $\pm 2\%$ was achieved in the measurement of carbohydrates containing 2.0–10 atom% of ^{13}C, and less than $\pm 1\%$ for a ^{13}C abundance above 10 atom%. However, in the measurement of compounds containing below 2 atom% of ^{13}C, the variance of the data was much larger.

The precision achieved in this work is not necessarily sufficient for the ^{13}C analysis of biological compounds. However, as mentioned before, in other methods of stable isotope analysis with much better precision, it is difficult to avoid completely the errors caused by the complex purification procedure or degradation of materials. Therefore, in most tracer experiments, the present method seems to be suitable for the routine ^{13}C analysis of many biological compounds, as shown for example in the experiments on corn leaves and soybean plants fed $^{13}CO_2$.

A higher precision of measurement would permit the use of ^{13}C in a much lower enrichment experiment. Several workers[12] have reported other approaches for improving the precision of the peak intensity, but the level achieved was approximately $\pm 1\%$. Possible improvements may be achieved with the development of quadrupole-type GC–MS systems possessing improved sensitivity in the high mass region.

It is useful to discuss the uniformity of the distribution of isotopes in labelled molecules. For this purpose, the measured abundance of isotopic molecular species must be compared with the abundance obtained by calculation on the assumption of a random distribution of the isotopes. The distribution of labelled molecules in authentic [U-^{13}C]glucose (Table III) was very different from the theoretically uniform distribution of 60.83 atom% of ^{13}C (results of calculation not shown). The possibility that the sample is a mixture of unenriched glucose and enriched glucose with a uniform ^{13}C distribution was examined following the method of Heron[13] (Table VII). The measured abundance of enriched glucose was obtained as follows:

TABLE VII

COMPARISON OF MEASURED AND CALCULATED VALUES FOR ENRICHED FRACTION OF AUTHENTIC [U-^{13}C]GLUCOSE

Data were calculated from intensities of isotopic species of MH^+ ions of TMS-oximes of non-labelled and labelled (60.83 atom%) glucose according to the method of Heron[11].

Species	*Measured abundance*	*Calculated*		*Difference*
		Expression	*Abundance*	
M	0.00000*	$(1-x)^6$	0.00001	−0.00001
M + 1	0.00688	$6(1-x)^5x$	0.00025	0.00662
M + 2	0.01779	$15(1-x)^4x^2$	0.00399	0.01380
M + 3	0.05542	$20(1-x)^3x^3$	0.03336	0.02206
M + 4	0.13552	$15(1-x)^2x^4$	0.15695	−0.00143
M + 5	0.35274	$6(1-x)\ x^5$	0.39378	−0.04104
M + 6	0.41165	x^6	0.41165**	

* Assumed to be zero.

** This value was used for calculation of x: $x = \sqrt[6]{0.41165} = 0.8625$.

initially, it was assumed that all of the intensity at M (m/e = 628) was due to the unenriched glucose, and the observed intensities above m/e 629 were corrected for the contribution of the naturally occurring isotopic species of the TMS-oxime of unenriched glucose followed by the correction of intensities from isotopes other than ^{13}C from the enriched glucose. The results also indicate the significant differences from the values calculated on the assumption of uniformity. Thus, this sample of [U-^{13}C]glucose is considered to be significantly different from the uniform labelling.

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