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A CONSTITUTIONAL ANALYSIS OF SOME ^{14}C-LABELED CARBOHYDRATES OF HIGH SPECIFIC ACTIVITY BY CARBON-13 N.M.R. SPECTROSCOPY AND FIELD-DESORPTION, MASS SPECTROMETRY*

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ABSTRACT

The conventional methods of determining the labeling pattern in organic molecules are very laborious and time-consuming, because they rely on carbon-by-carbon chemical degradations. The determination of labeling patterns in carbohydrates by means of ^{13}C-n.m.r. spectroscopy is now described for the first time. A rapid (30 min), micro ($\leqslant 1$ μg) method for determining the distribution of various isotopomers (which can be used to calculate specific activity), using f.d.m.s., is also described.

INTRODUCTION

The preparation of uniformly labeled, carbon-14 compounds has generally been accomplished by incorporation of ^{14}C-labeled precursors by enzymically active systems. Uniformly labeled carbohydrates have been prepared by light-catalyzed exposure of a variety of plants and leaves to CO_2 isotopically enriched with ^{14}C or ^{13}C. These have included leaves from canna[2], tobacco[3,4], bean[5], sweet potato[6], and cantaloupe[7]. Incorporation of non-radioactively labeled, endogenous, biochemical precursors might lead to a non-uniformly labeled compound. The factors affecting the isotopic labeling patterns[8–10] and degree of isotope incorporation have been reported.

The traditional methods for the determination of the labeling pattern have relied upon chemical degradation. We now present work on the determination of the uniformity of labeling of ^{14}C-labeled compounds by use of two complementary,

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*Quantitative Field-Desorption Mass Spectrometry, Part XX. For Part XIX, see ref. 1.

analytical techniques, namely, ^{13}C-nuclear magnetic resonance (^{13}C-n.m.r.) spectroscopy and field-desorption, mass spectrometry (f.d.m.s.).

The ^{14}C source normally used for labeling work is prepared by neutron irradiation of ^{14}N (typically $^{14}NH_4{}^{14}NO_3$), and thus contains no other isotopes of carbon. Endogenous precursors are the only source of either ^{12}C or ^{13}C. Because the presence of the ^{12}C isotope is attended by 1.1% natural abundance of ^{13}C, the ^{13}C-n.m.r. spectrum of a ^{14}C-labeled compound will reveal the ^{12}C-labeling pattern and, in reverse, the ^{14}C-labeling pattern.

The f.d.m.s. studies[11], the first to be reported for the direct determination of radioisotopes, provide data complementary to the ^{13}C-n.m.r. data. The discrimination of molecules by differences in molecular weight does not give the isotope distribution, but it does show the distribution of molecules containing 1, or more, ^{14}C atom(s) in them, information not available from the ^{13}C-n.m.r. studies. In addition, the molecular-weight distribution yields a direct determination of specific activity.

Both techniques provide information on the purity of materials, and they open up a new avenue for compositional analysis.

EXPERIMENTAL

The D-[1-^{14}C]2-deoxy-*arabino*-hexose was prepared from D-[1-^{14}C]2-amino-2-deoxyglucose, as described by Matsushima[12]. D-[^{14}C(U)]Glucose was prepared by exposure of tobacco leaves to $^{14}CO_2$, as described by Kollman *et al.*[3].

The mass-spectrometric investigations were performed in a Varian 731 double-focusing mass spectrometer equipped with a combined e.i./f.i./f.d. source, which was interfaced to the Varian SS-200 data system. The anode, a high-temperature, activated tungsten wire, was kept at +8 kV, and the slotted, counter electrode at −3 kV. The ion signals were electrically recorded by, and accumulated in, a Tracor Northern NS-570A multichannel analyzer which was triggered externally by the cyclic, magnetic scan of the mass spectrometer.

The ^{13}C-n.m.r. spectra were recorded with a Bruker WP-200 Fourier-transform spectrometer operating at 50.32 MHz for ~M solutions in D_2O. 1,4-Dioxane (67.4 p.p.m.) was used as the internal reference standard.

RESULTS

A. ^{13}C-N.m.r. results. — An examination of the ^{13}C-n.m.r. spectrum of D-glucose in D_2O (see Fig. 1) shows that, under the experimental conditions used (65° nutation angle: 1.64-s repetition rate; 8 k data points), all peaks assigned[13] to the α anomer (or the β anomer) have the same intensity (±10%). Hence, a ^{13}C-n.m.r. spectrum of D-[^{14}C(U)]glucose recorded under the same experimental conditions should reveal, by inspection, the ^{13}C distribution and, indirectly, the ^{14}C distribution. Inspection of the ^{13}C-n.m.r. spectrum (see Fig. 2) of D-[^{14}C(U)]-glucose [NEC-042X; specific activity, 300 mCi/mmol: New England Nuclear (NEN)]

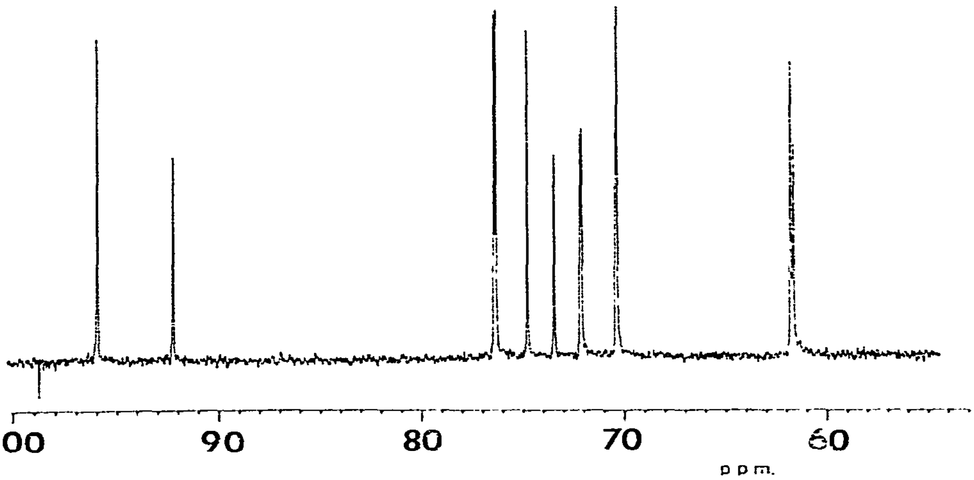

Fig. 1. ^{13}C-N.m.r. spectrum of D-glucose.

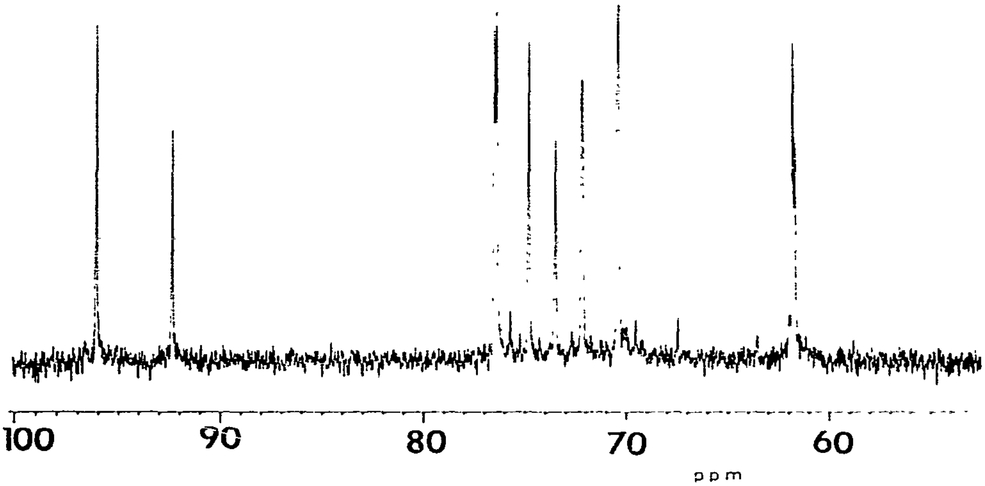

Fig. 2. ^{13}C-N.m.r. spectrum of D-[^{14}C(U)]glucose at 300 mCi/mmol.

shows only peaks ascribable to D-glucose. Furthermore, it is apparent that this material is, indeed, uniformly labeled. In order to check the specific activity of this product, it was converted into methyl α-D-[^{14}C(U)]glucopyranoside (specific activity, 300 mCi/mmol) using unlabeled methanol. The ^{13}C-n.m.r. spectra of ^{14}C-labeled and unlabeled methyl α-D-glucopyranoside are given in Figs. 3 and 4, respectively. Using the resonance at 55.87 p.p.m. due to -OCH$_3$ as the standard, the sum of the resonance intensities due to C-1–C-6 in the labeled product is only 16.3% of the sum of those in the unlabeled material. Therefore, the specific activity of methyl α-D-[^{14}C(U)]glucopyranoside would be 16.3% less than the theoretical value of 376.2

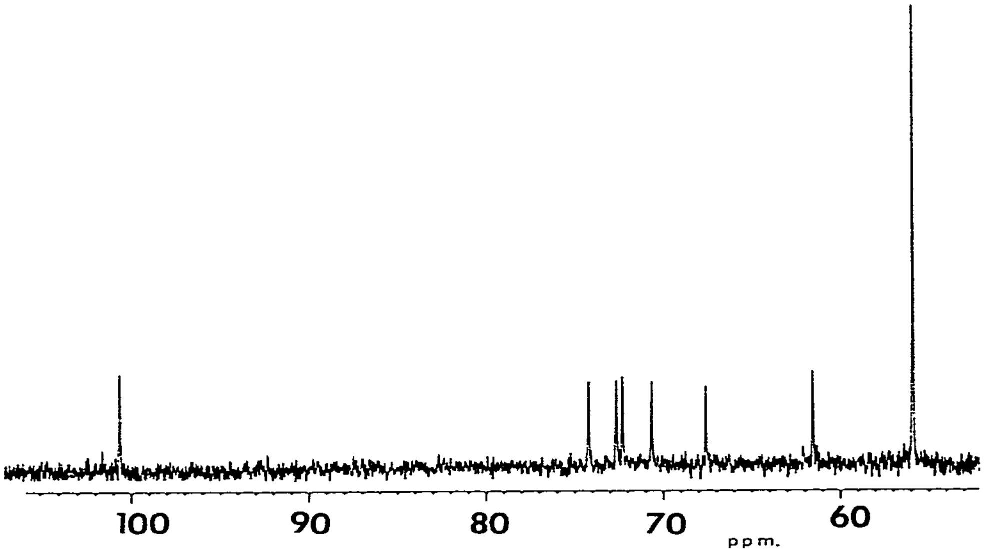

Fig. 3. ^{13}C-N.m.r. spectrum of methyl α-D-[^{14}C(U)]glucopyranoside at 300 mCi/mmol.

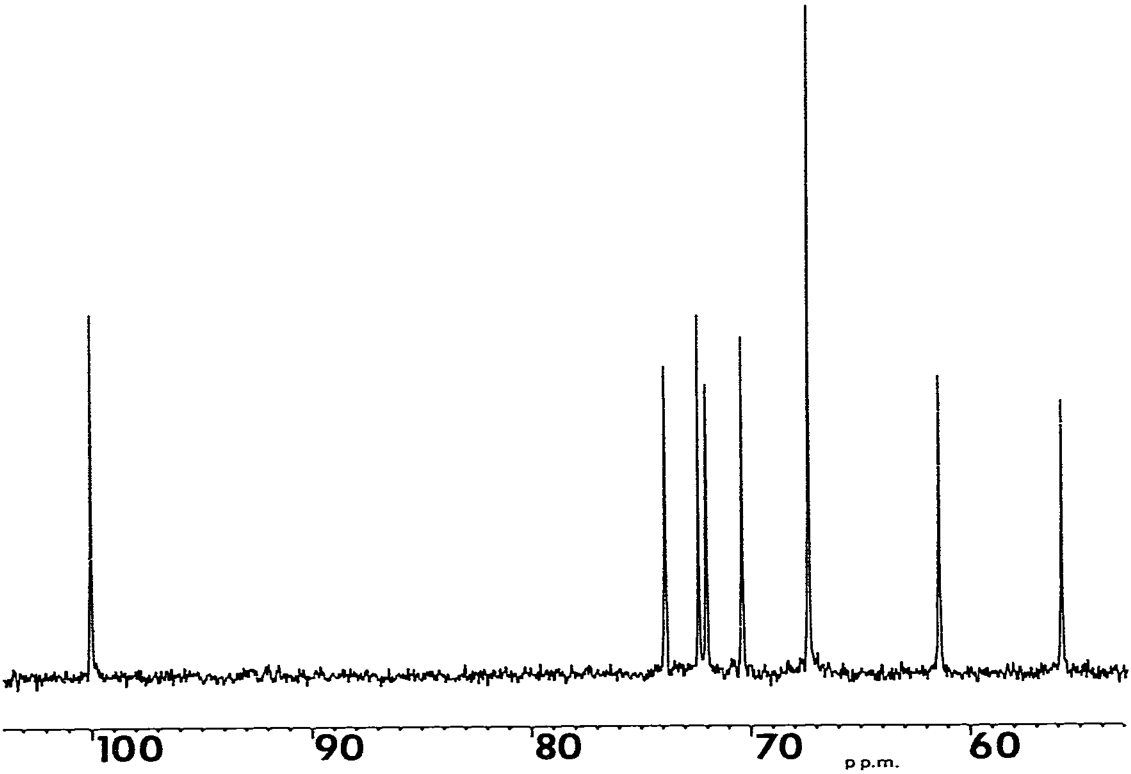

Fig. 4. ^{13}C-N.m.r. spectrum of methyl α-D-glucopyranoside.

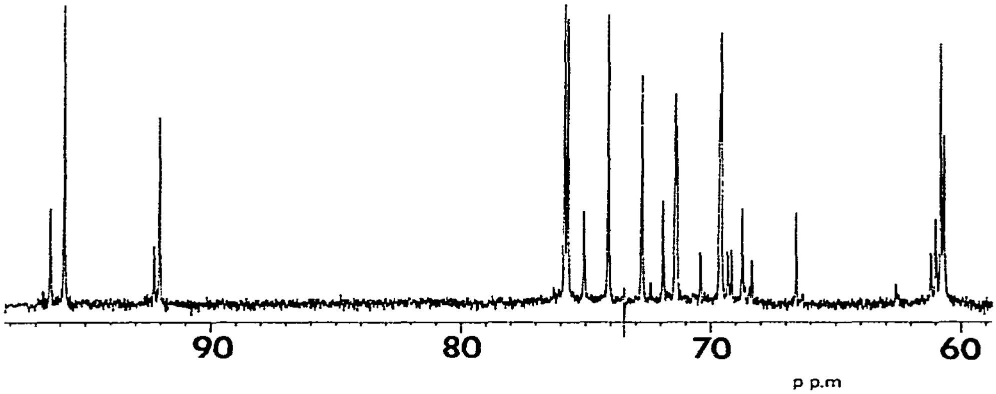

Fig. 5. ^{13}C-N.m.r. spectrum of D-[^{14}C(U)]glucose at 360 mCi/mmol, showing unlabeled D-galactose impurity.

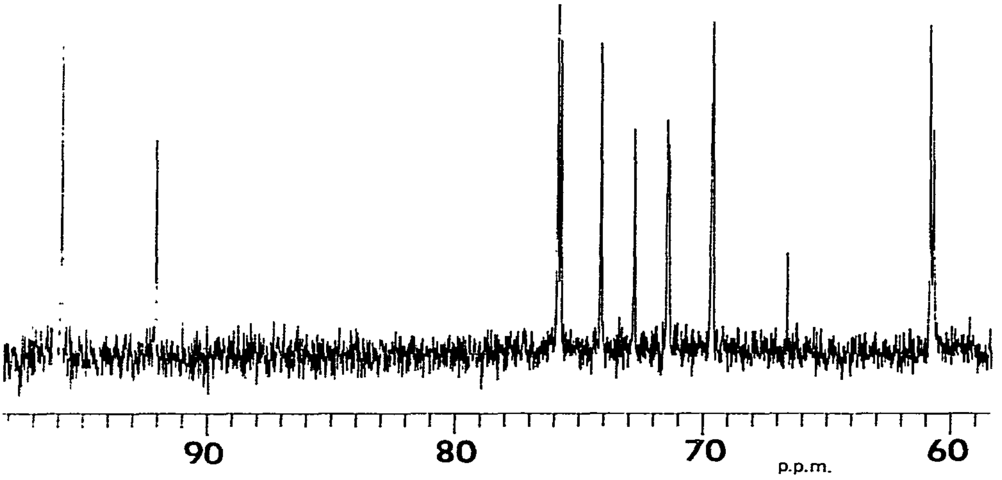

Fig. 6. ^{13}C-N.m.r. spectrum of D-[^{14}C(U)]glucose at 360 mCi/mmol, after removal of D-galactose impurity.

mCi/mmol (6 × 62.7 mCi/C atom), that is, 314.9 mCi/mmol, which is within 5% of the specific activity (300 mCi/mmol) determined colorimetrically. The accuracy of this analysis is estimated to be within ±10%.

An examination of the ^{13}C-n.m.r. spectrum (see Fig. 5) of D-[^{14}C(U)]glucose (NEN Lot 1088-117; 360 mCi/mmol) revealed the presence of extraneous peaks that could be ascribed to the presence of a small proportion of unlabeled D-galactose. It should be noted that the sensitivity of ^{13}C-n.m.r. spectra to unlabeled impurities increases with increase in the specific activity of the product. This may be seen from the present example, where the intensity of the ^{13}C-resonances for galactose are approximately one third of the intensity of the unlabeled D-glucose present in the sample. In D-[^{14}C(U)]glucose having a specific activity of 360 mCi/mmol, only

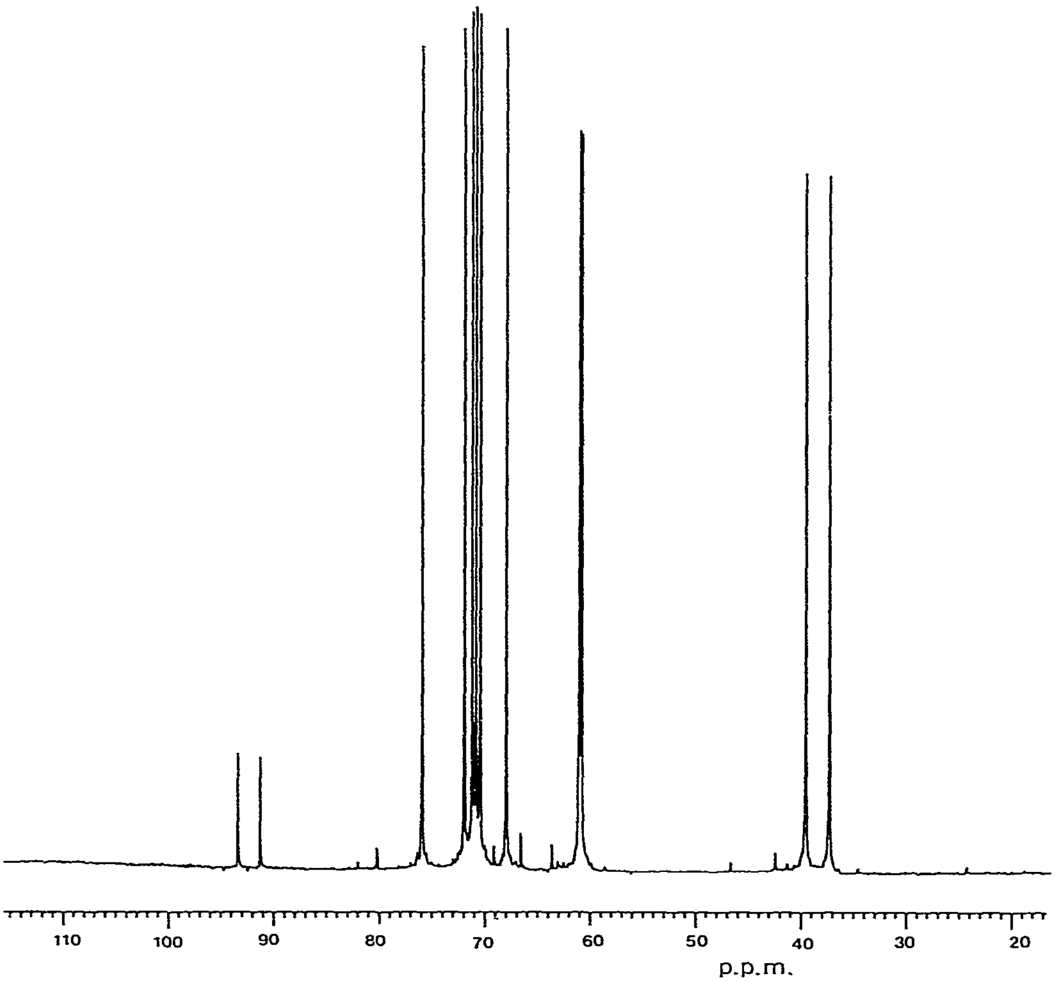

Fig. 7. ^{13}C-N.m.r. spectrum of D-[1-^{14}C]2-deoxy-*arabino*-hexose at 53 mCi/mmol.

4.3% of the carbon atoms are not labeled with carbon-14. Thus, the proportion of unlabeled galactose present as an impurity may be estimated to be ~1.1% of the total sample. This level of impurity is difficult to detect by conventional means (unless there is prior knowledge of the nature of the impurity). Chromatographic purification yielded material whose ^{13}C-n.m.r. spectrum (see Fig. 6) indicated the absence of galactose, and confirmed the uniformity of the ^{14}C-labeling.

The ^{13}C-n.m.r. spectrum (see Fig. 7) of D-[1-^{14}C]2-deoxy-*arabino*-hexose (NEN Lot 1088-100; specific activity, 53 mCi/mmol) reveals a diminution of intensity for only the C-1 resonances (94.2 p.p.m. for the α anomer, and 92.1 p.p.m. for the β anomer). Comparison of the intensity of the residual, C-1 absorptions with absorptions in the spectrum (see Fig. 8) of unlabeled 2-deoxy-D-*arabino*-hexose recorded under identical conditions of accumulation permitted direct determination of the

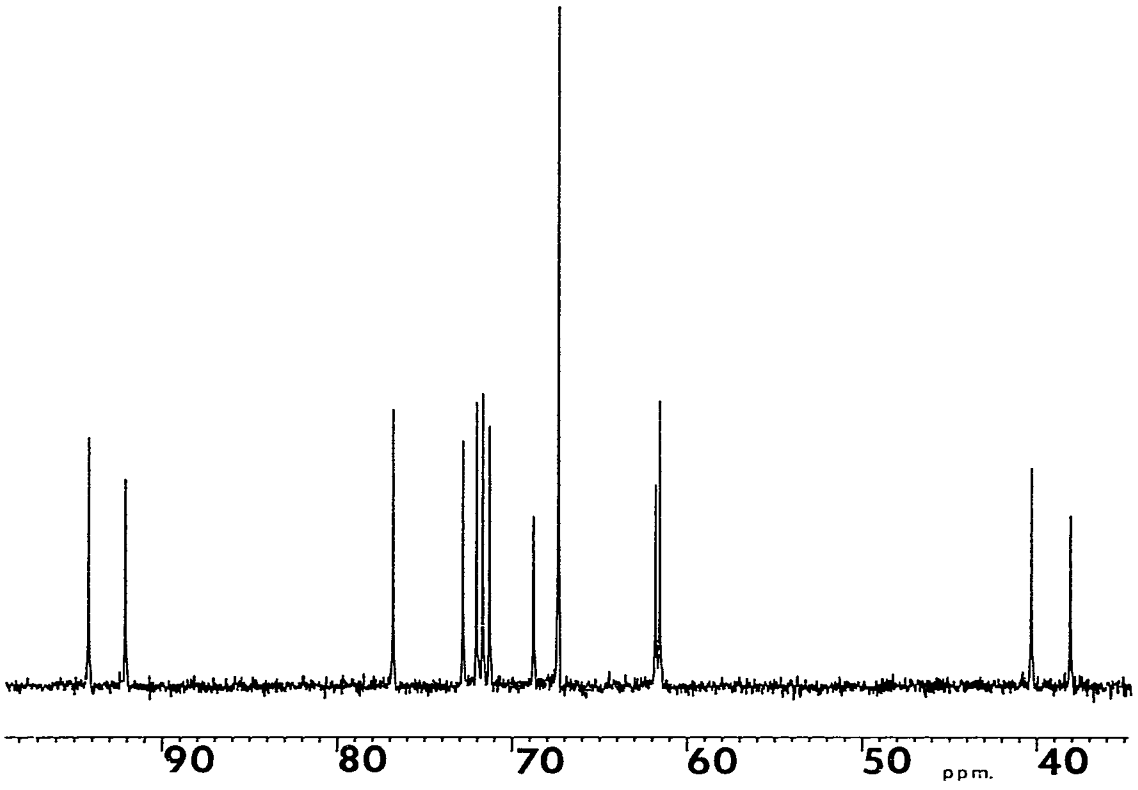

Fig. 8. ^{13}C-N.m.r. spectrum of 2-deoxy-D-*arabino*-hexose.

specific activity (53 mCi/mmol) of the D-[1-^{14}C]2-deoxy-*arabino*-hexose. As before, excellent agreement with the results of the more classical method for determination of specific activity was thus obtained.

B. F.d.m.s. results. — A sample of D-[^{14}C(U)]glucose (Lot 1088-117; 360 mCi/mmol) was examined by f.d.m.s. Small proportions of alkali-metal impurities (presumably leached from the glass storage-vessel) led to attachment of a cation to the organic molecule (cationization[14]) and produced abundant (D-glucose + ^{23}Na)$^+$ ions. This type of ion is ideally suited for direct, isotope determination[15], as proton-transfer reactions on the surface of the emitter [which would yield $(M + H)^+$, $(M - H)^+$, $(M - 2\,H)^+$...ions] no longer interfere with the isotopic determination. The use of repetitive, magnetic scanning, with accumulation of the signal in a multi-channel analyzer, led to a standard deviation of 0.30% in the reported distributions. As may be seen from the data presented in Table I, 97.25% of all molecules are labeled with at least one ^{14}C atom, and 87.3% of all carbon atoms have been labeled with ^{14}C.

The preponderant molecular species contain either five or six ^{14}C atoms. From the observed distribution, the specific activity of the sample was calculated to

TABLE I

DIRECT ISOTOPE DETERMINATION OF ^{14}C IN D-GLUCOSE BY F.D.M.S.

m/z	*Number of ^{14}C atoms*	*Ion distributions (%)*	
		360 mCi/mmol	*270 mCi/mmol*
203	0	2.75	26.70
205	1	0.20	0.30
207	2	0.45	0.30
209	3	2.55	0.85
211	4	6.75	4.55
213	5	35.80	27.25
215	6	51.55	40.05

TABLE II

ANALYSIS OF ^{14}C DISTRIBUTION IN LABELED GLUCOSE

Number of ^{14}C atoms	*Observed distribution (%)*[a]	*Corrected distribution (%)*	*Predicted distribution (%)*[b]
0	2.75	0	0
1	0.20	0.21	0.005
2	0.45	0.46	0.12
3	2.55	2.62	1.47
4	6.75	6.94	9.90
5	35.80	36.81	35.49
6	51.55	53.01	53.00

[a] The f.d. results provided the basis for the observed distribution. [b] The predicted distribution was derived by assuming a random distribution, with the probability of finding a single ^{14}C atom set to 0.8996 (to fit the percentage of molecules having six labels).

be 328.7 mCi/mmol. As this specific activity, as determined by f.d.m.s., differed from the results of Glucostat determinations (360 mCi/mmol), a check of the f.d.m.s. method was conducted, in which a new sample was prepared by dilution of the original sample with unlabeled D-glucose (to yield a final specific activity of 270 mCi/mmol). The distribution determined by f.d.m.s. is reported in Tables I and II. As may be seen, only the *m/z* 203 peak shows any significant increase. The derived, specific activity of the diluted (with unlabeled sugar) lot of D-glucose was 249.7 mCi/mmol. Thus, the f.d.m.s. data are internally consistent.

In Figs. 9A–9C, the shift in the isotopic pattern for uniformly labeled D-glucose, with increase in ^{14}C content, is illustrated by the f.d.m.s. spectra in the $(M + Na)^+$ region. The $(M + Na)^+$ signal for cationized, unlabeled D-glucose (*m/z* 203) decreased sharply with increase in specific activity. It was found to be 92% of the total, ionization current (t.i.c.) for unlabeled D-glucose, 26% of the t.i.c. for D-glucose having a

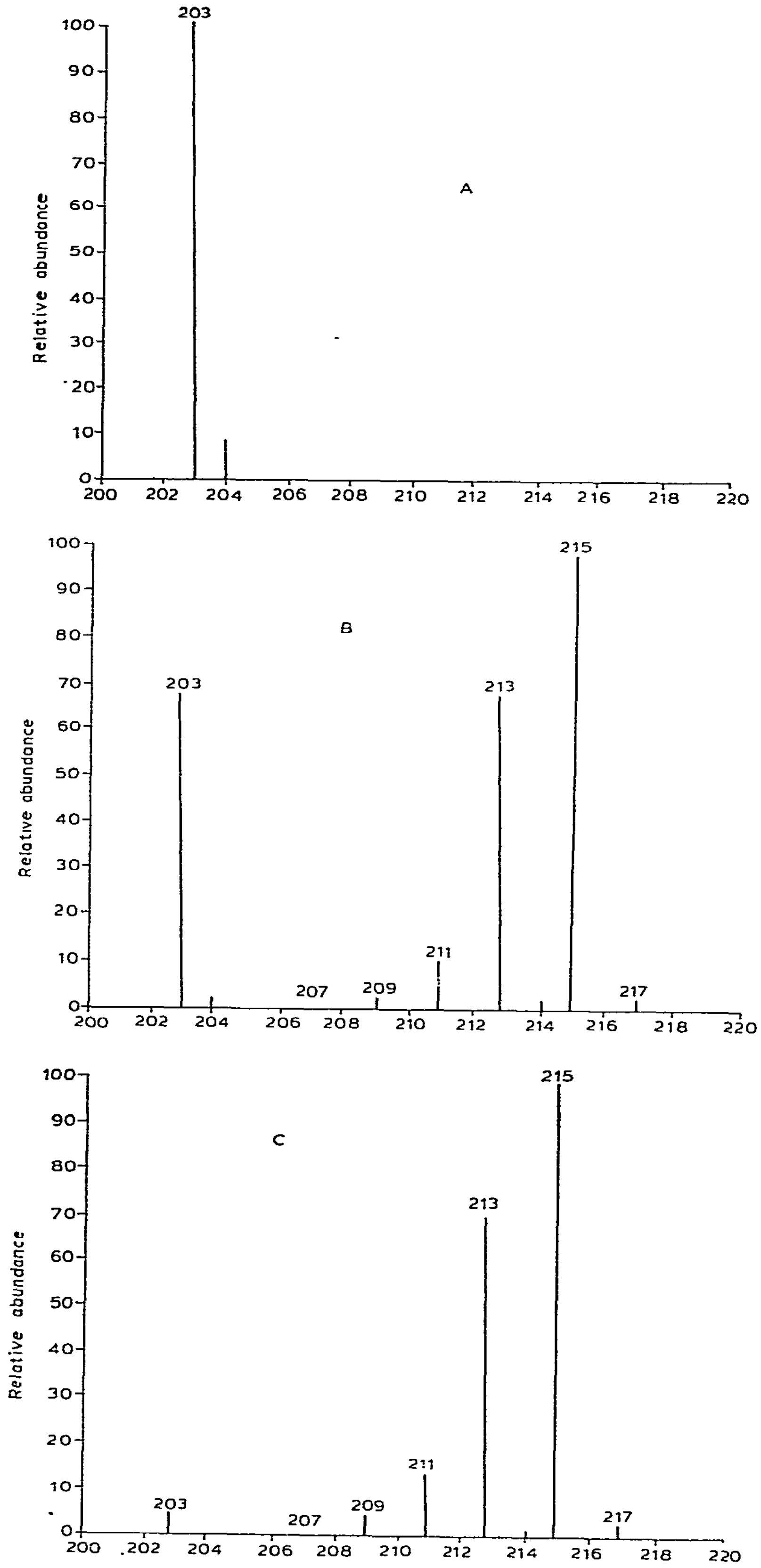

Fig. 9. F.d.m.s. spectrum of A, unlabeled D-glucose; B, D-[^{14}C(U)]glucose at 270 mCi/mmol; and C, D-[^{14}C(U)]glucose at 360 mCi/mmol.

specific activity of 270 mCi/mmol, and only 2% of the t.i.c. for D-glucose having a specific activity of 360 mCi/mmol.

DISCUSSION

The presence of non-labeled, endogenous precursors (other than those that contain only one carbon atom) would lead to a distribution among isotopomers which would differ significantly for the random, statistical distribution expected for carbohydrate photosynthesis solely from one-carbon precursors; in particular, a higher percentage of molecules having either two or three unlabeled carbon atoms would be expected. Interestingly, the distribution among isotopomers is not statistical. There is clearly an excess of unlabeled D-glucose, presumably arising from endogenous carbohydrate not utilized during the dark-leaf starvation-period. If the isotopomer distribution observed for the presence of unlabeled D-glucose is corrected, 89.84% of all carbon atoms in D-glucose that was synthesized during the exposure of tobacco leaves to $^{14}CO_2$ were labeled. This would lead to a predicted, isotopomer distribution as presented in Table II. Again, there are differences between the "corrected, observed" distribution and the predicted distribution. The main difference from a statistical distribution is that there is not enough material labeled with four ^{14}C atoms.

In summary, both ^{13}C-n.m.r. spectroscopy and f.d.m.s. investigations have yielded information on the distribution of isotopically labeled species that would be difficult to obtain by other means. The advantages of the f.d.m.s. method are that (*1*) very small sample-sizes (typically, one μg or less) are needed, and (*2*) the time for one f.d.m.s. measurement (including sample application, emitter adjustment, signal accumulation, and output) is ~30 min. Very accurate information concerning the distribution of isotopomers, which can be expressed as a specific activity, is directly obtained. However, information regarding the uniformity of labeling is not directly obtainable. ^{13}C-N.m.r. spectral measurements require large samples (the sample size needed depends on the specific activity), typically on the order of 100 mg of a compound of very high specific activity (90% of ^{14}C); this gives usable data collected in several hours. The uniformity of labeling may be directly obtained from the ^{13}C-n.m.r. data. In selected cases, determinations of specific activity are possible.

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