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Evidence for a Nonstatistical Carbon Isotope Distribution in Natural Glucose

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ABSTRACT

The relative carbon isotope content ($\delta^{13}C$ value) in each position of glucose from a C_4 plant (maize starch) and a C_3 plant (sugar beet sucrose) has been determined by stepwise chemical and biochemical degradation of the molecule and stable isotope ratio measurement of the fragments. The suitability of the degradation methods has been tested through their chemical yield and isotope balance. The results from both methods agreed perfectly, revealing a defined and reproducible ^{13}C distribution in glucose from both origins. Most prominent was a relative ^{13}C enrichment by 5 to 6 δ-units in position 4 and a depletion by about 5 δ-units in carbon 6. As possible reasons for these nonstatistical isotope distributions, isotope effects of the aldolase, the triose phosphate isomerase, and the transketolase reactions during carbohydrate biosynthesis are discussed. The practical importance of the results in regard to isotope distributions in secondary plant products as a means for food authenticity control is outlined.

For many years the average natural abundance of carbon isotopes has been used to assign plant natural products to the origin from C_3 and C_4 plants. Recently, determinations of intramolecular stable carbon isotope distributions in naturally occurring secondary compounds or in products of biochemical reactions have been used as a sophisticated tool to identify their biosynthetic origin as well as isotope effects in their formation (2–4, 10–13, 15). In this context it has sometimes been assumed that carbohydrates as the primary carbon sources in plants have a homogeneous and statistical intramolecular isotope distribution due to the scrambling of their carbon atoms during their synthesis via the Calvin cycle (4, 9). In contradiction to this assumption are observations on the ^{14}C distribution in sugars synthesized during tracer studies on photosynthetic reactions (5). However, in this case the observed isotopic variation could also have been an effect of the short reaction time and would disappear after a longer experimental period. Recently published results of the partial chemical degradation of glucose from maize starch (6) or of the intramolecular isotopic composition of lactate from the fermentation of beet sucrose (E Melzer, unpublished results) indicate a nonstatistical carbon isotope distribution even in natural glucose. Corresponding nonstatistical distributions have also been reported for the carbon bound hydrogen atoms in this substance (9).

Therefore, we have systematically studied the complete carbon isotope distribution in glucose from different origins, namely from a C_3 plant (sucrose from beet sugar) and from a C_4 plant (maize starch hydrolysate). By the combination of biochemical and chemical methods we have been able to determine the relative and absolute ^{13}C abundance in any of the six positions in the glucose molecule. The reliability of the methods used was controlled through the chemical and isotopic yields of the degradation procedures and by their assessment with standard substances.

MATERIALS AND METHODS

The assignment of the carbon atoms of glucose to the products of the biological degradations used are shown in Figure 1. The fermentation yields with thoroughly washed resting cells of *Lactobacillus bulgaricus* and *Leuconostoc mesenteroides* were in the range of 96 to 98 and 90 to 95%, respectively. The degradation of glucose by *Saccharomyces cerevisiae* and *L. bulgaricus,* in combination with the chemical degradation of the fermentation products (ethanol and lactic acid, respectively), led to the mean $\delta^{13}C$ values for the carbon atoms 1 + 6, 2 + 5, 3 + 4 of glucose. In spite of the fact that the two fermentation pathways are different, the results were absolutely identical. By the glucose fermentation with *L. mesenteroides* and a combined chemical degradation of the ethanol obtained, the isotope abundances in carbon atoms 1, 2, and 3 of the glucose molecule could be measured individually. Finally, from the combination of the results of the homofermentative and heterofermentative fermentations the $\delta^{13}C$ values for any individual carbon atom in the glucose molecule were obtained.

Position in glucose	Products from fermentation with		
	Saccharomyces cerevisiae	Lactobacillus bulgaricus	Leuconostoc mesenteroides
C-1	CHO	CH_3	CH_3	CO_2
C-2	HCOH	CH_2OH	CHOH	CH_3
C-3	HOCH	CO_2	CO_2H	CH_2OH
C-4	HCOH	CO_2	CO_2H	CO_2H
C-5	HCOH	CH_2OH	CHOH	CHOH
C-6	CH2OH	CH_3	CH_3	CH_3

Figure 1. Scheme of the assignment of the carbon atoms in the fermentation products obtained from glucose fermentation by resting cells of different microorganisms (according to Simon and Floss, ref. 16).

Biological Degradations of Glucose

Fermentations and Isolation of the Products

A solution of 50 g glucose in 550 mL water was incubated with 7 g of washed and dried bakers' yeast for 3 d at 25°C in a closed reaction vessel under an N_2 atmosphere. The evolving gas was passed through two absorption vessels filled with 3 N NaOH; at the end of the fermentation the remaining CO_2 was driven out by flushing the reaction mixture with N_2 for 5 min (during this time the bottle was cooled in order to avoid losses of ethanol). The yeast cells were then removed by centrifugation, and the ethanol was separated from the medium by slow distillation over a column after Rossmann and Schmidt (14). The CO_2 was isolated from the NaOH solution as $BaCO_3$ according to Simon and Floss (16).

The fermentations of glucose with *L. mesenteroides* and *L. bulgaricus* (washed cells from a 12 h culture of 2000 mL medium) were performed in two-necked reaction flasks containing 200 mL 0.1 N phosphate buffer, pH 6.0, and 1.5 g of the substrate at 35°C under a N_2 atmosphere. The pH value was maintained at 6.0 ± 0.2 under control of a pH electrode, by adding 1 N NaOH with a syringe. The reaction was complete when no further CO_2 was evolved from the medium and/or the pH value remained constant (about 2 h). Lactic acid was isolated from the media by liquid/liquid extraction with water/diethylether according to Simon and Floss (16), after the ethanol had been separated from the alkaline solution (pH 8.5 adjusted by addition of NaOH) by distillation as described above.

Degradation of Fermentation Products

The oxidative decarboxylation of L-lactic acid was performed with L-lactate oxidase (EC 1.13.12.4) using the method of Melzer and Schmidt (13), yielding CO_2 (C-1 of lactate) and acetate (C-2 and C-3 of lactate) which were separated and isolated as described (13). Ethanol was oxidized to acetic acid with a chromic acid/sulfuric acid mixture (75 g of CrO_3 and 75 mL of H_2SO_4 in 300 mL water); the acetic acid formed was separated by distillation and isolated as sodium acetate (Fig. 2) (14, 16).

Sodium acetate from the oxidation of ethanol and the degradation of lactic acid was submitted to pyrolysis with sodium hydroxide, and the carbon atom of the methyl group was isolated as CH_4, which was subsequently combusted to carbon dioxide (11, 13). The carbon atom 1, bound in the form of Na_2CO_3 after pyrolysis of the sodium acetate, was not analyzed because its $\delta^{13}C$ value could easily be calculated from the $\delta^{13}C$ difference between acetate and the CH_4 carbon atom (11, 13). The carbon isotope analysis of the individual different carbon dioxide samples was performed according to Winkler and Schmidt (19). D-Lactic acid from the glucose fermentation with *L. mesenteroides* could not be degraded with L-lactate oxidase; therefore, this product was directly combusted, yielding the mean isotope content of the molecule.

Chemical Degradation of Glucose

The chemical degradation of glucose was performed according to Simon and Floss (16). The method, originally developed for the determination of the ^{14}C distribution in glucose in connection with tracer work, had to be adapted to the present problem (degradation of g amounts of glucose and quantitative turnover rates).

This was attained by individual examination and optimization of each step with standard substances under isotope balance control. The final conditions used are displayed in the reaction scheme (Fig. 3), according to which glucose was first derivatized to glucose-phenylosotriazole, which was subsequently degraded by periodate to phenyltriazole-aldehyde (containing C-1 + C-2 + C-3 of glucose), formic acid (sum of C-4 + C-5), and formaldehyde (C-6). After distillative separation, the formaldehyde was precipitated with 2,4-dinitrophenylhydrazine (for detailed reaction conditions see Fig. 3) and analyzed as formaldehyde-2,4-dinitrophenylhydrazone. The formic acid was oxidized to CO_2, which was isolated as $BaCO_3$.

The methods were tested by submitting standard substances to the same procedures. A comparison of their isotopic composition before ($\delta^{13}C$ values HCOOH: −22, 94‰; H_2CO: −29, 12‰) and after ($\delta^{13}C$ values HCOOH: −23, 22‰; H_2CO: −29, 56‰) the treatment revealed negligible fractionations. Also, the high yield obtained for the phenyltriazole aldehyde guaranteed that the first degradation step did not involve an isotopic fractionation. Furthermore, the isotopic compositions of the phenylosotriazoles (−18, 08‰ for C_4-glucose; −25, 18‰ for C_3-glucose) matched those calculated from the $\delta^{13}C$ values of the cleavage products (−17, 83‰ and −24,

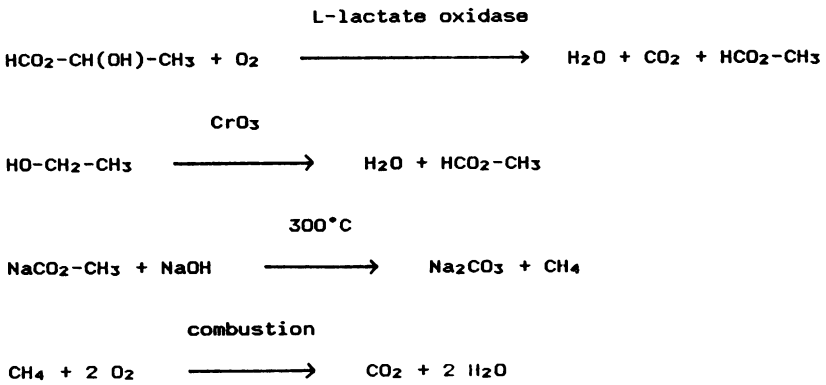

Figure 2. Scheme of the reactions used for the further degradation of the fermentation products (according to Melzer and Schmidt, refs. 11 and 13).

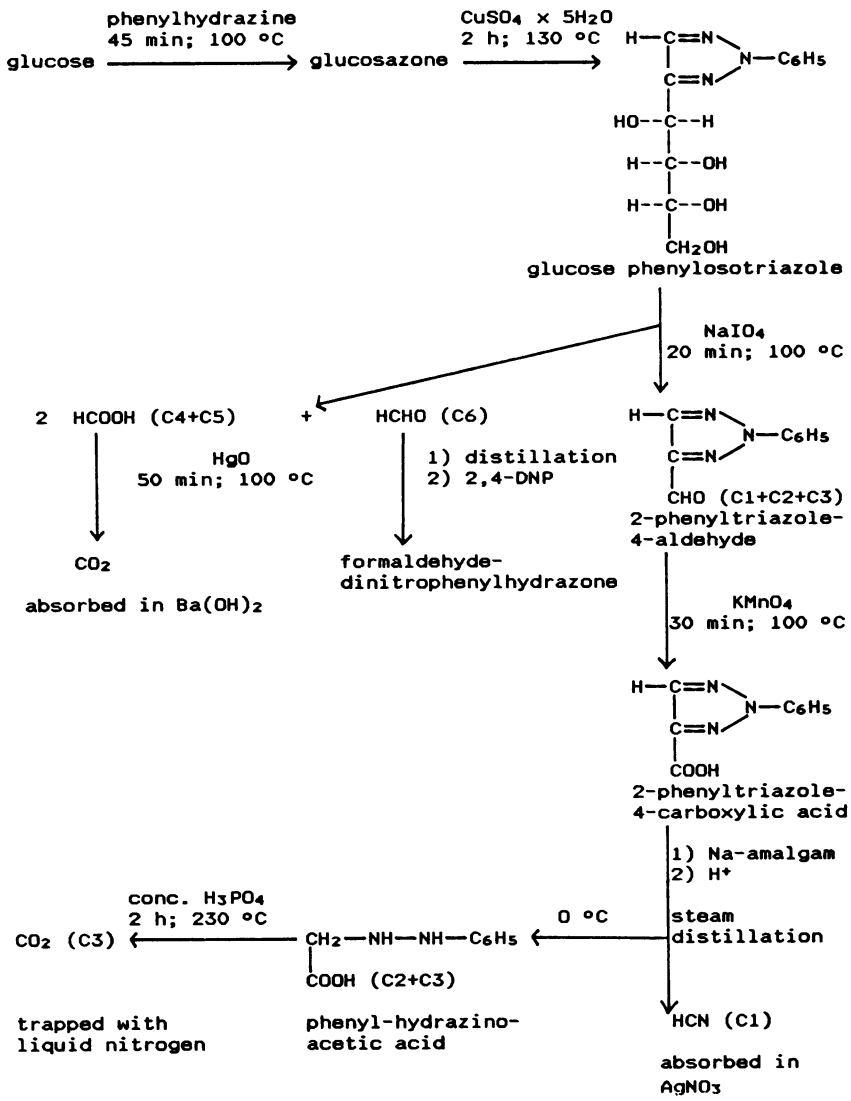

Figure 3. Chemical degradation of glucose for positional carbon isotope analysis (according to Simon and Floss, ref. 16).

82‰, respectively). Phenyltriazole aldehyde was oxidized to the corresponding acid without any shift of the isotopic composition. The product was further degraded with sodium amalgam, yielding HCN (C-1 of glucose) and phenylhydrazino acetic acid (C-2 and C-3 of glucose). The distillation of HCN and its isolation as AgCN was tested with standard KCN ($\delta^{13}C$ values: KCN: −35, 84‰; AgCN after distillation: −35, 68‰). Finally, the $\delta^{13}C$ value obtained for phenylhydrazino acetic acid agreed exactly with that calculated from the $\delta^{13}C$ values for triazolic acid and for HCN. The decarboxylation of phenylhydrazino acetic acid yielded CO_2 derived from C-3 of glucose. The $\delta^{13}C$ value of C-2 was calculated from that for C-2 + C-3 minus that for C-3.

RESULTS

On the basis of the chemical degradation of the glucose molecule, the $\delta^{13}C$ values for nearly all individual carbon atoms could be determined (for positions 4 and 5 the sum was obtained). The biochemical degradations yielded $\delta^{13}C$ values for any individual carbon atom. A comparison of the results from both methods for the two glucose samples (Table I) demonstrates excellent agreement for most positions (except positions 2 and 3 of the C_4 compound) and total identity of the measured and calculated mean values for the complete glucose molecule.

The results reveal a remarkable depletion of ^{13}C in position 6 for both glucose samples, independent of their origin from C_3 or C_4 plant material. Similarly, a reproducible relative enrichment of ^{13}C is found for position 4 of the glucose from both origins. The only discrepancy between the results of the chemical and biochemical/chemical degradations concerns carbon atoms 1 to 3 of maize glucose; but even here the differences are smaller than those between the δ values in these positions and that of carbon atoms 4 to 6, and the discrepancy may be due to an experimental error. Also, the results are in complete agreement with those reported for partial degradations of maize glucose by Ivlev *et al.* (6), and they meet the ^{14}C distribution in short time photosynthesis

Table I. *Intramolecular Carbon Isotope Distribution in Glucose from Maize Starch and Sugar Beet Sucrose*

Results obtained by chemical and biological/chemical degradation. $\delta^{13}C$ values as per mil *versus* PDB standard; $\Delta\delta^{13}C$ values are deviations relative to the measured average value for the glucose molecule.

C atom	Maize Glucose				Sugar Beet Glucose			
	Fermentation		Chemical degradation		Fermentation		Chemical degradation	
	$\delta^{13}C$	$\Delta\delta^{13}C$	$\delta^{13}C$	$\Delta\delta^{13}C$	$\delta^{13}C$	$\Delta\delta^{13}C$	$\delta^{13}C$	$\Delta\delta^{13}C$
C-1 to C-6 measured	−10.3		−10.8		−25.0		−25.2	
C-1	−9.4	+0.9	−12.4	−1.6	−26.3	−1.3	−26.5	−1.3
C-2	−10.4	−0.1	−11.0	−0.2	−25.9	−0.9	−26.0	−0.8
C-3	−11.0	−0.7	−9.6	+1.2	−23.1	+1.9	−23.0	+2.2
C-4	−5.1	+5.2			−18.7	+6.3		
			−8.9	+1.9			−22.8	+2.4
C-5	−10.4	−0.1			−26.1	−1.1		
C-6	−15.1	−4.8	−14.8	−4.0	−29.9	−4.9	−30.4	−5.2
C-1 to C-6 calculated	−10.2		−10.9		−25.0		−25.3	

experiments of Gibbs and Kandler (5), namely an enrichment of ^{14}C in carbon atoms 3 and 4. Hence, it can be concluded that the nonstatistical carbon isotope distribution is a reproducible, system-implied and natural phenomenon occurring in C_3 and C_4 plants.

DISCUSSION

The question arises: what may be the reason(s) for this nonstatistical carbon isotope distribution? The glucose samples investigated originated from corn starch and sugar beet sucrose, both storage carbohydrates of the corresponding plants. According to our present knowledge, storage carbohydrates are formed from cytoplasmic hexoses which by themselves are produced from DHAP[1] exported from the chloroplasts into the cytoplasm. Here, after DHAP is isomerized to GAP, the trioses condense to yield FbP. This compound is incorporated into sucrose or metabolized by the glycolytic pathway to pyruvate. If we assume a given isotope distribution for the DHAP exported from the chloroplasts, the reactions mentioned and the metabolic branching certainly provide opportunities for isotope discriminations. An isotope effect on the GAP dehydrogenation (GAP dehydrogenase reaction) would lead to an enrichment of ^{13}C in C-1 of the remaining triose phosphates. The competing aldolase reaction would consequently produce ^{13}C-enriched FbP in positions C-3 and C-4, an assumption that is actually confirmed by the results (the possible reason for the result on C-3 of corn glucose obtained by fermentation has been pointed out above). The large difference observed for the $\delta^{13}C$ values in these two positions could be due to an isotope effect on the aldolase reaction itself. As a matter of fact, ^{14}C tracer studies on this reaction revealed that an isotopic label of [^{14}C]GAP appears faster in FbP than in [^{14}C]DHAP (18). Another explanation for this difference between the trioses could be the occurrence of a nonequilibrium in the triosephosphate pool due to a partial inhibition of triosephosphate isomerase or to a low physiological concentration of GAP (1, 17) provoked by its preferential use in other synthetic reactions (8).

On the other hand, the observed nonstatistical ^{13}C distribution in glucose could also arise from kinetic isotope effects in the reductive pentose phosphate cycle within the chloroplasts. In a complete cycle, three molecules of RuBP are carboxylated to give six molecules of phosphoglyceric acid. Phosphorylation and reduction yield six molecules of GAP (Fig. 4). Only five of these are required to regenerate the three molecules of RuBP. The sixth GAP molecule is either exported (as DHAP) from the chloroplast to the cytoplasm or converted into fructose-6-phosphate for the synthesis of assimilatory starch. In this case the chloroplast aldolase reaction would be involved and be a possible source for an isotopic discrimination.

In addition, within the Calvin cycle itself an isotope effect may be linked to the transketolase reaction. The enzyme is a thiamine pyrophosphate protein like pyruvate decarboxylase and pyruvate dehydrogenase (7), for the reactions of which isotope effects connected to C-C bond fission have been described (4, 11). This possible isotope effect would result in an enrichment of ^{13}C in C-1 of the triosephosphates.

The ^{13}C depletion found for the carbon atom in position 6 of the glucose molecule cannot yet be directly connected to a possible isotopic fractionation implied in a reaction of the pentose phosphate cycle or cytoplasmic glucose formation. However, it is the only carbon atom of the hexoses which is never involved in any C-C bond formation or fission. It is therefore probably never equilibrated with C-1, which by its turn is implied in such a process in the oxidative pentose phosphate cycle. Hence the δ value of C-6 may represent the "true and original" carbon isotope abundance of carbohydrates.

Secondary plant products (lipids, phenolic compounds) are in general depleted in ^{13}C as compared to the primary photosynthesis products (carbohydrates) (4, 11). This depletion may in fact be partly a consequence of the ^{13}C distribution in glucose, because in the course of lipid biosynthesis the ^{13}C-enriched carbon atoms 3 and 4 of glucose are lost as CO_2, and the relatively depleted carbon atoms 1, 2, 5, and 6 form acetyl-CoA. Nevertheless, a further depletion on carbon atom

[1] Abbreviations: DHAP, dihydroxyacetone-phosphate; FbP, fructose-1,6-bisphosphate; GAP, glyceraldehyde phosphate; PDH, pyruvate dehydrogenase; RuBP, ribulose-bisphosphate.

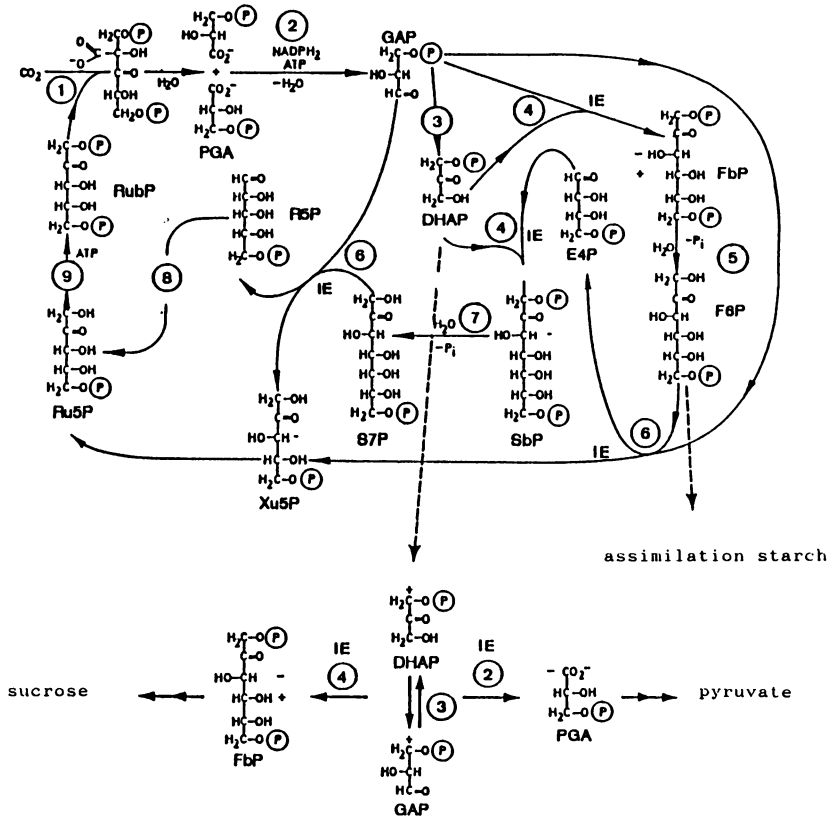

Figure 4. Reactions of the Calvin cycle with possible sources for the nonstatistical carbon isotope distribution in glucose (reactions with possible carbon isotope effects indicated by IE): 1 = RuBP-carboxylase; 2 = GAP-dehydrogenase; 3 = triosephosphate-isomerase; 4 = aldolase; 5 = FbP-1-phosphatase; 6 = transketolase; 7 = sedoheptulose-bisphosphate-phosphatase; 8 = pentosephosphate-isomerase; 9 = Ru5P-kinase; (+): ^{13}C enrichment; (−): ^{13}C depletion; IE = isotope effect. PGA = phosphoglyceric acid; F6P = fructose-6-phosphate; S7P = sedoheptulose-phosphate; Xu5P = xylulose-5-phosphate; Ru5P = ribulose-5-phosphate.

1 of acetyl-CoA (corresponding to carbon atoms 2 and 5 of glucose) must result from the isotope effect on the PDH reaction (11). The relative contribution of the two effects to the ^{13}C depletion in individual lipids must depend on the turnover rate of the PDH reaction (11). Indeed, the value for the isotope effect of this reaction ($k_{12}/k_{13} \approx 1.03$) suggests that its contribution must exceed significantly that from the nonstatistical isotope distribution in glucose. The isotope effect on the PDH reaction also demands an alternating relative ^{13}C abundance in products originating from acetyl-CoA.

This has in fact been found by us in isoprenoids (S Gradl and H-L Schmidt, unpublished results). Without any doubt the carbon isotope pattern of glucose must be taken into account to explain nonstatistical carbon isotope patterns in secondary plant products; however, isotope effects on defined steps of their very biosynthesis and on diffusion processes seem also to be of importance. Therefore, *in vitro* experiments on individual enzyme reactions and comparisons on carbon isotope patterns of intra- and extra-chloroplastic compounds are planned or in progress. The knowledge expected from these experiments will solve some of the open questions connected to photosynthesis; it will also be the base for assignments of secondary plant products to their origin and biosynthesis.

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