Plant Physiol. (1980) 65, 254–259
0032-0889/80/65/0254/06/$00.50/0

Characterization of Photorespiration in Intact Leaves Using ^{13}Carbon Dioxide Labeling

Received for publication April 30, 1979 and in revised form September 10, 1979

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ABSTRACT

The ^{13}C nuclear magnetic resonance spectra of sucrose extracted from intact soybean and corn leaves labeled with $^{13}CO_2$ for 5 to 180 minutes have been obtained at 22.6 megahertz. The spectra provide a direct determination of the relative concentrations of ^{12}C-^{13}C and ^{13}C-^{13}C pairs of carbons in the labeled triose components of sucrose. This distribution of label is strongly dependent on the O_2 concentrations used during labeling for soybean but not for corn. Based on the time dependence of the experimental labeling patterns, photorespiration in air appears to be a sizable fraction of the apparent photosynthetic rate for soybean, but not for corn.

Photorespiration is a light-stimulated oxidation of the products of photosynthesis to CO_2 (5). One of the more elaborate efforts to characterize photorespiration in the past has involved the simultaneous measurement of transient $^{12}CO_2$- and $^{14}CO_2$-labeling chamber concentrations in equilibrium with actively photosynthesizing leaves (12–15). However, this method has a limitation in that the specific activity of $^{14}CO_2$ at the site of carbon fixation need not approach the external specific activity so that no reliable estimate of internally refixed CO_2 is possible (3). This is a limitation shared by all gas exchange methods for measuring photorespiration (5).

Determinations of internally refixed CO_2 by the ^{14}C labeling of various early photosynthetic metabolites such as 3-PGA[1], glycine, and serine are complicated by the possible heterogeneity of these pools interfering with comparisons of absolute activity. For example, the specific activities of 3-PGA and serine in sunflower leaves after 15 min never reach more than about 0.85 and 0.6, respectively, of the specific activity of the applied labeled $^{14}CO_2$ (13). It is not clear whether this effect is due to the photorespiratory production of $^{12}CO_2$ from unlabeled substrate diluting $^{14}CO_2$ entering the leaf and so preventing the isotopic saturation of the Calvin cycle and its immediate products, or whether it is due to dilution by small isolated pools of metabolites not actively involved in photosynthesis. Kinetic modeling of labeled metabolites is not sufficiently precise to establish the presence of relatively small inhomogeneities (6). These complications have resulted in widely varying estimates as to the extent of internal refixation of photorespired CO_2 in both soybean and corn (2, 5, 11, 18, 26).

We have established that by labeling intact soybean and corn leaves as a function of time with 90% enriched $^{13}CO_2$, the distribution of label among *pairs* of carbons in triose products of the Calvin cycle can be determined from the ^{13}C NMR spectra of sucrose extracted from the leaves (21). The NMR spin-spin multiplet pattern of the protonated anomeric carbon of sucrose (the anomeric carbon of the glucose moiety) is simple because of the presence of only one nearest neighbor carbon (24). Thus, the ratio of singlet to spin-spin doublet can be interpreted directly as the ratio of ^{12}C-^{13}C concentration to ^{13}C-^{13}C concentration in labeled triose fragments of sucrose. Even though sucrose is remote from the Calvin cycle and its isotopic saturation may take hours, the saturation of an individual triose component formed during the labeling period will be as fast as that of the Calvin cycle itself. Thus, only one of the four triose components of sucrose may have any ^{13}C label at all, but that one component will be massively labeled; that is, all three of its carbons will reflect the isotopic composition of the Calvin cycle at the time of formation of the labeled triose. We detect this local isotopic saturation by means of ^{13}C-^{13}C splitting in the NMR spectra of the sucrose. Even though we are examining sucrose, we are analyzing the isotopic distribution in those labeled 3-carbon fragments which have been close to carbon cycling in the Calvin cycle during the labeling period.

The time dependence of these labeling distributions can be interpreted in terms of photosynthetic and photorespiratory processes. This leads to estimates for internally refixed CO_2 which do not depend on equilibration of gas concentrations internal and external to the leaf, and which are, in addition, internally standardized. That is, we derive information about carbon metabolism by analyzing ratios of NMR signal intensities, and so avoid many of the difficulties of standardizing activities to variations from leaf to leaf in photosynthetic rates, homogeneity of pools, and distribution of label among products. For example, the presence of an unlabeled fraction of an inhomogeneous metabolite pool does not affect our conclusions substantively inasmuch as we measure and analyze the *relative* concentrations of ^{12}C-^{13}C and ^{13}C-^{13}C pairs in *labeled species only*. The ^{13}C NMR experiment conveniently ignores all ^{12}C-^{12}C pairs, and the natural abundance of ^{13}C is too low to pose a serious problem. Absolute or specific ^{13}C determinations are not required. Based on the ^{13}C NMR measurement of label in pairs of carbons, photorespiration in air appears to be a sizable fraction of the APS rate for soybean, but not for corn.

MATERIALS AND METHODS

Growth of Plants. *Glycine max* (cv. Wayne) and *Zea mays* (cv. Golden Miniature) were grown in chambers having interior dimensions of approximately 1 × 1.5 × 2 m. The plants were grown at 30 C (day) and 20 C (night) with a photoperiod of 14 h. RH was maintained near 50%. Daytime CO_2 concentration was controlled at 325 μl/l. The plants were grown in 22.5-cm-diameter pots filled with a mixture of half Vermiculite and half sterilized soil fertilized with 1 g NH_4NO_3, 0.5 g superphosphate, and 1.5 g potash. Soybeans inoculated with rhizobia were not further fertilized. Corn was fertilized once a week with a solution containing

[1] Abbreviations: 3-PGA: 3-phosphoglyceric acid; NMR: nuclear magnetic resonance; APS: apparent photosynthesis.

the major inorganic nutrients. Soybeans were grown two plants per pot, 12 pots per growth chamber; corn, one plant per pot, 10 pots per growth chamber.

The growth chambers were fitted with four ceiling-mounted 1,000-w metal halide discharge lamps, separated from the interior of the chamber by a transparent thermal barrier, and 24 40-w 1.2-m white fluorescent lamps mounted on the walls. This combination produced, near the vertical midpoint of the chamber, light having about 50% of the total PAR of normal sunlight, and 25% of solar intensity measured as blue light (450 nm) using a calibrated spectroradiometer (Isco Instruments, Lincoln, Nebr.). Intense blue light (10, 17, 22) was essential in growing Wayne soybeans with internodal separations similar to those of uncrowded field-grown plants.

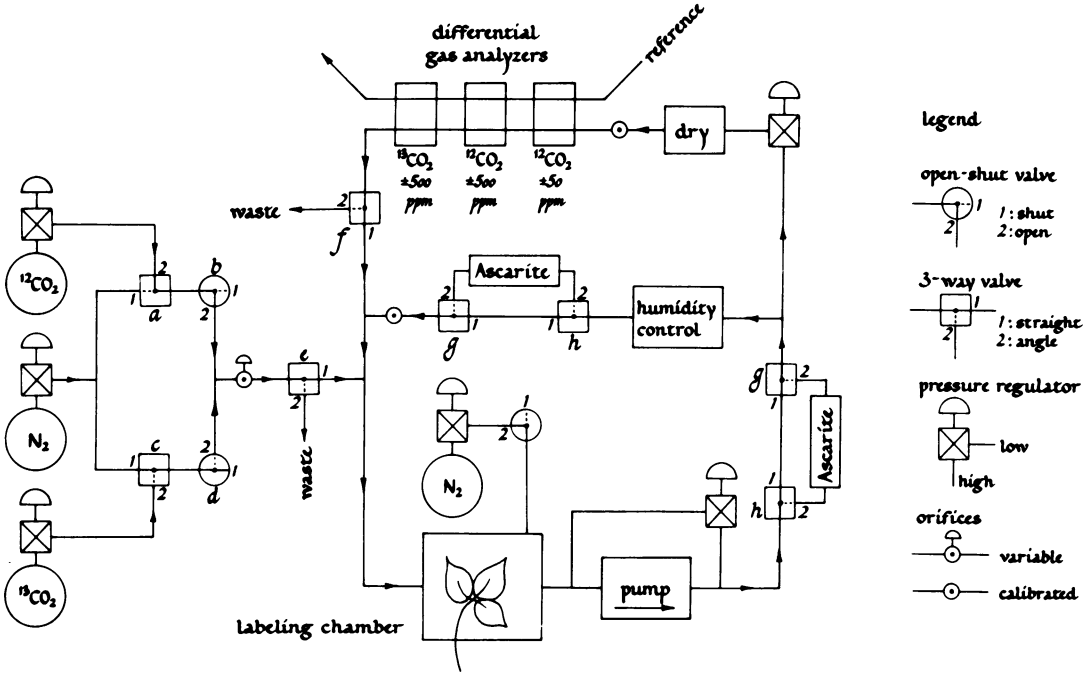
About 40 days after planting, whole soybean and corn plants were removed from the growth chambers for labeling experiments described below.

Operation of Labeling Chamber. The labeling chamber (Siemans Corp., Iselin, N.J.) was a Plexiglas box approximately 12 × 25 × 25 cm equipped with a Peltier temperature regulator, an internal variable speed fan, quantum sensor (Lambda Instruments, Lincoln, Nebr.), and RH and temperature sensors. The removable lid was sealed with pressure clamps and a rubber gasket. The labeling chamber was suspended by cables connected to a winch inside a growth chamber similar to those described above. An attached intact third or fourth corn leaf, or fourth or fifth soybean trifoliolate, was sealed inside the labeling chamber using a soft putty as sealant. Variable position louvers were mounted on the top of the labeling chamber so that light intensity inside the chamber could be varied without affecting the rest of the plant.

A simplified gas flow diagram of the labeling apparatus is shown in Figure 1. The total volume of the system was 20 liters. A diaphragm pump produced a net flow through the 7-liter chamber of 40 liters/min with a typical air flow rate across the leaf surface of 5 m/min. The chamber operated at a slight positive pressure (0.5 mm Hg) automatically maintained by a pressure-controlled valve and a N_2 source.

Prior to a labeling experiment, a measure of the APS rate was performed with the valves of the system in state I (Figure 1, inset, bottom). These two-position valves were all operated remotely by solenoids interfaced to computer-controlled relays. The variable orifice shown in Figure 1 was a voltage-variable valve also controlled by the same computer (Hewlett-Packard 9821A, Palo Alto, Calif.). Readings from the nondispersive IR gas analyzers (URAS 2T, Intertech, Indianapolis, Ind.), polarographic O_2 analyzer (not shown), temperature and humidity sensors, and various mass flow meters (not shown) were entered into the computer automatically using a multichannel scanner coupled to a programmable digital multimeter. The computer was programmed to maintain constant temperature (30 C) and RH (40%) using the Peltier regulator and solenoid-switched water bubblers and $CaCl_2$ drying traps. The computer also adjusted the flow of $^{12}CO_2$ into the chamber to maintain photosynthesis at a prescribed CO_2 concentration. The CO_2 in the gas cylinders was diluted with N_2 to simplify metering small amounts of CO_2. The CO_2 concentration inside the chamber was measured by a differential gas analyzer with a full scale sensitivity of 100 μl/l, referenced by a CO_2 and N_2 mixture generated continuously by a gas dynablender (Matheson Gas Products, E. Rutherford, N.J.). Using mass flow rates, changes in CO_2 concentration, and the known step response characteristics of the flow and detection system, the APS rate was calculated by the computer every 30 s and plotted. When the performance of the leaf to be labeled was satisfactory, the length of the labeling period was entered in the computer. The following events then occurred automatically under computer control.

Valves were switched to state II which cleared unlabeled CO_2 from the flow metering system shown at the left of Figure 1. $^{13}CO_2$ was then flushed through the metering system with the valves in



state	a	b	c	d	e	f	g	h	
I	2	2	1	1	1	1	1	1	steady-state APS measurement
II	1	2	1	1	2	1	1	1	purge
III	1	1	2	2	2	1	1	1	purge
IV	1	1	2	2	1	2	2	2	initial labeling
V	1	1	2	2	1	1	1	1	long-term labeling

FIG. 1. Gas flow diagram of the $^{13}CO_2$-labeling system.

state III. These two preparatory steps required 2 min during which photosynthesis was proceeding as before but with no new CO_2 entering the chamber. The CO_2 concentration never decreased more than 30 μl/l during this period. Labeled CO_2 entered the chamber with the valves in state IV, at which time the traps containing ascarite (NaOH adsorbed on silica) were also switched into the flow system on the high pressure side of the chamber (Fig. 1). The ascarite quantitatively removed CO_2. From measured mass flow rates, the concentration of CO_2 in the chamber was maintained by adjustment of the variable orifice to admit $^{13}CO_2$. During this period the system was operating open loop with respect to CO_2 (*i.e.* no CO_2 was recycled). Since the gas volume of the loop containing the chamber was replaced approximately six times/min, the change from $^{12}CO_2$ was 95% complete in 30 s.

The isotopic switch in the remainder of the system was considerably slower, especially through the loop containing the three gas analyzers in series, where a pressure regulator and orifice reduced the flow to 1 liter/min. Thus, valve state IV was maintained for 5 min. This was about three times as long as required to establish constant $^{13}CO_2$ concentration through the entire system, as measured by one of the gas analyzers in the series equipped with a $^{13}CO_2$ detection cell.

For labeling experiments lasting longer than 5 min, the valves were switched to state V, which removed the ascarite traps from the flow, conserved $^{13}CO_2$, and permitted accurate photosynthetic rate measurements, as well as measurement of the isotopic composition of the labeling gas. Labeling experiments were ended with a 20-s period (not counted as part of the labeling time) during which the $^{13}CO_2$ supply was closed and the ascarite traps were activated. This removed most of the $^{13}CO_2$ from the labeling chamber at which point the lid was removed, the stem cut, and the leaf immersed in liquid N_2. The latter procedure generally required about 10 s. The complete label termination procedure lasted one-tenth of the time of the shortest labeling period reported in these experiments. Following the labeling of a leaf the entire plant was destroyed.

Isolation and Enzymic Analysis of Sucrose. Neutral fractions, containing principally sucrose, were isolated from labeled leaves by the procedures described by Atkins and Canvin (1). Solutions containing these fractions were examined by NMR as described below. A small part of each solution was removed for analysis of total sucrose. Sucrose was first hydrolyzed with invertase by the method of Cerning-Beroard (4). Glucose was then determined by an enzymic procedure which converted glucose to 6-P-gluconate and generated NADPH, the optical absorption of which was measured at 340 nm. A standard curve based on known sucrose levels was used to calculate sucrose concentrations. All samples were analyzed with and without invertase hydrolysis to correct for the presence of sugars other than sucrose.

Conversion of Sucrose to Lactic Acid. A few sucrose samples were converted to lactic acid for examination by NMR. An enzymic procedure converted the sucrose in the neutral fractions to lactic acid. The neutral fraction, in less than 3 ml of water, was added to 5 ml of 0.02 M K-phosphate (pH 4.6) containing 300 units of invertase. After incubating the mixture for 30 min at 30 C, components were added to make the following concentrations in a total volume of 25 ml: 75 mM KCl, 10 mM $MgSO_4$, 50 mM Tris-HCl (pH 8.5), 0.1 mM 2,3-diphosphoglyceric acid, 1 mM DTT, 5 mM sodium ADP, 5 mM sodium ATP, and 5 mM NAD. The pH was adjusted to 8.1 with KOH. Enzymes (purchased from Sigma Chemical Co., St. Louis) were added in the following amounts: hexokinase (yeast), 40 units; P-glucose isomerase (yeast), 20 units; fructose-6-P kinase (rabbit muscle), 40 units; aldolase (rabbit muscle), 5 units; triose-P isomerase (yeast) 20 units; glyceraldehyde-3-P-dehydrogenase (yeast), 20 units; 3-P-glyceric phosphokinase (yeast) 28 units; P-glycerate mutase (rabbit), 40 units; enolase (yeast), 20 units; pyruvate kinase (rabbit muscle), 22 units; and lactic dehydrogenase (rabbit muscle), 20 units. The mixture was incubated for 3 h at 30 C. The yield of lactic acid from 10 mg of sucrose under these conditions was in excess of 85%. Ion exchange chromatography purified lactic acid from most reaction components. The reaction mixture was applied to a column (0.8 × 9 cm) of Dowex AG 1-X8 resin (formate form, 200–400 mesh). The column was then washed with 5 ml of water and lactic acid was eluted with 10 ml of 4 M formic acid. The material which did not adhere to the first column (flow-through and wash) was applied to a second resin column of the same type and lactic acid was eluted with 4 M formic acid. The combined formic acid eluates were taken to dryness at reduced pressure, residual formic acid removed with N_2 gas, and the residue was suspended in a small volume of water. Lactic acid was measured with a lactic dehydrogenase enzymic procedure (16).

^{13}C NMR Spectra. Fourier transform ^{13}C NMR spectra of sucrose and lactic acid solutions (1.8 ml in volume) were obtained at 22.6 MHz using a spectrometer equipped with an external ^{19}F field-frequency stabilizer, a quadrature detector, a double-tuned 10-mm-diameter single-coil insert, and a Nicolet 1080 data system. Data accumulation times varied from 0.5 to 8 h, the latter to obtain natural abundance spectra of dilute solutions.

The spectra of labeled sucrose were corrected for the natural abundance background. Approximately 50 mg of sucrose was isolated from the portion of corn leaf enclosed in the labeling chamber. This was about three times the amount for a typical soybean trifoliolate of 1.5 dm^2. The former produced a natural abundance background signal which was about 20% of the intensity of the singlet arising from labeled carbons in the 5-min labeling experiment. The NMR correction was made on the spectrum by computer subtraction of the strong natural abundance signal from a concentrated sucrose standard (180 mg/ml), scaled in intensity by the results of the enzymic sucrose assay performed on the solution to be examined by NMR. The correction was not important for any of the sugar solutions from the longer length labeling experiments, or for most soybean sugar solutions.

RESULTS

Photosynthesis Measurement. APS rates of leaves obtained from steady-state measurements made just prior to ^{13}C labeling are presented in Tables I and II. In agreement with earlier experiments (2), we find that APS in soybeans is enhanced by about 50% in the presence of either low O_2 or high CO_2 concentrations, with no substantial improvement by a combination of the two (Table I). Corn APS shows no strong dependence on O_2 concentration (Table II). Individual corn plants had APS rates completely unaffected by O_2. We ascribe the minor dependence reported in Table II to variations in APS for the group of corn plants used in the low O_2-labeling series. In general, reported APS rates are accurate to better than ±2%, but variations for different plants under similar conditions were on the order of ±10%. Both soybean and corn have APS rates which are strong functions of light intensity (Tables I and II).

^{13}C NMR Spectra. The ^{13}C NMR spectra of sucrose (and other neutrals) extracted from soybean leaves labeled under two sets of conditions are shown in Figure 2. The spectra are complicated by extensive spin-spin coupling due to the high concentration of label. The NMR signal from sucrose extracted from a trifoliolate carried through the labeling procedure but with no intentional exposure to $^{13}CO_2$, was about at the noise level. As described above, this minor natural abundance background was removed so that essentially all of the signal shown in Figure 2 is due to label.

The protonated anomeric carbon resonance of the glucose component of sucrose is chemically shifted from most of the other sugar resonances. Its spectrum is simple. If this anomeric carbon has its only carbon nearest neighbor labeled, a spin-spin doublet is observed (24). If this carbon nearest neighbor is unlabeled, the protonated anomeric carbon spectrum is a single line appearing at

Table I. *Soybean $^{13}CO_2$-labeling Parameters at 30 C*

[CO_2]	[O_2]	Time after Planting	Labeling Time	Light Intensity	APS	f_D[a]
$\mu l/l$	%	days	min	$mE\ m^{-2}\ s^{-1}$	$mg\ CO_2\ dm^{-2}\ h^{-1}$	
325	21	41	5	0.70	26	0.25
325	21	41	15	0.70	25	0.50
325	21	41	45	0.70	32	0.75
325	21	42	180	0.70	28	0.85
325	1.0	41	5	0.70	45	0.61
325	1.0	40	10	0.70	46	0.76
325	1.0	40	15	0.70	44	0.77
325	1.0	41	45	0.70	42	0.82
325	1.0	42	180	0.70	48	0.84
1,000	1.0	40	5	0.70	44	0.59
1,000	1.0	41	15	0.70	42	0.81
1,000	1.0	42	45	0.70	47	0.80
1,000	1.0	42	180	0.70	47	0.85
325	21	47	15	0.70	19	0.59
750	21	47	15	0.70	23	0.76
1,150	21	47	15	0.70	38	0.78
1,450	21	47	15	0.70	37	0.77
1,950	21	47	15	0.70	40	0.77
325	21	48	45	0.70	26	0.72
325	21	48	45	0.34	12	0.55
325	21	49	45	0.10	7	0.50
325	21	50	45	0.07	5	0.44

[a] Fraction of anomeric carbon signal contained in spin-spin doublet.

the center of the doublet (Fig. 2). (Structural formulas of glucose with arrows connecting the appropriate carbons to NMR lines for both natural abundance and labeled species can be found in Fig. 3 of ref. 21.) Longer range coupling creates minor line broadening which will be ignored (*cf.* below). Thus, the measure of the doublet to singlet integrated intensities provides an immediate and direct measure of the relative concentrations of ^{13}C-^{13}C and ^{12}C-^{13}C pairs in one of the triose fragments of sucrose. This result has been reported before (22). The doublet and singlet intensities of the protonated anomeric carbon NMR spectra of sucrose in all of the labeling experiments on both soybean and corn are presented in Tables I and II as f_D, the fraction of the protonated anomeric carbon signal contained in the doublet. Note that $f_D = x^2/[(1-x)x + x^2] = x$, where x is the mole fraction ^{13}C in the labeled triose (Table III). Thus, for sufficiently long labeling times, f_D approaches 0.9, the isotopic composition of the 90% enriched $^{13}CO_2$ used for labeling.

Table II. *Corn $^{13}CO_2$-labeling Parameters at 30 C*

[CO_2]	[O_2]	Time after Planting	Labeling Time	Light Intensity	APS	f_D[a]
$\mu l/l$	%	days	min	$mE\ m^{-2}\ s^{-1}$	$mg\ CO_2\ dm^{-2}\ h^{-1}$	
325	21	40	5	0.70	33	0.24
325	21	41	10	0.70	28	0.61
325	21	41	15	0.70	32	0.62
325	21	41	30	0.70	35	0.69
325	21	40	45	0.70	42	0.77
325	21	40	180	0.70	42	0.84
325	1.0	37	5	0.70	39	0.30
325	1.0	37	10	0.70	40	0.66
325	1.0	37	15	0.70	44	0.64
325	1.0	38	30	0.70	38	0.74
325	1.0	38	45	0.70	37	0.78
325	1.0	36	180	0.70	45	0.86
325	21	35	45	0.70	47	0.81
325	21	35	45	0.34	36	0.74
325	21	36	45	0.10	15	0.70
325	21	36	45	0.07	6	0.45

[a] Fraction of anomeric carbon signal contained in spin-spin doublet.

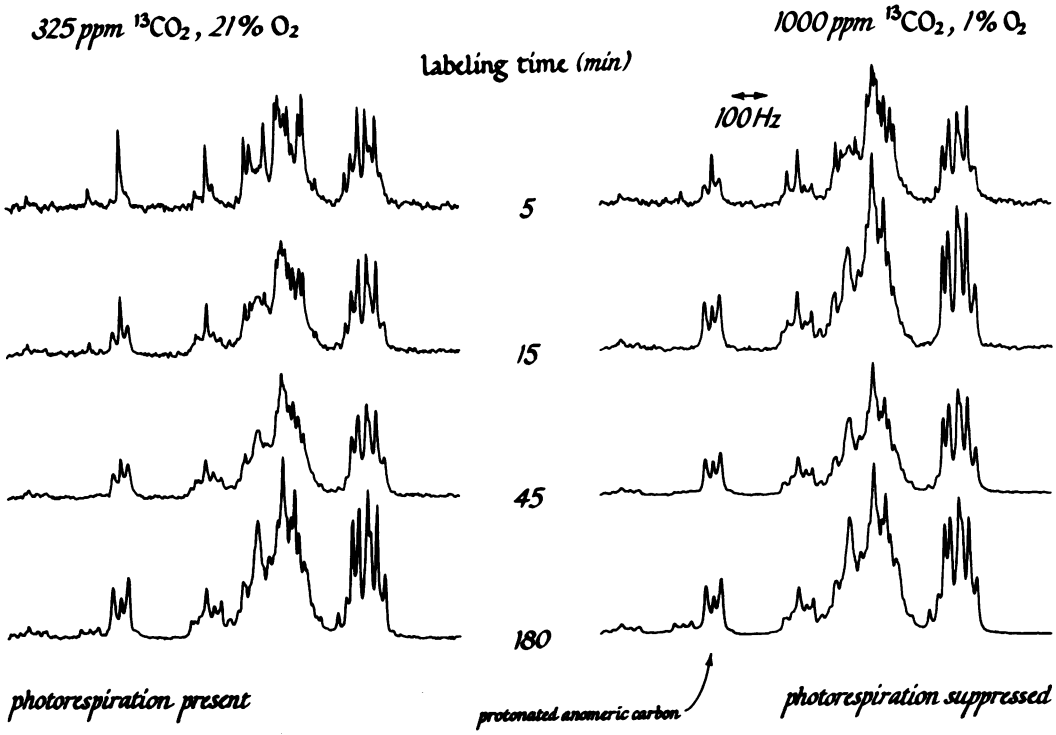


FIG. 2. ^{13}C NMR spectra of sucrose extracted from soybean leaves labeled with $^{13}CO_2$ for 5–180 min using two different gas compositions.

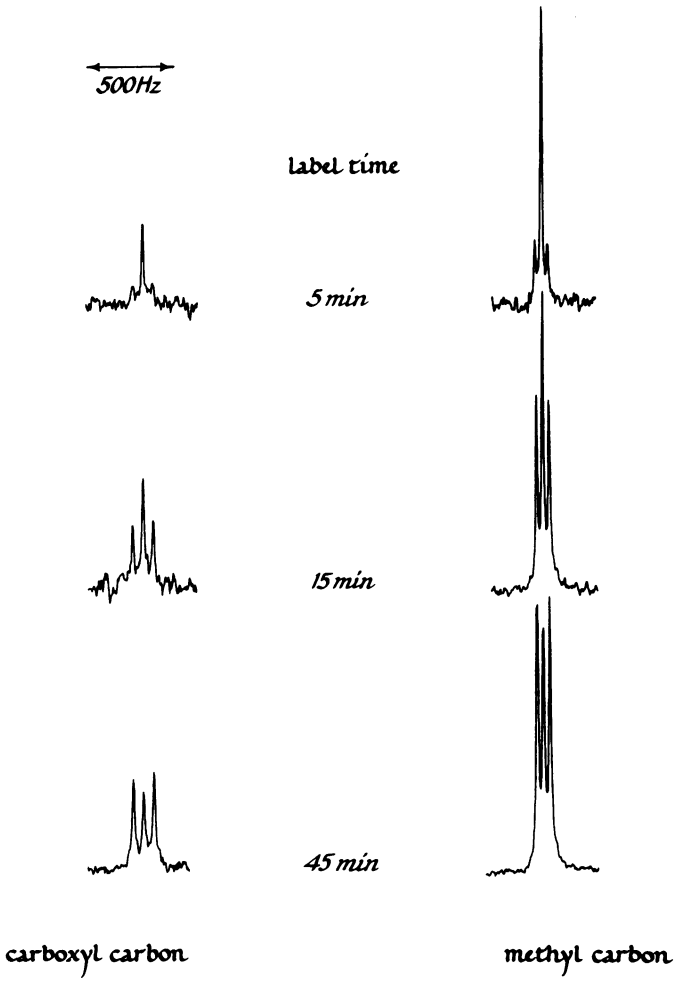


FIG. 3. ^{13}C NMR spectra arising from the carboxyl and methyl carbons of lactic acid derived enzymically from sucrose extracted from soybean leaves labeled for 5, 15, and 45 min at 325 μl/l $^{13}CO_2$ and 21% O_2.

Table III. *Labeling Patterns for Triose Fragments of Sucrose Containing Glucose Anomeric Carbon (Starred)*

Triose	Anomeric Carbon NMR Signal	Carbon Pair	Carbon Pair Probability in Terms of x, Mole Fraction ^{13}C of Labeled Triose
^{12}C-^{12}C-^{12}C*		^{12}C-^{12}C*	(1-x) (1-x)
^{13}C-^{12}C-^{12}C*		^{12}C-^{12}C*	(1-x) (1-x)
^{12}C-^{13}C-^{12}C*		^{13}C-^{12}C*	x(1-x)
^{13}C-^{13}C-^{12}C*		^{13}C-^{12}C*	x(1-x)
^{12}C-^{12}C-^{13}C*	Singlet	^{12}C-^{13}C*	(1-x)x
^{13}C-^{12}C-^{13}C*	Singlet	^{12}C-^{13}C*	(1-x)x
^{12}C-^{13}C-^{13}C*	Doublet	^{13}C-^{13}C*	x^2
^{13}C-^{13}C-^{13}C*	Doublet	^{13}C-^{13}C*	x^2

The values of f_D (*versus* time) for soybeans are strongly dependent on labeling conditions. Under either high CO_2 or low O_2 concentrations, f_D reaches a value of about 0.80 with 15-min labeling, but is 0.72–0.75 after 45-min labeling under normal CO_2 and O_2 concentrations (Table I and Fig. 2). After 5 min of labeling in low O_2, f_D is 0.60, whereas in normal gas concentrations it is only 0.25. Because of the dependence on O_2, this large difference in f_D cannot be attributed to $^{12}CO_2$ physically trapped either in the labeling chamber or in the leaf itself.

The integrated intensities of the spectra of lactic acid derived from massively labeled sucrose are consistent with a uniform distribution of labeled carbon. The doublet and singlet intensities are the same for both 1- and 3-position carbons (Fig. 3). Since these two carbon positions would be most likely to show the effects of a nonuniform distribution of label (23), it is safe to conclude that the spectrum of any carbon of sucrose, and in particular that of the anomeric carbon, can be used to determine the statistics of the ^{13}C distribution within pairs of carbons of labeled triose components. (Having established this fact, no further lactic-acid NMR spectra were obtained.) Integrated intensities must be used in this determination, however, since relative peak heights in sucrose are affected by differential line broadening due to difference in long range spin-spin coupling. Standard procedures for deconvolution of overlapping NMR lines can be used (20) resulting in intensity determinations accurate to better than ±10%.

The values of $f_D(t)$ for corn are not strongly dependent on O_2 concentrations. Approximately the same NMR patterns are observed for corn labeled at 21% O_2 and at 1% O_2, both using 325 μl/l CO_2 (Fig. 4).

Both soybean and corn anomeric carbon NMR spectra depend strongly on the light intensity used during the labeling (Tables I and II). For both systems, lower light intensities mean lower APS rates and smaller values of f_D, although for soybean the change in f_D below 0.2 mE m^{-2} s^{-1} is relatively small.

DISCUSSION

The ^{13}C-labeling patterns for sucrose extracted from soybean leaves under normal and low O_2 conditions (Fig. 2 and Table I) can be interpreted in a straightforward way. Photorespiration generates $^{12}CO_2$ (25) which dilutes $^{13}CO_2$ entering the Calvin cycle, is refixed, and thereby delays the isotopic saturation of the immediate triose products of photosynthesis for times longer than 45 min. Suppression of photorespiration, either by low O_2 or high CO_2 concentrations, results in much faster isotopic saturation. Because of the large differences between labeling patterns in low and normal O_2 conditions (compare, *e.g.*, the singlet and doublet intensities in Fig. 2 after 15-min labeling), the amount of unlabeled carbon generated by photorespiration and then internally refixed may be substantial, possibly as much as 20–40% of the net carbon fixation, depending upon whether other sources of unlabeled carbon are routed through the Calvin cycle under conditions of high photorespiration (12, 19).

The absence of a dependence of the ^{13}C-^{13}C and ^{13}C-^{12}C patterns on O_2 conditions during labeling of corn can be explained in either of two ways: first, there is no substantial O_2-dependent photorespiration in corn; or second, there is a massive pool of unlabeled carbon not present in soybean which overwhelms photorespired and refixed $^{12}CO_2$ in corn and so masks its influence on the observed labeling patterns. Certainly the possibility of such a pool exists. The 4-position of the active malate pool is one immediate source of unlabeled carbon not related to photorespiration which is present in C_4 species such as corn, but not in C_3 species such as soybean (8, 9). Furthermore, reasonably fast exchange processes with the inactive malate pool, or coupling between the Calvin cycle and the 1-, 2-, and 3-carbons of malate via pyruvate, P-enolpyruvate, and triose-P could provide other sources of unlabeled carbon flowing into the Calvin cycle (2).

We feel the second explanation is not consistent with all of the experimental results. If massive sources of unlabeled carbon were a factor in the corn experiments, the rate of incorporation of total label into triose products of the Calvin cycle would be much less for corn than for soybean. Comparing the singlet and doublet intensities of Figures 2 and 4, we see that the appearance of the ^{13}C-^{13}C doublet for corn, although somewhat slower than for soybean in low O_2, is substantially faster than for soybean in air. That is, the rate of isotopic saturation for corn is not subject to an unduly long time delay because of massive reserves of unlabeled

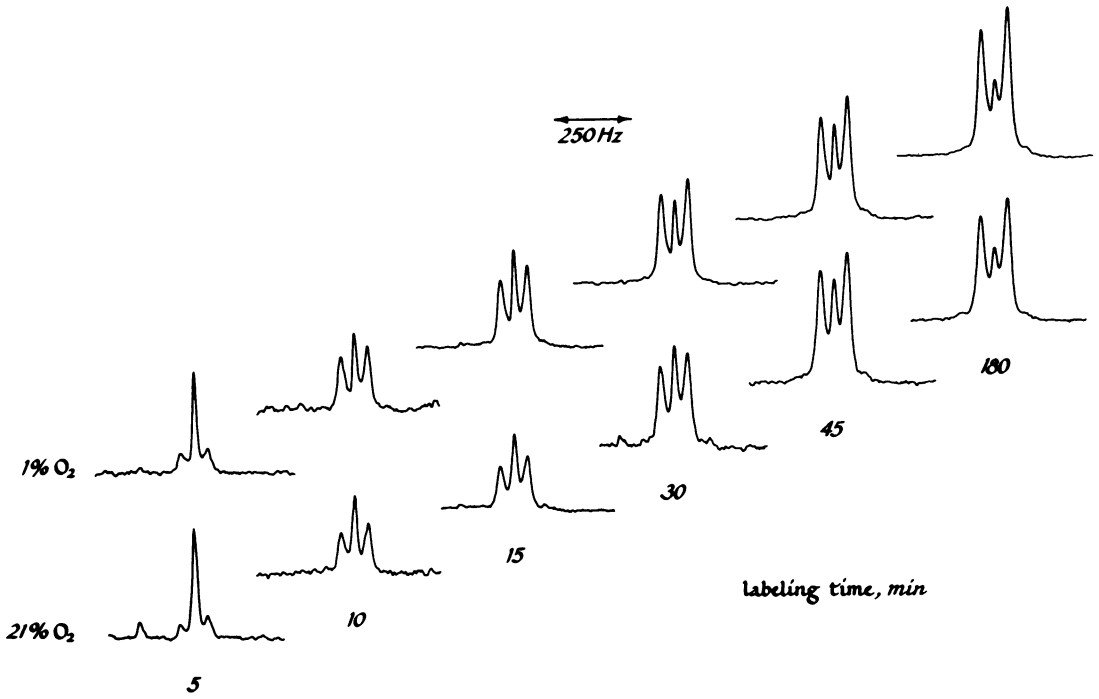


FIG. 4. Protonated anomeric carbon NMR spectra of sucrose extracted from corn leaves labeled with 325 μl/l $^{13}CO_2$ for 5–180 min at 1% and 21% O_2.

carbon entering the Calvin cycle. We conclude that the first explanation offered above is probably correct and that photorespiration in corn is substantially less than in soybean. This conclusion is consistent with the suggestion that photorespiration in corn is inhibited by high bundle sheath cell CO_2 concentrations generated by the malate transport acting as a pump (7).

The fact that for both soybean and corn f_D does not reach 0.9 even after 3-h labeling (Tables I and II) suggests the presence of a very slowly isotopically saturating source of unlabeled carbon, such as, *e.g.* a pool containing the substrates for mitochondrial respiration. The dependence of f_D for both soybean and corn on the light level reflects the slower rate of isotopic saturation of both the Calvin cycle and tightly coupled unlabeled carbon pools under conditions of reduced total carbon flux.

All of the experiments discussed above involved measuring the distribution of ^{13}C label in the early products of the Calvin cycle by sampling only the sucrose pool. Future experiments could involve sampling a variety of dynamic pools (serine, glycine, malate) the combined results for which would allow the specification of detailed metabolic pathways, and so produce more quantitative conclusions about internally refixed photorespiratory CO_2 than reached here.

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