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Synthesis of Glycolate from Pyruvate via Isocitrate Lyase by Tobacco Leaves in Light

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ISRAEL ZELITCH
Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, P.O. Box 1106, New Haven, Connecticut 06504

ABSTRACT

Tobacco (*Nicotiana tabacum* var Havana Seed) leaf discs were supplied tracer quantities of [2-^{14}C]- and [3-^{14}C]pyruvate for 60 minutes in steady state photosynthesis with 21% or 1% O_2, and the glycolate oxidase inhibitor α-hydroxy-2-pyridinemethanesulfonic acid was then added for 5 or 10 minutes to cause glycolate to accumulate. The [3-^{14}C]pyruvate was converted directly to glycolate as shown by a 50% greater than equal-labeled ^{14}C in C-2 of glycolate, and the fraction of ^{14}C in C-2 increased in 1% O_2 to 80% greater than equal-labeled. This suggests the pathway using pyruvate is less O_2-dependent than the oxygenase reaction producing glycolate from the Calvin cycle. The formation of glycolate from pyruvate in the leaf discs was time-dependent and with [2-^{14}C]- and [3-^{14}C]pyruvate supplied leaf discs the C-2 of glyoxylate derived from C-2 of isocitrate was labeled asymmetrically in a manner similar to the asymmetrical labeling of C-2 of glycolate under a number of conditions. Thus glycolate was probably formed by the reduction of glyoxylate. Isocitric lyase activity of tobacco leaves was associated with leaf mitochondria, though most of the activity was in the supernatant fraction after differential centrifugation of leaf homogenates. The total enzyme activity was at least 35 micromoles per gram fresh weight per hour. The relative contribution of the pathway to the glycolate pool is unknown, but the results support the existence of a sequence of reactions leading to glycolate synthesis during photosynthesis with pyruvate, isocitrate, and glyoxylate as intermediates.

The biosynthesis of glycolate by C_3 leaves during photosynthesis occurs largely from the oxygenase reaction associated with ribulose-1,5-bisP carboxylase activity that produces P-glycolate (2) which is converted to glycolate by a phosphatase located in the chloroplast (8). Glycolate is then further metabolized to generate CO_2 produced during photorespiration (8, 13, 22). This mechanism is attractive because in general terms it clearly explains the well known inhibition of CO_2 fixation by O_2, although the question of whether all of the glycolate is synthesized by this mechanism is more difficult to answer with certainty. Several authors argue that there is substantial evidence that it is probably the sole mechanism of glycolate formation (12, 13).

It is difficult to establish that this is the exclusive mechanism *in vivo* using $^{18}O_2$ incorporation into glycolate because other reactions may also incorporate $^{18}O_2$ into glycolate, and because of dilution by photosynthetically generated $^{16}O_2$. A relative enrichment of at least 59% was reported in some experiments (1). It is often stated (12, 17) that experiments with a mutant of *Arabidopsis* that is lethal in normal air and has less than 5% of normal P-glycolate phosphatase activity provide compelling evidence that P-glycolate is the major, or only, precursor of glycolate. In the presence of a suicide inhibitor of glycolate oxidase supplied through the roots, the *Arabidopsis* mutant accumulated P-glycolate greatly and glycolate only slightly, hence the above conclusions (17). This mutant, as well as a similar mutant of barley (5) produces little glycolate, serine, or glycine when provided with $^{14}CO_2$ in 21% O_2. The inhibition in these mutants of the alternative pathway of serine synthesis from P-glycerate (11) confirms the occurrence of additional metabolic disturbances. It is also well known that P-glycolate is a potent inhibitor of triose phosphate isomerase and the Calvin cycle (5), hence the mutant that accumulates P-glycolate has considerable shortcomings as an experimental tool for evaluating alternative sources of glycolate synthesis.

Although it is well known that $^{14}CO_2$ produced essentially equally labeled glycolate during photosynthesis. I previously presented evidence that supplying tobacco or maize leaf discs with [2-^{14}C]glyoxylate or [3-^{14}C]pyruvate produced glycolate labeled predominantly in C-2 (25), showing it did not arise by CO_2 fixation. Labeled glyoxylate will be converted directly to glycolate by reactions catalyzed by glyoxylate reductases (10, 28), and experiments on the stereochemical incorporation of ^3H_2O into glycolate raised the possibility that the reduction of glyoxylate can provide another source of glycolate (14). I previously suggested (25) that the incorporation of [3-^{14}C]pyruvate into C-2 of glycolate could occur by way of conversion to isocitrate and the action of isocitrate lyase (EC 4.1.3.1) to generate glyoxylate (Fig. 1), although direct evidence for such a sequence of reactions was then lacking.

There are still few published reports about isocitrate lyase activity in leaves. The enzyme is found in glyoxysomes of plant tissue but not in leaf peroxisomes (6). Godvari *et al.* (4) found inhibitors of the enzyme were present in leaf extracts, and after gel filtration with Sephadex to remove inhibitors, they showed activity in leaves of several species including tobacco (0.54 μmol/mg protein·h). Hunt and Fletcher (7) used Sephadex filtration to remove low mol wt inhibitors in extracts of young pea leaves and established a mitochondrial location for the enzyme based on co-migration with cytochrome oxidase in density gradient centrifugation. The activity was low (1 μmol/g fresh weight·h).

In the present study the incorporation of [2-^{14}C]- and [3-^{14}C]pyruvate into glycolate in the light was reexamined under conditions where glycolate accumulation was varied in tobacco leaf discs by changing the O_2 concentration and the time of exposure to α-HPMS,[1] an inhibitor of glycolate oxidase that causes rapid accumulation of glycolate (23). Net photosynthesis was measured and the specific radioactivity in C-2 of glycolate was determined and compared to the specific radioactivity of the pyruvate supplied. In one experiment the labeling of C-2 of isocitrate (that produces glyoxylate labeled in C-2 by the isocitrate lyase reaction) was examined. The activity of isocitrate lyase

[1] Abbreviations: α-HPMS, α-hydroxy-2-pyridinemethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

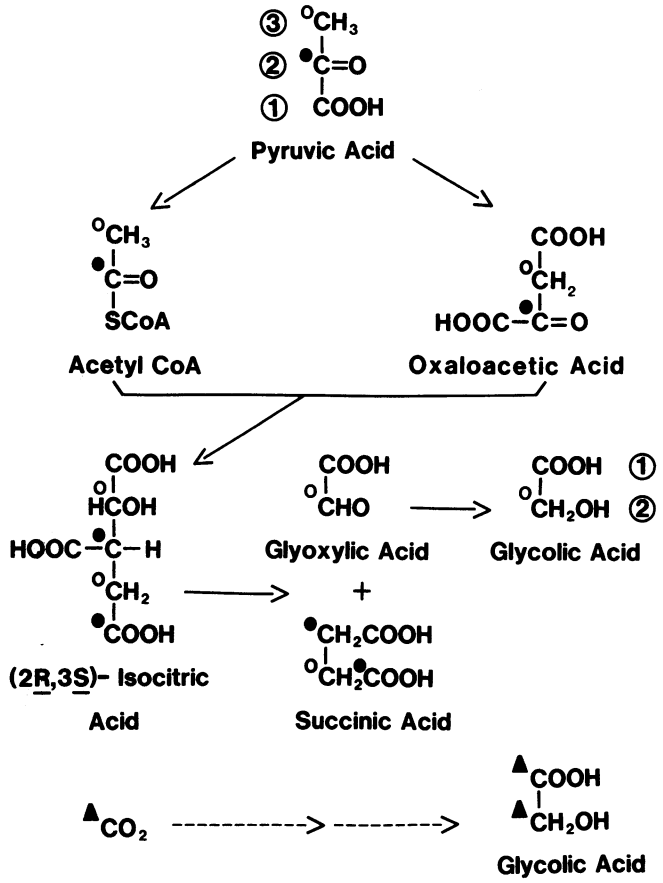

FIG. 1. Schematic diagram showing labeling from C-2 (●) and C-3 (○) of pyruvate to produce asymmetrical labeling of glycolate from [3-^{14}C]pyruvate and equal-labeling of glycolate from photosynthetically fixed $^{14}CO_2$. The sequence of reactions involves the enzymes pyruvate decarboxylase, malic enzyme, malate dehydrogenase, citrate synthase, aconitase, isocitrate lyase, and glyoxylate reductases.

in leaves was investigated using a sensitive assay that permits use of small amounts of enzyme in the reaction mixture and thereby minimizes the effects of endogenous inhibitors. Evidence is presented that a sequence of reactions occurs in the light for the conversion of pyruvate to glycolate with isocitrate and glyoxylate as intermediates.

MATERIALS AND METHODS

Net Photosynthesis of Leaf Discs and Incorporation of [^{14}C]Pyruvate. Leaf discs, 1.6 cm diameter, were cut with a sharp punch from greenhouse-grown leaves of tobacco (*Nicotiana tabacum*, var Havana Seed) and floated right side up on a thin layer of water in a Petri dish. The discs were selected by a Latin Square method and each sample comprised 18 discs with a fresh weight about 720 mg. The samples were placed in Plexiglas chambers (1 L) fitted with a stirring fan and serum stoppers, and were flushed continuously at a rate of 1 L/min with moistened 21% O_2, and in some instances later with 1% O_2, containing 370 μl CO_2/L while illuminated from above (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) with air temperature about 30°C. Periodically the chamber was closed with stopcocks and duplicate samples of the atmosphere were withdrawn with hypodermic syringes at zero time and at 60 to 120 sec later. Net CO_2 assimilation was determined by injecting the samples into an infrared CO_2 gas analyzer and measuring the rate of CO_2 depletion during the brief interval in the closed system (15). The stopcocks were opened and flushing of the chambers was resumed until the next sampling period.

When a steady rate of CO_2 assimilation was reached, after at least 60 min, the water in the Petri dish was withdrawn and replaced with 3.5 ml of 1 mM K [^{14}C]pyruvate. Flushing of the chambers was continued for 60 min and the photosynthetic rate was determined several times by CO_2 depletion as described above. The [^{14}C]pyruvate solution was then withdrawn and replaced with 5 ml of 10 or 20 mM α-HPMS, an inhibitor of glycolate oxidase (23). During treatment with α-HPMS, net photosynthesis was measured at least once. The experiment was terminated 5 or 10 min after the leaf discs were placed on the inhibitor solution by rapidly transferring the discs into 20 ml of boiling 20% ethanol containing 10 mM sodium bisulfite. The killed discs were ground in a Ten Broeck homogenizer, the suspension adjusted to 25 ml, and after a small sample was withdrawn to determine total radioactivity, the homogenate was centrifuged at 38,000g for 15 min and the residue was washed twice with water and centrifugation. Radioactive glycolate and isocitrate were separated and purified from the combined supernatant fluids.

Radioactive Substrates and Their Radiochemical Purity. The [2-^{14}C]pyruvate was obtained from Research Products International Corp. and the [3-^{14}C]pyruvate from DuPont NEN Research Products. These were dissolved in carrier 1.0 mM K-pyruvate (prepared from freshly distilled pyruvic acid and crystallized in 80% aqueous ethanol). Radiochemical purity of these substances was determined by analysis with an LDC/Milton Roy HPLC system connected to a Flo-One (Radiomatic Instruments and Chemical Co.) scintillation radioactive flow detector. Radiochemical purity was usually measured the day prior to each experiment. Compounds were separated on an Aminex Ion Exclusion HPX-87H column (Bio-Rad Laboratories) by elution with 0.015 N H_2SO_4 at a flow rate of 0.5 ml/min. Typical retention times for metabolites important in this study were (in min): citrate, 10.6; isocitrate, 10.8; glyoxylate, 12.2; pyruvate, 12.4; glycerate, 14.3; glycolate, 16.0; succinate, 16.2; and formate, 18.2. Better separation of glyoxylate from pyruvate was achieved using a C18 Reverse Phase HPLC column (Perkin-Elmer) eluted with 20 mM KH_2PO_4-0.75% H_3PO_4 (pH 1.8) at a flow rate of 0.8 ml/min. In this system typical mass retention times were (in min): glyoxylate, 4.1; and pyruvate, 4.9. No radioactive glyoxylate or glycolate could be detected in any sample of [^{14}C]pyruvate examined by these HPLC systems. The radiochemical purity of the [^{14}C]pyruvate used in the experiments ranged 94 to 97%, and the ^{14}C-containing contaminant was almost entirely in one compound assumed to be 'parapyruvate' (γ-methyl-γ-hydroxy-α-ketoglutarate), a well-known product of stored pyruvate solutions (21). It had a retention time on the Aminex column at 9.8 min and on the C18 column at 6.8 min, well separated from pyruvate, glyoxylate, or glycolate in both systems.

Radioactivity was determined by scintillation counting using an external standard ratio method to determine efficiency. Samples were assayed in plastic vials containing 10 ml of scintillation fluid (Opti-Fluor, Packard Instrument Co.). Released $^{14}CO_2$ during degradations was absorbed on paper wicks moistened with ethanolamine solution. The wicks were transferred to the scintillation fluid containing 0.1 ml of Protosol (DuPont NEN Research Products) to facilitate elution of radioactivity (24).

Isolation of Glycolate and Isocitrate by Ion Exchange Chromatography and HPLC. Leaf extracts were passed through a column of Dowex-acetate anion exchange resin 0.7 × 6 cm, and after the neutral and basic compounds were eluted with water, elution was continued with 4 N acetic acid (23). The first 4 ml were discarded and the next 10 ml, which contain mainly glycolate and glycerate, were collected in conical tubes. The rate of glycolate accumulation in the discs was established by color-

imetric determination of the glycolate concentration in a small sample of this fraction (25). Stepwise elution was continued with 4 N acetic acid, 2 N formic acid, and the citrate-isocitrate was collected in 20 ml of 4 N formic acid. Pyruvate would also normally be located in this fraction, but since a great excess of $NaHSO_3$ was present in the killing solution it reacted with pyruvate to give an addition product that was in a fraction collected later by elution with 10 ml of 1 N HCl.

The glycolate fractions were placed in a 45°C water bath, and the volume decreased to about 100 μl by blowing a stream of air on the surface. After clarification by microfiltration, the fraction was reduced in volume again to about 20 μl, and the glycolate was separated and purified by preparative HPLC using the Aminex column described above. The radioactivity and glycolate concentration were determined in the pure glycolic acid, and after it was degraded, the specific radioactivity in C-2 of glycolate was calculated.

The citrate-isocitrate fraction in 4 N formic acid was taken to dryness in a stream of air at 45°C. It was dissolved in a small volume of water, and after microfiltration and further reduction in volume, preparative HPLC using the Aminex column was used to isolate purified citrate-isocitrate.

Degradation of [^{14}C]Glycolate and [^{14}C]Isocitrate. Glycolate was degraded (23) by first oxidizing it in Warburg flasks to glyoxylate with glycolate oxidase (an ammonium sulfate fraction from spinach leaves) at pH 9.0 in the presence of excess catalase, followed by oxidative decarboxylation of glyoxylate with ceric sulfate in 2 N H_2SO_4 to obtain $^{14}CO_2$ (derived from C-1) and ^{14}C-formic acid (from C-2). The $^{14}CO_2$ was trapped in paper wicks in the centerwell, moistened with 5 M ethanolamine, and the ^{14}C-formic acid remaining in the main compartment was obtained by sublimation *in vacuo* in one arm of a two-armed sublimation apparatus and frozen in liquid N_2 (24). Eleven degradations carried out with [1-^{14}C]glycolate purified by HPLC gave a mean of 94.1% in C-1, and two determinations of [2-^{14}C]glycolate gave a mean of 96.4% in C-2. Values of ^{14}C in C-2 of glycolate varied by less than 2% in duplicate determinations in the experiments described.

To degrade isocitrate, the purified citrate-isocitrate obtained by HPLC was treated with highly purified isocitrate lyase from *Pseudomonas indigofera* (kindly provided by M. J. Conder and B. A. McFadden, Washington State University) to convert the isocitrate to glyoxylate and succinate (Fig. 1). The enzyme solution contained 10 mM MOPS buffer (pH 7.5); 1 mM Na EDTA (pH 7.5); 25 mM $MgCl_2$; and 1 mM DTT. The enzyme solution was incubated at 30°C for 10 min before 20 μl enzyme solution (about 0.01 unit) was added to neutralized purified citrate-isocitrate (20 μl) in a tube containing 20 μl of 200 mM MOPS (pH 7.5) and 25 μl water. The reaction was carried out at 30°C, and a control reaction mixture was carried out in which 20 μl of 10 mM K_3 isocitrate was added in place of the citrate-isocitrate samples. After 4 h an additional 20 μl of enzyme solution was added to all reaction mixtures, and after 20 h the samples were taken to dryness by blowing a stream of air on the surface while the tubes were kept at 45°C. Analysis of the control reaction mixture using the Aminex HPLC system showed about a 60% decrease in isocitrate and concomitant formation of equal molar quantities of glyoxylate and succinate.

Glyoxylate produced by the isocitrate lyase reaction was isolated by preparative HPLC using the Aminex column system. The purified glyoxylate was degraded with ceric sulfate as described above in the glycolate degradation to obtain $^{14}CO_2$ (from C-1) and ^{14}C-formic acid (from C-2). These originally came from C-1 and C-2 of isocitrate, respectively (Fig. 1). Control degradations of [1-^{14}C]glyoxylate purified by HPLC gave a mean 95.2% ^{14}C in C-1, and [2-^{14}C]glyoxylate gave a mean of 96.6% ^{14}C in C-2.

Subcellular Fractionation of Extracts of Tobacco Leaves and Assay of Isocitrate Lyase Activity. Isolation of a mitochondrial fraction from tobacco leaves was modified from methods previously described (18, 27). The midrib was removed and 3.0 g of lamina was ground for 75 s with 1.5 g sand in a cold mortar with a pestle in 30 ml of cold grinding medium. The grinding medium contained 0.4 M sucrose, 0.2 M Tris, 33 mM KH_2PO_4, 20 mM Na citrate, and 5 mM EDTA adjusted to pH 7.7 with 7 mM 2-mercaptoethanol added just before use. The suspension was squeezed through eight layers of cheesecloth and centrifuged in the cold at 6,000*g* for 10 min to obtain a fraction containing broken chloroplasts and peroxisomes. The resulting supernatant was then centrifuged at 38,000*g* for 10 min to obtain the mitochondrial fraction and final supernatant. Both residue fractions were suspended in about 3.0 ml of 20 mM MOPS (pH 7.5), 1 mM EDTA, and 7 mM 2-mercaptoethanol. The two particulate fractions and the supernatant liquid were sometimes dialyzed prior to assay (4). Dialysis of each fraction was carried out in 50 volumes of 10 mM MOPS (pH 7.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol with stirring at 11°C for 22 h.

The assays for isocitrate lyase activity were based on the method described in Jameel *et al.* (9). The reaction mixture in colorimeter tubes consisted of 200 mM MOPS (pH 7.5), 100 μl; 100 mM $MgCl_2$, 50 μl; 20 mM EDTA, 50 μl; water to make the final volume 1500 μl; 5 to 20 μl enzyme solution; and freshly prepared 1.6% phenylhydrazine hydrochloride, 100 μl. The components were incubated at 30°C for 10 min, and the reaction was initiated by addition of 15 mM natural K_3 (2*R*,3*S*)-isocitrate (20). After 20 to 40 min, the reaction was terminated by the addition of 500 μl of 12 N HCl. A zero time correction was always made by adding HCl to a control reaction mixture prior to adding isocitrate. Colorimetric determinations of the glyoxylate formed from isocitrate was made by adding 100 μl of 8% $K_3Fe(CN)_6$ and reading the absorbancy at 535 nm exactly 7 min later in a Bausch and Lomb colorimeter (0.01 μmol Na glyoxylate, Fluka Chemical Corp., gave an absorbancy of about 0.125). Protein was determined by a dye-binding method (3).

RESULTS

Incorporation of [2-^{14}C]- and [3-^{14}C]Pyruvate into [2-^{14}C]Glycolate in 21% and 1% Oxygen. Leaf discs were exposed to ^{14}C-labeled 1 mM K-pyruvate under steady state conditions of photosynthesis for 60 min to label the precursor pools of glycolate, and glycolate oxidase was then blocked with α-HPMS for 5 or 10 min to cause glycolate to accumulate (Tables I, II, and III). The rates of CO_2 assimilation and glycolate accumulation from CO_2 were greatly in excess of the rates of the tracer quantity of [^{14}C]pyruvate metabolized (<0.3 μmol/g fresh weight·h), hence there was ample opportunity for the pyruvate to be metabolized to $^{14}CO_2$ and produce equally labeled glycolate by the Calvin cycle (Fig. 1).

Asymmetrical labeling in glycolate was always observed when [3-^{14}C]pyruvate was supplied to the leaf discs. The C-3 position is of course more slowly converted to $^{14}CO_2$ during respiration than is C-2. In Table I, the fraction of [^{14}C]glycolate in C-2 in 21% O_2 was 58% greater and in 1% O_2 was 82% greater than equal labeling or 50%. In Table II the fraction in C-2 of glycolate in 21% O_2 was about 45% greater than symmetrical after 5 and 10 min with the inhibitor, and in 1% O_2 was 80% greater than equal-labeled. Table III shows about a 58% increase above equal labeling in C-2 of glycolate after 5 or 10 min with the inhibitor in 21% O_2 and a 78% increase in 1% O_2. Thus, labeling from [3-^{14}C]pyruvate was always greater in C-2 of glycolate in 1% O_2 than in 21% O_2.

When [2-^{14}C]pyruvate was the substrate, equal labeling of the glycolate carbons usually occurred (Table I), although occasionally this substrate was incorporated into glycolate with some

Table I. *Incorporation of Potassium [2-^{14}C]- and [3-^{14}C]Pyruvate into [2-^{14}C]Glycolate by Tobacco Leaf Discs in Light in 21% and 1% Oxygen*

Discs were floated on a thin layer of water and then on 1mM K [^{14}C]pyruvate (specific radioactivity 2.68 × 10^6 DPM/μmol for C-2 labeled pyruvate and 3.95 × 10^6 DPM/μmol for C-3 labeled pyruvate) for 60 min in light (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) at about 30°C while flushed with moistened 21% O_2 or 1% O_2 containing 370 μl CO_2/L. The pyruvate solution was then removed and replaced with 10mM α-HPMS for 10 min before the leaf discs were killed and the organic acids extracted and purified. Net photosynthesis, measured after discs were on α-HPMS solution for about 5 min, was about 95% of the previous steady rate. Controls with 1.07 × 10^6 DPM [2-^{14}C]pyruvate and 1.58 × 10^6 DPM [3-^{14}C]pyruvate, respectively, heated without leaf discs produced no detectable [^{14}C]glycolate.

Conditions and Position of ^{14}C-label in Pyruvate	Mean Net Photosynthesis Discs in Water and [^{14}C]Pyruvate	Total ^{14}C in Discs	Total ^{14}C in Glycolate	Fraction of [^{14}C] in C-2 of Glycolate	Specific Radioactivity in C-2 of [^{14}C]Glycolate	Specific Radioactivities [^{14}C]Pyruvate Supplied ÷ [2-^{14}C]Glycolate Formed
	μmol CO_2/g fresh wt · h	*dpm × 10^3*	*dpm × 10^3*	*%*	*dpm/μmol C-2*	*Ratio*
21% O_2						
[2-^{14}C]	32.6	671	12.2	49	6,700	401
[3-^{14}C]	26.4	1,510	16.4	79	13,400	295
1% O_2						
[2-^{14}C]	43.9	914	21.3	46	5,160	519
[3-^{14}C]	39.6	1,360	8.79	91	11,400	346

Table II. *Incorporation of Potassium [3-^{14}C]Pyruvate into [2-^{14}C]Glycolate by Tobacco Leaf Discs in Light in 21% and 1% Oxygen*

Discs were treated as in Table I with 1 mM [3-^{14}C]pyruvate (specific radioactivity 14.9 × 10^6 DPM/μmol C-3). The discs were then treated with 20 mM α-HPMS for 5 or 10 min in 21% O_2 and for 10 min in 1% O_2 before the discs were killed. Net photosynthesis measured after discs were on α-HPMS solution for about 4 min showed rates about 85% of the previously steady rate. A control in which 12.5 × 10^6 DPM of [3-^{14}C]pyruvate was heated without leaf discs after fractionation by anion exchange chromatography and analysis with the HPLC system produced 11,700 DPM (<0.1% of the ^{14}C supplied) in glycolate. It had a slightly later retention time than standard glycolate. This control provides a maximum value for any possible nonenzymic production of [^{14}]glycolate from [^{14}C]pyruvate. In the control sample, HPLC analysis showed no detectable ^{14}C in citric-isocitric acids.

Experimental Conditions	Mean Net Photo synthesis Discs in Water and [3-^{14}C]Pyruvate	Rate Glycolate Accumulation with α-HPMS	Total ^{14}C in Discs	Total ^{14}C in Glyocolate	Fraction of [^{14}]Glycolate in C-2	Specific Radioactivity in C-2 of [^{14}C]Glycolate	Specific Radioactivity [^{14}C]Pyruvate Supplied ÷ [2^{14}-C]Glycolate Formed
	μmol CO_2/g fresh wt · h	*μmol/g fresh wt · h*	*dpm × 10^3*	*dpm × 10^3*	*%*	*dpm/μatom C-2*	*Ratio*
21% O_2							
α-HPMS 5 min	215	40.0	6,500	81.7	74	28,100	530
α-HPMS 10 min	229	30.5	6,800	75.6	72	17,500	851
1% O_2							
α-HPMS 10 min	270	7.5	5,280	33.9	90	39,100	381

Table III. *Incorporation of Potassium [2-^{14}C]- and [3-^{14}C]Pyruvate into [2-^{14}C]Glycolate, and into [2-^{14}C]Glyoxylate Derived from [^{14}C]Isocitrate, by Tobacco Leaf Discs in Light in 21% and 1% Oxygen*

Discs were treated as in Table I with 1 mM K [^{14}C]pyruvate (specific radioactivity 4.40 × 10^6 DPM/μmol for C-2 labeled pyruvate and 6.82 × 10^6 DPM/μmol for C-3 labeled pyruvate). The discs were then treated with 10 mM α-HPMS, and 9 discs were removed after 5 min and the remaining discs after 10 min in 21% O_2; discs in 1% O_2 were kept on α-HPMS solution for 10 min.

Conditions and Position of ^{14}C-Label in Pyruvate	Mean Net Photosynthesis Discs in Water and [^{14}C]Pyruvate	Rate Glycolate Accumulation with α-HPMS	Fraction of [^{14}C] in C-2 of Glycolate	Sp. Radioactiv. in C-2 of [^{14}C]Glycolate	Sp. Radioactiv. [^{14}C]Pyruvate Supplied ÷ [2-^{14}C]Glycolate Formed	Fraction of [^{14}C] in C-2 of Glyoxylate Derived from [^{14}C] Isocitrate
	μmol CO_2/g fr wt·h	*μmol/g fr wt·h*	*%*	*dpm/μatom C-2*	*Ratio*	*%*
21% O_2						
[2-^{14}C]						
α-HPMS 5 min	68.0	19.6	35	2,580	1,710	
α-HPMS 10 min	68.0	14.9	34	2,060	2,140	38
[3-^{14}C]						
α-HPMS 5 min	67.0	18.5	78	5,350	1,270	75
α-HPMS 10 min	67.0	12.0	80	6,160	1,110	73
1% O_2						
[2-^{14}C] 10 min	107	7.1	36	2,910	1,510	33
[3-^{14}C] 10 min	110	6.5	89	5,210	1,310	73

asymmetrical labeling. Thus, Table III shows an experiment with [2-^{14}C]pyruvate in which the fraction of [^{14}C]glycolate found in C-2 is one-third less than the 50% that would be obtained from equal labeling. Perhaps [2-^{14}C]pyruvate sometimes shows a tendency to label the C-1 of glycolate preferentially because $^{14}CO_2$ produced labels C-4 of oxaloacetate by the malic enzyme and malic dehydrogenase reactions, hence C-1 of isocitrate and C-1 of glyoxylate and glycolate become labeled more than 50% (Fig. 1).

The ratio of the specific radioactivity of the [3-^{14}C]pyruvate supplied to the leaf discs compared to the specific radioactivity in accumulated [2-^{14}C]glycolate provides an indication of the efficiency of direct incorporation of the precursor into glycolate. I previously showed (25) that when $^{14}CO_2$ in 21% O_2 was added to tobacco leaf discs this ratio was 1.6, and with [2-^{14}C]glyoxylate the ratio was 94. As expected, [3-^{14}C]pyruvate produced a lower ratio than [2-^{14}C]pyruvate indicating it was a more effective precursor of C-2 of glycolate in either 21% O_2 or 1% O_2 (Tables I and III). Lowering the O_2 concentration from 21 to 1% increased net photosynthesis (Tables I, II, and III) and decreased the rate of glycolate accumulation (Tables II and III). The effect of O_2 concentration on the ratio with [3-^{14}C]pyruvate was variable. The ratio did not change in 1% O_2 compared to 21% O_2 when 10 mM α-HPMS was used (Tables I and III), and was decreased when 20 mM α-HPMS was supplied (Table II).

An experiment was conducted with 21% O_2 in which half the leaf discs exposed to 10 mM α-HPMS were killed after 5 min and the remaining discs after 10 min (Table III). When [3-^{14}C]pyruvate was the substrate, 30% more glycolate was found in the discs treated 10 min with α-HPMS than after 5 min, and yet the specific radioactivity in C-2 of glycolate was 15% higher after longer exposure with the inhibitor. Finding a greater increase in the specific radioactivity in C-2 of glycolate in comparison with the increase in glycolate concentration shows that a time-dependent increase in glycolate synthesis from pyruvate occurred in the leaf discs.

The pattern of labeling in isocitrate was studied when [2-^{14}C]- or [3-^{14}C]-pyruvate was added to leaf discs (Table III) since isocitrate was the presumed precursor of glyoxylate and hence glycolate (Fig. 1). Many manipulations were required to isolate, purify, and degrade isocitrate as described under "Materials and Methods." About 100 dpm were recovered in the glyoxylate ultimately degraded, and a long counting time (100 min) was required to determine radioactivity accurately. Nevertheless, there was remarkably good agreement between the fraction of ^{14}C found in C-2 of glyoxylate derived from [^{14}C]isocitrate and the fraction of ^{14}C found in C-2 of glycolate under a number of different conditions. Both molecules were labeled asymmetrically in a similar manner, providing evidence in support of the biochemical pathway (Fig. 1) from pyruvate to glycolate with isocitrate as an intermediate.

Isocitrate Lyase Activity in Tobacco Leaves. Previous workers have reported low activities of the enzyme in leaves (4, 7) and have shown that enzyme inhibitors are present in leaf extracts. Use of a more sensitive system (9) allowed smaller amounts of enzyme solution to be assayed and thereby minimized the effect of endogenous inhibitors. I have confirmed the report (7) that activity is associated with a mitochondrial fraction in leaves (Table IV), but the activity and specific activity shown here is far higher than previously described. I also observed that when an extract of leaves of *Nicotiana sylvestris* was fractionated by percoll gradient centrifugation (16), isocitrate lyase activity closely followed the cytochrome oxidase activity used as a mitochondrial marker. Table IV shows that by freezing a mitochondrial suspension and then thawing and centrifuging again, an amount of activity similar to that remaining in the residue may be extracted. The mitochondrial activity was thus at least 9 μmol/g fresh weight·h, and the specific activity in the mitochondrial supernatant fraction was enriched at least 3-fold compared to the residue. The K_m for natural isocitrate by the mitochondrial supernatant fraction was about 0.14 mM.

Although isocitrate lyase activity is associated with mitochondria, the greatest proportion of activity in dialyzed subcellular fractions obtained by differential centrifugation was found in the supernatant fraction (Table V). It is not known whether the activity in the supernatant represents enzyme extracted from mitochondria or whether more than one form of the enzyme exists in leaves. The total activity (35 μmol/g fresh weight) is sufficiently high to suggest that this enzyme could support the flux from isocitrate to glycolate at a reasonable rate.

DISCUSSION

It has been demonstrated with tobacco leaf discs supplied [3-^{14}C]pyruvate under steady state photosynthesis that pyruvate is metabolized and converted directly into C-2 of glycolate (Fig. 1). Glycolate formed in 21% O_2 had about 50% greater than equal-labeling in C-2 (Tables I–III), while glycolate produced photosynthetically from $^{14}CO_2$ is equally labeled (25). Under steady state photosynthesis in 1% O_2 the glycolate synthesized was labeled still more asymmetrically in C-2 (about 80% greater than equal-labeled), probably because at low O_2 the rate of glycolate synthesis from CO_2 and the Calvin cycle is much slower (though CO_2 fixation increases) and the pathway of glycolate formation from pyruvate is less O_2-dependent.

If on supplying [3-^{14}C]pyruvate the specific radioactivity in C-2 of glycolate were the same in 21% O_2 and 1% O_2, it would suggest that the pyruvate pathway had an O_2-dependency similar to the pathway involving ribulose-1, 5-bisphosphate carboxylase/oxygenase. However, if the specific radioactivity in C-2 of glycolate were greater in 1% O_2, it would indicate that the pyruvate pathway was less O_2-dependent than the mechanism associated with CO_2 fixation. In Tables I and II (as well as in another experiment not presented) 10 mM α-HPMS was added to leaf discs for 10 min to cause glycolate accumulation, and there was little difference in the specific radioactivity of C-2 in 21% and 1% O_2. In Table II, where 20 mM α-HPMS was supplied for 10 min, the specific radioactivity was twice as great in C-2 in 1% O_2 than in 21% O_2. In another similar experiment not presented here when 20 mM α-HPMS was also used, the specific radioactivity in C-2 in 1% O_2 was 69% higher than in 21% O_2. In all of these experiments there was little inhibition of net photosynthesis during the first 5 min of exposure to α-HPMS compared to the steady rate prior to addition of the inhibitor. The results with 20 mM α-HPMS demonstrate that the pyruvate pathway of glycolate formation is probably less O_2-dependent than the oxygenase pathway, a conclusion consistent with the observed increase in unsymmetrical labeling in 1% O_2 compared with 21% O_2 discussed above.

Heating a highly radioactive [3-^{14}C]pyruvate solution under conditions similar to those used to kill the leaf discs at the end of each experiment produced a maximum of 0.1% of the total ^{14}C as glycolate (Table II). This raised a concern about the possible contribution of such a radiochemical artifact to the results obtained. The amount of [^{14}C]pyruvate remaining in the leaf discs (Tables I and II) was approximately one-half of total ^{14}C of the homogenate, hence the radioactivity in glycolate was about 2% of the total ^{14}C in various experiments or 20-fold greater than the 'glycolate' obtained in heating highly radioactive [3-^{14}C]pyruvate. Finding (Table III) that discs killed after 10 min in 10 mM α-HPMS had a 30% higher concentration of glycolate and a 15% higher specific radioactivity in C-2 of glycolate than after 5 min shows there was a time-dependent increase in glycolate synthesis from [3-^{14}C]pyruvate in leaf discs. The consistent increase in 1% O_2 compared to 21% O_2 in the fraction of [^{14}C]glycolate in C-2 in discs given [3-^{14}C]pyruvate (Tables I–III)

Table IV. *Isocitrate Lyase Activity in Mitochondrial Fraction Obtained by Differential Centrifugation of Tabacco Leaf Extracts*

The mitochondrial fraction in suspending medium was frozen overnight. It was thawed and centrifuged again at 38,000*g*. The residue was then taken up in suspending medium.

Experiment No.	Mitochondrial Residue		Mitochondrial Supernatant	
	μmol/g fresh wt·h	*μmol/mg protein·h*	*μmol/g fresh wt·h*	*μmol/mg protein·h*
1	6.07	6.32	7.35	32.8
2	4.61	8.17	4.48	21.3

Table V. *Distribution of Isocitrate Lyase Activity in Fractions of Tobacco Leaves Obtained by Differential Centrifugation*

The fractions were dialyzed as described under "Materials and Methods" prior to assay.

Fraction	Isocitrate Lyase Activity	
	μmol/g fresh wt·h	*μmol/mg protein·h*
Broken chloroplasts and peroxisomes	2.24	0.64
Mitochondria	2.46	3.08
Supernatant	31.2	4.92

also demonstrates the metabolic origin of glycolate synthesis from pyruvate. Results showing that C-2 of glyoxylate derived from C-2 of isocitrate (Fig. 1) is labeled asymmetrically in a manner similar to C-2 of glycolate (Table III) under a number of different conditions with [2-^{14}C]- and [3-^{14}C]pyruvate as substrates provide further evidence that glycolate is synthesized from isocitrate by leaf discs.

Previous studies on isocitrate lyase in leaves reported low activities (4, 7), and perhaps this explains in part the failure by workers in this field to consider pyruvate as an alternative source of glyoxylate and glycolate (25). When [3,4-^{14}C]isocitrate was fed to excised tobacco leaves in the dark, the specific radioactivity in succinate was about one-quarter that of the isocitrate administered (19), a result consistent with the presence of isocitrate lyase activity. The enzyme appears to be associated with mitochondria (Tables V and VI) as previously suggested (7), although the largest amount of activity was found in the supernatant fraction (Table VI) and it may also be located in other subcellular sites. The total activity found, at least 35 μmol/g fresh weight·h (Table V), would be similar to the rate of dark respiration or greater. The data presented here demonstrate the existence of a sequence of reactions (Fig. 1) but provide no information about its rate in leaves in the light. If an appreciable portion of the isocitrate metabolized in the light were diverted to glycolate, this could well confound attempts to assess the role of light in dark respiration as well as attempts to reconcile the characteristics of ribulose-1, 5-bisphosphate carboxylase/oxygenase with respect to the effects of CO_2 and O_2 concentrations *in vitro* and *in vivo*. If the alternative sequence contributes significantly to the glycolate pool during photosynthesis, its regulation would provide another means of regulating photorespiration and net photosynthesis (26).

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