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Production of Isoprene by Leaf Tissue[1]

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ABSTRACT

Isoprene production by *Hamamelis virginiana* L. and *Quercus borealis* Michx. leaves was studied. When ambient CO_2 concentrations were maintained with bicarbonate buffers, the rate of isoprene production at 125 microliters per liter of CO_2 was approximately four times that at 250 microliters per liter of CO_2. Isoprene production was drastically inhibited by 97% O_2. Dichlorodimethylphenylurea (0.1 mM), $NaHSO_3$ (10 mM), and α-hydroxy-2-pyridinemethanesulfonic acid (10 mM) inhibited isoprene production but increased the compensation point of the tissue. Isonicotinic acid hydrazide neither inhibited isoprene emission nor increased the compensation point of the tissue significantly. Inhibition of isoprene production does not seem to correlate with stomatal resistance. Isoprene was labeled by intermediates of the glycolate pathway, and similarities are noted between the biosynthesis of isoprene and that of β-carotene.

The production[3] of significant quantities of free isoprene (2-methyl-1,3-butadiene) by leaf tissue requires conditions similar to those required for high rates of photorespiratory glycolate synthesis (4, 6, 7, 9–16). Both isoprene production and glycolate synthesis are characterized by an absolute light requirement; maximal production of isoprene and glycolate requires high light intensity, high temperature, and subambient CO_2 concentrations. In addition, DCMU inhibits both processes (4, 7) and both processes can use substrates other than recently fixed photosynthate (13, 16, 20).

Shah and Rogers (17) have proposed a biosynthetic pathway which implicates glycolate metabolism in chloroplastic terpenoid synthesis. According to their hypothesis, glycolate serves as the major precursor of the acetyl-CoA utilized in terpenoid synthesis. If the pools involved in glycolate metabolism serve as presursor pools for both photorespiratory CO_2 emission and terpenoid synthesis, the physiological requirements for maximal isoprene production might be explained in terms of the availability of glycolate.

The intent of this study is: (*a*) to examine the role of stomatal regulation in isoprene emission; and (*b*) to examine the implications of Shah and Rogers' (17) hypothesis with regard to the physiology and biochemistry of isoprene production.

MATERIALS AND METHODS

Isoprene Production and Quantitation. *Hamamelis virginiana* plants were grown in sand culture in a growth room under mixed fluorescent and incandescent lighting at 250 to 450 μeinsteins m^{-2} sec^{-1} between 400 and 700 nm. Daylength was 12 hr with a day-night temperature of 27:18 C. An automatic watering system supplied the plants with modified Hoagland's solution three times daily.

Leaf discs were cut from fresh, light-acclimated leaves on either side of the midvein. The anisolateral leaf discs were placed abaxial side up on distilled H_2O or inhibitor solutions. They were enclosed in cylindrical Lucite chambers or Petri plates modified for gas sampling (7). In some experiments tissue was incubated in a Gibson respirometer. The respirometer reaction vessels were fitted with a glass stopper in one sidearm and a No. 7 snap top serum cap over the other sidearm. The vent to the manometric apparatus was sealed with Parafilm. Normally, 0.5-ml air samples were removed through the serum cap. The glass stopper was then cracked to allow pressure equilibration. Twelve discs, 0.41 cm^2, or leaf slices, 300 μm × 1 cm, were floated on 3 ml of buffer containing 5 mM potassium biphthalate and 1 mM $CaSO_4$ (pH 5) within the respirometer reaction vessel. Leaves were sliced with a Spencer sliding microtome, washed in distilled H_2O, and uniformly blotted. Normally, 200-mg portions were incubated in each reaction vessel. Incubation temperature was 30 C, light intensity was 12 mw cm^{-2} (210 μeinsteins m^{-2} sec^{-1} between 400 and 700 nm).

Direct gas chromatographic techniques assayed isoprene content in the air surrounding the enclosed tissues (7). In some studies involving radioactive tracers, isoprene was separated and assayed with a Hewlett-Packard 57 A gas chromatograph equipped with a flame ionization detector. Columns were Hewlett-Packard 50.8 cm, 10% silicone, W98 on 80 to 100 WHP. The carrier gas was helium. Temperature programming from −40 to 150 C was used. The rate was 8 C min^{-1} for the first 5 min, 16 C min^{-1} for the next 5 min and 32 C min^{-1} for the remaining time.

Vertical bars in all figures denote one standard deviation from the mean.

Maintenance of Near Ambient CO_2 Concentrations in Closed Reaction Vessels. In order to determine accurately the effect of CO_2 concentration on isoprene production, it was necessary to maintain CO_2 concentrations near ambient levels within closed respirometer reaction vessels. In order to accomplish this, CO_2 buffer solutions were placed in the sidearms of the reac-

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[3] The term production is used to imply both the biosynthesis of isoprene and its movement from the tissue to the air phase surrounding the tissue.

tion vessels. Total buffer solution volume was 3 ml. Filter paper strips were used to increase surface area for CO_2 exchange. Buffer solution A consisted of 6 N KOH and maintained CO_2 concentrations between 125 and 150 μl/l throughout the experiment (CO_2 concentration data not shown). Buffer solution B consisted of 18 ml of diethanolamine, 9 g of $KHCO_3$, 45 mg of thiourea, 25 ml of H_2O, and 1 ml of 6 N KOH. It maintained CO_2 concentrations between 225 and 275 μl/l. Buffer solution C was identical to B except that 1.25 ml of 6 N HCl was used in place of the KOH. It maintained CO_2 concentrations between 485 and 520 μl/l. Control reaction vessels containing H_2O in the sidearms allowed mean CO_2 concentrations to vary between 275 and 375 μl/l over the course of the experiment. *Hamamelis virginiana* leaf slices were incubated under normal conditions in 3 ml of a solution consisting of 0.04 M MES and 0.1 mM $CaSO_4$, pH 6.

^{14}C Incorporation into Isoprene. In order to test Shah and Rogers' (17) pathway with regard to isoprene synthesis, it was necessary to use ^{14}C-labeled intermediates. These intermediates included L-mevalonic acid-2-^{14}C (5.03 mCi/mmole), which was a gift of P. E. Kolattukudy, glycine-2-^{14}C (5.93 mCi/mmole), which was obtained from New England Nuclear, acetate-1-^{14}C (K salt) (56 mCi/mmole), and glycolate-2-^{14}C (Ca salt) (55.5 mCi/mmole), which was purchased from International Chemical and Nuclear Corporation (ICN).

Respirometer reaction vessels were prepared with 3 ml of biphthalate buffer and the appropriate radioactive substrate in the main well. Two reaction vessels contained 87 μM glycolate-2-^{14}C (2.59×10^7 cpm), two contained 876 μM glycine-2-^{14}C (2.79×10^7 cpm), two contained 1.11 mM mevalonic acid-2-^{14}C (3.00×10^7 cpm), and four contained 105 μM acetate-1-^{14}C (3.13×10^7 cpm).

Fresh *Hamamelis virginiana* leaf slices were placed in all but two reaction vessels. Boiled tissue served as a control in two of the reaction vessels containing acetate-1-^{14}C. The reaction vessels were flushed with CO_2-free air upon attachment. After incubation under normal conditions for 2 hr, the closed reaction vessels were placed on ice in the dark. Analysis was conducted immediately. Isoprene concentration and retention time were determined with both columns of the Hewlett-Packard 57 A gas chromatograph in place. Radioactive isoprene was collected by removing one column from the detector fitting, attaching a short length of 0.318-cm stainless steel tubing to the end of the column, and bubbling the gas through vials containing 10 ml of scintillation fluid. Air (15 ml) was removed from each flask with a syringe, and the air was displaced with 15 ml of H_2O. Air samples from two reaction vessels containing identical substrate were combined and injected into the chromatograph. Fractions were collected at 30-sec intervals. A distinct peak in radioactivity corresponded to the isoprene retention time.

Scintillation fluid consisted of dimethyl POPOP (0.0003), PPO (0.005), and toluene (1) (w/w/v). Over 95% of radioactive isoprene was trapped in the scintillation fluid. A Packard 2009 Tri-Carb scintillation spectrometer was used. In most cases, the standard deviation attributable to counting was less than 3%; in no case was it greater than 6%.

Quantitation of Lipid Fractions. After analysis of isoprene content, leaf strips were removed from the flasks, washed with deionized H_2O, and frozen for later analysis of saponifiable and nonsaponifiable lipids. The frozen leaf strips were ground to a fine powder in liquid N_2 and extracted for 10 hr with chloroform-methanol (2:1, v/v) under N_2. All separations were carried out under N_2 and dim laboratory lighting. The chloroform-methanol extract was filtered through glass wool into a separatory funnel to remove cellular debris, and the filtrate was washed with 10 ml of chloroform-methanol and 30 ml of H_2O. The separatory funnel was gently shaken, acidified, and the phases were allowed to separate. The chloroform phase was removed and dried over anhydrous Na_2SO_4 overnight. The aqueous phase was set aside for counting with the total washes.

The Na_2SO_4 was filtered off and washed twice with 5 ml chloroform. The chloroform was evaporated to dryness under N_2 and the residue was taken up in 20 ml of absolute ethanol. Aqueous KOH (2 ml, 60% w/v) was added with shaking; the mixture was covered with N_2 and left for 16 hr in the dark at room temperature for saponification to occur. The solution was diluted with 20 ml of H_2O, acidified, and extracted three times with 10 ml of chloroform. The ethanol and chloroform phases were separated, the chloroform phase was washed twice with 10 ml of H_2O, and the washes were added to the ethanol phase and saved for counting as total washes. The chloroform phase was washed three times with 10 ml of 1% (w/v) $NaHCO_3$. Fatty acids were in the bicarbonate phase and nonsaponifiable fats were in the chloroform phase. The bicarbonate phase was acidified and extracted three times with 10 ml of chloroform. Fatty acids were in the chloroform phase. Aliquots of all fractions were counted and internal standards were used to correct for quenching.

The remainder of the nonsaponifiable fraction was assayed for counts in sterols by a slight modification of Grunwald's (3) technique. The nonsaponifiable fraction was evaporated with N_2, and the residue was dissolved in 10 ml of hot 2% digitonin in 80% (w/v) ethanol and 5 ml of hot water. The samples were allowed to cool and remain at room temperature overnight. The precipitate was washed three times with 80% ethanol and once with diethyl ether. The white sterol-digitonin precipitate was dried under N_2, broken with 1 ml of pyridine, and the digitonin was removed by precipitation with 20 ml of diethyl ether. The ether-pyridine layer was taken to dryness under N_2, weighed, and counted in toluene scintillation fluid.

Bray's solution (1) was used to assay the radioactivity in the extracted tissue fractions. Counting efficiency in Bray's solution was 0.86 times the counting efficiency in toluene-based scintillation fluid. Radioactivity in all cases was expressed as radioactivity in Bray's solution. Internal standards were used to correct for quenching.

Effects of Postulated Intermediates on Specific Activity of Isoprene Derived from Glycine-2-^{14}C. Either 150-mg *Hamamelis virginiana* leaf strips or 12 *H. virginiana leaf* discs were incubated in respirometer reaction vessels on biphthalate buffer containing glycine-2-^{14}C (10 μCi at 0.24 mM glycine for 2 hr with the strips, and 20 μCi at 0.48 mM glycine for 3 hr with the discs). Ascarite was used to remove CO_2. After incubation the flasks were placed on ice in the dark. A Carle Model 9000 gas chromatograph (7) was used for determination of isoprene concentration and for fraction collection. Fractions were collected by removing the detector, extinguishing the flame, and placing plastic tubing over the flame jet. The 4-ml air samples were taken from each reaction vessel. The fraction corresponding to the isoprene peak was collected and counted as described previously.

RESULTS

Isoprene Degradation. Very little if any isoprene escapes from or is degraded in Gilson respirometer flasks in the dark. Immediately after illumination the isoprene content was measured in flasks containing leaf discs. After 4 hr in the dark at 30 C, isoprene content was again measured. Both at the beginning and end of the dark period isoprene content was

Table I. *Effect of Leaf Damage on Isoprene Emission*

Large and small leaf discs from *Quercus borealis* leaves were floated with the abaxial side up in Gilson respirometer flasks on 3 ml of buffer consisting of 5 mM potassium biphthalate and 1 mM $CaSO_4$ (pH 5) for 3 hr. Eight large discs (one per flask) and 16 small discs (two per flask) were assayed.

Disc Diameter	Isoprene Emission					
	Based on circumference			Based on leaf disc area		
	Circumference	Mean	SD	Area	Mean	SD
mm	*mm*	*μl/l·mm*		*mm²*	*μl/l·mm²*	
15.0	47.1	0.482	0.036	177	0.128	0.010
10.4	65.4	0.344	0.046	170	0.132	0.018

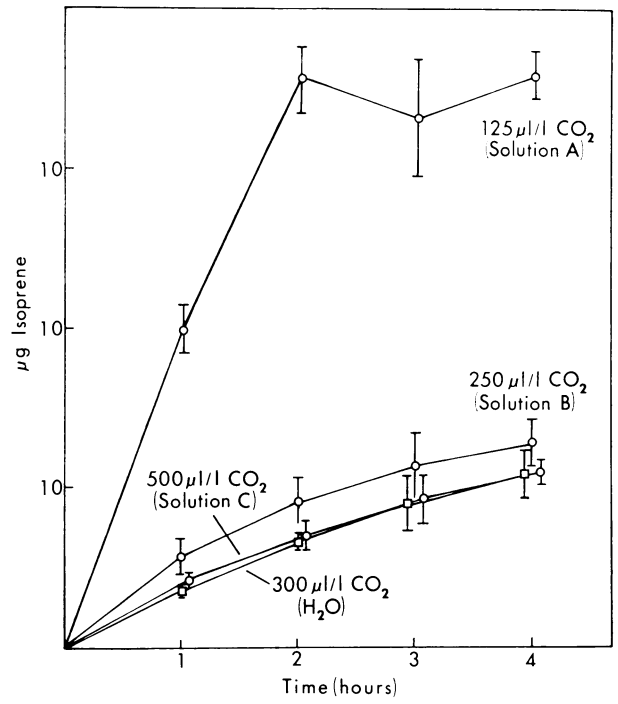

FIG. 1. Response of isoprene production to CO_2 concentration.

24.3 μl/l with standard deviations of 1.9 and 2.6 μl/l, respectively.

Effect of Leaf Damage on Isoprene Production. If isoprene production is primarily attributable to tissue damage, isoprene production by whole uninfiltrated leaves should be greater in damaged leaves than in undamaged leaves. Six *Hamamelis virginiana* leaves were carefully removed from the plant insuring that no part of the lamina was cut or crushed. Three leaves were uniformly damaged by crushing small portions of the leaf with the end of a rat tail file. This produced 20 small crushed areas distributed randomly over the surface of the leaf. The other three leaves were not damaged. The six leaves were incubated 1 hr in 140-ml Gilson reaction vessels. Isoprene was produced by the undamaged leaves at rates of 49.6, 43.9, and 56.3 μg/g fresh weight·hr. Isoprene was produced by damaged leaves at rates of 34.7, 35.1, and 41.1 μg/g fresh weight·hr. The results indicate that isoprene production is not the result of damage to the lamina.

If isoprene is primarily produced by damaged tissue, isoprene production by uninfiltrated leaf discs should be proportional to the circumference of the discs. If isoprene is not produced by damaged tissue, isoprene production should be proportional to disc area. Table I shows that isoprene production is proportional to disc area but not to cut surface area in uninfiltrated *Querus borealis* leaf discs. The results indicate that isoprene production is not the result of damage to the lamina.

Effect of CO_2 Concentration on Isoprene Production. Figure 1 summarizes the effect of CO_2 concentration on isoprene production. Note that sidearm solution A (6 N KOH) maintains CO_2 concentrations below 150 μl/l; this low CO_2 concentration stimulates isoprene production from the leaf strips. Sidearm solutions C, D, and H_2O maintain CO_2 concentrations above approximately 200 μl/l. These higher concentrations are apparently inhibitory to isoprene production. It is unlikely that volatiles from solutions C or D inhibit isoprene production, inasmuch as the control with H_2O in the sidearm showed essentially the same rate of isoprene production.

Leaf strips rather than leaf discs were used to reduce the probability that the inhibitory effect of high CO_2 concentrations on isoprene production is attributable to stomatal closure induced by high CO_2. The results suggest that, like glycolate synthesis, isoprene production is inversely related to CO_2 concentration at near-ambient CO_2 levels.

Effect of CO_2 Concentration on DCMU Inhibition of Isoprene Production. Rasmussen and Jones (7) showed that DCMU is a potent inhibitor of isoprene production. However DCMU caused a significant efflux of CO_2 from the tissue. It was unclear whether the inhibition of isoprene production caused by DCMU was the result of increased CO_2 concentrations within the tissues or direct inhibition of isoprene production by DCMU.

Both in the presence and absence of DCMU, 6 N KOH maintained the concentration of CO_2 below 100 μl/l. In the absence of KOH, the concentration of CO_2 rose to 3600 μl/l at 5 hr (data not shown). Maintenance of CO_2 concentrations below 100 μl/l did not significantly modify the inhibition of isoprene production by DCMU (Fig. 2). This indicates that, unless an extreme CO_2 concentration gradient exists across the stomata, the inhibitory effect of DCMU is attributable to its inhibition of photosynthetic electron transport.

Effect of α-HPMS, $NaHSO_3$, and INH on Isoprene Production and CO_2 Metabolism. Shah and Rogers (17) largely based their hypothesis that the glycolate pathway is a primary source of acetyl-CoA in β-carotene synthesis on the almost complete inhibition of $^{14}CO_2$ incorporation into β-carotene by α-HPMS[4] and INH.

Figure 3 shows the effects of a α-HPMS, INH, and $NaHSO_3$ on isoprene synthesis. Figure 4 shows the effects of these inhibitors on the compensation point of the tissue. The inhibitors α-HPMS and $NaHSO_3$, inhibitors of glycolate oxidase (19), both inhibit isoprene production; however, since they cause the CO_2 concentration in the reaction vessels to increase dramatically, they also seem to affect CO_2 metabolism. It is unclear whether they affect isoprene synthesis primarily by inhibiting glycolate oxidase.

INH has little effect on either isoprene production or CO_2 metabolism. It may cause a slight increase in the compensation point of the tissue; however, isoprene production is essentially unaffected.

Effect of $NaHSO_3$ on Isoprene Production in Dark. Leaf discs infiltrated with buffer or $NaHSO_3$ solutions were allowed

[4] Abbreviations: α-HPMS: α-hydroxy-2-pyridinemethanesulfonic acid; INH: isonicotinic acid hydrazide.

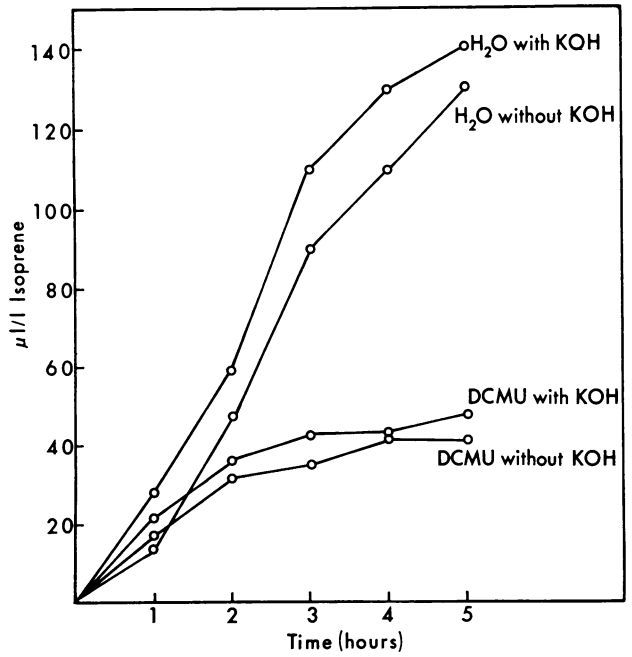

FIG. 2. Effect of DCMU on isoprene production under high and low CO_2. Six leaf discs of *H. virginiana* were vacuum infiltrated with either H_2O or 0.1 mM DCMU. Discs were incubated on a few ml of infiltrating solution in Petri plates modified for gas sampling. Shell vials containing 3 ml of 6 N KOH or H_2O were enclosed in the Petri plates.

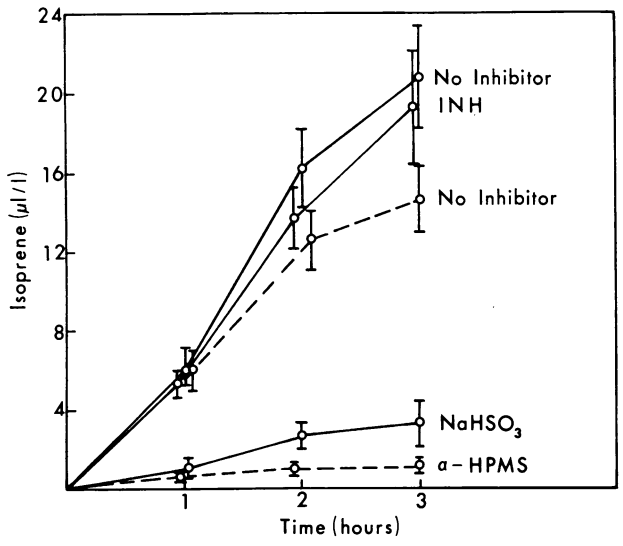

FIG. 3. Effects of α-HPMS, INH, and $NaHSO_3$ on isoprene production. Twelve *H. virginiana* leaf discs of 0.41 cm^2 were incubated in 5 mM potassium biphthalate and 1 mM $CaSO_4$ buffer solution with or without 10 mM inhibitor (pH 5) in Gilson reaction vessels containing INH, $NaHSO_3$, or buffer, and were assayed for isoprene. In another experiment seven flasks containing α-HPMS or buffer were assayed for isoprene under identical conditions.

to synthesize isoprene in the light for 1.5 hr in respirometer reaction vessels. They were then placed in the dark and the isoprene content of the chambers was monitored for another 1.5 hr. There was no change in isoprene content in the chambers during the dark treatment (Fig. 5).

^{14}C Incorporation into Isoprene. Shah and Rogers (17) hypothesized, partially on the basis of radioisotope incorporation

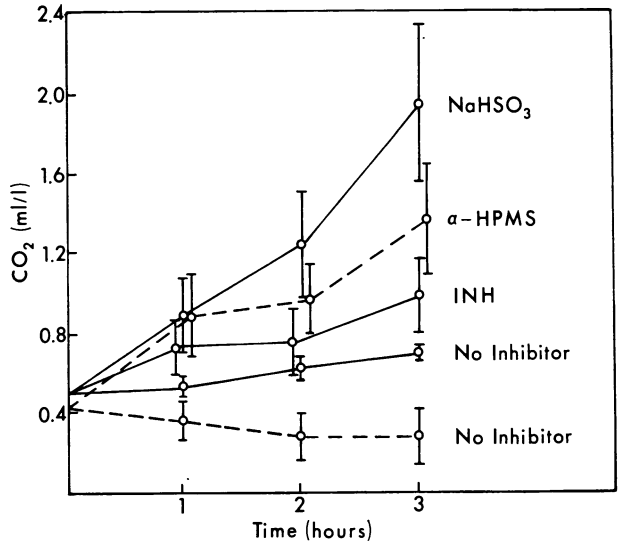

FIG. 4. Effect of α-HPMS, INH, and $NaHSO_3$ on CO_2 evolution. Twelve *H. virginiana* leaf discs of 0.41 cm^2 were incubated in 5 mM potassium biphthalate and 1 mM $CaSO_4$ buffer solution with or without 10 mM inhibitor (pH 5) in Gilson reaction vessels containing INH, $NaHSO_3$ or buffer solution, and were assayed for CO_2. In another experiment seven flasks containing α-HPMS or buffer were assayed for CO_2 under identical conditions.

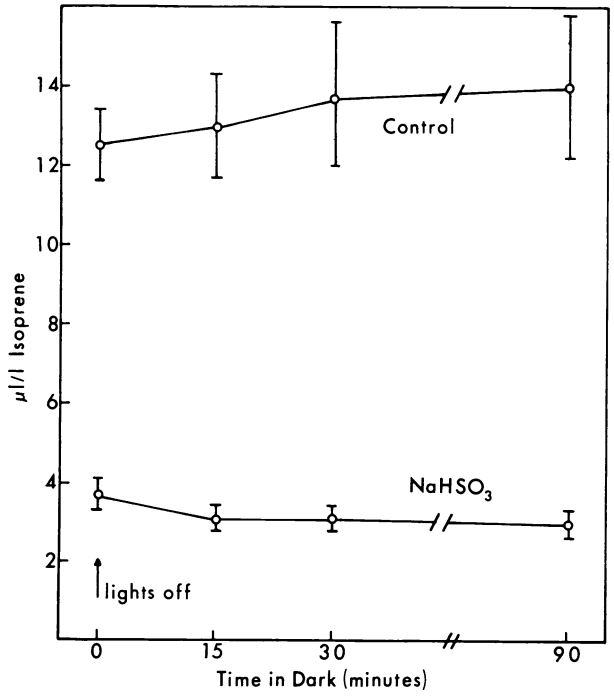

FIG. 5. Effect of $NaHSO_3$ on isoprene production in the dark. Discs were vacuum-infiltrated with a solution containing 5 mM potassium biphthalate and 1 mM $CaSO_4$. In addition, the indicated solution contained 10 mM $NaHSO_3$. After 1.5 hr of illumination the flasks were sampled and immediately covered with aluminum foil to prevent further illumination. Subsequent dark treatment was at 30 C with shaking. Ten respirometer flasks, each containing 12 discs of 0.41 cm^2, were assayed for each solution.

patterns, that in greening maize seedlings β-carotene is synthesized chloroplastically via the pathway glycolate → glyoxylate → glycine → serine → pyruvate → acetyl-CoA → β-carotene. We used radioactive intermediates of the pathway

Table II. *^{14}C Incorporation into Isoprene and Sterols*

Substrate	Acetate-1-^{14}C	Mevalonate-1-^{14}C	Glycine-2-^{14}C	Glycolate-2-^{14}C
CPM in isoprene	1090	190	7340	3390
CPM in sterols	3810	3370	474	454
CPM in isoprene/cpm in sterols	0.29	0.056	15	7.5
Specific radioactivity of isoprene/specific radioactivity of sterols	53	71	5200	3100

Table III. *Effects of Postulated Intermediates on Specific Radioactivity of Isoprene Derived from Glycine-2-^{14}C*

Unlabeled Substrate 10 mM	Tissue	Isoprene Concentration	Radioactivity in Isoprene	Specific Radioactivity
		nmoles	*nCi*	*nCi nmole*
None	Strips	8.4	0.089	0.011
None	Discs	12.0	0.14	0.012
None	Discs	12.0	0.10	0.0083
Glycolate	Strips	11.0	0.067	0.0061
Glycolate	Discs	13.0	0.12	0.0092
Glycolate	Discs	16.0	0.094	0.0059
Glyoxylate	Strips	13.0	0.13	0.010
Glyoxylate	Discs	13.0	0.15	0.011
L-Serine	Strips	4.0	0.0094	0.0023
L-Serine	Discs	10.0	0.032	0.0032
L-Serine	Discs	10.0	0.036	0.0036
Pyruvate	Strips	9.1	0.056	0.0061
Pyruvate	Discs	16.0	0.078	0.0049
Pyruvate	Discs	12.0	0.078	0.0065

proposed by Shah and Rogers (17) to determine whether their incorporation into isoprene in *Hamamelis virginiana* is similar to their incorporation into β-carotene in greening maize seedlings. When glycine-2-^{14}C or glycolate-2-^{14}C is used as the substrate, more radioactivity is incorporated into isoprene than into sterols (Table II). Conversely, when acetate-1-^{14}C or mevalonate-2-^{14}C is used as substrate, more radioactivity is incorporated into sterols than into isoprene. The ratio of the final specific radioactivity of isoprene to the final specific radioactivity of the sterol fraction is also calculated for each substrate (Table II). The same trend is evident. Relative to their contribution of radioactivity to the sterol fraction, glycolate-2-^{14}C and glycine-2-^{14}C contribute more radioactivity to isoprene than do acetate-1-^{14}C and mevalonate-2-^{14}C.

Isotopic dilution of isoprene was tested by measuring the change in the specific radioactivity in isoprene labeled with glycine-2-^{14}C in the presence of excess glycolate, glyoxylate, L-serine, and pyruvate (Table III). L-Serine caused a 4-fold decrease in the specific radioactivity of isoprene. Glycolate and pyruvate caused somewhat less dilution of the radioactivity in isoprene. Glyoxylate had no effect.

DISCUSSION

Stomatal Regulation of Isoprene Production. In this study, the quantity of isoprene measured has been the concentration of isoprene in the air phase surrounding the leaf discs or leaf strips. Many of the physical parameters and inhibitors that affect isoprene synthesis could also affect the resistance of the tissue to gas exchange. However, the hypothesis that high CO_2 concentrations, DCMU, $NaHSO_3$, and α-HPMS decrease the rate of isoprene production by causing stomatal closure is inconsistent with a number of experimental results. First, if CO_2 is not absorbed with strong base, infiltration of leaf discs with DCMU causes CO_2 concentrations in the air phase to rise to 3000 μl/l in 5 hr (data not shown) while reducing isoprene production by more than 60%. Likewise, infiltration of leaf discs with $NaHSO_3$ or α-HPMS causes dramatic inhibition of isoprene production while CO_2 concentrations in the air phase rise dramatically (Figs. 3 and 4). The argument that in these cases CO_2 evolution is the result of extremely high CO_2 concentration gradients across a high stomatal resistance is inconsistent with the complete inhibition of isoprene evolution when tissue infiltrated with $NaHSO_3$ is placed in the dark after incubation in the light (Fig. 5). If isoprene inhibition by $NaHSO_3$ were attributable to high stomatal resistance, or high resistance of any other type, the isoprene content in the air phase should continue to rise after the tissue is placed in the dark.

Another experimental result contradicting the hypothesis of stomatal regulation of isoprene production is the response of leaf strips (300 μm wide) to varying CO_2 concentrations (Fig. 1). The large ratio of cut surface area to stomatal area should render stomatal aperture insignificant with respect to gas exchange regulation in these leaf strips. However, CO_2 concentration had a material effect on isoprene production rates. Direct observation of cuticular impressions revealed that a significant number of stomates remained open even after inhibition of isoprene synthesis with α-HPMS. There was more variation in the percentage of open stomates between leaf discs than between treatments (data not shown). These results strongly suggest that regulation of isoprene synthesis, rather than regulation of stomatal resistance, is the primary mechanism controlling the rate of isoprene production.

Relation of Isoprene Production to Glycolate and Chloroplastic Terpenoid Synthesis. Shah and Rogers (17) based their hypothesis, that the pathway of glycolate metabolism is a primary source of acetyl-CoA in β-carotene synthesis, on two major considerations. First, INH and α-HPMS almost completely inhibit the incorporation of $^{14}CO_2$ into β-carotene. Second, ^{14}C intermediates of the pathway of glycolate metabolism contribute ^{14}C to the isoprene pool. The dramatic inhibition of isoprene production by α-HPMS and $NaHSO_3$ (Fig. 3) suggests that the pathway of glycolate metabolism is involved in isoprene synthesis. INH does not inhibit isoprene production; however, the lack of inhibition could be attributable to permeability barriers since INH does not change the compensation point of the tissue significantly (Fig. 4).

If the same pathway is involved in the synthesis of both isoprene and β-carotene, similar radioisotope incorporation patterns should be observed. The intermediates acetate-1-^{14}C, glycine-2-^{14}C, glycolate-2-^{14}C, and mevalonate-2-^{14}C were used, and the ratios of ^{14}C in isoprene to ^{14}C in sterols were calculated. Table II shows that, relative to their contributions of ^{14}C to the sterol fraction, glycolate-2-^{14}C and glycine-2-^{14}C contribute more ^{14}C to isoprene than do acetate-1-^{14}C and mevalonate-2-^{14}C. The relationship holds whether ratios of counts incorporated or ratios of specific activities of the final products are used. Shah and Rogers (17) found the same pattern of incorporation with regard to β-carotene and sterol synthesis in etiolated maize seedlings. We consider this to be evidence that similar pathways are involved in isoprene and β-carotene synthesis and that the pathway is able to utilize ^{14}C from intermediates of the pathway of glycolate metabolism. According to the pathway proposed by Shah and Rogers (17), little radioisotope dilution would be expected when tissue labeled with glycine-2-^{14}C is exposed to unlabeled glycolate or glyoxylate.

However, dilution of ^{14}C in isoprene would be expected when the unlabeled compound is serine or pyruvate. Serine caused a 4-fold reduction in ^{14}C in isoprene but pyruvate had no effect. Permeability barriers encountered by unlabeled pyruvate and serine could account for these results.

In isolated chloroplasts at rate-limiting CO_2 levels, increasing O_2 concentrations caused the percentage of $^{14}CO_2$ incorporated into glycolate to increase (2, 5). However, these high O_2 concentrations cause total $^{14}CO_2$ fixation and accumulation of ^{14}C-glycolate to decrease dramatically (2). Isoprene production is inhibited by 80% when leaf discs are incubated under 97% O_2 for 20 min (data not shown). This is consistent with the hypothesis that high levels of O_2 inhibit isoprene production by reducing total glycolate accumulation.

Rate-limiting CO_2 concentrations stimulate the rates of production of glycolate and isoprene (refs. 2, 21; Fig. 1). At rate-limiting CO_2 concentrations, isoprene is initially produced at high rates, but after 2 hr the rate decreases dramatically (Fig. 1). This is consistent with the hypothesis that both low CO_2 concentrations and some endogenous substrate are necessary for high rates of isoprene production. After several hours under low levels of CO_2, the endogenous reserves are depleted. The depletion effect would not be expected and was not observed under higher CO_2 concentrations (Fig. 1).

There are notable similarities between isoprene production and photorespiratory glycolate metabolism. Both processes are stimulated by high light intensity and high temperature; DCMU and α-hydroxysulfonates inhibit both processes (refs. 4, 7, 10–16, 18; Fig. 3). High CO_2 concentrations and high O_2 concentrations cause rapid inhibition of isoprene production in leaf discs (Fig. 1). They also decrease total $^{14}CO_2$ incorporation into glycolate in isolated chloroplasts (2). Glycolate production and isoprene production are enhanced by rate-limiting CO_2 concentrations (ref. 8; Fig. 1).

Similarities also exist between β-carotene biosynthesis in etiolated maize seedlings and isoprene biosynthesis. Both processes are inhibited by α-HPMS, and both isoprene and β-carotene are labeled by intermediates of the pathway of glycolate metabolism (refs. 7, 17; Table II).

Isoprene production is not inhibited by INH (Fig. 3), and serine and pyruvate do not cause the expected dilution of ^{14}C in isoprene from glycine-2-^{14}C. Otherwise our results are consistent with the hypothesis that the pathway involved in glycolate metabolism can contribute carbon to the pathway involved in isoprene and β-carotene synthesis.

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