

# Oxygen-18 and Deuterium Labeling Studies of Choline Oxidation by Spinach and Sugar Beet<sup>1</sup>

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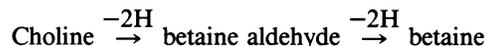
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## ABSTRACT

Chenopods synthesize betaine by a two-step oxidation of choline: choline → betaine aldehyde → betaine. The pathway is chloroplastic; the first step has been shown in isolated spinach (*Spinacia oleracea* L.) chloroplasts to be O<sub>2</sub>- and light-dependent, the role of light being to provide reducing power (P Weigel, EA Weretilnyk, AD Hanson 1988 Plant Physiol 86: 54-60). Here, we report use of *in vivo* <sup>18</sup>O- and <sup>2</sup>H-labeling in conjunction with fast atom bombardment mass spectrometry to test for two hypothetical choline-oxidizing reactions that would explain the observed requirements for O<sub>2</sub> and reductant: a desaturase or an oxygenase. Simple syntheses for <sup>2</sup>H<sub>3</sub>-choline, <sup>2</sup>H<sub>3</sub>,<sup>18</sup>O-choline, and <sup>2</sup>H<sub>3</sub>,<sup>18</sup>O-betaine are given. A desaturase mechanism was sought by giving choline deuterated at the 2-carbon, or choline unlabeled at this position together with <sup>2</sup>H<sub>2</sub>O and by analyzing newly synthesized betaine. About 15% of the <sup>2</sup>H at C-2 was lost during oxidation of choline to betaine, and about 10% of the betaine made in the presence of 50% <sup>2</sup>H<sub>2</sub>O was monodeuterated. These small effects are more consistent with chemical exchange than with a desaturase, because 10 to 15% losses of <sup>2</sup>H from the C-2 position also occurred if choline was converted to betaine by a purified bacterial choline oxidase. To test for an oxygenase, the incorporation of <sup>18</sup>O from <sup>18</sup>O<sub>2</sub> into newly synthesized betaine was compared with that from <sup>18</sup>O-labeled choline, in light and darkness. Incorporation of <sup>18</sup>O from <sup>18</sup>O-choline was readily detectable and varied from about 15 to 50% of the theoretical maximum value; the <sup>18</sup>O losses were attributable to exchange of the intermediate betaine aldehyde with water. In darkness, incorporation of <sup>18</sup>O from <sup>18</sup>O<sub>2</sub> approached that from <sup>18</sup>O-choline, but in the light was severalfold lower, presumably due to isotopic dilution by photosynthetic <sup>16</sup>O<sub>2</sub>. These data indicate that the chloroplast choline-oxidizing enzyme is an oxygenase.

betaine) in saline or dry environments (11, 12, 27). Much evidence (6, 26) now favors Wyn Jones' hypothesis (28) that in higher plants betaine is a major cytoplasmic osmolyte, and betaine levels as high as 300 mM have recently been reported for cytoplasm (13) and chloroplasts (19) of salinized chenopods.

In stressed or unstressed plants, and in other organisms, betaine is synthesized by a two-step oxidation of choline; both steps occur in the chloroplast in spinach and other chenopods (7):



In spinach chloroplasts, the second step is catalyzed by a specific betaine aldehyde dehydrogenase (1, 24). Although the enzyme for the first step has not yet been identified, experiments with chloroplasts have defined some of its characteristics. Thus, in isolated chloroplasts, the choline → betaine aldehyde reaction requires O<sub>2</sub> and light, with light most probably acting to supply reducing power (23). Because the light requirement found for choline oxidation in isolated chloroplasts distinguishes this choline-oxidizing reaction from the choline dehydrogenases (11, 16, 22) and oxidases (10, 29) of other organisms, it was of interest to establish whether choline oxidation in intact leaf tissue also required light. As this proved to be the case, we used stable isotope labeling experiments with leaf discs to probe the nature of the choline-oxidizing reaction *in vivo*. Specifically, as outlined below, we tested for two hypothetical choline-oxidizing reactions, an oxygenase or a desaturase, that would explain requirements for O<sub>2</sub> and photosynthetically generated reductant.

**Oxygenase.** Figure 1 is a scheme for choline oxidation catalyzed by an oxygenase; a monooxygenase reaction is shown, but the considerations below apply equally to a dioxygenase. An oxygenase reaction would incorporate an atom of <sup>18</sup>O from <sup>18</sup>O<sub>2</sub> into one of the hydroxyl groups of the hydrate form of betaine aldehyde, the form likely to predominate in aqueous solution (2). There are clear precedents for such alcohol → aldehyde oxidations, for example, in kaurenol metabolism (25). Also, the  $\gamma$ -butyrobetaine hydroxylases of rat liver and *Pseudomonas* are precedents for a hydroxylation at a carbon  $\beta$ - to a trimethylammonium group (8).

Several variables influenced the design and interpretation of our <sup>18</sup>O-incorporation experiments. (a) Betaine aldehyde, but not choline or betaine, is expected to undergo appreciable <sup>18</sup>O-exchange with water under the mild conditions obtaining *in vivo* (20). This would cause loss of <sup>18</sup>O introduced into betaine aldehyde; the loss is predicted to increase as the rate of turnover of the betaine aldehyde pool decreases. (b) Dehydrogenases typically act on the free aldehyde, not the hydrate (4, 17, 21); if this holds for BADH, no more than 50% of the <sup>18</sup>O incorporated into the hydrate could enter betaine, even in the absence of <sup>18</sup>O exchange with water. (c) For betaine quantification by FABMS<sup>3</sup> (18),

<sup>3</sup> Abbreviation: FABMS, fast atom bombardment mass spectrometry.

Members of the Chenopodiaceae and Gramineae, as well as certain cyanobacteria and bacteria, accumulate betaine (glycine

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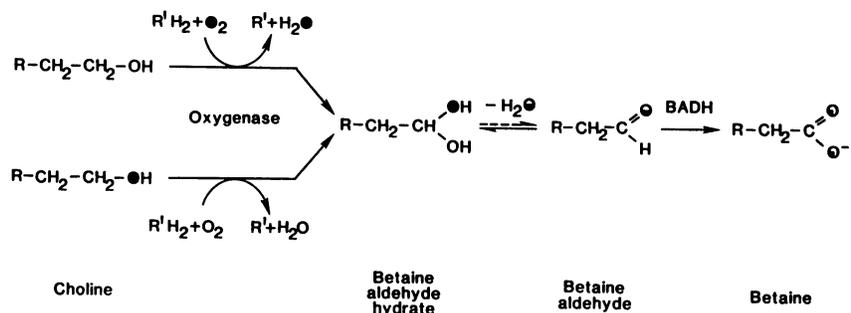


FIG. 1. Hypothetical scheme for oxidation of choline to betaine aldehyde by an oxygenase. The predicted patterns of  $^{18}O$ -incorporation are depicted by shading. R,  $(CH_3)_3N^+$ ;  $R'H_2$ , an electron donor such as NADPH; BADH, betaine aldehyde dehydrogenase.

preparation of the ester derivatives eliminates one of the carboxyl oxygens of betaine (15). Thus, as at neutral pH the carboxyl group of betaine is fully ionized and its two oxygens are chemically equivalent, no more than one-half of the  $^{18}O$  present in the betaine *in vivo* can be retained in the ester derivative. (d) Betaine could undergo some  $^{18}O$  exchange under the relatively harsh conditions required for sample purification and esterification (20). Note that it follows from (b) and (c) that the maximum  $^{18}O$  abundance in the betaine ester analyzed by FABMS would be 25%, even in the absence of exchange processes.

**Desaturase.** The hypothetical oxidation of choline by a desaturase is shown in Figure 2. In the upper reaction, the hydrogens of carbon atoms 1 and 2 of choline are replaced by deuterium; one deuterium atom from each carbon is lost in the formation of the enol, which spontaneously tautomerizes (30) to form the aldehyde, with the hydrogen at the 2-position coming from water. Assuming this water to be the bulk solvent, only one of the deuteriums at the 2-carbon of choline would be retained in betaine aldehyde and betaine. Thus, a desaturase would in the simplest case yield monodeuterated betaine from  $^2H_4$ -choline. Conversely, as shown in the lower reaction of Figure 2, if unlabeled choline is oxidized in the presence of  $^2H_2O$ , a desaturase would introduce one deuterium atom at the 2-position. Although a choline desaturase apparently lacks a direct precedent, an indirect one is chloroplastic stearyl-ACP desaturase, which requires  $O_2$  and photosynthetically-generated reductant (14).

An important variable for our deuterium labeling experiments was that exchange could occur *in vivo* between the solvent and deuterium or hydrogen atoms on carbon-2 of betaine aldehyde. However, such exchange is expected to be slow at physiological pH (2).

## MATERIALS AND METHODS

**$^2H$ - and  $^2H, ^{18}O$ -Labeled Choline.** Choline chloride (trimethyl- $^2H_3$ ) (98 atom %) was obtained from Cambridge Isotope Laboratories (Cambridge, MA), and choline-1,1,2,2- $^2H_4$  chloride (98 atom %) from MSD Isotopes (Montreal, Canada).  $^2H_3$ -Choline chloride and  $^2H_3, ^{18}O$ -choline chloride were prepared from be-

taine aldehyde (methyl- $^2H_3$ ) chloride (synthesized as described in Rhodes *et al.* [18]). Aliquots (0.2 ml) of 1.6 M  $^2H_3$ -betaine aldehyde in 0.2 N HCl were dried in a stream of air and redissolved in 1 ml of  $H_2^{16}O$  or  $H_2^{18}O$  (97 atom %, MSD Isotopes, Merck & Co., St. Louis, MO), to which was added 20  $\mu L$  of 2.5 N HCl. After stirring at room temperature for 2 h, reaction mixtures were neutralized with 6 N NaOH, and 40 mg  $NaBH_4$  was added to reduce betaine aldehyde to choline. After stirring for a further 15 min, 2.5 N HCl was added dropwise until effervescence ceased, and the reaction mixtures were taken to dryness in a rotary evaporator at 30°C. The dried reaction mixtures were extracted with 2  $\times$  5 ml acetonitrile:methanol (10:1, v/v); the extract was filtered through Whatman No. 1 paper and rotary evaporated to dryness. The dried sample was then reextracted with 2  $\times$  5 ml acetonitrile:methanol (20:1, v/v), and extract filtered and dried as above. The latter steps removed the major inorganic ion contaminants.

Authenticity and isotope abundance of the  $^2H_3$ -choline and  $^2H_3, ^{18}O$ -choline preparations were determined in several ways. First, only one quaternary ammonium compound, comigrating with authentic choline chloride, was detected by TLC on silica gel G developed with *n*-butanol/acetic acid/water (6/2/2, v/v) and sprayed with the Dragendorff reagent. Second, direct insertion probe chemical ionization MS (using a Finnigan Series 4000 quadrupole GC/MS system, probe temperature 350°C, isobutane reagent gas, mass range 65–250 amu) showed major choline fragment ions of mass 90 ( $M+H-CH_3$ ) for unlabeled choline standards, mass 90 ( $M+H-C^2H_3$ ) and 93 ( $M+H-CH_3$ ) for synthetic  $^2H_3$ -choline, and mass 92 ( $M+H-C^2H_3$ ) and 95 ( $M+H-CH_3$ ) for synthetic  $^2H_3, ^{18}O$ -choline, with approximately 90 atom %  $^{18}O$  abundance for the latter product, as determined from the ion ratios  $m/z$  90:92 and 93:95. Third, FABMS (18) of underivatized  $^2H_3$ -choline gave an intact molecular cation of mass 107 indicative of 98 atom %  $^2H_3$  abundance. For underivatized  $^2H_3, ^{18}O$ -choline, intact molecular cations of mass 109 and 107 were observed in the ratio 9.75:1, with no detectable ions at mass 104. This indicates a  $^2H_3$  abundance of >98% and an  $^{18}O$  abundance of 90.9%. Fourth, FABMS of O-heptafluorobutyryl choline derivatives (prepared by reacting dried samples with 100  $\mu L$  heptafluorobutyric anhydride at 120°C for 10 min) confirmed

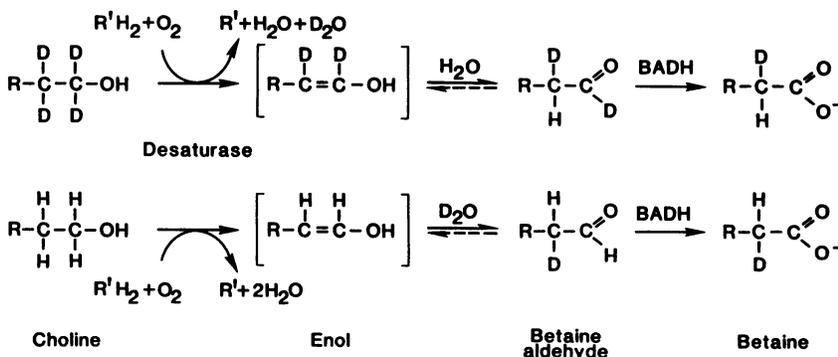


FIG. 2. Hypothetical scheme for oxidation of choline to betaine aldehyde via a desaturation step, followed by spontaneous tautomerization. The upper reaction shows the deuterium (D) labeling patterns expected when 1,1,2,2- $^2H_4$ -choline is supplied; the lower reaction shows those expected when the reaction is carried out in  $^2H_2O$ . Symbols as in Figure 1.

98 atom %  $^2\text{H}_3$  abundance in the  $^2\text{H}_3$ -choline preparation: 98% of the molecular cation choline heptafluorobutyrate signal was associated with an ion of mass 303. Similarly, the O-heptafluorobutyryl derivative of the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline preparation gave ions of mass 305 and 303 indicative of 98%  $^2\text{H}_3$  abundance and 90%  $^{18}\text{O}$  abundance; the  $^{18}\text{O}$  abundance did not decline during 12 months storage of the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline in aqueous solution at  $-20^\circ\text{C}$ . FABMS of heptafluorobutyryl derivatives was used to determine  $^2\text{H}_3$ -choline and  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline levels in the solutions supplied to leaf discs; aliquots of these solutions were spiked with known amounts of unlabeled choline as internal standard. Fifth, FABMS of *n*-butanol:acetyl chloride (5:1 v/v,  $120^\circ\text{C}$ , 20 min) derivatized preparations of  $^2\text{H}_3$ -choline and  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline confirmed that they were not contaminated with either unlabeled or  $^2\text{H}_3$ -labeled betaine or betaine aldehyde. The main impurity in the  $^2\text{H}_3$ -choline and  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline preparations was NaCl, residual from acidification of  $\text{NaBH}_4$ . The final NaCl levels in the 0.4 to 0.5 mM choline solutions supplied to leaf discs were, however,  $<0.1$  mM and these low impurity levels were shown not to influence metabolism of labeled choline by comparing rates of oxidation of synthetic  $^2\text{H}_3$ -choline and pure  $^2\text{H}_9$ -choline.

**$^2\text{H}_3$ ,  $^{18}\text{O}$ -Betaine.**  $^2\text{H}_3$ ,  $^{18}\text{O}$ -Betaine was prepared by enzymic oxidation of betaine aldehyde (methyl- $^2\text{H}_3$ ) chloride labeled with  $^{18}\text{O}$  by exchange with  $\text{H}_2^{18}\text{O}$ . Dry  $^2\text{H}_3$ -betaine aldehyde chloride (0.32 mmol) was incubated with 1 mL of  $\text{H}_2^{18}\text{O}$  (97 atom %) plus 20  $\mu\text{L}$  of 2.5 N HCl for 1.3 h at room temperature, followed by addition of 5  $\mu\text{L}$  of a saturated solution of imidazole to bring the pH to 7.5, and incubation for 1 h at room temperature with 100 units of choline oxidase (*Alkaligenes* sp., Sigma) to convert betaine aldehyde to betaine. The reaction mix was then applied to a  $6.5 \times 1.6$  cm column of Dowex-1 ( $\text{OH}^-$ ) and the aqueous eluate applied to a  $5 \times 1.6$  cm column of Dowex-50 ( $\text{H}^+$ ). After washing the column with 20 mL water, betaine was eluted with 20 mL of 6 N  $\text{NH}_4\text{OH}$  and rotary evaporated to dryness; 3 mg of betaine was recovered, dissolved in 0.5 mL of  $\text{H}_2^{16}\text{O}$ , and stored at  $-20^\circ\text{C}$ . Direct probe chemical ionization MS of a 600- $\mu\text{g}$  dried sample of this betaine preparation relative to unlabeled and  $^2\text{H}_9$ -betaine revealed an average  $^{18}\text{O}$  abundance of the two equivalent oxygens of the betaine carboxyl group of 79%, and a  $^2\text{H}_3$  abundance of the quaternary ammonium group of 98%. Thus, the major molecular ions ( $M+1$ ) of unlabeled betaine,  $^2\text{H}_9$ -betaine, and  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine were  $m/z$  118, 127, and 125, respectively. For the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine, the ion clusters in the mass range 121 to 125 were consistent with the following species and relative ion ratios:  $^2\text{H}_3$ ,  $^{16}\text{O}$ ,  $^{16}\text{O}$ -betaine: $^2\text{H}_3$ ,  $^{18}\text{O}$ ,  $^{16}\text{O}$ -betaine +  $^2\text{H}_3$ ,  $^{16}\text{O}$ ,  $^{18}\text{O}$ -betaine: $^2\text{H}_3$ ,  $^{18}\text{O}$ ,  $^{18}\text{O}$ -betaine (4.9%: 32.5%:62.6%, respectively) (*cf.* expected ratios for 79%  $^{18}\text{O}$ -betaine of 4.4%: 33.2%: 62.4%, respectively, assuming  $^{18}\text{O}$  to be equally distributed among the two oxygens of the carboxyl group of betaine). Note that the average  $^{18}\text{O}$  abundance of the betaine (79%) was somewhat lower than that of the reagent water (about 93%), presumably due to oxygen exchange during ion-exchange purification (see below). No detectable decline in  $^{18}\text{O}$  abundance was observed during up to 4 months of storage of the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine preparation in  $\text{H}_2^{16}\text{O}$  at  $-20^\circ\text{C}$ .

**$^{18}\text{O}$  Exchange of  $^2\text{H}_3$ ,  $^{18}\text{O}$ -Betaine during Derivatization and Purification.** Upon derivatization to the *n*-butyl ester, one oxygen is expected to be lost from betaine, with the further possibility of some exchange of the remaining  $^{18}\text{O}$  with the reagent alcohol. Tests showed that the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine preparation yielded an *n*-butyl ester of 98%  $^2\text{H}_3$  abundance, with a single oxygen originating from betaine of 72.2%  $^{18}\text{O}$  abundance, as determined by FABMS; 27.8% of the  $^2\text{H}_3$ -betaine signal was associated with an ion of mass 177 ( $^2\text{H}_3$ ,  $^{16}\text{O}$ -betaine *n*-butyl ester), and 72.2% with an ion of mass 179 ( $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine *n*-butyl ester). Exchange losses of  $^{18}\text{O}$  during esterification were thus approximately 10%.

As the procedure used to isolate betaine from leaf discs for FABMS involved eluting from Dowex-50 with 2.5 N HCl, and freeze-drying of this eluate, the extent of  $^{18}\text{O}$ -exchange under acid conditions was evaluated.  $^2\text{H}_3$ ,  $^{18}\text{O}$ -Betaine (0.5–1  $\mu\text{mol}$ ) was treated with 8 mL 2.5 N HCl and freeze-dried prior to esterification with *n*-butanol; the resulting betaine ester exhibited an  $^{18}\text{O}$  abundance of 66.2%, suggesting that only a small amount of  $^{18}\text{O}$  exchange (about 10%) took place. To evaluate the amount of  $^{18}\text{O}$  exchange in the whole extraction and purification procedure, leaf disc extracts of spinach were spiked with 0.5 to 1  $\mu\text{mol}$  of  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine (79%  $^{18}\text{O}$  abundance), processed as described below, and derivatized with *n*-butanol. The mean  $^{18}\text{O}$  abundance of the betaine spike recovered was 54.8% (SE 3.3%, 11 independent trials). Thus, the entire sequence of extraction, purification, and derivatization steps led to a reduction of 30% in the  $^{18}\text{O}$  abundance of betaine, some 10% of which was due to esterification, 10% due to freeze drying in HCl, and presumably a further 10% due to ion exchange chromatography *per se* and/or extraction. We sought to reduce the exchange losses in esterification by using methyl, ethyl, isopropyl, and isobutyl esters, but these gave almost the same  $^{18}\text{O}$  abundance values as the *n*-butyl ester, so that the rate of isotope exchange with the reagent alcohol during esterification was not affected by use of alcohols that undergo  $\text{SN}_1$  and  $\text{SN}_2$  reactions at different rates. We also attempted to eliminate exchange losses during Dowex-50 elution and freeze drying by eluting with 4 N  $\text{NH}_4\text{OH}$  in place of 2.5 N HCl; however, this change had no effect on  $^{18}\text{O}$  losses.

**Proton NMR and Isotope Exchange Reactions of Betaine Aldehyde.** Proton NMR spectra of betaine aldehyde chloride (Sigma) in deuterated DMSO were obtained with a Bruker WM-250 Fourier-transform NMR spectrometer. To study oxygen exchange of the carbonyl group with water, 0.16 or 0.32 mmol of dry  $^2\text{H}_3$ -betaine aldehyde chloride was dissolved in 1 mL  $\text{H}_2^{18}\text{O}$  (97 atom %), with or without neutralization with imidazole (pH 7 or 1, respectively). Aliquots (75 or 100  $\mu\text{L}$ ) were removed at short intervals and added to vials containing 10 or 40 mg  $\text{NaBH}_4$  to trap  $^{18}\text{O}$  incorporated into  $^2\text{H}_3$ -betaine aldehyde as  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline. After 15 min, the reaction mixes were acidified with 0.2 mL of 2.5 N HCl, and choline was extracted with acetonitrile/methanol (20:1, v/v). The isotope abundance of the choline was determined by FABMS of the O-heptafluorobutyl choline derivatives ( $m/z$  305 and 303) as above. Note that  $^{18}\text{O}$ -incorporation into betaine aldehyde could not be detected by our analytical methods, for two reasons. First, conversion to the acetal derivative via a hemiacetal intermediate (15) would be expected to displace any  $^{18}\text{O}$  in the aldehyde with  $^{16}\text{O}$  from the reagent alcohol. This was confirmed by preparing  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine aldehyde by exchange of  $^2\text{H}_3$ -betaine aldehyde with  $\text{H}_2^{18}\text{O}$  as above, and derivatization with *n*-butanol: acetyl chloride. FABMS analysis showed no detectable  $^{18}\text{O}$  in the di-*n*-butyl acetal derivative. Second, oxygen exchange during purification of the aldehyde prior to FABMS would have been very extensive. To measure the exchange of the hydrogen atoms on the carbon atom (C-2) adjacent to the carbonyl group, approximately 10  $\mu\text{mol}$  of betaine aldehyde chloride was dissolved in  $^2\text{H}_2\text{O}$ , and incubated for 2 h at  $22^\circ\text{C}$ . The sample was then freeze-dried and analyzed by direct probe chemical ionization MS.

**Plant Material.** Spinach plants (*Spinacia oleracea* L., cv. Savoy Hybrid 612) were grown singly in 600-ml pots of vermiculite/pea gravel (2:1, w/w) in the following growth chamber conditions: 8-h day,  $23^\circ\text{C}$ , 300  $\mu\text{E m}^{-2} \text{s}^{-1}$  PAR, 60% RH/ $20^\circ\text{C}$  night. Irrigation was with half-strength Hoagland solution. Sugar beet plants (*Beta vulgaris* L., cv Great Western D-2) were grown singly in 600-ml pots of organic potting mix in the following growth chamber conditions: 16-h day,  $21^\circ\text{C}$ , 180  $\mu\text{E m}^{-2} \text{s}^{-1}$  PAR, 70% RH/ $16^\circ\text{C}$  night. Beet plants were watered with Peters solution (N/P/K, 20/20/20).

**Preparation and Infiltration of Leaf Discs.** Three discs of 1.7 cm diameter (for stable isotope experiments) or 8 to 10 discs of 1.1 cm diameter (for  $^{14}\text{C}$ -labeling experiments) were cut from leaves 5 or 6 (counting from the lowest leaf) of plants 4 to 5 weeks old. The discs from each leaf were distributed systematically among experimental treatments. Sets of three 1.7-cm discs or five 1.1-cm discs were placed in 50-mL Erlenmeyer flasks containing 10 ml of 0.4 to 0.5 mM choline or betaine aldehyde in  $\text{H}_2\text{O}$ , or in up to 50%  $\text{D}_2\text{O}$ . The fresh weight of three 1.7-cm disks was 0.3 g for spinach, 0.2 g for beet; the fresh weight of five 1.1-cm spinach discs was 0.2 g. The  $^2\text{H}_4$ -,  $^2\text{H}_3$ -, and  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline and  $^2\text{H}_3$ -betaine aldehyde were as described above; [ $^{14}\text{C}$ ]choline (52  $\mu\text{Ci}/\mu\text{mol}$ , NEN-Dupont, purified as in Hanson *et al.* [7]) was diluted with unlabeled choline to a final specific activity of 40 nCi/ $\mu\text{mol}$ . In some experiments, the feeding solutions were unbuffered, in which case the initial pH was adjusted to 6.3 to 6.5; other experiments included 20 mM Hepes/KOH plus 8 mM  $\text{NaHCO}_3$ , final pH 7.7. During infiltration, discs were held flat on the flask bottom with a circle of nylon net (850  $\mu\text{m}$  mesh), and the flask was shaken manually. For [ $^{14}\text{C}$ ]choline experiments, flasks were connected to a vacuum system for 15 s; air was then admitted to the flask, which was then stoppered. For the infiltration of discs with  $^2\text{H}_3$ -labeled choline or betaine aldehyde, flasks were closed with a rubber stopper and connected via a needle to a two-line system through which 3 to 5 cycles of 5 s evacuation/5 s  $\text{N}_2$  flushing were applied. After applying a final 5-s vacuum, the head space ( $50 \pm 2$  ml) of the flask was filled by injecting either air or 10 mL  $^{18}\text{O}_2$  (99 atom %, Stohler Isotope Chemical Inc., Waltham, MA) plus about 40 mL  $\text{N}_2$ .

**Leaf Disc Incubation and Washing.** After the infiltration step, flasks were incubated for up to 6 h with continuous shaking (100 rpm) at  $29^\circ\text{C}$  under a bank of cool-white fluorescent tubes (140  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), or in darkness. In order to estimate total  $^{14}\text{C}$  uptake in experiments with [ $^{14}\text{C}$ ]choline, the following procedure was used to wash label out of the free space before discs were extracted. Discs incubated for various times were removed from the incubation medium, blotted dry, placed in 100-mL Erlenmeyer flasks containing 20 mL of unlabeled 0.5 mM choline, and shaken for 30 min (100 rpm) in darkness at room temperature. Time courses of  $^{14}\text{C}$  release to the medium showed that 30 min sufficed for free space washout. After washing, disks were again blotted dry and then extracted. Discs supplied  $^2\text{H}_3$ -labeled choline or betaine aldehyde in unbuffered conditions were not washed before extraction; when Hepes/KOH was used, discs were washed as above, except that water replaced 0.5 mM choline.

**Isolation of Quaternary Ammonium Compounds from Leaf Discs.** Sets of three 1.7-cm discs or five 1.1-cm discs were extracted in  $2 \times 2$  ml of methanol/chloroform/water (12:5:1 v/v), and aqueous and organic phases separated by adding 1 mL of chloroform plus 1.5 mL of water. The aqueous phases were drawn off, dried in a stream of  $\text{N}_2$  at  $45$  to  $50^\circ\text{C}$ , and redissolved in 1 mL of water. Samples labeled with  $^2\text{H}_3$ - or  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline, or  $^2\text{H}_3$ -betaine aldehyde received an internal standard of  $\gamma$ -butyrobetaine (1125 or 1233 nmol, prepared as in Rhodes *et al.* [18]) at this point, and were then fractionated by ion exchange chromatography, as follows. The sample was applied to a 1.5-mL Dowex-1 ( $\text{OH}^-$ )/Biorex 70 [ $\text{H}^+$ ], 2:1 [v/v] mixed resin column replaced Dowex-1.) The column series was washed with 8 ml of water and the Dowex-1 column was removed. The Dowex-50 column was washed with a further 8 mL of water and then eluted with 8 mL of 2.5 N HCl. The eluate, containing quaternary ammonium compounds, was collected in 25-mL vials, frozen in liquid  $\text{N}_2$ , and freeze-dried. The freeze-dried samples were mailed from Michigan State University to Purdue University for FABMS analysis.

In [ $^{14}\text{C}$ ]choline experiments, the redissolved aqueous phase was applied to a 1.5-mL column of mixed resin (Dowex-1 [ $\text{OH}^-$ ]/Biorex 70 [ $\text{H}^+$ ], 2:1 [v/v]) connected in series with a 1.5-mL column of Dowex-50 ( $\text{H}^+$ ). The column series was washed with 8 mL of water, and the mixed resin column was removed. The Dowex-50 column was also washed with 8 mL of water, and then the betaine fraction was eluted with 8 mL of 4 N  $\text{NH}_4\text{OH}$ . The eluate was evaporated to dryness under a heat lamp in a stream of air, and redissolved in 0.5 mL water. [ $^{14}\text{C}$ ]Betaine in the eluate was determined by scintillation counting; TLE analysis of representative eluates confirmed that the only labeled compound was betaine.

**Determination of Betaine and Betaine Aldehyde Levels and Isotope Abundance in Leaf Discs.** Betaine and betaine aldehyde in the freeze-dried HCl eluates from Dowex-50 chromatography were redissolved in 0.4 mL of 60% methanol, dried, and derivatized with *n*-butanol:acetyl chloride (5:1 v/v,  $120^\circ\text{C}$ , 20 min). Excess reagent was removed by evaporation to dryness in an air stream and the samples were then taken up in glycerol (50–100  $\mu\text{L}$ ) for FABMS analysis as described (18). Betaine was quantified by the ratio of ions at  $m/z$  174, 177, 178, 179, and 183 (unlabeled,  $^2\text{H}_3$ -,  $^2\text{H}_4$ -,  $^2\text{H}_3$ ,  $^{18}\text{O}$ - or  $^2\text{H}_5$ -, and  $^2\text{H}_9$ -betaine esters, respectively) relative to the internal standard  $\gamma$ -butyrobetaine ( $m/z$  202). The internal standard was calibrated against authentic betaine. The molecular cation of unlabeled betaine *n*-butyl ester gave relative ion intensities at  $m/z$  174:175:176 of 100:10.7-11.3:0.98-1.33, respectively. The  $^2\text{H}_3$ ,  $^{16}\text{O}$ -betaine *n*-butyl ester gave an ion cluster at  $m/z$  177:178:179 with the same relative intensities. Thus, we routinely subtracted from the ion intensity at  $m/z$  179 from 0.98 to 1.33% of the ion intensity at  $m/z$  177 to arrive at the ion intensity due specifically to the molecular cation of the  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine *n*-butyl ester. For the experiments of Table I, in which all  $^2\text{H}_3$ ,  $^{16}\text{O}$ - and  $^2\text{H}_3$ ,  $^{18}\text{O}$ - betaine synthesis rates were low, a further background subtraction was applied at  $m/z$  179 to correct for glycerol chemical noise generated in the FABMS process ( $1.7 \pm 0.7$  nmol  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine equivalents per  $\sim 50$   $\mu\text{L}$  sample). The lower limit of detection of betaine *n*-butyl esters above glycerol background chemical noise was approximately 2.5 nmol/50  $\mu\text{L}$  glycerol (18).

Betaine aldehyde was quantified by monitoring the ions at  $m/z$  232 and 235 (unlabeled and  $^2\text{H}_3$ -di-*n*-butyl acetal derivatives, respectively). As noted above,  $^{18}\text{O}$  in betaine aldehyde was not detectable. Betaine aldehyde was calibrated against the internal standard  $\gamma$ -butyrobetaine.

**$^{14}\text{CO}_2$  Fixation Measurements.** Five 1.1-cm leaf discs were infiltrated as above in 10 mL of a solution containing Hepes/KOH 20 mM, 8 mM  $\text{NaH}^{14}\text{CO}_3$  (25 nCi/ $\mu\text{mol}$ ) and 0.5 mM unlabeled choline, final pH 7.7. After sealing the flask, light  $^{14}\text{C}$  fixation (in the conditions given above for discs) was followed by taking 100- $\mu\text{L}$  aliquots of the medium at various times up to 6 h, and determining the remaining  $^{14}\text{C}$  by scintillation counting. Values obtained with a blank flask without discs were used to correct for  $^{14}\text{CO}_2$  partitioning into the head-space.

**Computer Simulations.** Computer simulations (described in "Results and Discussion") were carried out on a Zenith Data Systems microcomputer with dual disc drive, interfaced with a Star SD-10 dot matrix printer. Programs were written in BASIC.

## RESULTS AND DISCUSSION

**Effect of Light on Choline Oxidation by Spinach Leaf Discs.** Discs oxidized [ $^{14}\text{C}$ ]choline to betaine continuously during 6 h in the light (Fig. 3); the initial rate (about 100 nmol/h  $\cdot$  mg Chl) was similar to rates of choline oxidation in isolated chloroplasts (23). Choline oxidation in darkness was less than in light, and essentially ceased within 2 h. This cessation was not due to loss of viability, because after 4 h in darkness, discs synthesized betaine actively upon transfer to light. The effect of light can be

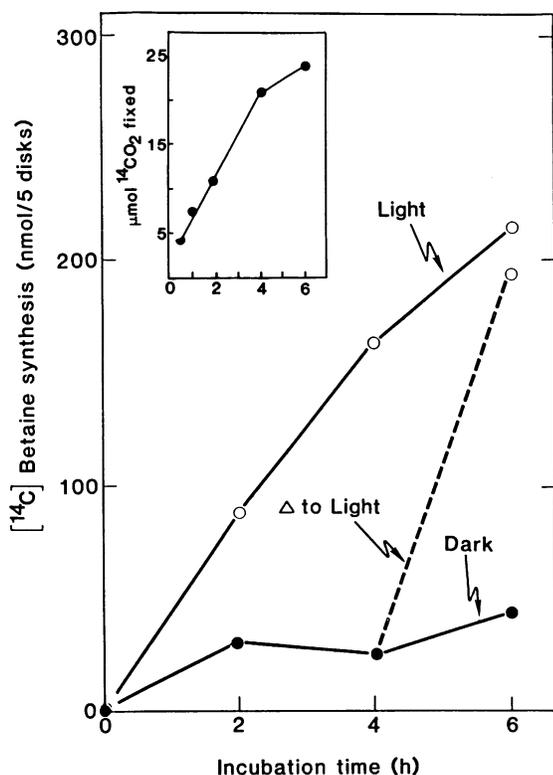


FIG. 3. Time courses for oxidation of [ $^{14}\text{C}$ ]choline to betaine by spinach leaf disks in light and darkness. The infiltration medium was: [ $^{14}\text{C}$ ]choline 0.5 mM, Hepes/KOH 20 mM (pH 7.7), and  $\text{NaHCO}_3$  8 mM. Data points are means of duplicates. Initial rates of [ $^{14}\text{C}$ ]choline uptake in light and dark were: 303 and 273 nmol/2 h, respectively. Inset shows a parallel experiment with unlabeled choline and  $\text{NaH}^{14}\text{CO}_3$  run in the light to estimate the rate of photosynthesis.

ascribed to increased choline oxidation rather than to increased uptake, because although initial betaine synthesis rates in light and dark differed by a factor of three, initial choline uptake rates were almost the same (Fig. 3, legend).

To confirm that light acts at the first step of choline oxidation, the labeled betaine aldehyde and betaine pools were determined in discs supplied with  $^2\text{H}_3$ -choline and incubated for 5 h in darkness or light.  $^2\text{H}_3$ -Betaine aldehyde pools were 0.91 and 7.3 nmol/3 discs in darkness and light, respectively, and  $^2\text{H}_3$ -betaine pools were 81 and 544 nmol/3 discs. The low level of  $^2\text{H}_3$ -betaine aldehyde in darkness shows that light promotes the oxidation of choline to betaine aldehyde, not the oxidation of the aldehyde to betaine.

The experiment of Figure 3 was carried out in the presence of Hepes/KOH buffer and  $\text{HCO}_3^-$ , with a parallel experiment including  $\text{H}^{14}\text{CO}_3^-$  (Fig. 3, inset) which showed that the discs photosynthesized throughout the incubation. This confirmed that the first step of choline oxidation *in vivo* resembles that in isolated chloroplasts (23) with respect to light stimulation in the simultaneous presence of photosynthesis. Because results very similar to those of Figure 3 were obtained when  $\text{HCO}_3^-$  and buffer were omitted, these components were not routinely used in subsequent experiments.

**Chemical Nature of Betaine Aldehyde.** Because interpretation of our stable isotope data required some background on the chemistry of betaine aldehyde, we investigated the dominant form in solution, and evaluated  $^{18}\text{O}$ - and  $^2\text{H}$ -exchange reactions. Aldehydes can exist in three forms in aqueous solution, thus:



The hydrate form (*gem*-diol) is expected to be prevalent in the case of a small aldehyde with an adjacent electron-withdrawing group such as a quaternary ammonium function (2), and proton NMR spectra of betaine aldehyde in deuterated DMSO before and after addition of  $^2\text{H}_2\text{O}$  confirmed that the compound exists in solution almost solely (>99%) as the hydrate. The aldehydal proton had a quintuplet splitting pattern in pure  $^2\text{H}$ -DMSO which reduced instantaneously to a triplet upon adding  $^2\text{H}_2\text{O}$  (not shown). Exchange between  $\text{H}_2^{18}\text{O}$  and the aldehyde (due to equilibrium A) was fairly rapid; the half-time for exchange was 0.6 min at neutral pH, and 0.8 min at pH 1. Exchange between the hydrogen atoms on C-2 and  $^2\text{H}_2\text{O}$  (due to equilibrium B) was relatively slow, with only 20 to 30% deuterium ( $^2\text{H}_1$ ) labeling occurring after 2 h.

**Deuterium-Labeling Tests for a Desaturase Reaction.** In initial tests for a desaturase mechanism, spinach leaf discs were supplied with  $^2\text{H}_3$ -choline in the presence of 50%  $^2\text{H}_2\text{O}$ , and the amounts of  $^2\text{H}_3$ -,  $^2\text{H}_4$ -, and  $^2\text{H}_5$ -betaine determined (Fig. 4A). The presence of  $^2\text{H}_2\text{O}$  had no discernible effect on the amounts of betaine produced, which were in the normal range. Approximately 10% of the betaine synthesized was  $^2\text{H}_4$ -labeled, a value which in principle might be given by a desaturase in which the proton lost from C-2 is strongly shielded from the bulk solvent water and hence tends to add back. However, some  $^2\text{H}_5$ -betaine was also

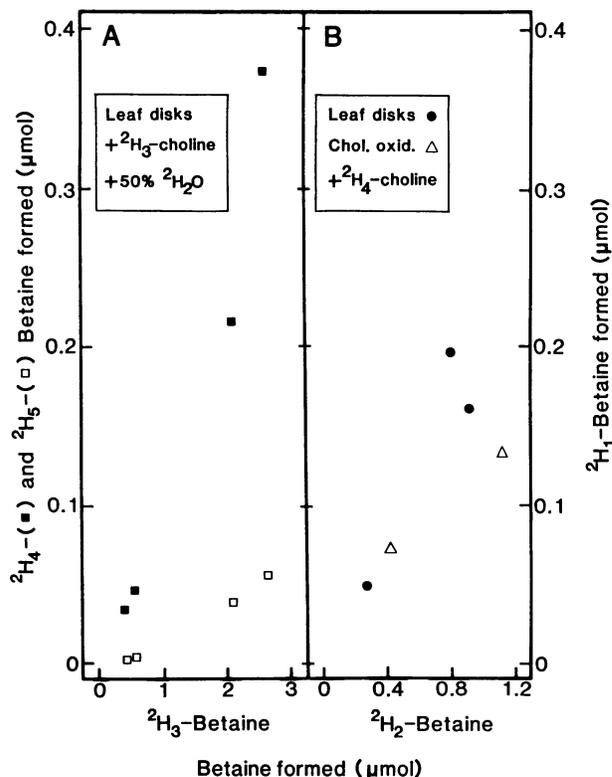


FIG. 4. Scatter plots showing (A) the extent of incorporation of  $^2\text{H}$  from  $^2\text{H}_2\text{O}$  into betaine by spinach leaf discs and (B) the loss of  $^2\text{H}$  during conversion of  $^2\text{H}_4$ -choline to betaine by spinach leaf discs or *Alcaligenes* choline oxidase. For the experiments in A, discs were supplied with  $^2\text{H}_3$ -choline in the presence of 50%  $^2\text{H}_2\text{O}$ , so that  $^2\text{H}$  incorporation from  $^2\text{H}_2\text{O}$  is detected as formation of  $^2\text{H}_4$ - and  $^2\text{H}_5$ -betaine. For the experiments in B, leaf discs or purified choline oxidase received 1,1,2,2- $^2\text{H}_4$ -choline; because both  $^2\text{H}$  atoms at the 1-position are lost in the formation of betaine, the amount of  $^2\text{H}_1$ -betaine formed is a measure of  $^2\text{H}$  loss from the 2-position. The ranges in total amount of labeled betaine formed reflect differences in substrate dose in experiments with choline oxidase, and biological variation in the case of leaf disc experiments, in which incubations were for 5 h in the light.

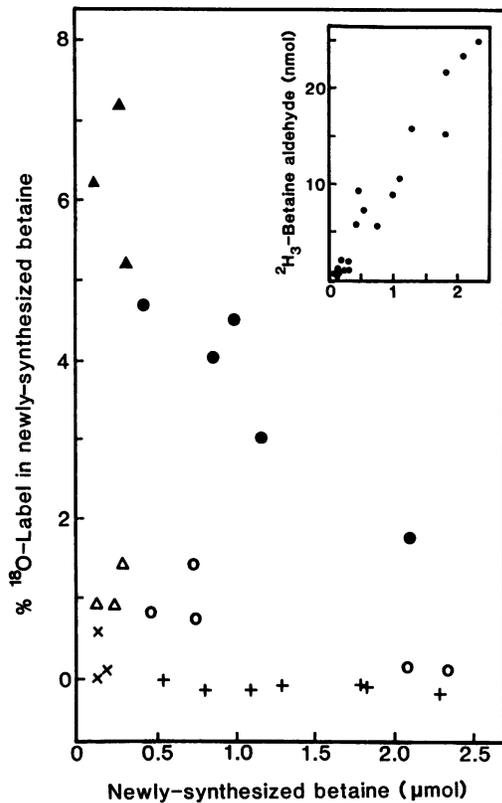


FIG. 5. Scatter plot relating the  $^{18}\text{O}$  content of betaine synthesized in the light to the total amount of newly synthesized (*i.e.*  $^2\text{H}_3$ -labeled) betaine, for leaf discs supplied  $^2\text{H}_3$ -choline under air (crosses),  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline under air (solid symbols), and  $^2\text{H}_3$ -choline under  $^{18}\text{O}_2$  (open symbols). Data points are from eight experiments, five with spinach (+, ●, ○) and three with sugar beet (×, ▲, △); incubations were for 5 h. Inset shows the relationship between pool size of  $^2\text{H}_3$ -labeled betaine aldehyde and the amount of newly synthesized betaine; data points are from three of the spinach experiments, and all the sugarbeet experiments.

produced, suggesting chemical exchange between water and the hydrogens at C-2 of betaine aldehyde as a more likely explanation, particularly because these hydrogen atoms exchanged measurably *in vitro*. Evidence in favor of chemical exchange came from comparing the betaine formed from 1,1,2,2- $^2\text{H}_4$ -choline by spinach leaf discs with that made by purified *Alcaligenes* choline oxidase (Fig. 4B). Consistent with the  $^2\text{H}_2\text{O}$  results, leaf discs

produced betaine that was about 85%  $^2\text{H}_2$ -labeled, 15%  $^2\text{H}_1$ -labeled; the betaine made by the choline oxidase enzyme had a similar composition. A further observation consistent with chemical exchange is that betaine aldehyde isolated from leaf discs and choline oxidase reaction mixes supplied with  $^2\text{H}_4$ -choline was predominantly  $^2\text{H}_1$ -labeled (not shown). Taken together, these results give no support for a desaturase mechanism.

**$^{18}\text{O}$ -Labeling Tests for an Oxygenase Reaction.** A series of experiments was conducted in light and darkness. Each comprised three treatments: spinach or sugar beet leaf discs were given  $^2\text{H}_3$ -choline under air;  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline under air; or  $^2\text{H}_3$ -choline under  $^{18}\text{O}_2$ . Figure 5 summarizes results of eight such experiments carried out in light; the *x*-axis is the total amount of  $^2\text{H}_3$ -labeled betaine formed, and the *y*-axis is the percentage of this newly synthesized betaine that contained an atom of  $^{18}\text{O}$ . The data show the following features. First, in both spinach and sugar beet, the amount of  $^2\text{H}_3$ -betaine formed was quite variable, presumably due to differences among leaf batches in the *in vivo* activity of the choline  $\rightarrow$  betaine aldehyde step. Second, the  $^{18}\text{O}$ -content calculated for the  $^2\text{H}_3$ -betaine synthesized from  $^2\text{H}_3$ -choline under air was always close to zero, validating the standard correction factor used. Third, treatments supplied  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline under air formed a considerable amount of  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine, with the extent of  $^{18}\text{O}$ -labeling decreasing as the activity of the pathway increased. Fourth, the betaine synthesized under  $^{18}\text{O}_2$  always contained appreciable  $^{18}\text{O}$ , but the  $^{18}\text{O}$  content averaged only 16% of that seen with  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline.

The repeatable but low incorporation of  $^{18}\text{O}$  from  $^{18}\text{O}_2$  in the light would be consistent with an oxygenase, were the  $^{18}\text{O}_2$  supplied to the infiltrated discs to be diluted severalfold at the enzyme site inside the chloroplast with photosynthetic  $^{16}\text{O}_2$  (3, 5). Results from experiments in the dark, where no such isotopic dilution of the supplied  $^{18}\text{O}_2$  would occur, support this explanation (Table I). Although the amount of  $^2\text{H}_3$ -betaine synthesized (about 0.13  $\mu\text{mol}$ ) was less than in the light (cf. Fig. 3), the extent of  $^{18}\text{O}$ -labeling from  $^{18}\text{O}_2$  approached that from  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline. To confirm that the incorporation of  $^{18}\text{O}$  from  $^{18}\text{O}_2$  in darkness occurred at the choline  $\rightarrow$  betaine aldehyde step, not at the betaine aldehyde  $\rightarrow$  betaine step, spinach leaf discs were supplied with 0.5 mM  $^2\text{H}_3$ -betaine aldehyde under  $^{18}\text{O}_2$ . The  $^2\text{H}_3$ -betaine synthesized after 5 h in darkness contained no detectable  $^{18}\text{O}$  (not shown).

**Computer Modeling of  $^{18}\text{O}$ -Labeling Data.** The above  $^{18}\text{O}$ -labeling results are clearly consistent in a qualitative sense with an oxygenase reaction. When oxygen exchange of betaine aldehyde is taken into account, the results are also quantitatively consistent with an oxygenase mechanism, as the following mod-

Table I. Incorporation of  $^{18}\text{O}$  into Betaine Synthesized in Darkness

In each of five experiments, spinach leaf discs were incubated for 5 h in darkness with  $^2\text{H}_3$ -choline in air,  $^2\text{H}_3$ ,  $^{18}\text{O}$ -choline in air, and  $^2\text{H}_3$ -choline in  $^{18}\text{O}_2$ . Betaine aldehyde pools (mean  $\pm$  SE) were  $4.00 \pm 0.57$ ,  $2.73 \pm 0.42$ , and  $3.63 \pm 0.34$  nmol/3 discs, respectively.

Substrate	Atmosphere	$^2\text{H}_3$ , $^{16}\text{O}$ -Betaine <sup>a</sup>	$^2\text{H}_3$ , $^{18}\text{O}$ -Betaine <sup>b</sup>	$^{18}\text{O}$	
				Abundance in $^2\text{H}_3$ -Betaine	%
		nmol/3 discs			
$^2\text{H}_3$ -Choline	Air	$139.2 \pm 10.5$	$1.7^c \pm 0.7$		0
$^2\text{H}_3$ , $^{18}\text{O}$ -Choline	Air	$130.8 \pm 17.9$	$8.6 \pm 1.9$		5.01 <sup>d</sup>
$^2\text{H}_3$ -Choline	$^{18}\text{O}_2$	$134.0 \pm 14.4$	$7.4 \pm 1.6$		4.08

<sup>a</sup> Calculated from ion intensity at *m/z* 177 relative to internal standard (*m/z* 202); mean  $\pm$  SE. <sup>b</sup> Calculated from ion intensity at *m/z* 179 (minus 1.33% of ion intensity at *m/z* 177) relative to internal standard (*m/z* 202), not corrected for glycerol chemical noise at *m/z* 179. <sup>c</sup>  $^2\text{H}_3$ ,  $^{18}\text{O}$ -Betaine equivalents due to glycerol chemical noise at *m/z* 179. <sup>d</sup> Average  $^{18}\text{O}$  abundance of  $^2\text{H}_3$ -betaine, subtracting 1.7 nmol/3 discs  $^2\text{H}_3$ ,  $^{18}\text{O}$ -betaine equivalents due to glycerol chemical noise at *m/z* 179.

eling studies will show. In addition, this modeling work indicates which form of the aldehyde may be bound by betaine aldehyde dehydrogenase.

In the case of betaine from discs given  $^2\text{H}_3, ^{18}\text{O}$ -choline, the highest possible  $^{18}\text{O}$  abundance would be 15.8%, assuming: (a) 90%  $^{18}\text{O}$  abundance in  $^2\text{H}_3, ^{18}\text{O}$ -choline; (b) a theoretical maximum yield of 25% (Fig. 1); (c) an average  $^{18}\text{O}$  loss from betaine by exchange during sample processing of 30% ("Materials and Methods"); and (d) no loss by *in vivo* exchange between betaine aldehyde and water. The observed  $^{18}\text{O}$  abundance in betaine made from  $^2\text{H}_3, ^{18}\text{O}$ -choline was at most 7 to 8% (Fig. 5; Table I). Hence, it is likely that betaine aldehyde exchange with water was substantial even at low pathway flux rates, and became more so as flux rose. The latter trend would be expected were the metabolic pool of betaine aldehyde to expand relative to the flux as flux increased, which is clearly possible given the large expansion in the total betaine aldehyde pool (Fig. 5, inset). The half-time estimated for betaine aldehyde  $^{18}\text{O}$  exchange *in vitro* (0.6 min at pH 7) fits well with the above interpretation. Thus, adopting a half-time of 0.6 min and further assuming a metabolic pool of betaine aldehyde of 10 nmol/3 discs, and a flux rate of 200 nmol/3 discs·h, leads to a predicted  $^{18}\text{O}$  content for betaine of 5.2% uncorrected for  $^{18}\text{O}$  loss from betaine during sample processing, and hence 3.64% when corrected for the latter loss. This value is close to the observed value of approximately 4%  $^{18}\text{O}$  at this betaine aldehyde pool size and flux (*c.f.* Fig. 5).

Simulation studies confirmed that in addition to the half-life of betaine aldehyde  $^{18}\text{O}$ , the betaine flux:betaine aldehyde pool ratio ( $\text{h}^{-1}$ ) was critical in determining the predicted  $^{18}\text{O}$  abundance of betaine derived from  $^2\text{H}_3, ^{18}\text{O}$ -choline. For example, to account for an observed average  $^{18}\text{O}$ -abundance of  $^2\text{H}_3$ -betaine in leaf discs supplied with  $^2\text{H}_3, ^{18}\text{O}$ -choline in darkness (Table I) a flux:betaine aldehyde pool ratio of  $31 \text{ h}^{-1}$  was required. Since the observed average betaine flux in these experiments was 28 nmol/3 discs·h, then the metabolic pool of  $^2\text{H}_3$ -betaine aldehyde must have been only 0.9 nmol/3 discs. The observed  $^2\text{H}_3$ -betaine aldehyde pool was  $2.7 \pm 0.42$  nmol (Table I), again indicating fairly close agreement between observed and theoretical values. Perhaps no more than 1.8 nmol of  $^2\text{H}_3$ -betaine aldehyde/3 discs exits from the metabolic pool during a 5-h incubation in darkness. This represents less than 1.3% of the betaine flux during the same 5-h incubation. Strict control over betaine aldehyde export from the metabolic pool *in vivo* is implied by these results.

For both choline and betaine aldehyde hydrate, carbon-1 is a prochiral center. Hence, in principle, a choline oxygenase could introduce  $^{18}\text{O}$  into one of the two hydroxyl positions, and a betaine aldehyde dehydrogenase which initially bound the hydrate could distinguish between this  $^{18}\text{OH}$  group and the  $^{16}\text{OH}$  from choline, and so discriminate against one or the other. However, if *in vivo* exchange of betaine aldehyde with water occurs, the  $^{18}\text{O}$  originally present in one hydroxyl group would tend toward random distribution between both, as the exchange reaction is not stereospecific. At low pathway flux rates in light or darkness, about one-half the  $^{18}\text{O}$  is apparently lost by exchange from the betaine aldehyde pool (Fig. 5; Table I). Under these circumstances, it can be shown that the remaining  $^{18}\text{O}$  would be substantially but not completely randomized, with about 60% present at the original position. In this connection, the small but consistent difference between  $^{18}\text{O}$ -labeling in darkness from  $^{18}\text{O}$ -choline and  $^{18}\text{O}_2$  (Table I) should be noted. The lower value obtained for  $^{18}\text{O}_2$  (about 4% versus 5%) would be explained were the oxygen introduced by the oxygenase also the one removed in a dehydration reaction taking place on the surface of the dehydrogenase. If this is the explanation, then chloroplast betaine aldehyde dehydrogenase, in binding the hydrate form of its substrate rather than the free aldehyde, would differ from other aldehyde dehydrogenases (4, 17, 21). An alternative explanation

for the difference between  $^{18}\text{O}$ -labeling in darkness from  $^{18}\text{O}$ -choline and  $^{18}\text{O}_2$  would be that the metabolic pool of  $^2\text{H}_3$ -betaine aldehyde was significantly greater in the  $^{18}\text{O}_2$  treatments relative to the  $^{18}\text{O}$ -choline treatments—a possibility that cannot be ruled out from the data of Table I and the preceding discussion. Thus, the metabolic pool of  $^2\text{H}_3$ -betaine aldehyde need only have expanded from 0.9 to 1.2 nmol/3 discs to account for the lower  $^{18}\text{O}$ -abundance of betaine in the  $^{18}\text{O}_2$  treatments.

**Evolutionary Implications.** Our results indicate that the chloroplast choline-oxidizing enzyme is an oxygenase, unlike the flavoprotein choline dehydrogenases (11, 16, 22) and oxidases (10, 29) known from mammals, fungi, and eubacteria. We therefore hypothesize that the betaine synthesis pathway arose more than once in evolution, and that the betaine pathway in plants has a distinct origin to that in other kingdoms. Perhaps only two origins need be invoked, for flavoprotein dehydrogenases can behave as oxidases after separation from a multienzyme system (9). Because betaine occurs widely among halophytic cyanobacteria (12), and because the betaine pathway is chloroplastic in the higher plants that have been studied (7, 23, 24), it is tempting to consider a cyanobacterial origin for the plant betaine synthesis genes.

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