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Betaine synthesis in chenopods: Localization in chloroplasts

(stress resistance/*Spinacia oleracea*/choline oxidation/betaine aldehyde oxidation/protoplasts)

ANDREW D. HANSON*, ANN MARIE MAY*, REBECCA GRUMET*, JANET BODE*, GENE C. JAMIESON†, AND DAVID RHODES†

*MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; and †Zoëcon Corporation, 975 California Avenue, Palo Alto, CA 94304

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ABSTRACT **Plants from several families (Chenopodiaceae, Gramineae, Compositae) accumulate betaine (glycine betaine) in response to salt or water stress via the pathway: choline → betainal (betaine aldehyde) → betaine. Betaine accumulation is probably a metabolic adaptation to stress. Intact protoplasts from leaves of spinach (*Spinacia oleracea*) oxidized [^{14}C]choline to betainal and betaine, as did protoplast lysates. Upon differential centrifugation, the [^{14}C]choline-oxidizing activity of lysates sedimented with chloroplasts. Chloroplasts purified from protoplast lysates by a Percoll cushion procedure retained strong [^{14}C]choline-oxidizing activity (1–3 nmol/mg of chlorophyll per hr), although the proportion of the intermediate, [^{14}C]betainal, in the reaction products was usually higher than for protoplasts. Isolated chloroplasts also readily oxidized [^{14}C]betainal to betaine (20–100 nmol/mg of chlorophyll per hr). Light increased the oxidation of both [^{14}C]choline and [^{14}C]betainal by isolated chloroplasts ≈3-fold; this light-stimulation was abolished by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Similar results were obtained with another chenopod (*Beta vulgaris*) but not with pea (*Pisum sativum*), a species that accumulates no betaine. The chloroplast site for betaine synthesis in chenopods contrasts with the mitochondrial site in mammals.**

Quaternary ammonium compounds such as betaine (glycine betaine) are accumulated by many organisms that face saline or dry environments (1–4); these organisms include halophilic bacteria and blue-green algae, invertebrates, vertebrates, and higher plants. It is probable that quaternary ammonium compounds are metabolically inert solutes that are compatible with normal cell function when present at high concentrations; this property fits them for use as internal osmotica in environments of low water potential (1, 2). Wyn Jones *et al.* have suggested that betaine is confined largely to the cytoplasm in higher plants (1, 5). Higher plants that accumulate betaine include chenopods, grasses, and composites (5). In most cases, there is a low constitutive level of betaine in unstressed plants, and water or salt stress provokes a several-fold increase (6). Much indirect evidence indicates that stress-induced betaine accumulation by higher plants is a metabolic adaptation to stress (7, 8).

In vivo radiotracer studies of both unstressed and stressed plants (6) show that betaine is synthesized in leaves by a two-step oxidation from choline:

$$\text{Choline} \xrightarrow{-2H} \text{betainal (betaine aldehyde)} \xrightarrow{-2H} \text{betaine}.$$

The betainal pool is very small (9, 10). Betaine is not detectably catabolized in vegetative tissues (11, 12), which implies that the betaine titer is governed mainly by the rate of synthesis.

The probable adaptive value of betaine accumulation, the two-step biosynthesis, and the advent of molecular-genetic tools for manipulating simple metabolic pathways have drawn attention to the genes for betaine synthesis. At this time they are among the few rational candidates for "stress resistance" genes in higher plants (7, 8). However, although mammalian and microbial enzymes that oxidize choline to betaine are well known (6, 8), the plant enzymes have not been demonstrated hitherto *in vitro*.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea* L., cultivar Savoy Hybrid 612) was grown in vermiculite or in a 1:1 mix of vermiculite and gravel containing 0.02% captan and was watered with half-strength Hoagland's solution. Growth chamber conditions were: 8-hr day, 250 μmol of photons per m^2·s, 24°C, 60% relative humidity; 16-hr night, 24°C. Sugarbeet (*Beta vulgaris* L., cultivar Great Western) and pea (*Pisum sativum* L., Argentum mutant) were grown in soil in growth chamber conditions as described (12, 13).

Protoplast Preparation. Young, pale-green, first to fifth leaves of 3- to 5-wk-old spinach plants were used. After the lower epidermis was removed, leaf pieces were incubated in the dark for 2.5–3 hr on a rotary shaker (60 cycles per min at 26°C) in 0.5 M sorbitol containing 5 mM 4-morpholineethanesulfonic acid/KOH, 1 mM $CaCl_2$, chloramphenicol at 10 μg/ml (=isolation medium), 1.5% Macerase (Calbiochem), 1% cellulase (Worthington), and 0.2% fatty-acid-free bovine serum albumin, adjusted to pH 5.5. Standard filtering, washing, and flotation steps (14) under sterile conditions were used to isolate intact protoplasts; these were resuspended in the isolation medium at pH 5.8. The same procedure was used for sugarbeet leaves but modified for pea leaves as follows: a 30-min digestion, with Macerase reduced to 1%, was used; and in the flotation step (14), the two lower layers were changed to 0.5 M sucrose with 23% and 11.5% Percoll. Photosynthetic activity of protoplasts was monitored with an O_2 electrode. Data from preparations were discarded if microbial contamination was more than two viable organisms per 10^4 protoplasts (nutrient broth agar and potato dextrose agar plates). Experiments were repeated with several protoplast preparations.

Organelle Preparation. Protoplasts were resuspended [*ca.* 1 mg of chlorophyll (Chl) per ml] in lysis medium (0.4 M sorbitol/50 mM Hepes/KOH, pH 7.6/1 mM Na_2EDTA/0.2% fatty-acid-free bovine serum albumin/10 μg of chloramphenicol per ml. The lysis procedure (14) used 15-μm nylon mesh. Lysates were fractionated by differential centrifugation or by a Percoll step procedure for intact chloroplasts (15). Particulate fractions were resuspended in lysis medium. Standard

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

methods were used to estimate Chl (16), catalase (17), cytochrome *c* oxidase (18), and chloroplast intactness (19).

[^{14}C]Choline Oxidation Assays. [^{14}C]Choline (58 or 59 μCi/μmol, Amersham; 1 Ci = 37 GBq) was purified by treatment with alkaline H_2O_2 (20), followed by electrophoresis on cellulose TLC plates in 1.5 M formic acid (9). Assays were in 12 × 75 mm glass tubes containing 14–17 nmol of [^{14}C]choline in 5 μl of H_2O and 50 μl of protoplast suspension (50–150 μg of Chl) or 50 μl of subcell fraction; incubation was at 26–29°C on a rotary shaker (60 cycles per min) in darkness or red light (Kodak FHS lamp, Corning filter 2424, 2000–4000 μmol of photons per m^2·s). Assays were stopped in liquid N_2 after adding 1 μmol of carrier betainal and stored at −20°C. For analysis, the reaction mix was divided. Half received 1 μmol of carrier betaine and was applied to 1-ml columns of mixed-bed ion-exchange resin and AG-50(H^+) (Bio-Rad) arranged in series (21); [^{14}C]betaine was eluted from the AG-50 column with NH_4OH and determined after electrophoresis on Gelman ITLC-SA strips (9). The other half received a further 0.5 μmol of betainal and was treated for 1–2 hr at 22°C with 1 ml of 0.17 M NaOH/10% H_2O_2 to convert betainal to betaine (20). [^{14}C]Betaine was then estimated as above. The [^{14}C]betainal present in the original reaction mix was obtained by difference. Samples stopped at time 0 were used to correct for traces of labeled betainal and betaine present in the [^{14}C]choline.

[^{14}C]Betainal Oxidation Assays. [^{14}C]Betainal was prepared as described (11). The specific activity was adjusted with carrier to 2.3 μCi/μmol. Assay mixtures contained 27 nmol of substrate and 50 μl of sample. Analysis of [^{14}C]betaine was as for [^{14}C]choline, save that the whole reaction mix was applied to the mixed-resin column, and ^{14}C in the effluent was taken as a measure of [^{14}C]betaine formation.

Determination of Quaternary Ammonium Compounds by Fast Atom Bombardment MS. Analyses were made of unlabeled protoplasts and chloroplasts and those to which [*N-trimethyl*-$^2H_9$]choline chloride (98%; Cambridge Isotope, Woburn, MA) had been supplied. Choline was determined as the heptafluorobutyryl derivative, betaine as the isobutyl ester, and betainal as the diisobutyl acetal (22).

RESULTS

[^{14}C]Choline Oxidation by Protoplasts. Spinach leaf protoplasts oxidized [^{14}C]choline to betainal and betaine in darkness and light; light approximately tripled the rate (Fig. 1). The amount of [^{14}C]choline oxidized per 100 μg of Chl in 4 hr of darkness was used as a standard measure of activity; preparations varied considerably for this parameter and for the percentage of [^{14}C]betainal formed. Mean values (±SEM) and ranges for 24 preparations were: choline oxidation, 0.24 ± 0.03 nmol/100 μg of Chl per 4 hr (range, 0.03–0.58); and betainal, 27 ± 6% (range, 0–76%). Small differences in developmental age of the material accounted for most of the variation in dark [^{14}C]choline oxidation rate; this rate was strongly and negatively correlated ($r = -0.82$, $P < 0.001$) with a measure of developmental age (μg of Chl per 10^6 protoplasts). Photosynthetic activity of preparations also was negatively correlated with developmental age and proved a useful predictor of choline-oxidizing activity. For example, protoplasts with O_2-evolution rates of <10 μmol/mg of Chl per hr oxidized little choline.

The effect of osmotic stress on the choline-oxidizing activity of protoplasts was tested using sorbitol (up to 1.3 M) or salts [NaCl/$CaCl_2$, 2:1 (mol/mol), up to 200 mM total]. Neither sorbitol nor salts promoted [^{14}C]choline oxidation at any concentration tested. However, [^{14}C]choline oxidation remained active at high sorbitol concentrations: with 1.3 M sorbitol (solute potential = −37 bars; 1 bar = 100 kPa), [^{14}C]choline oxidation in darkness was >80% that of the control (0.5 M sorbitol), although 1.3 M sorbitol abolished net O_2 evolution. Salts were more inhibitory: 200 mM total salts (final solute potential = −20 bars) cut [^{14}C]choline oxidation by ≈50%.

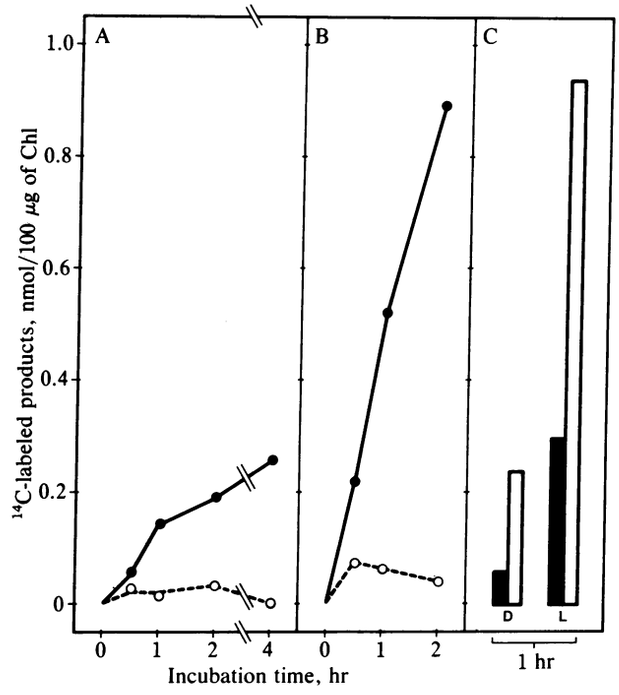

FIG. 1. Oxidation of [^{14}C]choline to betainal (open symbols) and betaine (solid symbols) by intact protoplasts. (*A*) Spinach protoplasts in darkness. (*B*) Spinach protoplasts in light; the protoplast preparation was the same as in *A*. (*C*) Sugarbeet protoplasts; [^{14}C]choline oxidation products formed during 1 hr in darkness (D) or light (L).

To help interpret ^{14}C-labeling data, endogenous pools of choline, betainal, and betaine were measured in spinach protoplast preparations (Table 1). The betainal pool was small, but the choline pool was comparable to the standard dose of [^{14}C]choline supplied in the assay (about 15 nmol/100 μg of Chl). Tests showed that protoplasts took up only ≈30% of the supplied [^{14}C]choline during a 4-hr dark incubation. Isotopic dilution of fed [^{14}C]choline by endogenous choline was thus potentially large.

Sugarbeet leaf protoplasts also oxidized [^{14}C]choline in darkness and light (Fig. 1*C*), but pea leaf protoplasts showed no detectable [^{14}C]choline oxidation (<4 pmol/100 μg of Chl per 4 hr). The lack of activity in pea was not a consequence of poor protoplast condition as the protoplasts were photosynthetically active (19 and 41 μmol of O_2/mg of Chl per hr in two experiments).

[^{14}C]Choline Oxidation by Protoplast Lysates. Spinach lysates were able to oxidize [^{14}C]choline; the oxidation rate was 25–50% lower than in the corresponding intact protoplasts, and the proportion of [^{14}C]betainal formed was higher. Upon

Table 1. Pool sizes of quaternary ammonium compounds in spinach leaves, protoplasts, and Percoll-purified chloroplasts

Exp.	Preparation	Pool size, nmol/100 μg of Chl		
		Choline	Betainal	Betaine
1	Leaves	—	0.37	119
	Protoplasts	31	0.27	46
2	Protoplasts	18	0.16	52
3	Protoplasts	28	0.42	69
	Chloroplasts	0.4	0.15	13

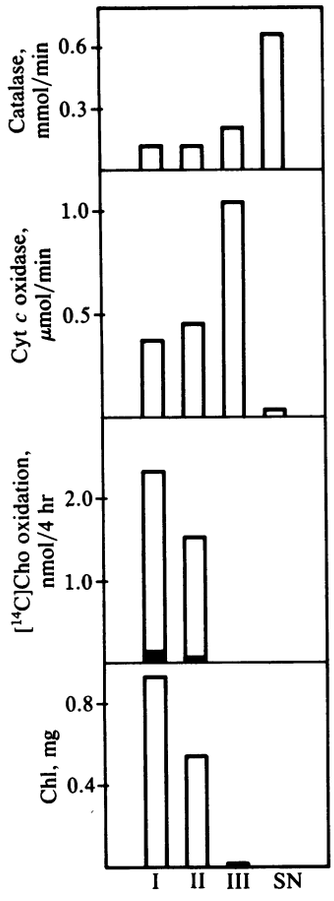

FIG. 2. Distribution of [^{14}C]choline-oxidizing activity and markers after differential centrifugation at 250 × *g* (bars I), 1000 × *g* (bars II), and 15,000 × *g* (bars III) of a spinach protoplast lysate. [^{14}C]Choline oxidation was assayed after a 4-hr dark incubation; solid and open parts of the [^{14}C]choline oxidation bars show conversion to betaine and betainal respectively. Enzyme activities and Chl contents are expressed as units per fraction. SN, supernatant; Cyt, cytochrome; Cho, choline.

differential centrifugation of lysates, choline-oxidizing activity all sedimented with Chl-containing fractions (Fig. 2). Chloroplasts purified by a Percoll step were greatly depleted in catalase and cytochrome *c* oxidase but rich in choline-oxidizing activity (Table 2). Note that apparent recoveries of [^{14}C]choline-oxidizing activity of >100% are a likely consequence of endogenous choline depletion in purified chloroplasts (Table 1). Light promoted [^{14}C]choline oxidation by spinach chloroplasts (Fig. 3 and Table 3); DCMU eliminated this promotion and also somewhat inhibited choline oxidation in darkness (Table 3). The products of [^{14}C]choline oxidation in spinach chloroplasts usually differed from those in corresponding intact protoplasts (compare Figs. 1 and 3): in chloroplasts, the proportion of [^{14}C]betainal was typically higher.

Chloroplasts from sugarbeet protoplasts had [^{14}C]choline-oxidizing activities similar to spinach chloroplasts (Table 3); pea chloroplasts lacked detectable [^{14}C]choline oxidation in light and darkness (<2 pmol/100 μg of Chl per 30 min).

Direct Evidence for Betainal Formation from Choline. Because the standard ^{14}C assay measured betainal indirectly (as an increase in betaine after oxidation), the presence of labeled betainal was verified in two ways. First, [^{14}C]choline oxidation products were eluted with HCl from the mixed-resin column and analyzed by TLC. A peak of ^{14}C that cochromatographed with authentic betainal was found when, and only when, the standard assay indicated that [^{14}C]betainal was present. Second, pairs of protoplast or chloroplast samples were given [^{14}C]choline or [*N-trimethyl*-$^{2}H_9$]choline. [*N-trimethyl*-$^{2}H_9$]Betainal (diisobutyl acetal derivative, m/z = 241) was quantified by fast atom bombardment MS. The levels of [^{14}C]betainal estimated by the standard assay were highly correlated with levels of [*trimethyl*-$^{2}H_9$]betainal measured directly in the sister samples (13 paired samples; r = 0.96, $P < 0.001$).

[^{14}C]Betainal Oxidation. Spinach protoplasts oxidized [^{14}C]betainal in darkness at least 10 times faster than they oxidized [^{14}C]choline (for four preparations, the mean [^{14}C]betainal oxidation rate ± SEM = 2.16 ± 0.64 nmol/100 μg of Chl per hr). Spinach protoplast lysates also readily oxidized [^{14}C]betainal; after differential centrifugation, activity was mainly in the chloroplast and supernatant fractions (Fig. 4 *Left*). Addition of 2 mM NAD^+ or $NADP^+$ to the supernatant increased [^{14}C]betainal oxidation 3- to 5-fold; this pyridine nucleotide-dependent activity could have come, at least in part, from broken chloroplasts (see chloroplast intactness values in the Fig. 4 legend). Chloroplasts purified by a Percoll step showed very active [^{14}C]betainal oxidation (Table 4); the dark oxidation rates for [^{14}C]betainal were more than 10 times those for [^{14}C]choline. Chloroplast oxidation of [^{14}C]betainal was increased about 3-fold by light; the light promotion was DCMU-sensitive (not shown). Anomalously high recoveries of [^{14}C]betainal-oxidizing activity in chloroplasts were consistently noted (Table 4). In a mixing experiment, 500 × *g* supernatant added back to washed chloroplasts depressed [^{14}C]betainal oxidation. This effect accounts for the aberrant recovery values, but its biochemical basis is obscure; it cannot be isotope dilution by endogenous betainal because the pool is so small (Table 1).

Table 2. Recovery of [^{14}C]choline-oxidizing activity and markers in Percoll-purified spinach chloroplasts

Exp.	Chloroplast intactness,* %	Chl, mg	[^{14}C]Choline oxidation† Betainal, nmol	[^{14}C]Choline oxidation† Betaine, nmol	Catalase, μmol/min	Cytochrome *c* oxidase, nmol/min
1 (Dark)						
Lysate	80	0.92	0.83	0.29	445	1370
Chloroplasts	90	0.29	1.03	0.31	7	14
2 (Light)						
Lysate	73	0.94	3.62	0.30	1065	1454
Chloroplasts	81	0.47	3.63	1.06	12	9

Data for Chl and enzyme activities are expressed as total units per sample.
*Estimated by light-dependent ferricyanide reduction in Exp. 1, and by phase-contrast microscopy in Exp. 2.
†Assayed after 4 hr of darkness for Exp. 1 and after 30 min of light for Exp. 2.

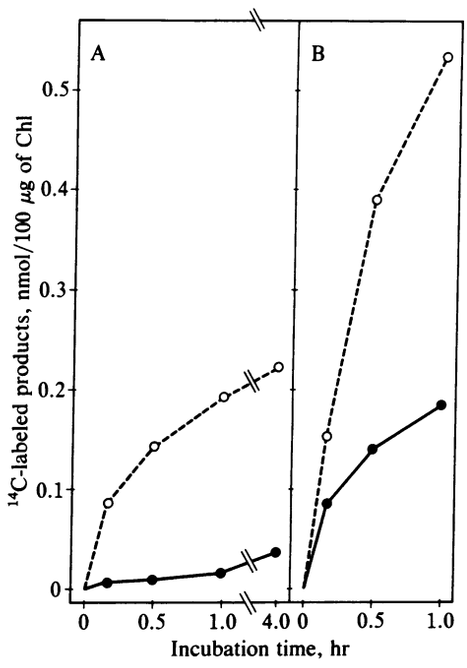

FIG. 3. Oxidation of [^{14}C]choline to betainal (○) and betaine (●) by spinach chloroplasts in the dark (*A*) and light (*B*). The chloroplasts were isolated from the protoplast preparation of Fig. 1. The chloroplast fraction was a washed 500 × *g* pellet; chloroplast intactness evaluated by phase-contrast was 78%.

Pea protoplasts oxidized [^{14}C]betainal at measurable rates in darkness (mean ± SEM for four preparations = 0.32 ± 0.19 nmol/100 μg of Chl per hr), even though—as noted above—they did not oxidize [^{14}C]choline. Pea lysates also oxidized [^{14}C]betainal, and upon differential centrifugation, most of the activity was recovered in the supernatant (Fig. 4 *Right*). The supernatant activity was increased up to 2-fold by 2 mM NAD^+; $NADP^+$ had little effect.

DISCUSSION

Sterilely prepared protoplasts were used as starting material for two reasons. First, the surface microflora of plants includes choline-oxidizing bacteria (23), and tests with leaf homogenates confirmed extensive microbial oxidation of [^{14}C]choline. Second, protoplast lysis is the method of choice for organelle isolation, and the *in vivo* activity of protoplasts can be directly compared with the *in vitro* activity of subcellular fractions. A drawback of protoplasts was that stressed leaves gave poor yields, precluding use of protoplasts to study *in vivo* regulation of the betaine pathway by stress.

Table 3. [^{14}C]Choline oxidation by spinach and sugarbeet chloroplasts (washed 500 × *g* pellets)

	[^{14}C]Choline oxidation, pmol/100 μg of Chl per 30 min			
	Spinach		Sugarbeet	
Treatment	Betainal	Betaine	Betainal	Betaine
Dark	100	11	70	154
Light	286	330	48	640
Dark + DCMU*	29	9	—	—
Light + DCMU*	38	9	112	120

*For DCMU treatment, chloroplasts were resuspended (≈ 100 μg of Chl per 50 μl) in lysis medium saturated with DCMU (≈180 μM).

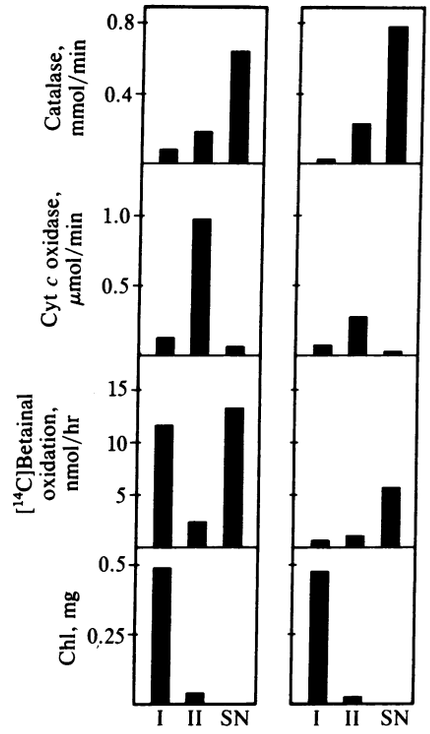

FIG. 4. Distribution of [^{14}C]betainal-oxidizing activity and markers after differential centrifugation at 500 × *g* (bars I) and 15,000 × *g* (bars II) of protoplast lysates from spinach (*Left*) and pea (*Right*). Particulate fractions were washed once in lysis medium. [^{14}C]-Betainal oxidation was assayed after a 1-hr incubation in darkness with no cofactor additions. Enzyme activities and Chl contents are expressed as units per fraction. Chloroplast intactness evaluated by phase-contrast was 75% for spinach and 53% for pea. SN, supernatant.

Betaine synthesis rates in our spinach leaves can be estimated as ≈0.5 nmol/100 μg of Chl per hr [taking the leaf betaine level as ≈120 nmol/100 μg of Chl (Table 1) and the growth rate as 10%/day]. The average rate of [^{14}C]choline oxidation by protoplasts was ≈60 pmol/100 μg of Chl per hr in darkness and about 3-fold higher in light. Allowing for isotopic dilution of absorbed [^{14}C]choline by endogenous choline, protoplasts clearly oxidized choline at physiological rates.

Our data for spinach show that the chloroplast is the sole site of choline oxidation to betainal and probably the main site of betainal oxidation to betaine. Similar results for sugarbeet imply that chloroplast choline oxidation is a general feature of chenopods. Failure to detect oxidation of choline or betainal by chloroplasts from pea—a species unable to accumulate betaine (5)—argues strongly that the activities found in spinach and sugarbeet chloroplasts are physiologically relevant. Chloroplast betaine synthesis in chenopods is consistent with synthesis of [^{14}C]betaine from labeled precursors by leaves, but not roots or hypocotyls, of sugarbeets (12, 24).

A chloroplast site for choline oxidation is interesting from the viewpoint of comparative biochemistry. In mammalian liver, choline oxidation is mitochondrial; the first enzyme is an inner-membrane flavoprotein (choline dehydrogenase), the second an NAD^+-linked betainal dehydrogenase present in the matrix (20, 25). Several microorganisms oxidize choline in an analogous way (8, 26), whereas in others a single soluble enzyme (choline oxidase) may catalyze both steps (27, 28). Thus, the enzymes of choline oxidation in cheno-

Table 4. Recovery of [^{14}C]betainal-oxidizing activity and markers in Percoll-purified spinach chloroplasts

Exp.	Chloroplast intactness,* %	Chl, mg	[^{14}C]Betainal oxidation,† nmol/hr	Catalase, μmol/min	Cytochrome *c* oxidase, nmol/min
1					
Lysate	59	0.67	27.7	671	2030
Chloroplasts	61	0.28	31.0	20	12
2					
Lysate	—	0.97	12.7	1540	2930
Chloroplasts	95	0.35	18.9	94	72

Data for Chl and enzyme activities are expressed as units per sample.
*Chloroplast intactness was estimated by phase-contrast microscopy.
†[^{14}C]Betainal oxidation was assayed in 1 hr of darkness.

pods may be unrelated evolutionarily to those in other kingdoms; this would preclude exploitation of sequence homologies at protein and nucleic acid levels in the isolation of chenopod genes for betaine synthesis. Furthermore, other plant families that accumulate betaine (grasses, composites) are not considered closely related to chenopods (29), so our findings for chenopods may not apply to these taxa.

Four further points can be made about the choline-oxidizing system of spinach. First, chloroplasts almost certainly oxidize choline in two discrete steps because label appeared in free betainal as well as in betaine. Since betainal is a trace intermediate *in vivo* (9, 10) but was often a major end product *in vitro*, it may be that protoplast and/or chloroplast isolation disengages the second oxidation step from the first. Disengagement rather than outright activity loss of the second step seems likely because, when assayed directly by supplying [^{14}C]betainal, the second step was far more active than the first. Analogous disengagement of choline oxidation steps can occur in isolated liver mitochondria (25). Second, it is likely that stress increases betaine synthesis by acting on the first step, as this step is rate-limiting in both isolated chloroplasts and intact protoplasts. Stress may increase the level of choline-oxidizing enzyme, because short-term osmotic stress did not promote the [^{14}C]choline-oxidizing activity of protoplasts. Third, we confirm a report (30) of pyridine nucleotide-dependent oxidation of betainal by a spinach supernatant. However, we also found supernatant activity in pea, indicating that the soluble spinach activity could—at least in part—be due to an aldehyde-oxidizing system unrelated to betaine synthesis. Cytosolic dehydrogenase(s) in mammalian liver can oxidize betainal (31). Last, the DCMU-sensitive light enhancement of choline oxidation connotes some link between betaine synthesis and photosynthetic electron transport. This link could be direct [e.g., via oxidant generation (32)] or indirect [via enzyme activation, stromal pH, or substrate uptake (33)].

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1. Wyn Jones, R. G., Storey, R., Leigh, R. A., Ahmad, N. & Pollard, A. (1977) in *Regulation of Cell Membrane Activities in Plants*, eds. Marrè, E. & Ciferri, O. (Elsevier, Amsterdam), pp. 121–135.
2. Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. & Somero, G. N. (1982) *Science* **217**, 1214–1222.?
3. Mohammad, F. A. A., Reed, R. H. & Stewart, W. D. P. (1983) *FEMS Lett.* **16**, 287–290.
4. Galinski, E. A. & Truper, H. G. (1982) *FEMS Lett.* **13**, 357–360.
5. Wyn Jones, R. G. & Storey, R. (1981) in *Physiology and Biochemistry of Drought Resistance in Plants*, eds. Paleg, L. G. & Aspinall, D. (Academic, Sydney, Australia), pp. 171–204.
6. Hanson, A. D. & Hitz, W. D. (1982) *Annu. Rev. Plant Physiol.* **33**, 163–203.
7. Hanson, A. D. & Grumet, R. (1984) in *Cellular and Molecular Biology of Plant Stress*, eds. Key, J. L. & Kosuge, T. (Liss, New York), pp. 71–92.
8. Le Rudulier, D., Strom, A. R., Dandekar, A. M., Smith, L. T. & Valentine, R. C. (1984) *Science* **224**, 1064–1068.
9. Hanson, A. D. & Scott, N. A. (1980) *Plant Physiol.* **66**, 342–348.
10. Coughlan, S. J. & Wyn Jones, R. G. (1982) *Planta* **154**, 6–17.
11. Ladyman, J. A. R., Hitz, W. D. & Hanson, A. D. (1980) *Planta* **150**, 191–196.
12. Hanson, A. D. & Wyse, R. (1982) *Plant Physiol.* **70**, 1191–1198.
13. Zeevaart, J. A. D. (1983) *Plant Physiol.* **71**, 477–481.
14. Edwards, G. E., Robinson, S. P., Tyler, N. J. C. & Walker, D. A. (1978) *Plant Physiol.* **62**, 313–319.
15. Mills, W. R. & Joy, K. W. (1980) *Planta* **148**, 75–83.
16. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15.
17. Luck, H. (1965) in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Academic, New York), pp. 886–888.
18. Hodges, T. K. & Leonard, R. T. (1974) *Methods Enzymol.* **32**, 397–398.
19. Lilley, R. M., Fitzgerald, M. P., Rienits, K. G. & Walker, D. A. (1975) *New Phytol.* **75**, 1–10.
20. Haubrich, D. R. & Gerber, N. H. (1981) *Biochem. Pharmacol.* **30**, 2993–3000.
21. Hitz, W. D. & Hanson, A. D. (1980) *Phytochemistry* **19**, 2371–2374.
22. Jamieson, G. C., Myers, A. C., Fitch, W. L. & Rhodes, D. (1984) in *Proceedings, 32nd Annual Conference on Mass Spectrometry and Allied Topics* (Am. Soc. Mass Spectrom., East Lansing, MI), pp. 830–831.
23. Kortstee, G. J. J. (1970) *Arch. Mikrobiol.* **71**, 235–244.
24. Hanson, A. D. & Rhodes, D. (1983) *Plant Physiol.* **71**, 692–700.
25. DeRidder, J. J. M. & VanDam, K. (1975) *Biochim. Biophys. Acta* **408**, 112–122.
26. Nagasawa, T., Kawabata, Y., Tani, Y. & Ogata, K. (1976) *Agric. Biol. Chem.* **40**, 1743–1749.
27. Ikuta, S., Imamura, S., Misaki, H. & Horiuti, Y. (1977) *J. Biochem.* **82**, 1741–1749.
28. Ohta-Fukuyama, M., Miyake, Y., Emi, S. & Yamano, T. (1980) *J. Biochem.* **88**, 197–203.
29. Jones, S. A. & Luschinger, A. E. (1979) *Plant Systematics* (McGraw Hill, New York).
30. Pan, S.-M., Moreau, R. A., Yu, C. & Huang, A. H. C. (1981) *Plant Physiol.* **67**, 1105–1108.
31. Wilken, D. R., McMacken, M. L. & Rodriquez, A. (1970) *Biochim. Biophys. Acta* **216**, 305–317.
32. Rabinowitch, H. D. & Fridovich, I. (1983) *Photochem. Photobiol.* **37**, 679–690.
33. Buchanan, B. B. (1980) *Annu. Rev. Plant Physiol.* **31**, 341–374.