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Betaine Synthesis from Radioactive Precursors in Attached, Water-stressed Barley Leaves[1, 2]

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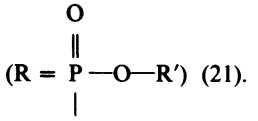
ABSTRACT

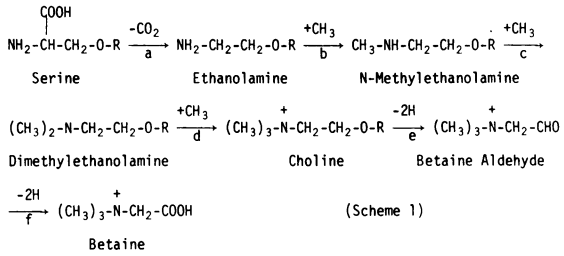
In wilted barley leaves, betaine accumulates at about 200 nanomoles per 10 centimeters leaf per day. Results with ^{14}C-labeled precursors were qualitatively and quantitatively consistent with *de novo* synthesis of this betaine from serine via ethanolamine, choline, and betaine aldehyde and indicated that water stress may increase the activities of all steps in this pathway except the last.

Doses (1 micromole) of each ^{14}C-labeled precursor were supplied as droplets to the tips of attached, 10-centimeter, second-leaf blades of turgid and wilted plants, and the incorporation of ^{14}C into betaine was followed. From the rates of betaine labeling, estimates were made of the potential capacities (nanomoles per 10 centimeters leaf per day) for the methylation and oxidation steps. Labeling of betaine from [^{14}C]choline, [^{14}C]ethanolamine, and [^{14}C]serine was about 7- to 10-fold greater in leaves wilted for 2 days than in turgid leaves, whereas label from [^{14}C]betaine aldehyde appeared in betaine at about the same rate in both turgid and wilted leaves. In leaves wilted for 2 days, the potential capacities for converting [^{14}C]-ethanolamine, [^{14}C]choline, and [^{14}C]betaine aldehyde to betaine all approached or exceeded the rate of betaine accumulation (about 200 nanomoles per 10 centimeters leaf per day); in turgid leaves, only the potential for converting betaine aldehyde to betaine exceeded this rate. The rate of conversion of [^{14}C]ethanolamine to betaine increased 4-fold after 6 to 10 hours of wilting, which was soon enough to account for the onset of betaine accumulation.

Betaine (*N,N,N*-trimethylglycine) occurs in turgid leaves of cultivated barley, wheat, and rye and of their wild relatives, at a concentration of about 20 μmol/g dry weight; the betaine concentration increases severalfold in most of these plants upon moderate water stress (4, 8, 22) and upon salination (15, 22). Betaine is also found in several wild salt-marsh plants, but at far higher concentrations (≥400 μmol/g dry weight under saline conditions), and for such halophytic species betaine is very likely of adaptive significance, perhaps as a cytoplasmic osmoticum (*e.g.* refs. 16 and 23). Are the more modest accumulations of betaine elicited in cereal crops by water and salt stress also of adaptive value? This question must be answered before selection for high capacity to accumulate betaine could be recommended in breeding crops for adaptation to saline or drought-prone environments (3). One possible approach to the question is to search for and exploit intraspecific variation for betaine content; an alternative, presented here, is to identify the metabolic causes for, and consequences of, betaine accumulation.

Qualitative evidence, based mainly on the metabolism of tracer amounts of [^{14}C]formate in detached barley leaf tissue (4), showed that the betaine which accumulated in wilted leaves probably did so as a result of complete *de novo* synthesis from 1- and 2-carbon precursors (steps b through f in Scheme 1). The pathway of Scheme 1 was first demonstrated in plants by Delwiche and Bregoff (2), using unstressed leaf tissue of *Beta vulgaris*. It is not clear whether this pathway in plants involves the free bases themselves (R = H) or some bound forms of these compounds





In the work reported here, we used attached leaf tissue; we sought to confirm the operation of the entire pathway (steps a through f) in stressed leaves, and to find which of the steps increase in activity during stress.

MATERIALS AND METHODS

Plant Material and Stress Regimes. Spring barley (*Hordeum vulgare* L., cv. Proctor CI 11806) plants were grown in growth chambers to the three-leaf stage and water-stressed as described (4). Briefly, plants were either grown in Perlite and stressed by flooding the Perlite with a solution of PEG[3] 6000 (osmotic potential, about −19 bars) or were grown in a soil mix (5) and stressed by withholding irrigation.

Rice (*Oryza sativa* L., California line ESD-7-1) plants were grown in Perlite or soil to the five- or six-leaf stage and stressed as above. Growth chamber conditions for rice were: 19-h day; photosynthetic irradiance, 23 mw cm^{-2}; day/night temperature, 27/21 C; day and night RH, 70 to 80%.

^{14}C-labeled Precursors. The L-[U-^{14}C]serine (171 mCi/mmol), [2-^{14}C]ethan-1-ol-2-amine (44 or 50 mCi/mmol), and [methyl-^{14}C]choline (52 or 59 mCi/mmol) were from Amersham Corp. Before use in leaf-feeding experiments, the [^{14}C]choline was puri-

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[3] Abbreviations: PEG: polyethylene glycol 6000; MCW: methanol-chloroform-water; ψ_{leaf}: leaf water potential; TLE: thin-layer electrophoresis.

fied by application to a 1-ml column of Bio-Rex 70-H^+; after washing the column with H_2O, the [^{14}C]choline was eluted with 1 N HCl. The [methyl-^{14}C]betaine aldehyde (59 mCi/mmol) was synthesized from [methyl-^{14}C]choline by using rat liver mitochondria (10, 12). The ^{14}C-labeled precursors were mixed with unlabeled carrier for most leaf-feeding experiments, to give final specific radioactivities in the range 0.16 to 0.80 μCi/μmol.

Leaf-feeding Experiments. For barley, ^{14}C-labeled precursors were supplied to the second leaf in 1-μmol doses of precisely known specific radioactivity, by a feeding technique similar to that given by Hanson and Tully (6). The second leaf blade (length, 20–25 cm) was left attached to the plant and trimmed to 10 cm in length. The basal 13-mm section of the removed portion of the leaf was used to estimate ψ_{leaf} with a Wescor HR-33T dew point microvoltmeter (6). The 1-μmol dose of ^{14}C-labeled precursor, dissolved in 3 μl K-phosphate (20 mM, pH 7.0), was applied to the cut end of the 10-cm blade. The 3-μl drop was drawn into the leaf within 2 to 10 min, after which time the cut blade end was sealed with Vaseline. Barley plants were incubated with ^{14}C-labeled precursors in the growth chamber during the light period for up to 9 h, and then the 10-cm second-leaf blade sections were harvested for extraction. Experiments with rice were similar, except that later leaves (four to six) were fed.

The amounts of ^{14}C-labeled precursors remaining in the free space of barley leaf blades were checked after 1 h of incubation as follows. The 10-cm blade sections were cut off, sliced into 5-mm segments, and shaken for 10 min in 10 ml of a 1 mM solution of the appropriate unlabeled precursor. Samples (0.1 ml) were taken at intervals for scintillation counting.

The distribution of label within the second leaf of barley was checked after 3 h incubation with [^{14}C]ethanolamine as follows. The entire second leaf (10 cm blade + sheath) was harvested and divided into 10- or 20-mm segments, which were then freeze-dried and assayed for ^{14}C content by scintillation counting after combustion in a Packard Tri-Carb sample oxidizer. Loss of $^{14}CO_2$ and other volatile ^{14}C-labeled products was checked in a similar experiment by passing an air stream (about 4 l/h) over three plants which had been fed with [^{14}C]ethanolamine and enclosed in a 20-liter glass tank. The exhaust gas stream from the tank was passed through traps of 0.1 N HCl and Carbosorb-II, as described by Ladyman *et al.* (10).

Extraction and Separation of Metabolites. In experiments with [^{14}C]choline and [^{14}C]ethanolamine, leaf blade tissue was extracted with a MCW procedure (5). In the [^{14}C]serine experiment, the MCW procedure was modified to accommodate an initial killing/extraction step in 2 ml boiling isopropanol. Extraction with MCW caused extensive degradation of [^{14}C]betaine aldehyde, so, in experiments with this precursor, blade tissue was extracted with cold 0.5 N formic acid. Blades were ground with sand in conical centrifuge tubes with 2 ml frozen 0.5 N formic acid. After thawing, tubes were centrifuged in a bench centrifuge; the supernatant was drawn off, and the residue was re-extracted with 2 ml 0.5 N formic acid at room temperature. The pooled supernatants then were clarified by centrifugation at 10,000*g* for 10 min and passage through Millipore filters (0.45 μm pore size).

The aqueous phases from MCW extractions and filtered formic acid extracts were evaporated to dryness in a stream of N_2 at 60 to 70 C and re-dissolved in 0.2 to 1.0 ml H_2O. To separate ^{14}C-labeled metabolites from the ^{14}C-labeled precursors supplied, the following three combinations (*a*, *b*, and *c*) of methods then were used. (*a*) TLE alone. Samples equivalent to about one-fifth blade were mixed with appropriate marker compounds and applied to 3-cm origins on 5- × 20-cm strips of either ITLC-SA (Gelman Inst. Co.) or 0.1-mm cellulose precoated TLC plates (E. Merck). Electrophoresis was on a Shandon model Q11 apparatus, with circulating coolant at about 5 C or about 12 C. The ITLC plates were run in 1.5 N formic acid at 2 kv for 10 to 16 min; the cellulose plates were run in 70 mM Na-tetraborate, pH 9.3, for 10 to 14 min. (*b*) One ion-exchange step + TLE. Samples were passed through a 1-ml column containing a mixture of AG 1-OH^- and Bio-Rex 70-H^+, as described (8). This column retained essentially all charged species except betaine. The effluent, containing betaine and neutral compounds, then was subjected to TLE on ITLC, as above. (*c*) Two ion-exchange steps + TLE. The first ion-exchange step was the same as that in *b*; in the second step, the effluent from the mixed-resin column was applied to a 1-ml column of AG 50-H^+. This column bound the betaine; after washing to remove uncharged compounds, the betaine was eluted with 4 N NH_3 (8). The eluate was subjected to TLE on ITLC, as above. Use of either of the ion-exchange procedures (*b* or *c*) improved the precision with which small amounts of [^{14}C]betaine could be measured in the presence of a large excess of unmetabolized ^{14}C-labeled precursor.

In all experiments, blanks (containing unlabeled blades to which 1 μmol of ^{14}C-labeled precursor had been added immediately before extraction) were included as checks for chemical degradation of the ^{14}C-labeled precursors during processing, particularly for any oxidation of choline and betaine aldehyde to betaine. The above procedures resulted in very little degradation of ^{14}C-labeled precursors, and the amount of ^{14}C activity in betaine that could be ascribed to chemical oxidation was negligible.

Chemical Analyses. Betaine was assayed by the combined ion-exchange/pyrolysis-GC method described elsewhere (8). Ethanolamine was determined by isotope dilution, by using an addendum of 10 nCi [^{14}C]ethanolamine (44 mCi/mmol)/leaf blade. Blade tissue was extracted in MCW, and the aqueous phase was passed through a 1-ml column of AG 1-OH^- to remove amino acids. The eluate from this column then was subjected to TLE on ITLC, as above. The ethanolamine zone was located by autoradiography and eluted from the ITLC strips (6). The ^{14}C content of eluates was found by scintillation counting, and the amine content was estimated with the Folin reagent (1).

Identification and Determination of ^{14}C-labeled Metabolites. Radioactive products were located on TLE plates by autoradiography and identified by reference to authentic marker compounds. The identity and purity of the [^{14}C]betaine zone on ITLC-TLE were checked for control and stressed leaf samples in experiments with all ^{14}C-labeled precursors, as follows. The [^{14}C]betaine zones from ITLC-TLE were eluted with 30% (v/v) ethanol and rerun in TLE on cellulose plates in Na-tetraborate as above and in TLC on Silica Gel G plates in methanol-acetone-concentrated HCl (90:10:4, v/v). In all cases, the original [^{14}C]betaine zone migrated with authentic betaine and was chromatographically homogeneous, in the two additional separatory systems. The identity of [^{14}C]phosphorylcholine was checked by hydrolysis with 4 N HCl at 100 C for 18 h; the single labeled hydrolysis product co-electrophoresed with choline in TLE.

The ^{14}C activity in TLE plate zones was determined by cutting out (ITLC) or scraping off (cellulose) the zones, shaking them in scintillation vials with 1 ml 30% (v/v) ethanol for 5 min, followed by scintillation counting in a dioxane-based scintillant (10 ml). Counting efficiencies were 81% for the ITLC samples and 86% for the cellulose samples.

RESULTS AND DISCUSSION

Barley leaves can accumulate betaine at a rate of about 200 nmol/10 cm leaf·day upon wilting (4); two considerations follow. (*a*) If the accumulating betaine is made via the pathway outlined in Scheme 1, then the *in vivo* potentials of all steps in this pathway must equal or exceed a rate of 200 nmol/10 cm leaf·day in water-stressed leaves. (*b*) If any step in this pathway limits the accumulation of betaine in unstressed leaves, then the *in vivo* potential of that step should increase markedly upon wilting, no later than the onset of betaine accumulation. Further, such a step might be

expected to have an *in vivo* potential well below 200 nmol/10 cm leaf·day in turgid leaves.

In principle, an estimate of the *in vivo* potential of any step in a biosynthetic pathway can be made by supplying the appropriate ^{14}C-labeled reactant at known specific radioactivity and measuring the incorporation of ^{14}C into the product of that step and/or subsequent steps in the path. To make such estimates for the steps of betaine biosynthesis in attached leaf tissue, both general and special conditions must be met. The general conditions include: (*a*) that addition of the ^{14}C-labeled precursor not perturb the metabolism of the leaf; (*b*) that the ^{14}C-labeled precursor mix freely with endogenous pools of precursors and that the sizes and turnover rates of such endogenous pools not appreciably reduce the specific radioactivity of the added ^{14}C-labeled precursor during the experiment; (*c*) that the immediate product of the ^{14}C-labeled precursor be further metabolized only by subsequent steps in the pathway and that the final pathway product (betaine) behave as an unmetabolized end product. Conditions special to attached leaves are: (*d*) that the ^{14}C-labeled precursor be readily taken up and evenly distributed throughout the leaf; (*e*) that [^{14}C]betaine and other labeled pathway products not be exported from the leaf during the experiment. Evidence that these conditions hold in barley leaves, at least to a first approximation, is given in the first three sections below. Estimates then are made for the potential *in vivo* capacities of several sequences of pathway steps, starting with the final step and working backwards along the path.

Absorption and Distribution of ^{14}C-labeled Precursors. Washout curves for control and wilted blade tissue, obtained after 1 h incubation with [^{14}C]choline and [^{14}C]ethanolamine, are given in Figure 1A. Similar curves were found for [^{14}C]betaine aldehyde and [^{14}C]serine. In all cases, a rapid efflux of ^{14}C in the first 2 to 4 min of washing was followed by a slower, steady release of label. The initial rapid efflux, which accounted for 5 to 15% of the ^{14}C label supplied, most probably represented unabsorbed precursor remaining in free space plus some label release from cut surfaces. It is likely that at least 85% of the supplied ^{14}C-labeled precursor doses had been removed from the free space of the blade within 1 h.

Figure 1B shows label distribution from fed [^{14}C]ethanolamine along control and wilted blades and sheaths after 3 h incubation.

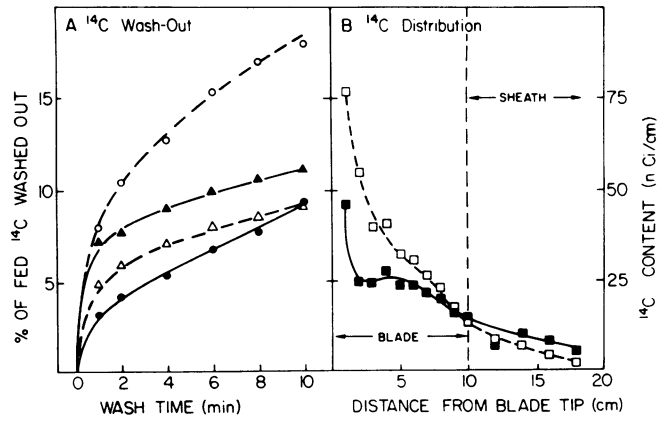

FIG. 1. Uptake and distribution of ^{14}C-labeled precursors in second leaves of barley. A, wash-out curves for blades from unstressed plants (△, ○) and from plants wilted with PEG for 2 days (▲, ●), 1 h after supplying 1 μmol [^{14}C]choline (○, ●; 0.75 μCi) or [^{14}C]ethanolamine (△, ▲; 0.71 μCi). B, curves showing location of ^{14}C within leaves of well-watered soil-grown plants (□- - -□) and soil-grown plants wilted for 2 days (■——■), 3 h after supplying 1 μmol (0.49 μCi) [^{14}C]ethanolamine. Recoveries of fed ^{14}C from the 10-cm blade sections were: well-watered, 73%; wilted, 50%. In both A and B, ψ_{leaf} was about −5 bars in unstressed leaves and −15 to −20 bars in wilted leaves.

Label within the 10-cm blade section was clearly not confined to the fed tip itself; 31% (control) and 40% (wilted) of the ^{14}C present in the blades was recovered from their basal halves. Although not uniform, these distribution patterns are sufficiently even to permit quantitative treatment of attached-leaf labeling data and are sufficiently similar to allow valid comparison of control and wilted treatments. With all ^{14}C-labeled precursors, the recoveries of label from wilted blades were lower than those from control blades; the results of Figure 1B (50% *versus* 73% recoveries, respectively) are typical. For [^{14}C]ethanolamine, disappearance of ^{14}C from turgid and wilted blades was attributable mainly to migration of part of the fed 3-μl droplet down the xylem to nonharvested parts (7). Loss of $^{14}CO_2$ and ^{14}C-labeled amines was negligible.

Free Pools of Endogenous Precursors. The contents of serine, ethanolamine, and choline fell within the range 0.04 to 0.18 μmol/10 cm leaf for turgid and wilted barley leaves (Table I). Betaine aldehyde could neither be detected chemically after TLE (Table I) nor identified as a labeled product of tracer [^{14}C]choline or [^{14}C]ethanolamine metabolism (not shown). However, when a trapping pool of 1 μmol unlabeled betaine aldehyde was supplied together with tracer [^{14}C]choline (396 nCi; 8 nmol) to a leaf blade wilted for 3 days, 28 nCi of [^{14}C]betaine aldehyde was recovered after 6 h incubation. The label in [^{14}C]betaine was reduced from 68 to 27 nCi by inclusion of the trapping pool. These results are consistent with the presence of a small free pool of betaine aldehyde that is quite accessible to applied betaine aldehyde.

Because the endogenous levels of the precursors were generally below 0.1 μmol/10 cm leaf, a standard dose of 1 μmol was chosen for added ^{14}C-labeled precursors. A 1-μmol dose of ^{14}C-labeled precursor never caused visible damage nor gave rise to ^{14}C-labeled products, unlike those observed when only a tracer amount of ^{14}C-labeled precursor was fed. In the cases of ethanolamine, choline, and betaine aldehyde, turnover of the endogenous pools was fairly slow. With these precursors, 30 to 70% of the added 1-μmol dose of ^{14}C-labeled precursor remained unmetabolized in the second

Table I. *Levels of Probable Precursors of Betaine in Turgid and Wilted Barley Leaf Tissue*

Water stress was imposed with PEG for choline estimates; in all other cases, irrigation was withheld. Literature values for seedling leaves were converted to units of μmol/10 cm leaf by taking a 10-cm second-leaf segment to have a fresh weight of 100 mg, or a dry weight of 20 mg, or to be one-half of an entire second-leaf blade.

Precursor	Time of Water Stress	Ψ_{leaf}	Precursor Content	Barley Cultivar	Reference
	days	*bars*	*μmol/10 cm leaf*		
Serine	0	−3	0.04	Prior	14
	3	−16	0.04	Prior	
Ethanolamine	0	−3	0.05	Proctor	
	2	−23	0.05	Proctor	
Choline	0	−3	<0.05	Proctor	4
	3	−26	0.05–0.10	Proctor	
	0		0.08	California Mariout	22
			0.18	California	
	2			Mariout	
Betaine aldehyde	0	−5	<0.01[a]	Proctor	
	2	−24	<0.01[a]	Proctor	

[a] Estimated semiquantitatively on TLE plates sprayed with $AgNO_3$/NaOH reagent, by comparison to authentic betaine aldehyde standards; detection limit on TLE plates was 0.01 μmol/10 cm leaf.

leaf blade at the end of experiments (6 or 9 h); longer experiments (up to 24 h, results not shown) indicated that ^{14}C-labeled precursors remaining at 6 or 9 h were eventually metabolized and, therefore, were unlikely to have been sequestered. Serine turned over much faster, so that very little free [^{14}C]serine remained after only 3 h (see below).

Metabolism and Export of Pathway Intermediates. When [^{14}C]betaine aldehyde, [^{14}C]choline, and [^{14}C]ethanolamine were supplied, betaine was a major labeled product and little label built up in free pools of the presumed intermediates (see Table II for representative results with [^{14}C]ethanolamine). We have reported elsewhere that betaine is not appreciably degraded in leaves or in other shoot tissues of turgid and wilted barley plants (10). Thus, it was usually necessary to measure only ^{14}C appearance in betaine to assess the potential capacities of various sequences of pathway steps.

For wilted plants, we have shown that phloem translocation had essentially ceased after 2 days (18), making [^{14}C]betaine export from wilted leaves improbable. For turgid plants, possible export of [^{14}C]betaine from second-leaf blades to other shoot organs during experiments was investigated as follows, for experiments in which [^{14}C]choline or [^{14}C]ethanolamine was supplied. In the experiment of Figure 2B, and in one similar to that of Figure 3A, the shoot tissues remaining after removal of the second leaf blades were taken at the 3-h and 9-h harvests for [^{14}C]betaine determination. The [^{14}C]betaine contents of these shoot tissues at 3 and 9 h were 0.2 and 1.6 nCi, respectively, and 0.5 and 1.2 nCi, respectively, for [^{14}C]choline and [^{14}C]ethanolamine experiments. The increases between 3 and 9 h were only 20 to 30% of the simultaneous accumulations of [^{14}C]betaine in the second-leaf blades. Thus, during these 9-h experiments, only a small amount of [^{14}C]betaine could have been exported from turgid blades because at least part of the increases in [^{14}C]betaine in the shoots must have arisen from *in situ* conversion of [^{14}C]choline and [^{14}C]ethanolamine. Accordingly, [^{14}C]betaine export from turgid blades was taken as negligible in calculations of *in vivo* capacities of pathway steps.

Metabolism of [methyl-^{14}C]Betaine Aldehyde. Trace amounts (about 3 nmol) of [^{14}C]betaine aldehyde were quantitatively oxidized to betaine by turgid and wilted barley leaves within 1 h; no label appeared in choline or other metabolites. A 1-μmol dose of [^{14}C]betaine aldehyde was also rapidly converted to betaine in both turgid and wilted barley leaves (Fig. 2A); neither [^{14}C]choline nor any other metabolite was found. The highest rates of [^{14}C]-betaine synthesis were during the 1st h of incubation and were similar in turgid and wilted leaves; if betaine synthesis is taken to proceed at the same rate throughout a day/night cycle, these rates correspond to 1,500 and 1,300 nmol/10 cm leaf·day, respectively. These estimates of potential capacity, and those made below with other ^{14}C-labeled precursors, are minimum estimates insofar as they assume specific radioactivities of endogenous precursors of betaine equal to that of the fed ^{14}C-labeled precursor. Although the rate of [^{14}C]betaine synthesis declined with time, and more sharply so in the unstressed leaves, it never fell below the equivalent of 350 nmol/10 cm leaf·day. There is an *in vivo* potential for betaine aldehyde oxidation in unstressed leaves which is sufficient to account for betaine accumulation upon wilting; there is little increase in this potential in wilted leaves. It follows that the potential for betaine aldehyde oxidation is unlikely to limit betaine accumulation in barley.

The potential for oxidizing betaine aldehyde was sought also in turgid and wilted leaves of rice, a cereal which contains very little betaine before or after wilting (8). After a 9-h incubation with 1 μmol betaine aldehyde, oxidation was not detectable in turgid leaves and was only just detectable in wilted leaves, at a rate corresponding to less than 100 nmol/10 cm leaf·day. This suggests that the *in vivo* betaine aldehyde-oxidizing potential of barley is physiologically significant to betaine accumulation and is not merely a nonspecific aldehyde oxidase active against applied substrates.

Metabolism of [methyl-^{14}C]Choline. When 1 μmol [^{14}C]choline was supplied to turgid and wilted leaves, the two main H_2O-soluble metabolites were betaine and phosphorylcholine. Wilting had little effect on ^{14}C incorporation into phosphorylcholine (not shown) but increased ^{14}C incorporation into betaine markedly (Fig. 2B). In turgid leaves, the rate of [^{14}C]betaine synthesis was low and fairly constant throughout the incubation and corresponded to about 35 nmol/10 cm leaf·day. In wilted leaves, the rate of [^{14}C]betaine synthesis rose during the incubation from 200 to 350 nmol/10 cm leaf·day. In similar experiments with turgid and wilted rice leaves, no oxidation of choline was detected after 9 h incubation, implying that the choline-oxidizing potentials measured in barley were physiologically significant.

The *in vivo* potential for choline conversion (via betaine aldehyde) to betaine appears adequate in wilted barley leaves to support betaine accumulation but inadequate in turgid leaves. The oxidation of choline to betaine aldehyde, therefore, could be one stress-sensitive control point in betaine biosynthesis.

Table II. *Metabolism of [^{14}C]Ethanolamine by Turgid and Wilted Barley Leaves*

Control leaf sections (Ψ_{leaf}, about −2 bars) were from well-watered plants, and stressed leaf sections (Ψ_{leaf}, about −27 bars) were from plants wilted for 3 days with PEG. After 9 h incubation with [^{14}C]ethanolamine (0.42 μCi/μmol), the labeled metabolites in the MCW aqueous phase were separated by TLE in the ITLC-formic acid system.

[^{14}C]Ethanolamine Supplied	Treatment	Total ^{14}C in MCW Aqueous Phase	^{14}C Distribution among TLE Plate Zones[a]					
			Ethanolamine	Monomethylethanolamine	Dimethylethanolamine	Choline/Betaine Aldehyde	Betaine	Other[b]
μmol/10 cm leaf		*nCi/10 cm leaf*	*% ^{14}C recovered*					
1.0	Control	330	78.6	0.8	0.5	0.5[c]	0.6	6.6
	Stress	214	63.2	1.3	0.4	1.6[c]	6.1	11.9
0.45	Control	82	76.7	1.1	0.9	1.4	0.9	10.9
	Stress	57	48.1	2.1	2.0	4.4	17.6	14.5

[a] Recovery of applied ^{14}C from TLE plates averaged 88%; the named TLE plate zones are arranged in order of decreasing mobility, from ethanolamine (highest) to betaine (lowest).

[b] Other products (not identified) included one migrating slightly ahead of betaine, and at least two products that remained close to the origin, in the region in which phosphorylated bases ran.

[c] Betaine aldehyde shown to be absent by TLE in cellulose/Na-tetraborate system.

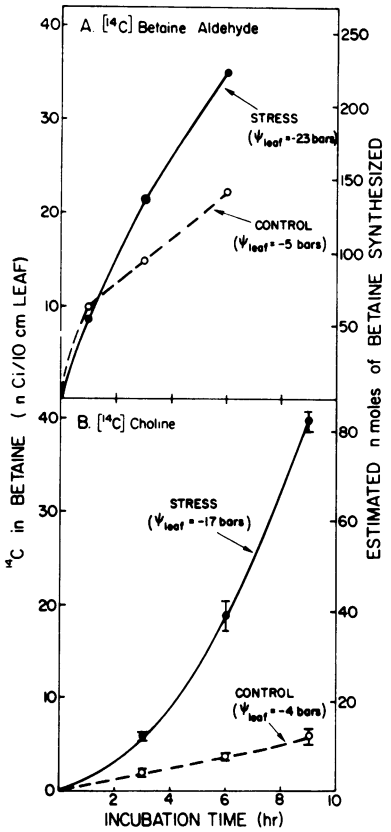

FIG. 2. Time courses for the conversion of [^{14}C]betaine aldehyde (A) and [^{14}C]choline (B) to betaine, in second leaves of barley plants. One μmol ^{14}C-labeled precursor was fed to 10-cm sections of turgid, control, and wilted leaves from plants stressed for 2 days with PEG. The specific radioactivities of fed precursors (betaine aldehyde, 0.16 μCi/μmol; choline, 0.48 μCi/μmol) were used to estimate the quantity of betaine formed (right-hand vertical axis). Leaf-water potentials at the start of the experiments are given in parentheses next to each curve. Each point is the mean of duplicate leaves in A and of triplicate leaves ± SE in B. The experiments of A and B were repeated, with similar results.

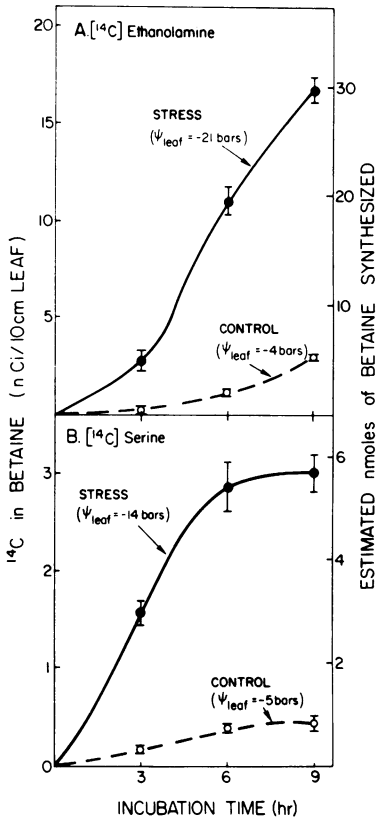

FIG. 3. Time courses for the conversion of [^{14}C]ethanolamine (A) and [^{14}C]serine (B) to betaine. One μmol ^{14}C-labeled precursor was fed to turgid or wilted leaves, as in Figure 2. Specific radioactivities of ^{14}C-labeled precursors: ethanolamine, 0.56 μCi/μmol; serine, 0.80 μCi/μmol. Points are means of triplicate determinations ± SE. The experiment of A was repeated, with similar results.

Metabolism of [2-^{14}C]Ethanolamine. The distributions of ^{14}C among free bases and other H_2O-soluble metabolites of turgid and wilted leaves are given in Table II. Because little ^{14}C accumulated in free choline (or the free methylethanolamines), the potential of the reaction sequence which produces free choline from ethanolamine is unlikely to exceed the potential for oxidizing choline in either turgid or wilted leaves. It is clear that some ^{14}C label must have been present also in bound forms of the bases such as CDP-bases or phosphatides (17, 21), which were not analyzed. Partitioning of ^{14}C-methylation products to these compounds could have been more active in turgid than in wilted leaves, resulting in a lower accumulation of ^{14}C in betaine without change in total methylating activity. Experiments with [^{14}C]formate, however, indicated that the sum of *N*-methylation reactions was about 3-fold greater in wilted than in turgid leaf tissue (4). It follows that the potential of the methylating reaction sequence, like the potential for oxidizing choline, increases upon stress because wilted leaves converted about 10-fold more [^{14}C]ethanolamine to betaine.

The difference in rates of [^{14}C]betaine synthesis is shown in more detail in Figure 3A. In turgid leaves, the rate of [^{14}C]betaine synthesis increased with time but rose no higher than about 25 nmol/10 cm leaf·day. In wilted leaves the rate increased and then fell slightly, reaching a maximum (between 3 and 6 h) corresponding to about 140 nmol/10 cm leaf·day. In assessing the total activity of the methylation steps, the small amount of ^{14}C present in free choline should be added to that in betaine; the amount of [^{14}C]choline was about equal to that of [^{14}C]betaine in turgid plants and was about one-fourth that of [^{14}C]betaine in wilted plants (Table II). Potentials for methylating ethanolamine, therefore, may be assigned minimum values of about 50 and 180 nmol/10 cm leaf·day for turgid and wilted plants, respectively.

From the sizes of free choline pools (Table I), from the incorporation of ^{14}C from [^{14}C]ethanolamine into free choline (Table II) and into betaine (Fig. 3A), and from the rate of betaine accumulation (Fig. 4B), it can be calculated that the specific radioactivity of the extracted [^{14}C]choline in wilted leaves was most probably lower at the end of a 9-h experiment than that of the betaine which accumulated during the experiment (<0.05 *versus* about 0.2 nCi/nmol, respectively). This indicates that the [^{14}C]choline derived from methylation of ethanolamine does not equilibrate completely with the bulk of the free choline in the leaf before oxidation to betaine.

Metabolism of [U-^{14}C]Serine. In the 6-h period following addition of 1 μmol [^{14}C]serine, wilted leaves synthesized about 7-fold more [^{14}C]betaine than turgid leaves; in both wilted and turgid leaves there was little further [^{14}C]betaine synthesis after 6 h (Fig. 3B). Qualitatively, these results suggest that the 2-carbon moiety of betaine is derived from serine and that the potential for serine decarboxylation equals or exceeds that for methylation of ethanolamine, at least in wilted leaves. Quantitative treatment of the [^{14}C]serine data was not attempted because [^{14}C]serine was extensively metabolized to products unrelated to the betaine pathway, and its specific radioactivity fell very sharply during the experiment. Of the 1-μmol dose of [^{14}C]serine supplied to turgid and

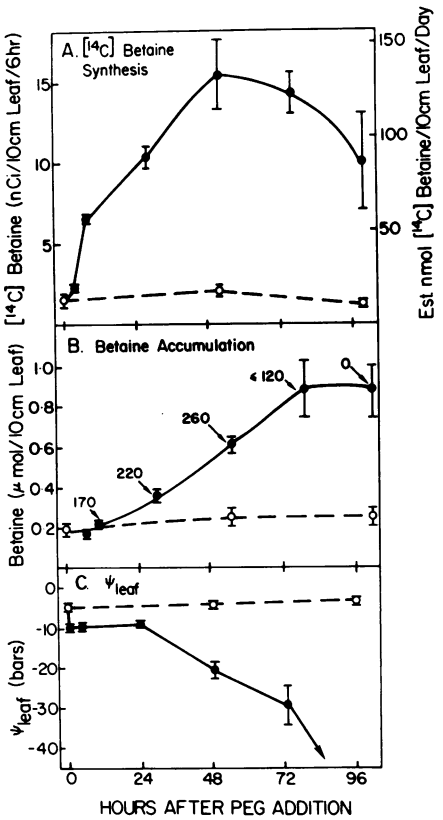

FIG. 4. The effect of various periods of PEG stress on the rate of [^{14}C]ethanolamine conversion to betaine (A), on the accumulation of endogenous betaine (B), and on leaf-water potential (C) in second leaves of barley. ●——●, wilted plants stressed with PEG; O- - -O, turgid, well-watered plants. In A, incubation was for 6 h with 1 μmol [^{14}C]ethanolamine (0.52 μCi/μmol); the time axis represents the midpoints of the 6-h incubation periods. In B, numbers next to points on the betaine accumulation curve are rates of betaine accumulation (in units of nmol/10 cm leaf·day) calculated from tangents to the curve. Betaine contents and ψ_{leaf} values are means ± SE of three or four replicates; [^{14}C]betaine data are means ± SE of three replicates.

wilted leaves, <0.5% remained as free serine after only 3 h; during this time, a number of compounds of intermediary metabolism became labeled (*e.g.* aspartic acid, glutamic-acid, and organic acids). The rapid disappearance of ^{14}C from free serine can account for the halt in [^{14}C]betaine synthesis after 6 h (Fig. 3B).

Betaine Accumulation and [^{14}C]Ethanolamine Metabolism after Various Periods of Stress. If the *de novo* synthesis pathway is to account for betaine accumulation during stress, and if the potentials of several of the pathway steps are indeed too low in turgid leaves to support the necessary flux of intermediates, then the potentials of such limiting steps should increase during stress and should do so no later than at the onset of betaine accumulation. To test this prediction, the rate of [^{14}C]ethanolamine conversion to betaine was determined at various times during a wilting treatment and compared with the chemically determined time course of betaine accumulation in the same leaf samples (Fig. 4). Betaine accumulation began within the first day of stress, and continued until the third day (Fig. 4B). The potential for converting [^{14}C]ethanolamine to betaine (Fig. 4A) increased only slightly during the first 6 h of wilting but rose markedly during the subsequent 4 h; this potential reached a maximum after 2 to 3 days of stress and was variable, but still quite high, on the 4th day of wilting, when betaine accumulation had stopped (Fig. 4B). These results are consistent with operation of the *de novo* synthesis

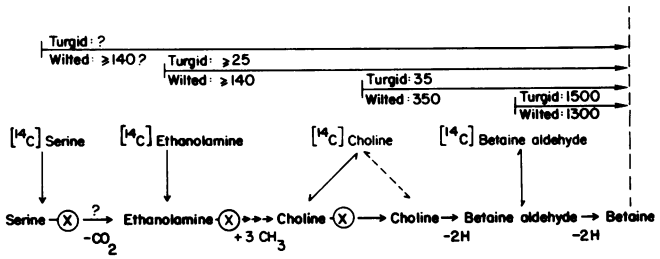

FIG. 5. Proposed scheme for betaine biosynthesis in water-stressed barley leaves, showing the estimated potential capacities for various sequences of steps in the pathway. In the biosynthetic pathway, those steps for which no experimental evidence is presented bear question marks. The horizontal arrows above the biosynthetic pathway designate the sequences of steps whose potential capacity was estimated. Numbers next to these arrows are the highest rates of [^{14}C]betaine synthesis observed for the sequences in turgid leaves and in leaves wilted for 2 days; the rates of [^{14}C]betaine synthesis are in units of nmol/10 cm leaf·day.

pathway during wilting and also suggest that the supply of substrates for the pathway, rather than failure of any of the enzymic steps, may restrict betaine accumulation during severe stress.

SUMMARY AND CONCLUSIONS

The scheme of Figure 5 offers a simplified but quantitative description of changes in the *de novo* synthesis pathway which could account for the accumulation of betaine in wilted leaves. This scheme also contains two features which are more speculative. First, derivatives of serine, ethanolamine, and choline (-⊗ suffix) are shown as the reactants and products for the decarboxylation and methylation steps. This feature could be included on the grounds of comparative biochemistry alone (*e.g.* ref. 13), but there is also some experimental evidence to support it. Although the absorption of all ^{14}C-labeled precursors was almost complete after 1 h (Fig. 1A), only with [^{14}C]betaine aldehyde (Fig. 2A) was there no tendency for an initial lag phase in the rate of [^{14}C]betaine synthesis; such lags were particularly clear with [^{14}C]ethanolamine (Fig. 3A). A lag could reflect the time required for a pool of an intermediate (*e.g.* ethanolamine-⊗; Fig. 5) to approach the specific radioactivity of the added ^{14}C-labeled precursor. Second, perhaps exogenous [^{14}C]choline enters that pool of free choline which is destined for oxidation to betaine aldehyde principally by way of choline-⊗. This speculation arises (*a*) because the time course for [^{14}C]choline conversion to betaine showed a definite lag in wilted leaves (Fig. 2B) and (*b*) because equilibration between the bulk of the endogenous free choline and the choline destined for oxidation was probably slow (see "Metabolism of [2-^{14}C]Ethanolamine," above).

The details of the biosynthetic pathways for the free bases ethanolamine and choline are not well known in plants. In mammals and bacteria, the decarboxylation of serine and the methylations of ethanolamine take place with the substrate in ester linkage as part of a phosphatide, and are catalyzed by membrane-associated enzymes (*e.g.* refs. 13 and 19). Available evidence suggests a similar chain of reactions in plants (9, 11, 17, 20, 21). It is possible that -⊗ in Figure 5 is a phosphatidic acid residue and that the increased rate of betaine synthesis during stress accompanies accelerated turnover of the base moieties of phospholipids in cell membranes.

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