Plant Physiol. (1991) 97, 1199–1205
0032-0889/91/97/1199/07/$01.00/0

Received for publication May 6, 1991
Accepted July 15, 1991

Comparative Physiological Evidence that β-Alanine Betaine and Choline-O-Sulfate Act as Compatible Osmolytes in Halophytic *Limonium* Species[1]

Andrew D. Hanson*, Bala Rathinasabapathi, Beverly Chamberlin, and Douglas A. Gage

Institut de recherche en biologie végétale, Université de Montréal, 4101 rue Sherbrooke est, Montréal, Québec, Canada H1X 2B2 (A.D.H.); and Michigan State University-Department of Energy Plant Research Laboratory (A.D.H., B.R.) and Department of Biochemistry (B.C. and D.A.G.), Michigan State University, East Lansing, Michigan 48824

ABSTRACT

The quaternary ammonium compounds accumulated in saline conditions by five salt-tolerant species of *Limonium* (Plumbaginaceae) were analyzed by fast atom bombardment mass spectrometry. Three species accumulated β-alanine betaine and choline-O-sulfate; the others accumulated glycine betaine and choline-O-sulfate. Three lines of evidence indicated that β-alanine betaine and choline-O-sulfate replace glycine betaine as osmoregulatory solutes. First, tests with bacteria showed that β-alanine betaine and choline-O-sulfate have osmoprotective properties comparable to glycine betaine. Second, when β-alanine betaine and glycine betaine accumulators were salinized, the levels of their respective betaines, plus that of choline-O-sulfate, were closely correlated with leaf solute potential. Third, substitution of sulfate for chloride salinity caused an increase in the level of choline-O-sulfate and a matching decrease in glycine betaine level. Experiments with ^{14}C-labeled precursors established that β-alanine betaine accumulators did not synthesize glycine betaine and *vice versa*. These experiments also showed that β-alanine betaine synthesis occurs in roots as well as leaves of β-alanine betaine accumulators and that choline-O-sulfate and glycine betaine share choline as a precursor. Unlike glycine betaine, β-alanine betaine synthesis cannot interfere with conjugation of sulfate to choline by competing for choline and does not require oxygen. These features of β-alanine betaine may be advantageous in sulfate-rich salt marsh environments.

Considerable evidence indicates that the QAC[2] glycine betaine accumulates in the cytoplasm and chloroplasts of species of the family Chenopodiaceae during osmotic stress and acts as a nontoxic or compatible osmolyte (for reviews, see refs. 15 and 28). Glycine betaine is likely to play the same role in the many other angiosperm families in which it accumulates in response to stress (27, 30), although in these cases the subcellular site of accumulation remains to be demonstrated (6, 20).

Certain higher plants, especially halophytes, accumulate QACs other than glycine betaine. *Limonium vulgare* (Plumbaginaceae) is one example. This species has been shown to contain high levels (>100 μmol·g dry weight^{-1}) of β-alanine betaine (12, 20) and choline-*O*-sulfate (7) when grown in saline conditions. Like glycine betaine, these two compounds exist as zwitterions with zero net charge at physiological pH (Fig. 1). Again, like glycine betaine, both are accumulated by certain marine algae and other marine organisms (1, 3), and neither is inhibitory at high concentrations to isolated enzymes (16, 20). On these grounds, it seems possible that β-alanine betaine and choline-*O*-sulfate could supplement or supplant glycine betaine as a cytoplasmic osmolyte.

However, there is no other evidence for this possibility and some against it. Differences in both *L. vulgare* material and in QAC analysis methods among investigators are further sources of uncertainty. β-Alanine betaine, glycine betaine, and choline-*O*-sulfate have never been assayed by unambiguous methods in the same plant material. The relationships between levels of these QACs and overall osmotic adjustment have not been determined, nor has it been clearly established that β-alanine betaine and choline-*O*-sulfate are compatible osmolytes. Finally, a large but poorly defined proportion of β-alanine betaine has been reported to be esterified to choline (11, 12). Such an ester, which would carry two fixed positive charges and require balancing anions, would lack the characteristics of a compatible solute (18, 30).

In this investigation, therefore, we sought to clarify whether β-alanine betaine and choline-*O*-sulfate have osmoregulatory functions in *Limonium*. Sensitive and specific FABMS methods (7, 17) were used to determine QACs. The surprising finding that various *Limonium* species accumulate either glycine betaine or β-alanine betaine facilitated a comparative physiological approach.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Leaves of *Limonium vulgare* were collected from a coastal salt marsh in the Bassin D'Arcachon, Gironde, France, and freeze dried within a few hours of harvest. Seeds of *Limonium*

[1] Supported by the Natural Sciences and Engineering Research Council of Canada and by the U. S. Department of Energy under contract DE-AC02-76ERO-1338.

[2] Abbreviations: QAC, quaternary ammonium compound; FABMS, fast atom bombardment mass spectrometry; M^+, molecular ion; m/z, mass to charge ratio; RWC, relative water content; Ψ_s, solute potential.

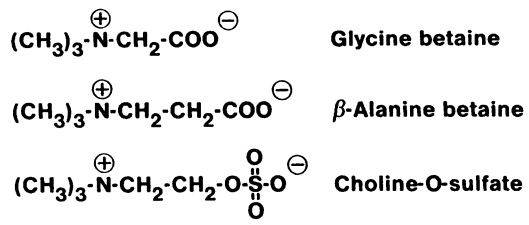

Figure 1. Structures of quaternary ammonium compounds.

sinuatum, Limonium perezii, Limonium latifolium, and *Limonium aureum* were purchased from Park Seed, Greenwood, SC. Plants were grown individually in pots of vermiculite in an environmental chamber (16-h day, 21°C, PPFD 300 μmol quanta $\cdot m^{-2} \cdot s^{-1}$; 8-h night, 16°C) and irrigated daily with two pot volumes of half-strength Hoagland solution. Salinization treatments were started when seedlings were well established. Salts were added to the irrigation solution every third day, in increments of 50 mM NaCl, or its osmotic equivalent for mixtures of NaCl and Na_2SO_4. In experiments in which several salt concentrations were used, plants were maintained at the various concentrations until at least 4 d after the highest concentration was reached, at which point leaves were harvested from all treatments. For root experiments, plants were grown hydroponically in 20-L tanks of half-strength Hoagland solution sparged continuously with air; NaCl was added in 50-mM or 100-mM increments every third day. Leaf and root material for QAC determination was freeze dried, ground in a Wiley mill to mesh size 40, and stored at −20°C.

Isolation and Determination of QACs

Internal standards of deuterated (d_9) glycine betaine and β-alanine betaine were prepared by N-methylation of the corresponding amino acid with d_3-iodomethane (99.5 atom % deuterium; MSD Isotopes, Montréal, Canada) according to the method of Rhodes *et al.* (17); d_9-choline-*O*-sulfate was synthesized from d_9-choline (99.2 atom % deuterium, MSD Isotopes) by the method of Stevens and Vohra (19). Samples (50 mg) of freeze dried and milled tissue received 448 nmol of d_9-glycine betaine, 200 nmol of d_9-β-alanine betaine, and 1000 nmol of d_9-choline-*O*-sulfate and were extracted using the methanol/chloroform/water procedure described previously (7). The aqueous phase from this extraction was fractionated on 1.5-mL columns of Dowex-1 (OH^-) and Dowex-50 (H^+) arranged in series. Choline-*O*-sulfate was isolated from the neutral fraction by TLC and determined by FABMS as described before (7); the detection limit for choline-*O*-sulfate was approximately 1 μmol $\cdot$ g dry weight^{-1}. Betaines were eluted from the Dowex-50 column with 8 mL of 2.5 M HCl, and the eluates were freeze dried. Betaines were derivatized with *n*-butanol and analyzed by FABMS using the procedures of Rhodes *et al.* (17); the detection limit for betaines was approximately 1 μmol $\cdot$ g dry weight^{-1}. Choline was estimated by the TLC method of Storey and Wyn Jones (21).

Analysis of Sulfate and Chloride

Samples (250 mg) of freeze dried and milled leaf material were extracted with 5 mL of water at 90 to 95°C for 1 h. The residue was removed by filtration and washed with 3 × 10 mL of water. The extract and washings were pooled and made to a final volume of 50 mL. A Dionex series 4000i HPLC apparatus with an HPIC AS4A analytical column was used to estimate sulfate and chloride in this solution; the eluant was 2.8 mM Na_2CO_3/2.8 mM $NaHCO_3$.

Water Relations Measurements

Measurements of Ψ_s were made on frozen/thawed leaf discs using a Wescor HR-33T dewpoint microvoltmeter equipped with C-52 sample chambers. One disc was taken from a young, fully expanded leaf of each plant in a treatment. RWC was estimated as described by Turner (25), using leaf discs taken from all plants in a treatment and a 4-h hydration time.

^{14}C-Labeling Experiments

Leaf discs (11 mm in diameter) or root segments (20 mm) were cut from salinized plants. Batches of 10 leaf discs were placed in 10 mL of H_2O containing either 74 kBq of [*methyl*-^{14}C]choline (2.1 GBq mmol^{-1}, NEN-DuPont) or 148 kBq of β-[1-^{14}C]alanine (2 GBq mmol^{-1}, NEN-DuPont), infiltrated under vacuum for 15 s, and incubated with shaking (100

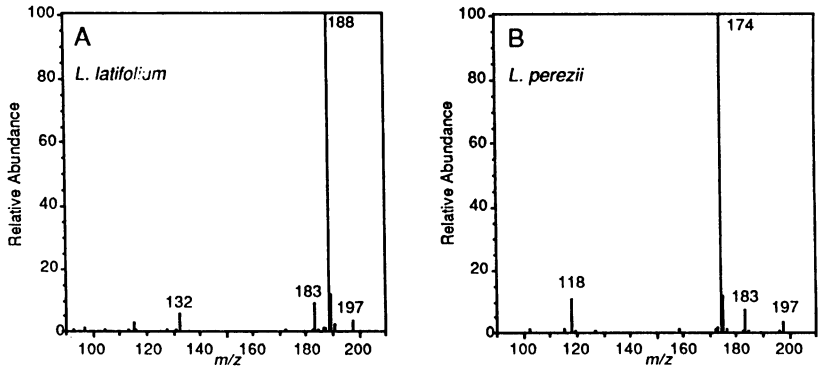

Figure 2. FABMS results for the betaine fractions from representative *Limonium* species, following esterification with *n*-butanol. The matrix used was glycerol. Betaines were extracted from 50-mg dry weight leaf samples from salinized plants. Each sample received internal standards of d_9-glycine betaine (448 nmol) and d_9-β-alanine betaine (200 nmol), whose *n*-butyl derivatives give the peaks seen at m/z 183 and 197, respectively. A, *L. latifolium*. Note the peaks at m/z 188 (M^+ of β-alanine betaine *n*-butyl ester) and m/z 132 (M^+ - butene, a fragmentation product equivalent to the protonated zwitterion). B, *L. perezii*. Peaks at m/z 174 and 118 represent the M^+ of glycine betaine *n*-butyl ester and the fragment ion formed by loss of butene, respectively.

Table I. *Levels of QACs in Leaves of Limonium Species Grown under Saline Conditions*

L. vulgare was collected from a coastal salt marsh; other species were salinized with 400 or 450 mM NaCl in controlled environments.

Species	QAC Level		
	Glycine betaine	β-Alanine betaine	Choline-*O*-sulfate
	μmol·g dry wt^{-1}		
L. vulgare Mill.	1.5	104	130
L. latifolium (Sm.) O. Kuntze	<1	91	97
L. aureum (L.) Hill ex O. Kuntze	<1	88	91
L. sinuatum (L.) Mill.	112	<1	40
L. perezii (Stapf) F.T. Hubb.	120	<1	51

rpm) for 9 h under a bank of cool-white fluorescent tubes (PPFD 150 μmol quanta·m^{-2}·s^{-1}) at 22°C. Root segments were rinsed in 5 × 10 mL of half-strength Hoagland solution containing 100 mM NaCl and shaken (100 rpm) in 3 mL of this medium for 1 h in darkness at 22°C. β-[1-^{14}C]Alanine (56 kBq) was then added, and the incubation was continued for 9 h. Thirty minutes before the end of incubations, the medium was replaced with a 0.5 mM wash solution of unlabeled choline or β-alanine. As a check for possible metabolism of β-[1-^{14}C] alanine by the microflora in experiments with roots, carbenicillin (500 μg mL^{-1}) was added to the incubation medium in some experiments. This treatment reduced the number of viable bacteria at least 10-fold but did not affect the ^{14}C-labeling patterns observed.

A methanol/chloroform/water procedure (13) was used for tissue extraction. In experiments with [^{14}C]choline, the ^{14}C recovered in the chloroform phase was taken as a measure of ^{14}C incorporation into phosphatidylcholine; TLC of representative samples confirmed that all ^{14}C in this phase was accounted for by phosphatidylcholine together with small amounts of its breakdown products. The aqueous phase was fractionated by ion-exchange chromatography on 1.5-mL mixed-bed and Dowex-50 (H^+) columns as described previously (9); choline-*O*-sulfate was recovered in the effluent from these columns, and betaines were eluted from the Dowex-50 column with 8 mL of 2.5 N HCl. These fractions were freeze dried, redissolved in water, and analyzed further by TLC or thin-layer electrophoresis and autoradiography. Labeled compounds in the Dowex-50 fraction were separated by TLC on silica gel G plates (0.25-mm layer thickness, Machery-Nagel Polygram) developed with methanol/acetone/concentrated HCl (90:10:4, v/v/v) and by thin-layer electrophoresis on ITLC-SA sheets (Gelman Sciences) in 1.5 N formic acid for 15 min at 1.8 kV. The effluent fraction was subjected to TLC on cellulose plates (0.1-mm layer thickness, Merck) developed with *n*-butanol/acetic acid/water (60:20:20, v/v/v). Scintillation counting was used to quantify ^{14}C in column fractions and in zones of chromatograms and electrograms.

Osmoprotection Bioassays with Bacteria

Bacterial strains were wild-type *Salmonella typhimurium* TL1, wild-type *Escherichia coli* K10 (from L. Csonka, Purdue University, West Lafayette, IN), and a *betA* mutant of *E. coli*, FF48 (24) (from A. Strøm, University of Tromsö, Tromsö, Norway). Cells were grown in liquid M63 medium containing 22 mM D-glucose (10) and 0.6 M NaCl. QACs were sterilized by filtration and added to give a final concentration of 1 mM. [^{14}C]Choline-*O*-sulfate (0.21 MBq mmol^{-1}) was synthesized from [*methyl*-^{14}C]choline by the method cited above. Growth was at 37°C with vigorous aeration. Cultures (3–5 mL) were inoculated with cells growing exponentially in M63 medium without NaCl, and growth was monitored by absorbance at 420 nm. For studies of intracellular accumulation of QACs, cells were harvested by centrifugation and washed in M63 medium containing 0.65 M NaCl; the cell pellet was then extracted in methanol/chloroform/water as described above. The aqueous phase was further analyzed by ion-exchange chromatography and TLC by methods cited above; [^{14}C] choline-*O*-sulfate was quantified by scintillation counting and β-alanine betaine by FABMS.

RESULTS AND DISCUSSION

QACs in Leaves of Salinized Plants

Five *Limonium* species were examined, including *L. vulgare*. When grown in saline conditions, all accumulated both choline-*O*-sulfate and a betaine (Table I). Three species contained >80 μmol·g dry weight^{-1} of β-alanine betaine and little or no detectable glycine betaine, whereas two accumulated >110 μmol·g dry weight^{-1} of glycine betaine but no detectable β-alanine betaine. Figure 2 shows FABMS results for *n*-butanol-derivatized betaine fractions for two representative species. The *L. latifolium* spectrum (Fig. 2A) has a strong signal at m/z 188 (β-alanine betaine *n*-butyl ester) and a smaller one at m/z 132 (the β-alanine betaine fragment ion formed by loss of butene from the ester) but shows no clear peak above the glycerol matrix background at m/z 174 (glycine betaine *n*-butyl ester). Conversely, the *L. perezii* spectrum (Fig. 2B) shows a large peak at m/z 174 and a smaller one from the glycine betaine fragment ion at m/z 118 but no signal distinguishable from the background at m/z 188. To confirm their identities, betaines were isolated by TLC and thin-layer electrophoresis and then analyzed by FABMS without derivatization. The expected protonated M^+ (*i.e.* the zwitterions + H) were observed at m/z 132 for β-alanine betaine and at m/z 118 for glycine betaine.

The labile choline ester of β-alanine betaine reported from *L. vulgare* by Larher and Hamelin (12) was not detected in any *Limonium* species. Ester hydrolysis cannot explain these negative results because pools of free choline were small and in the normal range for higher plants (<2 μmol·g dry weight^{-1}). We suggest that the choline ester in the study by Larher and Hamelin could have arisen by transesterification with choline-*O*-sulfate under the acidic ethanol extraction conditions used (5).

QACs in Roots

To find whether any of the QACs is organ specific, the QAC profiles of roots and leaves of two representative species were compared (Table II). No qualitative differences were found between roots and leaves; roots of both species accu-

Table II. *Levels of QACs in Roots and Leaves of Two Limonium Species*

Plants were salinized with 300 mM NaCl in hydroponic culture; root systems were divided into the thickened pigmented taproot and fine white roots.

Species	Organ	QAC Level		
		Glycine betaine	β-Alanine betaine	Choline-*O*-sulfate
		μmol·g dry wt^{-1}		
L. sinuatum	Tap root	50	<1	60
	Fine roots	40	<1	43
	Leaves	71	<1	36
L. latifolium	Tap root	<1	63	78
	Fine roots	<1	61	71
	Leaves	<1	84	113

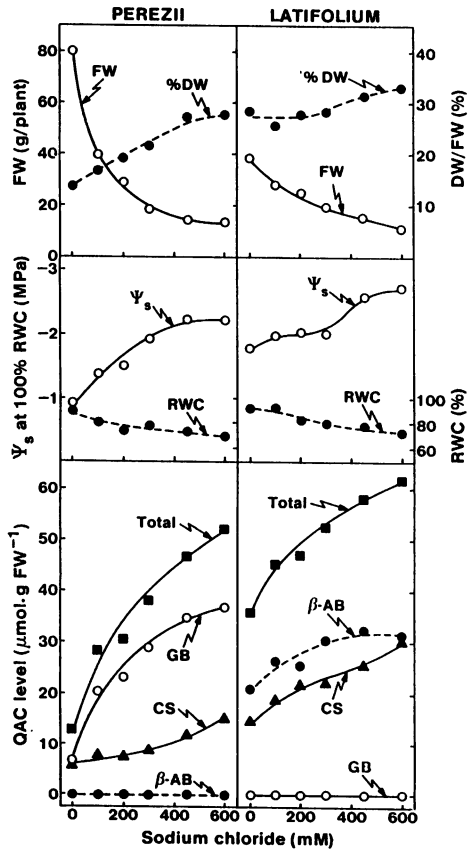

Figure 3. Effect of NaCl salinity on growth, osmotic relations, and the accumulation of QACs in leaves of representative *Limonium* species. Growth and osmotic relations data are means of four replicates; QACs were determined on pooled material from all replicates. Plants were harvested 13 weeks after sowing. GB, glycine betaine; CS, choline-*O*-sulfate; β-AB, β-alanine betaine; DW, dry weight; FW, fresh weight.

mulated choline-*O*-sulfate and the betaine found in their leaves. However, there were quantitative differences in the relative amounts of choline-*O*-sulfate and glycine betaine in *L. sinuatum*, with choline-*O*-sulfate more prominent in roots.

Effect of Salinity on QAC Levels, Osmotic Adjustment, and Growth

L. perezii and *L. latifolium* were salinized with nutrient solution containing 0 to 600 mM NaCl (Fig. 3). As expected, salinity depressed growth and elicited osmotic adjustment as measured by a decrease in Ψ_s at 100% RWC. *L. latifolium* maintained a lower Ψ_s than *L. perezii* in non-saline conditions and showed accordingly less osmotic adjustment. The patterns of QAC accumulation in leaves differed in the type of betaine but were otherwise similar in three respects. First, only one betaine was accumulated in each species, regardless of NaCl level. Second, whereas choline-*O*-sulfate continued to accumulate as salt concentration was increased above 300 mM, there was relatively little further increase in glycine betaine level and none in β-alanine betaine level. Third, the total QAC content (betaines + choline-*O*-sulfate) was closely correlated with Ψ_s ($r = 0.97$, $P < 0.001$), with data points for both species falling on the same regression line (Fig. 4). The levels of individual QACs were also significantly correlated with Ψ_s ($r = 0.81$–0.98, $P \leq 0.05$).

Effect of Na_2SO_4 Salinity on Levels of Sulfate, Chloride, and QACs

The media in the preceding salinity experiment contained only 0.5 mM sulfate, which might have limited choline-*O*-sulfate accumulation. Tests of this possibility were conducted with *L. sinuatum* by partially replacing chloride in the irrigation medium with sulfate, while keeping the osmolality constant at 740 mosmol kg^{-1} (equal to that of 400 mM NaCl). The molar ratios of sulfate to chloride used were 1:19 (similar to seawater), 1:2, and 2:1 (Fig. 5). Growth was comparable in all salinity treatments, and no foliar injury symptoms developed. On medium containing 400 mM NaCl and 0.5 mM sulfate, there was little free sulfate in leaves, and the choline-

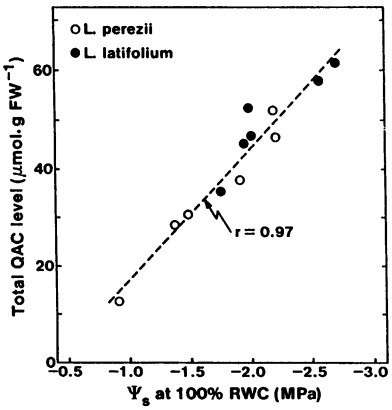

Figure 4. Relationship between total QAC content and Ψ_s at 100% RWC in leaves of *L. perezii* and *L. latifolium*. Data are from Figure 3. The coefficient of linear correlation is significant at $P = 0.001$.

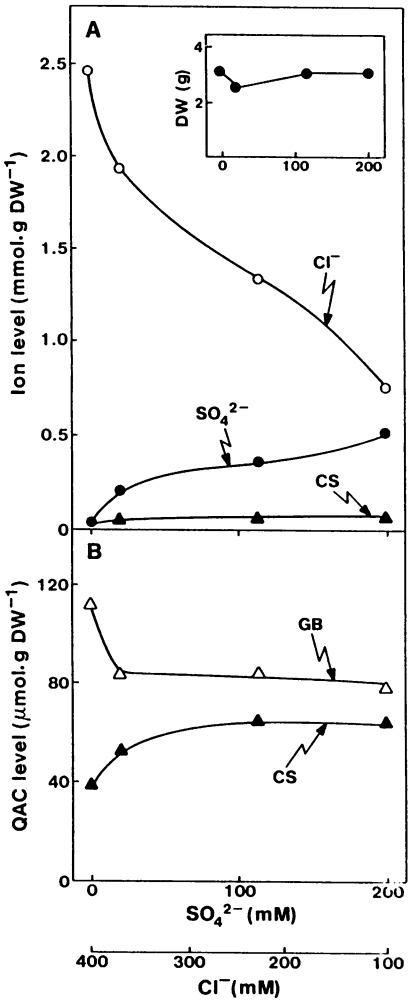

Figure 5. Effect of partially replacing chloride by sulfate on the levels of chloride and sulfate (A) and QACs (B) in leaves of salinized *L. sinuatum*. Data for choline-*O*-sulfate are replotted in A for direct comparison with sulfate levels. All salinization solutions were isoosmotic with 400 mM NaCl. Inset, response of shoot dry weight. GB, glycine betaine; CS, choline-*O*-sulfate; DW, dry weight. Data are for pooled material from four plants per treatment.

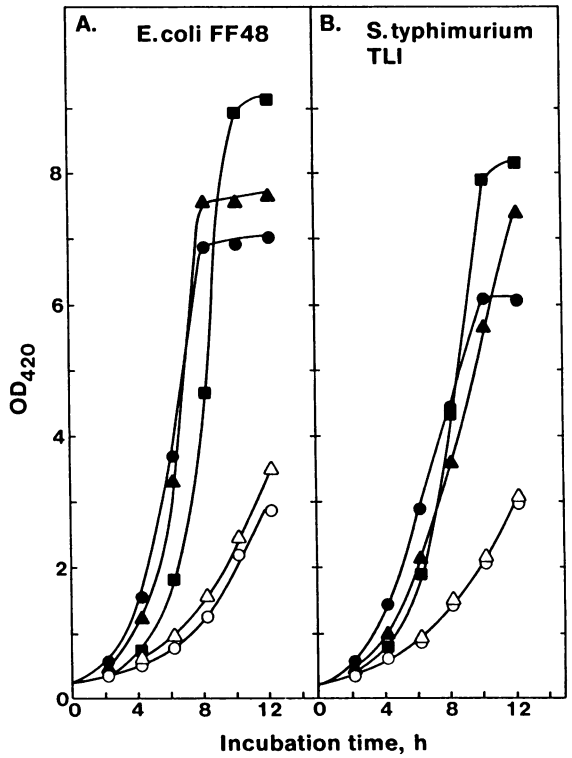

Figure 6. Osmoprotection by β-alanine betaine and choline-*O*-sulfate. *E. coli* mutant FF48 (*betA*, unable to convert choline to betaine) and *S. typhimurium* wild-type cells were grown aerobically at 37°C in M63-glucose containing 0.6 M NaCl alone (○) or with 1 mM choline-*O*-sulfate (▲), β-alanine betaine (■), choline (△), or glycine betaine (●). Growth was measured by absorbance.

O-sulfate level was 40 μmol·g dry weight^{-1}. As internal and external sulfate concentrations increased, the level of choline-*O*-sulfate increased to a plateau value of about 65 μmol·g dry weight^{-1}. There was a matching decline in glycine betaine level, so that total QAC content did not change.

Bacterial Osmoprotection Bioassays

Because the compatibility of a solute is determined by its chemical structure and is largely independent of the nature of the organism (18, 28), bioassays of stimulatory effects on the

Table III. *Accumulation of Choline-O-Sulfate and β-Alanine Betaine in Cells of E. coli Grown in 0.6 M NaCl*

Strain	Osmoprotectant	Dose Supplied	Recovered in Cells	Cell No.	Intracellular[a] Concentration
		μmol	μmol		M
FF48	[^{14}C]Choline-*O*-sulfate	5.24	3.33	1.3×10^{10}	0.71
K10	β-Alanine betaine	5.0	3.16	1.7×10^{10}	0.51

[a] Estimated from cell number assuming dimensions of 0.7 × 1.4 μm and a periplasmic space of 20% cell volume (26).

Table IV. *Metabolism of [^{14}C]Choline by Leaf Discs of Glycine Betaine- and β-Alanine Betaine-Accumulating Limonium Species*

Batches of 10 discs from salinized plants were incubated in 10 mL of water containing 74 kBq (35 nmol) of [^{14}C]choline for 9 h in the light.

Species	Endogenous Betaine	NaCl Level	^{14}C Distribution among QACs		
			Phosphatidylcholine	Glycine betaine[a]	Choline-*O*-sulfate
		mM	*kBq/10 discs*		
L. sinuatum	Glycine betaine	100	9.47	0.41	2.50
		200	5.25	0.17	1.81
L. latifolium	β-Alanine betaine	100	5.48	≤0.02	5.03
		200	9.25	≤0.02	2.55

[a] Radioactivity in glycine betaine corrected for 0.06% [^{14}C]glycine betaine present in samples spiked with [^{14}C]choline.

growth of bacteria in high-osmolarity media (osmoprotection) provide a general measure of compatibility (23). β-Alanine betaine and choline-*O*-sulfate acted as osmoprotectants for *E. coli* and *S. typhimurium* and were comparable in effectiveness to glycine betaine (Fig. 6). All three QACs decreased the doubling time to approximately 1.5 h, although the lag phases for β-alanine betaine and choline-*O*-sulfate were consistently longer than for glycine betaine. Such differences in lag phases may explain an earlier report (14) that choline-*O*-sulfate and β-alanine betaine have only weak osmoprotectant properties. Neither of the strains shown in Figure 6 can oxidize choline to glycine betaine (4, 24) and, as expected, neither was protected by choline, so that the effect of choline-*O*-sulfate is unlikely to be due to cleavage of the ester bond followed by oxidation of choline to glycine betaine. Both β-alanine betaine and choline-*O*-sulfate were accumulated to high levels (Table III). The choline-*O*-sulfate was labeled with ^{14}C to provide a sensitive means of detecting any metabolites. Chromatographic analyses demonstrated that >99% of the ^{14}C recovered from the cell pellet remained in the form of choline-*O*-sulfate, confirming that its osmoprotective effect does not require conversion to glycine betaine.

Metabolism of ^{14}C-Labeled Precursors

To determine whether β-alanine betaine accumulators lack the capacity to synthesize glycine betaine, the metabolism of choline in *L. sinuatum* and *L. latifolium* was compared. Leaf discs of both species readily incorporated [^{14}C]choline into phosphatidylcholine and choline-*O*-sulfate, but only the glycine betaine accumulator *L. sinuatum* synthesized [^{14}C]glycine betaine (Table IV). In root segments of both *L. sinuatum* and *L. latifolium*, [^{14}C]choline was actively metabolized to choline-*O*-sulfate and phosphatidylcholine. Substantial [^{14}C]choline oxidation by the root microflora (29) prevented determination of [^{14}C]glycine betaine formation in roots.

To establish whether glycine betaine accumulators are unable to synthesize β-alanine betaine, the precursor β-alanine (11) was supplied to leaf discs of *L. sinuatum* and *L. latifolium*. Methylation of β-[^{14}C]alanine to β-alanine betaine was very active in *L. latifolium* but undetectable in *L. sinuatum* (Table V). Root segments of *L. latifolium* also converted β-[^{14}C]alanine to β-alanine betaine, but *L. sinuatum* segments did not (Table V).

Table V. *Conversion of β-[^{14}C]Alanine to β-Alanine Betaine by Leaf and Root Tissue of Glycine Betaine- and β-Alanine Betaine-Accumulating Limonium Species*

Leaf discs or root segments from salinized plants were incubated with β-[^{14}C]alanine (2 GBq·mmol^{-1}) for 9 h.

Species	Endogenous Betaine	NaCl Level	Tissue	β-[^{14}C]Alanine Betaine Synthesis
		mM		*kBq·g fresh wt^{-1}*
L. sinuatum	Glycine betaine	100	Leaf	≤0.01
		200	Leaf	≤0.01
		100	Root	≤0.01
L. latifolium	β-alanine betaine	100	Leaf	67.1
		200	Leaf	31.4
		100	Root	27.0

CONCLUDING DISCUSSION

Our data build a strong circumstantial case that, like glycine betaine, β-alanine betaine and choline-*O*-sulfate act as compatible cytoplasmic osmolytes. First, that *Limonium* species accumulate either glycine betaine or β-alanine betaine, but not both, suggests that they have the same function. Second, the reciprocal changes in glycine betaine and choline-*O*-sulfate levels elicited by sulfate salinity imply that choline-*O*-sulfate can fill the same role as betaines. Third, the correlations between Ψ_s and total QAC concentration, and between Ψ_s and individual QACs, are very similar to those for glycine betaine in members of the Chenopodiaceae (8, 22), in which glycine betaine is known to be located in the cytoplasm (15). Finally, that bacterial growth in high-osmolarity media is promoted by β-alanine betaine or choline-*O*-sulfate, and that both compounds reach intracellular levels ≥0.5 M, establishes them as effective compatible solutes.

The β-[^{14}C]alanine- and [^{14}C]choline-feeding experiments demonstrate that glycine betaine and choline-*O*-sulfate share choline as a precursor and show that β-alanine betaine synthesis is active in roots of β-alanine betaine-accumulating species. These results suggest two metabolic factors that may favor β-alanine betaine accumulation over glycine betaine accumulation. Sharing choline as a precursor, choline-*O*-

sulfate and glycine betaine each potentially interferes with the synthesis of the other. Because choline-*O*-sulfate formation may serve to detoxify sulfate (3, 7), such interpathway competition may be disadvantageous in sulfate-rich habitats. The second metabolic factor is the oxygen requirement of the first step in choline oxidation to glycine betaine (2), which would impair glycine betaine synthesis in roots under the severe hypoxia that often prevails in salt marshes. Conversely, hypoxia would not affect the N-methylation reactions unique to β-alanine betaine synthesis because these do not involve oxygen. The habitats of glycine betaine- and β-alanine betaine-accumulating *Limonium* species are consistent with this: *L. sinuatum* and *L. perezii* are found in dry, sandy, or rocky soils, whereas *L. vulgare* and *L. aureum* colonize salt marshes.

ACKNOWLEDGMENTS

We thank the Michigan State University-National Institutes of Health Mass Spectrometry Facility (grant No. RR 00480 to J. T. Watson) for the use of mass spectrometers. We are indebted to Dr. Henri Parriaud and Dr. Renaud Brouquisse for help in collecting *L. vulgare* leaves.

LITERATURE CITED

1. **Blunden G, Gordon SM** (1986) Betaines and their sulphonio analogues in marine algae. Prog Phycolog Res **4:** 39–80
2. **Brouquisse R, Weigel P, Rhodes D, Yocum CF, Hanson AD** (1989) Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiol **90:** 322–329
3. **Catalfomo P, Block JH, Constantine GH, Kirk PW** (1973) Choline sulfate (ester) in marine higher fungi. Marine Chem **1:** 157–162
4. **Csonka LN** (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev **53:** 121–147
5. **Gorham J, McDonnell E, Wyn Jones RG** (1982) Determination of betaines as ultraviolet-absorbing esters. Anal Chim Acta **138:** 277–283
6. **Hanson AD** (1991) Compatible solute synthesis and compartmentation in higher plants. *In* GN Somero, CB Osmond, CL Bolis, eds, Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Levels. Springer-Verlag, Berlin (in press)
7. **Hanson AD, Gage DA** (1991) Identification and determination by fast atom bombardment mass spectrometry of the compatible solute choline-*O*-sulphate in *Limonium* species and other halophytes. Aust J Plant Physiol **18:** 317–327
8. **Hanson AD, Wyse R** (1982) Biosynthesis, translocation, and accumulation of betaine in sugar beet and its progenitors in relation to salinity. Plant Physiol **70:** 1191–1198
9. **Hitz WD, Hanson AD** (1980) Determination of glycine betaine by pyrolysis-gas chromatography in cereals and grasses. Phytochemistry **19:** 2371–2374
10. **Landfald B, Strøm AR** (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J Bacteriol **165:** 849–855
11. **Larher, F** (1976) Sur quelques particularités du métabolism azoté d'une halophyte: *Limonium vulgare* Mill. DSc thesis, Université de Rennes, Rennes, France
12. **Larher F, Hamelin J** (1975) L'Acide β-triméthylaminopropionique des rameaux de *Limonium vulgare* Mill. Phytochemistry **14:** 205–207
13. **Lerma C, Hanson AD, Rhodes D** (1988) Oxygen-18 and deuterium labeling studies of choline oxidation by spinach and sugar beet. Plant Physiol **88:** 695–702
14. **Mason TG, Blunden G** (1989) Quaternary ammonium and tertiary sulphonium compounds of algal origin as alleviators of osmotic stress. Bot Marina **32:** 313–316
15. **McCue KF, Hanson AD** (1990) Drought and salt tolerance: towards understanding and application. Trends Biotechnol **8:** 358–362
16. **Pollard A, Wyn Jones RG** (1979) Enzyme activities in concentrated solutions of glycine betaine and other solutes. Planta **144:** 291–298
17. **Rhodes D, Rich PJ, Myers AC, Reuter CC, Jamieson GC** (1987) Determination of betaines by fast atom bombardment mass spectrometry. Plant Physiol **84:** 781–788
18. **Somero GN** (1986) Protons, osmolytes, and fitness of internal milieu for protein function. Am J Physiol **251:** R197–R213
19. **Stevens CM, Vohra P** (1955) Occurrence of choline sulfate in *Penicillium chrysogenum*. J Am Chem Soc **77:** 4935–4936
20. **Stewart GR, Larher F, Ahmad I, Lee JA** (1979) Nitrogen metabolism and salt-tolerance in higher plant halophytes. *In* RL Jefferies, AJ Davy, eds, Ecological Processes in Coastal Environments. Blackwell Scientific Publications, Oxford, pp 211–227
21. **Storey R, Wyn Jones RG** (1977) Quaternary ammonium compounds in relation to salt resistance. Phytochemistry **16:** 447–453
22. **Storey R, Wyn Jones RG** (1979) Responses of *Atriplex spongiosa* and *Suaeda maritima* to salinity. Plant Physiol **63:** 156–162
23. **Strøm AR, LeRudulier D, Jakowec MW, Bunnell RC, Valentine RC** (1983) Osmoregulatory (Osm) genes and osmoprotective compounds. *In* T Kosuge, CP Meredith, A Hollaender, eds, Genetic Engineering of Plants—An Agricultural Perspective. Plenum Press, New York, pp 39–59
24. **Styrvold OB, Falkenberg P, Landfald B, Eshoo MW, Bjørnsen T, Strøm AR** (1986) Selection, mapping and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway. J Bacteriol **165:** 856–863
25. **Turner NC** (1981) Techniques and experimental approaches for the measurement of plant water status. Plant Soil **58:** 339–366
26. **Van Wielink JE, Duine JA** (1990) How big is the periplasmic space? Trends Biochem Sci **15:** 136–137
27. **Weretilnyk EA, Bednarek S, McCue KF, Rhodes D, Hanson AD** (1989) Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. Planta **178:** 342–352
28. **Wyn Jones RG** (1984) Phytochemical aspects of osmotic adaptation. Recent Adv Phytochem **18:** 55–78
29. **Wyn Jones RG, Rippin AJ, Storey R** (1973) Metabolism of choline in the rhizosphere and its possible influence on plant growth. Pestic Sci **4:** 375–383
30. **Wyn Jones RG, Storey R** (1981) Betaines. *In* LG Paleg, D Aspinall, eds, The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, pp 171–204