Determinations of Betaines by Fast Atom Bombardment Mass Spectrometry

IDENTIFICATION OF GLYCINE BETAIN DEFFICIENT GENOTYPES OF ZEA MAYS

Received for publication November 28, 1986 and in revised form March 3, 1987

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ABSTRACT

A rapid, sensitive, and selective method for the determination of betaines is described and discussed. The method entails derivatizing the quaternary ammonium compounds to increase their sensitivity to detection by fast atom bombardment mass spectrometry. Sensitivity of detection increases markedly as the length of the carbon chain of the alcohol used to esterify the betaine carboxylic acid group is increased (C4 > C3 > C2 > C1 > C0). The lower limit of detection of glycine betaine as the 5-propyl ester is 6.85 nanomole per microliter of glycerol. Betaine aldehyde can be readily derivatized to the di-n-butyl or di-n-propyl acetal derivatives which exhibit lower limits of detection of about 5 picomoles and 10 picomoles per microliter of glycerol, respectively. Accurate quantification of these compounds is accomplished by the use of deuterium labeled internal standards or quaternary ammonium compound homologs of distinct mass. Methods for the synthesis of these internal standards are reported. Some applications of these methods are illustrated with stable isotope tracer studies onatics of metabolism of choline to betaine aldehyde and glycine betaine in spinach leaf discs, and the identification of several Zea mays genotypes which appear deficient in glycine betaine. Tracer studies with deuterium labeled betaine aldehyde suggest that the deficency of glycine betaine in one sweet corn hybrid is probably not due to a deficiency in the capacity to oxidize betaine aldehyde.

In considering screening maize germplasm for genotypic differences in glycine betaine levels, and potential methods for characterizing putative betaine null genotypes, we noted a paucity of convenient methods which would lend themselves to both accurate quantification of low glycine betaine levels in the presence of other quaternary ammonium compounds (e.g. choline, phosphoryl-choline, and betaine aldehyde) and stable isotope tracer studies on the betaine biosynthetic pathway. Available methods for betaine determination lack either sensitivity, resolution, and/or amenability to stable isotope tracer work (4, 8, 12-14, 19, 20). We therefore sought sensitive mass spectral methods for quantifying glycine betaine and its stable isotope abundance.

Mass spectral analysis of quaternary ammonium compounds of synthetic and natural origin has been approached for the most part by desorption methods. FABMS (6), 252Cf fission-fragment and laser desorption (27), thermal ionization (11), field desorption (2, 21, 26), direct exposure chemical ionization (6), and secondary ion mass spectrometry (9, 31) have all been applied to determine general features of ionization and fragmentation common to quaternary ammonium compounds. While all of the above techniques produce intact molecular cations, FABMS is particularly well suited to the analysis of preformed ions which exhibit surface activity in liquid sample matrices (25). Generally, FABMS of quaternary ammonium salts produces molecular ions with the greatest relative abundance and allows monitoring of long-lived signal derived from stable ion emission. In this paper we demonstrate the application of FABMS in the quantitative determination of betaines in plant extracts, describing the optimization of derivatization conditions to increase the sensitivity of detection of betaines. The utility of these techniques is illustrated with stable isotope tracer studies on the glycine betaine biosynthetic pathway in spinach and Z. mays leaf discs, and the identification of several glycine betaine deficient genotypes of Z. mays.

MATERIALS AND METHODS

Chemicals. Unlabeled glycine betaine and betaine aldehyde chloride were obtained from Sigma Chemical Co. Deuterium labeled choline chloride (trimethyl-d3) (98%) was obtained from Cambridge Isotope Labs. (Cambridge, MA). Deuterium labeled glycine betaine (trimethyl-d3) (99.5%) was prepared by modifications of procedures outlined by Chen and Benoiton (5), by quaternizing glycine with iodomethane-d3 (CD3I) (99.5%) obtained from General Electric Co.

1 Purdue University Agricultural Experiment Station Article No. 10,888. This work was in part supported by a grant from the Corporation for Science and Technology in Indiana.

2 Abbreviations: FABMS, Fast atom bombardment mass spectrometry; gfw, gram fresh weight.

Quaternary ammonium compounds occur widely in nature and may serve important roles as compatible osmotic solutes or osmoprotectants (13, 17, 22, 28-30, 34). Glycine betaine (N,N,N-trimethyl glycine) is thus of considerable interest in terms of the osmotic stress tolerance of the Chenopodiaceae and Gramineae (7, 15-18, 20, 32, 33). But a key test of the adaptive significance of glycine betaine accumulation in osmoregulation in higher plants may well be the identification of detailed characterization of betaine deficient mutants of glycine betaine accumulating species (16, 17). A candidate species for the search for such mutants or naturally occurring variants is Zea mays, a species which is reported to have relatively low glycine betaine titers in comparison to many other grasses and cereals (20, 28).
tained from MSD Isotopes (Merck & Co., Inc.). Glycine (1 mmol) was first converted to the isobutyl ester by reacting with 5 ml isobutanol + 1 ml acetyl chloride at 120°C for 60 min. Excess reagent was evaporated under a stream of N₂ and the amino acid ester dissolved in 20 ml methanol to which was added 3 ml CD₃J and 1 g KHCO₃. After stirring with a magnetic stir bar in a closed vial for 48 h at 23°C the reaction mixture was filtered through Whatman No. 1 filter paper and the filtrate evaporated to dryness. The dried filtrate was extracted with 200 ml chloroform and the chloroform extract was brought to dryness and hydrolyzed with 10 ml 5.5 M HCl at 120°C for 8 h to convert the betaine ester (iodide salt) to the free acid (HCl salt). Following evaporation to dryness to remove excess HCl and free I₂, the sample was redissolved in 5 ml water and washed through a 5 x 2 cm column of Dowex-1-ΟH⁻ with 20 ml H₂O. The aqueous eluant was treated with 50 mg Ag₂O to precipitate any free halide ions, filtered through Whatman No. 1 filter paper, and the filtrate applied to a 10 x 2 cm column of Dowex-50W-H⁺. After washing the column with 20 ml H₂O, glycine betaine-d₃ was then eluted with 20 ml 6 M NH₄OH and evaporated to dryness. Integrity of the standard was verified by FABMS. These procedures were found necessary to recover glycine betaine-d₃, as the inner salt, free of halide ions. Other betaines synthesized by these methods include N,N-dimethyl proline (proline betaine; stachydrine), N, N-dimethyl piperidic acid (piperidac acid betaine; homostachydrine) and N,N-trimethyl γ-aminoobutyrate (γ-butyrobetaine) from the corresponding amino acids and iodomethane. Hydroxyproline betaine (betonicine) was obtained from Atonergic Chemmats Corp. (Plainview, NY).

Betaine aldehyde (methyl-d₃) chloride was synthesized by dissolving 2 ml (dimethylamino)acetalddehyde diethyl acetal (Fluka Chemical Corp., Hauppauge, NY) in 10 ml methanol and reacting with 5 ml CD₃J (99.5%) for 24 h at 23°C in a closed vial stirred with a magnetic stir bar. The reaction mixture was evaporated to dryness under a stream of N₂, redissolved in 5 ml H₂O, and applied to a 10 x 2 cm column of Dowex-1-CI⁻ to exchange I⁻ with Cl⁻. The aqueous eluant (25 ml) was then brought to a final concentration of 2.5 M HCl with concentrated HCl and then heated at 100°C for 8 h. After hydrolysis the sample was concentrated to dryness in a rotary evaporator, redissolved in 5 ml H₂O, and applied to a 10 x 2 cm column of Dowex-50W-H⁺. After washing with 25 ml H₂O, betaine aldehyde-d₃ chloride was eluted with 20 ml 2.5 M HCl and the sample again concentrated to dryness in a rotary evaporator. Integrity of the standard was verified by FABMS.

Esterification. Samples of betaines or betaine aldehyde chloride were generally redissolved in 0.4 ml 60% methanol prior to esterification and brought to dryness under a stream of N₂ in 1 ml microreaction vessels (Supelco, Inc., Bellefonte, PA). Methylenecetonic (100 μl) was added and again concentrated to dryness. Esters were prepared by adding 200 μl of desired reagent alcohol:acetvl chloride (5:1 v/v, freshly prepared on ice) and heating at 120°C for 20 min. Betaine esters prepared in this way included methyl, ethyl, n-propyl, isopropyl, n-butyl, and isobutyl. Under these same derivatization conditions betaine aldehyde was converted to the corresponding di-alcohol acetel derivatives. After reaction, excess reagent alcohol was evaporated under a stream of N₂ and the derivatives dissolved in 25 to 100 μl glycerol immediately prior to FABMS analysis. These derivatives were stable for over 1 month in the dried form at room temperature in sealed vials.

Isolation of Betaine(s) from Plant Tissues. Leaf tissue of Triticum aestivum (Probed winter wheat) and Spinacia oleracea (cv Bloomsdale) (1-2 gfw) was excised from 3 and 12 week old plants, respectively, grown in the Zeeon greenhouse in Palo Alto, CA without supplemental illumination in 10 cm diameter fiber pots containing supersoil (2:3:1 sand:perlite:1), fertilized with half strength Hoagland solution biweekly. Leaf or shoot tissue of Salicornia depressa, Cuscuta salina (parasitic on Salicornia), Grindelina humilis, Atriplex patula, Beta vulgaris, Cotula coronopifolia, Spargularia marina, Gnaphalium californicum, and Distichlis spicata (2-3 gfw) was collected on May 18, 1983, from the Baylands Nature Preserve Salt Marsh, Palo Alto, CA. Excised tissue was immediately extracted in preweighed vials containing 20 ml methanol. Vial weights before and after plant tissue addition were used to determine the fresh weight of plant tissue extracted.

Leaf tissue of Zea mays inbreds (all inbreds of Northrup King Co., Stanton, MN) was excised from rows (23 foot rows; 40 plants/row) grown at the Northrup King field station at Woodland, CA in the summer of 1984. Each sample was comprised of a random sample of 5 leaves per row (the youngest fully expanded leaf from 5 individual plants per row) from which a subsample of 37.5 cm² of leaf tissue (5 x 3 x 2.5 cm) was cut from the leaf lamina 15 to 20 cm from the leaf tip. The leaf tissue was cut into 15 x 1 x 2.5 cm sections and immediately placed in a vial containing 10 ml methanol. Sampling generally took less than 2 min/row. Samples were taken 8 weeks after planting (planting date, April 23, 1984; sampling date, June 20, 1984) at mid-day from 2 replicate rows of each of 22 inbred lines grown in randomized, bordered blocks. The trial was furrow irrigated on April 24, 1984, May 8, 1984, May 29, 1984, and June 19, 1984. Although no water stress was evident on the sampling date (June 20, 1984) substantial leaf rolling was observed in several of the inbred lines prior to the irrigation on June 19, 1984.

Methanol extracts of plant tissue were phase separated after at least 4 h of storage at 4°C by addition of chloroform and H₂O to give a final ratio of 10:5:6 (methanol extract:chloroform:H₂O, v/v/v) and the upper aqueous phase was concentrated to dryness, at which point a known amount of internal standard (glycine betaine-d₃, usually 1125 nmol) was added. The samples were redissolved in 1 ml H₂O and applied to a 2 x 1 cm column of Dowex-1-ΟH⁻ prepared from Dowex-1-CI⁻ by washing the latter column with 2 ml 6 M NaOH followed by 25 ml H₂O. Betaines were eluted with 6 ml H₂O and the aqueous eluants applied to 2 x 1 cm columns of Dowex-50W-H⁺. After washing the latter columns with 8 ml H₂O, betaines were eluted with 6 ml 6 M NH₄OH and concentrated to dryness. Samples were redissolved in 0.4 ml 60% methanol and esterified with n-propanol:acetyl chloride (5:1 v/v) prior to FABMS analysis as described above under Esterification.

Isolation of Betaine and Betaine Aldehyde from Spinach Leaf Discs Infiltrated with Choline-d₃ Chloride. Leaf discs (1.7 cm in diameter) were punched from 8 week old spinach plants and vacuum infiltrated with different concentrations of choline-d₃ chloride (in H₂O) for 1 min, and then incubated in the same infiltration solutions under fluorescent lights at 25°C for up to 4 h in glass Petri dishes (5 discs/25 ml of infiltration/incubation medium). Leaf discs were harvested at different time intervals by blotting dry on tissue paper and extracting in 5 ml methanol in preweighed vials. Each sample was comprised of 5 discs. The methanol extracts were processed for betaine and betaine aldehyde analysis exactly as described above except that γ-butyrobetaine (1125 nmol) was used as internal standard and that betaines and betaine aldehyde were eluted from Dowex-50W-H⁺ with 5 ml 2.5 M HCl rather than 6 ml 6 M NH₄OH.

Stable Isotope Tracer Studies with Sweet Corn Hybrids Differing in Glycine Betaine Levels. Four hybrids of Zea mays (sweet corn) (Silver Queen, Spirit, 1720 and 2708) obtained from Rogers Bros. Seed Co., Boise, ID were grown in 15 foot rows (20 plants/row) in W. Lafayette, IN in the summer of 1986 (planting date, May 11, 1986; sampling date, July 1, 1986). Leaf tissue (the youngest fully expanded leaf) was excised from 5 random plants per row of each genotype from which was punched 60 x
Determination of Betaines by FABMS

2.3 cm leaf discs (12 discs/leaf). Thirty leaf discs of each genotype were placed in 500 ml side-arm flasks containing 50 ml of either 1 mM \( d_2 \)-choline chloride or 1.6 mM \( d_2 \)-betaaine aldehyde chloride, vacuum infiltrated for 30 s, and subsequently incubated for up to 5 h at 25°C under fluorescent lights in the infiltmation medium. Samples of 5 leaf discs were removed at different time intervals (0, 1, 2, 3, 4, and 5 h) and extracted in 10 ml methanol. For the \( d_2 \)-betaaine aldehyde feeding studies \( d_2 \)-glycine betaine (1000 nmol/sample) was used as internal standard and betaines were eluted from Dowex-50W-H\(^+\) with 6 M NH\(_2\)OH. For the \( d_2 \)-choline feeding studies, \( \gamma \)-butyrobetaine (1125 nmol/sample) was used as internal standard, eluting betaines, and betaine aldehyde from Dowex-50W-H\(^+\) with 2.5 M HCl. In these studies, the samples were derivatized with \( n \)-butanol:acetyl chloride prior to FABMS analysis.

Mass Spectrometry. Aliquots of 1 \( \mu \)l of glycerol containing quaternary ammonium compounds or their derivatives were introduced into the mass spectrometer (HP5985A; Hewlett Packard) via the FAB probe and irradiated with Xenon (8 kv; 50 \( \mu \)amps, 50°C). The Fast Atom Capillaritron Source was by Pha- sor Scientific Inc., Duarte, CA (23).

RESULTS AND DISCUSSION

Method Development. In all analytical methodology, the objective of analyze derivatization is to confer particular physical or chemical properties which optimize selectivity and sensitivity. Desorption mass spectrometry methods are particularly well suited to preformed ionic species. Naturally occurring betaines are zwitterionic compounds with a carboxylic anion and quaternary ammonium cation in the same molecule. Two approaches can be taken to increase the net positive ion concentration. The glyceroic matrix in which the sample is dissolved can be acidified to impart a formal positive charge to the molecule, or the analyte can be esterified to the positively charged quaternary ammonium ester. The latter technique, termed 'reversed derivatization,' maximizes polarity which has been found to be desired in desorption mass spectrometry applications (3). Analysis of the zwitterionic form requires protonation in the condensed or gas phase, a reversible process, to form the observed adduct ion. The second case requires only a change of state from the condensed phase to the gas phase.

Ionization efficiency and therefore sensitivity depend as well on ionic size and interfacial surface properties. With compounds which contain distinct hydrophilic and hydrophilic character, surface activity in a liquid matrix can be affected by varying the hydrophilic aliphatic chain portions of the solute. For these reasons we sought to enhance method sensitivity by converting the zwitterionic betaines to the preformed ions of optimal ester chain length.

Significant effects of carbon number in the ester group can be observed by comparing positive ion FABMS sensitivity of equimolar mixtures of \( d_2 \)- and \( d_2 \)-glycine betaine esters relative to the glyceroic adduct ion (m/z 185) (Table I), and by comparing homologous \( d_2 \)-glycine betaine ester relative responses in an equimolar mixture (Table I). As seen in Table I, a relationship between sample/protonated glyceroic dimer (m/z 185) ratio and relative intensity versus carbon number of the ester group is strongly suggested. As the length of the carbon chain of the ester group is increased, signal intensity per nmol of betaine ester is markedly increased (Table I).

Selection of an appropriate derivative was based on signal intensity, competitive fragmentation via neutral elimination in the gas phase, minimal glyceroic background, and ease of derivative preparation. Esters greater than methyl undergo unimolecular decomposition to yield a fragment ion at m/z 127 for \( d_2 \)-glycine betaine and 118 for \( d_2 \)-glycine betaine esters, equivalent to protonated free acid (Table I). Straight chain esters fragmented to a lesser extent than \( \beta \)-branched structures (Table I) presumably due to the greater number of \( \gamma \)-hydrogens available for hydrogen migration in \( \beta \)-branched esters. Both the \( n \)-butyl and \( n \)-propyl esters of glycine betaine appear highly suitable for quantitative applications. A scan of FAB irradiated glyceroic produced a fairly clear spectrum from m/z 160 to m/z 170 (results not shown) suitable for quantifying \( d_2 \)- and \( d_2 \)-glycine betaine as the \( n \)-propyl esters at m/z 160 and 169, respectively. Although the cation of \( d_2 \)-glycine betaine \( n \)-butyl ester (m/z 183) gave the strongest signal (Table I), some background from irradiated glyceroic contributes to this signal at this mass, as shown by Field (10). The \( n \)-butyl ester of glycine betaine is not recommended for quantifying trace levels of \( d_2 \)-glycine betaine (i.e. less than 0.1 nmol/\( \mu \)l glyceroic). In our experience the lower limits of detection of the \( n \)-propyl and \( n \)-butyl esters of \( d_2 \)-glycine betaine are approximately 0.05 nmol/\( \mu \)l glyceroic for both esters. Signal observed at this level was approximately two times glyceroic chemical noise generated by the FAB process and blank background subtraction was necessary to acquire meaningful data (results not shown).

No attempt was made to look at higher alcohol homologs due to difficulties associated with removing excess reagent alcohol following the derivatization process.

Stable ion emission and long-lived signal duration have distinct advantages in statistical reliability and accuracy in this assay. We observed signal up to 15 min and there are reports of FABMS signal duration lasting hours (1). Scanning a narrow mass range of 25 mass units around the molecular cation region allows in excess of 300 data points to be acquired per minute. Transient sample signal such as occurs with gas or liquid chromatography-mass spectrometry can introduce bias in isotope ratio measurements through systematic mass cycling errors (24). Typically we acquired and integrated areas for 100 scans per sample. Figure 1 shows results of a linearity study, calibrating synthetic \( d_2 \)-glycine betaine against \( d_2 \)-glycine betaine as the \( n \)-propyl esters (m/z 169 and 160, respectively). The calibration curve was linear over two orders of magnitude from 45 to 0.45 nmol/\( \mu \)l glyceroic. It should be noted that the signal ratio for \( d_2 \) (m/z 160)/\( d_3 \) (m/z 169) was slightly less than 1 for an equimolar concentration (Fig. 1). The isotopically labeled derivatized produced a more stable cation with a lower degree of neutral elimination (see also Table I). This phenomenon was observed for several different ester isotope pairs which underwent gas phase unimolecular decomposition (Table I). Reliability of the assay was not affected, however, since the elimination occurs reproducibly.

\( \gamma \)-Butyrobetaine responded similarly to glycine betaine in that signal intensity per nmole increased markedly as the length of the carbon chain of the ester group was increased (results not shown). However, \( \gamma \)-butyrobetaine as the \( n \)-propyl ester exhibited a much lower degree of neutral elimination to the protonated free acid in comparison to the \( n \)-propyl ester of glycine betaine (Table II) (cf. Table I). Proline betaine-\( d_6 \) (stachydrine) and piperolic acid betaine (homostachydrine) \( n \)-propyl esters produced strong signals from the molecular cations but exhibited significant neutral elimination to the protonated free acids (Table II). Hydroxyproline betaine (betonicine) as the \( n \)-propyl ester (m/z 202) yields a fragment ion of mass 160 (results not shown). Thus, care should be taken in interpreting the origin of ions of mass 160 which could result from either the intact molecular cation of \( d_2 \)-glycine betaine \( n \)-propyl ester or neutral elimination of the hydroxyproline betaine ester to the protonated acid.

Betaine aldehyde yields di-alcohol acetate derivatives which exhibit exceptionally strong FABMS signals (Table III). Signal intensity again increases as the length of the carbon chain of the reagent alcohol is increased (Table III). The lower limit of detection above glyceroic background for the di-\( n \)-propyl and di-\( n \)-butyl acetate derivatives of betaine aldehyde correspond to
approximately 10 pmol and 5 pmol/μl glycerol, respectively (results not shown). Calibration of γ-butyrobetaine against α- and δ- glycine betaine and δ-beta- betaine aldehyde as the isobutyl derivatives gave the relative responses summarized in Table IV. The betaine aldehyde derivative was approximately 20 times more sensitive to detection than the γ-butyrobetaine derivative and 10 times more sensitive to detection than the glycine betaine derivatives (Table IV). It was possible to detect as little as 0.5 nmol betaine aldehyde in the presence of 1125 nmol γ-butyrobetaine, 11.25 nmol δ-glycine betaine, and 50 nmol δ-glycine betaine (Table IV).

Method Applications and Further Cautionary Notes. (a) Quantification of Betaine Levels in Halophytes. Betaines can be isolated from plant extracts free of amino acids, sugars, organic acids, choline, and betaine aldehyde in a simple two step ion exchange purification scheme, eluting betaines with 6 M NH₄OH from Dowex-50W-H⁺ in the last step (“Materials and Methods”). Removal of free amino acids on the first ion column (Dowex-1-OH⁻) is essential because in the derivatization process, amino acids also yield esters which can considerably complicate the mass spectra. Glutamate and aspartate (and their amides) yield di-carboxylic amino acid esters which give particularly strong FABMS signals as the protonated intact cations. The signals from di-carboxylic amino acid esters are much more intense than those for mono-carboxylic amino acids in the same molar ratios (results not shown). It is exceptionally important to remove valine which gives a molecular ion of identical mass to that of glycine betaine. By removing free amino acids, however, relatively clean mass spectra of the betaine fractions of glycine betaine containing plant species have been obtained. Glycine betaine n-propyl ester (m/z 160) and its protonated free acid fragmentation product (m/z 118) were virtually the only ions detected above glycerol background in the n-propyl esterified betaine fraction of several halophytes evaluated. Initial analyses were performed excluding any internal standard to verify that the plant extracts were free of ions of the same mass as the internal standard. Subsequent analyses were performed on extracts spiked with a known amount of δ-glycine betaine, scanning over a narrow mass range around the molecular cation region, calculating δ-glycine betaine levels from the ratio of ions...
Table III. Relative Responses of Betaine Aldehyde Alkyl Acetal Derivatives to FABMS

<table>
<thead>
<tr>
<th>Betaine Aldehyde Derivative</th>
<th>Relative Ion Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/z m/z m/z m/z m/z</td>
</tr>
<tr>
<td>Equimolar mixture of d5-betaine aldehyde di-alcohol acetal derivatives</td>
<td>102 148 176 185 204 232 235</td>
</tr>
<tr>
<td>d4-Betaine aldehyde di-n-propyl acetal</td>
<td>4 1 5 2 22 100 0</td>
</tr>
<tr>
<td>d4-Betaine aldehyde di-n-butyl acetal</td>
<td>0 0 0 1 0 0 100</td>
</tr>
<tr>
<td>d4-Betaine aldehyde di-n-propyl acetal</td>
<td>0 0 0 8 100 0 0</td>
</tr>
</tbody>
</table>

*a The ion of mass 185 corresponds to the protonated glycerol dimer.
*b Equimolar mixture of the isobutyl (m/z 232), n-propyl (m/z 204), ethyl (m/z 176), and methyl (m/z 148) di-alcohol acetal derivatives and underivatized (m/z 102) d4-betaine aldehyde each at 2 nmol/μl glycerol.
*c Betaine aldehyde-d3 di-n-butyl acetal (m/z 235) at 5 nmol/μl glycerol.
*d Betaine aldehyde-d4 di-n-propyl acetal (m/z 204) at 2 nmol/μl glycerol.

Table IV. Relative Responses of the Isobutyl Derivatives of Glycine Betaine, Betaine Aldehyde, and γ-Butyrobetaine to FABMS

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Concentration of the Quaternary Ammonium Compound Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ-Butyrobetaine isobutyl ester m/z 202</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>225* (100)#</td>
</tr>
<tr>
<td>2</td>
<td>225 (100)</td>
</tr>
<tr>
<td>3</td>
<td>225 (42.5)</td>
</tr>
<tr>
<td>4</td>
<td>112 (100)</td>
</tr>
<tr>
<td>5</td>
<td>112 (100)</td>
</tr>
<tr>
<td>6</td>
<td>112 (100)</td>
</tr>
<tr>
<td>7</td>
<td>225 (100)</td>
</tr>
<tr>
<td>8</td>
<td>225 (100)</td>
</tr>
<tr>
<td>9</td>
<td>225 (47)</td>
</tr>
<tr>
<td>10</td>
<td>112 (100)</td>
</tr>
<tr>
<td>11</td>
<td>112 (100)</td>
</tr>
<tr>
<td>12</td>
<td>112 (100)</td>
</tr>
</tbody>
</table>

*a Samples were as mixtures of γ-butyrobetaine, d4-glycine betaine, d4-glycine betaine, and d4-betaine aldehyde chloride, as indicated. Samples 1 to 6 were processed via Dowex-1-OH" and Dowex-50W-H" (eluting with 2.5 m HCl from the latter column) prior to derivatization and FABMS. Samples 7 to 12 were derivatized directly.
# Concentration of ester (nmol/25 μl glycerol).
* Relative ion intensity (of maximum ion intensity).
Table V. Glycine Betaine Levels Observed in a Range of Plant Species using Stable Isotope Dilution FABMS

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Origin of Plant Material</th>
<th>Glycine Betaine (\mu\text{mol/gfw})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salicornia depressa</em></td>
<td>Chenopodiaceae</td>
<td>Salt marsh</td>
<td>38*</td>
</tr>
<tr>
<td><em>Beta vulgaris</em></td>
<td>Chenopodiaceae</td>
<td>Salt marsh</td>
<td>22</td>
</tr>
<tr>
<td><em>Atriplex patula</em></td>
<td>Chenopodiaceae</td>
<td>Salt marsh</td>
<td>11</td>
</tr>
<tr>
<td><em>Spinacia oleracea</em></td>
<td>Chenopodiaceae</td>
<td>Salt marsh</td>
<td>5</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Gramineae</td>
<td>Greenhouse (unsalinized)</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Distichlis spicata</em></td>
<td>Gramineae</td>
<td>Salt marsh</td>
<td>40</td>
</tr>
<tr>
<td><em>Cuscuta salina</em></td>
<td>Convolvulaceae</td>
<td>Salt marsh (parasitic on</td>
<td>107</td>
</tr>
<tr>
<td><em>Patula</em></td>
<td></td>
<td><em>Salicornia</em>)</td>
<td></td>
</tr>
<tr>
<td><em>Catula coronopifolia</em></td>
<td>Compositae</td>
<td>Salt marsh</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Grindelia humilis</em></td>
<td>Compositae</td>
<td>Salt marsh</td>
<td>ND</td>
</tr>
<tr>
<td><em>Gnaphalium californicum</em></td>
<td>Compositae</td>
<td>Salt marsh</td>
<td>114</td>
</tr>
<tr>
<td><em>Spergularia marina</em></td>
<td>Caryophyllaceae</td>
<td>Salt marsh</td>
<td>ND</td>
</tr>
</tbody>
</table>

\* Glycine betaine determined from the ratio of ions 160:169 as described in Figure 1 in \(n\)-propanol esterified betaine fractions using \(d_9\)-glycine betaine as internal standard. \* Not detectable.

Table VI. Genotypic Variability in Glycine Betaine Levels in Maize Inbreds

<table>
<thead>
<tr>
<th>Inbred*b</th>
<th>Rep. 1</th>
<th>Rep. 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1506</td>
<td>0.74</td>
<td>0.47</td>
<td>0.61</td>
</tr>
<tr>
<td>644</td>
<td>0.66</td>
<td>0.72</td>
<td>0.69</td>
</tr>
<tr>
<td>338</td>
<td>0.96</td>
<td>0.56</td>
<td>0.76</td>
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\* Inbreds are ranked from low to high glycine betaine titer. \b One \text{cm}^2\text{ of leaf tissue is approximately equal to 0.018 gfw. Glycine betaine levels were determined from the ratios of ions 160:169 in }n\text{-propanol esterified betaine fractions using }d_9\text{-glycine betaine as internal standard.}

213) are clearly resolved from endogenous unlabeled glycine betaine \(n\)-propyl ester (m/z 160), the protonated glycerol dimer (m/z 185) and \(\gamma\)-butyrobetaine \(n\)-propyl ester as internal standard (m/z 188) in a single scan (Fig. 2B). The kinetics of conversion of \(d_9\)-choline to \(d_9\)-glycine betaine determined by this method (Fig. 2C) suggest a half-maximal velocity at an external concentration of 0.5 mM choline, and an apparent maximum velocity of about 330 nmol/h·gfw (Fig. 2C). Betaine aldehyde-\(d_9\) begins to accumulate substantially only when the supply of choline exceeds a concentration of 0.5 mM \(d_9\)-choline (Fig. 2D).

The apparent maximum velocity of choline oxidizing activity to glycine betaine in spinach agrees favorably with the highest rates of glycine betaine accumulation under extreme stress conditions in spinach (7.5 \(\mu\text{mol/d·gfw} = 312 \text{ nmol/h·gfw}\) (7)). These results support the view that betaine aldehyde is an inter-
mediate in the conversion of choline to glycine betaine in chen-
opods (7, 18).

Note that although choline and phosphoryl-choline coelute with betaine aldehyde and betaine(s) in the 2.5 M HCl eluant from Dowex-50W-H⁺, neither choline nor phosphoryl-choline derivatize with n-propanol:acetyl chloride. Thus, neither choline nor phosphoryl-choline appear in the FABMS spectra (Fig. 2B) presumably because these species are far less surface active than the betaine and betaine aldehyde derivatives in the glycerol matrix. In betaine/betaine aldehyde/choline/phosphoryl-choline fractions eluted from Dowex-50W-H⁺ with 2.5 M HCl, we have frequently observed Na⁺ and K⁺. Although this does not present a significant problem in the quantification of the betaine and betaine aldehyde derivatives, Na⁺ and K⁺ adduct ions with glyc erol are observed in the mass spectra (e.g. the ion of mass 223 in Fig. 2B corresponds to the K⁺ adduct ion of the glycerol dimer; 92 + 92 + 39⁺). With derivatized wizterionic species, H⁺, Na⁺, and K⁺ betaine adduct ions can occur, yielding complex spectra and distribution of betaine signal among several adduct ions. The formation of these betaine adduct ions relies upon the presence of a free carboxylic acid group (COO⁻) to which the cations can pair in the condensed phase. Reversed derivatization of betaines eliminates this problem of adduct ion formation in the condensed phase and represents a further rationale for adopting esterification for routine quantitation of betaines in plant extracts by FABMS.

(d) Kinetics of Metabolism of d₃-Choline and d₃-Betaine Alde-
hyde in Leaf Discs of Sweet Corn Hybrids Differing in Glycine
Betaine Levels. Analyses of several sweet corn hybrids (Rogers
Bros. Seed Co.) revealed a similar range of variability in glycine
betaine levels as observed for the field corn inbreds in Table VI.
Four sweet corn hybrids; Silver Queen, Spirit, 1720, and 2708,
were chosen for further characterization (Table VII). The hybrid
2708 exhibits exceptionally low glycine betaine titers (Table VII).
Feeding experiments with d₃-betaine aldehyde chloride revealed
that all four genotypes were capable of converting d₃-betaine
aldehyde to d₃-glycine betaine at similar rates in 5 h incubations
(Fig. 3). The FABMS spectra clearly revealed synthesis of d₃-
glycine betaine as the n-butyl ester (m/z 177) even in the d₀-
glycine betaine (i.e. m/z 174) deficient genotype, 2708 (results
not shown). Incorporation of d₃-betaine aldehyde into d₃-
glycine betaine was linear with respect to time in all four hybrids (Fig.
3). FABMS analysis of the synthetic d₃-betaine aldehyde chloride
revealed that this precursor was not contaminated with d₃-glycine
betaine (results not shown).

Unlike spinach, we were unable to detect incorporation of d₀-
choline into either d₀-betaine aldehyde or d₀-glycine betaine
(using γ-butyrobetaine as internal standard) in any of these four
genotypes in 5 h incubations (results not shown). These latter
analyses, undertaken using conditions in which betaine aldehyde
is not degraded, failed to reveal appreciable pools of unla beled
betaine aldehyde in any of the four sweet corn hybrids evaluated.
Betaine aldehyde levels (determined as the di-n-butyl acetal at
m/z 232) ranged from 0.2 to 0.5 nmol/gfw. If glycine betaine
deficiency in hybrid 2708 was due to an inherent deficiency in
the capacity to oxidize betaine aldehyde, accumulation of this
intermediate would be expected. Taken together, these results
suggest that the deficiency in glycine betaine in hybrid 2708 does
not reside at the level of the capacity to oxidize betaine aldehyde,
but indicate that exogenously supplied choline is not as effective
a precursor of glycine betaine in maize as in spinach. This may
be a consequence of the different compartmentation of choline
metabolism or the different pathways of choline biosynthesis en
route to betaine in chenopods and grasses (7, 17). Further work
is required to determine whether the phenotype of deficiency of
glycine betaine in maize is a function of genetic lesions in choline,
phosphoryl-choline, or phosphorytidyl-choline metabolism (17).

**CONCLUSIONS**

Derivatization of betaines and betaine aldehyde to impart
increased mass, a permanent positive charge (eliminating H⁺,
Na⁺, and K⁺ adduct ion formation in the condensed phase) and
hydrophobic properties (increasing surface activity in a glycerol
matrix) renders these quaternary ammonium compounds highly
sensitive to detection by FABMS. Purification of these com-
ponds from plant extracts requires a simple two-step ion ex-
change procedure. Quantification can be accomplished by the
use of deuterium labeled or homolog quaternary ammonium
compounds (e.g. γ-butyrobetaine) of distinct mass as internal
standards, with linearity maintained over a wide dynamic range
of sample/standard ratios. Sample analysis is facilitated: (a) by
the stability of the derivatives in the dried form, (b) stable ion
emissions when the samples are dissolved in glycerol and sub-
jected to positive ion FAB, and (c) rapidity of assay (less than 2
min/sample). These features of the assay method appear particu-
larly suited to stable isotope tracer studies on the pathway(s)
of betaine synthesis and for reliably and selectively quantifying low
mole amounts of betaines in complex mixtures of quaternary
ammonium compounds in plant extracts.

The present results reveal substantial genetic variability for
glycine betaine levels among maize inbreds and hybrids (>150-
fold) (cf. only 2- to 3-fold in barley cultivars [16]). We are

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**Table VII. Glycine Betaine Levels of Sweet Corn Hybrids**

<table>
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<tr>
<th>Sweet Corn Hybrid</th>
<th>Mean Glycine Betaine Level (nmol/gfw)</th>
<th>Standard Deviation</th>
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<tr>
<td>Silver Queen</td>
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<td>2415</td>
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<tr>
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<td>2708</td>
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</table>

*Means and standard deviations of eight independent analyses. Glycine betaine levels were determined from the ratios of ions 174:183 in n-butanol esterified betaine fractions using d₀-glycine betaine as internal standard.*

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**Fig. 3.** Time courses of incorporation of d₃-betaine aldehyde into d₃-
glycine betaine in leaf discs of four sweet corn hybrids (Silver Queen, C; Spirit, C; 1720, C; 2708, C). Linear regression equations of best-fit were as follows (where Y = mX + b, and Y = d₃-glycine betaine level (nmol/ gfw), m = rate (nmol/h-gfw). X = time (h), b = initial d₃-glycine betaine level (nmol/gfw), and r = correlation coefficient): Silver Queen—m = 127.51, b = 4.381, r = 0.9993; Spirit—m = 168.94, b = -4.857, r = 0.9975; hybrid 1720—m = 163.69, b = 3.286, r = 0.989; hybrid 2708—m = 127.66; b = -0.4763, r = 0.9952. Glycine betaine-d₃ levels were determined from the ratios of ions 177:183 in n-butanol esterified betaine fractions using d₀-glycine betaine as internal standard.
currently exploring the biochemical and genetic basis for the phenotype of glycine betaine deficiency and its contribution (or lack thereof) to stress susceptibility in maize. Preliminary results presented here indicate that the deficiency in glycine betaine in at least one sweet corn hybrid probably does not reside at the level of the capacity to oxidize betaine aldehyde.

Acknowledgments—We thank Dr. Andrew D. Hanson for stimulating discussions and for urging us to seek alternative methods for betaine and betaine aldehyde determination. We are grateful to Drs. Jim Mock, Larry Balko and Ruedi Sandmeier of Northrup Co., and Dr. Leon Hansen of Rogers Bros. Seed Co., for providing seed of Zea mays and for their encouragement in this project.

LITERATURE CITED