The Effect of Salt Stress on the Catabolism of Sugars in Leaves and Roots of a Mangrove Plant, *Avicennia marina*

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Z. Naturforsch. **52c,** 187–192 (1997); received October 29/November 25, 1996

Avicennia marina, Grey Mangrove, Respiration, Sucrose, Catabolism

Respiration and related aspects of metabolism were investigated in the roots and leaves of 2-year-old trees of the mangrove plant, *Avicennia marina* in the presence of 100, 250 and 500 mM NaCl. The rate of respiration of leaves increased with increasing concentrations of NaCl in the incubation medium, but respiration of roots was not similarly affected. In order to examine the relative rates of catabolism of glucose by the glycolysis-tricarboxylic acid (TCA) cycle and the oxidative pentose phosphate pathway (PP pathway), we determined the rates of release of $^{14}CO_2$ from [1–^{14}C]glucose and from [6–^{14}C]glucose in segments of roots and leaves. The ratios of rates (C_6/C_1) in roots varied from 0.30 to 0.44, while ratios of 0.85 to 0.99 were obtained when leaves were incubated in the presence of various concentrations of NaCl. It appeared that the PP pathway was more involved in sugar catabolism in the roots than in the leaves of *A. marina.* Uniformaly ^{14}C-labelled sucrose, incubated with segments of roots and leaves for 18 h, was converted to CO_2, amino acids (mainly glutamine), organic acids (mainly malic acid), sugars and ethanol-insoluble macromolecules. The incorporation of radioactivity into most of these components was not significantly affected by NaCl. However, in leaves (but not in roots) the release of $^{14}CO_2$ from [U–^{14}C]sucrose was enhanced by NaCl at 250 mM and 500 mM, while the rate of incorporation of radioactivity into macromolecules was reduced by high concentrations of NaCl. Incorporation of radioactivity from [U–^{14}C]sucrose into malic acid was enhanced in both roots and leaves by an increase in the concentration of NaCl from 100 mM to 500 mM (this concentrations is similar to that in sea water). Independent of the concentration of NaCl, more than half of the radioactivity in the neutral fraction from leaves was incorporated into an unidentified sugar, while in the same fraction from roots, the radioactivity was associated with glucose, fructose and sucrose. On the basis of these results, a discussion is presented of the characteristics of catabolism of sugars in *A. marina* in relation to salt resistance.

Introduction

Mangrove plants grow along in subtropical and tropical coasts. While there have been many ecological and morphological studies (for review, see Tomlinson, 1986), only limited information is available about the biochemical and metabolic properties of mangrove plants. *Avicennia marina,* which grows in tidal swamps, has been classified as a euhalophyte (Burchett *et al.,* 1989) on the basis of the criteria proposed by Glenn and O'Leary (1984). The salt resistance of this plant seems to be based on three different mechanisms, namely, salt avoidance by roots, the capacity for the maintenance of normal metabolic activity in the presence of high intracellular levels of salt, and recreation of some of the penetrating ions (Waisel *et al.,* 1986). Details of photosynthesis by *A. marina* and its responses to salinity were reported by Ball and Farquhar (1984 a,b). However, to our knowledge, no reports have been published about the metabolism of carbohydrates in this mangrove plant, although *A. marina* has been classified as a C_3 plants (Ball and Farquhar, 1984 a; Ball *et al.,* 1984; Bjorkman *et al.,* 1988). In the present study,

Abbreviations: LS medium, Linsmaire and Skoog (1965) medium; PP pathway, oxidative pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; TLC, thin-layer chromatography.

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0939–5075/97/0300–0187 $ 06.00

we examined respiratory metabolism and the related metabolism of carbohydrates in the leaves and roots of *A. marina*.

Materials and Methods

Plant material

Seeds (propagules) of *Avicennia marina* (Forstk.) Vierh. were collected on Iriomote Island, Okinawa, Japan, and seedlings were grown in a 0.3% solution of Hyponex (Murakami Bussan Co., Tokyo, Japan) supplemented with 100 mM NaCl under natural light at 15–35 °C in a greenhouse at the Forest and Forest Products Research Institute, Tsukuba, Japan. Leaves and roots used in this study were obtained from two-year-old plants.

Measurements of respiratory gas exchange

Uptake of O_2 was monitored by a revised version of Warburg manometric techniques with an "Oxigen uptester" (Taitec Co. Ltd., Tokyo, Japan). Samples (300 mg fresh weight) were placed in a flask that contained 3 ml of Linsmaier and Skoog (1965) medium (LS medium), pH 5.7, supplemented with various concentrations of NaCl, and shaken at 30 °C. The rate of O_2 uptake was measured at 5-min intervals for 60 min.

Administration of labelled compounds

Labelled compounds were administered by the methods of Ashihara and Matsumura (1977). All radiolabelled compounds were purchased from Amersham International plc (Amersham place, Little Chalfond, U. K.). Segments of leaves (5-mm squares) and roots (1 mm-thick, round slices) were incubated in 2 ml of LS medium supplemented with NaCl plus a labelled compound in a 30-ml Erlenmeyer flask with a centre well. In each well there was a small tube that contained a piece of filter paper wetted with 0.1 ml of 20% KOH. The flasks were incubated on an oscillating water-bath at 27 °C in darkness. At the end of each incubation, a small amount of perchloric acid (final concentration, 0.06%) was added to the main compartment of the flask to liberate solubilized $^{14}CO_2$. After 5 min, the small tube that contained a piece of filter paper wetted with KOH was removed from the centre well, and the leaf and root segments were washed with water and frozen with liquid nitrogen.

For determinations of ratio of rates of C_6 to C_1 metabolism, 400 mg of leaf segments and 500 mg of root segments were used as plant materials and 0.5 mM [1–^{14}C] glucose (specific activity, 1.6 MBq μmol^{-1}) and 0.5 mM [6–^{14}C] glucose (specific activity, 1.6 MBq μmol^{-1}) were used as radiolabelled tracers. The duration of each incubation was 90 min. For the determination of the metabolic fate of sucrose, 200 mg of leaf segments and 500 mg of root segments and 4.0 μM [U–^{14}C] sucrose (specific activity, 23.1 MBq μmol^{-1}) were used. The duration of each incubation was 18 hrs.

Analysis of radioactive metabolites

The $^{14}CO_2$ released from labelled compounds was collected in KOH in the centre wells of flasks. The solution of KOH that contained $KH^{14}CO_3$ was diluted with 10 ml of distilled water and aliquots (0.5 and 1.0 ml) were used for determinations of radioactivity.

Segments of leaves and roots were homogenized with 70% methanol that contained 20 mM sodium diethyldithiocarbamate, and each homogenate was incubated at 70 °C for 10 min. After cooling, each homogenate was centrifuged at 20,000 x *g* for 10 min. The precipitate was suspended in 5 ml of 70% methanol that contained 20 mM sodium diethyldithiocarbamate and centrifuged as above. The supernatants were combined and evaporated to dryness. The residue was dissolved in 1 ml of distilled water and separated into basic, acidic and neutral fractions on columns of Dowex 50W-X4 (H^+ form; Muromachi Kagaku Kogyo Ltd., Tokyo, Japan) and Dowex 1-X4 (Cl^- form; Muromachi Kagaku Kogyo Ltd., Tokyo, Japan). Samples (0.5 ml) were applied to the combination of two columns which contained 800 mg of Dowex 50W (upper column) and 800 mg of Dowex 1 (lower column). Columns were washed with 10 ml of distilled water, and unabsorbed compounds were designated the neutral compounds. Compounds that had adsorbed to the columns of Dowex 50W and Dowex 1 were eluted with 15 ml of 2 N NH_4OH and 15 ml of 5 N HCOOH, respectively, and the materials in the respective eluates were designated the basic and acidic compounds, respectively. Each fraction was evaporated to dryness and each resi-

due was dissolved in a small amount of 70% methanol (neutral and acidic compounds) or 0.1 N HCl (basic compounds).

Separation of the individual compounds in the three fractions was achieved by thin-layer chromatography on microcrystalline cellulose plates (Ashihara and Matsumura, 1977; Saito *et al.*, 1989). A mixture of 1-butanol, acetic acid and water (4:1:2, v/v) was used as the solvent system for the neutral and acidic compounds, and a mixture of phenol, water and formic acid (75:25:1, v/v) was used for the acidic compounds. For the separation of the neutral compounds, development of TLC plates was repeated with the same solvent system because the Rf values of sugars were low. The incorporation of radioactivity into individual amino acids was also analyzed with a fluorometric amino acid analyzer, which consisted of a type LC-4A HPLC system (Shimadzu Co., Kyoto, Japan) connected to a Shimpack ISC-07 (Li^+ type; Shimadzu) and ammonia trap column (S-1504; Shimadzu), a fluoro-metric monitor (type 530; Shimadzu) and an integrator (Chromato-Pack, type C-R1B; Shimadzu). Radioactivity was monitored with a radioanalyzer (type RCL-551; Aloka Co., Tokyo, Japan) connected to the system. Three different solvents: 0.15 N lithium citrate, pH 2.65, that contained 7% methylcellosolve; 0.3 N lithium citrate, pH 10.0; and 0.2 N lithium hydroxide were used as the mobile phase (Saito *et al.*, 1989; Ukaji and Ashihara, 1987). The total running time was 180 min. The recovery of amino acids was corrected by reference to measurements made with an internal standard.

Results and Discussion

Respiration in leaves and roots

Figure 1 shows the respiratory uptake of oxygen by leaves and roots of *A. marina*. The respiratory rate in roots was 40 nmol min^{-1} g^{-1} fresh weight, and concentrations of NaCl as high as 500 mM (this concentration is similar to that in seawater) did not influence the rate. By contrast, the rate in leaves increased with increasing concentrations of NaCl. The rates at 250 mM and 500 mM NaCl were 1.2-fold and 1.4-fold higher than that at 100 mM NaCl (the spciemens of *A. marina* used in these experiments had been grown at this concentration of NaCl). On a fresh weight basis, rates of respiration by leaves were much higher than those by roots. However, this difference was mainly due to the difference in structure between leaves and roots. Roots of *A. marina* contain large amounts of spongy tissue that lacks cytoplasm. Stimulation of respiration in leaves of *A. marina* by 100% seawater was also reported by Burchett *et al.* (1989).

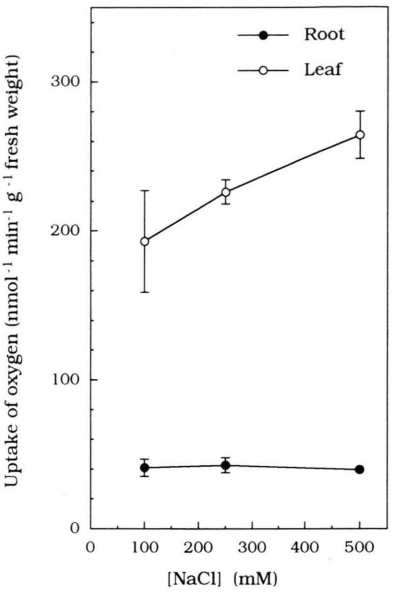

Fig. 1. Effects of NaCl on the rate of uptake of oxygen by leaves and roots of *Avicennia marina*. The values are expressed as nmol min^{-1} g^{-1} fresh weight with s.d. (bars; n=4).

Pathways for the oxidation of carbohydrates

In higher plants, glucose is catabolized by glycolysis and by the oxidative pentose phosphate (PP) pathway. The relative contributions of these pathways to total catabolism were estimated by measuring the rates of release of $^{14}CO_2$ from [1−^{14}C]glucose and [6−^{14}C] glucose (Fig. 2). When glucose is metabolized via glycolysis and the TCA cycle, the rates of release of $^{14}CO_2$ from [1−^{14}C]glucose and [6−^{14}C] glucose (C_1 and C_6) are the same. By contrast, the rate of release of CO_2 from [1−^{14}C] glucose is higher than that from [6−^{14}C] glucose if glucose is catabolized via the PP pathway. The radioactivity from [1−^{14}C] glucose is released specifically as $^{14}CO_2$ by the reaction catalyzed by 6-phosphogluconate dehydrogenase, the second dehydrogenase in the PP pathway. In

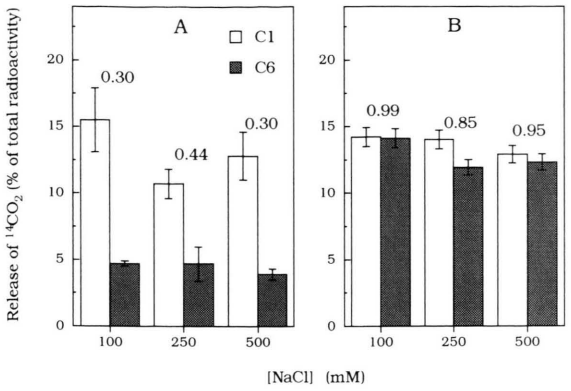

Fig. 2. Effects of NaCl on the release of $^{14}CO_2$ from roots (A) and leaves (B) of *Avicennia marina* after incubation with [1–^{14}C] glucose and [6–^{14}C] glucose. Release of $^{14}CO_2$, expressed as a percentage of the total radioactivity taken up by the samples and s.d. (bars; n= 4) are shown. The values shown above the column are the C_6/C_1 ratios. Total uptake of glucose by roots and leaves in the presence of 100 mM, 250 mM and 500 mM NaCl was as follows: Roots, 2.68±0.33, 2.21±0.18 and 2.61±0.37 nmol g^{-1} fresh weight; Leaves, 6.35±0.44, 7.68±0.33 and 8.55±1.21 nmol g^{-1} fresh weight.

roots, the rate of release of $^{14}CO_2$ from [1–^{14}C] glucose was more than twice that from [6–^{14}C] glucose, but similar rates of evolution of $^{14}CO_2$ were obtained when the two forms of labelled glucose were incubated with leaves. The ratios of rates (C_6/C_1) observed with roots varied from 0.30 to 0.44, while ratios of 0.85 to 0.99 were obtained with leaves that had been incubated in the presence of various concentrations of NaCl. Although many problems are associated with attempts to estimate the relative flux of the PP pathway and the glycolysis-TCA cycle from the C_6/C_1 ratio alone (ap Rees, 1980), the considarable difference in the ratios between the roots and the leaves suggests that hexose phosphates might be catabolized by different routes in roots and leaves. This result implies the greater involvement of the PP pathway in the oxidation of carbohydrates in roots than in leaves. This possibility is reasonable because the PP pathway is a major supplier of NADPH in non-photosynthetic tissues. In roots, the highest ratio of rates, accompanied by the lowest rate of release of $^{14}CO_2$ from [1–^{14}C] glucose, was observed at 250 mM NaCl. Inhibition of the release of $^{14}CO_2$ from [1–^{14}C] glucose by 250 mM NaCl was partly overcome at a higher concentration of NaCl (500 mM). These results suggest that the rate of catabolism of glucose by the PP pathway might be influenced by the concentration of NaCl. In root tips of the halophyte *Tamarix tetragyna*, salinity above 120 mM NaCl was reported to increase the proportion of absorbed glucose oxidized via the PP pathway, but such salinity did not affect the glycolytic pathway (Kalier and Poljakoff-Mayber, 1976).

Metabolism of sucrose in roots and leaves

In the majority of higher plants, the photosynthates are transported as the disaccharide sucrose (Smith, 1993). Thus, the oxidation of carbohydrates in plants usually involves sucrose as the starting material, and a large fraction of the fixed carbon enters the respiratory pathways and is used to provide carbon skeletons for biosynthesis (ap Rees and Dancer, 1987; Kubota and Ashihara, 1990). Therefore, [U–^{14}C]sucrose was chosen as the precursor for estimations of the metabolism of sugars in *A. marina*.

[U–^{14}C]Sucrose was administered to segments of roots and leaves of *A. marina* in darkness for 18 h in the presence of 100, 250 or 500 mM NaCl. The rates of total uptake of [U–^{14}C]sucrose by the segments and the rates of incorporation of radioactivity into various metabolites were determined and are shown in Table I. NaCl slightly enhanced the uptake of sucrose by both roots and leaves.

In roots, more than a half of the total radioactivity was recovered as CO_2, and 22–28% was recovered as ethanol-insoluble compounds, most probably proteins and polysaccharides. The rest of the radioactivity was distributed in ethanol-soluble, small molecules. The main neutral compounds were glucose, fructose and sucrose and the predominant acidic component was malic acid. Among the basic compounds, the most heavily labelled compound was glutamine. Analysis of this fraction by HPLC and TLC revealed that small amounts of radioactivity had also been incorporated into γ-aminobutyric acid and three unidentified amino acids which had not been incorporated into proteins. The distribution of radioactivity from [U–^{14}C]sucrose in most metabolites was similar even when the segments of roots had been incubated with high concentrations of NaCl. However, the rate of incorporation of radioactivity into acidic compounds, including malic

Table I. Metabolism of [U-^{14}C] sucrose by roots (A) and by leaves (B) of *Avicennia marina* in the presence of 100, 250 and 500 mM NaCl. Incorporation into metabolites is expressed as kBq g^{-1} fresh weight with s.d. (n=4). The values in parentheses show percentage of total radioactivity taken up by the samples.

(A) Roots

Fraction	100 mM	250 mM	500 mM
CO_2	93.8±0.6 (54.1)	100.5±1.5 (52.0)	107.0±9.4 (54.5)
Neutral compounds	15.7±1.0 (9.1)	16.2±0.8 (8.4)	21.1±1.8 (10.7)
Sucrose	5.4±2.6 (3.1)	4.5±0.4 (2.3)	6.4±1.9 (3.3)
Glucose	3.5±1.4 (2.0)	3.6±0.4 (1.9)	5.4±1.4 (2.8)
Fructose	5.8±1.9 (3.3)	5.7±0.2 (3.0)	7.0±1.9 (3.6)
Acidic compounds	7.1±0.1 (4.1)	11.9±0.2 (6.2)	15.9±0.1 (8.1)
Malate	4.5±0.1 (2.6)	8.3±0.3 (4.3)	11.7±0.3 (6.0)
Basic compounds	7.6±0.5 (4.4)	10.1±0.8 (5.2)	8.4±0.4 (4.3)
Glutamine	4.0±0.4 (2.3)	5.0±0.4 (2.6)	4.0±0.4 (2.0)
Insoluble comounds	49.1±3.6 (28.3)	54.5±5.6 (28.2)	43.9±0.2 (22.4)
Total uptake	173.3±3.8 (100)	193.2±8.5 (100)	196.3±7.9 (100)

(B) Leaves

Fraction	100 mM	250 mM	500 mM
CO_2	121.8±0.8 (43.1)	150.7±23.4(50.1)	168.5±1.8 (52.6)
Neutral substance	22.9±0.5 (8.1)	21.0±4.0 (7.0)	26.7±1.0 (8.3)
Sucrose	1.5±0.1 (0.5)	2.2±0.8 (0.7)	4.5±0.4 (1.4)
Glucose	3.0±0.1 (1.1)	2.3±0.7 (0.8)	3.2±0.1 (1.0)
Fructose	1.7±0.1 (0.6)	1.4±0.8 (0.5)	2.3±0.2 (0.7)
Unknown	12.3±0.3 (4.4)	11.8±1.1 (3.9)	12.4±0.9 (3.9)
Acidic substances	20.1±0.4 (7.1)	20.4±3.1 (6.8)	23.5±0.8 (7.3)
Malate	5.6±0.1 (2.0)	7.4±1.3 (2.5)	11.9±0.4 (3.7)
Basic substances	36.8±0.8 (13.0)	37.3±1.6 (12.4)	40.5±2.0 (12.6)
Glutamine	22.3±0.5 (7.9)	25.0±1.1 (8.3)	26.9±2.1 (8.4)
Insoluble substances	81.1±0.1 (28.7)	71.4±5.5 (23.7)	61.4±0.5 (19.2)
Total uptake	282.7±0.1 (100)	300.8±9.2 (100)	320.6±1.0 (100)

acid, increased at high concentrations of NaCl. It has been suggested that malic acid is present in the dianionic form (as malate) in the cytoplasm, although **Hmal**$^-$ or **H_2mal** may also be present in the acidic vacuoles (Martinoia and Rentsch, 1993). Kirhby and Knight (1977) reported that unequal uptake of cations or anions by plant roots can be balanced by the synthesis of malate in response to an excess of cation, or by the degradation of malate in the case of an excess of cation . Therefore, if *A. marina* were to absorb more Na^+ than Cl^- ions, synthesis of malate might be stimulated. However, the amount of Na^+ ions in leaves of *A. marina* grown with 50, 250 and 500 mM NaCl was reported to be similar to or slightly lower than that of Cl^- ions (Ball and Farquhar, 1984b).

In leaves, the rate of release of $^{14}CO_2$ from [U-^{14}C]sucrose increased at higher concentrations of NaCl and most $^{14}CO_2$ seemed to be released via the TCA cycle during respiration. This assumption is supported by the observation of a higher rate of respiratory uptake of O_2 at higher concentrations of NaCl. The results suggest that more sucrose was utilized for respiration in the presence of a high concentration of NaCl, which might result in production of more ATP, as is necessary for the removal of NaCl from the cytoplasm of leaves. Concomitantly, the rate of incorporation of radioactivity into ethanol-insoluble compounds decreased at high concentrations of NaCl. The rate of incorporation of radioactivity from [U-^{14}C]sucrose into ethanol-insoluble metabolites has often been considered to represent the total capacity for biosynthesis of macromolecules in plant tissues (Faust *et al.*, 1968; Ashihara and Komamine, 1975; Ashihara and Sato, 1993). The inhibition of growth of *A. marina* that is observed at high salinity (e.g., Burchett *et al.*, 1989) might be partially due to a reduction in biosynthetic capacity of leaf tissues caused by the salt. Less than 30%

of the total radioactivity was recovered as ethanol-soluble compounds. The distribution of radioactivity into individual metabolites was very similar to that observed in roots, but it is noteworthy that approximately 4% of the total radioactivity was found an unidentified neutral compound. The Rf value of this compound during TLC with repeated development with a mixture of butanol, acetic acid and water (see Materials and Methods) was 0.64. The value is much higher than the Rf values of fructose (0.45), glucose (0.43) and sucrose (0.38). Therefore, the compound seemd to be a neutral compound with a low molecular weight. Possible candidate compounds, such as glycerol, mannitol, stachyose and raffinose, had different Rf values. The rate of incorporation of radioactivity into this unidentified neutral compound was not influenced by the concentration of NaCl.

Acknowledgement

This research was supported by research grants from the Fujiwara Natural History Foundation and the Salt Science Research Foundation to H. A.

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