Metabolic Fate of ^{14}C-Labelled Nicotinamide and Adenine in Germinating Propagules of the Mangrove *Bruguiera gymnorrhiza*

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We studied the metabolic fate of [*carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine in segments taken from young and developing leaves, stem, hypocotyls, and roots of a shoot-root type emerging propagule of the mangrove plant *Bruguiera gymnorrhiza*. Thin-layer chromatography was used together with a bioimaging analyser system. During 4 h of incubation, incorporation of radioactivity from [*carbonyl*-^{14}C]nicotinamide into NAD and trigonelline was found in all parts of the propagules; the highest incorporation rates into NAD and trigonelline were found in newly emerged stem and young leaves, respectively. Radioactivity from [8-^{14}C]adenine was distributed mainly in the salvage products (adenine nucleotides and RNA), and incorporation was less in catabolites (allantoin, allantoic acid, and CO_2). Adenine salvage activity was higher in young leaves and stem than in hypocotyls and roots. Over a short time, the effect of 500 mM NaCl on nicotinamide and adenine metabolism indicated that NaCl inhibits both salvage and degradation activities in roots.

Key words: Bruguiera gymnorrhiza, Mangrove, Nucleotide Metabolism

Introduction

Bruguiera gymnorrhiza (black mangrove) is one of the most important and widespread mangrove species on the Ryukyu Islands of southern Japan (Takemura *et al*., 2000; Suwa *et al*., 2009). Mangrove plants have special morphological characteristics enabling them to withstand high levels of salt stress (Tomlinson, 1994; Hogarth, 1999; Kathiresan and Bingham, 2001). *Bruguiera gymnorrhiza* produces viviparous seeds (propagules); these are seeds that germinate on the parent plant. The mature propagules drop into the sea water, which can transport them over large distances. Once propagules are ready to root, they are likely to lodge in the mud. Although some morphological studies have been made (Tomlinson, 1994), little is known about the metabolism of mangrove propagules (Hogarth, 1999; Krauss and Allen, 2003). We have previously investigated the purine, pyrimidine, and pyridine nucleotide metabolism in the leaves of some mangrove plants (Ashihara *et al.*, 2003, 2010; Sato and Ashihara, 2008). However, the metabolism in individual organs of mangrove plants was determined only in *Avicennia marina* seedlings (Ashihara *et al.*, 2010).

Nicotinamide and adenine are degradation products of NAD and adenine nucleotides, and they are reutilized in the formation of these nucleotides by so-called salvage reactions (see Stasolla *et al*., 2003; Zrenner and Ashihara, 2011). Secondary metabolites, such as trigonelline and allantoin, are also produced from these compounds (see Ashihara, 2008; Zrenner and Ashihara, 2011). Activities of the salvage reactions and secondary metabolite formation differ widely between plant species and organs, and are often influenced by environmental stress, such as salt stress (Stasolla *et al.*, 2003; Ashihara, 2008). In the present work, the metabolic fate of [*carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine in different parts of shoot-root type emerging propagules of *Bruguiera gymnorrhiza* was studied so as to determine the profile of the nucleotide metabolism in these plant materials.

Material and Methods

Chemicals

[*Carbonyl*-^{14}C]nicotinamide (2.0 GBq/mmol) and [8-^{14}C]adenine (1.9 GBq/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Plant materials

Propagules of *Bruguiera gymnorrhiza* (L.) Lam. (Rhizophoraceae) were collected in December 2008 at Hoshidate on Iriomote Island, Okinawa, Japan. The propagules were cultured in distilled water with no nutrient for 1 year. A number of young and developed leaves, a stem, a cotyledon, and roots of a shoot-root type emerged propagule (Fig. 1) were separated, and segments of each organ were studied in ^{14}C-tracer experiments. The experiments were repeated using four propagules.

Administration of labelled compounds

[*Carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine were administered as described in our previous papers (Ashihara *et al.*, 2003, 2010). Segments of each organ [ca. 100 mg fresh weight (FW)] in 2.0 ml of 30 mM potassium phosphate buffer (pH 5.6), containing 10 mM sucrose and 0 or 500 mM NaCl, were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a glass tube containing a piece of folded filter paper that had been impregnated with 0.1 ml of 20% KOH in the centre well. Each reaction was started by adding 10 μl of a solution of [*carbonyl*-^{14}C]nicotinamide or [8-^{14}C]adenine (37 kBq) to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27 °C. After incubation, the glass tube was removed from the centre well and placed in a 50-ml Erlenmeyer flask containing 10 ml distilled water. At the same time the leaf disks were harvested and washed with distilled water, frozen, and then stored in liquid N_2 prior to extraction.

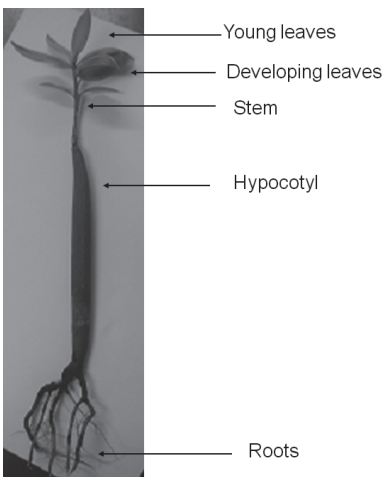

Fig. 1. Propagules of *Bruguiera gymnorrhiza* (black mangrove) used in this work. Mature propagules collected from adult trees on Iriomote Island were cultured in distilled water without nutrient in the university greenhouse for 1 year.

Analysis of $^{14}CO_2$ and ^{14}C-metabolites

Analyses of ^{14}C-labelled compounds were performed as shown in our previous papers (Ashihara *et al.*, 2010; Deng and Ashihara, 2010) with slight modifications as mentioned below. For [8-^{14}C]adenine metabolites, the precipitate [the perchloric acid (PCA)-insoluble fraction] was washed with a mixture of ethanol and ether (1:1, v/v) at 50 °C for 15 min to remove lipids. Then nucleic acids (DNA plus RNA) in the insoluble fraction were hydrolyzed with 6% PCA at 100 °C for 15 min. The radioactivity observed in the resulting hot PCA-soluble metabolites (purine bases derived from nucleic acids) was taken as the radioactivity incorporated into nucleic acids (mainly RNA). For the thin-layer chromatography (TLC) separation of PCA-soluble ^{14}C-labelled metabolites from [*carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine, the solvent systems IV (isobutyric acid/ammonia/water, 660:17:330, v/v/v) and I (*n*-butanol/acetic acid/water, 4:1:2, v/v/v) specified by Zheng and Ashihara (2004) were used, respectively.

Results and Discussion

Metabolism of [carbonyl-^{14}C]nicotinamide

Fig. 2 shows the results of the separation of the PCA-soluble metabolites derived from [*carbonyl*-^{14}C]nicotinamide by TLC, using solvent system IV (Zheng and Ashihara, 2004). Spots corresponding to nicotinic acid and nicotinamide and to nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NaMN) were clearly separated, and NAD was also separated from NADP by this system. The major spots corresponded to trigonel-

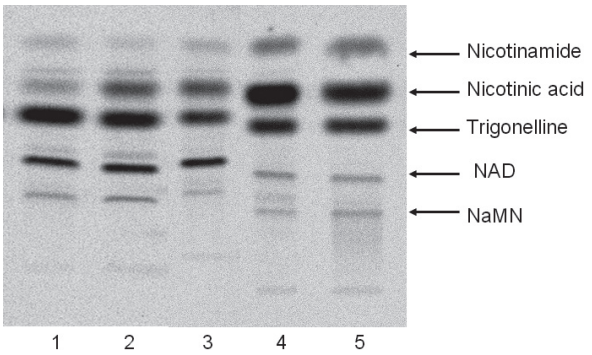

Fig. 2. Separation of the PCA-soluble ^{14}C-metabolites from [*carbonyl*-^{14}C]nicotinamide by TLC using solvent system IV (isobutyric acid/ammonia/water, 660:17:330, v/v/v). Samples were obtained from (1) young leaves, (2) developing leaves, (3) stem, (4) hypocotyls, and (5) roots of the propagules of *Bruguiera gymnorrhiza*. NaMN, nicotinic acid mononucleotide.

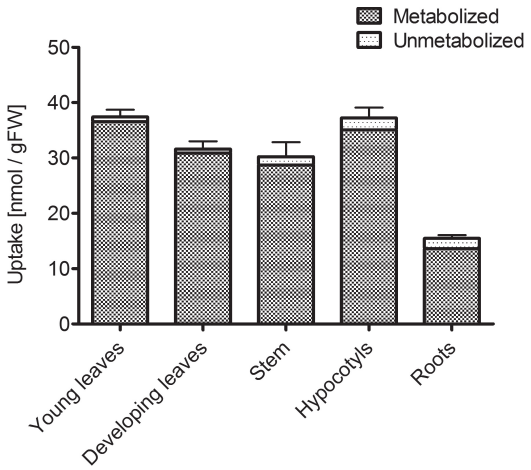

Fig. 3. Uptake of [*carbonyl*-^{14}C]nicotinamide by segments taken from various parts of *Bruguiera gymnorrhiza* propagules. The incubation time was 4 h. The total uptake by the segments was calculated by adding the radioactivity found in all cellular metabolites and in CO_2. Rates of total uptake are expressed as nmol/g fresh weight (FW) ± SD. The radioactivity recovered as nicotinamide (unmetabolized) and other compounds (metabolized) is shown separately.

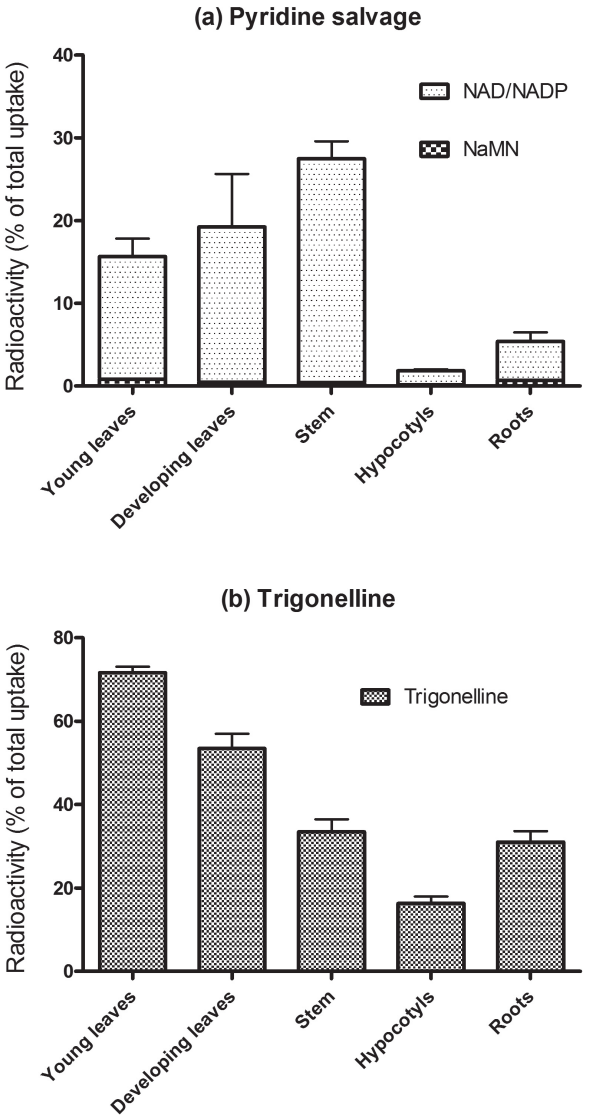

Fig. 4. Incorporation of radioactivity from [*carbonyl*-^{14}C]nicotinamide into (a) salvage products (NAD and NaMN) and (b) trigonelline in segments from various parts of *Bruguiera gymnorrhiza* propagules. The incubation time was 4 h. Incorporation of radioactivity into individual metabolites is expressed as percentage of total radioactivity taken up by the segments shown in Fig. 3. The SD values for the incorporation rates are also shown.

line, nicotinic acid, and NAD. Small amounts of radioactivity were found in NaMN and nicotinamide. Fig. 3 shows the total uptake of [^{14}C]nicotinamide by segments of the five different organs, expressed as nmol per g fresh weight. The uptake of [^{14}C] nicotinamide in roots was lower than in the other parts of the seedlings. Most of [^{14}C]nicotinamide taken up by the segments was metabolized 4 h after administration. Within 4 h of incubation, a large portion of [^{14}C]nicotinamide was converted to nicotinic acid and utilized for NAD and trigonelline synthesis. Figs. 4a and 4b show the results of the quantitative analysis of pyridine nucleotide (pyridine salvage) and trigonelline synthesis, re-

spectively, in different parts of the seedlings. Radioactivity was found in NaMN and NAD, but no or little activity was found in NADP in any part of the seedlings 4 h after initiation of the reaction. These results suggest that nicotinamide was converted to nicotinic acid, and that some nicotinic acid was salvaged by nicotinate phosphoribosyltransferase; NaMN was formed. NaMN was further converted to nicotinic acid adenine dinucleotide (NaAD), and NAD was then formed. Since [^{14}C]NaAD was not detected, the NaAD produced was converted immediately to NAD. No NADP was found in this experiment, but this is probably due to the short incubation time. In the preliminary experiments using the same plant materials, NADP and NAD were labelled 18 h after incubation (data not shown). Higher pyridine salvage activity was observed in stem and leaves than in roots and hypocotyls; the highest value was found in stem (28.5% of total radioactivity), and the lowest activity in hypocotyls (1.8%). These results suggest that pyridine salvage is operative in all parts of seedlings; high salvage activity was found in newly emerged organs from hypocotyls.

A large amount of radioactivity was found in trigonelline; the order of incorporation was: young leaves (73.4%) > developing leaves (54.7%) > stem (35.2%) ≒ roots (32.5%) > hypocotyls (17.3%). The young leaves of adult trees of *Bruguiera gymnorrhiza* also have high capability for trigonelline synthesis (Ashihara *et al.*, 2010). A major nicotinic acid conjugate in leaves of another mangrove species, *Avicennia marina*, is nicotinic acid glucoside (Ashihara *et al.*, 2010). The formation of trigonelline and of nicotinic acid glucoside is, reportedly, strictly alternative in cultured cells from different plant species (Willeke *et al.*, 1979). There are variations in nicotinic acid conjugate formation in different organs of the same plant species, however (Barz, 1985; Matsui *et al.*, 2007; Ashihara, 2008). Potato plants produce nicotinic acid glucoside in all organs, but trigonelline is also synthesized in green organs such as leaves and stems (Katahira and Ashihara, 2009). In general, trigonelline synthesis is more active in leaves than in roots (Matsui *et al.*, 2007). In *Bruguiera gymnorrhiza*, significant amounts of trigonelline were synthesized in all organs, although the highest activity was in young leaves. No nicotinic acid glucoside formation was detected after at least 4 h of incubation. Nicotinamide was immediately deaminated in the tissues, and nicotinic acid was accumulated particularly in hypocotyls (69.8% of total radioactivity) and roots (54.0%).

The short-term (90 min) effect of 500 mM NaCl (a concentration similar to that in sea water) on the metabolism of [*carbonyl*-^{14}C]nicotinamide in roots of *Bruguiera gymnorrhiza* was investigated; Table I shows the results. Uptake of [*carbonyl*-^{14}C]nicotinamide was reduced to approximately half, and the activity of pyridine salvage and trigonelline synthesis was greatly decreased. Although the incorporation rate was very low (2.7% of total radioactivity), release of $^{14}CO_2$ from [*carbonyl*-^{14}C]nicotinamide was stimulated by NaCl. It has been suggested that trigonelline accumulates in salt-stressed legumes (Cho *et al.*, 1999; Tramontano and Jouve, 1997; Wood, 1999) and in tomato (Rajasekaran *et al.*, 2001), as compatible solute. Suzuki-Yamamoto *et al.* (2006) reported that 100 mM NaCl stimulated trigonelline synthesis from labelled quinolinic acid, nicotinamide, and nicotinate in cultured cells of *Bruguiera sexangula*. The apparent discrepancy between results from cultured cells with a lower concentration of NaCl and the present results may be due to the difference in plant materials used and

Table I. Effect of 500 mM NaCl on the metabolism of [*carbonyl*-^{14}C]nicotinamide in roots of *Bruguiera gymnorrhiza* propagules.

Metabolite	0 mM NaCl	500 mM NaCl	% of control
NAD	3.2 ± 0.6	0.9 ± 0.4	28.1
NaMN	0.1 ± 0.1	0.0 ± 0.0	0.0
Trigonelline	9.7 ± 2.7	2.4 ± 0.8	24.7
Nicotinic acid	48.3 ± 1.4	67.4 ± 6.3	139.5
Nicotinamide	34.7 ± 2.0	26.1 ± 5.3	75.2
CO_2	0.4 ± 0.2	2.8 ± 1.0	700.0
Unidentified	3.5 ± 2.3	1.3 ± 0.4	37.1
Total uptake (nmol/g FW)	40.8 ± 3.1	20.5 ± 3.3	50.2

Incorporation of radioactivity into individual metabolites is expressed as % of total radioactivity taken up by the segments ± SD. Incubation time was 90 min.

in NaCl concentrations. Trigonelline is, however, an unlikely candidate for the major compatible solute in the propagules of *Bruguiera gymnorrhiza*, because no accumulation of trigonelline was detected in the seedlings (Yin, unpublished observation).

Metabolism of [8-^{14}C]adenine

Fig. 5 shows the results of the separation of PCA-soluble [8-^{14}C]adenine by TLC, using solvent system I (Ashihara and Nobusawa, 1981; Zheng and Ashihara, 2004). Four or five spots were detected, corresponding to ATP/ADP, AMP, allantoic acid, allantoin, and adenine. The ATP/

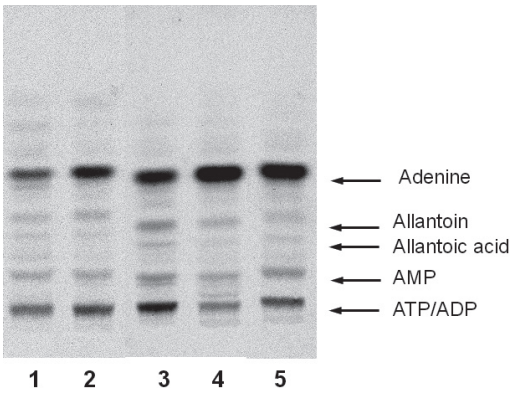

Fig. 5. Separation of the PCA-soluble ^{14}C-metabolites from [8-^{14}C]adenine by TLC using solvent system I (*n*-butanol/acetic acid/water, 4:1:2, v/v/v). Samples were obtained from (1) young leaves, (2) developing leaves, (3) stem, (4) hypocotyls, and (5) roots of the propagules of *Bruguiera gymnorrhiza*.

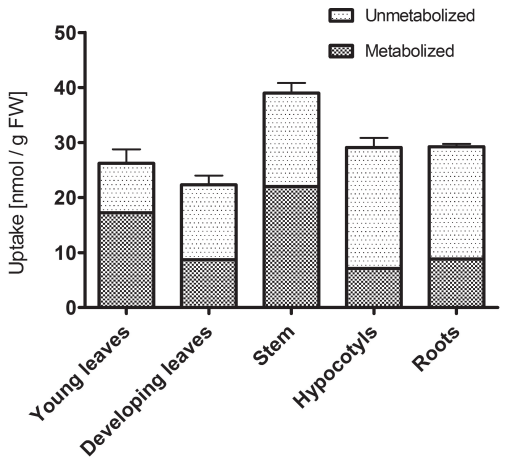

Fig. 6. Uptake of [8-^{14}C]adenine by segments taken from various parts of *Bruguiera gymnorrhiza* propagules. The incubation time was 4 h. The total uptake by the segments was calculated by adding the radioactivity found in all cellular metabolites and in CO_2. Rates of total uptake are expressed as nmol/g fresh weight (FW) ± SD. The radioactivity recovered as adenine (unmetabolized) and other compounds (metabolized) is shown separately.

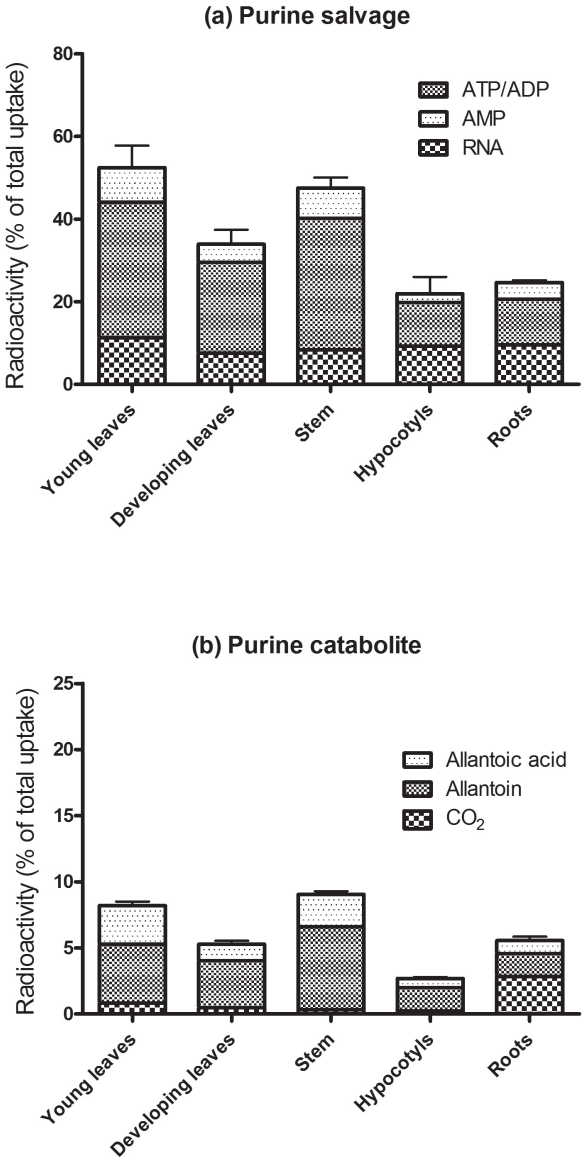

Fig. 7. Incorporation of radioactivity from [8-^{14}C]adenine into (a) salvage products (adenine nucleotides and RNA) and (b) catabolites (allantoic acid, allantoin, and CO_2) in segments from various parts of *Bruguiera gymnorrhiza* propagules. The incubation time was 4 h. Incorporation of radioactivity into individual metabolites is expressed as percentage of total radioactivity taken up by the segments shown in Fig. 6. The SD values for the incorporation rates are also shown.

Table II. Effect of 500 mM NaCl on the metabolism of [8-^{14}C]adenine in roots of *Bruguiera gymnorrhiza* propagules.

Metabolite	0 mM NaCl	500 mM NaCl	% of control
ATP/ADP	28.9 ± 1.1	17.8 ± 3.0	61.6
RNA	5.2 ± 0.3	3.9 ± 0.3	75.0
Allantoin	nd	nd	
Allantoic acid	nd	nd	
Adenine	64.9 ± 1.1	78.2 ± 3.2	120.5
CO_2	1.0 ± 0.2	0.1 ± 0.0	10.0
Total uptake (nmol/g FW)	37.0 ± 1.5	42.2 ± 1.5	114.1

Incorporation of radioactivity into individual metabolites is expressed as % of total radioactivity taken up by the segments ± SD. Incubation time was 90 min. nd, not detected.

ADP fraction may include very small amounts of ADP glucose and GTP and GDP, but we did not test for this. The total uptake of [8-^{14}C]adenine varied from 22 nmol/g fresh weight (developing leaves) to 39 nmol/g fresh weight (stem) (Fig. 6). More than half of the [8-^{14}C]adenine taken up by the segments was metabolized in young leaves (66% of total uptake) and stem (56%), but approximately 70–75% [8-^{14}C]adenine was retained unmetabolized in roots and hypocotyls (Fig. 6). Radioactivity from [8-^{14}C]adenine was incorporated mainly in the salvage products, including AMP, ADP, ATP, and nucleic acids (Fig. 7a). Our preliminary experiments indicated that radioactivity was distributed mainly in the adenine residues of RNA (data not shown). Adenine may therefore be converted to AMP by adenine phosphoribosyltransferase. AMP was further metabolized to ADP and ATP, which is then utilized for RNA synthesis. This adenine salvage activity was higher in leaves and stem than in hypocotyls and roots. The difference is due mainly to greater incorporation of free adenine nucleotides in leaves and stem. Incorporation of radioactivity into RNA was similar, or rather higher, in hypocotyls and roots than in developing leaves and stem. This is probably due to differences in the endogenous free adenine nucleotide pool between different organs; the pool may be larger in leaves and stem which are newly emerged from the hypocotyls. It was difficult to measure the endogenous pool sizes of adenine nucleotides by enzymatic analysis (Kubota and Ashihara, 1990; Shimazaki *et al.*, 1982) or HPLC (Ashihara *et al.*, 1987), because of the high concentration of phenolic compounds in the extract. Limited amounts of radioactivity from [8-^{14}C]adenine were recovered in some metabolites of the conventional purine catabolic pathway, including allantoic acid, allantoin, and CO_2 (Fig. 7b).

The short-term effect of 500 mM NaCl on the [8-^{14}C]adenine metabolism in roots was examined 90 min after administration (Table II). The total uptake of [8-^{14}C]adenine by the segments was slightly higher in salt-stressed roots, but incorporation into salvage products (nucleotides and RNA) was reduced by NaCl. Only very low radioactivity from [8-^{14}C]adenine was released as CO_2; this radioactivity was also less in roots treated with 500 mM NaCl.

Conclusion

The metabolic fate of [*carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine studied here suggests that the metabolic pathways shown in Fig. 8 are operative in all parts of the propagules of *Bruguiera gymnorrhiza*. Exogenously supplied nicotinamide was rapidly converted to nicotinic acid and accumulated, and some nicotinic acid was utilized in the biosynthesis of pyridine nucleotides and of trigonelline. In contrast to nicotinamide, supplied adenine was retained unchanged, especially in hypocotyls and roots, but some adenine taken up by the segments was utilized for the synthesis of nucleotides and RNA. Nicotinamide and adenine salvage activity were both higher in leaves and stem than in roots and hypocotyls. A limited amount of adenine was degraded by the oxidative purine catabolic pathway via allantoin. Since adenine deaminase activity is not found in plants (Yabuki and Ashihara, 1991; Stasolla *et al.*, 2003), the catabolism appears to begin after adenine has been converted to AMP (Fig. 8). The present results also show that, even in salt-resistant mangrove propagules, nicotinamide and adenine metabolism are inhibited by NaCl. We used segments of tissues so as to reflect the characteristics of the metabolism within the propagules. Mangrove plants have special morphological characteristics to reduce salt stress. Many mangrove

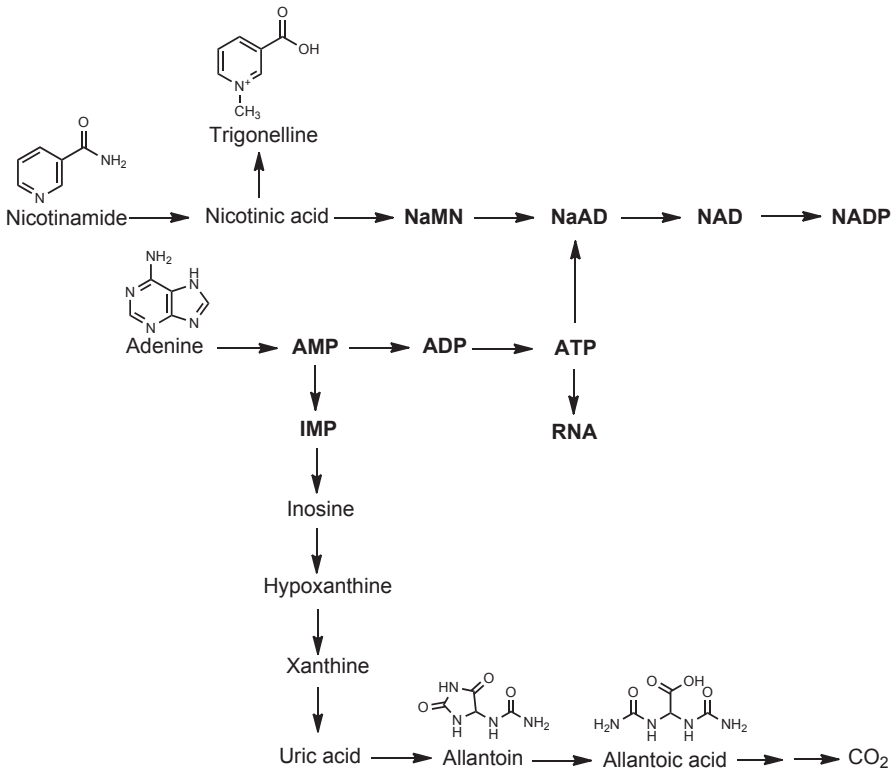

Fig. 8. Possible metabolic fate of [*carbonyl*-^{14}C]nicotinamide and [8-^{14}C]adenine in *Bruguiera gymnorrhiza* propagules. Nicotinamide and adenine are, respectively, the degradation products of NAD and adenine nucleotides, and are reutilized for nucleotide biosynthesis by the salvage pathways shown in this figure. Some nicotinamide and adenine are converted to trigonelline and to purine catabolites, respectively. No direct catabolic pathway of adenine is present in plants, so that purine catabolism appears to begin from AMP. ATP formed by adenine salvage is possibly utilized in NAD synthesis, but incorporation of radioactivity from [8-^{14}C]adenine into NAD was difficult to detect over the duration of the present experiments. IMP, inosine 5'-monophosphate; NaAD, nicotinic acid adenine dinucleotide; NaMN, nicotinic acid mononucleotide.

plants exclude salt by impermeable roots, and the concentration of NaCl inside the plants is not unduly high. Mangrove cells also store salt in vacuoles, excluding the salt from nucleotide metabolism. It appears that no metabolic adaptation against high salt concentration, at least in adenine and nicotinamide metabolism, is present in the propagules.

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