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BIOSYNTHETIC INCORPORATION OF THE AMINOBUTYL GROUP OF SPERMIDINE INTO PYRROLIZIDINE ALKALOIDS

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Key Word Index—*Senecio vulgaris*; Asteraceae; root cultures; pyrrolizidine alkaloid biosynthesis; homospermidine; spermidine; homospermidine synthase; polyamines.

Abstract—In short-term tracer experiments [^{3}H]putrescine and [^{14}C]spermidine were fed simultaneously to root cultures of *Senecio vulgaris*. The specific incorporation of the two tracers into putrescine, spermidine and pyrrolizidine alkaloids was followed over a period of 30 to 480 min. The results show that the aminobutyl moiety of spermidine is directly incorporated into the necine base of pyrrolizidine alkaloids. The same experiment carried out in the presence of the diamine oxidase inhibitor β-hydroxyethylhydrazine, which blocks the alkaloid biosynthesis after homospermidine, revealed the same specific incorporation into accumulating homospermidine. The results are in accordance with the substrate specificity of plant homospermidine synthase (EC 2.5.1.44) which uses both putrescine and spermidine as donor for the amino butyl group. Under physiological conditions more than half of the aminobutyl moiety of homospermidine comes directly from spermidine. This is the first report for the role of spermidine as aminobutyl donor in alkaloid biosynthesis. The importance of the linkage between spermidine and pyrrolizidine alkaloid biosynthesis is discussed.

INTRODUCTION

It has been long known that the polyamine spermidine is an efficient biogenetic precursor of the necine base (e.g. retronecine) of pyrrolizidine alkaloids [1–3]. It had been suggested that spermidine may be incorporated via degradation to putrescine the ultimate precursor of the necines or via a common intermediate (e.g. Δ_1-pyrroline) of the two amines. The role of putrescine as biogenetic precursor of necines and its incorporation via an intermediate with C_{2v} symmetry is well understood [4–9]. *N*-(4-Aminobutyl)-1,4-diaminobutane (homospermidine) was identified as such a symmetrical C_4-N-C_4 intermediate and its incorporation as intact molecule into retronecine has been demonstrated [4, 10, 11]. In enzymatic studies homospermidine was shown to be formed from two moles of putrescine in an NAD^+-dependent reaction catalysed by homospermidine synthase (EC 2.5.1.44) [12]. The role of free Δ_1-pyrroline as biogenetic intermediate was excluded. Homospermidine synthase was purified and characterized from pyrrolizidine alkaloid containing *Senecio* and *Eupatorium* species (Asteraceae) and the bacterium *Rhodopseudomonas viridis*. The plant and bacterial enzymes are very similar in their general and molecular properties [12–14]. The bacterial enzyme has recently been cloned and sequenced [15]. A remarkable feature of homospermidine synthase is that the first putrescine unit can be substituted by spermidine (Fig. 1), i.e. in the oxidation step spermidine instead of putrescine may provide the aminobutyl group which subsequently is combined with putrescine via an assumed imine intermediate to yield homospermidine.

These results strongly indicate that spermidine is a direct biogenetic precursor of pyrrolizidine alkaloids. However, *in vivo* experiments are necessary to prove this. Here we present the results of short-term tracer experiments with simultaneously applied [^{3}H]putrescine and [^{14}C]spermidine which show that in root cultures of *Senecio vulgaris* the aminobutyl group of spermidine is directly incorporated into homospermidine and thus retronecine.

RESULTS

Inhibitor experiments

In *Senecio vulgaris* root cultures putrescine is metabolized via three metabolic routes: (i) degradation

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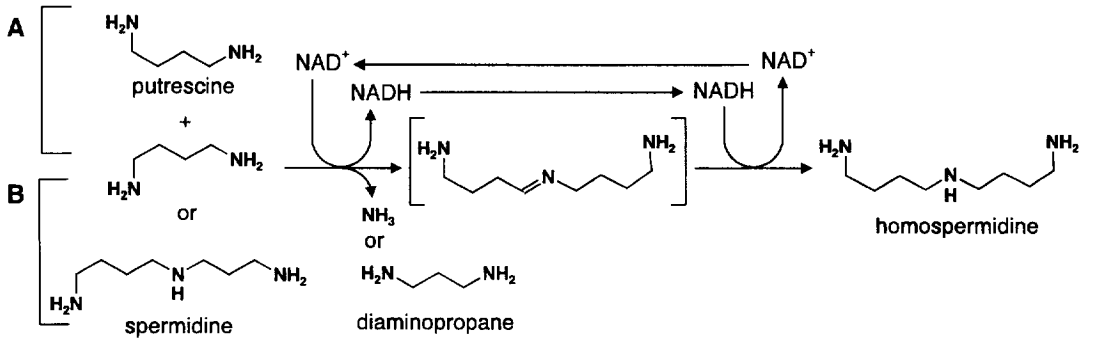

Fig. 1. Reaction catalysed by homospermidine synthase. A: formation of homospermidine from 2 moles of putrescine; B: formation of homospermidine from 1 mole putrescine and putrescine or spermidine as aminobutyl donor. NAD^+ acts catalytically, it catalyses the hydrogen transfer intramolecularly in a stoichiometric manner.

via Δ_1-pyrroline, (ii) conversion to spermidine and (iii) incorporation into homospermidine and thus the pyrrolizidine alkaloids (Fig. 2). *De novo* synthesis of putrescine proceeds exclusively from arginine via the agmatine-path [3, 16]. Both putrescine and spermidine are subjected to rapid turnover and they are reversibly connected, i.e. spermidine degradation leads to the formation of putrescine [3, 12] (Fig. 2). In a first attempt to demonstrate the role of spermidine as substrate of homospermidine formation we tried to interrupt the reversible connections between putrescine and spermidine by application of metabolic inhibitors. A number of inhibitors of spermidine formation such as cyclohexylamine (inhibitor of spermidine synthase) and methylglyoxal-bis-guanylhydrazone (inhibitor of SAM decarboxylase) were tested. All these inhibitors have been successfully applied in plant tissue cultures [17–19]. Our results, however, were always unreliable because of strong inhibitory effects on root growth and insufficient *in vivo* inhibition of the respective enzymes. The only useful inhibitor which does not affect root growth was β-hydroxyethylhydrazine (HEH) a potent inhibitor of diamine and polyamine oxidases. HEH was already successfully applied in previous studies [12]. It inhibits putrescine oxidase and blocks pyrrolizidine alkaloid biosynthesis after homospermidine (Fig. 2). As a consequence homospermidine which in the absence of HEH is almost undetectable in growing root cells accumulates. HEH does not affect the conversion of spermidine into putrescine, indicating that the respective enzyme (Fig. 2, step 3) is not a typical polyamine oxidase.

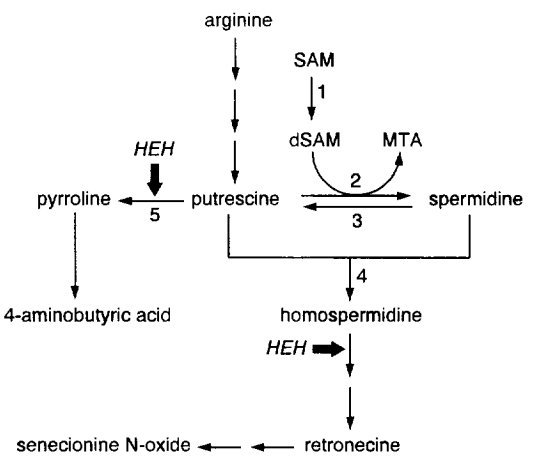

Fig. 2. Enzymatic links between putrescine, spermidine and homospermidine in *Senecio vulgaris* root cultures. SAM: *S*-adenosylmethionine, dSAM: decarboxylated SAM, MTA: 5′-methylthioadenosine, HEH: β-hydroxyethylhydrazine. 1: SAM decarboxylase (EC 4.1.1.50), 2: spermidine synthase (EC 2.5.1.16), 3: putrescine producing enzyme activity (HEH insensitive), 4: homospermidine synthase (EC 2.5.1.44), 5: diamine oxidase (EC 1.4.3.6).

Tracer experiments

Short-term tracer experiments were designed in which ^{3}H-labelled putrescine and ^{14}C-labelled spermidine were simultaneously fed to growing root cultures of *Senecio vulgaris*. In experiment A the specific incorporation of the two tracers was measured for intracellular putrescine, spermidine and total pyrrolizidine alkaloids (i.e. senecionine + integerrimine). Experiment B was performed under identical conditions but in the presence of 2 mM HEH to block the conversion of homospermidine into the alkaloids and to trigger its accumulation (Fig. 2). In experiment B the specific tracer incorporation was determined for putrescine, spermidine and homospermidine. Changes in the total tissue concentrations of the respective metabolites during the time course of the two experiments are summarized in Table 1. The relative proportions of ^{3}H- and ^{14}C-incorporation into alkaloids (exp. A) and homospermidine (exp. B) were calculated on the basis of the specific radioactivity established for putrescine and spermidine at the respective time of analysis. This was necessary to account for the differences in isotope dilution of the cellular pools of the two amines. Figure 3 summarizes the results and give the relative proportions of the specific tracer incorporation (specific tracer distribution). These data indicate that in both experiments a considerable proportion of the aminobutyl moiety of spermidine must

Table 1. Short-term tracer experiment with simultaneous feeding of 10 μM [^{3}H]putrescine and 10 μM [^{14}C]spermidine to root cultures of *Senecio vulgaris*: total tissue concentration of putrescine, spermidine and pyrrolizidine alkaloids (Exp. A) and putrescine, spermidine and homospermidine (Exp. B) during the time-course of the experiment

Experiment A (absence of HEH)

Time (min)	Putrescine	Spermidine (nmol g^{-1} fr. wt)	Alkaloid
30	61	79	1349
60	64	104	1430
120	89	101	1290
270	99	103	1330
480	105	79	1360

Experiment B (addition of 2 mM HEH 3 hr prior to tracer application)

Time (min)	Putrescine	Spermidine (nmol g^{-1} fr. wt)	Homo-spermidine
30	60	89	2.0
60	113	96	4.6
120	115	88	11.4
270	117	80	27.3

have been directly combined with putrescine to yield homospermidine. The specific ^{14}C-label in homospermidine and senecionine is much higher than the specific ^{14}C-label of putrescine caused by spermidine-putrescine interconversion. The shortest incubation times (i.e. 30 to 60 min) provide the most convincing data, due to the still almost neglectable amine interconversion. The pool concentrations of putrescine and spermidine do not change very much during the course of the experiment, although there is a slight increase in total putrescine especially in the presence of HEH (Table 1). The alkaloid level remains constant but homospermidine which is almost undetectable in the absence of HEH increases continuously with time in the presence of the inhibitor. The interconversion of putrescine and spermidine remains unaffected by HEH and the same is observed with the relative incorporation of the tracers into senecionine (absence of HEH) and homospermidine (presence of HEH) (Fig. 3).

DISCUSSION

Spermidine is precursor of a diverse variety of plant polyamine alkaloids and animal toxins [20, 21]. Spermidine as donor of the aminobutyl group, however, has not been reported before. The observation that approximately 50 to 70% of one of the two C_4-units of homospermidine are derived from spermidine (Fig. 3) are in good agreement with the substrate kinetics of plant homospermidine synthase [13, 14]. The enzyme incorporates putrescine and spermidine with same affinity and efficiency (i.e. identical K_m and V_{max} values) into homospermidine. The tissue concentrations of the two amines are in the same order of magnitude (Table 1). The direct participation of spermidine as donor of the aminobutyl group in homospermidine synthesis has been overlooked in all previous tracer studies because there is no way to distinguish between aminobutyl groups derived from putrescine and spermidine (Fig. 2) [8, 9, 22].

Why do plants use both putrescine and spermidine as ultimate substrates in pyrrolizidine alkaloid biosynthesis? A comparison of bacterial and plant homospermidine synthase revealed the bacterial enzyme less adapted to utilize spermidine as a substitute for putrescine. The efficiency (V_{max}/K_m) of the enzyme for spermidine is 10-fold lower than for putrescine. Since plant and bacterial homospermidine synthase are very similar in their general and kinetic properties the plant enzyme seems to be optimized in respect to the substrate properties of spermidine. A number of studies suggest that spermidine may play an important role in plant development, although as recently pointed out in a critical review [23], there is considerable murkiness about the roles of polyamines in plant development. One of the few points of agreement are the association of polyamines with growth and rapid cell division. In *Senecio* root cultures alkaloid biosynthesis is functionally and spatially linked to root growth; pyrrolizidine alkaloids do not undergo any significant degradation or turnover but their tissue concentration in a growing root culture is kept stable [3, 16]. Thus, a direct metabolic coupling of pyrrolizidine alkaloid biosynthesis to spermidine could provide a control mechanism to adjust the rate of alkaloid biosynthesis to growth intensity.

EXPERIMENTAL

Plant material. Root cultures of *Senecio vulgaris* L. (Asteraceae), established in 1993, were routinely grown in 250-ml conical flasks containing 75 ml MS medium with 4% sucrose and the phytohormones omitted [24]. The cultures were kept on gyratory shakers (120 rpm) at 25 in the dark and transferred every 2 weeks.

Tracer experiments. Roots (*ca* 1 g fr. wt) were collected from 10–14 day old cultures, transferred into 8 ml fresh MS medium and kept growing for 4 days until tracer addition. The tracers [1, 4n-^{3}H]putrescine (sp. act. 22.5 Ci · mmol^{-1}; Amersham, Braunschweig) and *N*-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine ([1,4-^{14}C]spermidine; spec. act. 118 mCi · mmol^{-1}, Amersham, Braunschweig) were added simultaneously at a final concn of 10 μM each and a sp. act. of $0.44 \cdot 10^6$ cpm · nmol^{-1} ([^{3}H]putrescine) and $0.046 \cdot 10^6$ cpm · nmol^{-1} ([^{14}C]spermidine), respectively. In experiments with HEH, the inhibitor (final conc 2 mM) was added 2–3 hr prior to application of the tracer. For extraction, the roots were harvested, rinsed with tap water, blotted between sheets of filter paper, weighed, and then ground in a mortar with 8

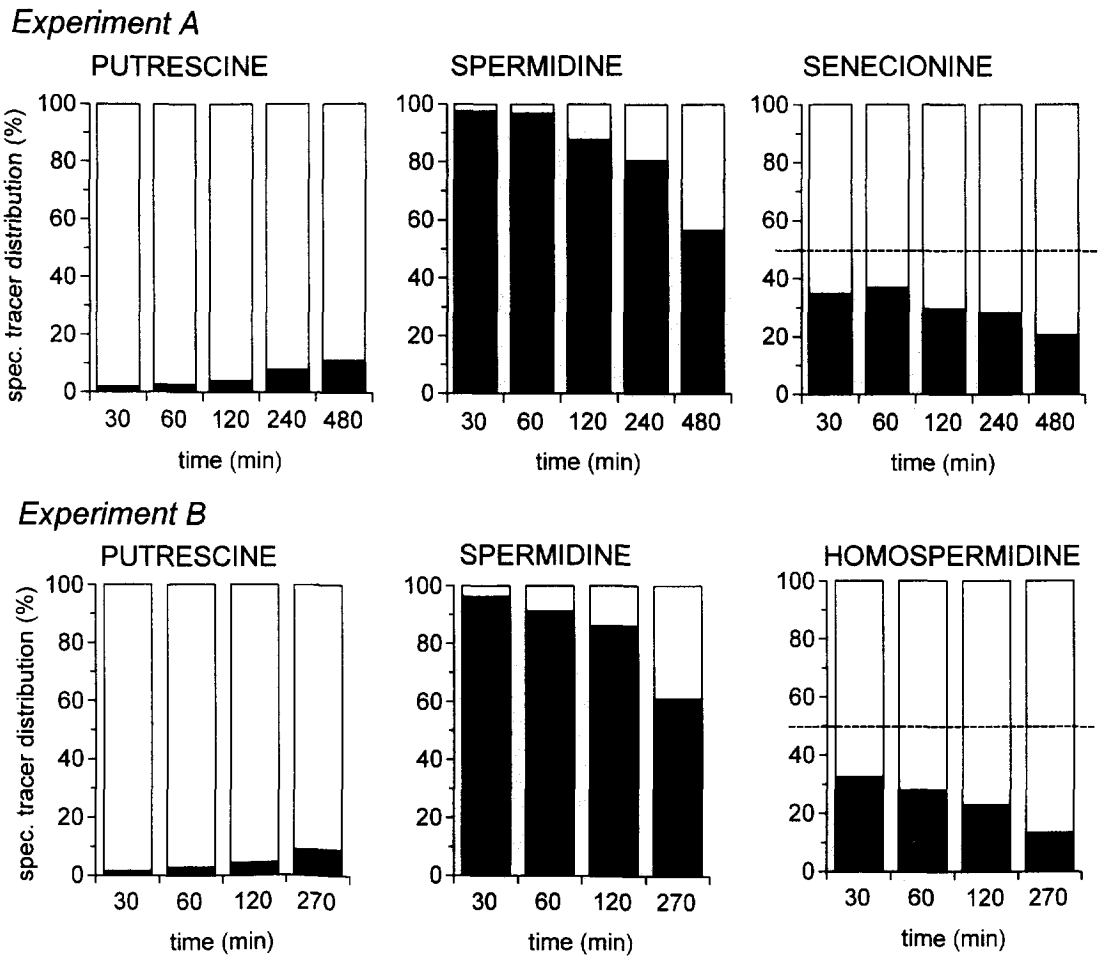

Fig. 3. Incorporation of [^{3}H]putrescine and [^{14}C]spermidine into senecionine (experiment A) and homospermidine (experiment B) in *Senecio vulgaris* root cultures. Standard feeding assays were performed with 10 μM [^{3}H]putrescine (spec. act. $0.44 \cdot 10^6$ cpm·nmol^{-1}) and 10 μM [^{14}C]spermidine (spec. act. $0.046 \cdot 10^6$ cpm·nmol^{-1}). In exp. B, 2 mM HEH were added. The relative specific tracer distribution (^{14}C, black columns and ^{3}H, white columns) were established on the basis of the specific activities at the time intervals indicated.

ml 0.05 M H_2SO_4 and homogenized in an ultrasonic bath for 30 min. After centrifugation the pellet was extracted again and the supernatants were combined and mixed with an excess of Zn dust. The mixt. was stirred at room temp for 5 hr to quantitatively reduce the pyrrolizidine alkaloid *N*-oxides. Then the soln was made basic (pH 11) with NH_4OH (25%) and extracted three times with *ca* 20 ml Et_2O each. The Et_2O extracts were dried, evapd and further purified by solid-phase extraction via Extrelut (Merck) columns as described in ref. [24]. The purified extracts contained the pyrrolizidine alkaloids as tertiary amines. The basic aq. soln of the Et_2O extraction was evapd, it contained the polyamines.

HPLC analysis of polyamines. Sepn and quantification of polyamines was achieved via their benzoyl derivatives according to ref. [25]. An RP-18 column (Nucleosil, 25 cm, 4 mm i.d.; Macherey & Nagel) was applied. Elution: isocratically using the solvent system MeCN–H_3PO_4 (1.5%) (40:60); parallel detection, (a) UV 230 nm, (b) radioactivity monitoring. The retention times (R_t; min) were as follows: putrescine, 5.5; cadaverine (internal standard), 6.4; spermidine, 8.4; homospermidine, 9.5.

Capillary GC of alkaloids. Quantification of the pyrrolizidine alkaloids was achieved by capillary GC according to ref. [26].

Radioactivity. All quantifications were performed by liquid scintillation counting.

Calculation of specific tracer distribution. Sp. radioactivity was determined for putrescine, spermidine and senecionine (exp. A) and putrescine, spermidine and homospermidine (exp. B) at the respective harvest times (t_x). The t_x values were chosen to calculate the respective tracer incorporation and to correct the data for isotope dilution. For a direct comparison of ^{3}H and ^{14}C incorporation (specific tracer distribution) all ^{3}H sp. radioactivities were corrected for differences in dilution to the ^{14}C sp. The ^{3}H-sp. radioactivities were corrected ($^3H_{cor}$) as follows:

$$^3H_{cor} = \frac{\text{sp. act. } ^3H}{([^3H]put_A):([^{14}C]spd_A)} \times \frac{([^3H]put_A):([^3H]put_B)}{([^{14}C]spd_A):([^{14}C]spd_B)}$$

$[^3H]put_A$ = sp. radioactivity of [^{3}H]putrescine applied (t_0)

$[^{14}C]spd_A$ = sp. radioactivity of [^{14}C]spermidine applied (t_0)

$[^3H]put_B$ = sp. radioactivity of [^{3}H]putrescine at t_x

$[^{14}C]spd_B$ = sp. radioactivity of [^{14}C]spermidine at t_x

t_x = time of root harvest and analysis.

Example, calculation of $^3H_{cor}$ for [^{3}H]putrescine at t_{30} and calculation of the specific tracer distribution in putrescine:

sp. radioactivity of [^{3}H]put at t_{30} $= 78 \times 10^3$ cpm nmol^{-1}.

sp. radioactivity of [^{14}C]put at t_{30} $= 0.28 \times 10^3$ cpm nmol^{-1}.

sp. radioactivity of [^{14}C]spd at t_{30} $= 15 \times 10^3$ cpm nmol^{-1}.

sp. radioactivity of [^{3}H]put at t_0 $= 425 \times 10^3$ cpm nmol^{-1}.

sp. radioactivity of [^{14}C]spd at t_0 $= 46 \times 10^3$ cpm nmol^{-1}.

$$^3H_{cor} = \frac{78 \times 10^3}{(425 \times 10^3):(46 \times 10^3)} \times \frac{(425 \times 10^3):(78 \times 10^3)}{(46 \times 10^3):(15 \times 10^3)}$$

$^3H_{cor} = 14.5 \times 10^3$ cpm nmol^{-1}.

Specific tracer distribution in putrescine at t_{30}: 98.1% ^{3}H and 1.9% ^{14}C, respectively.

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