Studies on the Biosynthesis of *sym*-Homospermidine in Sandal (*Santalum album* L.)

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The biosynthesis of the newly isolated polyamine, *sym*-homospermidine ($\text{NH}_2\text{--}[\text{CH}_2]_4\text{--NH--}[\text{CH}_2]_4\text{--NH}_2$), was studied by using radioactive amino acids. Arginine was the most effective precursor, being about 10 times as active as ornithine. Unlabelled agmatine and putrescine markedly inhibited the incorporation of [^{14}C]arginine into homospermidine. Similarly the incorporation of ornithine was inhibited by unlabelled arginine and putrescine. γ -Aminobutyraldehyde, the oxidation product of putrescine, was considered to be one of the intermediates in the biosynthesis of homospermidine. The biosynthesis may involve a Schiff-base formation of putrescine with γ -aminobutyraldehyde and subsequent reduction. A limited synthesis of spermidine also takes place under these conditions.

The polyamines spermine and spermidine are non-protein nitrogenous materials widely distributed in Nature. The distribution and the biological significance of these basic compounds have been reviewed by Tabor & Tabor (1964). Although the diamines occur in many plants (Smith & Richards, 1962), especially in K^+ -deficient conditions (Smith, 1968), only limited reports are available for the presence of spermine and spermidine in the plant kingdom (Moruzzi & Caldarera, 1964; Smith, 1970). An analogue of spermidine, bis-(3-aminopropyl)amine, has been shown to be present in very high concentrations in turnip leaves infected with virus, but not in healthy leaves (Johnson & Markham, 1962). Although it was thought that this compound was produced by the virus, it was later shown not to be present in the virus (Beer & Kosuge, 1970).

Kuttan *et al.* (1971) have reported the isolation and characterization of a new polyamine, *sym*-homospermidine ($\text{NH}_2\text{--}[\text{CH}_2]_4\text{--NH--}[\text{CH}_2]_4\text{--NH}_2$), in sandal leaves. It is present in the free state and in large amounts (0.5–1.5% of the dry weight of the leaves). Although the biosynthesis of polyamines has been studied extensively in bacteria (Tabor *et al.*, 1958) and in mammals (Pegg & Williams-Ashman, 1969), virtually no information is available on the biosynthesis of polyamines in plants. In bacteria and in higher animals the biosynthesis of spermidine proceeds by the condensation of putrescine with the isopropylamine moiety of decarboxylated *S*-adenosylmethionine. Such a possibility does not obviously exist for a symmetrical molecule like *sym*-homospermidine. An alternative pathway suggested by Kuttan *et al.* (1971), unsupported by experimental evidence, involved the oxidation of putrescine to form γ -aminobutyraldehyde, which can then con-

dense with a molecule of putrescine to form a Schiff base, which is ultimately reduced to form *sym*-homospermidine. In this paper we report the results of experiments with ^{14}C -labelled amino acids that act as precursors of homospermidine. Arginine and ornithine were found to be effective precursors of *sym*-homospermidine in sandal. On the basis of these results a pathway involving γ -aminobutyraldehyde as an intermediate has been postulated. A preliminary report of this work has appeared (Kuttan *et al.*, 1970).

Materials and Methods

Materials

All chemicals used were of A.R. grade. Unlabelled amino acids (A grade) and putrescine dihydrochloride were purchased from Calbiochem (Los Angeles, Calif., U.S.A.); putrescine dihydrochloride (Calbiochem) in the earlier experiments was a gift from Dr. L. K. Ramachandran (Department of Biochemistry, Osmania University, Hyderabad, India). Agmatine sulphate, spermine and spermidine were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). γ -Aminobutyraldehyde diacetal (K & K Laboratories, Plainview, N.Y., U.S.A.) was a gift from Dr. B. Witkop (National Institutes of Health, Bethesda, Md., U.S.A.). The free aldehyde was obtained by hydrolysis in 6% (w/v) HCl. The aldehyde was neutralized with NaHCO_3 solution to pH 8.0 just before the experiment. DL-[2- ^{14}C]Ornithine was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.), and L-[U- ^{14}C]arginine, L-[U- ^{14}C]proline and L-[U- ^{14}C]glutamic acid were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Infiltration experiments

Apical buds of sandal (*Santalum album* L.) with two adjoining leaves were used for most of the studies. A fresh cut was made on the stem, and the stem was immediately dipped in the test solution containing the precursor amino acid or amine in 0.2 ml of 0.2 M-sodium phosphate buffer, pH 7.0, and the infiltration was performed under vacuum as described by Kuttan & Radhakrishnan (1970). The bulk (95% or more) of the radioactive solution was infiltrated into the leaves within 3–4 h, as judged by the residual radioactivity in the tubes.

After the infiltration the leaves were transferred to a moist chamber and incubated with the stem dipping in water for 24 h at 24°C. The free amino acids and amines were then extracted with 75% (v/v) ethanol, and the extract was evaporated to dryness *in vacuo*. The residue was redissolved in 3 ml of 75% ethanol, 2 ml of chloroform was added and the mixture was shaken. The aqueous layer containing free amino acids and amines was obtained by centrifugation. The chloroform layer was twice washed with 0.5 ml of water, and the washings were pooled with the free amino acid fraction.

For competition experiments with unlabelled intermediates, the radioactive precursor was mixed with 50 μ mol of one of the suspected intermediates (unlabelled) in 0.2 M-sodium phosphate buffer, pH 7.0, in 0.2 ml. The infiltration time (5–6 h) was found to be longer than in the earlier experiment, but was complete, as checked by residual radioactivity in the tube.

Separation of homospermidine

Homospermidine was obtained pure after separation from other amino acids and diamines by using a Dowex 50 (X8; H⁺ form) resin column (0.6 cm $\times$ 5.0 cm, bed volume 1 ml) and eluting with HCl of different concentrations. The procedure is summarized in Scheme 1. Elution with 1.0 M-HCl removed the acidic and the neutral amino acids, and further elution with 2.5 M-HCl eluted all the basic amino acids, diamines and spermidine. Homospermidine was eluted in 3.0 M-HCl. Although spermine would be eluted in the same fraction, it had previously been shown that spermine is not present in sandal leaves, and separate radioautographic studies have shown that spermine is not synthesized under the conditions studied.

Homospermidine isolated by this procedure gave a single spot in two-dimensional chromatograms. The radiochemical purity of the isolated homospermidine after incorporation of ¹⁴C-labelled amino acid was checked by scanning chromatographic strips for radioactivity after development with the following two solvent systems: 1, butan-1-ol–acetic

Leaf extract

Acidified to pH 1.0; applied to Dowex 50 (X8; H⁺ form) column (0.6 cm $\times$ 5.0 cm, bed volume 1 ml);

Washed with water (20 bed volumes)	→	Acidic amino acids, urea and other products of metabolism
Washed with 1.0 M-HCl (20 bed volumes)	→	Neutral and acidic amino acids, including <i>cis</i> -4-hydroxyproline and proline
Washed with 2.5 M-HCl (20 bed volumes)	→	Ornithine, arginine, agmatine, putrescine and spermidine
Washed with 3.0 M-HCl (20 bed volumes)	→	Homospermidine

Scheme 1. Procedure for the separation of homospermidine from related compounds

acid–water (4:1:1, by vol.); 2, phenol in 0.2 M-KCl–HCl buffer, pH 1.0 (50:7, w/v).

Determination of homospermidine

After Dowex 50 chromatography, homospermidine in the eluates was determined by the ninhydrin procedure of Rosen (1957), standard curves being drawn for authentic homospermidine isolated from sandal leaves. The recoveries of homospermidine by the column procedure were usually $100 \pm 3\%$.

Paper chromatography and radioautography

The qualitative detection of amino acids was made by two-dimensional paper chromatography with buffered papers and buffered solvents (Subramanian & Rao, 1955). The chromatograms were developed by spraying with 0.4% (w/v) ninhydrin in acetone containing 2% (v/v) of 2,4,6-collidine, and heating at 60°C for 10 min. Radioautograms were developed after exposure of the photographic plates for 15 days to obtain a total of about 3×10^6 counts.

Measurement of radioactivity

Radioactivity was measured with either a Beckman LS-100 or a Packard Tri-Carb model liquid-scintillation counter with a dioxan-based scintillation mix (Ramaswamy & Radhakrishnan, 1964). To find the degree of quenching, internal standards of [¹⁴C]-benzoic acid were used with the Packard counter and the channels-ratio method with the Beckman counter.

Measurement of pool sizes of arginine and ornithine

An automatic amino acid analyser was used for the determination of these two amino acids (Benson & Patterson, 1965); proline was determined separately by the procedure of Schweet (1954).

Results*Incorporation of radioactive precursors into homospermidine*

The results obtained on the incorporation of radioactivity from various precursors into homospermidine are given in Table 1, which shows that both arginine and ornithine are good precursors of homospermidine. The incorporation of radioactivity into homospermidine was 7.5% with ornithine (assuming that only the L-isomer was active), 5.5% with L-arginine, 0.8% with L-glutamic acid and only 0.15% with L-proline. Determination of pool sizes in the leaf extracts gave the following values: arginine, 3.3 $\mu\text{mol/g}$ wet wt.; ornithine, 0.35 $\mu\text{mol/g}$ wet wt.; proline, 5.6 $\mu\text{mol/g}$ wet wt. Using these values for the pool sizes the calculated amount of homospermidine synthesized per g wet wt. of the leaf tissue was 26.7 nmol with ornithine and 181.5 nmol with arginine as precursors. Thus arginine appears to be a more effective precursor than ornithine. The labelling of homospermidine with [^{14}C]proline as precursor was insignificant. Although the homospermidine

fraction (3M-HCl eluate) was labelled, further fractionation by paper-chromatographic scanning for radioactivity showed no label in homospermidine, but label was present in another basic component (an alkaloid?).

After infiltration of the leaves with [^{14}C]arginine and [^{14}C]ornithine, the ethanolic extracts were subjected to two-dimensional paper chromatography and radioautography (Fig. 1). The ^{14}C -label was found in homospermidine and several other compounds metabolically related to the precursor employed. With arginine as the precursor the label was found in homospermidine, agmatine, putrescine, ornithine, proline and γ -aminobutyrate. With ornithine as the precursor the label was found in homospermidine, glutamate, proline, citrulline, putrescine, γ -aminobutyrate, aspartate and arginine.

Competition with unlabelled amino acids and amine intermediates

The nature of the intermediates in the biosynthesis of homospermidine starting from ^{14}C -labelled arginine or ornithine was further checked by competition with an excess of unlabelled intermediates suspected to be in the biosynthetic pathway (Table 1). With [^{14}C]arginine as the precursor the incorporation of radioactivity was severely inhibited by agmatine, ornithine and putrescine. Similarly the incorporation of radioactivity from [^{14}C]ornithine

Table 1. *Incorporation of radioactivity into homospermidine from amino acid precursors by sandal leaves and its inhibition in the presence of unlabelled intermediates*

In the competition experiments 50 μmol of the suspected intermediate was mixed with the radioactive precursor and infiltration was done as described in the text. The weight of the leaves in each case was 0.2–0.3 g and average values of two or three experiments are given. Homospermidine was isolated by ion-exchange chromatography and the radioactivity was determined.

Radioactive precursor (2×10^5 c.p.m.)	Unlabelled intermediate used (50 μmol)	Radioactivity in homospermidine (c.p.m.)	Incorporation of radioactivity	
			(% of initial)	(% of control)
L-[U- ^{14}C]Arginine	None	11500	5.5	100
L-[U- ^{14}C]Arginine	Agmatine	1400		12.7
L-[U- ^{14}C]Arginine	Putrescine	1873		17.0
L-[U- ^{14}C]Arginine	Ornithine	1620		14.7
DL-[2- ^{14}C]Ornithine	None	7500	7.5*	100
DL-[2- ^{14}C]Ornithine	Arginine	1500		20.0
DL-[2- ^{14}C]Ornithine	Putrescine	1090		13.2
DL-[2- ^{14}C]Ornithine	Proline	7600		100
L-[U- ^{14}C]Glutamic acid	None	1605	0.8	100
L-[U- ^{14}C]Glutamic acid	Ornithine	143		9.1
L-[U- ^{14}C]Proline	None	300	0.15	

* Calculated for the L-isomer only.

Fig. 1. Radioautograms after incorporation of [^{14}C]arginine and of [^{14}C]ornithine in sandal leaves

Tracing of the radioautograms obtained after incorporation of (a) [^{14}C]arginine and (b) [^{14}C]ornithine; (c) is a tracing of a ninhydrin-stained chromatogram. Two-dimensional chromatograms with Whatman no. 3 papers (50 cm $\times$ 40 cm) were prepared as described in the text, with the phenol system in direction 1 and the butan-1-ol system in direction 2. The amino acids are numbered as follows: 1 and 2, unidentified (traces); 3, spermidine; 4, homospermidine; 5, ornithine; 6, lysine; 7, asparagine; 8, arginine; 9, putrescine; 10, aspartate; 11, serine; 12, glycine

was inhibited by arginine and putrescine, but the inhibition with citrulline (not shown in Table 1) was not more than 50%. The incorporation of [^{14}C]glutamic acid, though small, was significantly inhibited by ornithine.

Competition with unlabelled γ -aminobutyraldehyde

Paper-chromatographic evidence obtained with a preparation of γ -aminobutyraldehyde showed that it was metabolized in the sandal plant, as shown by the increased formation of γ -aminobutyrate. If γ -aminobutyraldehyde is a free intermediate in the biosynthesis of homospermidine, the radioactivity from ornithine or arginine incorporated into homospermidine should be diluted by infiltrating with unlabelled γ -aminobutyraldehyde. In the actual experiments, as shown in Table 2, incorporation of the label from arginine was not inhibited by the preparation of unlabelled γ -aminobutyraldehyde. However, it was found that addition of bisulphite to the medium decreased the incorporation of [^{14}C]arginine, probably by trapping the γ -aminobutyraldehyde formed from putrescine. (It should be noted that the incorporation of arginine given in Table 2 is smaller than that given in Table 1, even after considering the lower initial radioactivity employed, and this may be related to seasonal variation of the concerned enzymes or variations in the pool sizes; however, this aspect has not been further investigated.) These results suggest the possibility that, if γ -aminobutyraldehyde is a precursor of homospermidine, it may not be a free intermediate; however, this will need further investigation.

Experiments with unlabelled putrescine showed increased formation of γ -aminobutyrate, presumably derived from the oxidation of γ -aminobutyraldehyde. This deamination was also reflected in the increased formation of the amides, glutamine and asparagine, and a decrease in the corresponding amino acids. This suggested the presence of a powerful diamine oxidase in the system. When the lower homologue of ornithine, 1,3-diaminobutyrate, was infiltrated, there was an increased formation of β -alanine, as might be expected. This conversion is similar to the formation of γ -aminobutyrate from ornithine via putrescine. Both these experiments suggested that the basic

(traces); 13, glutamine; 14, *cis*-4-hydroxy-L-proline; 15, agmatine; 16, glutamate; 17, threonine; 18, ethanolamine; 19, unidentified; 20, alanine; 21, γ -aminobutyrate; 22, proline; 23, tyrosine; 24 and 25, unidentified; 26, methionine and valine; 27, phenylalanine; 28, leucine and isoleucine; 29, citrulline. Radioactive spots which are not numbered are unidentified ninhydrin-negative materials.

Table 2. *Effects of γ -aminobutyraldehyde and sodium bisulphite on the incorporation of [14 C]arginine into homospermidine by sandal leaves*

The aldehyde was prepared by hydrolysis of the corresponding diacetal with 6% (w/v) HCl. It was neutralized with NaHCO₃, to pH 8.0, just before the experiment. The aldehyde or the bisulphite (50 μ mol) was mixed with [14 C]arginine (1×10^5 c.p.m.) and infiltration was carried out as described in the text. Average values of three experiments are given.

Radioactive precursor	Addition	Radioactivity in homospermidine (c.p.m.)
L-[14 C]Arginine	None	2385
L-[14 C]Arginine	γ -Aminobutyraldehyde	2841
L-[14 C]Arginine	Sodium bisulphite	1034

amino acids, ornithine and 1,3-diaminobutyrate, are metabolized by an initial decarboxylation followed by the action of diamine oxidase present in the system to yield the aldehyde intermediates.

Experiments on the formation of spermidine

Although the presence of spermidine in sandal leaves can be demonstrated, it is only a minor constituent. In view of the structural similarity, it was considered that the synthesis of spermidine may take place when putrescine (providing the C₄ moiety) condenses with a C₃ moiety derived from methionine, as noted in other systems (Tabor *et al.*, 1958), or with 1,3-diaminopropane (or its aldehyde oxidation product) derived from diaminobutyrate. With [14 C]ornithine (0.1 μ Ci) as a labelled precursor in the infiltration experiments, the radioactivity in spermidine was determined after paper-chromatographic separation followed by Dowex 50 ion-exchange chromatography, and the incorporation (413 d.p.m.) was found to be very low. It was not further enhanced by the addition of unlabelled methionine (460 d.p.m. incorporated) or of diaminobutyrate (635 d.p.m. incorporated). This suggested that the pathway for spermidine synthesis in sandal is a relatively minor one. It may be noted that diaminobutyrate was more effective than methionine, indicating that spermidine and homospermidine are probably biosynthesized by a similar pathway in the sandal.

Discussion

The present study has shown that homospermidine can be synthesized, starting from ornithine, arginine or glutamate as precursor. Considering the respective pool sizes of the amino acids, arginine was the most effective precursor. Competition experiments with unlabelled compounds and labelled precursors, [14 C]ornithine and [14 C]arginine, have thrown considerable light on the possible intermediates in the biosynthetic pathway for homospermidine. The pathway may be considered by reference to Scheme 2.

In the biosynthesis of homospermidine putrescine was the most important intermediate and occupied a central position. Unlike the situation in micro-organisms, direct decarboxylation of ornithine leading to putrescine (Gale, 1940) is not a favoured reaction in plants (Coleman & Hegarty, 1957). Another pathway demonstrated by Smith (1968) in barley leaves involves the conversion pathway arginine $\rightarrow$ agmatine $\rightarrow$ *N*-carbamoylputrescine $\rightarrow$ putrescine. Putrescine can also be formed by the hydrolysis of agmatine, as demonstrated by Morris & Pardee (1966) in *Escherichia coli*, in which there are multiple pathways for its formation. In sesame leaves Crocorno *et al.* (1970) showed that citrulline was a better precursor for *N*-carbamoylputrescine than was arginine, and an enzyme that decarboxylates citrulline to produce *N*-carbamoylputrescine has been shown in this plant. The higher incorporation of radioactivity from [14 C]arginine into homospermidine suggests that the formation of putrescine from arginine may be the preferred route in sandal leaves. *N*-Carbamoylputrescine has not been detected in the leaves in the free state or after arginine incorporation in sandal leaves. At the present time the pathway of arginine to putrescine in this plant has not been established.

The high incorporation of [14 C]ornithine into homospermidine shows that ornithine is directly converted into putrescine, because if ornithine were metabolized through arginine then the total radioactivity in homospermidine would have been tremendously diluted by the large pool size of arginine. On the other hand ornithine may also be acting via citrulline (Crocorno *et al.*, 1970), which has only a very small pool size in the leaves. However, this pathway is not a major one in this plant, because unlabelled citrulline did not substantially diminish the radioactivity in homospermidine after incorporation of ornithine.

In view of the symmetry of the homospermidine molecule, it has been considered that γ -aminobutyraldehyde, derived from putrescine by the action

Scheme 2. Postulated biosynthetic pathways for homospermidine in sandal leaves

of diamine oxidase, is the most probable intermediate. The biosynthesis of *sym*-homospermidine can therefore be visualized as the formation of a Schiff base between putrescine and γ -aminobutyraldehyde, followed by reduction (Scheme 2). Partial support for this mechanism rather than that for spermidine synthesis, i.e. linking of an activated C₄ moiety to a putrescine molecule, has been obtained in preliminary experiments showing incorporation of ³H from the medium. This incorporation of ³H from the medium suggests a nicotinamide nucleotide-linked reduction of the Schiff-base intermediate.

Evidence suggesting the formation of γ -aminobutyraldehyde from putrescine (see the Results section) was based on the observed increase in the formation of γ -aminobutyrate after putrescine infiltration. However, contrary to expectation, a preparation of unlabelled γ -aminobutyraldehyde did not inhibit the incorporation of [¹⁴C]arginine into homospermidine. γ -Aminobutyraldehyde in aqueous solutions is known to exist largely or exclusively in the cyclic pyrroline form and therefore may be unavailable for Schiff-base formation with putrescine. It is also possible that, in the enzymic

synthesis of homospermidine, γ -aminobutyraldehyde is not a free intermediate; further work is necessary to clarify this point.

If the putrescine moiety derived from arginine or ornithine provides one-half of the homospermidine molecule, the other half may be derived from putrescine itself (via γ -aminobutyraldehyde) or from glutamic semialdehyde (derived from proline, glutamate or ornithine), as postulated by Kuttan *et al.* (1971). It was observed that homospermidine was labelled either not at all or only to a very small extent with [¹⁴C]proline. Incorporation of [¹⁴C]ornithine into homospermidine was not inhibited by unlabelled proline (Table 1). Incorporation of [¹⁴C]glutamic acid, although small, was inhibited by ornithine, suggesting that glutamate incorporation is probably via ornithine. When the results are analysed from the point of view of the pool sizes, the incorporation of proline is very small and much lower than the value expected on the basis of dilution. The limited incorporation of proline into homospermidine may be similar to that of glutamate, via ornithine. These results thus preclude glutamic γ -semialdehyde as a possible intermediate in the biosynthesis of homo-

spermidine, and also suggest that the two halves of the *sym*-homospermidine molecule are both derived from putrescine.

From the above results the following pathway for the biosynthesis of homospermidine in sandal is suggested: arginine, ornithine $\rightarrow$ putrescine $\rightarrow$ γ -aminobutyraldehyde (+putrescine) $\rightarrow \rightarrow$ homospermidine.

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