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PUTRESCINE, SPERMIDINE AND SPERMINE IN HIGHER PLANTS

T. A. SMITH

Long Ashton Research Station, University of Bristol, BS18 9AF

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Abstract—The diamine, putrescine and the polyamines spermidine and spermine have been estimated quantitatively in the leaves of a number of higher plants. In six species grown in potassium-deficient conditions, by comparison with normal plants, the mean increase in the concentration of putrescine was 16·6-fold, for spermidine 1·9-fold and for spermine 1·2-fold. This is compatible with a metabolic pathway in which spermidine and spermine are derived consecutively from putrescine.

INTRODUCTION

THE DIAMINE putrescine (I) is closely related structurally to the two polyamines spermidine (II) and spermine (III).

(I) Putrescine $NH_2CH_2CH_2CH_2CH_2NH_2$

(II) Spermidine $NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2$

(III) Spermine $NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NHCH_2CH_2CH_2NH_2$

In micro-organisms[1] and animals[2] these three substances are synthesized by a metabolic pathway in which either one or both of the terminal amine groups of putrescine are substituted by propylamine residues derived from methionine, to form spermidine and spermine, respectively. Although all these amines are well-established as constituents of micro-organisms and animals, in plants most work has centred on putrescine and relatively little is known about the distribution of spermidine and spermine. Spermidine has been found in *Chlorella*,[3] tomato and Chinese cabbage leaves,[4] and together with spermine in *Petunia* pollen.[5] Moruzzi and Caldarera[6] have demonstrated the presence of a high concentration of spermidine and spermine in the embryo of cereals, and, with Bagni,[7] they have found these polyamines in all parts of the wheat plant except the roots and the anthers. Bagni also detected these substances in tomato juice,[8] Jerusalem artichoke (*Helianthus*) tubers[9] and seeds from a large variety of higher plants.[10] These polyamines were found in the alkaloids lunarine from *Lunaria annua* L. and *L. rediviva* L.,[11] palustrine from *Equisetum* sp.,[12] and pithecolobine

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from *Samanea saman* Merr.[13] A structurally related polyamine (diaminodipropylamine) has been demonstrated in certain plant viruses, but this substance could not be detected in the virus-free host plants.[14]

Spermine promotes the growth of *Helianthus* tuber explants *in vitro*[15] and polyamines activate protein synthesis in ribosomes extracted from this tissue.[10] It therefore appears that polyamines are of considerable importance in higher plants, as has also been shown in micro-organisms and animals.[4]

Since free polyamines had previously only been found in the leaves of three species of higher plants, it was of interest to investigate the occurrence of these substances in the leaves and other organs of a wider range of plants. Also, since the level of putrescine is greatly increased in potassium-deficient plants,[16,17] and as it is likely that putrescine is a precursor of spermidine and spermine in higher plants the effect of potassium deficiency on the polyamine content of leaves was also investigated.

RESULTS AND DISCUSSION

Development of the Procedure for the Estimation of Putrescine, Spermidine and Spermine

A method based on that proposed by Hammond and Herbst[18] was used for the analysis of the spermidine and spermine. In the present method, the trichloroacetic acid is allowed to remain after extraction and serves to reduce the affinity of the resin for the acidic and neutral amino acids. Using the cation exchange resin Dowex-50, the initial purification was effected by a batch process in place of the column method of Hammond and Herbst. Adsorption of the amines to the resin is rapid and is virtually complete in 30 min. Desorption with concentrated HCl is a slower process, probably because of the high cross-linking of the resin, and 2 hr were needed for the establishment of equilibrium. Since heavy metals catalyse the oxidation of amines,[19] the chelating agent ethylenediaminetetraacetic acid (EDTA) was incorporated in the plates, the standards and the HCl eluates prior to concentration, as recommended by Hammond and Herbst.

On the thin-layer chromatograms used for determining spermidine and spermine, the putrescine spot coincided with the spots due to arginine and lysine, and putrescine could not be estimated by this procedure, and although putrescine can be isolated and quantitatively estimated by gas chromatography,[20] it is not possible to determine the polyamines by this method. A combination of thin-layer and gas chromatography was therefore used for estimating the three amines quantitatively.

Known quantities (2·5 μmoles) of spermidine and spermine were subjected to the estimation procedure (see Experimental). In the absence of plant material the recovery of spermidine was 98 per cent (S.E. 2 per cent) and of spermine 92 per cent (S.E. 6 per cent). In the presence of an extract of 10 g of Cox's Orange apple leaves, the recovery for spermidine was 100 per cent (S.E. 2 per cent) and for spermine 81 per cent (S.E. 2 per cent). In the results presented for spermidine and spermine concentrations in leaves (Tables 1 and 2) no correction has been

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TABLE 1. SPERMIDINE AND SPERMINE CONTENT OF LEAVES, ROOTS AND FRUIT, EXPRESSED AS μmoles/g fresh wt.

Species	Cultivar	Age (days)*	Spermidine	Spermine
Leaves				
Wheat *Triticum aestivum* L.	Atle	50	0·125	0·038
Strawberry *Fragaria chiloensis* Duchesne var. *ananassa*	Cambridge Vigour	M	0·128	0·082
Apple *Malus sylvestris* Mill.	Beauty of Bath	M	0·229	0·044
Apple *Malus sylvestris* Mill.	Cox's Orange	M	0·200	0·057
Pyracantha coccinea Roem.		M	0·186	0·055
Tomato *Lycopersicon esculentum* Mill.	Outdoor Girl	M	0·156	0·038
Marrow *Cucurbita pepo* L. var. *medullosa* Alef.	Suttons improved green bush	M	0·158	0·040
Spinach *Spinacea oleracea* L.	Dominant 65 round	M	0·238	0·038
Willow *Salix babylonica* L.		M	0·191	0·085
Roots				
Pea *Pisum sativum* L.	Meteor	33	0·073	0·026
Pea *Pisum sativum* L.	Meteor	42	0·037	0·029
Fruit				
Apple *Malus sylvestris* Mill.	Cox's Orange	M	0·068	0·011

* M = Mature plant.

TABLE 2. PUTRESCINE, SPERMIDINE AND SPERMINE CONTENT OF THE LEAVES OF PLANTS GROWN UNDER NORMAL AND POTASSIUM-DEFICIENT CONDITIONS, EXPRESSED AS μmoles/g fresh wt.

Species	Cultivar	Age (days)	Normal potassium status			Potassium-deficient		
			Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Maize *Zea mays* L.	Hy2X07	31	0·441	0·115	0·032	3·380	0·313	0·058
Barley *Hordeum vulgare* L.	Zephyr	29	0·134	0·188	0·046	1·745	0·337	0·035
Radish *Raphanus sativus* L.	Saxa	31	0·083	0·314	0·039	0·980	0·472	0·049
Pea *Pisum sativum* L.	Meteor	31	0·020	0·358	0·204	0·473	0·829	0·270
Pea *Pisum sativum* L.	Meteor	47	0·014	0·402	0·115	0·625	0·537	0·109
Black currant *Ribes nigrum* L.	Baldwin	200	0·052	0·100	0·036	1·085	0·199	0·031
Tobacco *Nicotiana tabacum* L.	Burley	58	0·080	0·074	0·040	0·480	0·159	0·042
Ratio of mean amine contents (spermine = 1)			1·6	3·0	1	14·7	4·8	1
Mean ratio (potassium-deficient : normal plants)			16·6×	1·9×	1·2×			
Significance of ratio of means			<0·1%	0·1–1%	>5%			
Significance for differences between ratios of means				<0·1%	1–2%			

made for experimental losses. Even in the presence of amounts of putrescine greater than those encountered in the extracts (4 μmoles/g fresh weight), no interference was caused in estimating the spermidine or spermine.

Using this procedure the mean recovery of known quantities of putrescine (6·25 μmoles) was 93 per cent and of ethanolamine (6·25 μmoles) 7·2 per cent. Although ethanolamine has a retention time on gas chromatography which is not much greater than that of putrescine, it seems that interference from ethanolamine in putrescine estimation is unlikely to be great.

Occurrence of Putrescine, Spermidine and Spermine in Higher Plants

Two spots obtained on TLC of the amine fraction corresponded exactly with spermidine and spermine in R_f and ninhydrin colour and usually no other major ninhydrin positive spot was found near them. In some extracts of the pea leaves, however, a brown spot was found immediately above spermidine and overlapping it slightly. This spot, which was probably due to histidine, may have resulted in an over-estimation of spermidine in these extracts, though this error is unlikely to have been very great. Chromatography of the apple leaf amine fraction by quadruple displacement in the methyl cellosolve solvent indicated that the spermidine and spermine spots were homogeneous. The R_f values of these two compounds were then 0·56 and 0·33, respectively. Further confirmation of the identity of spermidine and spermine spots on thin-layer chromatograms of the amine fractions run in the methyl cellosolve solvent was obtained by applying the naphthalene black spray of Raina,[21] which is highly specific for these polyamines.

The spermidine and spermine content of the leaves of a number of species is given in Tables 1 and 2. Table 2, which includes data on putrescine, gives the results of estimations made on tissue taken from normal and potassium-deficient plants. Spermidine and spermine occurred in all the tissues investigated. The levels found for wheat leaves are about twice as high as those given for this tissue by Bagni *et al.*[7] (calculated as 0·062 and 0·027 μmoles/g fresh weight, respectively).

Combining the results for the leaves of all plants investigated (Tables 1 and 2), the mean concentrations of spermidine and spermine were respectively 0·198 (S.E. 0·093) and 0·062 (S.E. 0·044) μmoles/g fresh weight. The spermidine and spermine contents of the pea roots and apple fruit pulp were lower than in the leaves. The greatest putrescine accumulations with potassium deficiency were found in the monocotyledonous plants, maize and barley, while the greatest spermidine and spermine contents were found in the pea plants. The data presented in Table 2 were subjected to a logarithmic transformation before statistical analysis, since they were not normally distributed.

The relative changes in putrescine, spermidine and spermine content with potassium deficiency are consistent with a hypothesis that these three amines are linked in a metabolic sequence. The putrescine accumulated in the potassium-deficient plants presumably causes an increase in spermidine and a lesser increase in spermine by a mass-action effect. Additional evidence for this pathway has been obtained by feeding putrescine to the cut shoots of pea and barley seedlings. In each case the polyamine content was increased, the spermidine most, the spermine less so.

Occurrence of Diaminopropane in Wheat

After thin-layer electrophoresis of the amine fraction of wheat leaf extracts, the plates were dried thoroughly and sprayed with ninhydrin. On heating, a brown spot was found with

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a mobility identical to that of diaminopropane. If water was present before spraying, this spot was pink and could not be distinguished from putrescine. This behaviour was also found with authentic diaminopropane. Gas chromatography of these extracts with the same column parameters as those used for putrescine estimation, showed a peak (retention time 3·5 min) with a mobility corresponding to that of diaminopropane. This substance has been detected previously in extracts of pea seeds,[11] bovine brain,[22] and on oxidation of polyamines by *Mycobacterium smegmatis*[23] and *Serratia marcescens*.[24]

Other Peaks found by Gas Chromatography

In addition to putrescine with a retention time (r.t.) of 5·2 min, the extracts of the plants shown in Table 2 contained several unknown peaks. One peak (r.t. 4·7 min) occurred in all leaf extracts and, in barley and maize extracts, peaks occurred with retention times of 3·5 and 4·2 min, the first probably being due to diaminopropane. A further unknown (r.t. 6·8 min) was found in maize, barley, pea and black-currant extracts. None of these peaks could be identified with cadaverine or Δ^1-pyrroline.

The size of one peak (r.t. 9·6 min) ,which was also described previously in maize extracts,[25] was correlated with the size of the putrescine peak. The origin of this peak was eventually traced to a reaction between putrescine and a substance diffusing from a polythene bottle used for storing the methanolic KOH. In some extracts, this peak occupied an area equal to 25 per cent of the putrescine peak and for this reason some of the putrescine levels are slightly under-estimated.

EXPERIMENTAL

Plant Material

This was grown as described previously.[25] All samples were taken during the summer of 1969. K was supplied to the deficient plants at 1/10 of the normal concentration.

Extraction and Preliminary Purification of Amines

Plant material (10 g) was macerated in 5% trichloroacetic acid (80 ml) in a Vertis homogenizer. The extract was left for 18 hr at 2° and then centrifuged. After measuring the volume, the supernatant recovered was added to 1 g of Dowex-50 × 8 20–50 mesh, ion-exchange resin (H^+ form) in a 250 ml glass-stoppered narrow-necked bottle. This was shaken for 1 hr, and the solution was then removed by suction. The resin was then briefly washed in water (20 ml) and, after removing the water by suction, 36% HCl (10 ml) was added to the resin. Shaking was then continued for a further 2 hr. This eluate was removed and the resin was washed in about 3 ml of distilled water, the washings being combined with the acid eluate. EDTA (1 ml of 0·1%) was then added and the solution (the amine fraction) was concentrated to dryness at 60° under vacuum.

Estimation of Spermidine and Spermine

After cooling, the amine fraction was dissolved in 1 ml of 0·1 N HCl. Samples (normally 5 μl) of this solution were applied to an air-dried 20 × 20 cm CC41 cellulose plate, together with spermidine and spermine standards (normally 12·5 mμmoles of each). This plate was run in methyl cellosolve–propionic acid–water (70:15:15, by vol.) saturated with NaCl (the methyl cellosolve solvent).[18] When the solvent had reached the top of the plate (2½ hr), the solvent was dried at 100° for 15 min and the plate allowed to cool to room temp. The lower half was then sprayed with 10 ml of ninhydrin reagent (1% ninhydrin and 1% collidine in absolute ethanol). After drying under a cold air stream, a further 10 ml was sprayed on the lower half of the plate and the colour was developed at 60° for 15 min. The R_fs are given in Table 3. The areas of cellulose containing the coloured ninhydrin spots of spermidine and spermine were removed, and eluted with 70% ethanol (2 ml). The cellulose powder was dispersed with a Rotamixer and the solutions were centrifuged. After decanting, the solutions were centrifuged again. The absorbance was then read at 575 nm using 4-cm micro-cells. Response was almost linear for up to 50 mμmoles of spermidine or spermine applied.

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TABLE 3. NINHYDRIN COLOUR, ELECTROPHORETIC MOBILITY AND THE R_f VALUES FOR PUTRESCINE AND RELATED COMPOUNDS

Compound	Ninhydrin colour	Electrophoretic mobility* (in cm)	R_f in butanol–ketone solvent	R_f in methyl cellosolve solvent
Histidine	Brown	5·7	0·09	0·28
Arginine	Purple	5·4	0·05	0·42
Lysine	Purple	6·1	0·05	0·45
Methylamine	Pink	10·5	—	0·63
n-Propylamine	Blue	8·3	—	0·76
iso-Butylamine	Blue	7·7	—	0·79
iso-Amylamine	Purple	7·3	—	0·81
Ethanolamine	Purple	8·0	0·61	0·59
β-Phenylethylamine	Blue	6·7	0·95	0·79
Agmatine	Purple	7·3	0·22	0·43
N-Carbamylputrescine	Purple	6·3	0·50	0·50
1,3-Diaminopropane	Brown	8·7	0·80	0·36
Putrescine	Purple	8·5	0·75	0·43
Cadaverine	Purple	8·3	0·78	0·47
Diaminodipropylamine	Red	7·5	0·60	0·18
Spermidine	Purple	7·5	0·63	0·23
Spermine	Purple	6·6	0·50	0·13

* Distance travelled in 20 min at 25 V/cm, pH 3·5.

A number of amines and related compounds were also chromatographed on MN 300 cellulose plates in *n*-BuOH–MeCOEt–NH_4OH–H_2O (5:3:1:1, by vol.) (the butanol–ketone solvent). The R_fs are given in Table 3.

Estimation of Putrescine

After determining the spermidine and spermine, the amine fraction was evaporated to dryness at 60° under vacuum and subjected to gas chromatography following the method of Smith.[20] The methanolic-KOH amine solvent used in gas chromatography was modified since it was found that absolute methanol tails; this was inhibited by addition of 5% water. The nitrogen flow rate was decreased in the present study, the retention time of the putrescine at 150° being 5·2 min.

Occasionally, after injecting the extracts of the K-deficient plants containing large quantities of putrescine, "ghosting" was found in a subsequent blank injection.[26] Normally therefore a blank injection was made to confirm clearance of the column before further samples were applied.

Electrophoresis

MN 300 cellulose plates (300 μ thick) were spotted with the amine fractions of the plant extracts dissolved in 0·1 N HCl and the plates were sprayed with 2·5 ml of 0·05 M pH 3·5 citrate buffer. The plates were immersed in petroleum ether (80–100° boiling fraction) during electrophoresis for cooling. Separation was effected in 20 min at 25 V/cm. Relative mobilities are given in Table 3.

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