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Note

Paper chromatographic resolution of aliphatic guanidino amines, diamines and polyamines

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Of the several analytical procedures described previously for the separation and quantitation of polyamines such as spermine, spermidine, putrescine and cadaverine, most have definite limitations in that they are either non-specific or too cumbersome for routine use[1-3]. More recently, some sensitive methods have been devised for the detection and determination of these amines, such as fluorimetric[4,5] and enzymic assays[6-8], thin-layer[9] and gas chromatographic[10] techniques, thin-layer electrophoresis[11] and automated ion-exchange methods[12]. However, these procedures also do not assay all the amines present and are not entirely specific[3], thus necessitating further separation by other methods such as paper chromatography and electrophoresis. While spermine, spermidine and putrescine have been resolved by such techniques[13], difficulties were encountered when the mixed amine fractions from biological sources contained structurally related compounds and/or their biosynthetic precursors[12].

During an investigation on polyamine metabolism in *Lathyrus sativus* seedlings during development, we encountered in the amine fraction of the whole seedlings closely related and pairs of homologous amines, such as spermine–spermidine, putrescine–cadaverine and agmatine–homoagmatine. The simultaneous clear-cut resolution of these amines could not be achieved by using any of the reported paper chromatographic or electrophoretic systems. This paper describes a simple paper chromatographic system that affords the unequivocal separation of all of the above amines in a single run, thus making it possible to follow quantitative changes in their levels during the growth of the plant embryo. A method of preparing the mixed amine fraction free from trace amounts of amino acids and salts, prior to the chromatographic separation of the various amines, is also described.

EXPERIMENTAL AND RESULTS

Amines

Spermidine trihydrochloride, putrescine dihydrochloride and agmatine sulphate were obtained from Sigma, St. Louis, Mo., U.S.A., and cadaverine dihydrochloride and spermine tetrahydrochloride from the California Foundation for Biochemical Research*. The N-carbamyl derivatives of putrescine and cadaverine were synthesized

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according to the method of Smith and Garraway[14], and diaminopropane was prepared according to the method of Aspinall[15]. Homoagmatine was prepared by guanidation of cadaverine with *o*-methylisourea[16]. Symmetrical homospermidine (1,9-diamino-5-azanonane) from sandal (*Santalum album*)[17] was a gift from Prof. A. N. Radhakrishnan of Vellore and synthetic non-symmetrical homospermidine (N-(3-aminopropyl)-1,5-diaminopentane) was kindly donated by Dr. Donald F. Worth of Parke Davis, Ann Arbor, Mich., U.S.A. These substances were purified and used as reference standards.

Preparation of the amine fraction

About 0.5–1.0 g of *L. sativus* seedlings (any other biological specimen could be used) was finely ground and extracted with 3 volumes of cold 0.4 *M* $HClO_4$. After clarifying the extract by centrifugation at 2–4°, excess of perchlorate was removed as $KClO_4$. The amines were extracted into 3 volumes of *n*-butanol under the conditions described by Russel *et al.*[18], *i.e.*, at pH 13.0 in the presence of a saturating concentration of an alkaline salt mixture (Na_2SO_4–$Na_3PO_4 \cdot 12H_2O$, 7:1 w/w). The *n*-butanol extract was acidified with HCl and concentrated, and was found to contain trace amounts of amino acids[13] and substantial amounts of salt, which interfered in the paper chromatography. This difficulty was obviated by passing the *n*-butanol fraction,

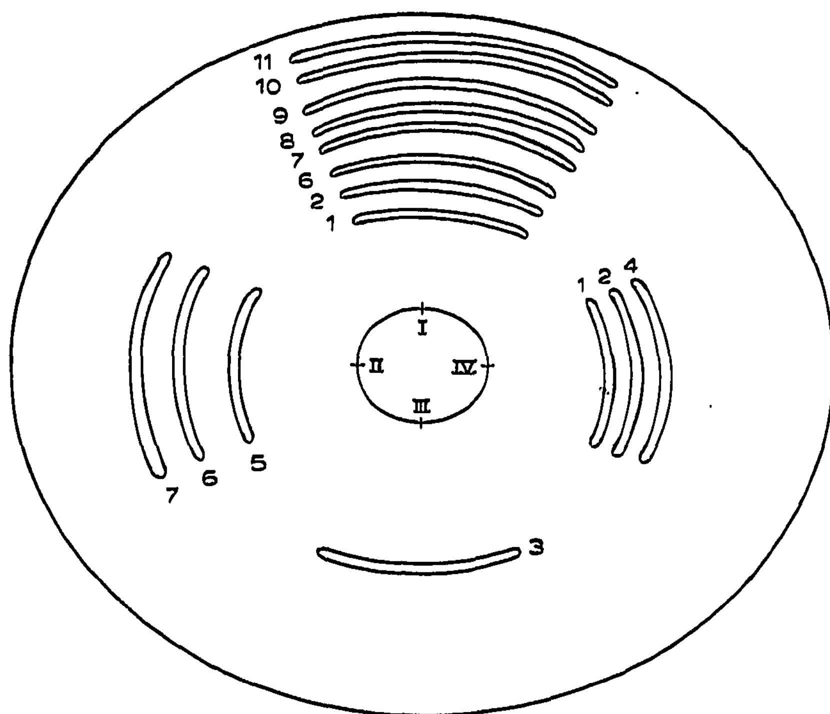

Fig. 1. Schematic representation of the separation of aliphatic amines by circular paper chromatography. I = Mixture of guanidino amines, diamines and polyamines and N-carbamyl derivatives of diamines; II = mixture of diamines; III = symmetrical homospermidine; IV = mixture of polyamines. 1 = Spermine; 2 = spermidine; 3 = symmetrical homospermidine; 4 = non-symmetrical homospermidine; 5 = diaminopropane; 6 = putrescine; 7 = cadaverine; 8 = agmatine; 9 = homoagmatine; 10 = N-carbamylputrescine; 11 = N-carbamylcadaverine.

in water (adjusted to pH 5.0), through a small column of Dowex 50-X8 (H^+) (5 ml bed volume). Amino acids and aromatic amines were eluted from the resin with 2.0 *M* ammonia solution (10 bed volumes) followed by washing with 100 bed volumes of water. Elution with 15 bed volumes of 0.4 *M* HCl removed the salt completely, while elution with 8 bed volumes of 6 *M* HCl resulted in the complete recovery of the amines from the resin. The 6 *M* HCl eluate was evaporated to dryness *in vacuo*, dissolved in a known volume of water and an aliquot of the solution was subjected to paper chromatography.

Paper chromatography

The circular paper chromatographic technique described by Giri and Rao[19] was followed. Whatman No. 1 filter-paper (45 × 45 cm) buffered with 0.067 *M* KCl–HCl buffer (pH < 2.0) and air-dried at room temperature was used. A solvent system consisting of a 4:1 (v/v) mixture of freshly distilled phenol and 0.067 *M* KCl–HCl (pH < 2.0) was used[20]. The chromatogram was run for 16 h in an air-tight glass chamber saturated with the solvent containing 2% of KCN. After the run, the chromatogram was dried in air and then at 80° for 1 h. Trace amounts of residual phenol were removed by washing with diethyl ether and the amines were rendered visible by spraying with a 0.3% solution of ninhydrin in acetone and heating the paper for 90 min at 70°. A typical chromatogram showing the resolution of the amines is depicted schematically in Fig. 1. It can be seen that spermine, spermidine, putrescine, cadaverine, agmatine and homoagmatine present in the plant seedlings, as well as exogenously added N-carbamyl derivatives of putrescine and cadaverine, are separated in this system and are well resolved. However, it was found that this chromatographic system did not separate the higher homologues of spermidine, *viz.* symmetrical and non-symmetrical homospermidine from each other and from putrescine, although they were well separated from spermidine. This lack of separation, however, is not normally a problem as these compounds are rarely encountered in nature. Further, diaminopropane was found to be poorly resolved from spermine in this chromatographic system, although it could be well separated from its higher homologue, putrescine, and also from spermidine. As is evident from Fig. 1, the chromatographic method used is capable of giving a clear-cut resolution among homologous amines, as well as among those amines that are connected by biosynthetic pathways, *e.g.* agmatine, N-carbamylputrescine and putrescine[14]. The R_F values obtained for these amines are recorded in Table I.

For the quantitative determination of these amines, the separated coloured bands were cut out and eluted with 3 ml of acetic acid–ethanol–water (4:3:1) containing cadmium acetate (20 mg/ml)[21], and the colour intensity was measured at 510 nm in a Beckman DU spectrophotometer. The λ_{max} of the ninhydrin colour for all of the above amines was about 510 nm. The colour yield per micromole of the amines was proportional to the number of primary amino groups present in the molecule. A linear relationship between the intensity of the ninhydrin colour and concentration was obtained for all of the amines in the range of 10–140 nmole. The percentage recoveries obtained are listed in Table I.

With several of the paper chromatographic systems described earlier for the separation of polyamines, multiple bands have been reported for many of these amines[20,22]. In the present study we did not encounter such a phenomenon. The

TABLE I

R_F VALUES AND RECOVERIES OF VARIOUS ALIPHATIC AMINES IN THE PHENOL–HCl–KCl SOLVENT SYSTEM

Recoveries listed represent amounts recovered after extraction with *n*-butanol, treatment with Dowex 50-X8 (H^+) and circular paper chromatography.

Amine	*R_F value*	*Recovery* (%)
Spermine	0.34	104
Symmetrical homospermidine	0.50	—
Non-symmetrical homospermidine	0.49	—
Spermidine	0.43	92
1,3-diaminopropane	0.35	—
Putrescine	0.51	60
Cadaverine	0.62	99
Agmatine	0.69	80
Homoagmatine	0.79	84
N-Carbamylputrescine	0.86	—
N-Carbamylcadaverine	0.93	—

above procedure for the preparation of the mixed amine fraction and the chromatographic technique have been used successfully in order to separate and quantitate di- and poly-amines found in other biological systems, such as *Neurospora crassa*, chick embryo and *Phaseolus radiatus* seedlings.

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