A column and paper chromatographic method for separation of N-dimethylamino-succinamic acid in plant extracts

Recently N-dimethylamino-succinamic acid (B-9) has been used as a size-controlling compound on certain plant species[1-3]. To determine the distribution pattern and the ultimate fate of B-9 in plant tissue, it was necessary to develop a procedure for its chemical analysis. The purpose of this investigation was to devise a method of analysis for the compound by using column and paper chromatographic techniques.

Materials and methods

Initial studies were conducted on standards of ^{14}C-labeled B-9 which had not been subjected to plant metabolism. A water solution containing 0.8 μC of ^{14}C B-9 was passed through ion exchange resins attached in series. The upper column contained IR-120*, a cation exchange resin in the H^+ form, and the lower column contained IR-45*, an anion exchange resin in Cl^- form. The columns measured 8 mm inside diameter and were packed to a height of 15 cm. The resins were prepared essentially as described by ROMBERGER[4].

After passing the ^{14}C B-9 through the resins, 25 ml of distilled water was added as a resin wash. The columns were then disconnected and the IR-120 resin was eluted with 30 ml of 7.5 *N* ammonium hydroxide and concentrated to 1 ml under a warm air stream. The IR-45 resin was eluted with 7.5 *N* formic acid and concentrated to 1 ml under a warm air stream. Aliquots were taken from each fraction including the non-polar material that passed through both resins, and assay of the recovered radioactivity was accomplished in a windowless gas-flow counter.

One gram aliquots of freeze-dried plant material previously treated with non-labeled B-9 and 1 μC of ^{14}C-labeled B-9 were extracted for 4 h at 90° with 50% ethanol. The extract was filtered hot under suction and brought to a constant volume. The total extract was then passed through the ion exchange resins attached in series as previously described.

After elution and concentration, 100 λ of the material from the IR-120 resin was spotted on Whatman 3 MM paper and developed two-dimensionally. The solvent systems employed were as follows: the first dimension in *n*-butanol–methyl ethyl ketone–water (2:4:1, v/v/v) plus 2 ml of ammonium hydroxide per 100 ml of solvent, developed for 15 h; the second dimension was developed in isopropanol–ammonium hydroxide–water (20:1:4, v/v/v) for 15 h. The dried chromatograms were sprayed or dipped in a 1:1 solution of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$, a reagent commonly used in higher concentration for the detection of phenols[5]. The color reagent is sensitive to 0.5 μg of B-9. Autoradiographs were made of the same two-dimensional chromatograms to verify the color test.

Results and discussion

To test the separation efficiency of the ion exchange resins, ^{14}C-labeled B-9 was passed through the columns of resins attached in series. The eluate from the columns was concentrated and aliquots were taken for radioactive assay. The results of this test may be seen in Table I, which shows that of the total radioactivity recovered, 98.1%

* Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A. Mention of trade names does not imply endorsement by the U.S. Department of Agriculture over similar materials not so named.

TABLE I

DISTRIBUTION OF ^{14}C B-9 STANDARD SOLUTION ON ION EXCHANGE RESINS ATTACHED IN SERIES

Fraction	*Recovered activity*	
	*CCPM**	%
IR-120	55760	98.1
IR-45	80	0.1
Through resins	1000	1.8

* Corrected counts per minute.

was contained on the IR-120 resin, against only 0.1 % on the IR-45 resin, with 1.8 % passing through both resins. The efficiency of recovery of the ^{14}C B-9 on the IR-120 resin was so high that it was selected for use. Since B-9 is a positively charged molecule, one would expect it to adsorb on IR-120 with other positively charged molecules such as amino acids. The efficiency of the IR-120 resin for B-9 was not altered when plant extracts containing the chemical were passed through the column.

In recovering B-9 from a sample plant extract, positively charged molecules other than B-9 are adsorbed and eluted from the IR-120 resin. Therefore, further separation of B-9 after elution was necessary, and this was accomplished by employing two-dimensional paper chromatography. The separation of B-9 from other components of a plant extract eluted from IR-120 such as amino acids by using the two-dimensional paper chromatography is shown in Fig. 1. Positive location and identification of B-9 was done by comparing the standard B-9 solution with a plant extract containing radioactive B-9 (direct counting and autoradiograph) and with a plant extract containing nonradioactive B-9. The amino acids tryptophan and tyrosine, which color blue with the B-9 reagent, do not interfere, but rather aid in the location of B-9.

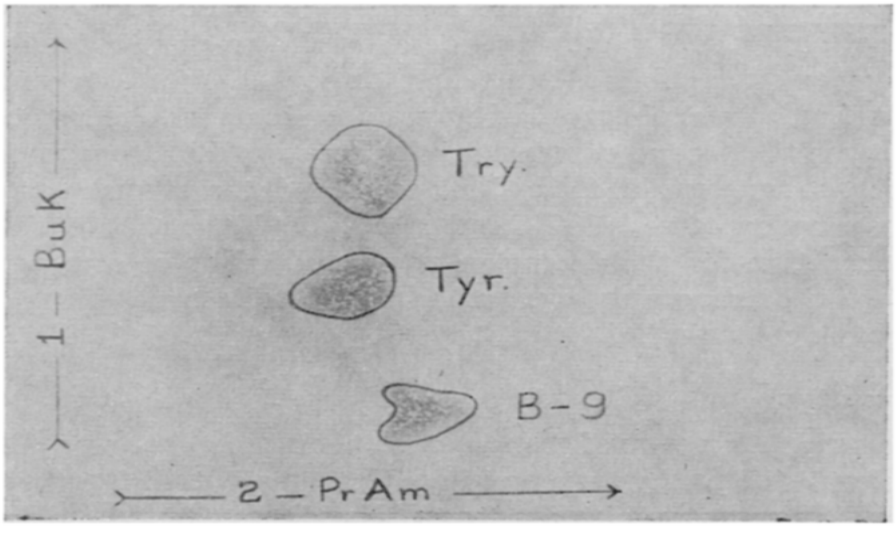

Fig. 1. Separation of B-9 from other positively charged components from IR-120. First dimension (1) developed in *n*-butanol–methyl ethyl ketone–water (2:4:1, v/v/v) plus 2 ml of ammonium hydroxide per 100 ml of solvent for 15 h, R_F 0.06. Second dimension (2) in isopropanol–ammonium hydroxide–water (20:1:4, v/v/v) for 15 h, R_F 0.33. Try = tryptophan; Tyr = tyrosine.

The B-9 extract separated on paper reacts with the reagent and gives a dark blue spot against a yellow background. In a very short time the background turns dark blue, and location of B-9 is difficult or impossible. This problem can be prevented by spraying immediately with 2 *N* ammonium acetate.

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Received January 24th, 1964

J. Chromatog., 15 (1964) 95–97

Countercurrent distribution of D-lyxose-1-^{14}C*

During the course of countercurrent distribution studies, we observed that D-lyxose-1-^{14}C migrated less rapidly than its unlabeled counterpart during countercurrent distribution. These data are similar to those previously reported[1] for D-arabinose-1-^{14}C and extend to these aldopentoses, during countercurrent distribution in cyclohexane–ethanol, PIEZ AND EAGLE'S[2] caution concerning the use of coincidence of radioactivity and an index of mass as the criterion for identity in studies of labeled amino acids. Implicit in such migration of solutes during countercurrent distribution as well as chromatography is the considerable error that can result in the selection of a single fraction rather than the peak for determinations of specific activity.

Materials

D-Lyxose-1-^{14}C and D-xylose-1-^{14}C with specific activities, respectively, of 0.18 and 0.21 mC per millimole were purchased from Calbiochem. The radiochemical purity of all compounds was found to be higher than 98 % when mass calculated from observed characteristic absorbance and absorbance index[3] was compared with mass computed from radioactivity and sample specific activity. *o*-Aminobiphenyl, purchased from Chemical Procurement Laboratories, College Point, New York, was purified by recrystallization.

Experimental

Countercurrent distribution. Twenty to 30 mg of a mixture of inert pentose and radioactive isomer with a final specific activity of 35–40 μC/mmole were dissolved in 100 ml of lower phase of the cyclohexane–ethanol system described in Fig. 1. The solution, after 24 h to permit anomeric equilibrium, was introduced into the first five tubes of the 100 tube countercurrent train. At the end of the indicated number of transfers, sampled tubes were dried in moving air at 22°. Dried samples were counted for

* Supported by a U.S. Public Health Training Grant.