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Note

Assay of polyamines by thin-layer chromatography and a combination of double-isotope dilution and scintillation methods

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The sensitive and precise determination of polyamines is of importance in the investigation of their biochemistry[1–3]. The most widely used methods are based on quantitation by high-performance liquid chromatography of the native of dansylated samples[4,5]. These methods are excellent but rely on the complete recovery of the sample and require special instrumentation. Regier and Kafatos[6] and Airhart *et al.*[7] have used radiolabelled dansyl chloride to analyse the quantity and specific activity of amino acids by a combination of double-isotope dilution and the fluorometric methods. I have adapted this technique to the quantitation of polyamines in biological samples. The main advantage of this method is that it does not require complete sample recovery nor special instrumentation, and relies primarily on the availability of a liquid scintillation spectrometer.

EXPERIMENTAL

Chemicals

^{14}C-labeled polyamines and [^{3}H]dansyl chloride were purchased from New England Nuclear. Thin-layer chromatography plates (silica 60 F254) were obtained from E. Merck. All reagents used were analytical grade.

Materials

Rat liver homogenates, *Escherichia coli* sonicates and L1210 leukemia cell lysates in 50 m*M* Tris–HCl were extracted for 3 min at 0°C with an equal volume of 0.4 *M* perchloric acid, and centrifuged at 2000 *g* for 15 min at 4°C prior to use. Etiolated pea internodes (6 days old) were similarly extracted and centrifuged at 48 000 *g* for 20 min at 4°C.

Procedure

To the tissue extracts (100 μl) was added a mixture of known amounts of ^{14}C-labeled polyamines (1 nmol [^{14}C]putrescine, $1 \cdot 10^{-2}$ μCi/nmol; 15 nmol [^{14}C]spermidine or [^{14}C]spermine, $9 \cdot 10^{-4}$ μCi/nmol) followed by 100 μl [^{3}H]dansyl chloride (0.46 mg/100 μl acetone, $1 \cdot 10^{-3}$ μCi/nmol). A ten-fold excess (in terms of

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neutralizing equivalents) of saturated sodium carbonate was added and the mixture incubated in the dark at 37°C for 2 h. The unreacted dansyl chloride was decomposed with 0.4 mmol proline, and the reaction mixture extracted three times with benzene or diethyl ether saturated with a sodium carbonate (saturated)–acetone–water (1:1:1). The benzene or ether layers were pooled and evaporated to dryness under a stream of nitrogen. To the dry residue, benzene or ether was added, and a portion of the resulting solution was spotted on silica gel thin-layer plates. These were developed in the dark, preferably in two dimensions. The solvents for the first dimension were cyclohexane–ethyl acetate (4:5) and for the second dimension, chloroform–triethylamine (100:8). The fluorescent spots were scraped off the plate, and placed in 0.5 ml New England solubilizer (NES) plus 5.0 ml Econofluor and counted in a liquid scintillation spectrometer for ^{14}C and ^{3}H.

Controls

Standards were prepared by danyslating the original ^{14}C-labeled polyamines with [^{3}H]dansyl chloride. These were added to a separate sample of the tissue extract and isolated in parallel with the experimental samples.

Calculations

The amount of unlabeled polyamine in the samples was calculated by the equation

$$U + S = S(A/B) \tag{1}$$

where U = amount (mol) of the unknown polyamine in the sample, S = amount (mol) of the radioactive polyamine added, A = ratio of $^{14}C/^{3}H$ in the control dansylated polyamine sample and B = ratio of $^{14}C/^{3}H$ in the experimental dansylated polyamine sample.

RESULTS AND DISCUSSION

The two dimensional chromatographic solvent systems described enable an excellent separation of the dansylated derivatives of putrescine (put), spermidine (spd) and spermine (spm) (Fig. 1).

The reliability of the method is indicated by Table I, which shows the recovery of known amounts of polyamines added to *E. coli* extracts. The recovery was optimal when two-dimensional chromatography was used.

Eqn. 1 takes into account the modification of the concentration of the unknown polyamine, U, by that of the added polyamine, S. It is, therefore, applicable to values of S that are larger, within certain limits, than U. Experimentally, reliable results can be obtained for $S \leqslant U$.

Another limitation of the assay is imposed by the spillover of ^{14}C into the ^{3}H channel in the Beckman spectrometer. This spillover is 20% in Econofluor; consequently, approximate limits for accurate determination of B are $0.4 \leqslant B \leqslant 7$. These limits can be greatly extended, especially at the lower values, if in each experiment the value of A is determined experimentally by TLC rather than theoretically. In this way the various experimental fluctuations, including quenching, are taken into account.

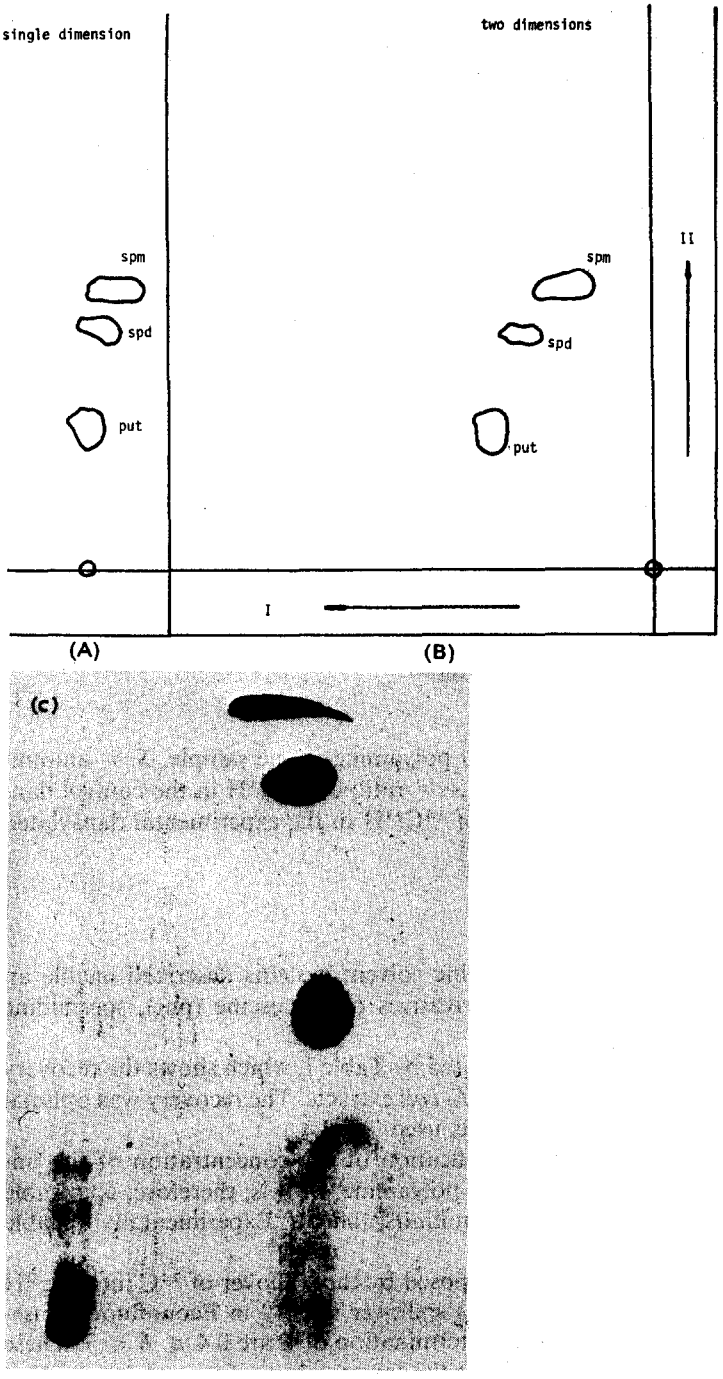

Fig. 1. Chromatography of dansylated derivatives of putrescine, spermidine and spermine. (A) One-dimensional TLC of dansylated polyamines. (B) Two-dimensional TLC of dansylated polyamines: I, cyclohexane–ethyl acetate (4:5); II, chloroform–triethylamine (100:8). (C) Autoradiography of dansylated [^{14}C]polyamines after two-dimensional TLC; the sample used in dansylation and chromatography was a mixture of [^{14}C]polyamines and *E. Coli* extract.

TABLE I

RECOVERIES OF KNOWN AMOUNTS OF POLYAMINES ADDED TO *E. COLI* EXTRACTS FROM SINGLE REPRESENTATIVE EXPERIMENTS

Polyamine	*Amount added (nmol)*	*Content in cell extract, A (nmol)*	*Cell extract + known amount of polyamines B (nmol)*	*Determined value (B−A) (nmol)*	*Recovery (%)*
(A) One-dimensional TLC, chloroform–triethylamine (100:8)					
Putrescine	2	1.16	3.03	1.87	93.5
Spermidine	20	0	16.92	16.92	84.6
Spermine	20	0.18	17.30	17.12	85.6
(B) Two-dimensional TLC					
Putrescine	2	1.84	3.82	2.02	101
Spermidine	20	0	18.39	18.39	91.95
Spermine	20	0	18.80	18.80	94.0

The stated specific activities of commercial samples of dansyl chaloride were found to be reliable. Should verification be required, because of radiation damage of the sample, this can be done by dansylating a commercial sample of a ^{14}C-labeled amino acid which is more stable than the commercially available polyamines and by determining the $^{14}C/^{3}H$ ratio. For most routine assays, the specific activity of commercial [^{3}H]dansyl chloride is too high, and should be diluted 20–100 fold. For many purposes, it may be considered adequate to add a known amount of radioactive

TABLE II

APPLICATION OF DOUBLE-ISOTOPE DILUTION METHOD TO THE DETERMINATION OF POLYAMINES IN DIFFERENT BIOLOGICAL MATERIALS

The values are the means ± S.D. for two or three separate experiments, except for the single determination in L1210 cells.

Material	*Polyamine content (nmol per 0.1 mg cell dry weight)*		
	put	*spd*	*spm*
E. Coli			
Sample 1	5.96 ± 0.21	7.03 ± 0.28	No
Sample 2	5.72 ± 0.015	6.22 ± 0.00	No
(nmol per 10 mg fresh weight)			
Etiolated pea internodes			
Sample 1	2.8 ± 0.1	4.4 ± 0.0	0.8 ± 0.01
Sample 2	5.3 ± 0.1	2.8 ± 0.3	0.9 ± 0.1
Sample 3	5.0 ± 0.1	4.4 ± 0.3	0.8 ± 0.0
(nmol per 10^5 cells)			
L1210 cells	0.18	0.72	0.29
(nmol per ml homogenate)			
Rat livers	1.8 ± 0.6	210 ± 0.00	224 ± 10.0

[^{3}H]dansyl chloride to a known quantity of non-radioactive dansyl chloride in order to establish a specific activity for the stock solution of dansyl chloride with an error of 1–5%.

In the present experiments, polyamines were determined in the nanomol and picomol range (Table II). Theoretically, the limits of detection are much lower and are determined by the specific activities of the undiluted radioactive reagents.

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