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Note

Separation of deoxythymidine and deoxythymidine nucleotides by column and thin-layer chromatography

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During studies on thymidine, thymidylate and nucleoside diphosphate kinases in extracts of the extremely halophilic bacterium *Halobacterium cutirubrum*, we needed to separate deoxythymidine (dT), and its 5′-mono-, di-, and triphosphates (dTMP, dTDP, and dTTP) rapidly by both column and thin-layer chromatography (TLC). However, most established methods for the column chromatography of mixtures of nucleoside mono-, di- and triphosphates have been developed for use with ribonucleotides[1–7] and generally involve gradient elution[2–6], which is not convenient in enzyme assays. Okazaki and Kornberg[8] have described the preparative-scale separation of deoxythymidine and its corresponding nucleotides on Dowex 1, but although their method is effective the volumes of eluant required were too large for our purposes. Similarly, one-dimensional TLC systems for the separation of these particular compounds have not been published.

We now describe (i) a modification of Randerath's[9,10] PEI-cellulose procedure in which the LiCl concentration and development times have been altered to give a good separation of deoxythymidine and its 5′-mono-, di- and triphosphates, and (ii) a rapid, small-scale method for the analysis of such mixtures on Dowex 1 columns. The latter was developed using the TLC procedure to verify the separations and is a combination of parts of several earlier methods[1,7,8].

EXPERIMENTAL AND RESULTS

Materials were purchased from the following suppliers: PCS liquid scintillation medium and radioactive compounds from Amersham/Searle (Oakville, Canada); Dowex AG 1-X4 from Bio-Rad Labs. (Richmond, Calif., U.S.A.); MN-300 PEI-cellulose, plastic-backed TLC sheets from Brinkmann (Rexdale, Canada); non-radioactive nucleotides and deoxythymidine from P-L Biochemicals (Milwaukee, Wisc., U.S.A.); all other chemicals and disposable Pasteur pipettes, from Fisher Scientific (Ottawa, Canada).

Ascending TLC was carried out at room temperature in Shandon Universal TLC Chromatanks (Johns Scientific, Toronto, Canada) fitted with stainless-steel holders that permitted rapid transfer of the chromatograms between tanks. The latter contained sufficient solvent to cover the bottom 1 cm of the chromatograms. Plastic-backed PEI-cellulose sheets (20 × 20 cm) were used and were stored at 4° in

the dark. For good results, it was essential that the sheets be thoroughly dried over silica gel. Old or discoloured sheets were washed well with distilled water and then dried. The samples (40 μl, max.) were applied 2 cm from the bottom of the sheets as a thin band, which was then dried in a stream of cool air. The chromatograms were developed successively in the following solutions for the times indicated: (i) 0.2 *M* LiCl, 2 min; (ii) 1 *M* LiCl, 6 min; (iii) 1.6 *M* LiCl, 22 min. In these conditions, deoxythymidine and the deoxythymidine 5′-nucleotides had the following R_F values relative to the solvent front: dT, 1.00; dTMP, 0.56; dTDP, 0.45; dTTP, 0.36. Fig. 1 illustrates a typical separation of the mixture of radioactive, acid-soluble compounds obtained during *in vivo* phosphorylation of deoxythymidine by *H. cutirubrum*. The products were identified by comparison with non-radioactive standards; the chromatograms were then cut into strips (2.5 × 0.7 cm) and the latter were counted in plastic vials containing 10 ml of a 2:1 (v/v) mixture of PCS and xylene, using a Beckman LS-230 liquid scintillation counter. The maximum concentration of KCl and/or NaCl in the samples that did not affect the separation was 0.04 *M*.

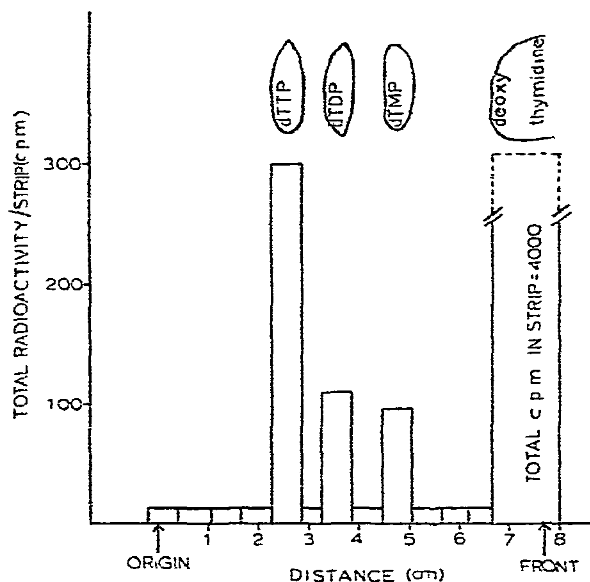

Fig. 1. TLC separation of dT, dTMP, dTDP and dTTP. The experiment was performed as described in the text. A mixture of the authentic compounds was added to the radioactive sample before chromatography and the spots corresponding to each compound were identified as indicated above the graph by comparison with a spot of each material run on its own. Strips slightly wider than the spots were cut out as indicated and the intermediate sections of the chromatogram were divided into further strips where possible. All the strips were counted as described in the text. [N.B. The radioactivity in the intermediate strips was $\leqslant$ background in all cases.]

Column chromatography was performed in disposable Pasteur pipettes fitted with a cottonwool plug to support the resin. Dowex 1 (Bio-Rad AG 1-X4) was suspended in 3 *N* HCl (8 ml/g). The suspension was left for 1 h at room temperature and the acid was then decanted. The resin was washed repeatedly with distilled water (10 ml/g/washing) until the pH of the washings was 5. Fines were removed during this

treatment. The resin was then suspended in distilled water (5 ml/g) and 2 ml of the suspension were transferred to the Pasteur pipette to give a column approx. 0.4 × 3 cm. The resin was washed twice with 2 ml of distilled water and was then ready for use. The sample (20 μl) containing up to 12.5 nmoles of radioactive deoxythymidine and/or deoxythymidine nucleotides was diluted with 0.04 M sodium acetate, pH 4.4 (0.98 ml) and the mixture was applied to the column. Deoxythymidine was washed from the resin with a further 10 ml of 0.04 M sodium acetate, pH 4.4, and the nucleotides were then eluted successively with the following solutions: (i) dTMP, 0.02 N HCl–0.02 M KCl (16 ml); (ii) dTDP, 0.02 N HCl–0.2 M LiCl (10 ml); (iii) dTTP, 0.2 N HCl–0.2 M LiCl (10 ml). Fractions (2 ml) were collected directly ino plastic vials containing Anderson and McClure's[11] liquid scintillation medium (6 ml) and counted. The maximum KCl or NaCl concentration that could be present in the diluted samples without affecting the separation was 0.02 M, but since the volume of diluted sample applied to a column was not critical this was not a limitation.

The results in Table I describe a series of experiments in which radioactive samples of the four compounds concerned were analysed by both the column and TLC procedures. The radioactive nucleotides had been stored for varying times and contained hydrolysis products: they therefore provided convenient mixtures for testing the column chromatographic method, while TLC permitted identification of the components of the mixtures by comparison with standards. It can be seen that the results obtained by the two methods are in excellent agreement, thus demonstrating the reliability of the column chromatographic procedure.

TABLE I

SEPARATION OF DEOXYTHYMIDINE AND DEOXYTHYMIDINE NUCLEOTIDES BY COLUMN AND THIN-LAYER CHROMATOGRAPHY

No.	*Sample*	*Method*	*Radioactivity recovered (%)*				*Total radioactivity (cpm)*
			dT	*dTMP*	*dTDP*	*dTTP*	
1	[^{3}H]dT	Column	98.0	1.1	0.8	0.1	16,132
		TLC	98.1	0.1	0.1	<0.1	371,923
2	[^{3}H]dTMP	Column	5.0	95.0	<0.1	0	25,256
		TLC	1.5	97.5	0.3	0.1	591,210
3	[^{3}H]dTDP	Column	1.5	5.0	92 0	1 5	10,328
		TLC	1.6	3.6	93.0	1.0	152,615
4	[^{14}C]dTTP	Column	3.0	2.0	10.0	85.0	11,267
		TLC	2.0	2.0	12.3	83.0	10,782

The column chromatographic method is particularly useful with samples of high ionic strength, because they can be diluted sufficiently to prevent the salts from affecting the separation and the whole sample can still be applied to the column. Samples with comparatively low radioactivity can therefore be used without loss of sensitivity as a result of dilution.

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