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An improved separation procedure for nucleoside monophosphates on polyethyleneimine- (PEI-)cellulose thin layers.

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ABSTRACT

A procedure is described for the two-dimensional separation of the 4 major and 16 modified nucleoside-(5') monophosphates on anion-exchange thin layers of polyethyleneimine- (PEI-)cellulose. The method, which is simple and less time-consuming than existing partition chromatographic methods, may be used for the identification of 5'-termini of RNA and RNA fragments.

For the separation of ribonucleoside-(5') monophosphates, obtained by nuclease S_1 digestion of oligoribonucleotides (see the preceding paper[1]), a PEI-cellulose thin-layer procedure has been developed, which is the subject of the present paper.

MATERIALS

The monophosphates, pA, pC, pU, pG, pI, and pCm, and the diphosphates, ppAm and ppUm, were purchased from Sigma Chemical Co. or P-L Biochemicals. Monophosphates of other nucleosides were prepared by a combination of chemical and enzymic methods from RNase T_1, A or U_2 fragments of $tRNA^{Leu}_{CUA}$ (yeast), $tRNA^{Leu}_{UUG}$ (yeast) and $tRNA^{Phe}$ (human placenta) as described in Methods. For the sources of alkaline phosphomonoesterase, snake venom phosphodiesterase, nuclease S_1, polynucleotide kinase, $(^3H)NaBH_4$, $(\gamma\text{-}^{32}P)ATP$, and PEI-cellulose thin-layer sheets, see the preceding paper[1]. Cellulose sheets were from E. Merck (EM Laboratories # 5502). Other chemicals used were of analytical reagent grade. Button-type permanent Alnico magnets (1/2" x 3/8") were from General Hardware Manufacturing Co., Inc., New York.

METHODS

Preparation of nucleoside-(5') monophosphates. The monophosphates, pAm, pUm, and (^{32}P)pGm, were prepared from ppAm, ppUm, and Gm-A-A-Y-A-Ψp, respectively[2]. 5'-Monophosphates of most modified nucleosides were prepared in ^{32}P-labeled form. pT, pm^{5}C, pm^{1}G, and pΨ were prepared from T-Ψ-C-Gp, m^{5}C-A-A-Gp, m^{1}G-C-Ψ-C-U-Gp, and Ψ-U-U-Ap[3], respectively; pD and pac^{4}C, from D-C-D-A-A-Gp and C-ac^{4}C-Gp[4], respectively; pm^{1}A, pm^{7}G, pm$_2^2$G, and pm^{2}G, from m^{1}A-U-C-C-C-Gp, m^{7}G-(D,U)-C-m^{5}C-C-U-Gp, m$_2^2$G-Ψp, and A-m^{2}G-Cp[5], respectively.

The monophosphates, pT, pm^{5}C, pm^{1}G, pΨ, pD, pm^{1}A, pm^{7}G, and pm$_2^2$G, were obtained by subjecting the corresponding oligonucleotide to 5'-terminal labeling by the (γ-^{32}P)ATP/polynucleotide kinase reaction[6,7], followed by digestion with nuclease S$_1$[8]. The reaction mixture contained 0.02 - 0.05 mM oligonucleotide, 0.12 mM (γ-^{32}P)ATP (3 - 5 Ci/mmole), 15 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM $MgCl_2$, and 0.14 U/µl polynucleotide kinase in a total volume of 15 µl. The incubation was carried out at 38° for 30 min. The labeled terminus was released as nucleoside-(5') monophosphate by incubating the kinase reaction mixture with nuclease S$_1$ (60 U/µl), sodium acetate, pH 4.5 (60 mM), LiCl (0.15 M), and $ZnCl_2$ (0.1 mM). After 20 - 50 min at 38°, the samples were applied to a PEI-cellulose thin-layer sheet and developed with acetic acid/formic acid or Tris-HCl using conditions similar to those described under Chromatography (see below). The monophosphate spots were located by autoradiography and eluted with 4 M pyridinium formate (pH 4.0) or 2 M LiCl and purified by passing the eluates through phosphocellulose[9]. 50,000 - 500,000 cpm of each monophosphate was isolated in this way to serve as a marker compound.

The monophosphates, pac^{4}C and pm^{2}G, were prepared by converting the trinucleotides, C-ac^{4}C-Gp and A-m^{2}G-Cp (0.05 - 0.1 mM), to the corresponding (^{3}H)-labeled oligonucleotide-(3') dialcohols, C-ac^{4}C-G'-T and A-m^{2}G-C'-T, as follows: (1) dephosphorylation with alkaline phosphomonoesterase (0.2 µg/µl), (2) oxidation with $NaIO_4$ (1 mM); and (3) reduction with (^{3}H)$NaBH_4$ (about 5 mM). C-ac^{4}C-G'-T and A-m^{2}G-C'-T were then digested with snake venom phosphodiesterase (0.25 µg/µl) in the presence of 20 mM bicine (pH 8.0), 10 mM $MgCl_2$, and 0.05 µg/µl alkaline phospha-

tase at 50° for 6 - 7 hrs to yield ac^{4}C-G'-T and m^{2}G-C'-T, respectively. This reaction appears to be due to endonuclease activity present in snake venom phosphodiesterase preparations. The dinucleotide derivatives appear to be resistant to this endonucleolytic activity. (^{32}P)pac^{4}C and (^{32}P)pm^{2}G were prepared from these derivatives by treatment with (γ-^{32}P)ATP/polynucleotide kinase, followed by digestion with nuclease S_1 as described above.

The monophosphate, pm^{6}A, was prepared by incubation of (^{32}P)pm^{1}A (130,000 cpm) in 30 mM bicine (pH 9.5) at 50° for 5 hrs.

Chromatography. Separations were carried out on PEI-cellulose thin layers that had been prepared in the laboratory. For two-dimensional mapping of nucleoside monophosphates, about 1,000 - 2,000 cpm of each labeled compound (pm^{1}A, pm^{6}A, pm^{5}C, pac^{4}C, pGm, pm^{1}G, pm^{2}G, pm$_2^2$G, pm^{7}G, pD, pΨ, and pT) and 10 - 20 nmole of unlabeled compounds (pA, pC, pU, pG, pI, pAm, pCm, and pUm), in a volume of 12 µl, was applied in 3-µl portions to the layer at 2.5 cm each from the left-hand and the bottom edge. Before chromatography, the sheet was soaked in 200 ml of methanol for 10 min and dried in a stream of cool air. Development was in stepwise fashion (without intermediate drying) with (1) water to the origin, (2) 0.25 N acetic acid to 9 cm, and (3) 0.8 N formic acid to 4 cm on a Whatman # 1 wick attached at 17 cm from the origin (first dimension). The sheet was dried thoroughly (10 - 15 min with cool air, then 5 min with warm air), soaked in a solution of 600 mg of tris(hydroxymethyl)aminomethane in 500 ml of methanol for 10 min, dried with cool air, and finally soaked in 500 ml of methanol for 10 min. The Tris/methanol treatment serves to adjust the pH of the layer so as to obviate the formation of pH fronts during development in the second dimension[10]. For the second dimension, chromatography was with (1) water to the origin and (2) 0.22 M Tris-HCl, pH 8.0, to 5 cm on a Whatman # 1 wick. Spots were located by examination under a short-wave ultraviolet lamp and by autoradiography.

The location of each individual compound on the map relative to the major compounds was determined by cochromatography

with a mixture of nonradioactive pA, pC, pU, and pG (10 - 20 nmole each). All compounds are resolved in this system except pU and pΨ, which may, however, be readily resolved on cellulose layers (see below). Using the same chromatographic conditions, a similar separation of monophosphates was achieved on commercial PEI-cellulose sheets but the spots were more diffuse than on "homemade" sheets, resulting in incomplete resolution of some compounds, for example the pm^2G/pGm pair.

For separating pU and pΨ, the area containing these compounds was cut from the chromatogram and soaked in methanol. The 2 compounds were then transferred by direct contact to a cellulose thin-layer sheet using magnets to hold the PEI-cellulose cut-out in contact with the cellulose layer[11]. To transfer the monophosphates quantitatively to the cellulose sheet, the chromatogram was first developed with 2 N ammonia to 2.5 cm above the origin. Magnets and cut-out were removed and the layer dried thoroughly. The sheet was then developed with acetonitrile/t-amyl alcohol/4 N ammonia (5 : 1 : 5, by vol.) to 4 - 5 cm on a Whatman # 1 wick attached at about 17 cm from the origin.

RESULTS AND DISCUSSION

An autoradiogram of a nucleotide fingerprint is shown in Fig. 1. The location of the nonradioactive compounds chromatographed has been indicated by drawing. Relative R_F values are compiled in Table 1. The combination of the two solvent systems thus affords an excellent resolution of major and modified nucleoside-(5') monophosphates, except that pΨ partially overlaps pU. Following contact transfer of the pU/pΨ area to a cellulose thin layer (see Methods), these 2 compounds are readily resolved by chromatography with acetonitrile/t-amyl alcohol/4 N ammonia (5 : 1 : 5). Relative R_F values are 0.91 for pΨ and 1.0 for pU. This solvent also separates pT and pUm from pU (relative R_F values, 1.10 for pT and 1.26 for pUm).

As expected, compounds carrying a positively charged base moiety (pm^1A, pm^7G) migrate very fast on the anion-exchange thin layer (see Fig. 1). For the separation of pm^7G from pCm and of

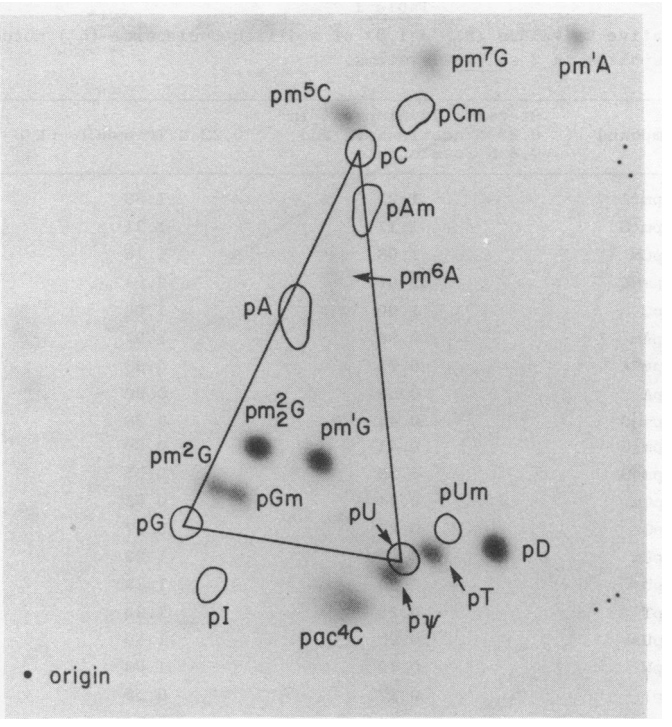

Fig. 1

Two-dimensional separation of a model mixture of nonradioactive and (^{32}P)-labeled nucleoside-(5') monophosphates on a PEI-cellulose thin layer. Development with acetic acid/formic acid in the first dimension (bottom to top) and Tris-HCl, pH 8, in the second dimension (left to right). For details of the procedure, consult text.

pm^5C from pC, 0.25 N acetic acid was found to be optimal. The spot of pm^6A is somewhat diffuse under these conditions but pm^6A gives a sharp spot in 0.4 - 0.6 N acetic acid (not shown). In general, optimal separation of derivatives of pA and pC is being achieved by development with a low concentration of acetic acid while pG and pU derivatives best separate at low concentrations of Tris-HCl, pH 8 (0.2 - 0.3 M).

The triangle drawn through the positions of the major nucleotides (Fig. 1) may be used for reference purposes to locate the modified derivatives.

The procedure described in the present communication enables one to resolve complex mixtures of nucleoside monophosphates.

Table 1

Relative R_F values (R_{pC} = 1.0) of modified nucleoside-(5') monophosphates in 2 solvent systems.

Compound	Stepwise development in 0.25 N acetic acid and 0.8 N formic acid	0.22 M Tris-HCl, pH 8
pm^1A	1.21	1.63
pm^7G	1.17	1.21
pCm	1.08	1.16
pm^5C	1.07	0.94
pC	1.00	1.00
pAm	0.90	1.01
pm^6A	0.75	0.93
pA	0.68	0.80
pm_2^2G	0.43	0.68
pm^1G	0.41	0.86
pm^2G	0.36	0.55
pGm	0.34	0.63
pG	0.29	0.47
pUm	0.27	1.23
pD	0.24	1.38
pT	0.23	1.20
pU	0.22	1.10
pΨ	0.19	1.08
pI	0.17	0.55
pac^4C	0.14	0.94

Since the total development time is only 4 - 5 hrs the procedure is less time-consuming than existing partition chromatographic procedures for two-dimensional separation of nucleoside monophosphates on thin layers or paper[12-14]. Also, since the present ion-exchange method does not entail the use of strongly acidic or basic solvents it should be suitable for the analysis of labile RNA constituents.

The separation procedure presented has been applied to the identification of 5'-terminal positions of oligonucleotides (see the preceding paper[1]); it may also be useful in base composition studies on (^{32}P)-labeled RNA.

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ABBREVIATIONS

Abbreviations used are as recommended (1970) by the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochim. Biophys. Acta 247, 1 (1971) or J. Mol. Biol. 55, 299 (1971)). Additional abbreviations: PEI-cellulose, anion-exchange cellulose material obtained by treating cellulose with polyethyleneimine[15]; C-ac^{4}C-G'-T, A-m^{2}G-C'-T, etc., (^{3}H)-labeled oligonucleotide-(3') dialcohols derived from C-ac^{4}C-G, A-m^{2}G-C, etc.; bicine, N,N-bis-(2-hydroxyethyl)glycine; Y, wybutosine[16], the fluorescent nucleoside from tRNA^{Phe} of Saccharomyces cerevisiae[17,18].

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