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AN IMPROVED METHOD FOR THIN-LAYER CHROMATOGRAPHY OF NUCLEOTIDE MIXTURES CONTAINING ^{32}P-LABELED ORTHOPHOSPHATE

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SUMMARY

Development of poly(ethylene)imine cellulose thin layers with phosphate solutions gives improved resolution of complex mixtures of nucleotides. Phosphate development also minimizes the tailing of highly radioactive orthophosphate present in the mixtures and thus facilitates chromatographic analysis of crude acid extracts of phosphate-labeled bacteria. Conditions employing phosphate development are described which give semi-quantitative resolution of the ribonucleoside triphosphate components of such extracts after one-dimensional chromatography as well as two-dimensional systems for quantitative resolution of the major nucleotide components.

INTRODUCTION

Recently a number of procedures for ion-exchange thin-layer chromatographic resolution of complex nucleotide mixtures have been developed (reviews[1,2]). Among these the use of poly(ethylene)imine cellulose anion exchanger (PEI) has been shown by RANDERATH *et al.*[3-5] to be particularly well suited for nucleotide analysis. However with many of the solvent systems affording the best separation of nucleotidic compounds, we have encountered problems with "tailing" of sizeable amounts of $^{32}P_i$** during TLC on PEI cellulose layers. This contamination precludes the application of unidimensional procedures for the analysis of acid extracts of $^{32}P_i$-labeled bacteria without prior removal of the P_i. Furthermore, even after two-dimensional separations, the quantitative estimation of some nucleotides from ^{32}P radioactivity was impaired by the tailing of $^{32}P_i$. We have minimized this problem through the use of ortho-

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** Abbreviations used: Only 5'-nucleotides were employed in this study. AMP = Adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GMP, GDP, GTP = guanosine mono-, di-, and triphosphate; UMP, UDP, UTP = uridine mono-, di-, and triphosphate; CMP, CDP, CTP = cytosine mono-, di-, and triphosphate; ITP = inosine triphosphate; XTP = xanthosine triphosphate; TTP = thymidine triphosphate; dCTP = deoxycytosine triphosphate; dGTP = deoxyguanosine triphosphate; dATP deoxyadenosine triphosphate. P_i = orthophosphate.

phosphate developing solutions and thus obviated the necessity for purifying such extracts free of $^{32}P_i$ prior to chromatography.

In this communication we describe the properties of phosphate chromatography of nucleotides on PEI cellulose thin layers. In addition, two-dimensional systems are described which we feel give improved resolution of the major nucleotide components of soluble bacterial pools.

METHODS

In general the methods employed are described by RANDERATH AND RANDERATH[6]. Poly(ethylene)imine cellulose thin layers on plastic sheets were obtained from Brinkmann Instruments, Inc., Westbury, N. Y. Prior to chromatography the PEI cellulose sheets were soaked in trays containing 1 l distilled water for 30 min and dried at room temperature with a fan. Chromatography was carried out by ascending development (dry start) in closed plexiglass chambers at room temperature.

Phosphate solutions were prepared from analytical grade phosphoric acid and its potassium salts. Nucleoside mono-, di-, and triphosphates were obtained from Sigma Chemical Co., St. Louis, Mo., and Schwarz BioResearch, Inc., Orangeburg, N. Y. Adenosine tetra- and pentaphosphates were purified from a commercial preparation of adenosine tetraphosphate (Sigma Chemical Co.) by preparative TLC and DEAE Sephadex column procedures[7].

Extraction of $^{32}P_i$-labeled acid-soluble materials from bacterial cultures was accomplished by the addition of an equal volume of 2 *M* formic acid[8]. The cell suspension was then held in ice for at least 15 min, and then clarified by centrifugation for 1 min at room temperature in a Beckman microfuge. Aliquots of the cell-free supernatant were applied 3 cm from the lower edge of the chromatogram and fan-dried prior to development. The identification of radioactive materials was confirmed by their co-migration with authentic nucleotides which were added to the extracts. The ^{32}P-labeled materials were visualized by exposing X-ray film to the dried chromatograms for 18 h, and developing the exposed film[8].

Where indicated in the text, nucleotidic materials were removed from the formic acid extracts by the addition of 20 mg of acid-washed Darco G-60 charcoal. After 5 min, the charcoal was removed from the extract by centrifugation. Elution of the nucleotidic materials from the charcoal was accomplished with 4 ml of an ethanol–H_2O–NH_4OH solution (65:35:0.3). The eluate was freed of charcoal by filtration through Celite and concentrated on a warm hot plate under a stream of warm air.

RESULTS

Fig. 1 shows radioautograms of chromatograms developed in one dimension using each of the procedures previously described for two-dimensional resolution of complex mixtures of nucleotides[4,5] as well as the results of chromatography of identical preparations in various concentrations of phosphate, pH 3.4. In each case 2×10^5 c.p.m. of $^{32}P_i$ was spotted at the origin. It may be seen that phosphate development markedly diminishes $^{32}P_i$ tailing, as compared to the other procedures, as well as diminishing the size of the spots. This effect is relatively independent of the phosphate concentration in the range examined.

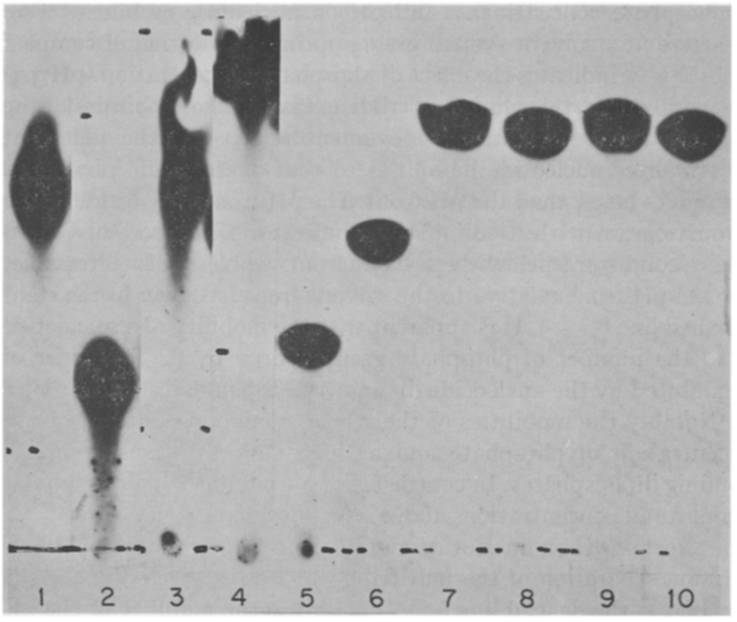

Fig. 1. Comparison of orthophosphate migration and tailing on PEI cellulose thin layers. Approximately 2 × 10^5 c.p.m. of $^{32}P_i$ were applied to each chromatogram and developed in one dimension under the following conditions: sample 1, step formate[4]; sample 2, step lithium chloride[4]; sample 3, step acetate–lithium chloride[5]; sample 4, step borate–acetate[5]; samples 5 through 10 were chromatographed in KH_2PO_4 (pH 3.4) at 0.25 *M*, 0.5 *M*, 0.75 *M*, 1.0 *M*, 1.25 *M*, and 1.5 *M*, respectively. In each case the chromatograms were developed such that the solvent front was 15–17 cm from the origin.

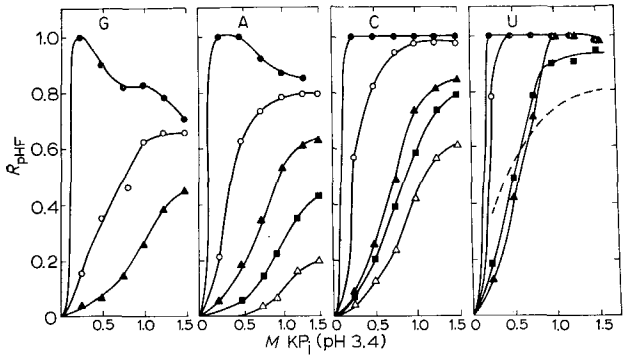

Fig. 2. The effect of potassium phosphate concentration on nucleotide mobilities. Standard nucleotide solutions were chromatographed in phosphate solutions (pH 3.4) at concentrations ranging from 0.25 *M* to 1.5 *M*. The mobilities of the nucleotides are expressed relative to that of the pH front (R_{pHF}). The effect of phosphate concentration on the mobility of the pH front relative to the solvent front (R_F) is the same in all panels but is shown only in panel U as a dashed line. Panel G: GMP, closed circles; GDP, open circles; GTP, closed triangles. Panel A: AMP, closed circles; ADP, open circles; ATP, closed triangles; adenosine tetraphosphate, closed squares; adenosine pentaphosphate, open triangles. Panel C: CMP, closed circles; CDP, open circles; CTP, closed triangles; ITP, closed squares; XTP, open triangles. Panel U: UMP, closed circles; UDP, open circles; UTP, closed triangles, TTP, closed squares.

The effects of phosphate concentration and pH on nucleotide mobilities were explored in order to arrive at a solvent system giving optimal resolution of complex mixtures of nucleotides. Fig. 2 indicates the effect of phosphate concentration (pH 3.4) on the mobilities of several nucleotides found in crude extracts. The mobilities of the compounds are expressed as their fractional movement relative to the pH front (R_{pHF}) since (1) it normalizes nucleotide mobilities to that of inorganic phosphate and (2) no nucleotide moves faster than the pH front. The pH front may be identified by spraying the chromatogram with a suitable pH indicator (Bromocresol Green) or more simply as the second front below the solvent front visible under ultraviolet light. The position of the pH front relative to the solvent front is shown in the right panel of Fig. 2 as a dashed line (- - - -). It is apparent that the mobility of a nucleotide is inversely related to the number of phosphate groups borne by it. The order of increasing mobility exhibited by the nucleoside di- and triphosphates is G, A, C, U, T at 0.75 *M* KH_2PO_4. Notably the mobilities of the purine monophosphates decrease with increasing concentration of phosphate and at high concentrations approach those of the corresponding diphosphates. In contrast, the pyrimidine monophosphates move with the pH front at all concentrations above 1 *M* phosphate.

Fig. 3 shows the effect of pH on nucleotide mobilities in 0.75 *M* potassium phosphate solution. Maximum separation of the four triphosphates is affected in the pH range of 3 to 5. As in Fig. 2, the dashed line (- - - -) describes the mobility of the pH front relative to the solvent front.

The resolution of the nucleotide triphosphates from ^{32}P-labeled compounds not adsorbed to charcoal is shown in Fig. 4. An acid extract of $^{32}P_i$-labeled *E. coli* cells

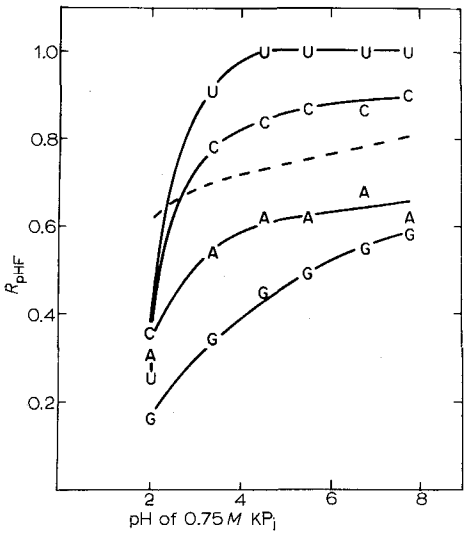

Fig. 3. The effect of pH on nucleotide mobilities. Standard solutions of ribonucleoside triphosphates were chromatographed in 0.75 *M* potassium phosphate solutions at a pH ranging from 2.0 to 7.8. As in Fig. 2 the R_F of the pH front is indicated as a dashed line. The mobilities of the nucleotides relative to the pH front are expressed as R_{pHF} and are plotted against pH. The mobilities of the following nucleotides are shown: G, GTP; A, ATP; C, CTP; U, UTP.

was chromatographed before and after removal of the nucleotidic materials by treatment with charcoal. For comparison the adsorbed nucleotides were eluted from the charcoal and also chromatographed. The four ribonucleoside triphosphates are well resolved from each other and P_i. In this uni-dimensional separation, pyrimidine nucleoside mono- and diphosphates migrate with P_i at the pH front. The area between the pH front and UTP contains AMP, ADP, GMP, and TTP. The material migrating slightly faster than CTP is dCTP. Under the conditions employed dATP and dGTP co-migrate with ATP and GTP respectively. The results obtained with the charcoal-treated extract indicate that the contamination of the triphosphate areas with non-adsorbable $^{32}P_i$-labeled materials is very low; the compound co-migrating with GTP is pyrophosphate.

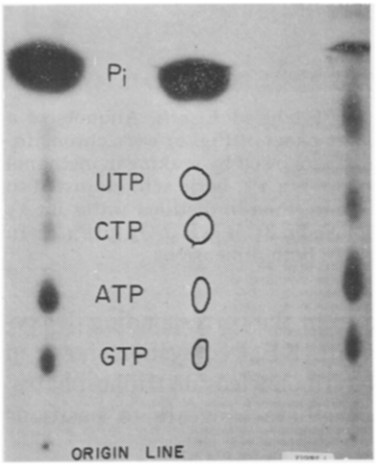

Fig. 4. One-dimensional chromatography of acid extracts of ^{32}P-labeled *E. coli* with 0.85 *M* KH_2PO_4 (pH 3.4). Aliquots of a formic acid extract of ^{32}P-labeled *E. coli* were chromatographed on PEI cellulose thin layers before (left sample) as well as after (middle sample) adsorption of nucleotidic material with charcoal. Chromatography of nucleotides eluted from charcoal, as described in Methods, is shown in the right sample. The mobility of marker nucleotides is indicated in the center panel. The figure shows the radioautogram obtained after 18 h exposure of X-ray film to the chromatogram.

The small discrepancies between the mobility of the triphosphates in the first two columns in Fig. 4 and those shown in Fig. 2 is attributed to the fact that the acid extracts were not adjusted to pH 3.4 before application. The nucleotides eluted from charcoal were applied as an unbuffered neutral solution and consequently their mobilities are the same as those in Fig. 2.

When higher resolution of the pyrimidine triphosphates than can be obtained with the simple one-dimensional system is required, a step formate[4]–0.85 *M* KH_2PO_4 two-dimensional system may be employed to considerable advantage. A typical separation of a crude $^{32}P_i$-labeled extract is shown in Fig. 5a. By first developing the chromatogram with a formate gradient, the nucleoside mono- and diphosphates as well as most of the P_i are resolved from the triphosphates[4]. Separation of the triphosphates is achieved in the second dimension with 0.85 *M* KH_2PO_4, pH 3.4, after removing the formate buffer from the PEI sheets by soaking in methanol for 15 min.

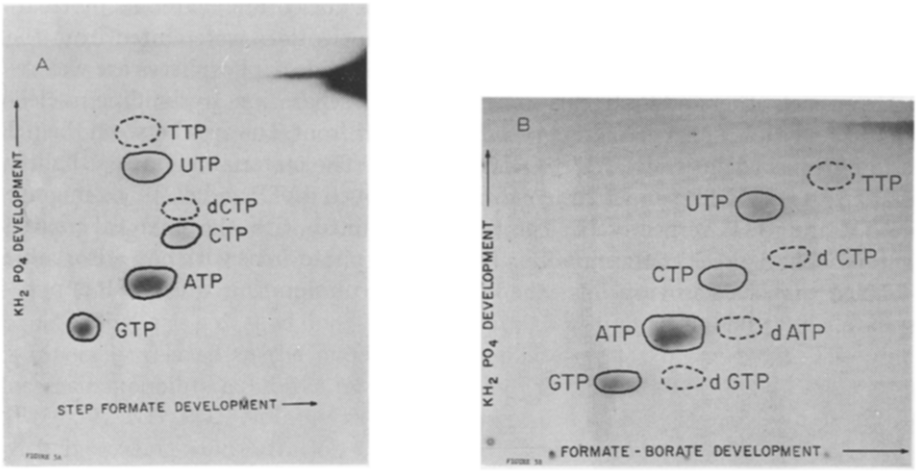

Fig. 5. Two-dimensional chromatography of acid extracts of ^{32}P-labeled *E. coli*. Aliquots of a formic acid extract (similar to that chromatographed in the left panel of Fig. 2) were chromatographed in two dimensions. Panel A: first dimension, step formate followed by soaking in methanol and drying[4]. Panel B: first dimension, 3.3 *M* ammonium formate + 4.2% boric acid (adjusted to pH 7.0 with NH_4OH) followed by soaking in methanol for 5 min, then in distilled water for 15 min, then dried. In panels A and B the second dimension employed 0.85 *M* KH_2PO_4 (pH 3.4). In each case the solvent fronts reached 15–17 cm above the origin in both dimensions.

The purine nucleoside triphosphates are not resolved from the corresponding deoxynucleoside triphosphates. The ammonium borate–acetate–KH_2PO_4 system shown in Fig. 5b affects the separation of all eight ribo- and deoxyribonucleoside triphosphates. However in the latter system, the pyrimidine triphosphates migrate to positions near the nucleoside mono- and diphosphates.

DISCUSSION

Analysis of crude ^{32}P-labeled extracts by thin-layer chromatography is difficult because of excessive tailing of highly radioactive $^{32}P_i$ and the large variety of phosphorylated compounds contained in such extracts. The application of KH_2PO_4 developing solutions minimizes these difficulties. The tailing of P_i observed during phosphate development is sufficiently low and the P_i spot sufficiently compact to allow accurate quantitation of materials moving close to P_i. At the concentrations of potassium phosphate greater than 0.75 *M*, most mono- and diphosphonucleosides as well as the majority of non-charcoal absorbable materials, migrate with P_i at the pH front. Under these conditions it is possible to estimate, semiquantitatively, the four major nucleoside triphosphates in crude extracts using one-dimensional development. Accurate estimation of ribonucleoside triphosphate is easily achieved with either of the two-dimensional systems presented in Fig. 5.

The change in mobilities of the nucleotides with increasing phosphate concentration of the developing solution shown in Fig. 4 is sufficient to mobilize higher homologs of purine nucleotides. Using 1.5 *M* potassium phosphate pH 3.4, we have identified adenosine tetra- and pentaphosphates and guanosine tetraphosphate as contaminants in several commercial preparations of ATP and GTP[7]. The mobiliza-

tion of these highly charged molecules suggests that this system may find application in the separation of oligonucleotides from RNA digests.

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