RESOLUTION OF COMPLEX NUCLEOTIDE MIXTURES BY TWO-DIMENSIONAL ANION-EXCHANGE THIN-LAYER CHROMATOGRAPHY*,**

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No rapid analytical method has been described in the literature so far which permits the complete separation of complex nucleotide mixtures containing microgram and submicrogram amounts of individual compounds. Most systems described for two-dimensional paper chromatography of nucleotides require development times of 3 to 4 days. Moreover, the unequivocal detection of these, to some extent very similar, compounds in tissue extracts presents a difficult problem of analysis that cannot be completely solved by the present techniques of paper chromatography and paper electrophoresis. On the other hand, anion-exchange column chromatography is not suitable for the analysis of very small amounts of nucleotides. The complete separation of complex mixtures by this technique requires elution periods of 1 to 10 days.

We feel that a new sensitive and rapid chromatographic technique by which complex nucleotide mixtures can be resolved might be valuable in studies concerning composition and metabolism of nucleotides in bacterial, plant or animal cells. This paper describes such a technique. The separations are carried out by two-dimensional anion-exchange chromatography on PEI-cellulose thin layers. For a discussion of the general behavior of mononucleotides on PEI-cellulose layers see the preceding paper[1].

METHODS

Poly(ethyleneimine) and nucleotides, with the exception of ADPG, were obtained commercially (see the preceding paper[1]). PEI-cellulose layers (capacity about 1.5 mequiv. N per g cellulose) were prepared on 20 × 20 cm glass plates with the Desaga-Brinkmann apparatus***, as described in the preceding paper[1]. Layers prepared from commercial PEI-cellulose powders give separations that are different from those described in this paper.

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** The abbreviations used in this paper are explained in the preceding paper[1].

*** It is also possible to prepare the layers without a spreader by pouring on the PEI-cellulose suspension and distributing it with a glass rod and by gentle tilting. Because such layers, though appearing homogeneous, give somewhat poorer and less reproducible separations, the commercial applicator was used throughout this investigation.

To remove impurities which could interfere with the chromatography in the second direction, each plate is given a preliminary development with 10% NaCl solution instead of with distilled water[1] in the following way. The plate is placed in a rectangular tank with 10% NaCl solution which is allowed to rise to a height of about 5 cm and is then developed without intermediate drying in a second tank with distilled water up to the upper edge. After drying in a current of cold air the plate is washed again with distilled water to the top edge. The plate is then allowed to dry at room temperature for 12–15 h. All plates are stored in darkness in the cold[1].

Chromatographic procedure

First dimension (perpendicular to the coating direction). After applying the nucleotide solution at the starting point S (Fig. 1) the chromatogram is developed in a closed rectangular jar for 2 min with 0.2 *M* LiCl, for 6 min with 1.0 *M* LiCl, and finally with 1.6 *M* LiCl up to a dividing line previously scratched through the layer 13.0 cm above the start (stepwise elution[1]; no intermediate drying). The plate is then dried in a current of warm air below 50°. The development time is about 75 min. It can vary slightly from batch to batch of the cellulose.

Examination of the chromatogram under a short-wave ultraviolet lamp reveals a partial resolution of the complex mixture (Fig. 1).

Second dimension. All parts of the layer which are not needed for the chromatography in the second dimension are scraped off with a sharp spatula. This includes a strip of 2.5–3 cm width below the finishing line of the first dimension.

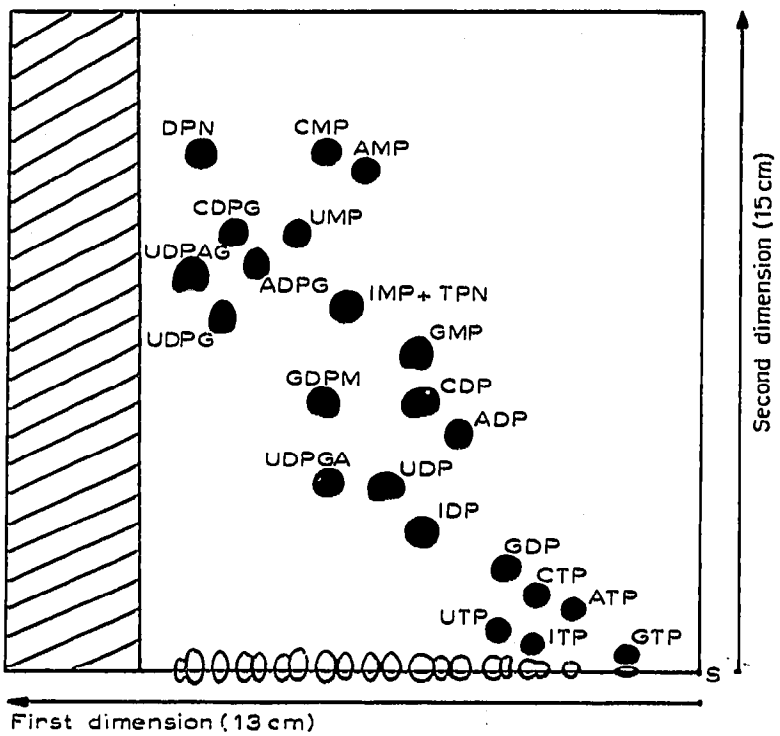

Fig. 1. Two-dimensional anion-exchange thin-layer chromatogram of mononucleotides. 0.5 mm thick PEI-cellulose layer. 0.1 ml of a solution containing 23 ribonucleotides (10–15 mμmoles each) was applied slowly in two 0.05 ml portions with intermediate drying from a micropipette to the layer at S. Elution and detection were carried out as described in the text. The hatched area was removed after the elution in the first dimension (see text).

In order to remove LiCl which would interfere with the elution in the second direction the plate is laid for about 15 min in a flat dish (25 × 25 cm) filled with 1 l anhydrous methanol. The dissolution of the salt is accelerated by occasional agitating.

After drying and scratching parallel lines into the bottom part[1] the chromatogram is developed in the second direction with formic acid–sodium formate buffers, pH 3.4[1], by a stepwise elution procedure. Solvents: 0.5 *M* buffer for 30 sec, 2.0 *M* buffer for 2 min, and 4.0 *M* buffer up to a finish line previously scratched through the layer 15.0 cm above the start of the second dimension. The development time is about 60 min. Finally the plate is dried in a current of hot air and examined under short-wave ultraviolet light.

In case of less complex mixtures the elution procedure can be simplified (see for example Fig. 2). Generally speaking, the duration of each "step" and the composition of the solvents depend on the particular compounds to be separated, see also the preceding paper[1].

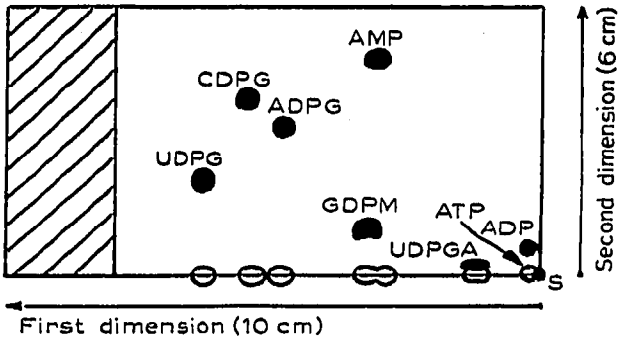

Fig. 2. Two-dimensional anion-exchange thin-layer chromatogram of nucleotide sugars, AMP, ADP, and ATP (8–15 mμmoles each). General procedure as described in the text, but no stepwise elution. The hatched area was removed after the elution in the first dimension. First dimension: 0.35 *M* LiCl, development time 55 min. Second dimension: 0.80 *M* formate buffer (pH 3.4), development time 12 min.

In order to identify a nucleotide X on the map a solution containing an excess of X was run together with the mixture. Only enough of the mixture was applied to the point of origin to make the compounds just detectable on the chromatogram. The presence of X was then apparent from the distinctly greater intensity of its spot compared with those of the other compounds.

In addition, the guanine nucleotides GDPM, GMP, GDP, and GTP were used as markers. When the plate is exposed to hydrochloric acid fumes for several minutes, nucleotides containing guanine fluoresce a brilliant light-blue in short-wave ultraviolet light, whereas derivatives of adenine, hypoxanthine, cytosine, and uracil appear as dark-blue spots.

RESULTS AND DISCUSSION

As is to be seen from Fig. 1, the 23 ribonucleotides applied at S are resolved into 22 distinct spots by the two-dimensional anion-exchange technique. IMP and TPN which are not separated under the conditions used can be resolved by one-dimensional chromatography with solvents containing formic acid, see Tables II and III of the preceding paper[1]. Fig. 2 demonstrates the resolution of a less complex model mixture containing several nucleotide sugars, AMP, ADP, and ATP.

The method is very sensitive: 0.5–2 mμmoles (0.15–1.2 μg) of each compound can be detected with a suitable ultraviolet lamp in a dark room. The sensitivity depends on the specific extinction coefficient of each nucleotide; it is, for example, lower for cytidine than for adenosine nucleotides. On the other hand, the technique can be used for micropreparative separations; mixtures containing 100 mμmoles or more of individual compounds can be resolved by two-dimensional chromatography on PEI-cellulose layers. The completeness of the resolution depends not only on the composition of the mixture being analyzed, but also on the thickness of the layer. For the separation of larger quantities 1–1.5 mm thick layers are superior to the 0.5 mm thick layers used in the present investigation.

With application of the technique described in this paper, it should be possible to detect and isolate unknown nucleotides in extracts from biological materials.

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SUMMARY

Very complex mononucleotide mixtures can be resolved by two-dimensional anion-exchange chromatography on poly(ethyleneimine)-cellulose thin layers. The complete resolution of a model mixture containing DPN, TPN, six nucleotide sugars, and fourteen common nucleoside-5'-mono-, di-, and triphosphates takes less than three hours. This separation cannot be achieved by the present techniques of paper chromatography and paper electrophoresis. The method is more sensitive than paper chromatography and column chromatography of nucleotides.

REFERENCE

1 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.