ION-EXCHANGE CHROMATOGRAPHY OF NUCLEOTIDES ON POLY-(ETHYLENEIMINE)-CELLULOSE THIN LAYERS[*],[**]

K. RANDERATH AND E. RANDERATH

*John Collins Warren Laboratories of the
Huntington Memorial Hospital of Harvard University
and
Biochemical Research Department,
Harvard Medical School
at the
Massachusetts General Hospital,
Boston, Mass. (U.S.A.)*

Although it has clearly been shown by COHN and his collaborators[1],[2] (for reviews see refs. 3 and 4) over ten years ago that for preparative separations of nucleic acid derivatives ion-exchange column chromatography on polystyrene resins is the best method, no effective analytical ion-exchange technique was known until recently by which traces of nucleotides ($0.05-1$ μg) can be separated and identified. Partition paper chromatography (for reviews see refs. 5 and 6) and paper electrophoresis (for reviews see refs. 6 and 7) are also not applicable in case of such small amounts. Because these methods give less sharp resolutions than ion-exchange column chromatography, they cannot be used to analyze very complex nucleotide mixtures in extracts from bacterial, plant and animal cells.

As we could demonstrate[8] (for a review see ref. 9), excellent and rapid separations of very small amounts of nucleic acid derivatives are obtained on ion-exchange thin layers. Chromatography on commercial cellulose ion-exchange papers gives more diffuse substance spots and hence poorer resolutions[10].

During the past three years special finely powdered cellulose cation- and anion-exchange materials have been developed which can be used in thin-layer chromatography. The following cellulose ion-exchangers for thin-layer chromatography

[**] The following abbreviations will be used: PEI-cellulose = a cellulose anion-exchange material obtained by impregnating chromatography cellulose with poly(ethyleneimine); ECTEOLA-cellulose = a cellulose anion-exchange material obtained by treating sodium cellulose with epichlorohydrin and triethanolamine; DEAE-cellulose = diethylaminoethyl-cellulose; QA-cellulose = a cellulose anion-exchange material containing quaternary ammonium groups; P-cellulose = cellulose phosphate; SE-cellulose = sulfoethyl-cellulose; CM-cellulose = carboxy-methyl-cellulose; PP-cellulose = cellulose cation-exchange material containing polyphosphate. AMP, GMP, IMP, CMP, UMP = adenosine-, guanosine-, inosine-, cytidine-, and uridine-5'-mono-phosphates; ADP, GDP, IDP, CDP, UDP = the corresponding diphosphates; ATP, GTP, ITP, CTP, UTP = the corresponding triphosphates. The prefix d- indicates that the compound is a deoxyribonucleotide. TMP = thymidine monophosphate; TTP = thymidine triphosphate. ADPG = adenosine diphosphate-glucose; GDPM = guanosine diphosphate-mannose; CDPG = cytidine diphosphate-glucose; UDPG = uridine diphosphate-glucose; UDPGA = uridine di-phosphate-glucuronic acid; UDPAG = uridine diphosphate-N-acetyl-glucosamine.

are at present commercially available: ECTEOLA-cellulose[*,**,***], DEAE-cellulose[*,**,***,§], PEI-cellulose[*,**], and QA-cellulose[§] (anion-exchangers); P-cellulose[*,**,***], SE-cellulose[*,§], and CM-cellulose[*,**,***,§] (cation-exchangers). PP-cellulose[**], a strongly acid cation-exchanger which is obtained by treating cellulose anion-exchange materials of low capacity with a polyphosphate solution[11], is suitable for separating nucleic acid bases, nucleosides, and other cationic compounds.

Ion-exchange thin-layer chromatography is more sensitive than partition paper chromatography, ion-exchange paper chromatography, paper electrophoresis, and partition thin-layer chromatography on unmodified cellulose layers. Amounts of 0.1 μg or less of nucleic acid bases, nucleosides, and nucleotides can be detected by examining the ion-exchange thin-layer chromatograms under a suitable short-wave ultraviolet lamp[§§] in a dark room.

Thin-layer chromatography of nucleotide coenzymes and of constituent nucleotides of nucleic acids can be carried out on layers of silica gel[8,12], unmodified cellulose[13-15], ECTEOLA-cellulose[8,9], DEAE-cellulose[9,16-18], and PEI-cellulose[9,10,19,20]. Also the separation on DEAE-Sephadex thin layers of a mixture of 5'-AMP, ADP and ATP has been reported[21]. For reasons which have been mentioned elsewhere[9], partition thin-layer chromatography of nucleotides on inorganic layers (silica gel, aluminum oxide) cannot be recommended. Layers of unmodified cellulose give sharper separations than chromatography paper[14]. They can be used for the resolution of not too complex mixtures. Many solvents developed for paper chromatography of nucleic acid derivatives are suitable in cellulose thin-layer chromatography[9,13-15].

As we showed earlier[11,22], cellulose ion-exchange materials can be prepared by treating unmodified or modified celluloses with high-molecular-weight basic or acid compounds. Anion-exchange materials which give especially sharp separations in thin-layer chromatography[9,10,19,20] are obtained[22] by impregnation with poly(ethyleneimine) (molecular weight 30,000–40,000) which is fixed substantively on cellulose fibers[23].

The separation of a number of mono-[9,20] and oligonucleotides[10,19] on PEI-cellulose thin-layers has been described previously. The present paper gives a detailed description of the behavior on PEI-cellulose layers of ribo- and deoxyribomononucleotides. The layers are prepared by suspending unmodified cellulose powder for thin-layer chromatography in a poly(ethyleneimine) hydrochloride solution and coating the suspension on glass plates in the usual manner[§§§].

MATERIALS AND METHODS

Materials

ADPG was a kind gift of Prof. L. F. LELOIR, Buenos Aires, to Prof. H. M. KALCKAR, Boston. D-Arabinosyl CMP, D-arabinosyl CDP, and D-arabinosyl CTP were kindly

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provided by Prof. S. S. COHEN, Philadelphia. The other nucleotides were commercial products obtained from Sigma Chemical Company, St. Louis, and from California Corporation for Biochemical Research, Los Angeles.

A 50 % (w/v) solution of poly(ethyleneimine) (free base) in water, manufactured by Badische Anilin- und Soda-Fabrik, Ludwigshafen, Germany, was obtained from the Chemirad Corporation, East Brunswick, N.J. All solvents were prepared from analytical reagent grade materials.

Preparation of plates

A dialyzed 1 % poly(ethyleneimine) hydrochloride solution is prepared from a 10 % poly(ethyleneimine) hydrochloride solution (pH about 6.0) as described earlier[9]. A suspension of unmodified cellulose powder for thin-layer chromatography[9] (30 g) in the dialyzed solution (200 ml) is homogenized in an electric mixer for about 30 sec. Approximately 0.5 mm thick layers are then prepared on degreased glass plates (10 × 20 or 20 × 20 cm) by means of the Desaga-Brinkmann applicator model S II or by means of the Camag-Thomas apparatus, see ref. 9. In case of narrow plates (5 × 20 or 10 × 20 cm) the Serva applicator[9] can be used.

In order to avoid edge effects the plates should be separated from each other immediately after coating. They are allowed to dry overnight on a horizontal support at room temperature. The resulting layers are mechanically very stable so that one can write on them with pencil or ball-point. They have a capacity of approximately 1.5 mequiv. N per g cellulose.

Layers of lower capacity (< 1 mequiv. N per g cellulose) can be prepared without previous dialysis of the poly(ethyleneimine) solution in the following manner. 50 % poly(ethyleneimine) solution (10 g) is diluted with distilled water (700 ml), brought to pH 6 with concentrated hydrochloric acid, and finally made up with distilled water to 1 l. Cellulose powder MN 300 (30 g) is suspended in the poly(ethyleneimine) solution (200 ml). Subsequently the layers are prepared according to the procedure described above. These layers have a capacity of 0.7–0.8 mequiv. N per g cellulose.

In order to obtain a straight solvent front dividing lines are scratched through the layer at a distance of 4–5 mm from the edges, and parallel lines are scratched into the bottom parts of the plates as described earlier[9]. Each plate is given a preliminary ascending development with distilled water[9,20]. The plates are then allowed to dry at room temperature for at least 12 h. Drying at elevated temperature is not advisable.

If the plates are not used within a few days they should be stored in darkness in the cold (0–4°) where they can be kept for several months. Whereas poly(ethyleneimine) solutions are stable, suspensions of cellulose in poly(ethyleneimine) solutions and PEI-cellulose layers can be kept for only a limited period at room temperature.

Chromatography

The samples are applied on a starting line drawn with a soft pencil 3.0 cm from the lower edge of the plate. 0.002 *M* solutions of sodium or lithium salts of nucleotides in distilled water were used throughout this investigation. After spotting all plates are exposed to a current of cold air for about 3 min.

Ascending chromatography is carried out in closed tanks filled with solvent to a height of 0.7–1.0 cm. Chromatography in open vessels gives results which are only slightly different from those described in this paper.

For the determination of R_F values 1 μl of the stock solutions (2 mμmoles) was spotted on layers impregnated with a dialyzed 1 % poly(ethyleneimine) solution. All chromatograms were developed up to a dividing line previously scratched through the layer 10.0 cm above the starting line. The elution was carried out perpendicular to the coating direction. The development time was 40–65 min depending on the composition of the solvent. Subsequently the plates were dried in a stream of hot air. The compounds were located by examining the plates in incident short-wave ultraviolet light and marked on the plate with a pencil.

Recording the chromatograms by photography in incident ultraviolet light

In order to obtain permanent records the chromatograms can be photographed in incident ultraviolet light, *cf.* Fig. 3. This photograph was obtained in the following way. A Polaroid MP-3 Land Camera (Polaroid Corporation, Cambridge 38, Mass.) was erected vertically over the chromatogram at a distance of 40–50 cm. An ultraviolet filter (Kodak daylight filter for type F color films No. 85 C) was placed in front of the lens (Rodenstock-Ysaron 1:4.7, $f = 127$ mm). Two short-wave ultraviolet lamps (Mineralight Model No. R 51), mounted at a distance of about 20 cm from the plate, were used to give even illumination in an otherwise completely darkened room. The exposure was 3 sec (lens opening f: 8; PolaPan 200 type 52 film). The conditions will of course depend on the properties of the ultraviolet lamps used.

Removal of interfering salts

Although generally speaking ion-exchange chromatography is less sensitive than partition chromatography to the presence of salts in the samples to be analyzed, a large excess of undesirable anions does interfere seriously with the separations on PEI-cellulose layers (see Figs. 5 and 6). Interfering salts are best removed by adsorption and subsequent elution of the nucleotides from activated charcoal (see *e.g.* refs. 24 and 25). A drawback of this technique is that recovery of nucleotides, especially in case of compounds containing guanine[25], is not quantitative. A further possibility is as follows. The samples are spotted in the usual manner, and after drying in a stream of air the plates are laid in a flat dish for about 10 min with anhydrous reagent grade methanol (300–600 ml). Subsequently the layers are dried, and chromatography is carried out as described above. This treatment removes excess salts, whereas in general more than 90 % of the nucleotides remain on the points of origin (see Figs. 5 and 6).

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RESULTS AND DISCUSSION

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General behavior of mononucleotides on PEI-cellulose layers

In Figs. 1 and 2 the R_F values of various derivatives of adenine and uracil are plotted against the lithium chloride concentration of the solvent used for elution. R_F values higher than 0.8 are not given because compounds migrating close to the solvent front have a tendency to form elongated spots so that exact R_F values cannot be measured. As can be seen from Figs. 1 and 2, the migration rate of nucleotides depends on the electrolyte concentration of the solvent in a way characteristic for each compound.

In general, the slope of the curves of pyrophosphoric acid diesters (I) (*e.g.*, ADPG, TPN) is steeper than that of monoesters of phosphoric, pyrophosphoric, or
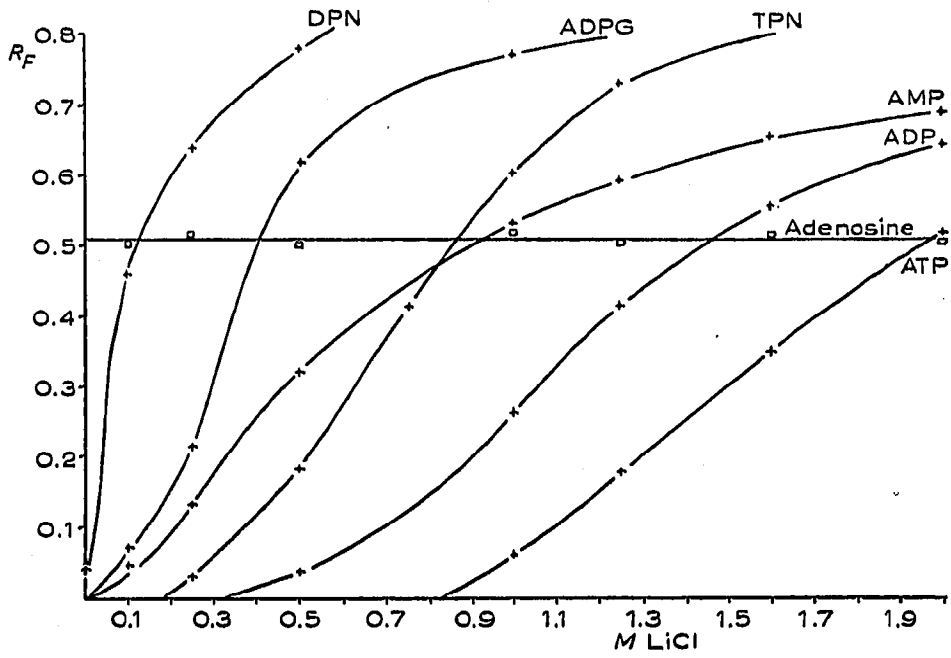
Fig. 1. Relationship between R_F values of adenine compounds and LiCl concentration of the solvent. Chromatography on 0.5 mm thick PEI-cellulose layers as described in the text.
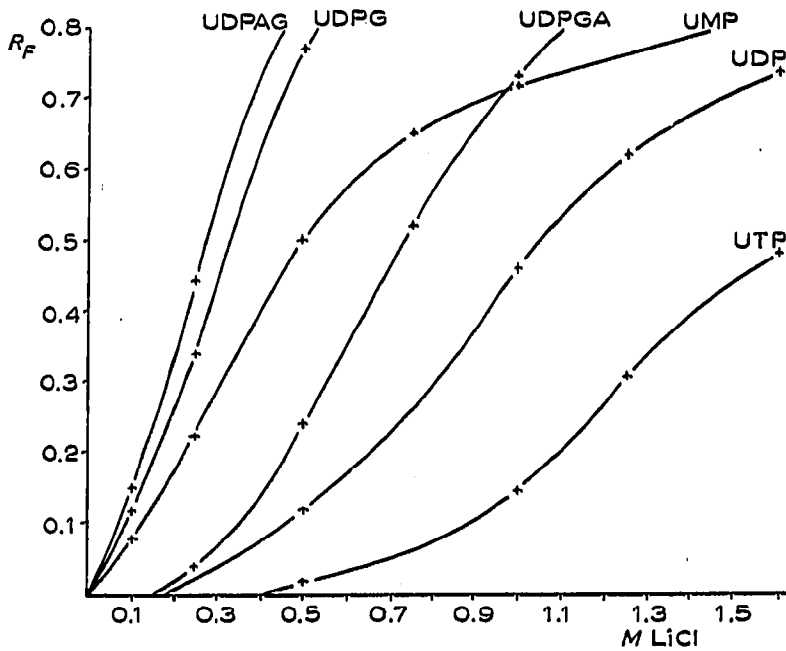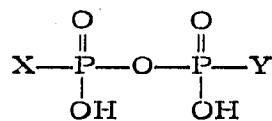
Fig. 2. Relationship between R_F values of uracil compounds and LiCl concentration of the solvent. 0.5 mm thick PEI-cellulose layers.

triphosphoric acid (e.g., AMP, ADP, ATP). In some cases (see Figs. 1 and 2) a crossing of monoester and diester curves can be noticed.

$$X—\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}—O—\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}—Y$$

(I)

The reproducibility of the R_F values is good if they are measured on plates obtained with a single poly(ethyleneimine) preparation. Slight variations (up to about $\pm$ 0.05) are observed if different poly(ethyleneimine) preparations are compared. The spot pattern, however, remains the same in each case.

In contrast to the nucleotides, R_F values of nucleic acid bases and nucleosides do not depend on the salt concentration of the solvent. This is correct for salt concentrations up to about 2 M NaCl or LiCl, see the data given for adenosine in Fig. 1. Uridine (not shown in Fig. 2) migrates with a R_F value of about 0.85 throughout the concentration range given in Fig. 2. At very high salt concentrations a decrease of the R_F values of some bases and nucleosides is observed. This is presumably a salting out phenomenon.

Figs. 1 and 2 show that it is possible to separate basic (or neutral) substances from nucleotides (or other acid substances) by a preliminary development with distilled water. Under these conditions all nucleotides, with the exception of DPN (Fig. 1), remain at the origin, whereas all water-soluble basic or neutral compounds migrate. The second development with electrolyte solutions for separating the nucleotides can be carried out in the direction of the first development or, preferably, perpendicular to it.

In Tables I–IV are listed the R_F data of a number of naturally occurring ribo- and deoxyribomononucleotides. It is apparent from these data that, under a given set of eluting conditions, the behavior of each nucleotide is influenced by the base, the sugar, and the phosphate residue of the molecule.

Elution under neutral conditions

At neutral pH (Table I and Figs. 1 and 2) the rate of migration decreases in the order: nucleotide sugars* (+ DPN) > monophosphates (+ TPN) > diphosphates > triphosphates containing the same base. Differences in net charge form the principal basis for this separation.

The order of elution predicted by net charge considerations is, however, subject to modifications. For example, the great rate of migration of the sugar nucleotide group cannot be explained by net charge considerations alone, since both sugar nucleotides (e.g., UDPG) and nucleoside monophosphates (e.g., UMP) carry a net charge of —2 at neutral pH. Furthermore, although the bases carry no net positive charge at neutral pH[3], the rate of migration in each group decreases in the order: uracil > cytosine > hypoxanthine > adenine > guanine derivatives.

Size, physical properties, and arrangement of nonionic substituents seem to in-

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* Oxidation of a nucleotide sugar (e.g., UDPG) to the corresponding uronic acid (UDPGA) results in a decreased migration rate, see also Fig. 3.

fluence the strength of the bonds established between compound and adsorbent. For example, one has to assume that purine compounds are adsorbed to the impregnated cellulose ion-exchanger more strongly than pyrimidine compounds of the same structural type, see also Fig. 3. Moreover, the net surface charge density on the molecule which is determined by the spatial arrangement of ionizable groups can be expected to be an important factor.

Elution under acid conditions

At pH 3.4 (Table II) the rate of migration decreases in the order: monophosphates (+ DPN) > nucleotide sugars (+ TPN) > diphosphates > triphosphates, and

TABLE I

R_F VALUES OF NUCLEOTIDES AT NEUTRAL pH

Compound	Solvent: LiCl in water		
	0.25 M	1.0 M	1.6 M
5'-AMP	0.11	0.52	0.65
5'-IMP	0.13	0.59	0.74
5'-GMP	0.06	0.40	0.51
5'-CMP	0.15	0.64	0.75
5'-UMP	0.20	0.74	0.80
ADP	0.00	0.26	0.54
IDP	0.00	0.30	0.63
GDP	0.00	0.17	0.45
CDP	0.00	0.33	0.64
UDP	0.00	0.41	0.71
ATP	0.00	0.06	0.34
ITP	0.00	0.09	0.39
GTP	0.00	0.05	0.25
CTP	0.00	0.11	0.41
UTP	0.00	0.14	0.49
d-AMP	0.11	0.52	—
d-GMP	0.06	0.41	—
d-CMP	0.18	0.65	—
TMP	0.24	0.74	—
d-ATP	0.00	—	0.35
d-GTP	0.00	—	0.26
d-CTP	0.00	—	0.43
TTP	0.00	—	0.52
DPN	0.64	0.80	> 0.80
DPNH	0.20	0.71	—
TPN	0.03	0.60	—
TPNH	0.00	0.34	—
ADPG	0.22	0.77	> 0.80
GDPM	0.12	0.72	> 0.80
CDPG	0.27	> 0.80	> 0.80
UDPG	0.34	> 0.80	> 0.80
UDPAG	0.44	> 0.80	> 0.80
UDPGA	0.04	0.73	—

— = not investigated.

TABLE II

R_F VALUES OF NUCLEOTIDES AT pH 3.4

Compound	Solvent: formic acid–sodium formate buffer pH 3.4			
	0.5 M	1.0 M	2.0 M	4.0 M
5'-AMP	0.68[a]	> 0.80	> 0.80	> 0.80
5'-IMP	0.40	0.60	0.73	> 0.80
5'-GMP	0.28	0.45	0.57	0.65[b]
5'-CMP	0.70[a]	> 0.80	—	—
5'-UMP	0.51	0.72	> 0.80	—
ADP	0.03	0.10	0.32	0.75[b]
IDP	0.00	0.04	0.14	0.49
GDP	0.00	0.02	0.09	0.34
CDP	0.08	0.20	0.45	> 0.80
UDP	0.02	0.07	0.24	0.60
ATP	0.00	0.00	0.04	0.24
ITP	0.00	0.00	0.02	0.11
GTP	0.00	0.00	0.00	0.07
CTP	0.00	0.02	0.05	0.29
UTP	0.00	0.00	0.02	0.17
DPN	0.68[a]	> 0.80	> 0.80	—
TPN	0.14	0.43	> 0.80	—
ADPG	0.30	0.57	> 0.80	—
GDPM	0.07	0.18	0.51	—
CDPG	0.45	0.67	> 0.80	—
UDPG	0.17	0.39[b]	0.77	—
UDPAG	0.32	0.55[b]	> 0.80	—
UDPGA	—	0.03	—	—

— = not investigated.
[a] Spot in second front.
[b] Elongated spot.

cytosine > adenine > uracil > hypoxanthine > guanine derivatives. At this pH the primary phosphoric acid groups are fully dissociated, and the secondary groups are totally undissociated. That monophosphates (e.g., UMP) precede nucleotide sugars (e.g., UDPG) under these conditions can be predicted from net charge considerations: because uracil carries no positive charge at pH 3.4, the net negative charges of UMP and UDPG are —1 and —2, respectively. However, the rather great differences between nucleotide sugars and nucleoside diphosphates (Table II) are not due to charge differences. The net charge of both UDPG and UDP is —2 at pH 3.4.

Since the pK_a values of hypoxanthine, uracil, guanine, adenine, and cytosine are different[3], the net charge on the individual nucleotides within each group is different. The order cytosine > adenine > guanine is predicted by net charge considerations. The relatively slow migration rates of hypoxanthine and guanine derivatives must, however, be explained by the greater attraction of the poly(ethyleneimine)-cellulose for these purines over the pyrimidines.

Good separations are obtained at pH 4.4 (R_F values not given). Acetic acid–sodium acetate buffers, with or without addition of lithium chloride, can be used as solvents. In this pH region uracil derivatives precede adenine derivatives of the same type.

At pH 2 (Table III) the rate of migration with regard to the bases is cytosine > adenine > guanine > uracil > hypoxanthine derivatives of the same type.

In general, smaller and sharper spots are obtained at acid pH values than under neutral or alkaline conditions. 1–3 N formic acid gives particularly sharp group separations. The addition of LiCl (Table III) increases the eluting power considerably.

TABLE III

R_F VALUES OF NUCLEOTIDES AT pH 2

Solvent 1 = 1.0 N HCOOH.
Solvent 2 = 2.0 N HCOOH–0.5 M LiCl (1:1).
Solvent 3 = 2.0 N HCOOH–2.0 M LiCl (1:1).

Compound	Solvent		
	1	2	3
5′-AMP	> 0.80	> 0.80	> 0.80
5′-IMP	0.19	0.53	0.78
5′-GMP	0.41	0.50[a]	0.72[a]
5′-CMP	> 0.80	> 0.80	> 0.80
5′-UMP	0.20	0.64	> 0.80
ADP	0.03	0.29	0.70
IDP	0.00	0.08	0.55
GDP	0.00	0.13	0.61
CDP	0.04	0.35	0.73
UDP	0.00	0.11	0.60
ATP	0.00	0.04	0.33
ITP	0.00	0.02	0.17
GTP	0.00	0.02	0.24
CTP	0.00	0.04	0.37
UTP	0.00	0.02	0.20
DPN	> 0.80	> 0.80	> 0.80
TPN	0.10	0.44[a]	> 0.80
ADPG	0.09	0.51[a]	> 0.80
GDPM	0.01	0.34	0.73
CDPG	0.13	0.60	> 0.80
UDPG	0.01	0.27[b]	> 0.80
UDPAG	0.02	0.38[b]	—
UDPGA	0.00	0.09	0.66

— = not investigated.
[a] Spot in second front.
[b] Elongated spot.

The formation of so-called second fronts is sometimes observed in the chromatography with acid solvents. These second fronts correspond to sudden pH changes within the layer, as can be detected by spraying the plates with suitable indicator solutions.

Because they do not interfere seriously with the chromatography, equilibration of the layers with solvent prior to chromatography is seldom necessary. It must be mentioned, however, that anomalies of R_F values can be observed sometimes if nucleotides migrate with, or close to, a second front. An example is the R_F value of GMP determined after chromatography with HCOOH–LiCl mixtures, see Table III.

Separation of deoxyribo- and D-arabinosylnucleotides from ribonucleotides

As shown in Table IV, deoxyribonucleotides can be separated from their ribonucleotide-analogues on PEI-cellulose layers by chromatography with solutions of LiCl in aqueous boric acid[20]. Under the conditions indicated in Table IV some ribonucleotide spots appear slightly elongated. This might be due to a partial dissociation of the borate complexes formed by these compounds.

TABLE IV

R_F VALUES OF NUCLEOTIDES IN THE PRESENCE OF BORIC ACID

Solvent 1 = 2 % H_3BO_3–2 M LiCl (2:1).
Solvent 2 = 4 % H_3BO_3–4 M LiCl (4:3).

Compound	Solvent	
	1	*2*
AMP	0.24	—
d-AMP	0.42	—
GMP	0.08	—
d-GMP	0.30	—
CMP	0.28	—
d-CMP	0.55	—
UMP	0.37	—
TMP	0.63	—
ADP	0.04	0.56
ATP	—	0.33
d-ATP	—	0.46
GTP	—	0.17
d-GTP	—	0.37
CTP	—	0.36
d-CTP	—	0.61
UTP	—	0.48
TTP	—	0.70

— = not investigated.

Also D-arabinosyl CMP (II), D-arabinosyl CDP, and D-arabinosyl CTP are separated from their ribo- and deoxyribo-analogues with solvents containing boric
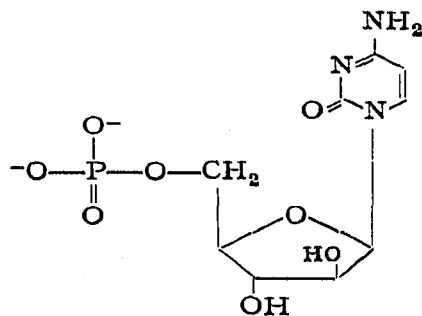
(II)

acid. The rate of migration decreases in the order deoxyribo- > D-arabinosyl- > ribonucleotides of the same structural type.

Stepwise elution

As can be seen from Fig. 1, a mixture of DPN, TPN, ADPG, AMP, ADP, and ATP cannot be resolved with one and the same solvent, since the adsorption affinities of these compounds for PEI-cellulose are too different. In such a case the chromatogram is developed with a series of solvents of increasing electrolyte concentrations. In Fig. 3 examples are given showing separations of adenine and uracil nucleotide mixtures. The discontinuous, stepwise procedure used for elution comprised three transfers of the plate without intermediate drying from one tank to another tank containing a higher lithium chloride concentration.

When compared with constant concentration elution, development with increasing concentrations gives sharper and more circular substance spots.
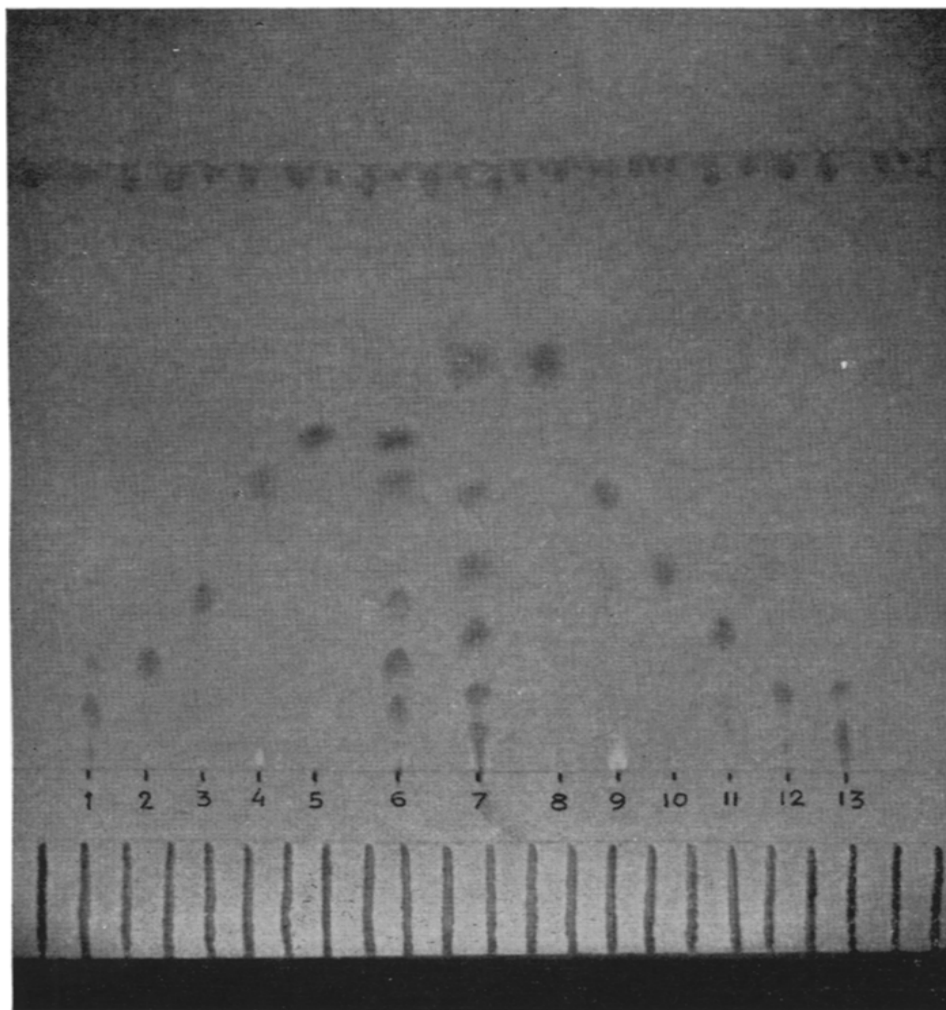

Fig. 3. Separations of adenine and uracil nucleotides on a 0.5 mm thick PEI-cellulose layer. The plate was developed (stepwise elution, see text) for 1 min with 0.1 M LiCl, for 5 min with 0.3 M LiCl, for 15 min with 0.7 M LiCl, and for 25 min with 1.5 M LiCl. Development distance: 9.3 cm in 46 min. Amounts applied: 5–10 mμ moles of each compound. 1 = UTP; 2 = UDP; 3 = UDPGA; 4 = 5'-UMP; 5 = UDPG; 6 = 1–5 together; 7 = 8–13 together; 8 = DPN; 9 = ADPG; 10 = 5'-AMP; 11 = TPN; 12 = ADP; 13 = ATP. Some impurities are visible, for example, ADPG contained a small amount of AMP, and ATP contained ADP and an unidentified compound (close to the start). Photographed by short-wave U.V. light. To increase the contrast, the plate was soaked in anhydrous methanolic 0.002 % fluorescein solution for $1^{1}/_{2}$ min and dried before photography.

The choice of the solvent depends on the composition of the mixture to be analyzed: for example, if the mixture contains only nucleotide sugars and nucleoside monophosphates, LiCl molarities between 0.1 and 1.0 are suitable. On the other hand, if a mixture of nucleoside di- and triphosphates is to be analyzed, the concentration range would be between 0.6–0.8 M for the first solvent and 1.7–2.0 M for the last solvent. The number of "steps" sufficient in most cases is 2–5.

Influence of the poly(ethyleneimine) concentration

Fig. 4 shows that the chromatographic results depend on the poly(ethyleneimine) concentration of the solution used for preparing the plates. In general, lowering of the concentration results in larger, more elongated spots and in higher R_F values. Whereas a 0.5 % (w/v) poly(ethyleneimine) solution gives good and for many purposes sufficient separations, the resolution becomes very poor if a 0.1 % solution is used (Fig. 4).
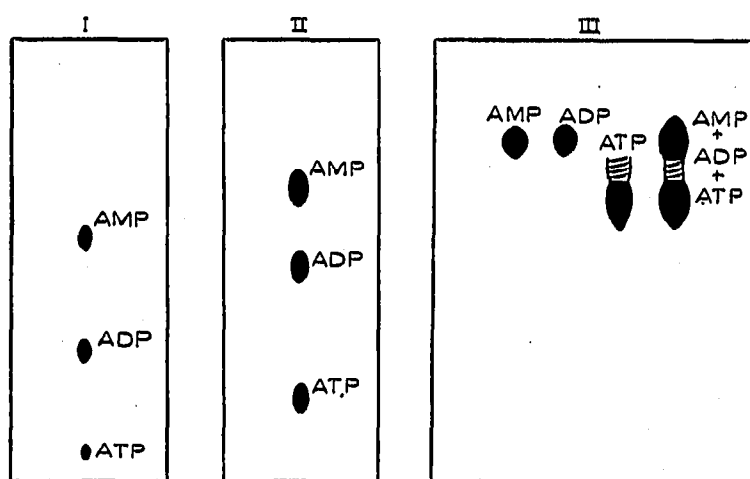

Fig. 4. Influence of poly(ethyleneimine) content (capacity). Chromatography with 1.0 M LiCl. The layers (0.5 mm thick) were prepared with dialyzed 1 % (I), 0.5 % (II) and 0.1 % (III) poly-(ethyleneimine) hydrochloride solutions.

As mentioned in the section *Preparation of plates*, layers of a capacity < 1 mequiv. N per g cellulose can be prepared without previous dialysis of the poly-(ethyleneimine) solution. Good separations are obtained on these layers; spot size and R_F values are similar to those observed with a 0.5 % dialyzed solution (Fig. 4) if an undialyzed solution of the same concentration is used, see section *Preparation of plates*. However, the poly(ethyleneimine) concentration should not exceed 0.7 % (undialyzed) and 1.5 % (dialyzed), because otherwise it is difficult to remove low-molecular-weight impurities which are incorporated in the layer.

Interference by salts

An excess of salts or buffers in the samples to be analyzed can interfere seriously with the separations (Figs. 5 and 6). By the methanol treatment described above (see section *Removal of interfering salts*) excess electrolytes are removed. A separation of AMP, ADP, and ATP can be obtained even if the sample contains a very large excess of sodium chloride (Fig. 5b). As shown in Figs. 5 and 6, slightly higher R_F values and more elongated spots are observed on plates treated with methanol.
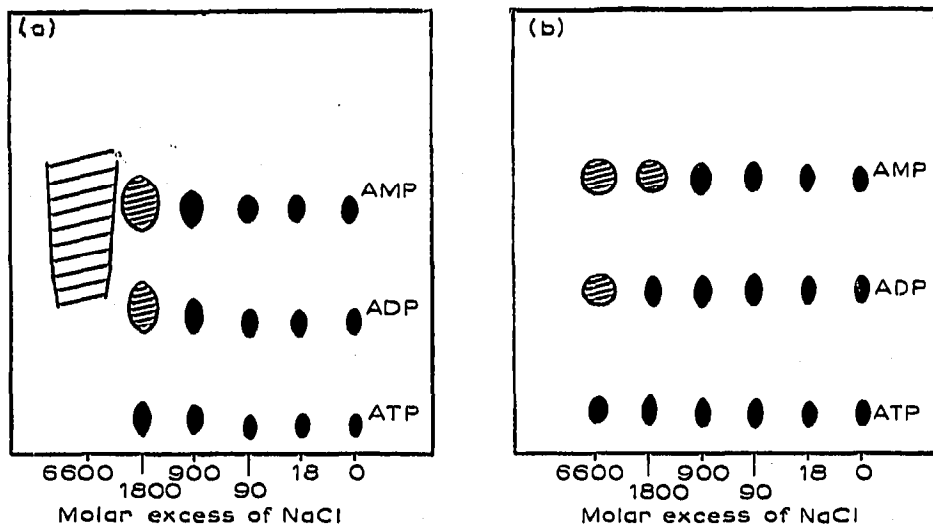
Fig. 5. Effect of interfering electrolytes. Chromatography with 1.0 M LiCl on 0.5 mm thick PEI-cellulose layers. a = without methanol treatment; b = with methanol treatment as described in the text.

CONCLUSIONS

In Table V the various methods for separating nucleotides are compared. When compared with paper chromatography[5] and paper electrophoresis[7] of nucleotides, the following advantages of ion-exchange thin-layer chromatography are evident:

1. Thin-layer chromatography is more sensitive than paper chromatography and paper electrophoresis[9]. Especially in biochemical analysis, where often only traces of the compounds being studied are available, the more than tenfold decrease in the scale of analysis is a particularly important advantage. The sensitivity is further increased if the compounds are labeled and the detection is carried out by autoradiography[27] or by scanning in a suitable instrument[9].
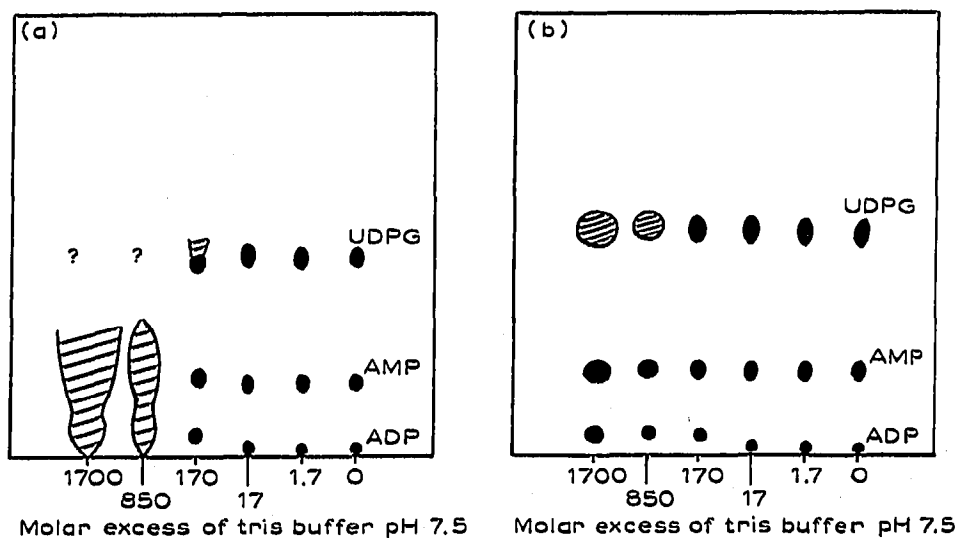

Fig. 6. Effect of interfering electrolytes. Solvent: 0.3 M LiCl. a = without methanol treatment; b = with methanol treatment as described in the text.

TABLE V

COMPARISON BETWEEN DIFFERENT METHODS FOR SEPARATION OF NUCLEOTIDES

Method	Optimum amounts	Type	Time required	Resolution of complex mixtures[a]
Paper chromatography	10–200 μg per spot	Partition (ion-exchange)	12 h–5 days	Moderate
Paper electrophoresis	100–500 μg per spot	Ionophoresis	Several h	Moderate
Column chromatography	50 μg–several hundred mg per column	Ion-exchange	Several h–10 days	Very good
Thin-layer chromatography	0.2–30 μg per spot	Ion-exchange (partition)	Several min–3 h	Very good

[a] See the following paper[28].

2. Complex mixtures of nucleotides that cannot be resolved at all by the present techniques of paper chromatography and paper electrophoresis can be separated by anion-exchange thin-layer chromatography[28].

3. The time required for separation is very short[9] (Table V).

4. Ion-exchange thin-layer chromatography can be carried out under milder conditions than paper chromatography and paper electrophoresis, sharp separations being obtained even at 0° (ref. 9). There is thus scarcely any danger of decomposition of sensitive nucleotides.

It should be kept in mind that the unequivocal identification of nucleotides is greatly facilitated by using anion-exchange thin-layer chromatography in conjunction with other methods which are based on different separation principles.

A comparison with anion-exchange column chromatography on polystyrene resins (see refs. 1–4, 25, 29) shows:

1. Both ion-exchange methods give nearly identical elution patterns at the same pH. The electrolyte concentrations required to obtain identical mobilities of individual compounds are, however, different.

2. The thin-layer chromatographic procedure results in an at least equal sharpness of resolution.

3. Thin-layer chromatography is less laborious than ion-exchange column chromatography and is therefore especially suitable for micropreparative separations (amounts of up to 2 mg per substance on a 20 × 20 cm plate). The nucleotides can be eluted with electrolyte solutions after removing the substance zones from the glass plates[9]. As in column chromatography, activated charcoal is used to reclaim the nucleotides from the eluting electrolytes.

4. In both methods a quantitative determination can be carried out by ultraviolet spectrophotometry[9]. A more detailed description of quantitative evaluation of the PEI-cellulose plates will be given in the near future.

The most important advantages of thin-layer chromatography on PEI-cellulose are excellent sharpness of separation, good reproducibility, high sensitivity, and great speed. Furthermore, the regular behavior of the nucleotides facilitates the identification of unknown compounds.

The high resolving power of the impregnated cellulose anion-exchange material is, in our opinion, to be attributed to its (a) high capacity, (b) great density of functional groups along the poly(ethyleneimine) chain, and (c) lack of cross linkage resulting in a high rate of the ion-exchange process.

SUMMARY

A great number of naturally occurring mononucleotides can be separated and identified by poly(ethyleneimine)-cellulose thin-layer chromatography. R_F data for 33 compounds are given, and the factors are discussed which influence the mobility under different elution conditions. The method is compared with other present techniques for separating nucleotides.

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