THIN-LAYER CHROMATOGRAPHY OF NUCLEIC ACID BASES, NUCLEOSIDES, NUCLEOTIDES AND RELATED COMPOUNDS

IV. SEPARATION ON PEI-CELLULOSE LAYERS USING GRADIENT ELUTION AND DIRECT FLUOROMETRY OF SPOTS*,**

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In a previous communication[1] a method was described for the characterization of more than sixty nucleo-derivatives and related compounds on cellulose thin layers. This technique is especially suitable for the detection of nucleo-derivatives in biological material. However, the separation of some groups of nucleotides is poor; nucleoside tri- and diphosphates show some tailing. Fortunately nucleotides can be separated very sharply on PEI-cellulose layers according to RANDERATH[2–4]. For the chromatography of nucleotides on PEI-cellulose layers stepwise elution with different solvents has been used[3–6] (for a review, see ref. 7). The quantitative estimation of these compounds, using a micro-technique, has also been described[6].

It was felt that gradient elution[8] may be useful for the chromatography of nucleo-derivatives on PEI-cellulose layers, when separation and detection of nucleic acid bases, nucleosides and nucleotides on the same plate is required. Since direct fluorometry of spots was found to be suitable for the quantitative analysis of compounds, *e.g.* nucleo-derivatives[9,10] and amino acids[11,12], resolved on silica gel or on cellulose layers, there is no doubt that this method can also be used for direct scanning of spots on PEI-cellulose chromatograms.

In this communication we would like to describe some results concerning gradient elution chromatography of nucleo-derivatives on PEI-cellulose layers. Additionally the direct fluorometry of these compounds will also be reported.

EXPERIMENTAL

Preparation of sheets

RANDERATH's method[13] was used with slight modifications. Plastic sheets (Type DC, GA 1190, Galenopharm, Geneva, Switzerland) 20 × 130 cm are cleaned

* For Part III of this series, see ref. 1.
** 6th communication on "Quantitative Thin-Layer Chromatography". 5th communication, see ref. 12.

and fixed on the plastic holder of the applicator (Desaga, Heidelberg, Germany) using scotch tape. A suspension of 22 g MN-300-cellulose (*Procedure a*) or 22 g purified MN-300-cellulose[1,10] (*Procedure b*) in 145 ml of a 1 % PEI-solution (cellulose powder supplied by Macherey, Nagel & Co., Düren, Germany, preparation of PEI-solution according to RANDERATH[13]) is homogenized for 1 min in an electric mixer. After stirring for about 1 min in a beaker to remove air bubbles, the suspension is placed in the applicator, the sheet coated immediately (slot width 0.5 mm) and allowed to dry overnight at room temperature.

Pretreatment of sheets

Ascending chromatography on the sheets (20 × 20 cm sheets are fixed with a rubber band on glass plates) is carried out in Shandon multiplate-chromatotanks (Shandon, London). In order to avoid loss of layer and to remove the impurities, filter paper strips (*e.g.* prefolded paper strips for BN-chamber, Desaga, Heidelberg Germany) are attached to the top and to the bottom. The sheets are developed with 10 % NaCl for 5 cm, and then, without intermediate drying, with distilled water up to 20 cm. After drying for about 3 h the sheets are redeveloped with water for 20 cm. After drying overnight at room temperature they are ready for use. For quantitative estimations it is necessary to remove impurities as far as possible. It is therefore advisable to examine the sheets in U.V. light before use, and if there are still visible impurities, another development with water should be carried out. *All runs are made perpendicular to the coating direction.*

Preparation of plates

RANDERATH's method[2–4] has been used with slight modifications. 100 g Polymin P (Badische Anilin- und Sodafabrik, Ludwigshafen, Germany) and 250 ml distilled water are stirred, neutralized with conc. HCl to pH 6, and made up to 500 ml with distilled water. 25 ml of this solution is dialyzed against 4 l distilled water (using a magnetic stirrer). After 24 h, the dialyzed solution is made up to 250 ml. 40 g purified MN-300-cellulose[1,10] (*Procedure c*) and the dialyzed PEI-solution are homogenized in an electric mixer for 1 min. The slurry is spread over 10 glass plates, 20 × 20 cm (slot width 0.5 mm) using *e.g.* STAHL's applicator (Desaga, Heidelberg, Germany), followed by drying overnight at room temperature.

Pretreatment of plates

In order to obtain an even solvent front it is advisable to remove a 0.5 cm strip from both edges of the plate. To avoid cracking it is also advisable to scratch lines into the layer as described by RANDERATH[14]. In addition, the attachment of filter paper strips to the top and the bottom is also necessary (*cf.* pretreatment of sheets).

Ascending chromatography, drying, etc. is made as described under "Pretreatment of sheets", however, *all runs should be in the coating direction.* Before spotting and chromatography all parts of the layer into which lines were scratched are scraped off.

Chromatography

For chromatography, a sandwich type chamber (BN-chamber, Desaga, Heidelberg, Germany) cooled with tap water and combined with a device for gradient elution previously described[8] is used. A similar apparatus for gradient elution thin-layer

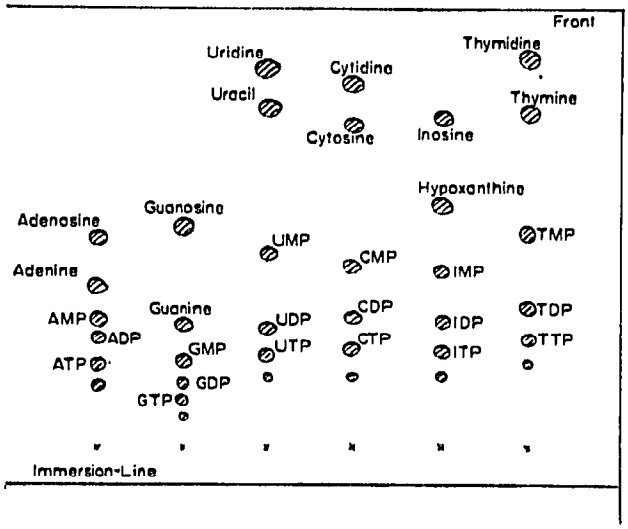
chromatography is now available (Desaga, Heidelberg, Germany). Total volume of the capillary, 4 ml; length, 276 cm, inner diameter, 1.5 mm. In this study we have used mainly the following solvent systems (designed as standard gradient) in which the capillary is filled (speed, 0.05 ml/sec), successively, with:

(1) 0.6 ml 0.1 *M* LiCl
(2) 0.6 ml 0.2 *M* LiCl
(3) 0.6 ml 0.5 *M* LiCl
(4) 0.6 ml 1 *M* LiCl
(5) 1.2 ml 2 *M* LiCl.

combined with the feed pipe in such a way that chromatography starts with the solvent of lowest salt concentration (here 0.1 *M* LiCl). Chromatography is perpendicular to the coating direction on the sheets. Development on plates is in the coating direction. Distance between immersion line and starting points was 1.5 cm.

Scanning of spots

For direct fluorometry[9–12] a Turner-Fluorometer 111 fitted with a door for thin-layer chromatograms (Camag, Muttenz, Switzerland) is used. Spots on the plates or sheets can be scanned by illuminating the chromatograms with a short wave-length



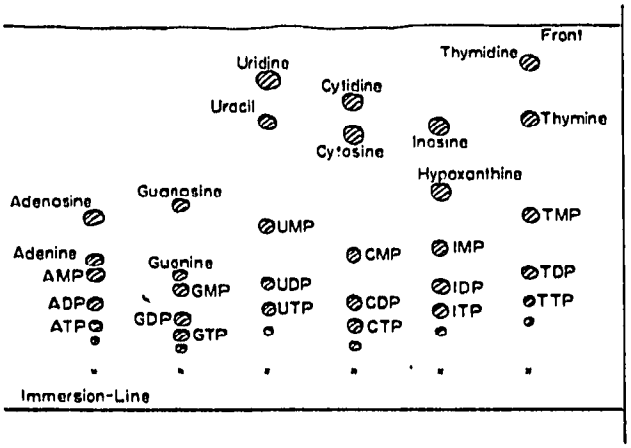


Fig. 1. Separation of the adenine, guanine, uracil, cytosine, hypoxanthine and thymine group on PEI-cellulose sheet (*cf.* procedure *b* in Experimental). Standard gradient (capillary is filled with: 0.6 ml 0.1 *M* LiCl; 0.6 ml 0.2 *M* LiCl; 0.6 ml 0.5 *M* LiCl; 0.6 ml 1 *M* LiCl; and 1.2 ml 2 *M* LiCl). Load: base, nucleoside and monophosphate 1 μg each; diphosphate and triphosphate 2 μg each. Distance between starting point and immersion line: 1.5 cm. Running time for 3.6 ml solvent: 160 min; relative air humidity: 65%.

Fig. 2. Separation of the adenine, guanine, uracil, cytosine, hypoxanthine and thymine group on PEI-cellulose plate (*cf.* procedure *c* in Methods). Experimental conditions as in Fig. 1. Running time: 95 min; relative air humidity: 37%.

fluorescent lamp (110–851) at 254 nm and by using a Corning 7–54 filter as primary filter (transmitting between 230–400 nm) and a Kodak-Wratten 2A (transmitting only above 405 nm) as secondary filter. The conditions are*: at door full open;

* Recently, some improvements were introduced by the manufacturer. The new apparatus probably requires the same variations in the experimental conditions as given in this paper and published previously[9–12].

sensitivity 10 ×; recording of fluorometer units by a Hi-Speed-Recorder 201, supplied by Kontron/Zürich (speed, 8 cm/min; voltage, 10 mV). Before scanning the chromatograms are dried 30 min in a stream of cold air (designed as "$t = 0$").

RESULTS AND DISCUSSION

The chromatographic behaviour of nucleo-derivatives on PEI-cellulose *sheets* prepared according to procedure *b* and on PEI-cellulose *plates* prepared according to procedure *c* (*cf.* Methods) with our standard gradient is shown in Figs. 1 and 2. As can be seen, there is a good separation in every group between nucleoside-mono-, di- and triphosphates as well as between nucleoside and the corresponding base. It is especially remarkable that in each group phosphates, nucleosides and the corresponding bases can be detected on the *same plate*, a fact which makes possible the analysis of substances from three different polarity classes.

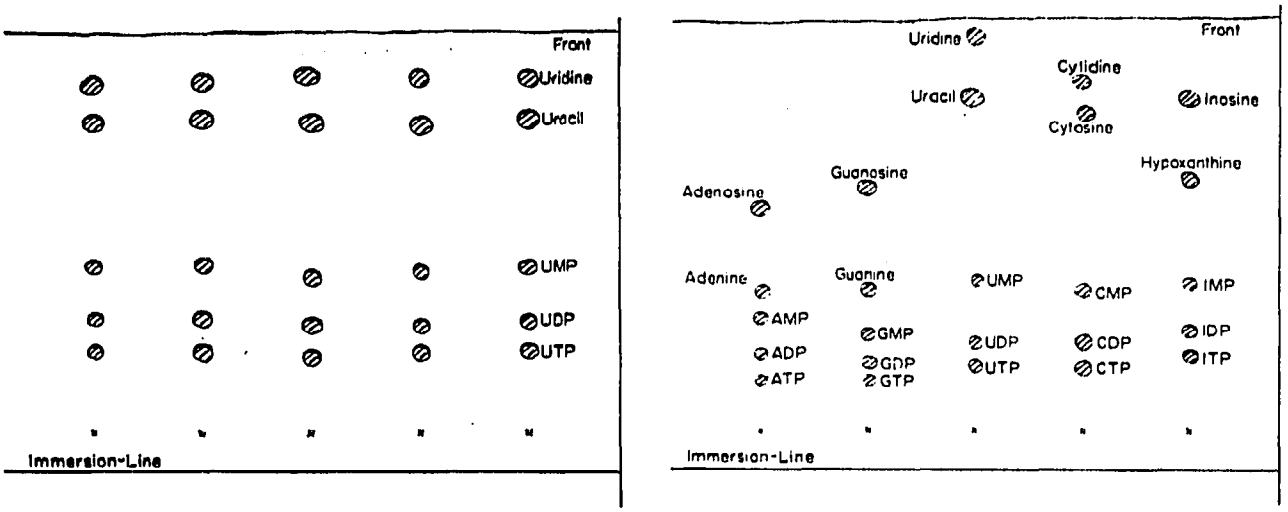


Fig. 3. Reproducibility of the standard gradient (*cf.* Methods) on the same PEI-cellulose sheet (*cf.* procedure *b* in Methods). Experimental conditions as in Fig. 1. Running time: 165 min; relative air humidity: 45%.

Fig. 4. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 0.4 ml water; (2) 0.6 ml 0.1 *M* LiCl; (3) 0.6 ml 0.2 *M* LiCl; (4) 0.6 ml 0.5 *M* LiCl; (5) 0.6 ml 1 *M* LiCl; (6) 1.2 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 120 min for 4 ml solvent; relative air humidity: 50%.

The separation will not be altered if we use PEI-cellulose sheets prepared according to procedure *a* (impregnation of unpurified cellulose with poly(ethyleneimine); *cf.* Methods). The reproducibility of the standard gradient (*cf.* Methods) on the same sheet is shown in Fig. 3. R_F values of compounds observed on *different sheets* under *different conditions* suggest that the variance of R_F values in gradient elution thin-layer chromatography is nearly of the same order of magnitude as that generally encountered in thin-layer chromatography[15].

The separation of nucleotides from their corresponding bases and nucleosides in each group is given in Figs. 1 and 2. However, adenine and guanine move approximately as fast as IMP and CMP and slower than UMP (*e.g.* Figs. 1 and 2). When we use gradient elution for the separation of complex mixtures containing nucleotides nucleic acid bases and nucleosides a distinct differentiation is required between *all*

nucleotides on the one hand, and *all* nucleosides and nucleic acid bases on the other hand. Since the R_F values of nucleic acid bases and nucleosides do not depend on the salt concentration of the solvent, these compounds can be separated from nucleotides and other acidic substances by a development with distilled water[3]. We have therefore modified our standard gradient slightly to obtain a better separation between the compounds discussed above. Starting with 0.4 ml distilled water instead of 0.1 *M* LiCl causes only a minor change (compare the positions of adenine, guanine, UMP, CMP and IMP in Figs. 2 and 4). Gradients containing 1 and 1.8 ml water, resp., as starting solvent are capable of separating all the nucleic acid bases and nucleosides

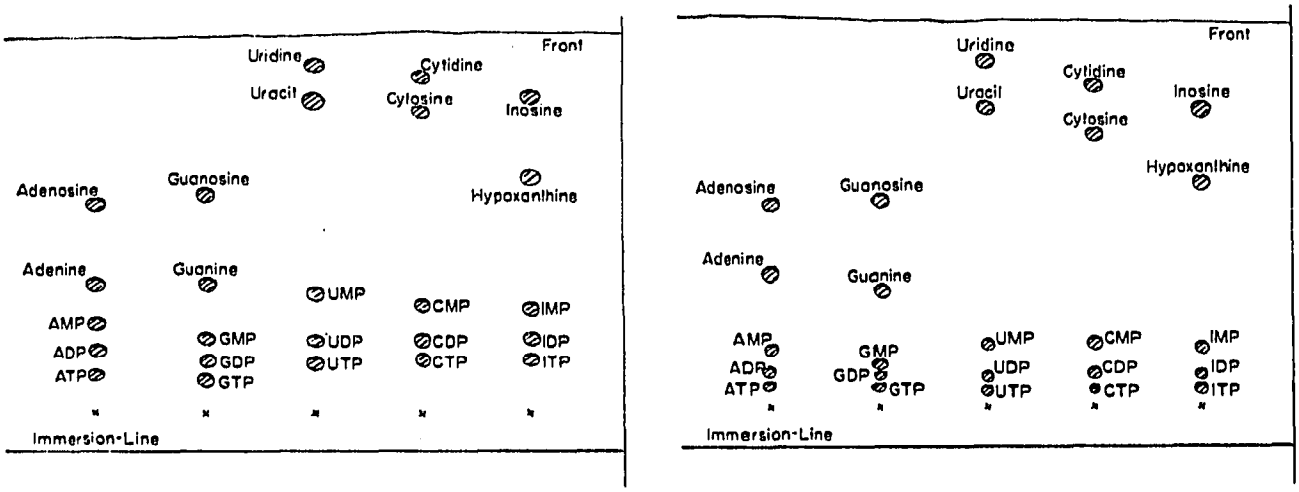


Fig. 5. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1 ml water; (2) 0.5 ml 0.1 *M* LiCl; (3) 0.5 ml 0.2 *M* LiCl ;(4) 0.5 ml 0.5 *M* LiCl; (5) 0.5 ml 1 *M* LiCl; (6) 1 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 95 min for 4 ml solvent; relative air humidity: 33%.

Fig. 6. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1.8 ml water; (2) 0.4 ml 0.1 *M* LiCl; (3) 0.4 ml 0.2 *M* LiCl; (4) 0.4 ml 0.5 *M* LiCl; (5) 0.4 ml 1 *M* LiCl; (6) 0.6 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 105 min for 4 ml solvent; relative air humidity: 33%.

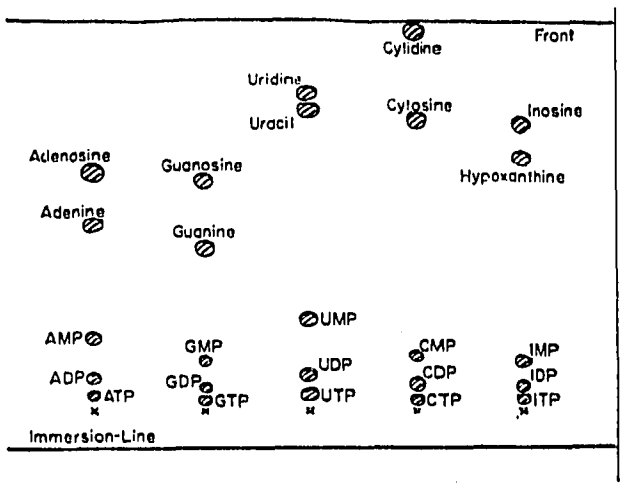
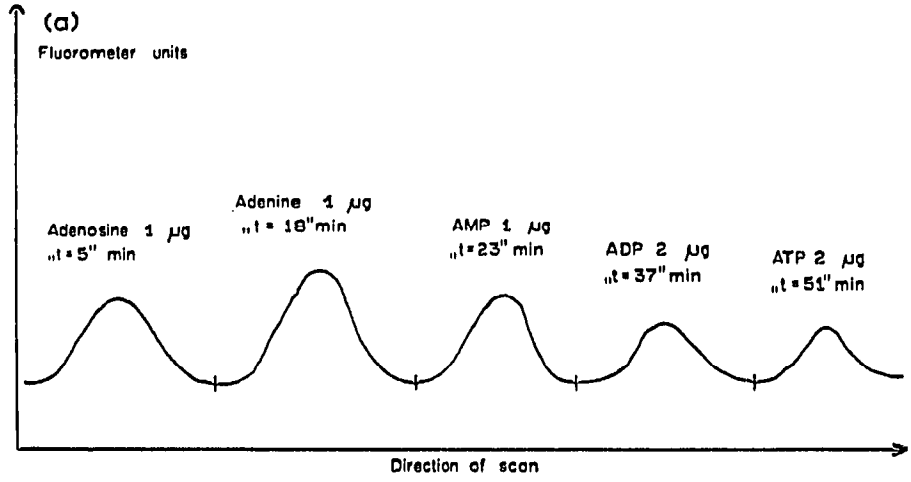
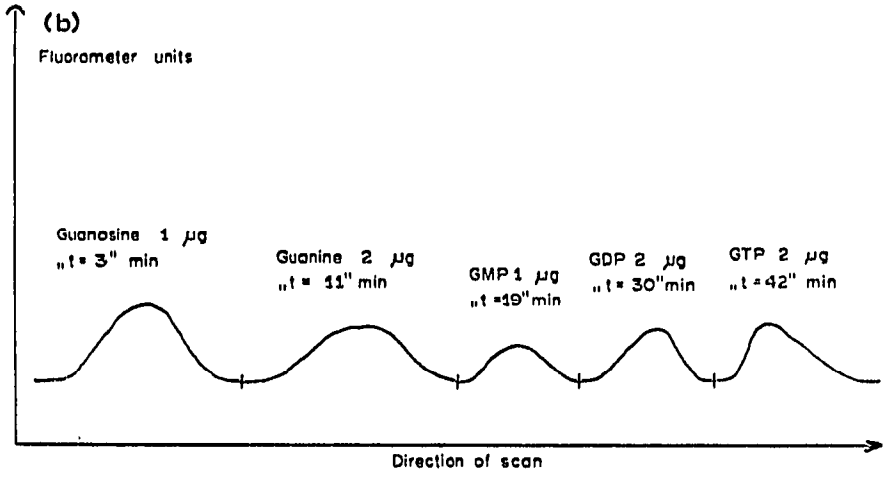
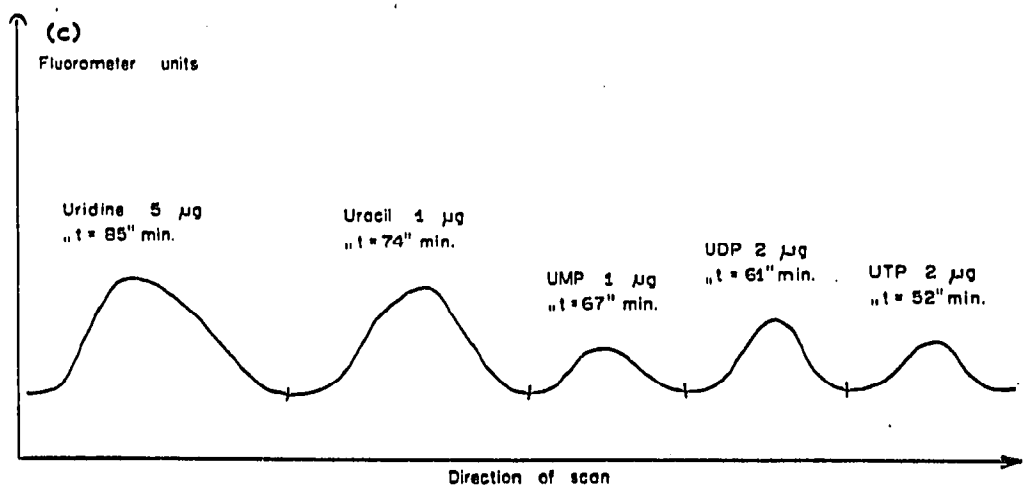


Fig. 7. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1 ml methanol–water (1:1); (2) 0.6 ml 0.1 *M* LiCl; (3) 0.6 ml 0.2 *M* LiCl; (4) 0.6 ml 0.5 *M* LiCl; (5) 0.6 ml 1 *M* LiCl; (6) 0.6 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 180 min for 4 ml solvent; relative air humidity: 36%.


(a)
Fluorometer units
Adenosine 1 µg
„t = 5" min
Adenine 1 µg
„t = 18" min
AMP 1 µg
„t = 23" min
ADP 2 µg
„t = 37" min
ATP 2 µg
„t = 51" min
Direction of scan


(b)
Fluorometer units
Guanosine 1 µg
„t = 3" min
Guanine 2 µg
„t = 11" min
GMP 1 µg
„t = 19" min
GDP 2 µg
„t = 30" min
GTP 2 µg
„t = 42" min
Direction of scan


(c)
Fluorometer units
Uridine 5 µg
„t = 85" min.
Uracil 1 µg
„t = 74" min.
UMP 1 µg
„t = 67" min.
UDP 2 µg
„t = 61" min.
UTP 2 µg
„t = 52" min.
Direction of scan

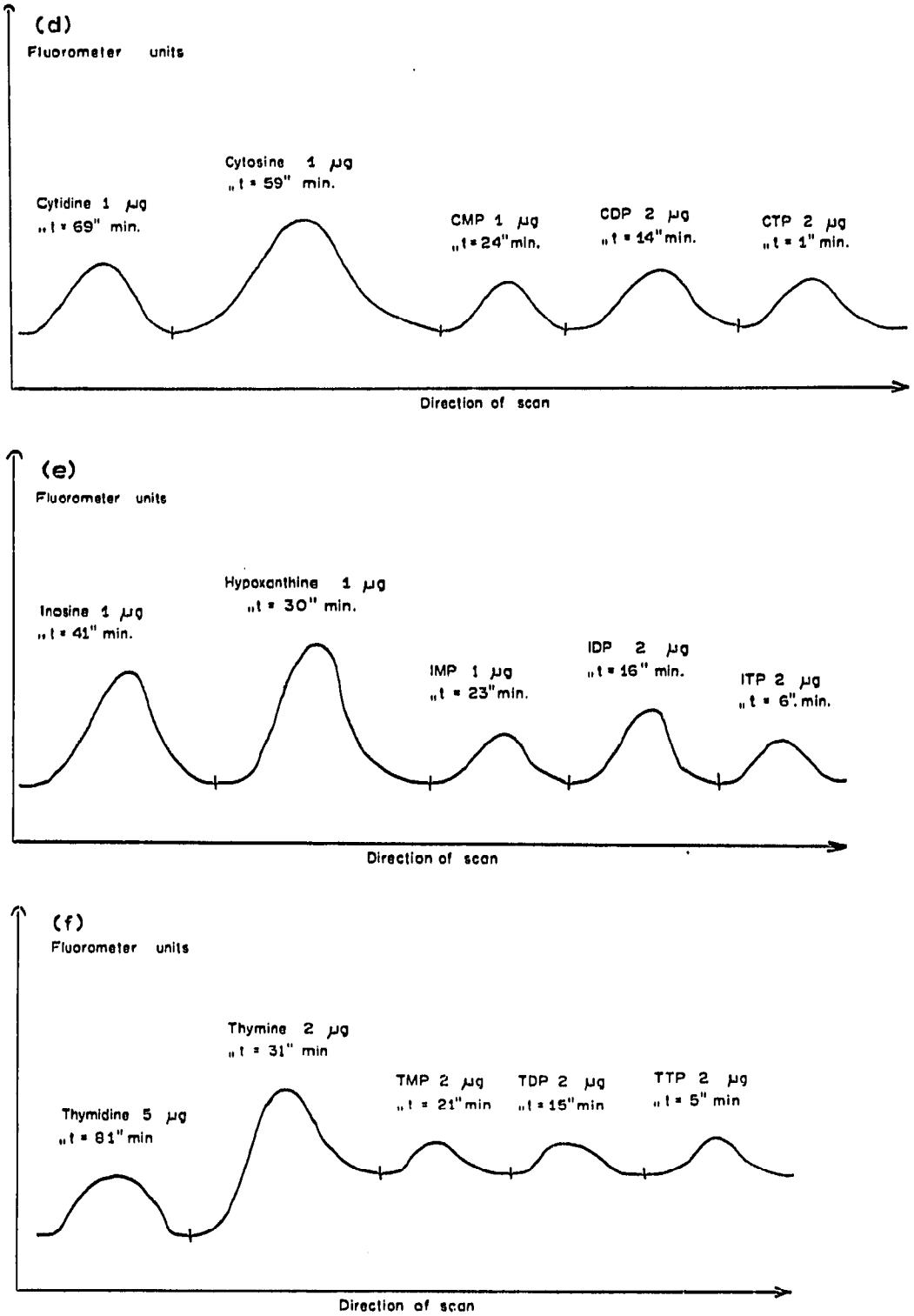


Fig. 8. Scanning of spots (perpendicular to the direction of run) on PEI-cellulose sheets (*cf.* procedure *b* in Methods). The sheet with the substances was covered, except a small strip containing the spot under investigation, with another plain PEI-cellulose sheet. Before scanning ("t = o" min) the chromatogram was dried 30 min in a stream of cold air. For the remaining conditions *cf.* Methods. Impurities in nucleotide di- and triphosphates are not given here. Loads are given in micrograms (μg). a = Adenine group; b = guanine group; c = uridine group; d = cytosine group; e = hypoxanthine group; f = thymine group.

under investigation from the nucleotides. However, the resolution of the nucleotides in these cases is somewhat less effective (Figs. 5 and 6). Finally Fig. 7 shows a chromatogram with a gradient containing 1 ml methanol–water (1:1) as starting solvent. Higher amounts of methanol give chromatograms in which the nucleic acid bases and nucleosides tail.

As already mentioned, attempts have been made to apply the direct fluorometric procedure previously reported[9–12] for scanning of spots on PEI-cellulose chromatograms. Earlier experiments* showed that direct fluorometry cannot be performed on unpurified cellulose layers. In order to overcome the difficulties caused by the inconstancy of the zero line, we have developed a purification procedure[1,10] (*cf.* Methods), which partly rectifies this shortcoming. Experiments not reported here, have shown that scanning of the spots is impossible on a PEI-cellulose layer prepared according to procedure *a* (*cf.* Methods) and that purification of the cellulose powder is indispensable. Fortunately the separation of the nucleo-derivatives is not altered by use of purified cellulose for the preparation of PEI-cellulose layers (procedures *b* and *c* in Methods). We have therefore carried out all measurements on PEI-cellulose layers prepared from purified cellulose powder (procedures *b* and *c*). The spots are scanned by illuminating the chromatogram with a fluorescent lamp (254 nm) and by using a primary filter transmitting between 230 and 400 nm (*cf.* refs. 9, 10, 11 and 12). Typical peaks** produced by the scanner are shown in Fig. 8. The fluorescence quenching, that means the peak area, as reported earlier[12] for amino acid derivatives, will be altered by the time of drying***. The drying time therefore has to be standardized. Thus the peak areas can only be compared with reservations. Nevertheless, they give an idea of the sensitivity of the method. In our opinion about 1 μg nucleoside phosphate and about 0.5 μg nucleoside or nucleic acid base can be estimated by this method. In some cases, however, it may be useful to increase the peaks by scale expansion.

It has to be pointed out that impurities in the layer, which, in some cases, can be seen in the region of the front, may disturb the scanning of substances with high mobilities.

All the precautions given under Pretreatment of sheets and Pretreatment of plates must therefore be followed. The scanning should, of course, be made with care as described previously[10] (*cf.* also ref. 7).

The application of gradient elution thin-layer chromatography on PEI-cellulose

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* Unpublished results.

** The amounts of nucleoside phosphates, given in Fig. 8, correspond to the compounds listed here: Adenosine-5′-monophosphoric acid monohydrate; adenosine diphosphate sodium salt; adenosine triphosphate disodium salt; cytidine-5′-monophosphate disodium salt; cytidine diphosphate sodium salt·$4H_2O$; cytidine triphosphate sodium salt·$4H_2O$; thymidylic acid ammonium salt; thymidine diphosphate trisodium salt; thymidine triphosphate trisodium salt; uridine monophosphate disodium salt; uridine diphosphate disodium salt; uridine triphosphate trisodium salt; guanosine monophosphate sodium salt·H_2O; guanosine diphosphate sodium salt; guanosine triphosphate sodium salt; inosine-monophosphoric acid; inosine diphosphate disodium salt·$5\frac{1}{2}H_2O$; inosine-triphosphoric acid.

Nucleoside di- and triphosphates contain the corresponding mono- and diphosphates, resp. The amounts of these impurities, however, vary from one lot to another. Some samples of nucleoside triphosphates also contain a substance with a lower mobility on PEI-cellulose layers. These spots are probably nucleoside tetraphosphates and their amounts again differ from one lot to another.

*** The standard deviation between "t = 150" min and "t = 600" min (31 measurements in intervals of each 15 min) is 6.9%.

and the direct fluorometry of spots to the separation and quantitative estimation of complex mixtures is to be investigated in our laboratories.

The combined use of the technique described in this paper and our earlier published method[1] for the analysis of nucleo-derivatives in biological material will be published in a subsequent communication.

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SUMMARY

The separation of nucleic acid bases, nucleosides and nucleotides on PEI-cellulose layers using gradient elution technique has been investigated. The standard gradient (0.1 *M* LiCl → 2 *M* LiCl) described in this paper allows the detection of nucleic acid base, nucleoside and nucleotide on the same plate or sheet.

Special reference is made to the direct fluorometry of compounds resolved on PEI-cellulose layers.

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