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

III. SEPARATION OF COMPLEX MIXTURES ON CELLULOSE LAYERS*

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Thin-layer chromatography (TLC) has been initially developed for the analysis of lipophilic substances[1,2]. This technique is now also, however, a standard method in the separation of hydrophilic substances, *e.g.* amino acids and derivatives[3] or constituents of nucleic acids[4]. The separation of nucleotides has been investigated by RANDERATH *et al.*[5–18] in great detail. These authors have used TLC on ion-exchange cellulose for the separation of complex mixtures of nucleotides. Other investigators have also published a number of papers on TLC of nucleo-derivatives (for review *cf.* ref. 4). In connection with our work concerning the isolation, detection and structure elucidation of substances in organ extracts we wanted a chromatographic technique which allows the separation and characterization of a very great number of nucleo-derivatives. Earlier experiments in our laboratory have shown that two-dimensional paper chromatography is very useful for the separation of nucleo-derivatives in organ extracts. It was felt that this technique may also be applicable, when cellulose thin layers are used instead of paper as carrier. The TLC method, reported in this communication, allows the characterization of more than sixty nucleo-derivatives and related compounds on cellulose layers, and is especially suitable for the detection of nucleo-derivatives in biological material. Moreover this technique allows the detection of nucleic acid bases, nucleosides and nucleotides in the same chromatogram**.

EXPERIMENTAL

Purification of the cellulose powder

60 g MN-300 cellulose powder (Macherey, Nagel & Co., Düren, Germany) are suspended in 500 ml *n*-propanol–25 % ammonia–water (6:3:1, v/v) and mixed by vigorous shaking (30 min). After filtration, the powder is suspended in 400 ml *n*-propanol, shaken vigorously for 15–30 min and filtered again. The solid residue is purified by washing with *n*-propanol and is again filtered. The powder is homogenized, vacuum-dried at 60° until all the ammonia is removed, and dried again 2 × 12 h (60°, high vacuum).

* For Part II of this series, see ref. 19.
** For preliminary report, *cf.* ref. 19.

Preparation of the plates

15 g purified cellulose powder are suspended in 90 ml water and homogenized 30 sec with an electric mixer. The slurry is spread, after removing air bubbles (about 5 min), over 5 glass plates (20 × 20 cm) with 0.5 mm slot width, using STAHL's apparatus (Desaga, Heidelberg, Germany), followed by drying overnight at ambient temperature.

Application of the solutes

Standard solutions as well as organ extracts are applied in portions of 5 μl 3 cm from the edges of the plate and each portion is dried in a stream of cold air. For studying the chromatographic behaviour, the chromatograms are loaded with 1–5 μg of pure compound dissolved in 5 μl solvent (we use generally 0.04 % NaOH for nucleobases and nucleosides or 10 % isopropanol for nucleotides).

Separation procedure and location of spots

For bidimensional chromatography (descending technique) the following solvent systems were used:

1st direction: *n*-propanol–25 % ammonia–water (6:3:1, v/v)

2nd direction: isopropanol–saturated ammonium sulphate–water (2:79:19, v/v).

Shandon multiplate-chromatotanks (Shandon, London) were filled with 500 ml of the first solvent system and 6 plates (layer always against layer) were immediately introduced. The solvent system for the first dimension migrated about 18 cm after 3 h. The chromatograms are then dried 20 min in a stream of cold air, turned through 90° and put into the second Shandon multiplate-chromatotank, which is at the same time filled with 500 ml of the second solvent system.

In this solvent the front again migrates about 18 cm within 3 h. The chromatograms are taken out, dried 20 min in a stream of cold air and put under U.V. light* at 254 and 360 nm (U.V. lamp supplied by Camag, Muttenz, Switzerland). The absorption spots are outlined and hatched with a pencil, the fluorescent spots are surrounded by an interrupted line. Much better for documentation is, of course, direct photography under U.V. light. The layer is illuminated with two U.V.-lamps (Camag, Muttenz, Switzerland) at 254 nm. Distance between sheet and lamps: 7 cm. The film (Agfa-Isopan IFF 13 DIN) is exposed for 4 min. Distance between camera (Leica M3, aperture 5.6 with filter No. 302/5—Omag/Switzerland) and back of the glass plate: 50 cm (using Leitz diapositiv repro DIN A4 for M cameras with Elmar-objectiv 50 mm). *All photographs were taken from the back.* Development of photographs was carried out with Neofin-blau (Tetenal, Hamburg/Germany) for 8 min at 20° using Agfa BW-1. The fluorescence spots remain, of course, invisible or almost invisible.

RESULTS AND DISCUSSION

Various techniques have been described for TLC of nucleo-derivatives[4]. The separation of complex mixtures, *e.g.* nucleotides, can be carried out on ion-exchange cellulose layers[9,13–18]. However, as far as we know, there are no methods in the literature on the separation of complex mixtures containing nucleic acid bases, nucleosides, nucleotides and related compounds. It was felt that a technique, which allows the

* The chromatograms should be viewed not only on the surface, but also from the back.

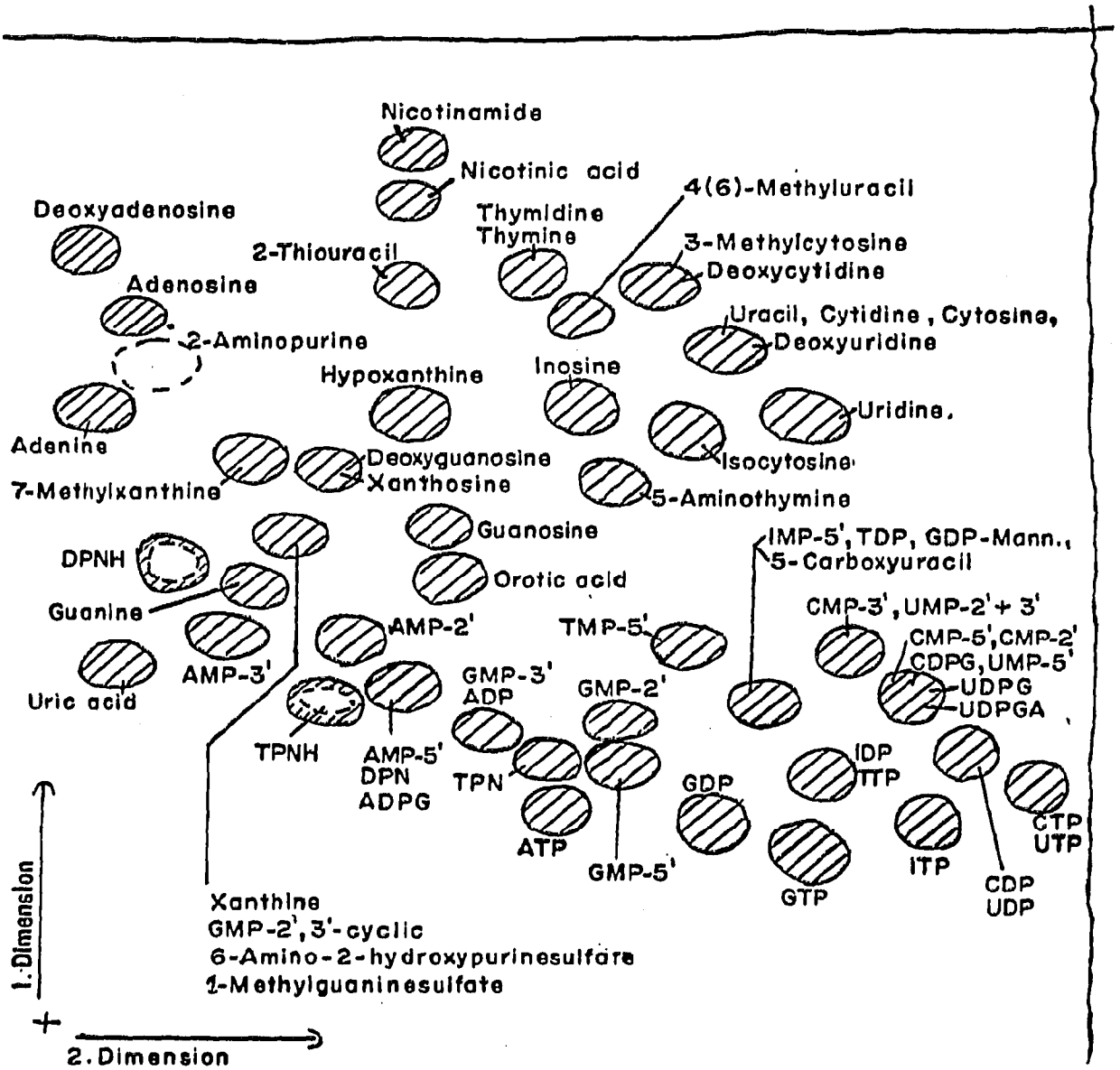

Fig. 1. Separation of nucleo-derivatives on purified cellulose layers. 1st dimension: *n*-propanol–25% ammonia–water (6:3:1, v/v); 2nd dimension: isopropanol–saturated ammonium sulphate–water (2:79:19, v/v). Absorption spots are hatched, fluorescence spots are surrounded by an interrupted line. For remaining conditions *cf.* experimental part. Note: DPNH and TPNH show fluorescence, which changes after several hours drying to absorption. R_F value of CTP in the second dimension: 0.83. (schematical)

separation of the compounds considered above, will be very useful for characterization of nucleo-derivatives in biological material as well as for the detection of nucleo-derivatives isolated from organ extracts by column chromatography. Since in our laboratory paper chromatography is a well established technique for the separation of nucleo-derivatives, we began to use our solvent systems for TLC on cellulose layers. Nucleic acid bases, nucleosides, nucleotides and related compounds can be separated on cellulose layers using the solvent systems given in the experimental part. It is a requisite that chromatography is carried out *without* chamber saturation (*cf.* Experimental). The ammonium sulphate solution, which is used for development in

the second direction, must be *completely saturated*, otherwise the separation in the second dimension will be altered. *These two conditions must be strictly fulfilled.* When MN-300-cellulose is used for the chromatography of nucleoside mono-, di- and triphosphates good separations are obtained. However, the resolution of nucleic acid bases and nucleosides on such layers is less effective and the spots tail. In order to

TABLE I

SEPARATION OF DIFFERENT GROUPS IN CHROMATOGRAMS ACCORDING TO FIG. 1

Group	*Spots*
Adenine	Adenine, adenosine, deoxyadenosine, AMP-3′, AMP-2′, AMP-5′+ADPG, ADP, ATP
Guanine	Guanine, guanosine, deoxyguanosine, GMP-3′, GMP-2′, GMP-5′, GDP, GTP, GDP-Mann
Hypoxanthine	Hypoxanthine, inosine, IMP-5′, IDP, ITP
Cytosine	Cytidine + cytosine, deoxycytidine, CMP-3′, CMP-2′ + CMP-5′, CDP, CTP, CDPG
Uracil	Uracil + deoxyuridine, uridine, UMP-2′ + 3′, UMP-5′, UDP, UTP, UDPG + UDPGA
Thymine	Thymine + thymidine, TMP-5′, TDP, TTP

overcome the difficulties caused by the cellulose, a purification procedure has been developed which rectifies this shortcoming*. The chromatographic behaviour of more than sixty nucleo-derivatives is shown in Fig. 1**.

The resolving power of our chromatographic system can be judged *e.g.* by the quality of the separation of substances which belong to the same base (see Table I). *Nucleic acid bases* and the *corresponding ribosides* are, apart from cytidine and cytosine, well separated. It is remarkable that the *ribosides* and *deoxyribosides* under investigation show different chromatographic behaviour. On the other hand, uracil and deoxyuridine as well as thymine and thymidine, that means the *base* and its corresponding *deoxyriboside*, travel together. Nucleoside mono-, di- and triphosphates are separated in every group. It should be mentioned that di- and triphosphates show tailing in the first dimension. Finally, the pyridine nucleotides are also resolved in this chromatographic system. As we can see in Fig. 1, some spots contain more than one substance. For the differentiation of compounds which are not resolved in chromato-

* For quantitative estimation of nucleo-derivatives by direct fluorometry, the use of purified cellulose is indispensable[19–21].

** Abbreviations: adenosine-5′-monophosphate=AMP-5′; adenosine-2′-monophosphate=AMP-2′; adenosine-3′-monophosphate=AMP-3′; adenosine diphosphate=ADP; adenosine triphosphate=ATP; adenosine diphosphate glucose=ADPG; guanosine-5′-monophosphate=GMP-5′; guanosine-2′-monophosphate=GMP-2′; guanosine-3′-monophosphate=GMP-3′; guanosine-2′,3′-cyclic-phosphate=GMP-2′,3′-cyclic; guanosine diphosphate=GDP; guanosine triphosphate=GTP; guanosine diphosphate mannose=GDP-Mann; inosine-5′-monophosphate=IMP-5′; inosine diphosphate=IDP; inosine triphosphate=ITP; cytidine-5′-monophosphate=CMP-5′; cytidine-2′-monophosphate=CMP-2′; cytidine-3′-monophosphate=CMP-3′; cytidine diphosphate=CDP; cytidine triphosphate=CTP; cytidine diphosphate glucose=CDPG; uridine-5′-monophosphate=UMP-5′; uridine-2′+3′-monophosphate=UMP-2′+3′; uridine diphosphate=UDP; uridine triphosphate=UTP; uridine diphosphate glucose=UDPG; uridine diphosphate glucuronic acid=UDPGA; thymidine-5′-monophosphate=TMP-5′; thymidine diphosphate=TDP; thymidine triphosphate=TTP; diphosphopyridine nucleotide=DPN; diphosphopyridine nucleotide red.=DPNH; triphosphopyridine nucleotide=TPN; triphosphopyridine nucleotide red.=TPNH.

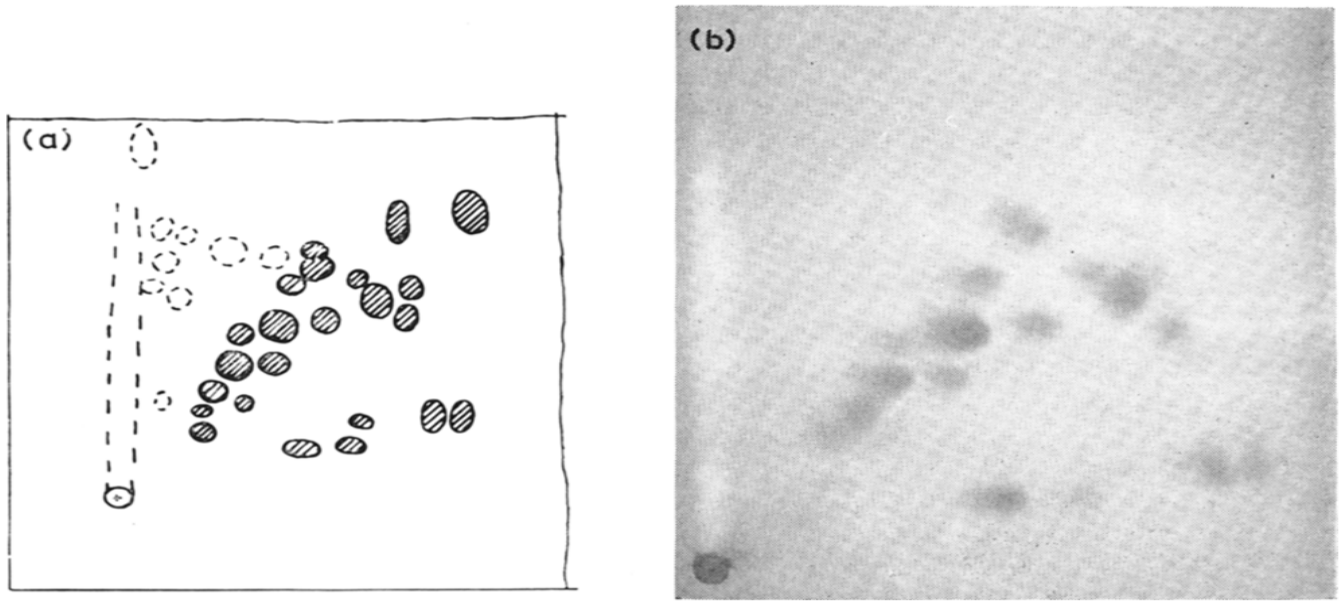

Fig. 2. (a) Detection of nucleo-derivatives in Robuden UD. Load: 5 μl 10:1 concentrated solution. For remaining conditions *cf.* Fig. 1 and the experimental part. (b) U.V. photography of chromatogram corresponding to Fig. 2a (*cf.* experimental part). Note: Some of the minor spots are not visible on the reproduction.

grams according to Fig. 1, additional chromatographic systems are necessary. The combined use of the technique described in this paper together with chromatography on poly(ethyleneimine) layers[9,13–18,21] is the method of choice for the identification of nucleotides and will now be investigated in our laboratory. Chromatographic systems for separation of the other groups not resolved on chromatograms according to Fig. 1 are mostly known from the literature (*cf.* ref. 4). Finally, it should be pointed out that the direct rechromatography of spots as suggested by RANDERATH[18] allows rapid and easy identification; however, chromatography must be performed on sheets

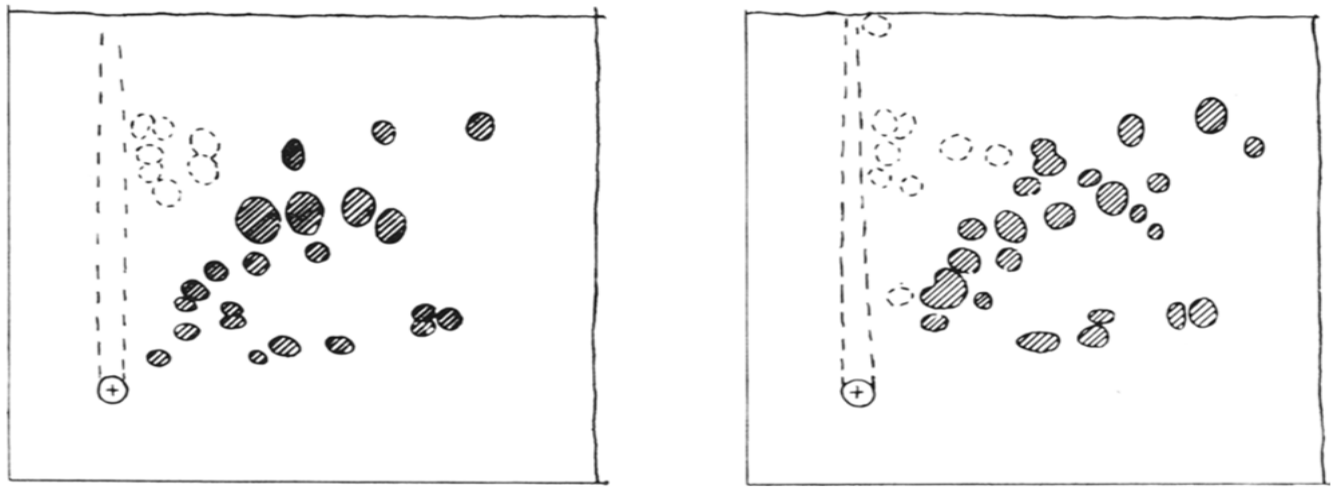

Fig. 3. Detection of nucleo-derivatives in Robuden UV. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 4. Detection of nucleo-derivatives in Raveron. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

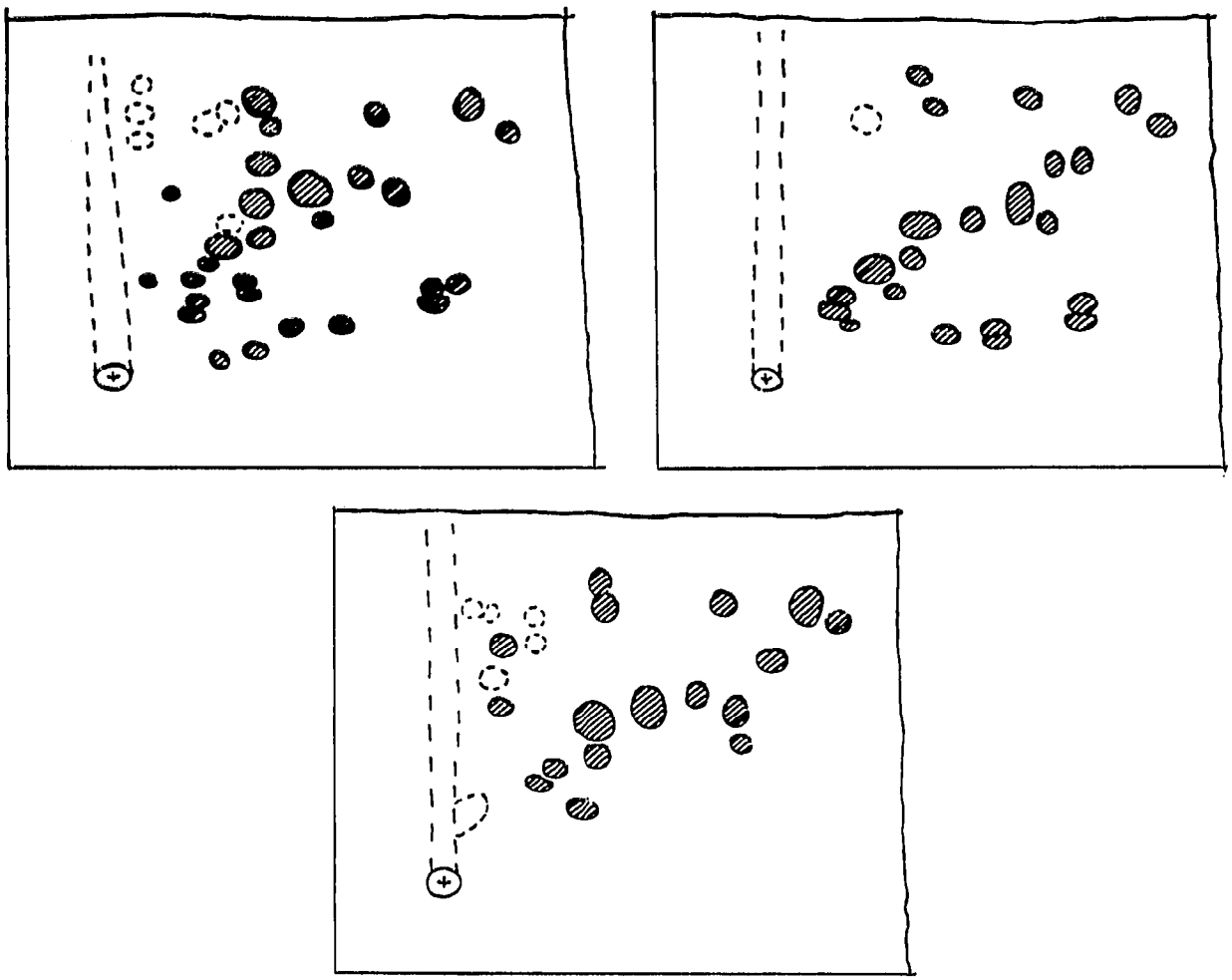

Fig. 5. Detection of nucleo-derivatives in Ripason. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 6. Detection of nucleo-derivatives in Rumalon. Load: 15 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 7. Detection of nucleo-derivatives in Recosen. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

instead of glass plates. Preliminary investigations have shown that cellulose thin layer sheets* can be used for the separation of nucleo-derivatives. Such sheets give chromatograms in which the separation is the same as on unpurified cellulose layers[19]. The same is true for precoated cellulose plates, which are now available**.

As we have already mentioned in the introduction to this paper, experiments have been made to apply our technique to the detection of nucleo-derivatives in biological material. Figs. 2–7 are schematic chromatograms and show the detection of nucleo-derivatives in different organ extracts***.

Fig. 2b is an original photograph (*cf.* Methods) corresponding to Fig. 2a.

* Macherey, Nagel & Co., Düren, Germany.
** Camag, Muttenz, Switzerland.
*** Recosen, Raveron, Robuden UD, Robuden UV, Ripason and Rumalon. Products of Robapharm Ltd., Basle, Switzerland.

Chromatograms from organ extracts, as shown in Figs. 2–7, were made *without* any pretreatment. The extracts were applied to the layer in portions of 5 μl (intermediate drying with a stream of cold air between each application). The quality of separation depends on the diameter of the starting spot. When the starting spot is too large the separation is less effective. On the other hand, if the spot applied to the chromatogram is too small, we again obtain poor resolution. In our experience the best chromatograms result when we use the procedure given above for spotting the organ extracts. However, the technique of application may depend on the nature of the biological material. Identification can be made by co-chromatography with known substances and by rechromatography in other chromatographic systems as discussed above. In this connection infrared spectroscopy on a microgram scale should be mentioned (*cf. e.g.* ref. 22).

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SUMMARY

A method is described for the characterization of more than sixty nucleo-derivatives on purified cellulose layers using *n*-propanol–25 % ammonia–water (6:3:1, v/v) in the first and isopropanol–saturated ammonium sulphate–water (2:79:19, v/v) in the second dimension. The technique is suitable for detection of nucleo-derivatives in biological material without any pretreatment.

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