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# The non-destructive detection of amino acids on thin-layer chromatograms using 1-Fluor-2,4-Dinitrobenzene[1])

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(Eingegangen am 4. Juni 1968)

An improved method for the *non-destructive* detection of amino acids separated on thin-layer chromatograms is described. The reaction of amino acids with 1-Fluor-2,4-Dinitrobenzene *in situ* allows the detection of 10 nMoles on *two-dimensional* and 0.5 to 1 nMoles on *one-dimensional* chromatograms. The technique makes possible *reaction chromatography* and *multidimensional chromatography* of amino acids, i. e. the *more unambiguous* identification in complex mixtures.

Eine verbesserte Methode zum *zerstörungsfreien* Nachweis von Aminosäuren auf Dünnschicht-Chromatogrammen wird beschrieben. Die Umsetzung von Aminosäuren mit 1-Fluor-2,4-Dinitrobenzol *in situ* erlaubt den Nachweis von 10 nMolen in *zweidimensionalen* und 0,5 bis 1 nMolen in *eindimensionalen* Chromatogrammen. Die Technik ermöglicht die *Reaktionschromatographie* und die *multidimensionale Chromatographie* von Aminosäuren, d. h., eine *eindeutige* Identifizierung in komplexen Gemischen.

The non-destructive detection of amino acids on thin-layer chromatograms, using 2,4-Dinitrofluorbenzene (DNFB), was reported in a preliminary communication (2). The technique described in this paper (2) has been subsequently used, in an improved version, to detection and reaction chromatography of amino acids (3, 4). In the present paper the *improved procedure* will be described.

## Material and Methods

### Material

MN-300-cellulose (Macherey, Nagel + Co. Düren)
Kieselgel G (Merck, Art. 7731)
Plastic foils for TLC, DC GA 1190 (Galenopharm, Geneva)
Toronto starch (Bender + Hobein, Zurich)
1-Fluor-2,4-Dinitrobenzene puriss (DNFB) (Fluka, Buchs, Switzerland)
Ninhydrin (Hoffmann La Roche, Basle)
Sodium carbonate (Merck, Art. 6391)
Sodium hydrogen carbonate (Merck, Art. 6329)
n-Butanol (Merck, Art. 9628)
Isopropanol (Merck, Art. 9634)
Formic Acid (Merck, Art. 264)
tert-Butylalcohol (Merck, Art. 9629)
Ethylmethylketone (Merck, Art. 9708)
Glacial Acetic Acid (Merck, 90063)
25% Ammonia (Merck, Art. 5432)
$\beta$-Naphthoquinone-4-sulfonic acid-sodium (Merck, Art. 6531)
DNP-amino acids (Serva, Entwicklungslabor, Heidelberg and Mann Research Laboratories, New York)
Amino Acid Standard solutions set No. 1, Catalogue No. 2058 (Shandon, London)
Methylalcohol (Merck, Art. 6009)
Sodium hydroxide (Merck, Art. 6496)
Chloroform (Merck, Art. 2445)

### Reagents

DNFB-solution: 1% (w/v) in absolute methyl alcohol (the solution must be freshly prepared!) (5)
1M Bicarbonate-buffer pH 8.8: 8.4 g $NaHCO_3$ dissolve (without heating!) in distilled water, pH adjusted to 8.8 with 2N NaOH, and made up to 100 m*l* with distilled water (5)
Ninhydrin-solution: 0.3 g Ninhydrin + 100 m*l* n-Butanol + 3 m*l* glacial acetic acid (5)
Folin's Reagent: 0.2 g $\beta$-Naphtoquinone-n-sulfonic acid-sodium + 100 m*l* 5% $Na_2CO_3$-solution (5)

### Preparation of the layers

Glass plates as well as plastic foils are coated with *purified* MN-300-cellulose (6, 7), and with silica gel G-Starch (5).

### Chromatography

*Two-dimensional* development on cellulose was carried out according to Jones and Heathcote (8). *First dimension*: isopropanol/formic acid/water (80:4:20 v/v), and after drying with a stream of cold air (1 hour), tert- butylalcohol/ethylmethylketone/25% ammonia/water (5:3:1:1 v/v) in the *second dimension*. All runs were *without* chamber saturation.
*One dimensional* chromatography on silica gel was performed with chloroform/methanol/25% ammonia (2:2:1 v/v) (5) All runs were *with* chamber saturation.

### Detection with DNFB

When the chromatography is complete, the layer is dried (15 min. in a stream of warm air) and cooled to room temperature. The

[1]) 9th communication on "Application of thin-layer chromatography in sequence analysis of peptides". 8th communication cf. Reference (1).

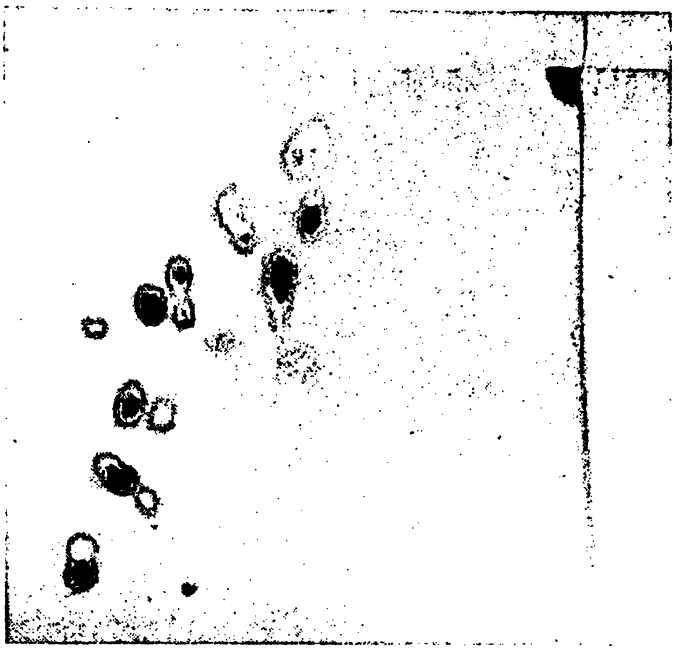

Fig. 1
Detection of amino acids with DNFB on two-dimensional chromatograms (Original UV-photograph)
Carrier: plates or plastic sheets coated with purified MN-300-cellulose, according to PATAKI (6, 7):
Solvents: Isopropanol/formic acid/water (80:4:20 v/v) in the first dimension and tert-Butylalcohol/ethylmethylketone/25% ammonia/water (5:3:1:1 v/v) in the *second* dimension, according to JONES and HEATHCOTE (8). 1 = Alanine, 8 = Arginine, 10 = Aspartic Acid, 12 = Cystine, 16 = Glutamic Acid, 18 = Glycine, 20 = Histidine, 22 = impurity, 23 = Isoleucine, 24 = Leucine, 25 = Lysine, 26 = Methionine, 32 = Phenylalanine, 33 = Proline, 35 = Serine, 37 = Threonine, 38 = Tryptophan, 39 = Tyrosine, 40 = Valine

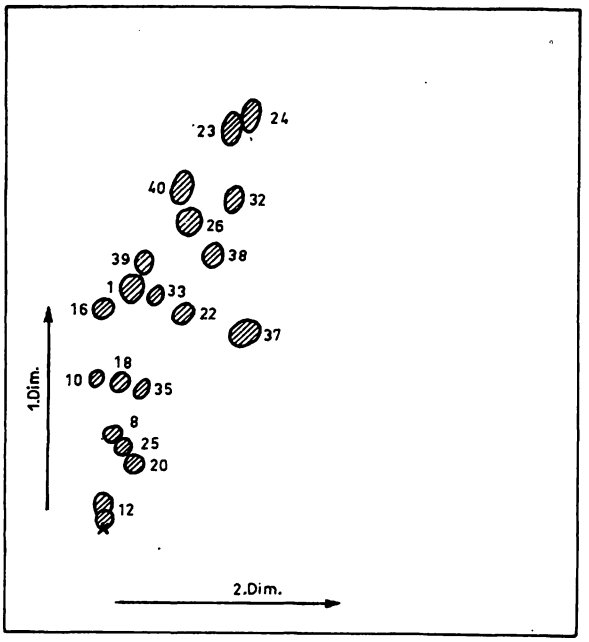


Fig. 2
Interpretation of chromatograms, according to Figure 1. Note that the spot distribution on *purified* cellulose layers (plates as well as sheets) (6, 7) is nearly the same as reported previously (8). Note that aspartic acid was not run in this particular chromatogram

chromatogram is sprayed with a 1% DNFB-solution for exactly 1 min., and subsequently with a 1M $NaHCO_3$ buffer for exactly 20 sec. The *covered* plate (2) (or sheet) ist incubated in the dark at 40° C for 1 h, and dried 15 min. in a stream of warm air. Subsequently the chromatogram is laid for 15 min. in a tank, which contains three vessels each filled with 20 m*l* conc. HCl. Of course, there is no direct contact between the layer and the HCl-solutions. After the layers have been aired (3—5 min. with a stream of cold air) the spots can be copied or photographs can be taken. After the HCl-procedure, 2,4-dinitrophenol is no longer visible in *day-light* and does not interfere with the spots.

## Results

The technique described previously (2) consists of spraying with a 10% DNFB-solution followed by washing the layer with ether. Although this procedure was capable of eliminating some of the 2,4-dinitrophenol which causes a yellow background and also interferes with the spots, the yellow colour of the layer was still a disturbing factor. Using a 1% DNFB-solution on the one hand, and replacement of the washing-procedure by a treatment with HCl on the other (cf. Methods), it is now possible to eliminate 2,4-dinitrophenol. Thus, the technique given in the experimental part allows the *non-destructive* detection of as little as about 10 nMole of an amino acid, separated on *two-dimensional* chromatograms. Using *one-dimensional* chromatography and the detection technique described above, it is even possible to detect 0.5 to 1 nMoles of amino acids (cf. Tab. 1). Figure 1 shows a number of amino acids detected with DNFB on cellulose layers. Figure 2 represents the interpretation of chromatograms according to Figure 1.

Table 1
Detection limits of amino acids with DNFB on silica gel layers
One-dimensional chromatography of amino acids with chloroform/methanol/25% ammonia (2:2:1 v/v) (5), detection with DNFB and HCl-treatment (cf. Methods)
The sensitivity is considerably lower as in the case of Ninhydrin- or Folin-reaction (cf. (4))

| Amino Acid | Detection limit µg | Amino Acid | Detection limit µg |
|---|---|---|---|
| Alanine | 0.05 | Isoleucine | 0.15 |
| β-Alanine | 0.1 | Leucine | 0.15 |
| α-Amino-butyric acid | 0.1 | Lysine · HCl | 0.1 |
| γ-Amino-butyric acid | 0.1 | Methionine | 0.15 |
| β-Amino-iso-butyric acid | 0.05 | Methioninesulfoxide | 0.1 |
| Arginine · HCl | 0.2 | Methioninsulfone | 0.1 |
| Asparagine · $H_2O$ | 0.15 | 1-Methyl-Histidine | 0.2 |
| Aspartic Acid | 0.15 | Ornithine · HBr | 0.1 |
| Citrulline | 0.2 | Phenylalanine | 0.2 |
| Cystine | 0.25 | Proline | 0.15 |
| Cysteic Acid | 0.2 | Sarcosine · HCl | 0.1 |
| Di-Iodo-Tyrosine | 0.25 | Serine | 0.1 |
| Ethanolamine | 0.1 | Taurine | 0.1 |
| Glutamic Acid | 0.15 | Threonine | 0.1 |
| Glutamine | 0.15 | Tryptophan | 0.2 |
| Glycine | 0.1 | Tyrosine | 0.2 |
| Histamine · 2HCl | 0.1 | Valine | 0.15 |
| Histidine · 2HCl | 0.2 | | |
| Homocystine | 0.3 | | |
| Hydroxyproline | 0.15 | | |

This chromatographic system according to JONES and HEATHCOTE (8) was used, due to its high resolving power; even *leucine* and *isoleucine* are separated. The replacement of cellulose by *purified* cellulose (6) has only a minor influence on the separation (cf. Fig. 1 and Ref. (8)). On the other hand, the use of *purified* cellulose (6) has been found advantageous if *rechromatography* (i. e. chromatography in several systems: *multidimensional-Chromatography*) or *reflectance spectroscopy* of DNP-amino acids formed on the layer is to be carried out (4, 9). Moreover, the *non-destructive* detection technique described in this paper allows the *reaction chromatography* of amino acids (3, 4); i. e. conversion of amino acids into DNP-derivatives *at the origin* followed by *one* or *two-dimensional* separation of DNP-compounds formed, or *one-dimensional* chromatography of amino acids followed by treatment with DNFB and chromatography of DNP-derivates formed in situ in the *second dimension.* These techniques, which allow the *more unambiquous* identification of amino acids, especially from biological sources, are reported elsewhere (4). The application of *non-destructive* detection techniques in sequence analysis of peptides and in the analysis of amino acids in biological material (e. g. urine or blood) will be described in a subsequent paper.

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# Saure Glycosaminoglycane des normalen und cirrhotischen Leberbindegewebes vom Menschen

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(Eingegangen am 10. Juni 1968)

Es wird über ein Verfahren zur Isolierung saurer Glycosaminoglycane aus Lebergewebe vom Menschen und die Auftrennung in ihre Einzelkomponenten berichtet. Dabei ergibt sich, daß etwa 90% der sauren Glycosaminoglycane des normalen Leberbindegewebes zu etwa gleichen Teilen aus Heparan-Sulfat und Dermatan-Sulfat bestehen. Die cirrhotische Leber zeigt einen starken Anstieg dieser Komponenten bei gleichzeitig hohem Gehalt an Hyaluronat. Die Ergebnisse werden im Hinblick auf die angewandte Methodik und die pathophysiologische Aussage diskutiert.

*The acidic glycosamine glycans in the connective tissue of normal and cirrhotic human liver*

A method is reported for the isolation of acidic glycosamine glycans from human liver, and their separation into individual components. It was found that approximately equal quantities of heparan sulphate and dermatan sulphate together account for about 90% of the acidic glycosamine glycans of normal liver. These components are markedly increased in cirrhotic liver, and there is also a high concentration of hyaluronate. The methods used and the patho-physiological significance of the results are discussed.

Den sauren Glycosaminoglycanen kommt eine wesentliche Bedeutung für die Struktur und Funktion der Interzellularsubstanz der Bindegewebe zu. Ihre qualitative und quantitative Verteilung bestimmt in den verschiedenen Bindegeweben die für deren spezifische Funktionen typischen physikalisch-chemischen Eigenschaften.

Die sauren Glycosaminoglycane als Komponenten der interzellulären Strukturs substanz werden damit zu Parametern, welche durch ihre Verteilungsmuster den jeweiligen Zustand der Gewebe zu charakterisieren vermögen. Physiologische und pathophysiologische Prozesse am Bindegewebe gehen mit qualitativen und quantitativen Veränderungen der Verteilungsmuster saurer Glycosaminoglycane einher (1—13).

Die Lebercirrhose ist geprägt durch eine unkontrollierte Proliferation des Bindegewebes, die u. a. mit dem Auftreten reifer kollagener Fasern an Stelle des feinen reticulären Fasergerüstes und einer Vermehrung der

[1]) *Abkürzungen:* saure GAG (s. GAG) = saure Glycosaminoglycane; CPC = Cetylpyridiniumchlorid; DS = Dermatan-Sulfat; HPS = Heparan-Sulfat; HP = Heparin; CH-4-S = Chondroitin-4-Sulfat; CH-6-S = Chondroitin-6-Sulfat; CHS = Chondroitinsulfate; Hyal = Hyaluronat.