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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

THIN-LAYER CHROMATOGRAPHY OF AMINO ACID DERIVATIVES
WITH KIESELGEL G. N-(2,4-DINITROPHENYL)-AMINO
ACIDS AND 3-PHENYL-2-THIOHYDANTOINE*

/Following is the translation of an article by M. Brenner,
A. Niederwieser, and G. Pataki**, in the German-language
periodical Experientia, Vol. XVII, No. 4, 15 April 1961,
pages 145-153./

Dinitrophenyl-amino acids (DNP-amino acids) and phenylthiohydantoines (PTH-amino acids) are formed when proteins or peptides are treated with dinitrofluorobenzene or phenyl-mustard-oil and the condensation product is decomposed in an appropriate manner (1, 2). Their separation from the reaction mixture and, particularly, their identification are of considerable practical significance because, systematically applied, the reaction sequences named permit sequential analysis of peptide structures. Numerous authors have dealt with this problem.

The following processes were recommended for separation and characterization of DNP-amino acids: chromatography with neutral silica gel (1), buffered silica gel (3), silicic acid celite (4), kieselguhr (5), buffered Hyflo-Super-Cel (6), paper (7), acetylated paper (8), chlorinated rubber (9), Amberlite IRC-50 and Duolite C-25 (10), polyamide (11), ionophoresis with silica gel (12) and, finally, inverse current distribution (13). All the above methods require either a substantial amount of apparatus or a considerable amount of time.

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Detection of the individual PTH-amino acids presents additional effort. The original proposal for an alkaline (2) or acid (14) hydrolysis for the purpose of subsequent identification of the liberated amino acids was unsatisfactory, because this process destroys Arg, CysH, (Cys)₂, Ser, Thr, Try, Asp(NH₂), and Glu(NH₂). In 1953, Landmann et al. (15) as well as Sjöquist (16) reported the unidimensional paper-chromatographic separation of PTH-amino acids proper. However, the distinguishability failed, on the one hand (15), with respect to Met, Val, and Phe, as well as for Leu and Pro, and Lys and Tyr, and, on the other (16), for Thr and Val, Ileu and Val, as well as for Asp(NH₂) and Glu(NH₂). Recently, Sjöquist (17) has reported a new procedure which permits the separation and quantitative determination of the most important PTH-amino acids in four concurrent unidimensional paper chromatograms, using four different solvent systems, Asp, Lys, Pro, and Hypo can be determined only indirectly, while only the sum of Leu and Ileu can be determined. The detection limit is near 0.5-1 µg PTH-amino acid / Grote reagent (15, 18), iodine azide (16, 19), fluorescence test (20). Sjöquist, continuing an investigation by Röver, Fabre, and Desnuelle (21), also developed a columnar-chromatic separation and determination procedure for PTH-amino acids (22).

Both in the analysis of amino acid solutions (23, 24) and in the identification of DNP- and PTH-amino acids, the thin-layer chromatography method is by far the most advantageous. Primary advantages are the saving in time and sensitivity that is increased by a factor of 10 (25). Particularly in the case of DNP-derivatives, better separation is achieved in which, for example, DNP-Leu and DNP-Ileu can be easily distinguished.

DNP-Amino Acids

(Prepared by A. Niederwieser)

A. Acid- and Water-Soluble DNP-Amino Acids, Extractable with Ether

DNP-Arg, DNP-CySO₃H, Mono-DNP(Cys)₂, alpha-DNP-His (26), Di-DNP-His, alpha-DNP-Lys, and O-DNP-Tyr can be identified through chromatography in the system n-propanol/NH₃34% (7:3 v/v) either individually or, to the extent that this is practically feasible, side by side. Duration of the process is approximately 2 hours. Table I gives R_f-values and spot identification. In spite of the fact that DNP-Arg and delta-DNP-Lys cannot be fully separated, it is possible to detect both side-by-side on the basis of their differences in the ninhydrin reaction. DNP-CySO₃H and Mono-DNP(Cys)₂ in actuality would never occur side-by-side. The sample (1 µg pro DNP-amino acid) is applied to 1 µl glacial acetic acid or 0.5 n HCl. It is important to remove excess acid before the chromatographic process is started. After the solution is applied, the plate should be heated to 60° for a period of 10 minutes.

B. Acid-Insoluble DNP-Amino Acids, Extractable with Ether

The certain identification of a DNP-derivative which belongs to this relatively large group usually requires a two-dimensional chromatogram. Here we use, in the first dimension, the "Toluene" System of Biserte and Osteux (27) and, in the second dimension, either: chloroform/benzene alcohol/glacial acetic acid, or: chloroform/t-amyl alcohol/glacial acetic acid, or: benzene/pyridine/glacial acetic acid, or: chloroform/methanol/glacial acetic acid in the composition given below. Use of the two-phase "Toluene"-System involves pre-treatment of the plates and results in spots with so-called "beards", (28) but, in view of its excellent separation properties, this process, for the present, is considered indispensable.

To characterize the above-named systems, we shall first describe their separation effect in uni-dimensional chromatograms. It should be noted, however, that in the case of uni-dimensional chromatograms of DNP-amino acid mixtures, a not insignificant influence of the quantity ratio on the Rf-values can be determined (29). In the second dimension of two-dimensional chromatograms this is fortunately not the case. Here Rf values are influenced less by the quantitative ratio of the components than by the "prior history" of the layer (chromatography in the first dimension, intermediate drying). The effect mentioned proceeds to the point that one must differentiate between the Rf values which result directly or indirectly from systems #2 to 5 (this means after the action of the "Toluene" system in two-dimensional chromatograms) (see Table II). Since the "prior history" may be extensively standardized, the Rf values of the second dimension are easily reproducible. Consequently two-dimensional chromatography mixtures yield characteristic spot patterns which can hardly be falsified by Rf value fluctuations. An unknown sample is therefore not chromatographed alone, but together with a standard mixture which contains a sufficient quantity of each DNP-amino acid in question (0.2 μ g) so that individual components are just visible after two-dimensional separation (Figure 1). The composition of the sample solution may then be detected immediately and satisfactorily in most cases, by the intensity of the corresponding spots.

Solvent Systems and Rf Values

Solvent Quality. Toluene: Shake twice with 1/10 part by volume conc. sulfuric acid, with water, with 2N soda solution and water, dry over calcium chloride, and distill through a short column. Pyridine: Boil over barium oxide for 24 hours and distill through a short column. Ethylene chlorhydrin: 2-chlorethanol purified "Fluka"³¹. Ammonia: 25% "Merck" ammonium hydroxide diluted with distilled water. Benzyl alcohol: Shake with saturated bisulfite solution, wash with 2N soda, dry over sodium sulfate and vacuum distill under N₂ through a short column. Benzaldehyde mixed in alters Rf values rather strongly. Methanol and glacial acetic

acid: distill through a short column. Chloroform: distill twice through a short column. t-amyl alcohol: distill the fraction 100.5-102.0° from "Fluka" practical t-amyl alcohol. Benzene: similar to toluene.

The specifications with regard to mixture ratios of systems #1 to 5, listed below, refer to volumes at ca. 20° C.

Solvent Systems: #1: Toluene-pyridine-ethylene chlorhydrin - 0.8N ammonia (100:30:60:60). ("Toluene" system)²⁷. The upper phase serves for chromatography, the lower for pretreatment of the thin-layer (compare experimental method). Spots with large "beards"²⁸ result from this system. A certain loss in matter is involved.

#2: Chloroform-benzyl alcohol-glacial acetic acid (70:30:3). Symmetrical spots result from this system. 2,4-dinitrophenol³² and 2,4-dinitroaniline migrate to the top.

#3: Chloroform-t-amyl alcohol-glacial acetic acid (70:30:3). This system separates similarly to system #2. 2,4-dinitrophenol and DNP-Val migrate more closely to DNP-Leu. Instead t-amyl alcohol is more stable and more volatile than benzyl alcohol.

#4: Benzene-pyridine-glacial acetic acid (80:20:2). In filter chromatography³³ this system is very suitable for separation of the less polar DNP-amino acids. 2,4-dinitroaniline³² migrates to the top:
 $R_{DNP-Leu} = 1.28$.

#5: Chloroform-methanol-glacial acetic acid (95:5:1). Di-DNP-Tyr and Di-DNP-Lys, which have not been separated in any of the previously described systems, may be clearly distinguished by this system by filter chromatography.

Table II gives the R_f values in the solvent systems described. Table III summarizes the reaction time and several peculiarities which merit particular mention.

Separation effects in two-dimensional methods. Figure 1 shows separation of a standard mixture of 0.2 μ g DNP-amino acids each in a combination of systems #1 and #2. Separation does not occur for the leucine group, the valine group and for Di-DNP-Lys/Di-DNP-Tyr.

For separation of the latter a combination of #1 with #5 (Figure 2) or even #5 alone may be used. A combination of #1 and #4 (Figure 3) permits distinguishing isomeric leucine derivatives and isomeric valine derivatives.

C. Experimental Method

General Remarks

We have already described the general experimental method²⁴. The instructions therein should be followed exactly. In addition the following should be noted:

1. Layer thickness applied with the same coating instrument and with equal ratio of Kiesel gel G to water depends on the coating speed.
2. Relatively hard layers are obtained if the coated plates can be air dried overnight at 20° C. At temperatures only slightly over 100° C the gypsum contained in Kiesel gel G can no longer accomplish its function as binder. The layer becomes soft, almost powdery. Plates "activated" at higher temperatures (for example 2 hrs at 140° according to Cherbuliez, et al²⁵) become sensitive to atmospheric moisture³⁴, and it is not surprising if Rf value fluctuations occur with their use.
3. Irregular layers may be obtained with small plates (200 X 50 mm) despite the use of coating instruments. They serve excellently for orientation but less so for exact experiments.
4. Drawing of a dividing line for limiting the migration distance and time was recently discontinued without damage to the experiment. The advantage of a less rigid time schedule is gained hereby.

Some special hints are required by the use of the "toluene" system.

The "toluene" System.

Pretreatment of the thin layer. A filter paper lined separation chamber (DESAGA Co.) is prepared with the lower phase of the "toluene" system. On the bottom of the chamber a thick bent glass rod is placed as a grid. Two plates prepared with Kiesel gel G are placed in the middle of the grid with layer coated sides to the outside and each upper edge leaning against a chamber wall. To prevent the solvent on the filter paper from reaching the layer, these are separated by a thick pencil stroke parallel to the upper edge. This should stand overnight. The Kiesel gel accepts much moisture which is not noticeable by its appearance but by the fact that substances put on the plate during this treatment may diffuse considerably.

Chromatography on a plate pretreated in this manner probably depends on diffusion between two liquid phases. In any case the effect of pretreatment to the plates which remain in air, is practically lost after 45 minutes. This effect must be taken into consideration during use of the plates.

Applying the substances. If a plate is left unprotected in air for various periods between pretreatment and chromatography, and the logarithms of Rf values are plotted against the logarithm of corresponding times, after even 2 minutes, linear loss sets in. Therefore, the pretreated layer is immediately covered with a glass plate after its removal from the chamber leaving a strip of only 1.5 cm width free at the lower edge. The substance can now be applied calmly. If no more than about 5 minutes are required, the disturbance is of no importance. Application may be speeded up by the use of relatively concentrated solutions. Volume should not exceed 1.

possible, because evaporation speed of solutions applied to pretreated moist layers is smaller than on dry ones. When the application is finished the protective plate is carefully removed and the chromatogram immediately initiated.

Intermediate drying with two-dimensional chromatograms. It should remain in an air current (a well ventilated cupola) for 10 minutes, warmed in an oven for 10 minutes at 60° and cooled in air for 10 to 15 minutes. The second dimension can then immediately be chromatographed. Longer drying is not advisable because partial destruction of DNP-amino acids takes place with air contact: oxidation of DNP-Met may simulate the presence of Di-DNP-His with the chromatography that follows. If longer storage is necessary after intermediate drying, the layer must be covered with a glass plate and stored in the dark.

Visibility of spots, Application of UV light, Documentation. With the exception of O-DNP-Tyr all DNP-derivatives are yellow. 0.1 μ g amounts (one-dimensional chromatograms) or 0.5 μ g (two-dimensional chromatograms) give spots well visible when the plate is studied in daylight. The chromatogram must be copied within several hours since spots will fade with time.

The chromatogram is usually copied in counterlight with ink (soft point) on glassine, which has been fastened tightly to the layer by means of two paper clips. With practice even the weakest yellow spots can be recognized on the white Kiesel gel layer. Nevertheless in many cases it is easier to observe the plate by transmitted UV light (light source -- layer -- glass plate -- glasses -- eye). Here the DNP-amino acids can be observed as very dark spots. Substances in small concentrations which absorb weakly are hard to see, since the Kiesel gel-gypsum layer, itself absorbent, appears dark. If 0.5 μ g zinc silicate³⁵ is added to 25 μ g Kiesel gel G, the layer is lightened by fluorescence and O-DNP-Tyr even in small amounts (ca. 0.06 μ g) is visible.

Preparation of a UV photoprint 36 is particularly recommended. Gevaert GEVACOPY paper is placed with its sensitive side directly on the layer and secured to it with a glass plate. For several seconds UV light is applied through the layer onto the photo paper which is developed in the normal fashion to a positive. Figures 1 to 3 were prepared in this manner. Greatest sensitivity is obtained with an exposure time in which the background

of the negative does not appear completely black, but grey. With longer exposure the background of the negative becomes progressively darker; simultaneously the diameter of the lighter substance spots is reduced, to disappear completely with overexposure. Use may be made of this for separation of large spots which partially overlap, by dissolving them into smaller ones.

PTH-Amino Acids

(Prepared by G. Pataki)

Solvent systems used in paper chromatographic separation of PTH-amino acids 15-17, 20, are not usable with Kiesel gel G. Satisfactory separations were obtained however, with the mixtures shown in table IV; in proper combination systems #6 to 9 permit direct identification of 14 of 19 quoted PTH-derivatives in table IV.

PTH-Val, PTH-Phe, PTH-Met on one hand, as well as PTH-Leu and PTH-Ileu on the other, migrate together without exception. Generally they have to be identified after hydrolysis in free amino acid form; if PTH-Met is present alone, it is identifiable directly after treatment with H_2O_2 without hydrolysis. Often determining gross composition of a peptide (total hydrolysis) furnishes sufficient information to shorten the identification process of Val and Phe or Leu and Ileu.

Solvent Systems, Color Reactions of PTH-Amino Acids and Rf Values

Solvent quality. Chloroform: Stabilized with 1.5% ethanol ("Fluka" 31); Formic Acid: anhydrous ("Fluka" 31); Glacial acetic acid, methanol, methyl ethyl ketone: commercial qualities, distilled through a short column; Pyridine: boil 24 hours over barium oxide and distill through a short column.

The specifications with regard to mixture ratios of systems #6 to 10 refer to volumes at ca. 20° C.

Solvent systems. #6: chloroform. #7: chloroform-methanol (9:1). #8: chloroform-formic acid (100:5). #9: chloroform-methanol-formic acid (70:10:2). #10: methyl ethyl ketone-pyridine-water-glacial acetic acid (70:15:15:2).

Color reactions. Iodine azide used by Edman and Sjöquist²⁰ and newly by Cherbuliez²⁵ for visibility is not the ideal reagent for PTH-amino acids, according to our experience. With Kiesel gel G white spots are obtained on a light blue background, which are hard to see and disappear quickly. Grote's reagent would be an improvement, but is impractical in use and too time consuming. The chlorotolidine test works well. After chlorination, protection from air currents, it rests for 3 to 5 minutes.

Very careful spraying results almost instantaneously in almost perfect dots; further spraying increases the size of the spots and under certain conditions they will overlap with neighboring spots (compare dots 10 and 11 in Figure 4). PTH-Gly is the only PTH-amino acid which has a characteristic spot shape which immediately identifies it (Figure 4).

Spot size and Rf values. The dependence of spot size on substance amount was tested for PTH-Pro in system #7. If the compound is applied each time in 0.5 μ l methanol, the spot surface with loads of 0.55 to 72 μ g is about proportional to the log of the substance quantity. Rf value remains practically constant.

Table IV gives the Rf values which are observed when the substances are individually applied (0.5 μ g in 0.5 μ l methanol) and chromatographed. In the presence of mixtures, applied also in a total volume of 0.5 μ l, deviations in Rf values occur. Identification as in the case of DNP-amino acids should depend therefore on comparison with control substances or on spot patterns of a two-dimensional chromatogram (loaded as a single-dimensional chromatogram).

Identification of PTH-Amino Acids

A) Individual PTH-amino acids or simple mixtures. In view of the above mentioned uncertainty with regard to Rf values it is advisable to chromatograph unknown PTH-amino acids two-dimensionally in systems #7 and 8, and to make a temporary assignment on basis of spot patterns in Figure 4. This is confirmed by a second test in which several neighboring PTH amino acids in the spot patterns are also chromatographed³⁸. 11 substances may be directly identified.

Special cases: Asp, Glu: appearance of a spot in area 2 (Figure 4) may be due to PTH-Asp or PTH-Glu or both. Differentiation is obtained by a one-dimensional chromatogram in system #9; the correlation is not difficult if both substances are run as controls (compare Figure 5).

Pro: If the unknown lies in the upper right area of large spot 13, a one-dimensional chromatogram in system #6 using as control substances the PTH-derivatives of Val, Met, Phe, Ileu, Leu and Pro (compare Figure 6) is indicated. The test permits within one half hour a decision between PTH-Pro and (PTH-Ileu and PTH-Leu) and (PTH-Val and PTH-Met and PTH-Phe).

Met: If it is a matter of the last of these three possibilities, the presence of PTH-Met may be determined by a further one-dimensional chromatogram in the same system, if the sample is first treated with H_2O_2 to PTH-amino acid. A new plate is therefore charged 4 times (A, B, C, D) with equal quantities of the unknown, it is dried for 60 seconds (solvent methanol), and 0.5 μ l H_2O_2 added each time: to A 30/4, B 30/8, C 30/16, D 30/32 per cent H_2O_2 . It may be chromatographed after 2 minutes. Figure 7 shows the resulting substance distribution in comparison to oxidation.

Evaluation indicates where oxidation caused the fewest by-products (e.g., C in Figure 7). If a spot appears only at the starting point and perhaps just a faint spot further up, the presence of PTH-Met is proven. If clear spots appear above the starting point, then PTH-Val or PTH-Phe or a mixture of both is also present. No spot at the starting point indicates the absence of PTH-Met; as in the case of all negative proofs, care should be taken.

Leu, Ileu, Phe, Val (and Met): If on the basis of figures 4 and 6 one or more of these amino acids are present, 1 μ g of the sample is hydrolyzed for 12 hours with 6N HCl at 120° in a tube, evaporated to dryness, dissolved in water and chromatographed one-dimensionally in n-BuOH-AcOH-H₂O (60:20:20) or phenol-H₂O (75:25); Phe, Val and Met may be differentiated from each other as well as from the leucines²⁴. To differentiate the leucines in the absence of Phe, Val and Met, a transmission chromatogram according to Figure 8 in system #10 is used³³. This method for leucine separation can be utilized also if the PTH-leucine mixture must first be separated from other PTH-amino acids by a one-dimensional chromatogram according to Figure 6 (compare complex mixtures). In this case the leucine spot is extracted from the one-dimensional chromatogram with methanol²⁴, hydrolyzed, and chromatographed by the transmission method³³. For positive identification the sample used for the one-dimensional chromatogram must contain a minimum of 1 μ g PTH-Leu or PTH-Ileu.

Remarks. If hydrolysis is unnecessary the above described experiments need a maximum of 1 μ g material per PTH-amino acid. It is therefore often desirable to save time at the cost of some substance and to perform these chromatograms simultaneously rather than subsequently. Time requirements for the various operations are: application 5 min., operating time 10 to 30 min., drying 5 min., chlorination 25 min., toluidine 2 min., H₂O₂ 12 min.

A) Complex mixtures. If the two-dimensional chromatogram (Figure 4) shows many spots, extraction may eventually be necessary. A new chromatogram is prepared, chlor-toluidine treatment is omitted, and the area of spots 1 and 2 is stripped in one direction and 13 in the other from the plate. For extraction Kiesel gel G is suspended in about 1 ml methanol. Temperature is held at 40 to 50° for 2 minutes, then filtration, followed by 3 washes with hot methanol and evaporation to dryness.

The combined material from spots 1 and 2 are chromatographed one-dimensionally in system #9 according to Figure 5 with PTH-Arg, PTH-Asp and PTH-Glu as controls.

The extract from spot 13 is treated according to paragraph A for Pro, Met, Leu, Ileu, Phe, Val (and Met).

Conclusion

DNP-Amino acids. Except for DNP-Hydro which was not examined, the DNP-derivatives of all protein-amino acids (Table I and Table II) may be simply separated by three simultaneous chromatograms in different systems (Table I, Figures 2 and 3). The time involved is only one to three hours. For positive proof of the presence of a DNP-amino acid by means of a UV-photocopy only 2×10^{-3} M. even 10^{-4} M is necessary for one-dimensional chromatograms: for example 0.02 μ g DNP-Ser is sufficient. For large loading (approximately 100 μ g) it is often possible to identify a 0.05% (1) of an unknown DNP-amino acid.)

PTH-Amino acids. One two-dimensional chromatogram (Figure 4) and two one-dimensional chromatograms (Figures 5 and 6) without protracted balancing, and operating simultaneously, can separate 14 of the 19 PTH-amino acids of Table IV. Aided by a simple oxidation technique (Figure 7) and by acid hydrolysis with subsequent thin layer chromatographic identification of the freed amino acid, which is stable with hydrolysis in any case, the missing five substances may be identified without further efforts (Figures 8 and 24). A modified chlor-tolidine test is suitable for making the PTH-amino acids visible; the detection limit lies at ca. 3×10^{-4} M, for example 0.05 μ g PTH-Pro(1).

FOOTNOTE APPENDIX

1. F. Sanger, *Biochem. J.*, 39, 507, 1945; 40, 261, 1946. - R. Porter and F. Sanger, *Biochem. J.*, 42, 287, 1948; F. Sanger, *Biochem. J.*, 45, 562, 1949.
2. Edman, P., *Acta chem. scand.*, 4, 283, 1950.
3. Blackburn, S., *Nature*, 163, 955, 1949. - W. R. Middlebrook, *Nature*, 164, 501, 1949.
4. Green, F. Ch. and Kay, L. M., *Anal. Chem.*, 24, 726, 1952.
5. Mills, G. L., *Biochem. J.*, 50, 707, 1952. - Braunitzer, G., and Reuther, K. - H., *Makromol. Chem.*, 18-19, 501, 1956.
6. Bell, P. H. et al., *Ann. N.Y. Acad. Sci.*, 51, 897, 1949.
7. Summary by G. Biserte et al. in *J. Chromatogr.*, 2, 225, 1959 and 3, 85, 1960.
8. Burton, H. S., *Chem. and Ind.*, London, 1954, 576.
9. Partridge, S. M. and Swain, T., *Nature*, 166, 272, 1950.
10. Seki, T., *J. Biochem. (Japan)*, 47, 253, 1960.
11. Steuerle, H. and Hille, E., *Biochem. Z.*, 331, 220, 1959. - Hille, E., *Biochem. Z.*, 333, 269, 1960. - Hoermann, H. and H. von Portatius, *Z. physiol. Chem.*, 315, 141, 1959.
12. Syngé, R. L. M., *Biochem. J.*, 44, 542, 1949.
13. Woolley, D. W., *J. biol. Chem.*, 179, 593, 1949.
14. Fraenkel-Conrat, H., *The Chemical Structure of Proteins*, a Ciba Foundation Symposium (Little, Brown & Co., Boston, 1953), page 102.
15. Landmann, W. A., Drake, M. P., and Dillaha, J., *J. Amer. chem. Soc.*, 75, 3638, 1953.
16. Sjöquist, J., *Acta chem. scand.*, 7, 447, 1953.
17. Sjöquist, J., *Biochem. Biophys. Acta*, 41, 20, 1960.
18. Grote, J. W., *J. biol. Chem.*, 93, 25, 1931.
19. Chargaff, E., Levine, C., and Green C., *J. biol. Chem.*, 175, 67, 1948.
20. Edman, P. and Sjöquist, J., *Acta chem. scand.*, 10, 1507, 1956.
21. Röver, M., Fabre, C., and Desnuelle, P., *Biochem. Biophys. Acta*, 12, 547, 1954.
22. Sjöquist, J., *Arkiv Kemi*, 11, 151, 1957.
23. Nuernberg, E., *Arch. Pharm.*, Berlin, 292/64, 610, 1959.
24. Brenner, M., and Niederwieser, A., *Exper.*, 16, 378, 1960. - Niederwieser, A., and Pataki, G., *Chimia*, 14, 378, 1960.
25. See also Cherbuliez, E., Baehler, B., and Rabinowitz, J., *Helv. chim. Acta*, 43, 1871, 1960.
26. im-DNP-His basically belongs to the group of acid soluble DNP-derivatives. According to H. Zahn and J. Pfannmueller, *Biochem. Z.*, 330, 97, 1958, it is not detectable of corresponding DNP-peptides because of its instability. Our research is therefore limited to α -DNP and Di-DNP-His.
27. Biserte, G. and Osteux, R., *Bull. Soc. Chim. biol.*, 33, 50, 1951.
28. Cf. Hais, I. M. in Hais and Macek, *Handbuch der Papierchromatographie / Handbook of Paper Chromatography*, VEB Gustav Fischer Publishers, Jena, 1958, page 147.

29. We know from our work with paper chromatography that particularly dinitrophenol (31) has this kind of disturbing effect (27).
30. A solution of 1 mg DNP-amino acid, each, in 5 ml acetone can be kept in the refrigerator for at least 4 weeks. For one test we need 1 μ l.
31. Buchs, SG, Switzerland.
32. 2,4-dinitrophenol and 2,4-dinitroaniline are byproducts of synthesis and of acid hydrolysis of DNP-peptides.
33. Brenner, M. and Niederwieser, A., *Durchlaufende Duennschicht Chromatographie /Continuous Thin-layer Chromatography*, Exper., in print, 1961.
34. Kirk, R. E. and Othmer, D. F., *Encyclopedia of Chemical Technology*, Interscience Publishers, Inc., New York, 1948, Vol. 2, page 773.
35. Zinc silicate luminous substance P 1, type 118-2-7, General Electric, Cleveland, Ohio.
36. Cf. also Gruenewald, H., *Photographie und Wissenschaft /Photography and Science*, 7, 8, 18, 1959.
37. Reindel, F. and Hoppe, W., *Chem. Ber.*, 87, 1103, 1954. For modification, see Brenner, M. et al., *Helv. chim. Acta*, 40, 1497, 1957. Chlorine treatment should here be extended to 25 minutes.
38. A direct comparison with a standard mixture (cf. DNP-amino acids) is expensive because of the limited stability (72 hours) of methanolic PTH-amino acid solutions.

TABLE APPENDIX

TABLE I

IDENTIFICATION OF ACID AND WATER SOLUBLE DNP-AMINO ACIDS
BY THIN-LAYER CHROMATOGRAPHY IN THE SYSTEM
n-PROPANOL/AMMONIA 34% (7 : 3 v/v)

	Rf • 100	Color	Absorption in UV	Color With Ninhydrin
Mono-DNP-(Cys) ₂	29	yellow	+	brown
DNP-CySO ₃ H	29	yellow	+	yellow
α-DNP-Arg	43	yellow	+	yellow
ε-DNP-Lys	44	yellow	+	brown
O-DNP-Tyr	49	colorless	+ ^a	violet
α-DNP-His	57	yellow	+	yellow
Di-DNP-His	65	yellow	+	yellow

(a) See Visibility of Spots, Application of UV Light, Documentation.

TABLE II

Rf VALUES (a) of ETHER SOLUBLE DNP-AMINO ACIDS IN SOLVENT SYSTEMS 1 to 5 WITH ONE-DIMENSIONAL, RISING OR HORIZONTAL CHROMATOGRAPHY

	1 ^a	2		3		4 ^c			5 ^e		
	(1)	(1)	(2)	(1)	(2)	(1)	horizontal	(2)	(1)	horizontal	(2)
DNP-AAB ^b	46	72	44	73	42	52	52	55	79	85	75
DNP-AAC ^f	79	92	66	83	57	105	108	109	108	101	106
DNP-Ala	34	54	35	60	34	32	33	38	59	66	58
DNP-β-Ala	27	71	57	73	50	89	98	100	99	95	102
DNP-Asp	02	13	08	09	13	06	05	11	07	06	06
DNP-Glu	01	26	17	31	21	12	12	23	12	12	14
DNP-Gly	27	32	22	40	23	17	18	22	31	38	31
DNP-Ileu	64	83	63	81	57	107	107	107	100	101	104
DNP-Leu	66	82	62	80	54	100	100	100	100	100	100
DNP-Nieu	69	82	60	80	52	86	90	88	101	100	98
DNP-Met	55	70	39	69	38	43	43	47	72	81	74
DNP-Met. O ₂ ^g	17	—	—	—	04	03	03	02	10	10	07
DNP-Phe	67	75	46	74	41	44	46	52	81	86	76
DNP-Pro	29	65	41	67	38	58	59	62	78	84	75
DNP-Sar	23	56	35	57	32	34	35	41	59	65	60
DNP-Ser	15	11	10	11	10	09	10	14	07	08	07
DNP-Thr	20	17	13	15	12	12	14	20	09	11	11
DNP-Try	65	69	38	69	31	23	25	33	54	61	49
DNP-Val	53	79	56	77	51	76	81	85	91	98	86
DNP-Nval	56	77	52	76	48	65	70	75	86	95	89
Di-DNP-(Cys) ₂	—	03	02	01	01	00	00	02	00	02	02
Di-DNP-His	53	11	09	08	04	05	04	08	12	16	14
Di-DNP-Lys	74	56	35	60	30	12	13	19	66	73	65
Di-DNP-Orn	70	34	23	40	20	06	06	10	39	46	39
Di-DNP-Tyr	76	58	35	60	30	17	16	19	57	65	57
2,4-DNP-OH ^h	41	100	76	83	55	22	21	23	148	102	111
2,4-DNP-NH ₂ ^h	90	90	84	72	63	115	128	129	131	101	115

(a) Rf . 100 shown here. (b) Cf. text on experimental method. (c) Rf values related to DNP-Leu. (d) After "pretreatment" of layer with chromatography in "toluene" system (No. 1) and intermediate drying (see text). (e) AAB = α-amino butyric acid. (f) AAC = α-amino caprylic acid. (g) Met. O₂ = methionine sulfon.

Legend: (1) rising
(2) indirect

TABLE III

NOTEWORTHY SEPARATION EFFECTS AND OPERATING TIMES FOR
ONE-DIMENSIONAL CHROMATOGRAPHY IN SOLVENT SYSTEMS 1 - 5

System Nr.	(1)		(2)		(3)	(4)
	Dinitro-anilin ²²	Dinitro-phenol ²²	Leu, Ileu, Nleu	Val, Nval	Tyr, Lys	
1	+	- ^a	-	-	-	1 h/15 cm
2	+	+	-	-	-	1 1/2 h/10 cm
3	- ^a	- ^b	-	-	-	1 h/15 cm
4 ^c	+	- ^c	+	+	-	2-3 h
5 ^d	- ^d	- ^d	-	+	+	2-3 h

- (a) 2,4-dinitrophenol lies between DNP-Val and DNP-Ala.
 (b) 2,4-dinitrophenol lies near DNP-Leu.
 (c) 2,4-dinitrophenol lies between DNP-Ala and DNP-Gly.
 (d) 2,4-dinitrophenol and 2,4-dinitroaniline lie slightly above DNP-Leu.
 (e) 2,4-dinitroaniline lies between DNP-Phe and DNP-Met.
 (f) Horizontal chromatography (cf. note 33). DNP-Leu moves about 10 cm in 2-1/2 hours.

Legend: (1) separation of DNP-amino acids from
 (2) separation of DNP-derivatives from
 (3) separation of Di-DNP-derivatives from
 (4) running time

TABLE IV

RF VALUES OF PTH-AMINO ACIDS IN SOLVENT SYSTEMS 6-9
WITH ONE-DIMENSIONAL RISING CHROMATOGRAPHY.
FOR EXPERIMENTAL METHOD SEE (24)

PTH	Nr. 6 CHCl ₃	Nr. 7 CHCl ₃ - CH ₃ OH (9:1 v/v)	Nr. 8 CHCl ₃ - HCOOH (100:5 v/v)	Nr. 9 CHCl ₃ -CH ₃ OH- HCOOH (70:30:2 v/v)
Ala	0.16	0.68	0.39	~1
Arg	0	0.01	0	0.24
Asp	0	0.01	0.13	0.70
Asp(NH ₂)	0	0.23	0.07	~1
Glu	0.01	0.04	0.17	0.75
Glu(NH ₂)	0.01	0.28	0.08	~1
Gly	0.10	0.56	0.33	0.90
His	0.01	0.29	0	~1
Ileu	0.40	0.77	0.57	~1
Leu	0.40	0.77	0.60	~1
Lys	0.12	0.71	0.34	~1
Met	0.33	0.75	0.51	~1
Met-Sulfoxyd	0.01	0.40	0.12	~1
Phe	0.28	0.74	0.50	~1
Pro	0.60	0.82	0.65	~1
Thr	0.04	0.45	0.15	~1
Try	0.13	0.62	0.39	~1
Tyr	0.03	0.47	0.21	~1
Val	0.32	0.74	0.55	~1

FIGURE APPENDIX

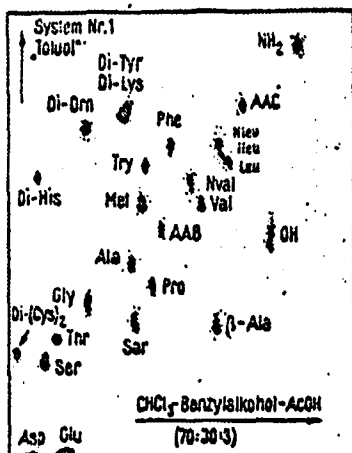


Figure 1. Two-dimensional chromatogram of a standard mixture of 0.2 μ g DNP-amino acids, each, in systems 1 and 2. Symbols: OH = 2,4-dinitrophenol (32), NH₂ = 2,4-dinitroaniline (32), Di = Di-DNP-derivatives, cf. also Table II. UV photocopy, original 12 X 10 cm.

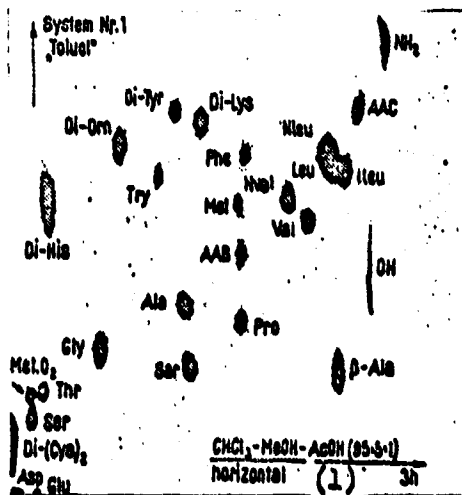


Figure 2. Two-dimensional chromatogram of 1 μ g DNP-amino acid each, in systems 1 and 5. Symbols as in Figure 1. Original 13 X 13 cm.

Legend: (1) continuous

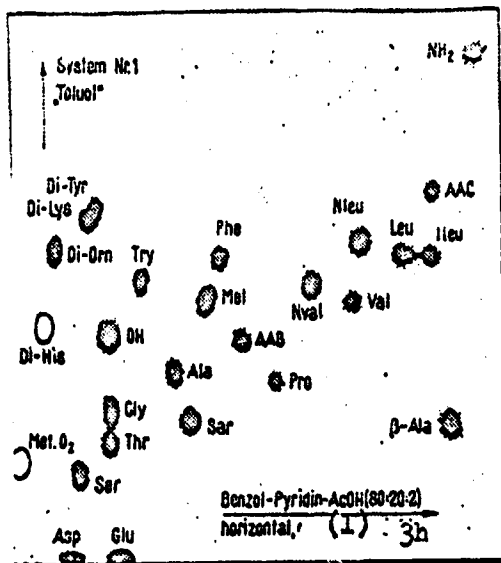


Figure 3. Two-dimensional chromatogram of a mixture of 1 μ g DNP-amino acid, each, in systems 1 and 4. Symbols: cf. Figure 1; Di-His and Met.O₂ were not run in the chromatogram shown.

Legend: (1) continuous

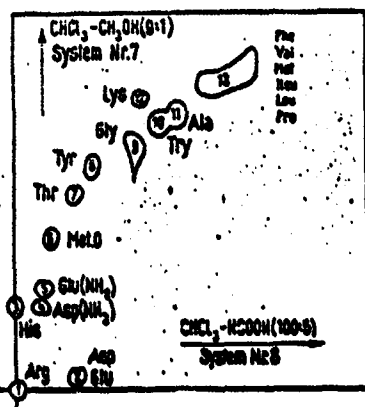
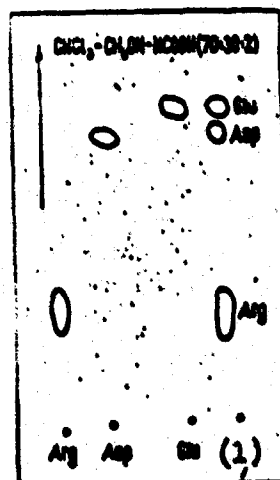


Figure 4. Two-dimensional chromatogram of a mixture of 0.5 μ g PTH-amino acid in systems 7 and 8. (Met. O = methionine sulfoxide) original 10 X 10 cm.

Figure 5. One-dimensional chromatogram in system 9 for proof of presence of PTH-Asp and PTH-Glu. Controls: 0.5 μ g PTH-Arg in the sample only in the special case of complex mixtures (see text). Migrating distance 11 cm.

Legends: (1) sample



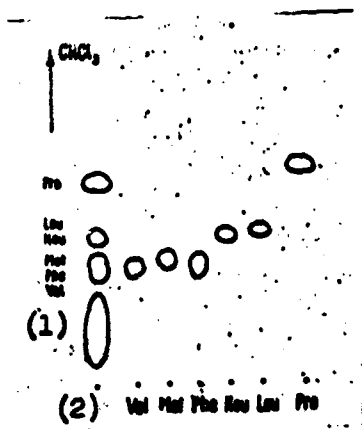
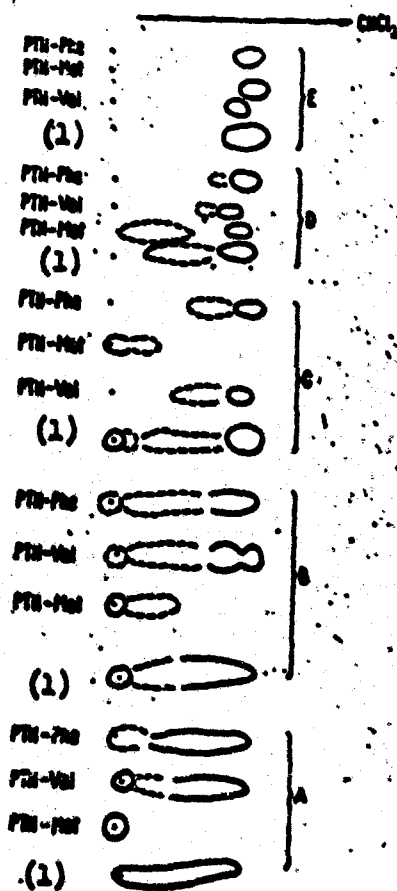


Figure 6. One-dimensional chromatogram in system 6 for proof of presence of PTH-Pro, PTH-Leu and PTH-Ileu, and PTH-Met, PTH-Phe and PTH-Val. Control: 0.5 μ g each. Migrating distance 10 cm.

Legend: (1) all other PTH-amino acids
(2) sample

Figure 7. Oxidation of PTH-amino acids (1 μ g each) of the "methionine group," by increasing amounts of H₂O₂ by itself or in mixtures (cf. test). E = comparison without H₂O₂. Migrating distance 10 cm.

Legend: (1) all three.



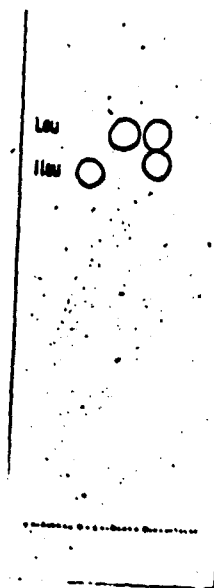


Figure 8. Transmission chromatogram in system 10 for separation of Leu and Ileu (5 hrs, 1 μ g Leu and Ileu, each). Migrating distance of Leu 10 cm.