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107. Yukiho Kubota : Separation of N-2,4-Dinitrophenylamino Acids by Paper Chromatography.

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The method for the N-terminal group analysis of peptides and proteins, first applied to insulin by Sanger,[1] has since been used extensively. The N-terminal amino acids of peptide chains are converted into their N-2,4-dinitrophenyl (DNP) derivatives with 1-fluoro-2,4-dinitrobenzene (FDNB), and after hydrolysis, the resulting DNP-amino acids are separated by chromatography on silica gel.[1,2] Resolution of a mixture of DNP-amino acids has also been achieved on chromatographic columns of kieselguhr,[3] Celite,[4] chlorinated rubber,[5] silicic acid-Celite,[6] and ion-exchange resin.[7]

On the other hand, some 25 different solvent systems have so far been proposed for paper chromatography. Most of them, however, have met with only limited success in separating all the ether-soluble DNP-amino acids owing to the tailing of spots and similarity of Rf values. Their separation on a single piece of paper has been achieved by Levy[8] using a two-dimensional techinique, which employs the toluene-chloroethanol-pyridine-0.8*N* ammonia system for the first dimension, and 1.5*M* phosphate buffer for the second. The ether-soluble DNP-amino acids are well separated with these solvent systems which are now being widely used for the amino acid sequence analysis of proteins. Despite the extensive applicability, however, several disadvantages have been experienced in this method. First, DNP-aspartic and -glutamic acids cannot be separated completely with these solvent systems, unless the concentration of the phosphate buffer is increased to 2.0 or even to 2.5*M*. At this concentration, however, the separation of other DNP-amino acids is impaired. Second, the separation of DNP-hydroxyproline is not always satisfactory. This often makes it difficult to apply this method to the analysis of amino acid in the protein of connective tissue which usually contains a large amount of hydroxyproline. Third, the two-dimensional procedure is somewhat troublesome when the sample solution to be chromatographed contains only a few kinds of DNP-amino acids.

Although the paper chromatography of DNP-amino acids has thus far encountered such difficulties, it has some advantages over column chromatography. In the first place, several chromatograms can be run in a single apparatus. This makes it possible to run chromatograms of the unknown and control simultaneously, and consequently eliminate errors due to variation in the experimental conditions which may take place if the chromatograms are run separately. In the second place, both one- and two-dimensional techniques are available. Combined with a variety of solvent systems, this offers an effective means for amino acid analysis.

In a previous paper,[9] the author reported that alanine, aspartic acid, and glycine are the N-terminals of parotin, and outlined a method for the separation of DNP-amino acids

*1 Hongo, Tokyo (久保田幸穂).

1) F. Sanger : Biochem. J., **39**, 507 (1945).
2) R.R. Porter, F. Sanger : *Ibid.*, **42**, 287 (1948).
3) G.L. Mills : *Ibid.*, **50**, 707 (1952).
4) J.L. Perrone : Nature, **167**, 513 (1951).
5) S.M. Partridge, T. Swain : *Ibid.*, **166**, 272 (1950).
6) F.C. Green, L.M. Kay : Anal. Chem., **24**, 726 (1952).
7) T. Seki : J. Biochem. (Tokyo), **47**, 253 (1960).
8) A.L. Levy : Nature, **174**, 126 (1954).
9) Y. Kubota : Endocrinol. Japon., **7**, 175 (1960).

by serial use of one-dimensional paper chromatography. This method separated most of the ether-soluble DNP-amino acids, dinitroaniline, and dinitrophenol. The separation of DNP-tryptophan and the water-soluble DNP-amino acids, however, was not attained by any combination of the solvent systems reported. Moreover, the previous method did not employ a two-dimensional technique. This makes the procedure somewhat complex, if a mixture of more than ten kinds of DNP-amino acids is submitted to chromatographic analysis.

For above reasons, attempt was made to improve the experimental procedure and solvent system for the paper chromatography of DNP-amino acids. The present paper describes one- and two-dimensional techniques available for the separation of the ether- and water-soluble DNP-amino acids. They are applied, as examples, to the estimation of the N-terminal amino acid of bovine plasma albumin and the qualitative analysis of amino acids of parotin.

Experimental

Filter Paper——Sheets of 10×40 or 40×40 cm. Whatman No. 1 filter paper were used instead of Schleicher and Schüll No. 2043a or Toyo Roshi No. 50 papers which had been employed in the previous work.[9]

Chromatographic Jar——Since the solvent systems used were composed of both volatile and nonvolatile liquids, proper equilibrium was much more difficult than with the usual chromatographic solvents. Some special devices were, therefore, made in constructing the apparatus. Cylindrical glass jars, 45 cm. in height and 18 cm. in diameter, with a ground-glass rim at the top were used. The rim was greased with white petrolatum or silicone grease, and covered with a well-fitting glass lid in order to obtain an air-tight seal. At the center of each lid, a 200-cc. separatory funnel and a glass valve which keeps the pressure of the atmosphere in the jar constant were attached through a rubber stopper. In the ascending run, a small funnel with a long glass stem which also serves as a supporter of the filter paper was placed beneath the separatory funnel. The chromatographic solvent was introduced into the jar through these funnels without opening the lid. The inside wall of the jar was covered with a cylinder of filter paper, whose lower end was dipped about 1 cm. into the water-rich phase covering the bottom of the jar. The paper cylinder soaked up the liquids continually and saturated the atmosphere with vapor of the solvent. A large glass petri dish, 15 cm. in diameter, containing 100 cc. of the organic solvent-rich phase was placed at the bottom of the jar in order to ensure saturation. A smaller glass petri dish, 12 cm. in diameter, was placed concentrically in the large one. The chromatographic filter paper, curled into a vortical cylinder, if necessary, was placed in the smaller petri dish by means of the supporter. In the descending run, the filter paper was suspended from a glass trough, 12.5 cm. in length, 6.5 cm. in width, and 3.5 cm. in depth, which was placed on a stainless steal stand.

Temperature Control——All jars were enclosed in a thermostatically regulated box and maintained at 20°±1°. The air in the box was circulated by a fan.

Solvent Systems——The introduction of a large DNP-group into the free amino group of amino acids diminishes their structural characteristics and enhances the tendency of tailing of spots. This leads to the difficulty in separating DNP-amino acids on a filter paper. This difficulty could be overcome by the use of solvent systems prepared by mixing polar and nonpolar solvents, and adding AcOH or NH_4OH. In order to facilitate the elution and rechromatography procedures, non-volatile buffers were not added with the exception of salting-out type chromatography.

The following solvent systems (in volume ratio) were used successfully.

(1) Toluene-AcOEt-EtOH-1.5*N* NH_4OH (1:1:1:1)
(2) Toluene-glacial AcOH-H_2O (2:1:1)
(3) Toluene-$CHCl_3$-5% AcOH (1:1:2)
(4) $CHCl_3$-BuOH-1% AcOH (4:1:5)
(5) BuOH-cyclohexane-0.2% AcOH (9:1:10)
(6) BuOH-cyclohexane-1.5*N* NH_4OH (9:1:10)
(7) 0.5*M* Na citrate and 0.1*M* citric acid, pH 5.4
(8) Toluene-1% AcOH (1:1)

With the exception of system (7), they were prepared by mixing the solvents in a separatory funnel at the operating temperature and allowing the two phases formed to separate. The organic solvent-rich phases were used as the chromatographic solvent.

Running Solvents——When the chromatogram was run with a buffer solution, the descending technique was employed to avoid an uneven distribution of the salts on the filter paper. In other

cases, the ascending technique was used. Since most of the solvents used are fast-running, disturbance of the chromatogram occurs owing to the rush of the solvents on the paper if they are run downward.

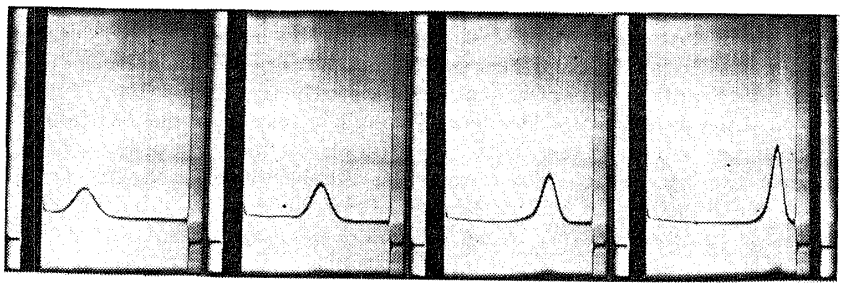
Prior to running the solvents, the filter paper on which sample solution was placed was allowed to equilibrate for more than 5 hr., preferably overnight, in the jar. The solvents were then poured into the smaller petri dish as already described.

Solutions of DNP-Amino Acids——Et_2O-soluble DNP-amino acids, dinitroaniline, and dinitrophenol were dissolved in MeCOEt to 20~50 m*M* concentration. H_2O-soluble DNP-amino acids were dissolved in HCOOH to about 30 m*M* concentration because of their scarce solubility in MeCOEt.

Placing Sample Solution on the Filter Paper——The sample solution was transferred to the filter paper with a glass capillary pipet or 'Agla' micrometer syringe. The spot was placed 6 cm. from the lower end of the filter paper in the ascending run, and 11 cm. from the upper end in the descending run. In the two-dimensional chromatography, the spot was placed at a point 6 cm. from the two sides of the paper. When, however, the chromatogram was run by the ascending technique in the first dimension and by the descending in the second, the spot was placed 6 cm. from the side perpendicular to the first solvent flow and 11 cm. from that to the second.

Estimation of DNP-Amino Acids——The spot was cut out about 5 mm. beyond its periphery which was traced with a pencil under ultraviolet ray of 360 mμ in a Chromato-Vue apparatus (Ultra-Violet Products, Inc.). The DNP-amino acid was extracted with 4 cc. of 1% $NaHCO_3$ in a test tube at 55~60° for 15 min.[10] After cooling for 15 min. at room temperature, the optical density was read at 360 mμ for typical DNP-amino acids and at 385 mμ for DNP-proline and -hydroxyproline.

Determination of N-Terminal Amino Acid of Bovine Plasma Albumin——A commercial preparation of bovine plasma albumin (Fraction No. V of Cohn, Daiichi Pure Chemicals Co.) was used. Ultracentrifugal analysis revealed the presence of a trace of a faster sedimenting component, globulin, in the preparation as shown in Fig. 1. Its purity, however, was sufficiently high for the check



126 min. 86 min. 54 min. 22 min.

Fig. 1. Sedimentation Pattern of Bovine Plasma Albumin
(The direction of contrifugal force is to the left)
Buffer : 0.01*M* AcONa and 0.01*M* AcOH in 0.10*M* KCl, pH 4.6, ionic strength, 0.1
Protein concentration : 1.0 g./100 cc.
Rotor speed : 59,780 r.p.m. Angle of phase plate : 70°

of the present method. The dinitrophenylation of the protein was made according to the procedure reported previously.[9] 100 mg. of DNP-albumin was hydrolyzed with 10 cc. of 6*N* HCl at 105~110° under reflux for 8 hr. The hydrolyzate was extracted with four 5-cc. portions of Et_2O. The combined Et_2O extract was evaporated to dryness *in vacuo* and the residue was quantitatively transferred to the filter paper with MeCOEt. Four chromatograms were run with solvent systems (1), (2), (4), and (5).

Detection of Amino Acids of Parotin——The same preparation of parotin that had been submitted to the molecular weight measurement[11] was used. A mixture of 20 mg. of parotin sealed in a small glass tube with 2 cc. of 20% HCl was heated in an oil bath at 105~110° for 8 hr. The hydrolyzate was evaporated to drypess *in vacuo* over P_2O_5 and solid NaOH. The dried residue was dissolved in 1 cc. of H_2O, roughly neutralized with $NaHCO_3$, and dinitrophenylated with 2 cc. of 4% EtOH solution of FDNB and 80 mg. of $NaHCO_3$ according to the method of Sanger.[1] After shaking

10) H. Fraenkel-Conrat, J. I. Harris, A. L. Levy : "Method of Biochemical Analysis," **2**, 359 (1959), Academic Press Inc., New York.
11) Y. Ito, Y. Kubota, Y. Shibuya : J. Biochem. (Tokyo), **47**, 422 (1960).

for 2 hr., EtOH was distilled off *in vacuo* and the residual aqueous solution was extracted with three 1-cc. portions of Et_2O. This removed excess FDNB and dinitrophenol. The aqueous layer was acidified with conc. HCl and extracted again with Et_2O in the same manner. The combined Et_2O extract was evaporated to dryness *in vacuo* and the residue was dissolved in MeCOEt. The aqueous layer was evaporated to dryness *in vacuo* over P_2O_5 and NaOH, and the residue was dissolved in HCOOH. The chromatographic detection of DNP-amino acids in the two solutions was made according to the procedures described below.

Results

Rf Values of DNP-Amino Acids——The Rf values of 20 DNP-amino acids, dinitroaniline, and dinitrophenol are listed in Table I. In a separate run, these values were highly

Table I. Rf Values of DNP-Amino Acids

Solvent system / DNP-amino acid	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Alanine	0.24	0.71[b]	0.59	0.95	0.71	0.58	0.37	
Arginine (α-mono)	0.26	0.0	0.0	0.03	0.60	0.63[a]	0.42	
Aspartic acid	0.01	0.04	0.01	0.69[c]	0.40	0.03	0.55*e*	
Cystine (bis)	0.13*e*	0.17*e*[a]	0.0	0.95	0.58	0.39	0.18	
Glutamic acid	0.01	0.10	0.01	0.88	0.52	0.03	0.51*e*	
Glycine	0.15	0.39[a]	0.18[a]	0.89	0.49	0.42	0.29	
Histidine (α, Im-bis)	0.65	0.0	0.0	0.01	0.0[a]	0.75[a]	0.0	
Hydroxyproline	0.07	0.05	0.02	0.72	0.49	0.27	0.54	
Isoleucine	0.60	0.95	0.93	0.97	0.94	0.85	0.38	
Leucine	0.62	0.95	0.91	0.97	0.94	0.87	0.43	less
Lysine (α, ε-bis)	0.89[a]	0.91	0.41[a]	0.97	0.88	0.87	0.04	than
Lysine (ε-mono)	0.14*e*	0.0	0.0	0.05	0.56	0.47[a]	0.43	0.70
Methionine	0.53[a]	0.91	0.79	0.96	0.85	0.76	0.37	
Phenylalanine	0.62	0.95	0.76*e*	0.96	0.92	0.85	0.27	
Proline	0.21	0.79[a]	0.72	0.95	0.75	0.50	0.46	
Serine	0.10	0.03	0.01	0.55[a]	0.40	0.34	0.40	
Threonine	0.14	0.08	0.03	0.82[a]	0.56	0.45	0.48	
Tryptophan	0.55	*t*	*t*	0.95	0.92	0.81	0.12	
Tyrosine (O, N-bis)	0.94[b]	0.95	0.60	0.95	0.93	0.92	0.0	
Valine	0.47[a]	0.94	0.87	0.96	0.92	0.77	0.41	
2,4-Dinitroaniline	0.95	0.70	0.95	0.96	0.93	0.91	0.0	0.84[a]
2,4-Dinitrophenol	0.24	0.96	0.96	0.96	0.82	0.62	0.28	0.99[a]

e: Elongated spot.
t: Tailing.
a) Separable from all others sufficiently for identification.
b) Separable from all others if dinitroaniline is absent.
c) Separable from all others if DNP-hydroxyproline is absent.

reproducible, if the temperature was kept at about 20° and the filter paper was saturated completely with vapor of the solvent. When, however, a mixture of DNP-amino acids was chromatographed, the Rf values varied slightly although it did not impair the effectiveness of the separation.

Removal of Dinitrophenol——The hydrolyzate of DNP-protein usually contains a considerably large amount of dinitrophenol which is extracted into the ether fraction. Since its spot partly overlaps those of several DNP-amino acids, it is preferable to remove it before chromatography. Its removal on filter paper was achieved with solvent system (8), as shown in Fig. 2-A. This process simultaneously removed dinitroaniline from DNP-amino acids. After cutting out both spots, DNP-amino acids were extracted with acetone slightly acidified with conc. hydrochloric acid. The extract was evaporated to dryness *in vacuo*, dissolved in methyl ethyl ketone, and chromatographed. In most cases, however, this procedure resulted in about 10% loss of each DNP-amino acid. For quantita-

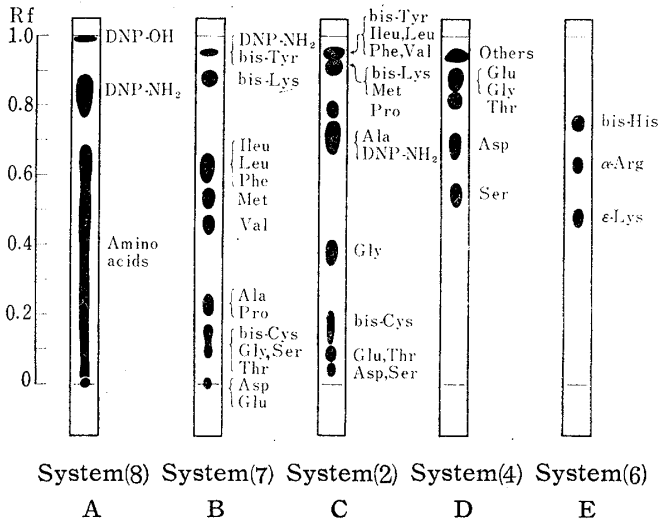


Fig. 2. One-dimensional Chromatograms of Synthetic Mixtures of DNP-Amino Acids with Various Solvent Systems

A : Ether-soluble DNP-amino acids, dinitroaniline, and dinitrophenol
B～D : Ether-soluble DNP-amino acids and dinitroaniline
E : Water-soluble DNP-amino acids

tive purposes, it is recommended to remove dinitrophenol by a cold-finger condenser of Mills[3] although it does not remove dinitroaniline.

Separation of Ether-soluble DNP-Amino Acids by One-dimensional Chromatography—Some 12 DNP-amino acids have characteristic Rf values and can be separated from all others by a single one-dimensional chromatography, as shown in Fig. 2 and noted by the superscripts *a*, *b*, and *c* in Table I. The ether-soluble DNP-amino acids thus separated are DNP-aspartic acid,bis(DNP)-cystine, DNP-glycine, bis(DNP)-lysine, DNP-methionine, -proline, -serine, -threonine, and -valine. If dinitroaniline is absent, DNP-alanine and bis(DNP)-tyrosine are also separable on one-dimensional chromatogram.

Solvent system (1) separates the ether-soluble DNP-amino acids into 8 groups which are (1) DNP-aspartic and -glutamic acids, (2) bis(DNP)-cystine, DNP-glycine, -serine, and -threonine, (3) DNP-alanine, and -proline, (4) DNP-valine, (5) DNP-methionine, (6) DNP-isoleucine, -leucine, and -phenylalanine, (7) bis(DNP)-lysine, and (8) bis-(DNP)-tyrosine and dinitroaniline. This solvent system, therefore, is convenient for the grouping of DNP-amino acids in both one- and two-dimensional chromatography. Fig. 3

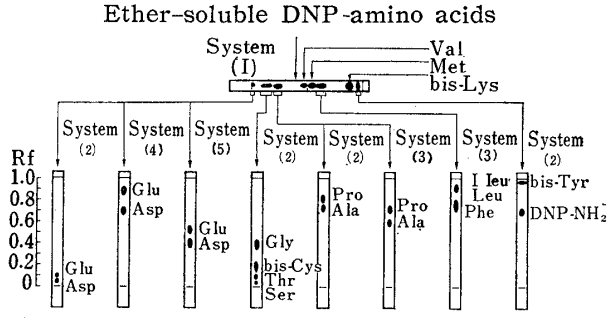


Fig. 3. Separation of Ether-soluble DNP-Amino Acids and Dinitroaniline by One-dimensional Chromatography

illustrates a method for the identification and separation of ether-soluble DNP-amino acids by serial use of one-dimensional chromatography. The extraction and rechromatography of spots were made by the same procedure as described in the removal of dinitrophenol. Since the Rf value is more reliable on the one-dimensional than two-dimensional chromatogram, this method is suited for the purpose of identification.

DNP-methionine and -valine can be separated from each other with solvent systems (3) and (5), as well as (1). Among these, solvent system (3) gives the most clear-cut separation because of the large difference between their Rf values. On the contrary, solvent system (5) is used for an overnight run, since it takes about 20 hours to finish one run of chromatography.

Resolution of a mixture of DNP-hydroxyproline, -serine, and -threonine which usually migrate overlapping is made with solvent system (4). This process also resolves a mixture of these and bis(DNP)-cystine. If, therefore, separation latter with solvent system (2) is incomplete, it is recommended to extract the spot and submit to rechromatography with solvent system (4). By this, bis(DNP)-cystine is completely separated from others.

Separation of Ether-soluble DNP-Amino Acids by Two-dimensional Chromatography ——Typical two-dimensional chromatograms of the ether-soluble DNP-amino acids are presented in Figs. 4 and 5, in which the first runs were made with solvent systems

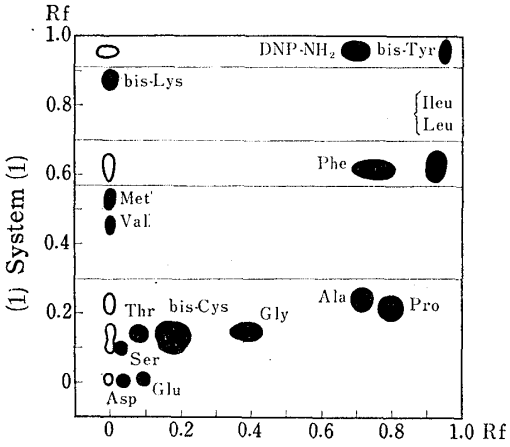


Fig. 4. Two-dimensional Chromatogram of a Synthetic Mixture of Ether-soluble DNP-Amino Acids and Dinitroaniline run with System (1) in the First Dimension

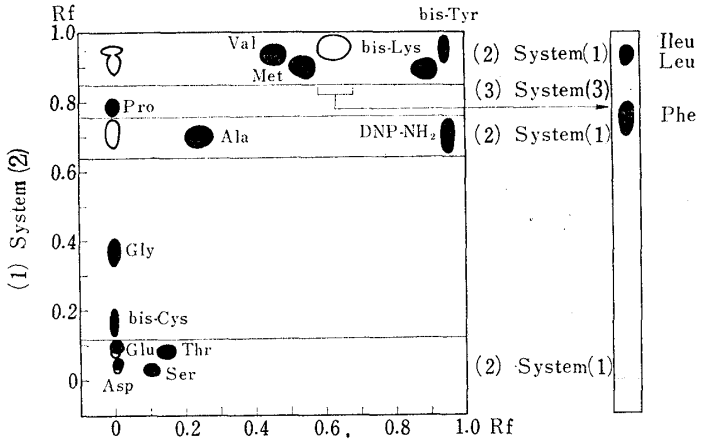


Fig. 5. Two-dimensional Chromatogram of a Synthetic Mixture of Ether-soluble DNP-Amino Acids and Dinitroaniline run with System (2) in the First Dimension

(1) and (2), respectively. In order to obtain the most effective separation, both chromatograms were run with several solvent systems in the second dimension. After the chromatograms had been run for about 5 hours with solvent system (1) or (2) in jars, the filter papers were taken out and dried at room temperature. Each filter paper was cut into 5 separate strips, three of which were run in the second dimension with the solvent systems indicated on the right side of the chromatogram. The outlined spots represent the positions at which DNP-amino acids were located in the first run.

Bis(DNP)-lysine, DNP-methionine, and -valine were separated by a first run on the chromatogram shown in Fig. 4. This excluded the necessity of a second run for them. This method, therefore, is convenient, especially when their detection or accurate estimation is necessary. Other DNP-amino acids were separated by the second run with solvent system (2), except DNP-leucine (-isoleucine) and -phenylalanine which were separated with solvent system (3). The disadvantage of this method is that the spot of bis-

(DNP)-cystine is not well defined, and accordingly its recovery is considerably low (below 70%) compared with those of other DNP-amino acids (80~90%). Bis(DNP)-cystine, DNP-glycine, and -proline were separated by the first run whose chromatogram is shown in Fig. 5. Contrary to the former, this method is suitable for the detemination of these DNP-amino acids. Others were separated by the second run with solvent system (1), again with the exception of DNP-leucine (-isoleucine) and -phenylalanine which were separated by rechromatography with solvent system (3).

Separation of DNP-Hydroxyproline——Fig. 6 represents a two-dimensional chromatogram on which DNP-hydroxyproline was separated from the others employing solvent systems (1) and (4) in the first and second dimensions, respectively.

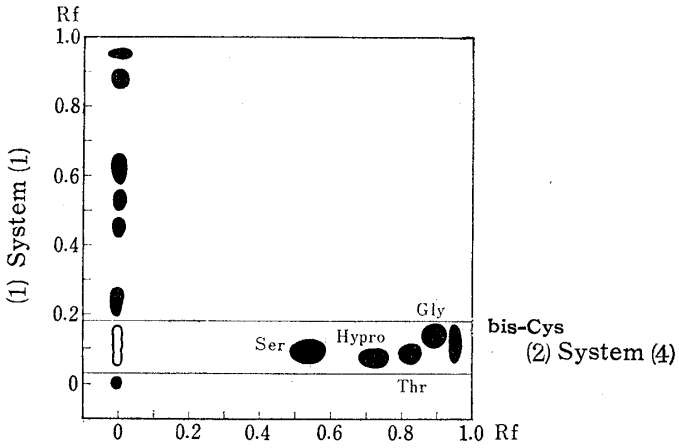


Fig. 6. Separation of DNP-Hydroxyproline by Two-dimensional Chromatography

Separation of DNP-Tryptophan——Since tryptophan is almost completely destroyed under the usual condition of hydrolysis with conc. hydrochloric acid, the separation of its DNP-derivative is not included in the chromatographic procedures described above. If a protein contains tryptophan, it must be detected from the alkaline hydrolyzate. In the present work, the separation of DNP-tryptophan was achieved by two-dimensional chromatography as shown in Fig. 7. The chromatogram was run with solvent system (1) in the first dimension and with solvent system (7) in the second.

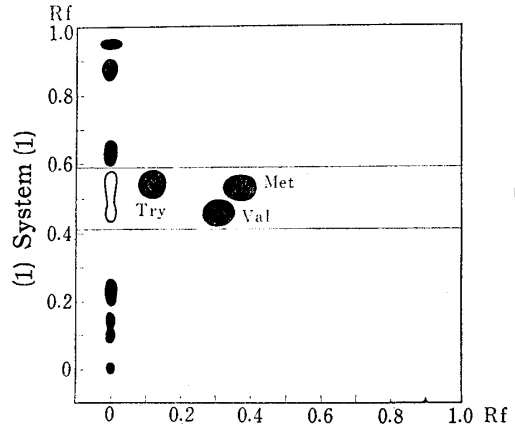


Fig. 7. Separation of DNP-Tryptophan by Two-dimensional Chromatography

Separation of Water-soluble DNP-Amino Acids——If a protein hydrolyzate is dinitrophenylated with FDNB and sodium hydrogencarbonate, lysine is converted to its bis(DNP)-derivative which is extracted into ether from an acidified aqueous solution. In this case, the aqueous layer usually contains α-DNP-arginine and bis(DNP)-histidine. Their separation was made most effectively with solvent system (5), in which the former gave an Rf value of 0.60 and the latter 0.

If a protein, whose free amino groups are located on non-basic amino acids except the ε-amino group of lysine, is dinitrophenylated, hydrolyzed, and extracted with ether, the remaining aqueous layer contains ε-DNP-lysine alone. For its quantitative separation, solvent system (5) can be used most successfully because of the compactness of the spot.

On the contrary, the aqueous layer of the hydrolyzate of a DNP-protein, which also carries arginine and histidine as N-terminals, contains ε-DNP-lysine, α-DNP-arginine, and, on rare occasions, bis(DNP)-histidine, because the last is usually destroyed by hydrolysis with hydrochloric acid. The resolution of a synthetic mixture of the three was achieved with solvent system (6), as shown in Fig. 2-E.

N-Terminal Amino Acid of Bovine Plasma Albumin——DNP-aspartic acid was separated from the ether-soluble fraction with solvent system (2), as shown in Fig. 8. This

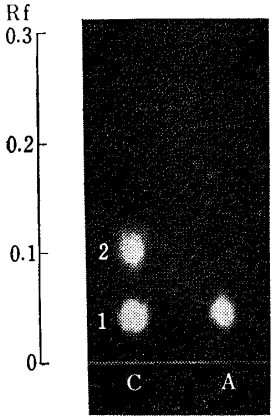


Fig. 8. One-dimensional Chromatogram of the N-Terminal Amino Acid of Bovine Plasma Albumin run with System (2)

A : Ether-soluble fraction of hydrolyzed DNP-albumin
B : Controls 1. DNP-aspartic acid
2. DNP-glutamic acid

was also confirmed with solvent systems (1), (4), and (5), in which the spot gave Rf values of 0.01, 0.69, and 0.41, respectively. From the optical density reading, it was estimated to be 0.69 mole per mole of protein at molecular weight of 69,000. Dividing by the hydrolytic and chromatographic recovery factors (83 and 90%, respectively), it was calculated to be 0.93 mole per mole of protein. This value agrees well with that of Thompson.[12]

Amino Acids of Parotin——Amino acids detected in the hydrolyzate of parotin together with the solvent systems used are listed in Table II. Although qualitative, the result was in good agreement with that obtained by the usual paper chromatography of free amino acids using the phenol-0.1% ammonia and butanol-acetic acid-water systems, as shown in Fig. 9. The following specific color reactions were also used and all were found

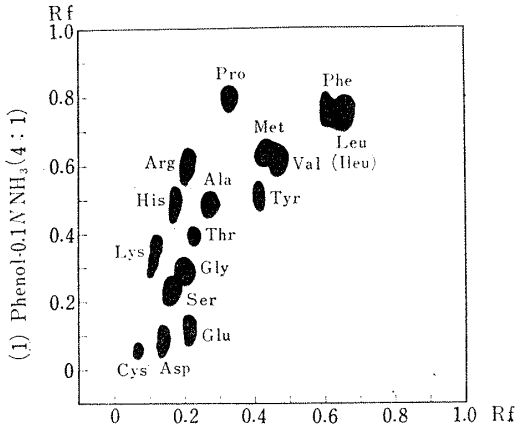


Fig. 9. Two-dimensional Chromatogram of the Hydrolyzate of Parotin

(2) BuOH-AcOH-H_2O (4:1:2)

12) E. O. P. Thompson : J. Biol. Chem., **208**, 565 (1954).

Table II. Rf Values of Spots and Corresponding DNP-Amino Acids detected on Chromatograms of the Dinitrophenylated Hydrolyzate of Parotin[a]

Solvent system / DNP-amino acid	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Alanine	—	0.71	0.60	—	—	—	—
Arginine (α-mono)	—	—	—	—	0.60	0.62	—
Aspartic acid	0.01	0.04	—	—	—	—	—
Cystine (bis)	—	0.18	—	0.95	—	—	—
Glutamic acid	0.01	0.10	—	—	—	—	—
Glycine	—	0.39	0.17	—	—	—	—
Histidine (α, Im-bis)	—	—	—	—	0.0	0.75	—
Leucine[b]	0.62	—	0.92	—	—	—	—
Lysine (α, ϵ-bis)	0.89	—	0.41	—	—	—	—
Methionine	0.54	—	0.78	—	—	—	—
Phenylalanine	0.62	—	0.75	—	—	—	—
Proline	—	0.79	0.72	—	—	—	—
Serine	0.10	—	—	0.54	—	—	—
Threonine	0.14	—	—	0.82	—	—	—
Tryptophan[c]	0.56	—	—	—	—	—	0.12
Tyrosine (O,N-bis)	0.94	0.94	—	—	—	—	—
Valine	0.47	—	0.88	—	—	—	—

a) The Rf value was measured with each individual DNP-amino acid separated by the procedures shown in Figs. 2, 3, and 7.
b) And/or DNP-isoleucine.
c) Separated from the alkaline hydrolyzate.

positive : Diazo reaction for histidine and tyrosine, isatine for proline, potassium iodide-chloroplatinic acid reagent for cystine and methionine, and Sakaguchi reaction for arginine. Tryptophan was detected on the chromatogram of the alkaline hydrolyzate with Ehrlich reaction.

Table II also serves as a guide in which the combinations of solvent systems are given for the identification of individual DNP-amino acid. For example, DNP-alanine is identified with certainty by a couple of one-dimensional chromatographies using solvent systems (2) and (3), and no further confirmation with other solvent systems is necessary. When a sample solution contains only one kind of DNP-amino acid, its identification can easily be made by choosing an adequate combination of solvent systems. This method, however, cannot be applied to a mixture of more than two kinds of DNP-amino acids, unless some of them have characteristic Rf values, such as DNP-glycine has in solvent system (2). In that case, identification must be made according to the precedures described above.

Discussion

Difficulty has been encountered in the paper chromatography of DNP-amino acids owing to the tailing of spots, before Monier, *et al.*[13] used with success the cyclohexane (or carbon tetrachloride)-isopropanol-*M*/20 potassium benzoate system. The addition of potassium benzoate diminished the tendency of tailing of spots to a large extent. Blackburn, *et al.*[14] also overcame the difficulty by using several buffered solvent systems. When, however, the extraction and rechromatography of spots are necessary, or when the second run on the same filter paper is required, use of salts is inexpedient, because they often disturb the proper migration of DNP-amino acids on the second chromatogram. In the present work, buffer or salt solutions were not employed except solvent system (7). This makes it possible not only to separate DNP-amino acids by

13) R. Monier, L. Pènasse : Compt. rend., **230**, 1176 (1950).
14) S. Blackburn, A.G. Lowther : Biochem. J., **48**, 126 (1951).

serial use of one-dimensional chromatography as shown in Fig. 3, but also to use any of the solvent systems, except the solvent systems (7) and (8), for two-dimensional one, irrespective of the dimension of run. Solvent system (7) is available only for the second run. Solvent system (8) can be used successfully for the removal of dinitroaniline and dinitrophenol but not for the separation of DNP-amino acids, since the latter tails strongly in this system. Compared with the method of Levy,[8] the present two-dimensional technique is somewhat more complex. The advantage of the present method, however, is that the solvent systems reported can be used effectively for the one-dimensional as well as for the two-dimensional run and that some 12 DNP-amino acids are separable on a single one-dimensional chromatogram. It may therefore be applied with convenience to the identification and estimation of the N-terminal amino acids of peptides and proteins. The result of analyses of bovine plasma albumin and parotin indicates the wide applicability of this method.

In order to obtain reliable Rf values and well-defined spots, it is essential to saturate the filter paper with vapor of the solvent. It the saturation is incomplete, one may obtain extremely elongated spots. Saturation for 5 hours is usually sufficient for this purpose. It is preferable, however, to equilibrate the filter paper overnight in the jar especially when solvent systems (1), (2), (3), and (4) are used because they are fast-running.

It must be pointed out that the chromatographic recovery varies slightly fron run to run. The variation is especially large with bis(DNP)-amino acids, and also depends on the distance traveled by the spot. It should therefore be determined in a given case by running the chromatogram of an appropriate control simultaneously with the unknown in the same jar.

DNP-leucine and -isoleucine could not be separated from each other with any of the solvent systems reported here. The identification of each individual DNP-leucine isomer, however, can be made with solvent systems (2) and (7) although not always satisfactory. The method for their separation is now under investigation.

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Summary

A method was described whereby the ether- and water-soluble DNP-amino acids are separated by one- and two-dimensional paper chromatography. The method was applied, as examples, to the N-terminal group analysis of bovine plasma albumin and qualitative amino acid analysis of parotin.

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