A TWO-DIMENSIONAL SEPARATION OF ACID, NEUTRAL AND BASIC AMINO ACIDS BY THIN LAYER CHROMATOGRAPHY ON CELLULOSE

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INTRODUCTION

The use of alumina or silica gel spread on thin layer plates has found an extensive application, whereas the use of cellulose powder as adsorbent in thin layer chromatography has so far met with only limited success. This also applies to the separation of amino acids. Thus, the procedures preferred for the thin layer chromatographic separation of amino acids are those using silica gel[1,2]. However, we experienced some difficulty with this adsorbent in separating certain basic amino acids and were unable to perform the SAKAGUCHI reaction for arginine on it. We therefore switched to the use of cellulose, whose separating ability for amino acids has already been mentioned by MUTSCHLER AND ROCHELMEYER[3]. WOLLENWEBER[4] examined nine solvent mixtures for amino acid chromatography on cellulose and VON ARX AND NEHER[5] developed a multidimensional technique based on four solvent systems. DITTMANN[6] investigated the separation of some amino acids of clinical interest on thin layers of cellulose. Originally, we were seeking to obtain a good separation of the basic amino acids as a group, but the procedure developed proved to be very suitable for a general separation of the amino acids*.

EXPERIMENTAL

Materials

Glass plates 20 cm × 20 cm × 0.4 cm.
Spreader: standard (STAHL) type from Desaga G.m.b.H., Heidelberg.
Adsorbent: standard cellulose without gypsum MN 300 from Macherey, Nagel & Co., Düren-Rl.
Solvents: Merck A.G., Darmstadt; Fluka, Buchs (St. Gallen).

Preparation of chromatoplates

14–15 g of cellulose were homogenized with 90 ml distilled water in a Waring blendor for about 2 min and the slurry formed was applied to 5 glass plates to a thickness of 250 μ using the Desaga spreader. The plates were dried in an oven (105°) for 10–15 min. The cellulose adheres so well to the surface that the plates may be stored without any special precautions.

* A preliminary and partial account of the method was given at the IIIrd Symposium on Chromatography, Brussels, 1964.

Developers

The solvent systems used were as follows (minutes required for development in parentheses):

First dimension. Two solvent systems were used:

(Ia) methanol–chloroform–17 % ammonium hydroxide, 2:2:1 (100)[2];

(Ib) *tert.*-amyl alcohol–methyl ethyl ketone–water, 6:2:2 (240)[7].

(Ia) is a general purpose solvent, (Ib) is used for the separation of leucine and isoleucine and gives a better performance in the separation of amino acids of high mobility. It was introduced by MUNIER AND SARRAZIN[7] in paper chromatography.

Second dimension. A single solvent is used:

(II) methanol–water–pyridine, 20:5:1 (100)[4].

We chose this solvent because it combines a high mobility for most amino acids with a small displacement of the basic amino acids.

The volume of solvent system used for development was 200 ml.

Chromatographic procedure

10 to 30 μl, corresponding to about 1–3 μg of each amino acid was applied with a micropipette in 2 μl drops on the starting point, at a distance of 2 cm from the edges of the plate, care being taken to evaporate the solvent in an air stream between each application.

The chromatoplates were placed in the chromatography tanks, previously saturated with the solvent vapours. The walls of the tanks were lined with filter paper soaked in solvent to ensure uniform saturation of the enclosed space. The solvent was allowed to ascend to a height of 15–17 cm. For the first dimension, the solvent should always ascend in the direction of the cellulose spread. The plates were then removed from the tanks and the solvent completely evaporated at room temperature.

In order to obtain a still better separation of leucine and isoleucine, solvent Ib may be run a second time in the same direction, the chromatoplates having been well dried in the interval.

For the second dimension, the plates are dipped into solvent II and the solvent is allowed to ascend until the convex front barely reaches the edge of the plate. The plates are again air-dried at room temperature.

Detection methods

(1) The cellulose plates were sprayed with a ninhydrin reagent made up of 50 ml 0.2 % ninhydrin in abs. ethanol, 10 ml acetic acid and 2 ml 2,4,6-collidine (solution I of MOFFAT AND LYTLE[8]).

The amino acids appeared after heating at 100° for 15 min. The detection of individual amino acids was facilitated by the specific colour shades developed by some of these acids. Aspartic acid was green-blue, histidine, cystine, phenylalanine, tryptophan and the 1- and 3-methylhistidines were grey-brown, glycine and tyrosine brownish, proline, hydroxyproline and asparagine yellow. These specific colours fade gradually and either disappear or turn into the classical blue-violet colour of the amino acids.

(2) In order to detect arginine and homoarginine separately, the plates were first sprayed with a solution containing α-naphthol and urea in ethanol (16 % urea in

water–0.2% α-naphthol in ethanol, in proportions of 5:1), and then, after having been dried at 40°, were sprayed with a solution of bromine in NaOH (3.3 ml Br_2 in 500 ml 5% NaOH) (SAKAGUCHI reagent[9]).

The pink spots of the guanidino amino acids appeared clearly.

Nomenclature

This is based on the polarity of the amino acids[10], which are distinguished as follows:

(1) The *neutral* amino acids, with an isoelectric point (p*I*) between 5 and 7. They may be divalent (1 COO^-; 1 NH_3^+), trivalent (1 COO^-; 1 NH_3^+; 1 OH^-) or tetravalent (2 COO^-; 2 NH_3^+).

(2) The trivalent amino acids of *basic* type (1 COO^-; 2 NH_3^+) with a p*I* above 7.

(3) The trivalent amino acids of *acid* type (2 COO^-; 1 NH_3^+) with a p*I* below 4.

RESULTS AND DISCUSSION

R_F values

The mean R_F values for 36 amino acids were determined and are shown in Table I.

TABLE I

R_F VALUES × 100*

Solvents: (Ia) methanol–chloroform–17% NH_4OH (2:2:1); (Ib) *tert.*-amyl alcohol–methyl ethyl ketone–H_2O (6:2:2); (II) methanol–pyridine–H_2O (20:1:5).

Amino acid	*Solvent*			*Amino acid*	*Solvent*			
	Ia	*II*	*Ia/II*		*Ia*	*Ib*	*II*	*Ia/II*
L-Arginine·HCl	34	4.2	8.1	DL-Serine	41		46	0.9
L-Homoarginine	42	5.1	8.2	Glycine	42		46	0.9
DL-Ornithine·HCl	44	3.4	13	β-Alanine	46		46	1.0
DL-DL-*allo*-δ-Hydroxylysine·HCl	51	3.2	16	L-Hydroxyproline	46		46	1.0
L-Lysine·HCl	56	4.5	12.5	γ-Aminobutyric acid	53		48	1.1
L-Histidine·H_2O·HCl	48	27	1.8	DL-Methionine sulfoxyde	54		45	1.2
L-1-Methylhistidine	63	34	1.85	DL-Methionine sulfone	67		49	1.37
L-3-Methylhistidine	63	44	1.43	DL-Threonine	51		53	0.96
				DL-α-Alanine	64		63	1.01
DL-Aspartic acid	21	46	0.46	L-Tyrosine	62	18	60	1.03
L-Glutamic acid	26	62	0.42	DL-Tryptophan	68	34	50	1.36
DL-α-Aminoadipic acid	29	61	0.47	Taurine	71		52	1.37
L-Cysteic acid·H_2O	31	47	0.66	L-Proline	75	11	64	1.17
				DL-Methionine	81	25	66	1.23
DL-α,α'-Diamino-pimelic acid	19	13	1.45	DL-Valine	85	17	72	1.18
L-Cystine	24	22	1.1	DL-Phenylalanine	89	39	71	1.25
DL-Homocystine	34	20	1.7	L-Leucine	85	34	82	1.04
				DL-Isoleucine	90	30	81	1.1
L-Asparagine	32	24	1.33	Phenol red	75	65	90	
L-Glutamine	39	34	1.15					
DL-Citrulline	36	35	1.03					

* Mean of 6–15 determinations.

We experienced some variability in the R_F values, but it was less important than anticipated. The boundary or "border" effect was almost negligible. We attribute this to the fact that the atmosphere in our tanks was well saturated. We could find no advantage in working in an unsaturated atmosphere, as proposed by some authors[5], nor in shaking the tanks before the introduction of the chromatogram, a procedure which is called "over-saturation" by STAHL[11].

We did not relate each R_F value to a reference substance, but we mention for orientation purposes the R_F values of phenol-red run with the three solvents.

In order to correlate the chromatographic behaviour with the chemical constitution of the amino acids, we also indicated in Table I the ratio of the R_F values in solvent Ia and II. The neutral amino acids show a ratio with an average slightly above 1 (0.9–1.7). The values for the acid amino acids are near 0.5 (0.4–0.6), whereas the ratios for the strongly basic amino acids (pI above 9) are above 8. The weakly basic histidines (pI ~ 7.5) show a ratio (1.4–1.8), which is somewhat above the average for neutral amino acids.

As expected, the elongation of the aliphatic chain or the introduction of an aromatic ring in the molecule increases the R_F value, while the presence of a hydroxyl group decreases it. This is confirmed especially with solvent Ia. When homologous amino acids are compared, it is seen that the elongation of the molecule by a single $-CH_2$ increases the R_F value, while the introduction of a hydroxyl group diminishes it without exception.

Pattern of amino acid distribution

Fig. 1 shows a photograph and Fig. 2 a diagram of a chromatoplate developed with the solvents Ia and II, on which 28 amino acids can be identified: these consist of the usual 18 amino acids present in proteins, excepting isoleucine and leucine, which overlap, together with 10 others, the choice of which was suggested by current investigations. The amino acid distribution follows the pattern of a Latin cross. Neutral amino acids (R_F ratio slightly above 1) remain in the general line of the diago-

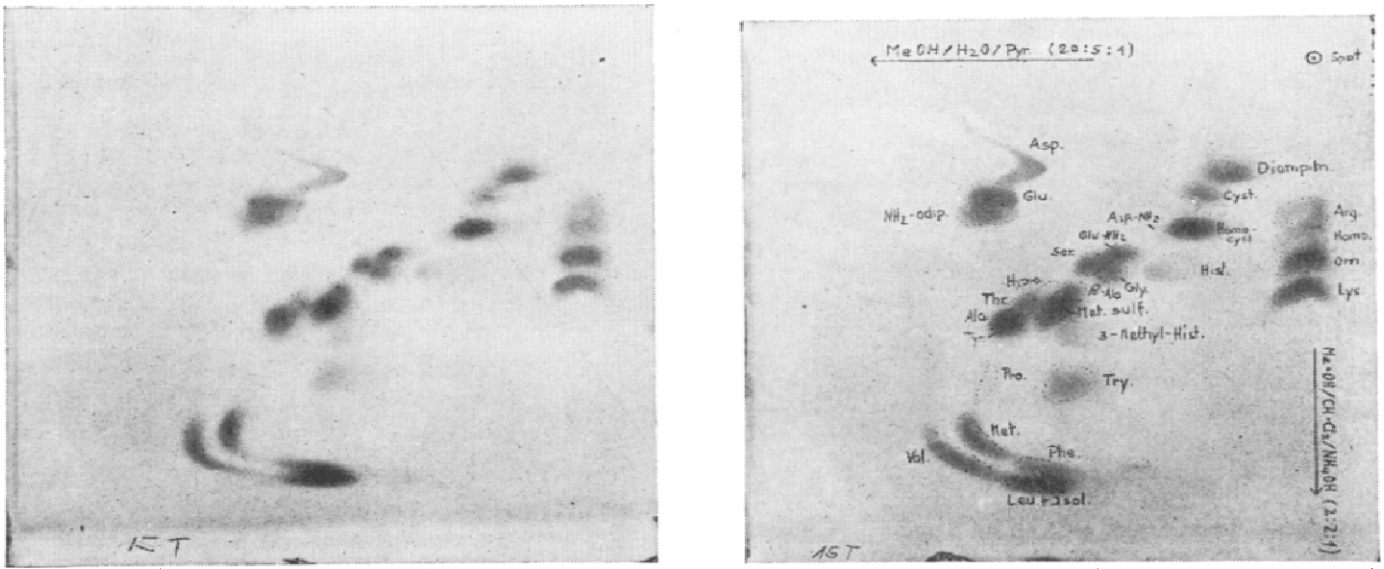

Fig. 1. Photographs showing the two-dimensional separation of amino acids with solvents Ia and II.

nal starting from the spot. The tetravalent neutral amino acids with four charges (diaminopimelic acid, cystine, homocystine) move least, followed by the monoamides (glutamine, asparagine) and ureides (citrulline). The neutral, divalent amino acids are displaced more and more according to the increasing importance of their lipophilic side-chain (aliphatic or aromatic). Tyrosine, although nominally

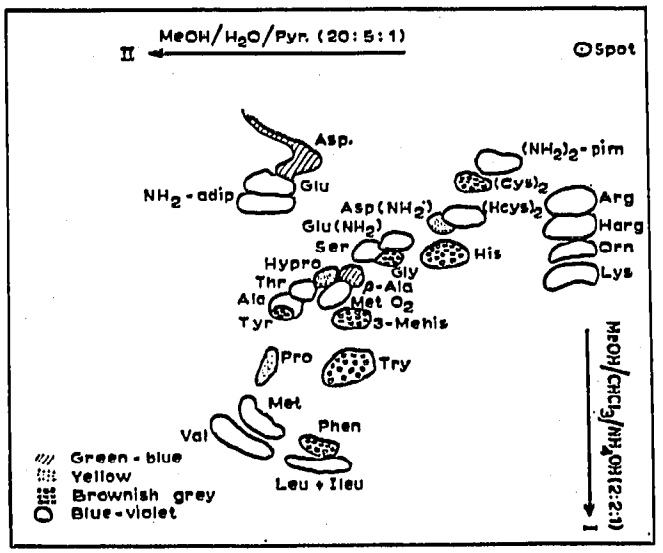

Fig. 2. Diagram of the amino acid separation with solvents Ia and II.

trivalent, is such a weak acid that it moves with the neutral amino acids. The trivalent amino acids with an uneven distribution of the charges tend to be displaced away from the diagonal, the negatively charged (glutamic, aspartic, amino-adipic acids) to the left, the positively charged (arginine, homoarginine, ornithine, lysine) to the right. The good separation of these strongly basic amino acids as a group is worth mentioning. The weakly basic histidines are closer to the main axis of the diagonal.

In view of the inherent logic of this amino acid distribution pattern, the location of an individual amino acid can be predicted to a certain extent. The factors which can be partly evaluated are: the number and balance of the charges (p*I*), the length of the side-chain, the presence of aromatic substituents and hydroxyl groups.

In Fig. 3, a chromatoplate developed with the solvents I b and II is shown, and on this the good separation of the fast-moving neutral amino acids such as leucine, isoleucine, phenylalanine, tryptophan, methionine, valine and tyrosine can be observed.

Thus, with two plates and three solvents it is possible to separate all the amino acids present in proteins plus a number of others.

Applications

The procedure may be applied to protein hydrolysates, as shown by the chromatography of an acid hydrolysate (after refluxing with 6 *N* HCl for 24 h) of the whey proteins ("lactalbumin"). For this purpose, most of the HCl was removed by repeated concentration at low temperature in a rotating evaporator and the remainder by drying in a vacuum desiccator over NaOH for 3 days. Better results were obtained when the slightly acid solution (pH 2–3) was passed through an Amberlite IR 120

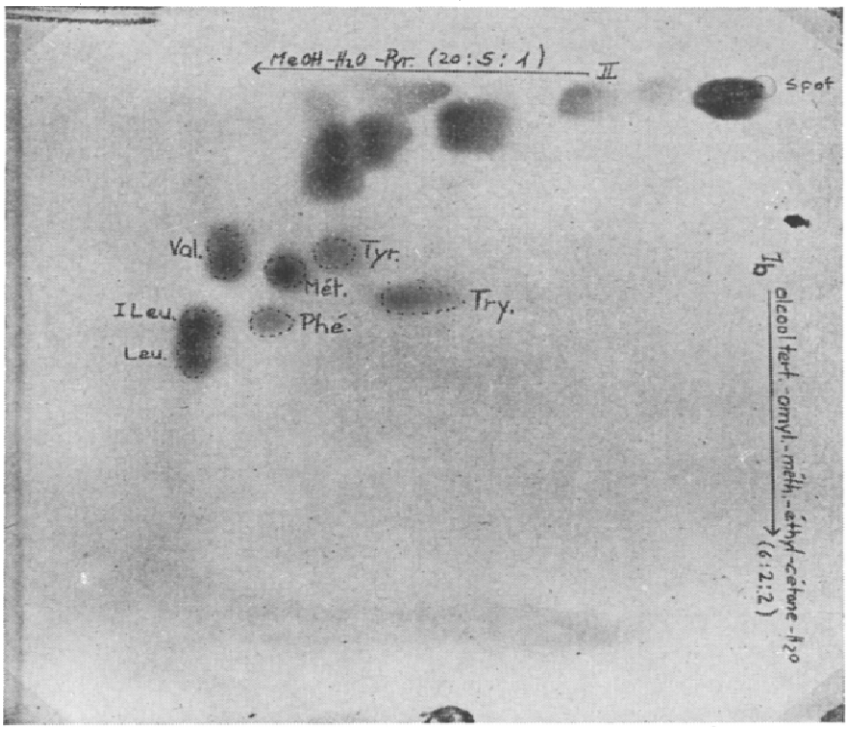

Fig. 3. Photograph of the separation of the fast-moving amino acids with solvents Ib and II.

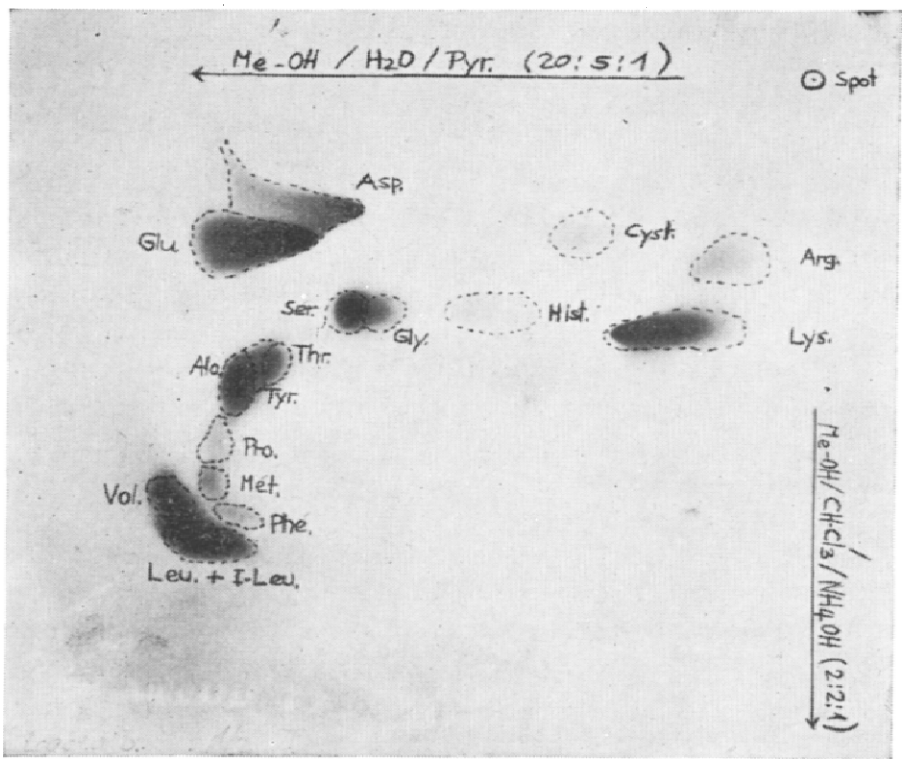

Fig. 4. Photograph of a chromatoplate of a "lactalbumin" hydrolysate with solvents Ia and II.

column (H^+ form), from which the amino acids are subsequently eluted with ammonium hydroxide (Figs. 4 and 5).

We also used the method for the determination of free amino acids in milk, after having removed the proteins either by precipitation with trichloroacetic acid which is subsequently extracted with ether, or by ultrafiltration. The amino acids

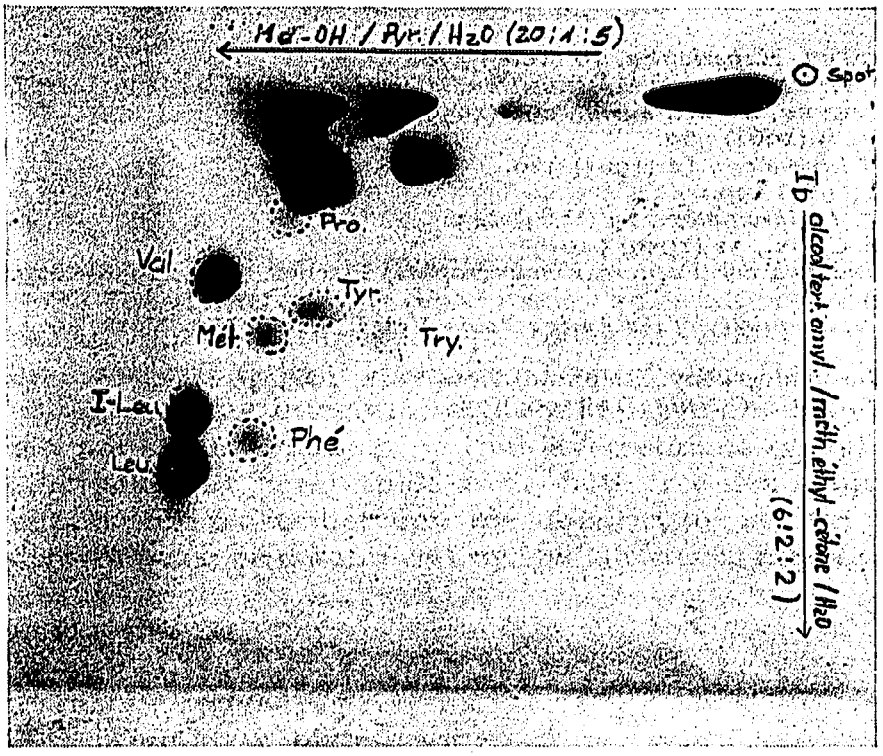

Fig. 5. Same as Fig. 4 with solvents Ib and II.

are concentrated on a cation exchange column as described above. We confirmed the findings of GHADIMI AND PECORA[12] that the free amino acid concentration in human native milk was considerably higher than that of heat processed cows' milk.

CONCLUSION

The procedure described in this paper is simple and reproducible and yields a logical amino acid distribution pattern in three distinct groups which extend over a large area of the chromatoplate.

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SUMMARY

A novel, two-dimensional, thin layer chromatographic separation of amino acids on cellulose is described. The neutral amino acids are well distributed along a

diagonal starting from the spot; the acid ones form a distinct group to the left and the basic amino acids are especially well separated in a row to the right of the diagonal. For better resolution of the fast-moving amino acids, another solvent is used in the first dimension which even allows the separation of leucine and isoleucine.

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