Teflon tubing, keep the solvent cool as it approaches the substrate. Without these coils the solvent would become warm at the top of the column. When the level of solvent has risen to G, stopcock A is carefully opened to allow the eluting solvent to flow into flask D. When the solvent at the top of the column is no longer colored by the material being chromatographed, the level may be permitted to rise until it returns to flask D by way of the insulated glass side arm. At this point, the solvent is being cooled at the top of the column, and excess solvent is being returned to D by "spill-over". Solvent passes through the column performing the chromatographic separation and flows back into D by way of stopcock A. In D, pure solvent is redistilled to the top of the column, while any eluted compound remains in flask D. With the choise of suitable solvents, *i.e.*, those in which the compound being chromatographed moves very slowly, the column can be operated overnight without attention. The continuous modification may be used to separate components on the column. As the bands approach the bottom of the column, with stopcock A closed, they may be collected through stopcock B. This procedure is necessary with heat-sensitive compounds. Alternatively, the eluting bands may be collected in flask D. After each band has eluted, both stopcocks A and B are closed; flask D, containing the eluted band, is cooled and is replaced with a flask containing pure solvent. In the same way, a change to more polar solvents can be effected. The column can be used with most common substrates and solvents.

This column has been successfully used in the chromatography of meso-tetrathienylporphin and metalloporphins. These results will be the subject of a forthcoming paper.

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Separation and identification of amino acids on starch thin layers

Thin-layer chromatography (TLC) has been successfully applied to the separation and identification of amino acids. BRENNER *et al.*[1,2] separated and identified 22 amino acids by a two-dimensional technique using silica gel as sorbent. By using several solvent mixtures they separated all the amino acids examined except leucine and isoleucine. In four systems of solvent mixtures on cellulose thin layers, VON ARX AND NEHER[3] separated and identified 52 amino acids of the 57 examined.

SYNGE[4] and MOORE AND STEIN[5] used starch in separations of amino acids by column chromatography, and taking this into account, we tried to separate amino acids by two-dimensional chromatography on thin layers of starch.

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TABLE I

R_F-VALUES AND COLOUR OF SPOTS OF AMINO ACIDS*,**

No.	Amino acid	$R_F \times 100$					Colour with	
		I 3.5 (2.15) h	*II* 8 (5) h	*III* 4 (2.5) h	*IV* 4 (2.5) h	*V* 5 (3.5) h	*NL*	*DEAN*
1	Alanine	26 (27)	28 (35)	27 (29)	44 (52)	14 (18)	Blue	Blue
2	Arginine·HCl	3 (3)	70 (75)	8 (7x)***	36 (44)	11 (15)	Blue	Blue
3	Asparagine	18 (20)	15 (21)	7 (9x)	26 (31)	7 (11)	Brown	Yellow-brown
4	Aspartic acid	10 (13)	5 (7)	11 (16)	25 (33)	8 (13)	Blue	Green-blue
5	Cystine	13 (19)	11 (18)	1 (3)	13 (15)	3 (7)	Blue-gray	Brown
6	Glutamic acid	12 (16)	7 (9)	24 (23)	42 (45)	13 (20)	Blue	Blue
7	Glycine	22 (23)	18 (23)	11 (11)	29 (35)	9 (13)	Purple	Reddish-brown
8	Histidine·HCl	35 (40)	45 (50)	6 (3)	33 (39)	10 (16)	Blue-gray	Brown
9	Isoleucine	58 (58)	70 (72)	75 (xx)	68 (77)	39 (54x)	Blue	Purple
10	Leucine	62 (60)	76 (80)	80 (xx)	71 (79)	43 (xx)	Blue	Purple
11	Lysine·HCl	25 (27)	63 (72)	5 (5)	31 (36)	8 (17)	Blue-gray	Gray-blue
12	Methionine	50 (49)	57 (64)	41 (xx)	56 (66)	29 (39)	Blue	Purple
13	Norvaline	49 (48)	60 (61)	60 (xx)	64 (70)	33 (40)	Blue	Purple
14	Norleucine	62 (60)	75 (82)	80 (xx)	74 (83)	46 (xx)	Blue	Purple
15	Ornithine·HCl	20 (26)	52 (57)	2 (3)	25 (33)	8 (13)	Blue	Blue
16	Phenylalanine	62 (58)	78 (78)	55 (xx)	62 (73)	35 (xx)	Blue-gray	Brown
17	Proline	33 (35)	77 (81)	31 (30)	52 (61)	23 (31)	Yellow	Yellow
18	Serine	37 (42)	15 (21)	11 (12)	30 (37)	9 (17)	Blue	Brown
19	Threonine	62 (58)	24 (29)	20 (20)	38 (48)	14 (27)	Blue	Gray-blue
20	Tryptophan	62 (60)	69 (72)	50 (xx)	56 (64)	32 (xx)	Blue	Brown-red
21	Tyrosine	40 (40)	35 (43)	43 (xx)	53 (64)	27 (37)	Blue-gray	Brown-gray
22	Valine	46 (46)	59 (59)	56 (xx)	59 (69)	31 (37)	Blue	Blue

* R_F values shown in parentheses are for MS thin layers.
** The developing time of chromatograms for 15 cm in one dimension is given (in parentheses for MS thin layers).
*** x = extended spot; xx = very extended spot.

Recent usage of maize starch for TLC has given good results in separations of some cations[6,7] and anions[8] as well as condensed phosphates[9].

Experimental

Procedure. For preparation of the thin layers commercial maize starch (MS)* and rice starch (RS)** were used. After purification, which has already been described[6,9], the MS was dried in the air overnight and then in a vacuum oven at 60°. The dried MS was milled in a Waring Blender and sieved through a 325 mesh (ASTM) sieve. RS was used without purification.

(a) 27 g of MS and 3 g of gypsum were suspended in 35 ml distilled water and the suspension was coated on to glass plates (20 × 20 cm) with the Desaga equipment***, thickness *ca.* 0.2 mm after drying (5 plates).

(b) 18 g of RS and 2 g of gypsum were suspended first in 20 ml 96% ethanol and then in 40 ml distilled water and the suspension was applied to glass plates as described in (a); thickness *ca.* 0.12 mm after drying (5 plates). The previous suspension of RS in ethanol is necessary in order to prevent the formation of bubbles which appear when RS is suspended only in water.

The thin layers were dried in the air at room temperature. The addition of gypsum is not necessary but it binds the starch more firmly to the glass plate and prevents the thin layer from forming very tiny capillaries which disturb the regular migration of the solvent mixture through the thin layer.

Twenty-two amino acids**** were examined (Table I). Spots of 1 μl containing 1 μg of each amino acid (2 μg of asparagine) in 0.1 *N* HCl were applied by micropipette on a corner of the glass plate at a distance of 2 cm from the edges. The chromatograms were developed in a glass chamber, which contained 50 ml of solvent mixture. The chromatograms were run both ways at room temperature without previous saturation of the chamber with solvent. After developing in the first dimension the chromatograms were dried for 20 min at 80°.

Solvents. In all cases the solvent mixture for the first dimension was:

(I) *n*-butanol–acetone–diethylamine–water (10:10:2:5).

For the second dimension the following solvent mixture were used:

(II) phenol–water (75:25, w/w); the vapour phase is equilibrated with 25% aqueous ammonia,

(III) isopropanol–formic acid–water (40:2:10),

(IV) isopropanol–water–acetic acid–pyridine (50:40:10:4),

(V) *n*-butanol–acetic acid–water–pyridine (40:10:50:2); upper layer.

Detection. (1) Ninhydrin–lutidine reagent (NL) consists of 0.25 g of ninhydrin, 95 ml of methanol and 5 ml of 2,4-lutidine. The developed and dried chromatograms were sprayed with NL reagent and heated in an oven for 10–20 min at 80°. Coloured spots of the amino acids appeared (Table I). If the chromatograms are allowed to remain a few hours at room temperature in the dark, the spots become more intensively coloured.

(2) Diethylamine–ninhydrin reagent (DEAN)[10] consists of 0.25 g of ninhydrin

* Produced in "Servo Mihalj", Zrenjanin, Yugoslavia.
** Powder white, "Carlo Erba", Milan, Italy.
*** Desaga GmbH, Heidelberg, Germany.
**** E. Merck A. G., Darmstadt, Germany.

and 100 ml of *n*-butanol. After development the chromatograms were dried and sprayed with *n*-butanol saturated with a 20% aqueous solution of acetic acid. The wet chromatograms were exposed to diethylamine vapour for 2 min and dried in a stream of warm air and then sprayed with reagent. After 10–20 min heating in an oven at 80° the coloured spots of the amino acids (Table I) appeared.

Results and discussion

(a) The maize starch was not found to be a suitable sorbent for the separation and identification of amino acids by TLC. The spots of a great number of amino acids are diffuse so that they overlap and with their close R_F values it is impossible to identify them. This is especially true for amino acids with high R_F values where the spots are a little extended, in the solvent mixtures (I), (II), and (IV), and very extended, in the solvent mixtures (III) and (V), in the direction of development. With a two-dimensional technique the spread increases a good deal and it is impossible to separate them.

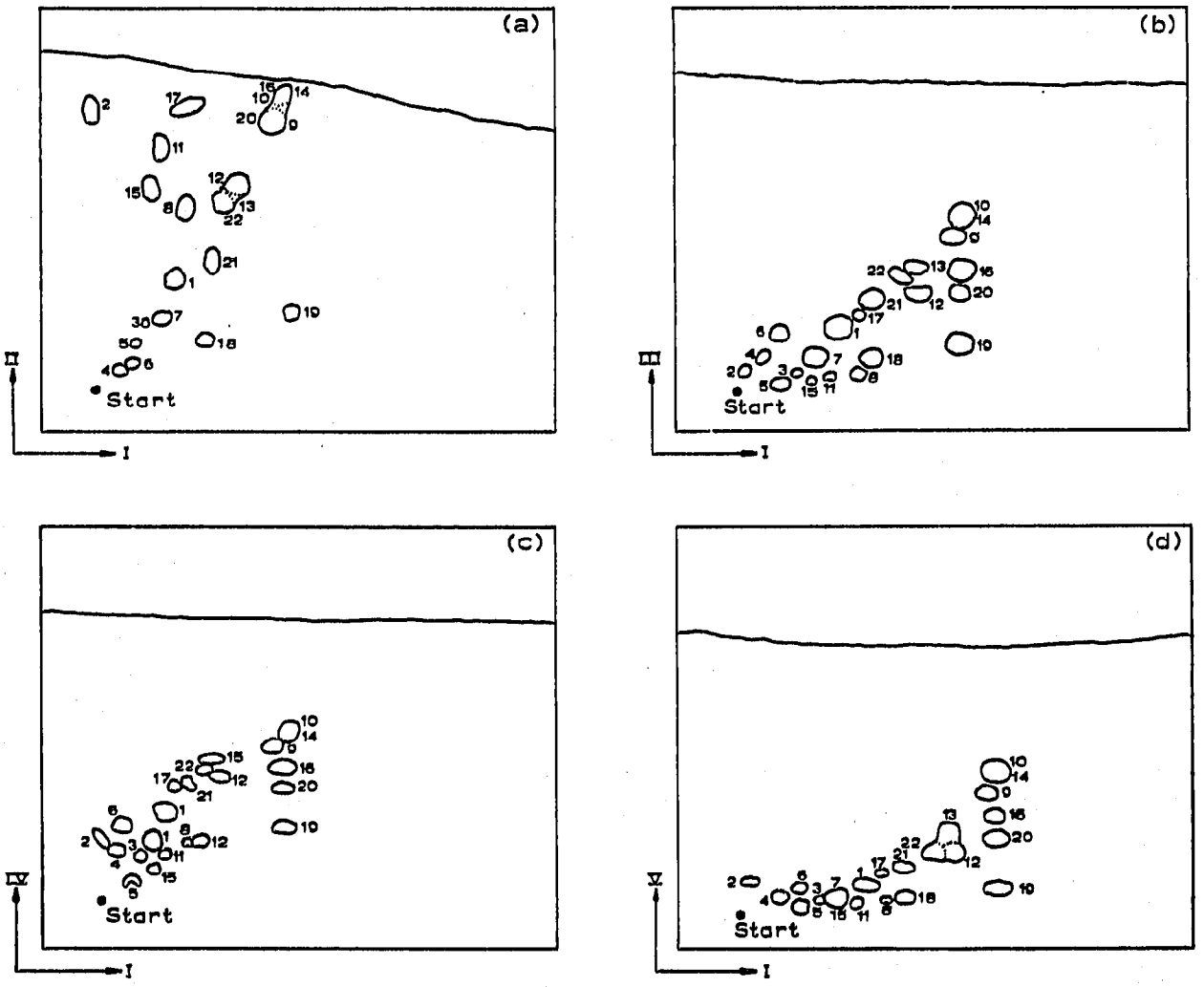

Fig. 1a–d. Thin-layer chromatograms on rice starch (RS) of 22 amino acids (see Table I) obtained by solvent mixtures combinations I + II, I + III, I + IV, and I + V. Start point and solvent front are traced.

(b) Rice starch proved to be a suitable sorbent for the separation and identification of amino acids by TLC (Figs. 1a–d). By using RS all the amino acids examined except leucine and norleucine (Figs. 1b and c) were separated by the combinations of solvent mixtures I + III and I + IV. The solvent mixtures I + II do not clearly separate the following groups of amino acids: methionine, valine and norvaline, and tryptophan, isoleucine, leucine, phenylalanine and norleucine. The combination I + V neither separated glycine from ornithine nor methionine, valine and norvaline from each other. The combinations of solvent mixtures I + III, I + IV and I + V resolved isoleucine from leucine and norleucine. The advantages of combinations I + III and I + IV are perceptible especially if the speed of migration is taken into account (Table I). The spots of the amino acids are much smaller than on MS, have a regular shape, and are not too diffuse. By combined use of the detection reagents NL and DEAN it was possible to identify reliably every amino acid which was separated.

Comparison of the results obtained on RS with the results obtained on silica gel[1,2] and cellulose[3] as carriers showed that RS was not inferior. It is possible to use the same or slightly modified solvent mixtures. There is no appearance of double solvent fronts whether the solvent mixture is acidic or basic. The speed of migration of the solvent mixtures is less than that on cellullose or silica gel thin layers but still good in comparison with paper chromatography. The sensitivity of detection is very great and 0.1 to 0.5 μg (asparagine) of various amino acids could be detected when the chromatograms were sprayed with NL reagent. In two-dimensional chromatography it is not recommended that the spots of amino acids should contain less than 0.5 μg (1 μg for asparagine). The DEAN reagent is less sensitive. Good reproducibility in separation can be obtained under similar working conditions.

The lack of separation of the amino acids on the MS layers can be explained by examination of the sizes of the various starch granules. MS has a bigger (20–30 μ longitudinal axis) granule than RS (2–10 μ, longitudinal axis), which causes a more rapid migration of the solvent mixture through the layers of MS and hence a worse separation.

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1 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 16 (1960) 378.
2 A. R. FAHMY, A. NIEDERWIESER, G. PATAKI AND M. BRENNER, *Helv. Chim. Acta*, 44 (1961) 2022.
3 E. VON ARX AND R. NEHER, *J. Chromatog.*, 12 (1963) 329.
4 R. L. M. SYNGE, *Biochem. J.*, 38 (1944) 285.
5 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 337; 176 (1948) 367; 178 (1949) 53.
6 V. D. CANIĆ AND S. M. PETROVIĆ, *Z. Anal. Chem.*, 211 (1965) 321.
7 V. D. CANIĆ, S. M. PETROVIĆ AND A. K. BEM, *Z. Anal. Chem.*, 213 (1965) 251.
8 S. M. PETROVIĆ AND V. D. CANIĆ, unpublished results.
9 V. D. CANIĆ, M. N. TURČIĆ, S. M. PETROVIĆ AND S. E. PETROVIĆ, *Anal. Chem.*, 37 (1965) 1576.
10 R. CIRCO AND B. A. FREEMAN, *Anal. Chem.*, 35 (1963) 262.

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