AMINO ACID ANALYSIS BY HORIZONTAL PAPER CHROMATOGRAPHY

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INTRODUCTION

The advantages of circular paper chromatography have been discussed by several authors[1–5] and could be confirmed by our initial trials, notably with respect to well defined sharp bands obtained with this method. They contrast favourably with the diffuse spots frequently observed with the two-dimensional technique[6]. Besides, the circular technique permits the comparison of different proteins or different hydrolytic fractions on one and the same paper[7,8]. GIRI and co-workers[8] had achieved this on ordinary chromatographic paper but the K.C.T. technique[9], in which a paper with five machine made slits is used, constitutes an additional improvement.

In confirmation of GIRI and co-workers[5] it was found that the solvent system *n*-butanol–acetic acid–water (4:1:5) causes a number of amino acids to run in pairs so that nineteen amino acids are separated into nine groups. These authors proposed two methods for separating the groups into their individual constituents. The first one[5] consists of rechromatographing the sample up to 6 times, the second one[10] of eluting the group bands by capillary action onto another paper and developing same with another solvent, *e.g.* pyridine–water (80:20), pyridine–isoamyl alcohol–water (10:10:7) or pyridine–amyl acetate–water (10:5:5). KRISHNAMURTHY AND SWAMINATHAN[11] had proposed a third method, in which they used five different solvent systems, determining from each chromatogram only those amino acids which are separated into a single and homogeneous band. RAO AND WADHWANI[12,13] also use several solvent systems but could reduce the number of these to three by adopting GIRI'S method of rechromatography.

The conventional circular method, in which the spot is applied near the centre and in which the solvent system *n*-butanol–acetic acid–water (4:1:5) is employed did in our experience not separate the lysine–histidine group adequately from the arginine–asparagine group and even the next band, aspartic acid–glycine–serine frequently was too near the preceding one. This point is of particular importance, because the location of the groups which are to be eluted and chromatographed with another solvent system can only be determined from their ninhydrin-developed counterparts on another segment of the circle, but the calendering of the paper often produces a solvent front of oval shape instead of an ideal circle, so that instead of the desired band only, parts of the neighbouring bands also are easily cut out. Probably for similar

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reasons GIRI[14] emphasized the need for increasing the size of filter paper from 15 to 35 cm in one of his later publications.

Neither modifications in the size of the paper tongue (0.3 to 1.0 cm) nor replacing same by a capillary[9], alleviated the difficulty mentioned above. Thus the problem was formulated as follows: (a) to find a solvent system and other conditions, which would separate 18 amino acids into a small number of well defined and well separated groups, (b) in which the amino acids are distributed in such a pattern that they can be easily separated by using not more than two other solvent systems, so that a maximum number of groups can be eluted and chromatographed on one and the same K. C. T. paper.

PRELIMINARY EXPERIMENTS

Initial trials with butanol–acetic acid–water (4:1:5), *tert.*-butanol–formic acid–water (75:0.8:24.2), collidine–water, lutidine–water, phenol–water and phenol–isopropyl alcohol–water (70:5:25) failed to meet all requirements postulated above. The failure with phenolic systems, in spite of vacuum distillation, NaCN-addition to the chamber, purification with petroleum ether–water[15], and aluminium treatment[16] was especially disappointing, since these solvents produce by the ascending or descending technique R_F values which differ widely from those obtained with other solvent systems. Initial trials with butanone–water appeared promising, but the highest R_F values were so low that even after three rechromatograms the groups were too crowded. Pyridine additions to this system gave better results but the solvent system *n*-butanol–butanone–water–ammonia 17 *N* (5:3:1:1), proposed by WOLFE[17] for two-dimensional procedures appeared most suitable and was used for a more detailed investigation.

Chemicals

n-Butyl alcohol, AnalaR, B.D.H.
2-Butanone, Eastman Kodak.
Ammonia, concentrated, analytical quality.
tert.-Butanol, Eastman Kodak.
2,4-Lutidine, refined, Koopers Co., Ltd., Pittsburgh, Pa.
Formic acid, 85–90%, Fisher Certified Reagent.

All organic solvents, except formic acid, were redistilled, those of boiling points over 100° *in vacuo*.

Solvent systems

n-Butanol–butanone–water–ammonia conc. (5:3:1:1, v/v), *tert.*-butanol–formic acid–water (75:0.8:24.2, v/v) and lutidine–water (2:1, v/v) give one phase only when their constituents are mixed.

Paper

Sheets of Whatman chromatographic paper No. 1 were used for the separations into 6 groups. They were washed according the procedure of KAY and co-workers[18] and

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cut into circles of 34 cm diameter. With a double razor three slits are made, producing two centre segments, each with an angle of 30° (Fig. 1).

The centre slit should run parallel to the direction in which the paper is calendered

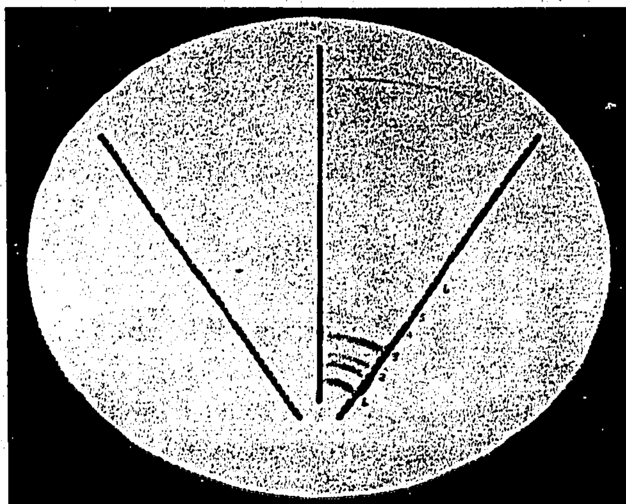

Fig. 1

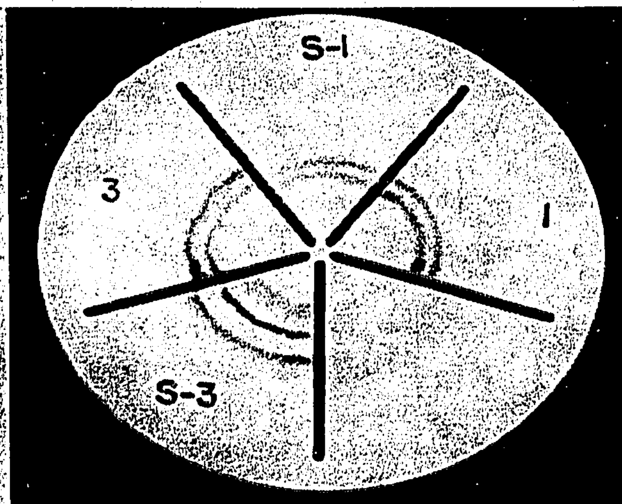

Fig. 2a

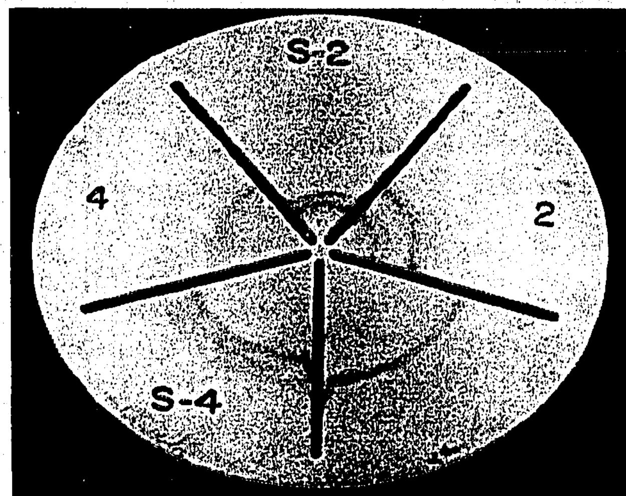

Fig. 2b

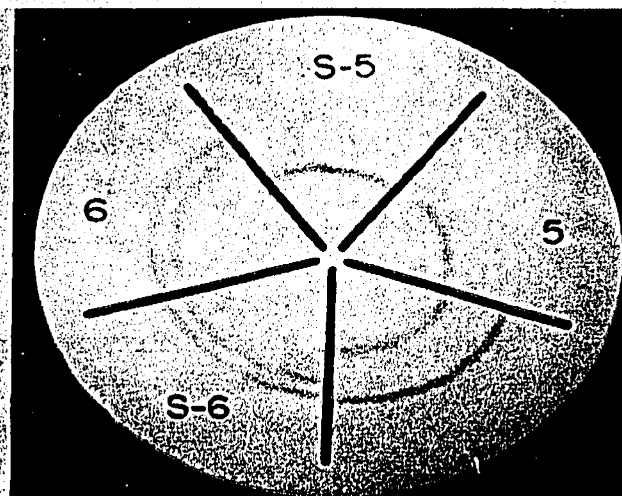

Fig. 2c

Figs. 1, 2a, 2b and 2c. Separation of a protein hydrolysate into six groups and of these into their individual amino acids. 2a: S1, glutamic and aspartic acid; S3, histidine, alanine, serine. 2b: S2, lysine, arginine, glycine; S4, valine, methionine, tyrosine. 2c: S5, threonine; S6, leucine, isoleucine, phenylalanine. The figures 1 to 6 indicate the groups, S1–S6 the corresponding pure standard samples. K.C.T.-paper 2a and 2c have been developed with BuOH:HCOOH:H_2O, 2b with lutidine:H_2O. Experiments 2a and 2c can be carried out on one paper, the addition of standard samples on the same paper not being necessary, once the technique has been established.

in order to avoid complications from the oval shape of the chromatogram.

Subseparation of the groups into individual amino acids was carried out on Whatman No. 1, K.C.T. paper of 26.5 cm diameter which contains 5 equally spaced radial slits (Figs. 2, a, b and c).

Investigations

In order to find such conditions as to produce the widest possible space between the

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TABLE I

VARIOUS MODIFICATIONS OF THE CIRCULAR AND TRIANGULAR METHOD

Expt.	*Method*	*Amount of solvent in ml**	*Number of runs*	*Length of runs = R_F 100 in mm*	*Total time required at 18–20°*	*Results*
A	Circular	35	1	140	12	Groups too crowded
B	Circular	35	2	130	24	Better than A
C	Circular	35	4	145	48	Better than A and B
D	Circular, but paper divided by 4 slits	35	1	135	13	Worse than A
E	*ditto*	35	2	150	26	Worse than B
F	*ditto*	35–12	1	160	13	Inadequate for fast moving bands
G	*ditto*	35–12	2	150	26	Inadequate for fast moving bands
H	Triangular, angle 37°	35	1	200	12	Crowded, but spacing more regular than above
J	Triangular, angle 45°	35	2	220	39	Quite good
K	Triangular, angle 37°	35–1	2	215	37	Inadequate for group of medium R_F-values
L	Triangular, angle 45°	35–5	2	180	30	Inadequate for group of medium R_F-values
M	Triangular, angle 42°	35–15	2	180	26	Widening of bands reduces spaces between them
N	Triangular, angle 30°	35	2	238	50–60	Satisfactory, 5 mm or more between the 6 groups

* The first figure indicates the amount of developing solvent. The second one additional amounts, put in separate dishes.

six groups obtained with the solvent system n-BuOH–2-butanone–NH_4OH–H_2O, various modifications of the horizontal technique were tried (Fig. 4 and Table I).

These tests permit the following conclusions: With the circular method proposed by GIRI two rechromatograms are required as a minimum (A, B). Four rechromatograms (C) give the best results as regards separation but the arcs of the fast moving groups extend to more than 15 cm, which is a detrimental factor for the capillary elution. This difficulty may be overcome by cutting the group bands in half and eluting each of them separately, which however constitutes a loss of time.

When the circular method was modified by dividing the paper into four equal segments, similar to the K.C.T.-technique, lower R_F values were obtained than with the GIRI-technique. Saturation of the atmosphere by putting dishes with additional solvent in the chamber increased the R_F values, but some bands widened and the intervals were reduced. GANGULI[21] had proposed to modify the circular technique by putting the starting point near the periphery, which increases the absolute length of the run. In order to prevent the widening of the bands into dimensions which render capillary elution impractical, two triangular segments were made in the paper (Fig. 1). Saturating the atmosphere by placing additional solvent in the cabinet produced also with this modification wide bands and small spaces in between (K, L, M). The best separations were obtained with a segment angle of 30° resulting in an R_F 100 of 240 mm (C, N). This confirms experiment J in which already quite satisfactory spacing was observed and in which R_F 100 was equal to 220 mm.

Method

The separation into 6 groups is effected in a wooden box, 37.5 × 37.5 × 5 cm, which is covered by a glass plate. The inside and the upper rim are impregnated with molten paraffin. The glass plate is affixed with adhesive tape. The paper is supported by 30 glass needles, vertically inserted in small pieces of cork and regularly spaced.

The solvent, 35 ml, is placed in a flat-bottomed dish, 2.5 cm high and 5 cm wide. A glass tube, provided with a groove at the lower end, serves as an additional support for the paper and as a leader for the paper tongue, which has a width of 1 cm. The latter is inserted into a razor slit at a distance of 5 cm from the periphery of the paper. The amino acid mixture is applied on both segments at a distance of 1 cm from the paper tongue. After the spots have been allowed to dry, the chromatogram is run till the solvent front reaches the end of the slits at the right and left side. At a temperature of 18–20° this usually takes 22–30 h. The paper is allowed to dry, one segment is developed with ninhydrin, the corresponding zones on the other segment are marked with a compass and cut out for capillary elution.

Capillary elution and subseparation of groups

This is nearly identical to the method of GIRI AND RAO[10], except that the leaderstrip and the cut out group band are placed between two microscope slides, which are held together with paper clips (Fig. 5). This modification reduces the time required for the

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elution process and can be accomplished in 30 instead of 90 min. Leaderstrip and cut out group band should be of equal width.

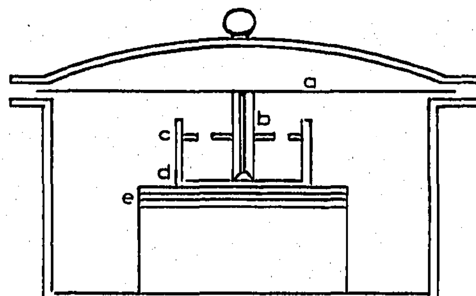

Fig. 3. Chromatography with K.C.T.-paper. a, K.C.T.-paper; b, capillary; c, perforated glass plate, resting on pieces of glass tube not shown in sketch; d, solvent vessel; e, glass plates for height adjustment.

The eluting technique is critical, an excess of water may flood the paper. Two ml in the upper vessel of Fig. 5 usually gave a suitable speed which, if necessary, can be increased by dropwise addition of water from a pipette. The K.C.T.-paper should be in contact with the lower tip of the cut out zone. When the eluted spot has extended

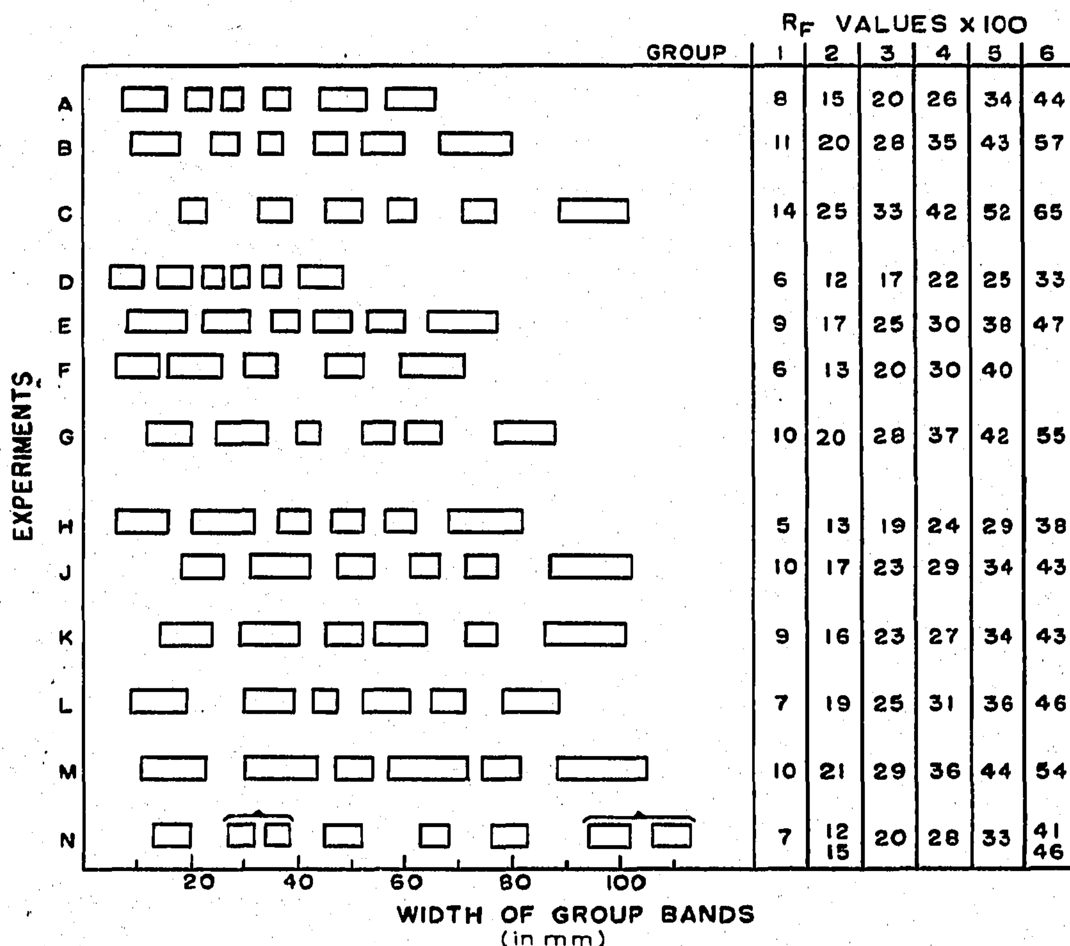

Fig. 4. Spacing, width and R_F values of six groups of amino acids, obtained under various conditions with the solvent n-butanol–butanone–ammonia–water. See also Table I.

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to 4 mm diameter, the K.C.T.-paper is detached by a turn of the screw (Fig. 5), dried at 3 cm distance from a hot plate for 30 sec and replaced. Fifteen to twenty repetitions are usually adequate, as proved by ninhydrin tests with the eluted paper, twenty-five can be made for good measure. Zone 1, 3 and 6 are transferred to different segments of one K.C.T. paper, which is chromatographed with *tert.*-butanol–formic acid–water and zone 2 and 4 are eluted on different segments of another paper which is chromatographed with lutidine–water.

The separation of the groups into individual amino acids is carried out in desiccators of 28 cm width (Fig. 3). The solvent reaches the paper through a capillary, 3 by 0.6

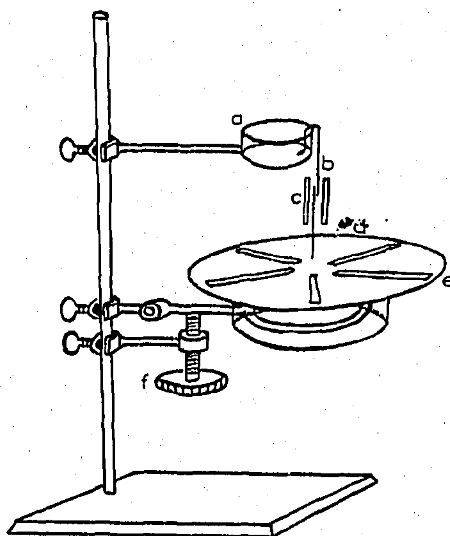

Fig. 5. Chromatography with K.C.T.-paper and capillary elution. a, solvent vessel; b, leaderstrip; c, microscope slides; d, cut out band from first chromatogram; e, K.C.T.-paper.

cm with a 0.36 mm bore. The capillary has a groove at its lower end, permitting access of the solvent and is held in vertical position by a perforated glass plate, which rests on 3 small pieces of glass tube or small weighing glasses. The glass vessels for the developing solvents are identical to those used in the wooden cabinet and contain 35 ml of liquid. In the case of *tert.*-butanol–formic acid–water, 130 ml of solvent are placed in a large petri dish at the bottom of the desiccator. This increases the speed of this slow-moving solvent system. For lutidine–water the addition of 20 ml was adequate to saturate the atmosphere.

The R_F values obtained by this method differ sufficiently for cutting out and eluting the ninhydrin compounds of the various amino acids, especially as the bands are only 3 to 5 mm wide when the two solvents mentioned above are used with 133 μg hydrolysate (Table II). As observed by other authors, the R_F values vary to a certain extent, but the intervals between them are practically constant. Groups 2 and 6 show each a tendency to split up into two bands. As regards group 2 no useful purpose would be served by cutting its bands out and eluting them separately, because lutidine–water separates all four amino acids of this group adequately (Table II). In group 6 isoleucine may be separated from the last group, containing leucine and

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TABLE II

SEPARATION OF A PROTEIN HYDROLYSATE INTO SIX GROUPS AND SUBSEPARATION OF THESE INTO INDIVIDUAL AMINO ACIDS ($R_F \times 100$)

Group	Amino acids	*n*-BuOH–methyl ethyl ketone–water–ammonia, 17 N (5:3:1:1), after 2 runs	*tert.*-BuOH–HCOOH–H_2O (75:0.8:24.2)	Lutidine–water (2:1)
1	Cysteine		26*	
	Glutamic acid	7	39	
	Aspartic acid		48	
2	Lysine	12		17
	Arginine			25
	Glycine	15		39
	Hydroxyproline			—
3	Histidine		76	
	Alanine	20	50	
	Serine		38	
4	Tyrosine			65
	Valine	28		44
	Methionine			51
	Proline			—
5	Threonine	33	37	
6	Isoleucine–leucine	41	63	
	Phenylalanine	46	53	

* The eluted spot was treated with a spot of H_2O_2. Non observance of this will produce from reagent cystine one more band at R_F 16. Proline[22], hydroxyproline[23] and trytophan[24] were determined by specific tests.

phenylalanine, but since the two leucine isomers are usually reported together, this subseparation is not absolutely necessary. If the removal of acid from the hydrolysate is inadequate, as may happen in case only one vacuum destillation has been applied, some threonine may be found in the 6th group.

After the completion of each run, the chromatograms are put in a ventilated fume hood till the translucency of the paper disappears, which usually takes five minutes. They are then washed twice with chloroform, dried at 60° and developed with ninhydrin according to the technique of KAY and co-workers[18]. Omitting the chloroform treatment of chromatograms run with lutidine may give low values, especially for glycine and methionine.

The effect of temperature variations

The temperature of our laboratory usually fluctuates between 19 and 22°, which did not affect the results described here. Higher fluctuations (15–22°), which occurred occasionally on account of breakdowns of the electric supply, interfered strongly. As a precaution the chromatographic chambers and desiccators may be placed inside a cabinet.

DISCUSSION

The fact that difficulties have been experienced in duplicating the results of other workers in the field does not in our opinion justify the statement of PROOM AND WOIWOD[19] who regard circular paper chromatography as "admirable for the separation of two or three substances, (but) not a satisfactory substitute for large scale

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paper chromatography in the separation of----protein hydrolysates". Although we felt it necessary to modify some details of the technique proposed by GIRI, we fully agree with this author's conclusion[20]: "The method is well suited to the separation, identification and even quantitative estimation of----amino acids----".

Reduced laboratory space, controlled solvent flow, small amounts of solvents, sharp bands, the facts that the centre of gravity of each zone is clearly defined[3] and that reference samples can be applied to the same sheet of paper as the test sample are advantages, which outweigh the additional step of capillary elution. It is true that a certain amount of crowding occurs, since no solvent system has as yet been found which spaces 18 amino acids evenly. Therefore it is in the opinion of the authors more important to have a small number of widely spaced groups in the first chromatogram than to have a greater number of crowded ones, provided the eluted groups can be easily separated into their individual amino acids. For the latter purpose the pyridine-containing solvent systems of GIRI did not always give ideal results on K.C.T. paper, probably because its radius is smaller than that used by GIRI. The combination of solvent systems described in this paper permits adequate separations. The fact that PROOM AND WOIWOD could not confirm GIRI's results may have also been caused by different amounts of solvents used. The latter author mentions only a "provision, where necessary for irrigation after the atmosphere was completely saturated----by attaching a glass tube with stopcock at the side of the basin"[5]. The amounts of solvents in relation to the volume of the cabinets used in both laboratories would be of interest, because our experiments G, K, L and M show that increased amounts of solvents widen the bands.

Occasionally the egg shape formation, resulting in distorted bands, made itself adversely felt with the K.C.T. paper. A typical case is shown in Fig. 2b with respect to group 4. One can of course draw conclusions as regards identity from the R_F values, but if a similar paper could be manufactured with an even number of slits, for instance four, arranged in positions vertical and parallel to the calendering of the paper, this interference would be less disturbing, because test sample and standard sample could be spotted opposite each other, so that the location of each spot of a band would correspond to that of its antipode.

Since the length of the run appears to be one of the most important factors, the triangular technique has been tried also with the solvent system *n*-butanol–acetic acid–water (4:1:5). Ten groups are obtained after two runs, but the intervals between five of them are only 3 mm wide. This solvent system may nevertheless be suitable for proteinaceous substances, containing a limited number of amino acids only. The distribution of amino acids is under investigation and will be reported separately.

SUMMARY

Various modifications of the circular paper chromatographic method for the quantitative determination of amino acids in protein hydrolysates have been investigated. With the system *n*-butanol–2-butanone–water–ammonia (5:3:1:1) six groups are obtained. Two groups are separated into their individual components with the system lutidine–water, three others with *tert.*-butanol–formic acid–water (75:0.8:24.2). Threonine appears as a single band. Length of the run is increased and separation is improved by dividing the paper into triangular segments and by putting the starting point near the periphery.

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