# COMBINED COLUMN–PAPER CHROMATOGRAPHY
## II. A NEW SYSTEM OF SOLVENTS FOR THE CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS

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Difficulties in obtaining satisfactory separation of all amino acids of biological origin by means of paper chromatography have necessitated the application of two-dimensional systems. Nowadays this often consists of a combination of ionophoresis and chromatography. Recent adaptations of electrochromatography to new systems of solvents were described by BOTHMAN AND HIGA[1] and, as applied to the analysis of peptides, by RICHMOND AND HARTLEY[2].

The use of combined column–paper chromatography gave the desired separation and had, in addition, certain other advantages. Preliminary separation of amino acids on chromatographic columns and direct placing of the eluate from the columns on moving paper sheets are the principal characteristics of this method. The paper chromatograms are subsequently developed by the ascending method.

In our previous work[3], an apparatus and procedure were described for serial analyses of body fluids. In the present work, we describe further modifications of the procedure and the results of analysing amino acids present in blood, urine and cerebrospinal fluid.

## EXPERIMENTAL

### *Apparatus*

(1) Chromatographic columns 0.9 by 45 cm filled with starch.

(2) Containers 100 ml in volume connected with particular columns directly or by means of igelite tubes.

(3) An automatic device for moving sheets of paper at a rate of 0.75 cm/h (*i.e.* 54 cm, the length of a paper sheet, during a 72-hour period). Columns, from which the eluate flows on to single paper sheets that are moving along, are fastened to this device. To evaporate an eluate deposited on the paper, a stream of warm air is blown under each column.

(4) Bell glasses for ascending chromatography.

### *Reagents, solvents and adsorbents*

(1) Ninhydrin reagent consisting of 0.5 g of ninhydrin dissolved in 100 ml of absolute ethanol with the addition of 0.5 ml of 1 *N* NaOH.

(2) Alcohol solutions: (a) 10 % (v/v) aqueous solution of ethanol, (b) 10 % acidic solution of ethanol (up to 90 ml of distilled water, 10 ml of ethanol and 1.0 ml of 1 *N* HCl), (c) 90 % (v/v) aqueous solution of ethanol, (d) 98 % (v/v) aqueous solution of methanol.

(3) 10 % (v/v) aqueous solution of ammonia.

(4) Potato starch, thoroughly washed with water, dried in air, ground in a mortar and sieved.

(5) Whatman No. 4 paper.

(6) Amberlite IR-120, Wofatite KPS-200, or other sulphonated cation exchangers.

## *Procedure*

### *Preparing columns*

Ethanol (90 %) is added to starch in such a quantity as to form a thick suspension after thorough mixing. This suspension is subsequently poured into the columns up to the point where the level of starch reaches the desired height (40 cm). A column filled with starch is washed with an acidified 10 % solution of ethyl alcohol until the eluate flowing out of it begins to show an acidic reaction.

The adsorbent in a column can be acidified by a previously prepared acidic solution of alcohol. However, in order to save time, it is better to apply a more strongly acid solution (*e.g.*, 0.5 ml of concentrated HCl in 10 ml of 10 % (v/v) ethyl alcohol). The excess of acid is subsequently washed out of the column by passing 20 ml of 10 % ethyl alcohol through it.

The solution should flow out of the column at a rate of 14–18 ml per day. This can be achieved by appropriate tamping of the starch in the column, as well as by placing the solvent container at various heights above the column. When analysing samples containing a large number of amino acids, or biological material in which the presence of many other ninhydrin-positive compounds may be expected, the rate of elution should be adjusted to about 14 ml per day.

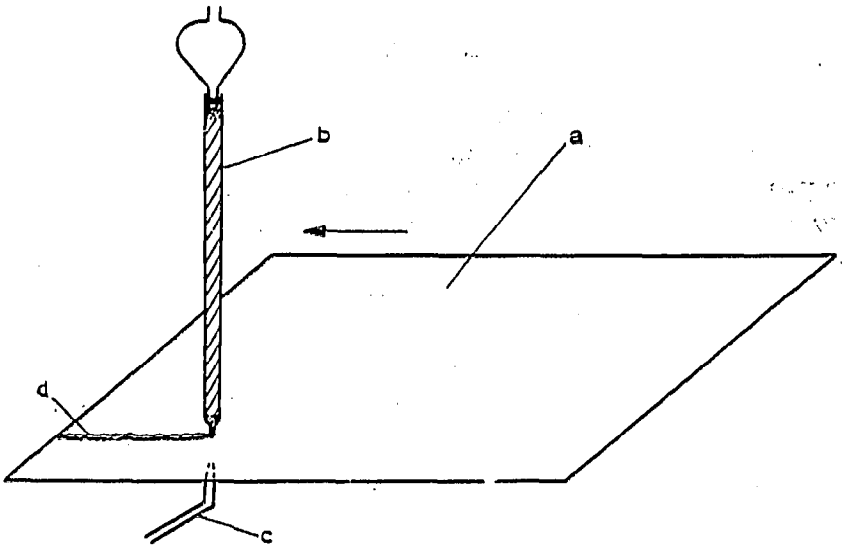


Fig. 1. Diagram showing the apparatus for placing an eluate from the chromatographic column on a moving paper sheet. (a) sheet of paper; (b) column; (c) tube blowing warm air under the column outlet; (d) trace formed by the eluate which dropped and dried.

### *Separation*

From 0.3 to 3.0 mg of a mixture of amino acids dissolved in a small volume (max. 1.5 ml) of 10 % ethyl alcohol is placed in the column prepared in the manner described above. Immediately after the solution has soaked in, about 0.5 ml of 10 % ethyl alcohol is added to rinse the internal walls of the column and, when this portion has flowed down to the adsorbent, the column should be connected to a vessel containing 100 ml of 10 % ethanol. In the final stage of the outflow of this solvent (on the sixth day), 110 ml of 10 % ethanol with 1 ml of 1 *N* hydrochloric acid solution are added to the container. From the moment the amino acid mixture is added until the outflow of the acidified 10 % ethanol, the lower end of the column should touch a sheet of paper moving beneath it (Fig. 1).

The separation on the column lasts 13 days and the eluate is collected on 4 sheets of paper. After the column separation is finished, these sheets, on which the fractions have been automatically placed in the form of a narrow band, are subjected to ascending chromatography in 98 % methanol.

### *Preparation of biological material*

Prior to chromatographic analysis, protein, inorganic salts and non-polar compounds which interfere with the chromatographic separation of amino acids, should be removed from the biological material. To this end, ethanol was added to serum, urine or other biological fluid up to a final concentration of 60 % (v/v). The protein precipitated was filtered off and washed twice with small portions of 60 % ethanol. The combined filtrates were diluted with an equal volume of water and desalted by passing through on Amberlite IR-120 ion exchanger, using the generally accepted technique. Amino acids bound on the ion exchanger were eluted with 60 ml of 6 *N* ammonia, the eluate thus formed was evaporated to dryness on a water bath, *ca.* 0.5 ml of distilled water was added and the mixture was evaporated once more. The dry residue was dissolved in 1 ml of 10 % ethanol and transferred to a starch column.

## RESULTS

### *Standard mixture*

In using column–paper chromatography, about 1.5 mg of a standard mixture of amino acids, dissolved in 1 ml of 10 % ethanol, were subjected to separation. The quantities of the particular amino acids varied in this mixture and fluctuated between 0.0025 and 0.017 mmoles. After staining with ninhydrin, chromatograms were obtained on which the amino acids formed distinct, clearly separated spots, except α-aminobutyric acid, which partially overlapped methionine, and ornithine, which touched lysine (Fig. 2). The distribution of all spots was characteristic and occurred repeatedly in several experiments. Slight irregularity was shown only by glutamic and aspartic acids, which tended to elute down the column slightly faster than other compounds. These minor differences did not, however, cause overlapping of spots and did not present difficulties in identification.

### *Serum*

Nine millilitres of human serum were analysed in the protein-free and desalted form. The presence of many compounds stained with ninhydrin was observed on the chro-

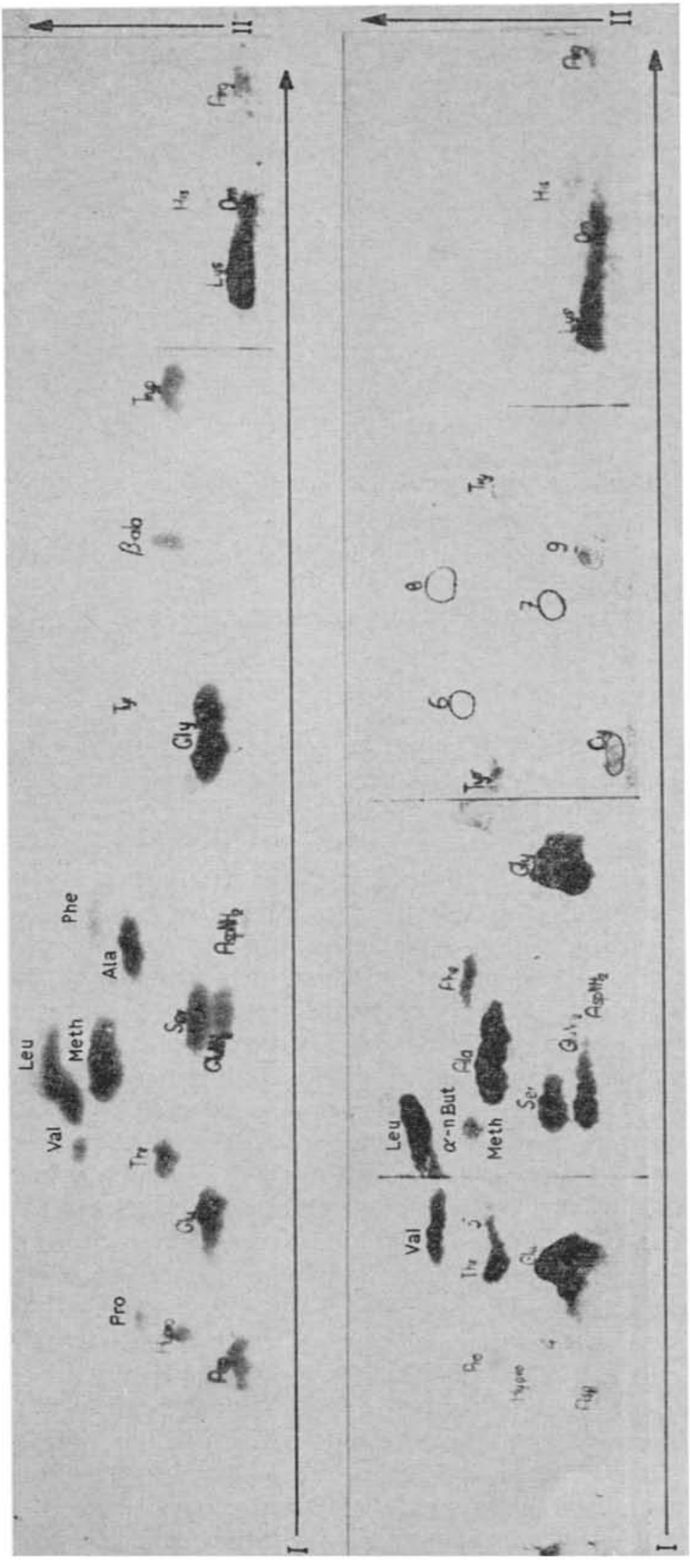


Fig. 2. Chromatogram of a standard mixture of amino acids. (I) direction of placing the eluate from the column; (II) ascending chromatography. Abbreviations: Asp = aspartic acid; Hypro = hydroxyproline; Pro = proline; Glu = glutamic acid; Thr = threonine; Val = valine; Leu = leucine; Meth = methionine; α-n But = α-aminobutyric acid; Ser = serine; Glu $NH_2$ = glutamine; Ala = alanine; Phe = phenylalanine; Asp $NH_2$ = asparagine; Gly = glycine; Tyr = tyrosine; β-ala = β-alanine; Tryp = tryptophan; Lys = lysine; Orn = ornithine; His = histidine; Arg = arginine.

Fig. 3. Chromatogram of amino acids contained in human serum. Abbreviations as in Fig. 2. 1,2,3,4,5,6,7,8,9 unidentified spots.

matograms (Fig. 3). Amongst them, in addition to the amino acids occurring in large quantities in serum (glycine, glutamic acid, glutamine), there were also amino acids that are present in low concentration (hydroxyproline, aspartic acid, asparagine, α-amino-*n*-butyric acid). Besides amino acids, spots were recorded corresponding to other chemical compounds (in the photographs, they are denoted by the numbers 1–9).

*Urine*

The chromatogram shown in Fig. 4 was obtained from 3 ml of the urine of a healthy adult individual. An almost full spectrum of amino acids was recorded here despite the fact that they occurred in considerably varying concentrations. In addition, the presence of 17 other ninhydrin-positive compounds, most of them located in places distant from the spots formed by amino acids, were visible on the chromatogram.

*Cerebrospinal fluid*

Since the concentration of free amino acids is lower in cerebrospinal fluid than in blood and urine, 45 ml of a fluid obtained from a lumbar puncture of an individual suffering from epilepsy was subjected to a single analysis. Clear and distinctly separated spots, of which 19 corresponded to common amino acids and 8 were formed by unidentified compounds, were recorded on the chromatogram (Fig. 5).

## DISCUSSION

The two-dimensional chromatograms obtained by combined column–paper chromatography are similar as those in the coloured photographs shown by FOWDEN[4]. The difference between his method and ours is that, instead of the toilsome work of transferring substances collected in numerous fractions on a collector, to the paper, we used an apparatus[3] which directly places the eluate of several analyses on the sheets simultaneously. This method extends the possibilities of using various adsorbents and various solvent systems for developing chromatograms.

Chromatograms of the analysis of one mixture only are placed on 2–4 sheets of paper. Under such conditions, particular components are separated into clearly defined spots by the use of a larger quantity of a mixture of amino acids (0.3–3 mg) than in two-dimensional paper chromatography. This enables us to detect components occurring in a quantity of a few μg, as well as those occurring in excess (> 300 μg). The results depend on the selection of suitable solvents. In the present work, two solvents, 10 % ethanol and 10 % ethanol with 1 ml of 1 *N* hydrochloric acid, were used for separating amino acids on the column; the eluate was collected on 4 sheets of paper. The chromatograms were subsequently developed with methanol by the ascending method. Quantitative values of particular components were determined by the method of elution and colorimetry developed by KAY *et al.*[5].

This method is easier in practice compared with the procedure previously described[3], since it does not require a concentration gradient of solvents and gives a more accurate separation of neutral amino acids. In particular, glycine separates from serine and phenylalanine from methionine, while proline and hydroxyproline appear at some distance from the remaining neutral amino acids. This is important, as there is only slight staining of imino acids with ninhydrin.

With the intention of separating as many components as possible during a

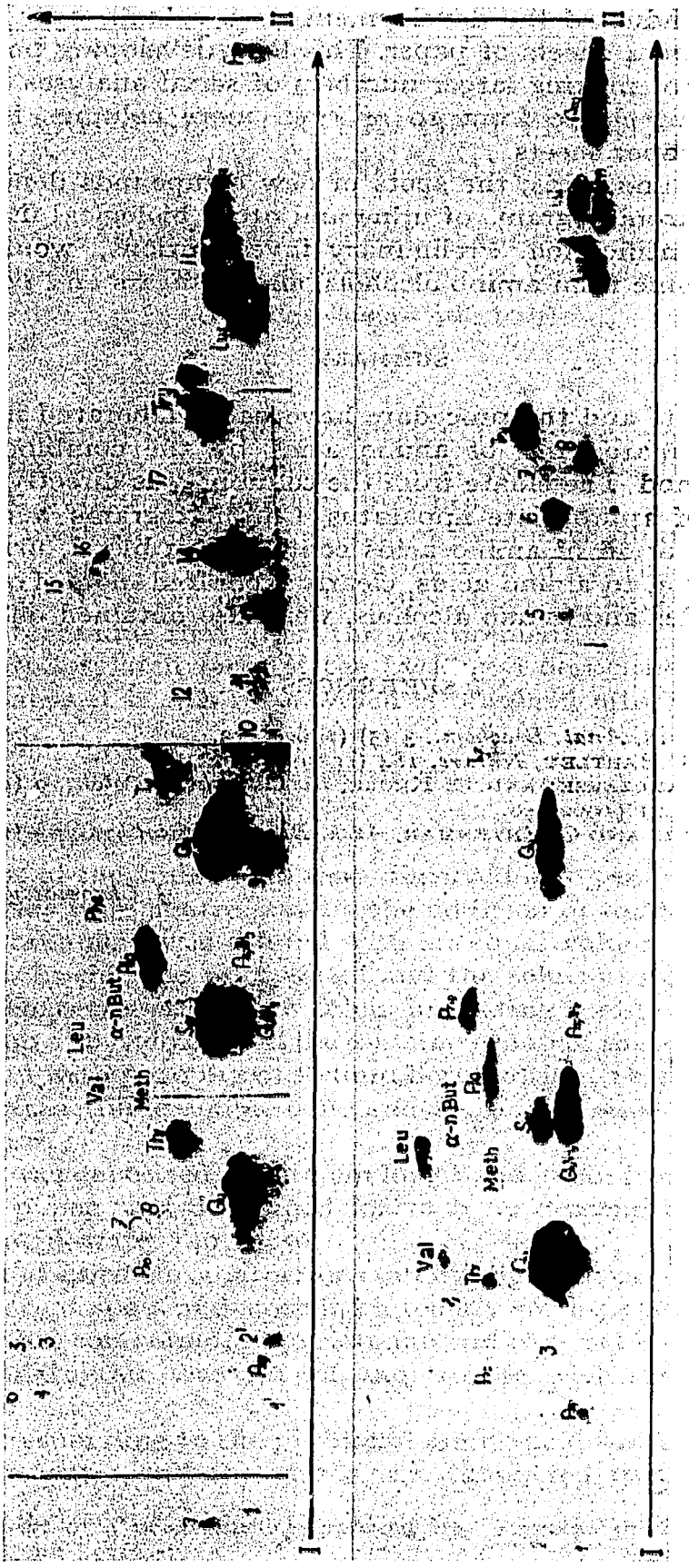


Fig. 4. Chromatogram of amino acids contained in human urine. Abbreviations as in Fig. 2. 10,11,12,13,14,15,16,17 unidentified spots.

Fig. 5. Chromatogram of amino acids contained in human cerebrospinal fluid. Abbreviations as in Fig. 2.

single analysis, we conducted the development on a column over a 13-day period, collecting the eluate on 4 sheets of paper. This long developing period is, however, used simultaneously for making larger numbers of serial analyses. By means of an appropriate device, an eluate from 10 or even more columns is simultaneously placed on particular paper sheets.

In addition to amino acids, the spots of new compounds denoted by Fig. 3–5 were discovered on chromatograms of urine and other biological fluids, this enabled us to begin their identification. Preliminary investigations, would indicate that some of these spots come from amino alcohols, amino sugars and peptides.

## SUMMARY

The systems of solvents and the procedure have been elaborated in order to obtain two-dimensional chromatograms of amino acids by the combined column–paper chromatography method. The eluate from the columns was directly placed on sheets of paper by means of appropriate apparatus. Chromatograms were obtained for a quantitative determination of amino acids contained in blood, urine and cerebrospinal fluid. In addition to amino acids, clearly separated spots of other amino derivatives, such as peptides and amino alcohols, were also obtained on chromatograms.

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