

Two-Way Separation of Amino Acids and Other Ninhydrin-Reacting Substances by High-Voltage Electrophoresis Followed by Paper Chromatography

By MARY L. EFRON

Medical Unit, University College Hospital Medical School, London, W.C. 1

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One-way separation of amino acids by electrophoresis at high voltage is now a well-established procedure (Gross, 1955). With the large number of amino acids often found in biological fluids, it is, however, very difficult to obtain satisfactory separation without overlap of spots.

Several investigators have used techniques for the separation of amino acids and other substances by a combination of paper electrophoresis and chromatography. Durrum (1950, 1951) developed the so-called 'strip-transfer' method. This consisted in cutting out the strip of paper on which chromatography had previously been carried out, blotting the spots on to a second sheet and carrying out ionophoresis at right angles. This technique was further elaborated by Honegger (1956). Consden & Glynn (1955) described another two-dimensional method in which chromatography followed by ionophoresis on the same paper was used.

All these methods utilize low-voltage electrophoresis, which does not give a good separation of the neutral amino acids. Since this paper was written, it has come to the author's attention that Dixon, Kauffman & Neurath (1958) have described the two-way separation of amino acids by using a combination of electrophoresis at 1500 v in one direction and chromatography in the other. They found this method more satisfactory than previously-used chromatographic methods. By modifying the apparatus described by Gross (1956) for one-way high-voltage electrophoresis in such a way that a square paper can be used, amino acids can be separated by high-voltage electrophoresis along one edge of the paper, and chromatography subsequently carried out in the other direction. The two-way separation thus obtained has several advantages over previously described chromatographic methods, especially with biological fluids.

METHODS

The apparatus described by Gross (1956) is used, except that the cooling plates are 16½ in. × 17 in. Whatman no. 4 filter paper, 16½ in. × 18½ in., is wetted with formic acid-acetic acid buffer, pH 2 (see Gross, 1956), and the edges are placed between paper wicks fed from polythene beakers

containing the electrodes, partially immersed in the same buffer. The solution containing the amino acid mixture is applied to the lower right-hand corner of the paper near the anode. If the quantity of solution to be examined is 25 µl. or less in volume, it can be applied directly to the wet paper. If the volume is greater than 25 µl. it should be applied to the dry paper in 25 µl. portions, the paper being dried in a hot-air stream between applications. The sheet is then dipped in buffer, care being taken not to wet the lower right-hand corner where the specimen has been applied. After being blotted to remove excess of moisture the paper is placed between the wicks and the dry corner then sprayed lightly with the same buffer. In this way elution of the applied amino acids is avoided during the wetting of the paper. With this apparatus the electrophoresis run can be carried out on two papers simultaneously, provided that each paper is placed between separate wicks and that the two papers are separated from each other by a sheet of polythene 0.005 in. thick. In this case a current of approximately 200 ma is produced when 3.5 kv is applied to the electrodes. The positions of the spots and the time required for the separation are the same as with a single sheet.

A potential difference of 2.5 kv is then applied to the electrodes, producing a current of approximately 120 ma. The amino acids travel towards the cathode, i.e. from right to left. A rapid and reproducible one-way separation of amino acids in urine, sweat or standard solution is obtained in 30–35 min. Electrophoresis of plasma samples (625 µl.) requires about 50 min., and spinal fluid (625 µl.) 60 min., twice as long as with the smaller-volume urine specimens. The paper is dried either in an oven or at room temperature. It is then ready for chromatography in the right-angle direction, i.e. from bottom to top.

If square cooling plates are not available, the apparatus similar to that described by Gross (1956), which is now available commercially (Locarte Co., London; Shandon Automation and Electronics Ltd., London), can be used. In this case the amino acid mixture is applied to the lower right-hand corner of a standard Whatman no. 4 paper, 18½ in. × 22 in., and the top 4½ in. of the paper moistened with buffer. Only this wet strip (4½ in. × 22 in.) is placed under the cooling plates (6 in. × 19½ in.); the remainder of the sheet is kept dry, so that no current passes through it. Electrophoresis is then carried out in the usual way, producing a good one-way separation of amino acids along the wet strip. The paper is then dried and is ready for chromatography from bottom to top. This method is less convenient than using square cooling plates, but the results are quite satisfactory.

In this Laboratory the standard descending paper-chromatographic technique is used, with a lutidine-water mixture (2:1, v/v) as the solvent. This requires 7-8 hr. for a sheet of this size, but the paper can be left overnight if the amount of solvent is limited to 30 ml./paper.

After being dried in an oven the paper is sprayed with a 0.1% (w/v) solution of ninhydrin in butanol, and then heated at 60° for 2 hr. or 100° for 1-2 min.

Preparation of biological fluids for electrophoresis

Urine. Urine can be applied directly to the wet paper, provided that it is free from protein. As a small amount of protein causes considerable streaking of amino acids during high-voltage electrophoresis, it is necessary to deproteinize any specimens giving a positive test for protein. This can rapidly be accomplished by adding 50 mg. of crystalline

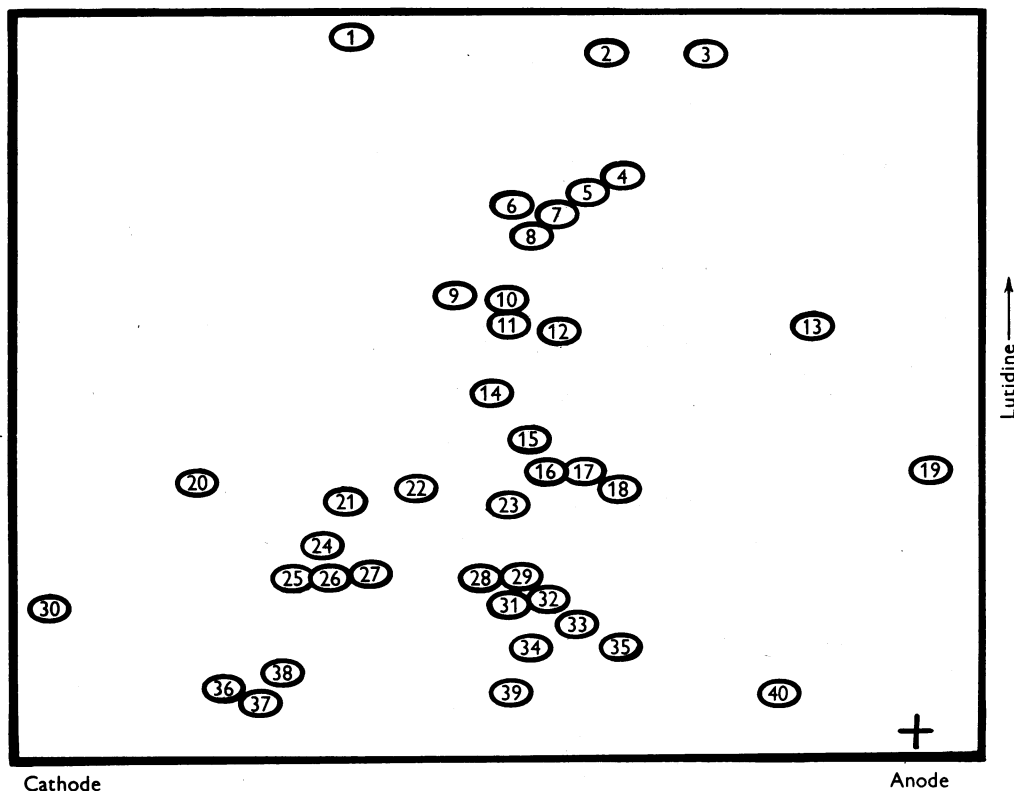


Fig. 1. Map of the location of amino acids and other ninhydrin-reacting substances. This shows the positions of spots, not tracings of their outlines. Each substance (5 μ g.) in standard solution was applied at the lower right-hand corner (+) near the anode. Electrophoretic separation was from right to left, toward the cathode, a pH 2 buffer being used. A potential difference of 3.5 kv was applied, developing a current of 100-120 ma for 30 min. Lutidine was then run from bottom to top. The identifications are:

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|----------------------------------|---|-------------------------------|
| 1. Tyramine | 15. Threonine | 28. Homocystine |
| 2. α -Amino-octanoic acid | 16. Proline | 29. Glutamine |
| 3. 3:5-Di-iodotyrosine | 17. Hydroxyproline | 30. Putrescine |
| 4. Tryptophan | 18. Methionine sulphoxide | 31. Asparagine |
| 5. Tyrosine | 19. Cysteic acid | 32. Glutamic acid; citrulline |
| 6. Leucine | 20. Ethanolamine | 33. Aspartic acid |
| 7. Phenylalanine | 21. β -Aminoisobutyric acid | 34. Cystathionine |
| 8. Methionine | 22. Alanine | 35. Glutathione |
| 9. Glucosamine | 23. Serine | 36. Ornithine |
| 10. Norvaline | 24. Histidine; 1- and 3-Methylhistidine | 37. Lysine |
| 11. Valine | 25. β -Alanine | 38. Arginine |
| 12. Methionine sulphone | 26. γ -Aminobutyric acid | 39. Argininosuccinic acid |
| 13. Taurine | 27. Glycine | 40. Phosphoethanolamine |

picric acid to 2 ml. of urine, centrifuging for 3 min. at 2500 rev./min. and decanting the supernatant. If this supernatant is clear yellow, the specimen has been adequately deproteinized. In an occasional specimen with heavy proteinuria the supernatant may still be cloudy. In this case an additional 50 mg. of picric acid should be added and the mixture centrifuged again. This will usually produce a clear supernatant. Picric acid does not interfere with electrophoresis, as it moves towards the anode when a buffer (pH 2) is used, whereas the amino acids move towards the cathode. A volume of urine containing 250 μ g. of total nitrogen is the standard quantity used in this Laboratory for routine purposes. If overloading is desired, however, 5-10 times this amount can be used without desalting. Desalting is accomplished automatically during the electrophoretic separation and there is therefore no interference by urinary salts with the subsequent chromatography. With such an overloaded urine the electrophoresis must be continued for 50 min.

Plasma. Plasma can also be deproteinized with picric acid and run without further preparation. Most 2 ml. plasma samples are adequately deproteinized by the addition of 150 mg. of picric acid. An occasional sample, particularly one containing haemolysed red cells, may require the addition of a further 50 mg. of picric acid to produce a clear supernatant. Approximately 1.3 ml. of protein-free plasma is recovered from the initial 2 ml. sample. Of this volume, 625 μ l. has been used as the standard quantity for routine study. It can be evaporated to a volume of 25-50 μ l. before being applied to the paper, or can be applied in 25 μ l. portions.

Spinal fluid. Spinal fluid is deproteinized by the addition of 25 mg. of picric acid to 1 ml. of fluid. As with plasma, desalting is unnecessary. The standard quantity for routine study is 625 μ l.

Sweat. Sweat is treated in the same way as spinal fluid. The supernatant in this case, though adequately deproteinized, never becomes completely clear. The standard quantity for study is 125 μ l.

RESULTS

A map of the positions taken by 43 ninhydrin-reacting substances in pure solution is reproduced in Fig. 1. Fig. 2 is a tracing of the spots due to ninhydrin-reacting substances present in the urine of a patient with typical Fanconi syndrome and gross amino aciduria. It can be seen that this method produces compact, round spots. Because of the short time required for electrophoretic separation, and the absence of salt interference, there is very little diffusion of the amino acid spots. Figs. 3 and 4 are tracings of the spots due to

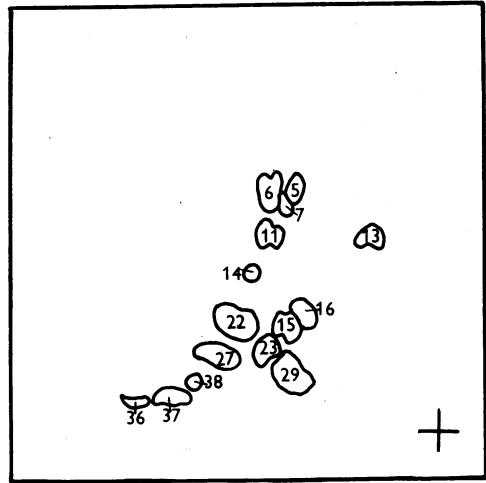


Fig. 3. Ninhydrin-reacting substances in normal plasma. Deproteinized plasma (625 μ l.) is applied. For numbering of spots, see Fig. 1.

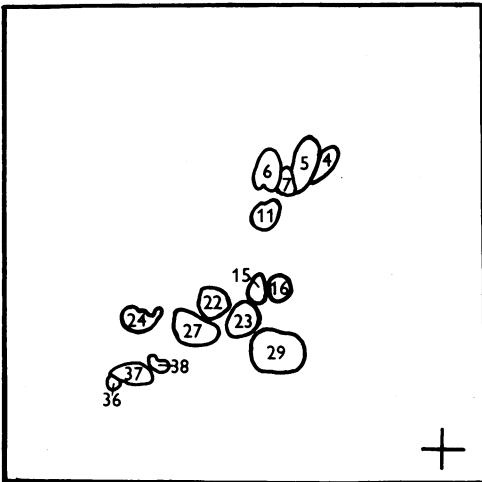


Fig. 2. Tracing of the spots in urine of a patient with Fanconi syndrome. An amount of urine equivalent to 250 μ g. of nitrogen was applied. Spot identification as in Fig. 1.

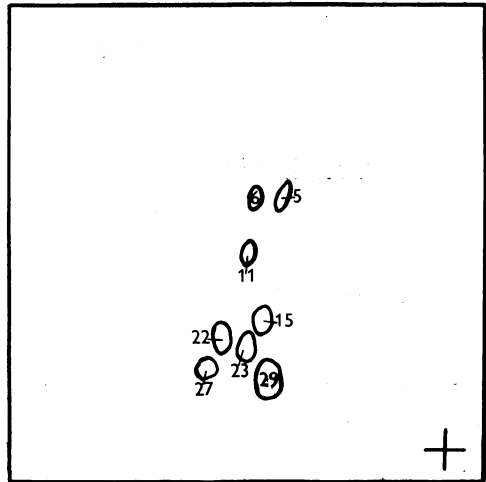


Fig. 4. Ninhydrin-reacting substances in normal cerebrospinal fluid. Deproteinized cerebrospinal fluid (625 μ l.) is applied. For numbering of spots, see Fig. 1.

ninhydrin-reacting substances present in a normal plasma and in normal spinal fluid, prepared as described above.

DISCUSSION

The combined ionophoretic-chromatographic technique has certain advantages over conventional two-way chromatography. This method is rapid. Time is saved by: (a) replacing the 24 hr. first run in phenol with high-voltage electrophoresis taking $1\frac{1}{2}$ hr.; (b) eliminating the need for desalting; (c) for protein-containing fluids (plasma etc.), avoiding the tedious process of ultrafiltration, which is replaced by a simple deproteinization with picric acid followed by centrifuging. The entire operation can be completed in 9–10 hr. instead of the 48 hr. required by two-way chromatography.

There is better separation of certain amino acids. For example, methionine separates from leucine, whereas in the phenol-lutidine chromatogram these spots overlap, and oxidation is necessary to detect the presence of methionine (Dent, 1947). Asparagine produces a distinct spot and does not overlap glycine. Cystathionine, argininosuccinic acid and phosphoethanolamine are more distinctly separated from each other. β -Aminoisobutyric acid separates well from histidine. On the other hand, glutamine and glutamic acid are not so well separated as they are by two-way chromatography, and the 1- and 3-methylhistidines overlap histidine.

The spots are rounder and more compact than with two-way chromatographic techniques. The electrophoretic separation allows very little diffusion of the spots, because of the short time required to traverse a given distance. As a result of the automatic desalting during electrophoresis, the final chromatogram shows little distortion of the

spots. Another advantage is that volatile substances such as ethanolamine are often found in pathological urines when not detected in the same specimen by routine chromatography.

SUMMARY

1. A technique for two-way separation of amino acids and other ninhydrin-reacting substances by high-voltage paper electrophoresis followed by paper chromatography is described, which gives more rapid separation than two-way paper-chromatographic methods.

2. The method is particularly useful for the study of plasma and spinal fluid, because these fluids can be deproteinized in a few minutes with picric acid, thus eliminating the tedious process of ultrafiltration. Moreover, desalting is not necessary, as it is automatically accomplished during the electrophoresis run.

3. A map is presented showing the positions taken by 43 ninhydrin-reacting substances when separated by this method.

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The Isolation and Characterization of Acetylcholine-Containing Particles from Brain

By V. P. WHITTAKER

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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Chemical transmission at nerve endings is now being increasingly discussed in terms of the 'synaptic vesicle' theory (del Castillo & Katz, 1956; Eccles, 1957; Hebb, 1957). Synaptic vesicles are small bodies about $0.05\ \mu$ in diameter observable in electron micrographs of thin sections of nervous tissue and concentrated in large numbers

at many different kinds of nerve endings (de Robertis & Bennett, 1955; de Robertis & Franchi, 1956; Robertson, 1956; Palay, 1956; Luft, 1956); they are assumed to contain chemical-transmitter substances (e.g. acetylcholine in cholinergic neurones) which are released in response to the arrival of a nerve impulse.