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P. Delmotte[a]
[a] National Center for Multiple Sclerosis, Melsbroek, Belgium

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SEPARATION OF PROTEINS AND OTHER COMPOUNDS BY CAPILLARY ISOTACHOPHORESIS

P. Delmotte
National Center for Multiple Sclerosis
B-1910 Melsbroek, Belgium

Electrophoresis, as the name implies, consists essentially in moving charged particles under the influence of an electric field. Since the first practical experiments by Tiselius[1], most of the variations in electrophoretic techniques have been based on the principle of zone electrophoresis. Only in the last decade did electrophoretic separation techniques,based on other principles, become available: isoelectric focusing and isotachophoresis. It is becoming more and more clear that neither of these techniques can provide the ideal solution to every protein separation problem but that each of them has its special field of application where it can give the researcher a maximum of information.

In zone electrophoresis (1,2), the sample ions are brought into a buffered solution bridging from the anode to the cathode. Each ion moves with its own characteristic speed, resulting under ideal conditions, in complete resolvement of the different species. The net electrophoretic mobility of a charged molecule depends principally on:

- the net electric charge on the molecule itself. This charge depends essentially on the difference between the pH of the buffer and the isoelectric point of the protein molecule.
- the strenth of the electric field
- the ease with wich the molecule can move through the supporting medium
- the magnitude of the water counterflow caused by electroendosmosis.

Zone electrophoresis has some drawbacks:

- a real equilibrium stage is never reached. To arrive at reproducible separation patterns, all experimental conditions must be strictly controlled.
- there is no concentrating effect: during the whole separation experiment, ordinary diffusion tends to spread out the zones.

Isoelectric focusing (3) makes use of a pH gradient. The principle of formation of natural pH gradients was already discussed at the beginning of this century. But, only a decade ago, the chemicals suited to construct, under the influence of an electric field, stable pH gradients became available (Svensson[3]). Protein molecules, brought into the same system, will under the influence of the same electric field, migrate until they reach a place in the gradient where the pH corresponds to their own isoelectric point. All the proteins with the same isoelectric point are focused in the same place of the gradient.

The main advantages of this technique are:

- very reproducible separation patterns are obtained
- the powerful concentrating effect permits detec-

tion of very small amounts of protein
- the isoelectric point of a given protein can serve as an aide in its identification.

Allthough the principle of isotachophoretic separation of charged molecules was already discussed a long time ago (Whetham[4]), the practical application of this electrophoretic principle became only possible during the last few years. As is often the case, it is because of progres made in other fields, like electronics and synthesis of new materials, that make possible the final step from theory to practice (10,11,12).
Contrary to zone electrophoresis and isoelectric focusing, in isotachophoresis use is made of two electrode solutions with different composition. In both of these solutions, the principal ion must have the same charge sign as the sample ions to be separated. But, in the first solution, called the "leading electrolyte solution", this ion must have, under the experimental conditions chosen, a net electrophoretic mobility higher than that of the fastest of the sample ions to be separated. The second solution, called the "terminating electrolyte solution", must have an ion with a net electrophoretic mobility below that of the slowest of the sample ions of interest. To complete these electrolyte solutions, a counter-ion, having a good buffering capacity at the chosen working pH, is selected.
At the start of the experiment, most of the separation chamber and one of the electrode vessels is filled with the leading electrolyte solution. The rest of the separation chamber and the other electrode vessel is filled with the other electrolyte solution. The sample solution is brought in the separation chamber, exactly at the interface of the two electrolyte solutions (see fig.1A).

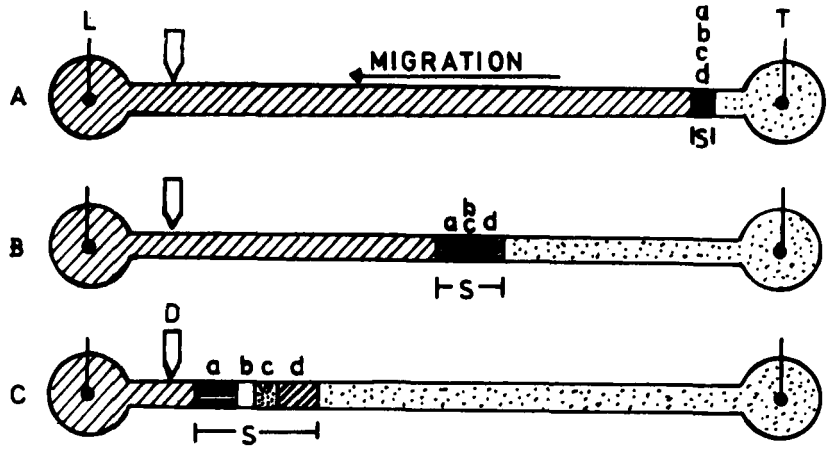

FIGURE 1

Principal stages of an isotachophoretic separation experiment. A= start - B = partial separation of sample ions - C = complete separation of sample ions just prior to detection.

On applying the electric potential, all charged particles start to move. Their displacement speed is limited by the net electrophoretic mobility of the fastest ion , namely that of the ion of the leading electrolyte solution. Under constant current conditions, all ions move with the same speed. At any stage, the sample ions of interest remain sandwiched between the two electrolyte ions, but, they order themselves according to their electrophoretic mobility (see fig.1B).
The conditions at true dynamic equilibrium (fig.1C) can be predicted from the Kohlrausch[5] formula. These conditions confer to isotachophoresis several interesting properties:

- inherently, the system has a pronounced zone boundary sharpening effect: if an ion lags behind

its own zone, then it comes automatically into a zone made up of ions with lower electrophoretic mobility, with a higher electric resistance and under the constant current condition, a higher electric field strength. The wandering ion is thus speeded up until it again reaches its own zone. Exactly the reverse happens when an ion accidently wanders into a preceding zone where a lower electric field strength reignes. This phenomenon results in very sharp zone boundaries.

- A second very important property of isotachophoresis resides in the fact that the concentration of all the subsequent ions in their respective zones adjusts itself to the concentration of the ion in the leading electrolyte solution. This has two interesting consequesces: firstly, isotachophoresis has a very strong concentrating effect. Sample ions present in very low concentrations are at equilibrium pushed together into very narrow zones. Secondly, as the concentration of ions in all the following zones is a function of the concentration of the leading ion and all zones move with the same constant speed, the length of the zones can serve as a direct measure of the amounts present in each zone.

Two other conditions can present themselves in an isotachophoretic experiment:

- If the sample contains ions with a net electrophoretic mobility higher than that of the leading electrolyte, than these ions will move "zone electrophoretically" through the leading electrolyte. If their concentration is not too high, this generally will not influence seriously the outcome of the separation experiment.

- If the sample contains ions with a net electrophoretic mobility lower than that of the ion in the terminating electrolyte solution, then these ions are continuously overtaken by the terminating ions and never reach the detector. In some instances this can be an advantage: the choice of the experimental conditions can be such that only those sample ions that are of interest remain sandwiched between the two electrolytes, thus simplifying the separation pattern.

In Fig.2 a block diagram of the different components of a complete isotachophoretic set-up are presented. The separation chamber, a teflon tube of 0,2 to 0,5 mm internal diameter is kept at a constant temperature by immersion in a thermostated fluid. On each side the capillary is connected to an electrode vessel with platinum electrodes.

On the leading electrode side, the fluids in the electrode vessel and capillary are separated by a semi-permeable membrane, so as to prevent hydrodynamic flow of fluid. On the terminating side of the capillary, provision is made for introducing the sample solution, either through a septum or with a special stopcock. On either side stopcocks and seringues are arranged to facilitate rinsing and filling of the electrode vessels and capillary.

The detection of the separated zones must be made during the dynamic equilibrium stage: the zones pass by the detectors at a constant speed. In most cases a combination of two or more detectors are installed in the same instrument and they can be used concurrently.

The thermometric detector is based on the fact that different amounts of heat are produced in each zone (constant current but different resistances). This heat

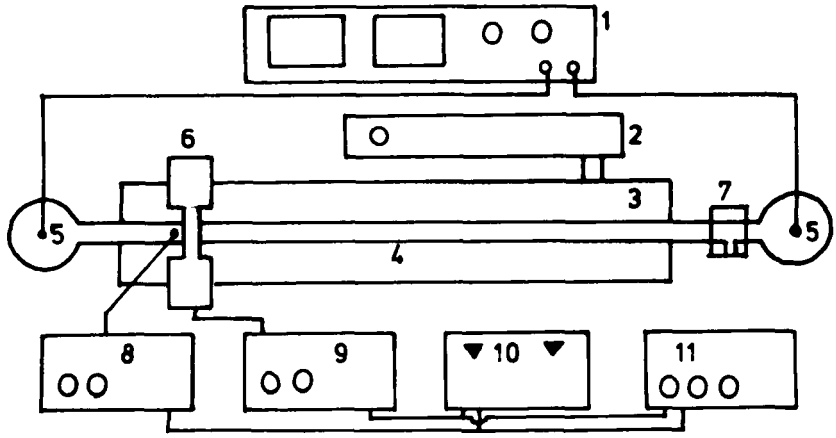

FIGURE 2

Bloc diagram of an isotachophoretic set-up. 1 = power supply - 2 = thermostat for capillary - 3 = thermostating fluid - 4 = capillary - 5 = electrode vessels - 6 = U.V. detector - 7 = injection port - 8 = amplifier for thermocouple signal - 9 = amplifier for U.V. signal - 10 = recorder - 11 = integrator.

is detected by a thermocouple glued to the outside of the capillary. Allthough of general use, this detector has a slow response and short zones can pass undetected. This detector does not lend itself well for zone length measurements.

In the U.V. detector, a very narrow beam of U.V. light passes directly through the capillary and its intensity is monitored by a phototube and amplifier. Due to its very rapid response, this detection system can even be used for compounds without U.V. absorbance: trace amounts of U.V. absorbing compounds are allways present in the electrode solutions and sample and these show up as very narrow spikes between the non-U.V. absorbing zones.

For potentiometric detection, very narrow platinum-iridium electrodes are passed through the walls of the capillary so as to make direct contact with the fluid inside. AC or DC potentiometric measurements can be made while the zones pass through the capillary at constant speed. The potentiometric detector has a sensitivity and and speed of response comparable to the U.V. detector.

Zone electrophoresis and isoelectric focusing are techniques that lend themselves nearly exclusively to the study of proteins. Isotachophoresis on the contrary was only applied to protein separation studies after it had already proved its worth for the analysis of smaller molecules.

Since most proteins have isoelectric points below pH 8,5 , best results are often obtained with a leading electrolyte pH of 9. A relatively slow moving leading ion like morpholinoethanesulfonic acid (MES), at a concentration of 0,005 M is of general use. With a 23 cm long capillary and a constant current of 40 microamperes during the detection stage, a complete separation experiment takes about 15 to 20 minutes. During this stage an electric field strength of more than 500 volts per cm can be reached.

Fig.3A shows a typical example of a separation experiment obtained after the injection of only 0,6 microliter of normal human serum, a very complex mixture of proteins. Only the albumin fraction, of which enough is present in the sample so that the concentration can adjust itself to the concentration of the leading ion, can readily be identified at the plateau formation, both in the U.V. and the thermometric tracings. The non-concordance in time of these two tracings is due to the physical distance between the two detectors along the capillary. All other proteins, although arranged according to their electrophoretic mobility, are only separated by

the traces of non-U.V. compounds present in the sample. It is evident that to render isotachophoresis suitable for protein analysis, this interspacing effect by non-U.V. absorbing compounds must be accentuated. This can conveniently be done by the use, in combination or alone, of discrete spacers, like amino-acids, and more complex mixtures (6,8). For this latter purpose, the amphoteric synthetic polypeptides, namely the ampholines used in isoelectric focusing, are suitable. It must however be remarked that the earlier preparations of ampholines, which contained fewer individual compounds, are more suitable for this purpose (the contrary is true for isoelectric focusing).

Figure 3B shows the separation pattern obtained when the same serum sample was injected together with a small amount of the amino-acid glycine. The complex mixture of proteins is now split up into two subgroups.

The separation pattern shown in figure 4, was obtained after the simultaneous injection of the serum sample, the amino-acid glycine and an ampholine preparation with isoelectric points between 9 and 11. The package of proteins with electrophoretic mobilities lower than that of the amino-acid glycine are now split up into a number of well defined subfractions. Depending on the problem one is interested in, a well defined portion of the protein mixture can be spaced between two discrete spacers and also be further split up by the use of a suitable preparation of ampholines.

This approach is illustrated by the separation patterns presented in figure 5: human serum and the three amino-acids, glycine, valine and beta-alanine were injected together with four different, 0,25 pH units ranges, of ampholines with mean isoelectric points of 10, 9,35 , 9,06 , and 8,66.

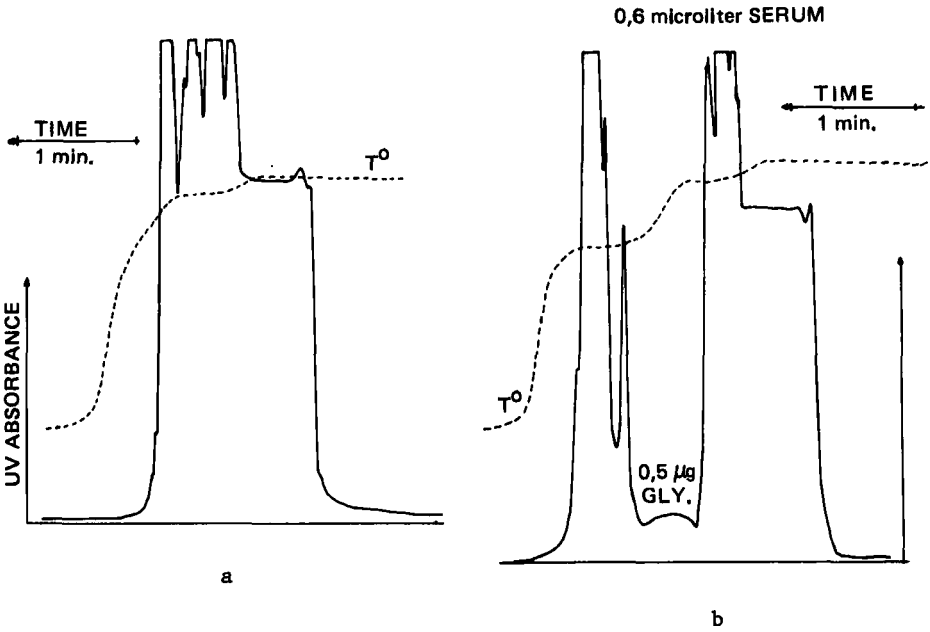

FIGURE 3

a) Isotachophoretic separation pattern of normal human serum. b) Same sample with amino-acid glycine added to sample before injection.

It is essential to draw attention to the differences in split-up obtained in isotachophoresis and isoelectric focusing. In isoelectric focusing, the protein fractions arrange themselves in the pH gradient according to their own isoelectric points: the separation is a completely natural one. In isotachophoresis on the contrary, the split-up depends on two parameters: the composition of the sample itself and the composition of the spacer mixture used. Two different proteins, with closely spaced isoelectric points, when sandwiched between two molecular species of the spacer mixture used, can give rise to a single mixed zone. It is up to the experimen-

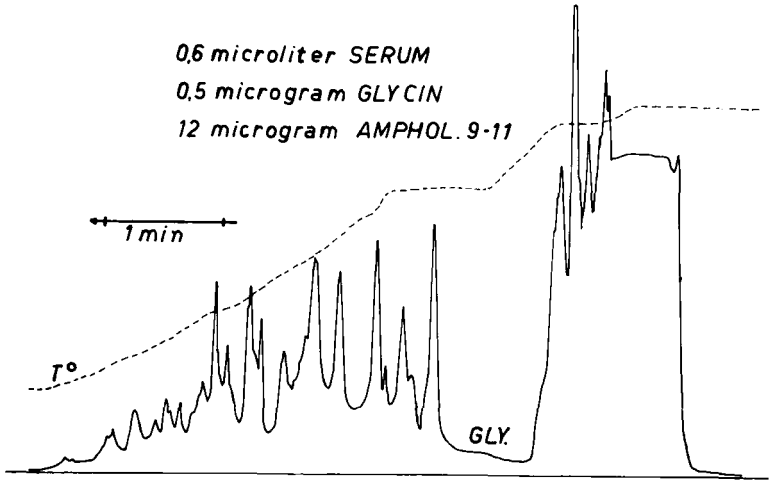

FIGURE 4

Isotachophoretic separation pattern of normal human serum when injected together with discrete spacer glycine and an ampholine preparation with a pH range from 9 to 11.

ter to make out if this is the case or not. One way to do this is to make use, for the same sample, of two ampholine preparations of different manufacturers, and to examine the changes in separation patterns that do occur.

The use of spacer mixtures without well defined composition, like the ampholines, although permitting very interesting separations, has also some drawbacks:

- It is not always possible to find the right mixture of ampholines, containing enough of the two non-U.V. compounds, one just faster and one just slower than the protein one wishes to isolate from the rest of the protein mixture.
- In most cases, the spacer mixture is so complex that it contains many molecules that have electrophoretic mobilities very close to one or more

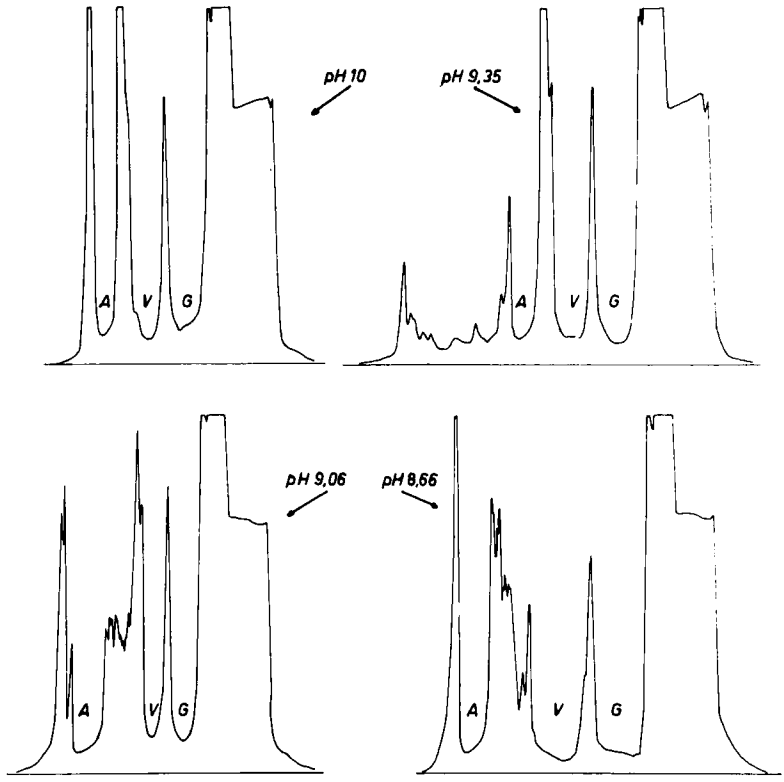

FIGURE 5

Separation patterns of normal human serum with discrete spacers glycine (G), valine (V), beta-alanine (A) and injected together with four different narrow-range ampholine mixtures.

of the proteins of the sample: stable mixed zones result, broadening and lowering some of the protein peaks.

- Care must always be taken when comparing separation patterns obtained with spacer mixtures of different composition. However, this can also be of an advantage to make out wether a protein peak is due to one or more molecular species or not.

An important problem in capillary isotachophoresis is the identification of the different fractions obtained. Most other electrophoretic techniques make use of some sort of supporting medium which permits, after the actual separation experiment, to apply immunological or other identification techniques to the separated fractions. This is not possible in capillary isotachophoresis: the separation takes place in free solution and the separation patterns remain only intact as long as the electric field remains applied. Other approaches are possible:

- Separation patterns obtained after injection of samples wtih or without a well defined protein fraction can be compared
- Separation patterns of two samples, one of which is enriched or depleted in a well defined fraction, can be compared.
- The situation of a fraction in the separation pattern with respect to the thermometric tracing can be compared to its situation, under the same conditions, when only the pure fraction and the spacer mixture are injected.
- Use can be made of a special fraction collector. This device permits the continuous collection of the separated fractions on a strip of cellulose acetate after they have passed the detectors. Immunological or other identification techniques can then be applied to the cellulose acetate strips. This technique is also very suitable in case of radioactively tagged proteins.

Figure 6 presents the separation patterns of the serum and cerebrospinal fluids of the same person. The three discrete spacers, glycine, valine and beta-alanine, together with a wide-range ampholine preparation

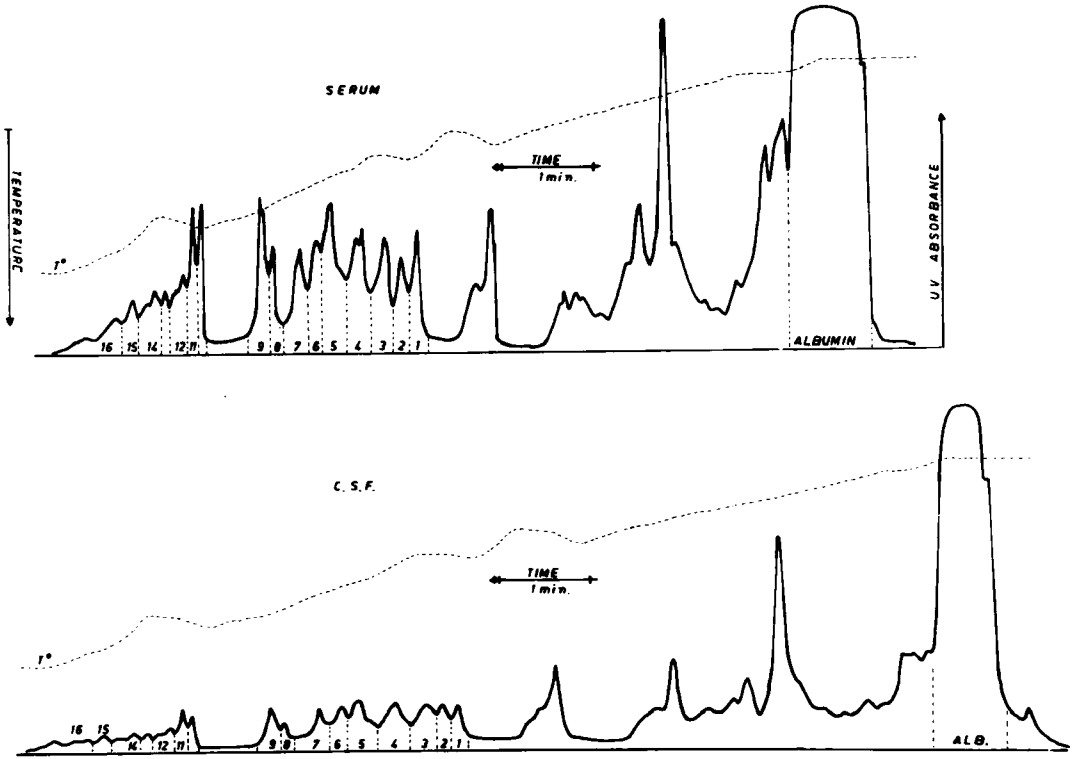

FIGURE 6

Isotachophoretic separation pattern of serum and cerebrospinal fluid from the same normal individual. Spacer mixture contained 3 amino-acids and a wide range ampholine mixture.

were used as spacer mixture. The peaks, numbered 1 to 16, are mobility subfractions of immunoglobulin G. The sharp peak, half-way between the albumin fractions and the amino-acid glycine is due to the protein transferrin.

One of the most interesting properties of capillary isotachophoresis for the analysis of proteins lies in the direct quantitative information it can provide (7,9). As already explained earlier, small ions can be quantified in isotachophoresis by zone length measurement. This is nearly impossible for proteins:

- Only rarely is enough of one protein species present so that its concentration can adjust itself to the concentration of the leading ion. So, there is no plateau formation and no measuring of zone length.
- The complex nature of the spacer mixtures that are normally used has a deleterious effect on zone boundary sharpness.
- The sagging of the boundaries of the protein zones, due to their high density, also negatively influences zone boundary sharpness.

However, the integration of the areas under the protein peaks provides very valuable quantitative information. Provided that a given peak is composed essentially of one protein species, than there is a linear relationship between the quantity of protein and the area under the peak of that protein (Delmotte[7]). Under suitable conditions, amounts of protein down to about 10 nanograms can readily be quantified by this technique.

To illustrate the usefulness of this new electrophoretic technique for the analysis of protein mixtures, a few practical examples are presented. Figure 7 shows the separation patterns of two cerebrospinal fluid specimens, one of a normal individual and one from a Multiple Scle-

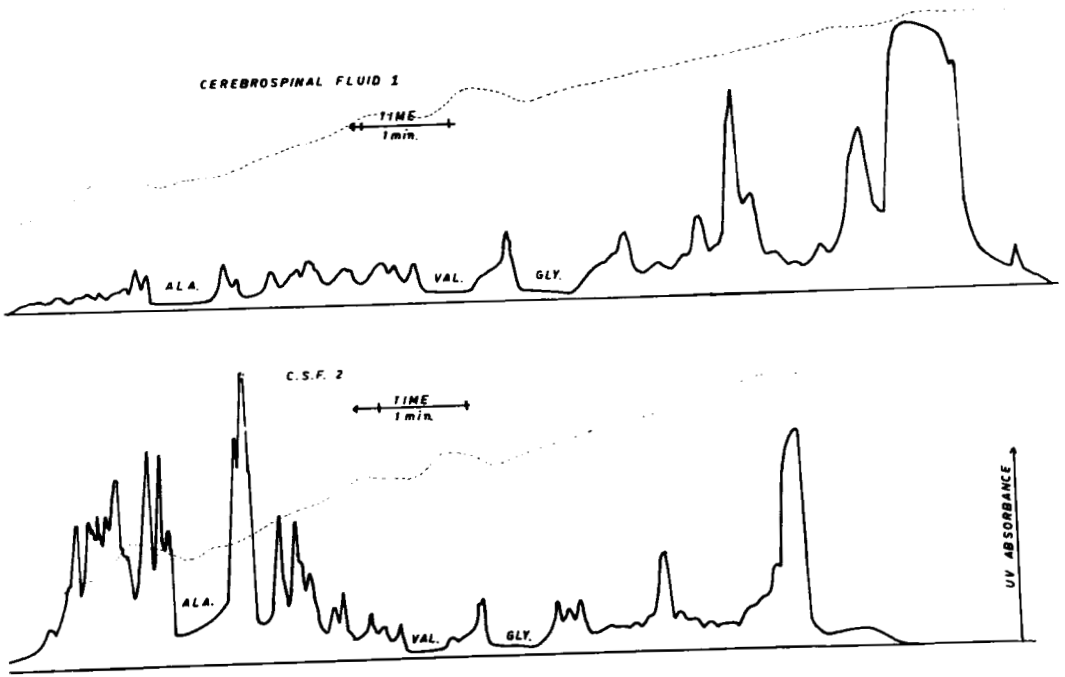

FIGURE 7

Separation patterns of cerebrospinal fluid specimens: fluid 1 is from normal individual, fluid 2 is from a Multiple Sclerosis patient.

rosis patient. In each case the same spacer mixture (3 amino-acids plus ampholines) and the same operating conditions were used.
The sample volume was 3 microliters of ten times concentrated cerebrospinal fluid. Striking differences in the two patterns are evident:

- In the pathological sample (n°2), the relative amounts of albumin and the immunoglobulin G subfractions (moving behind the amino-acid valine), are very different, when compared to the normal sample.
- Among the immunoglobulin G mobility subfractions, those with the lowest electrophoretic mobility show the greatest increase.

By using this technique, the daily intrathecally produced immunoglobulin G can be estimated (Delmotte[9]).

When only minute amounts of sample are available, capillary isotachophoresis is a powerful tool for obtaining quantitative information. This is illustrated in figure 8. The first separation pattern (a), comes from the water-soluble proteins from the eye lens of a young mouse. The second pattern corresponds to a similar sample, but from a very old mouse. With this technique, the quantitative changes in soluble protein composition of the mouse lens with respect to age could be quantified (13).

Finally, figure 9 shows the separation patterns of water soluble proteins from human brain white matter (14,15). The first sample was obtained after normal cell disruption, the second after ultrasonic treatment of the insoluble residue from the first extraction. For the mouse lens proteins and the brain proteins, the same spacer mixture was used as that for the serum and cerebrospinal fluid experiments.

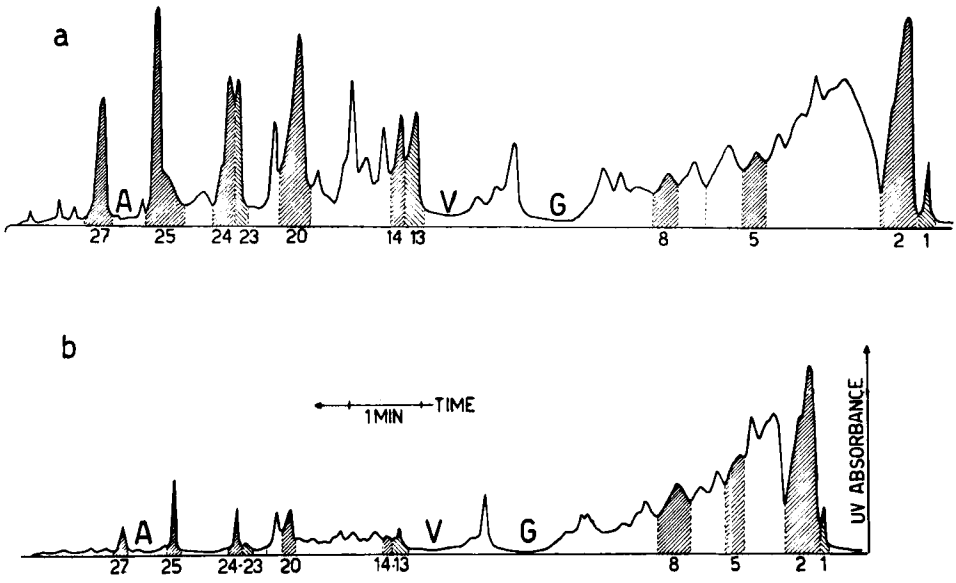

FIGURE 8

a) Isotachophoretic separation pattern of water soluble proteins from the eye lens of a young mouse. b) pattern obtained under exactly the same conditions with sample coming from a very old mouse. (Spacer mixture the same as for human serum).

The least that can be said of capillary isotachophoresis for the analysis of proteins, is that it is a valid addition to the battery of electrophoretic techniques allready available for this purpose. In addition, capillary isotachophoresis can, with proper care, provide very valuable quantitative information. Although only one sample can be analysed at a time, one complete experiment takes only 10 to 20 minutes. The technique is especially useful when quantitative information is needed on one or more proteins in a series of samples with roughly the same qualitative composition. One very in-

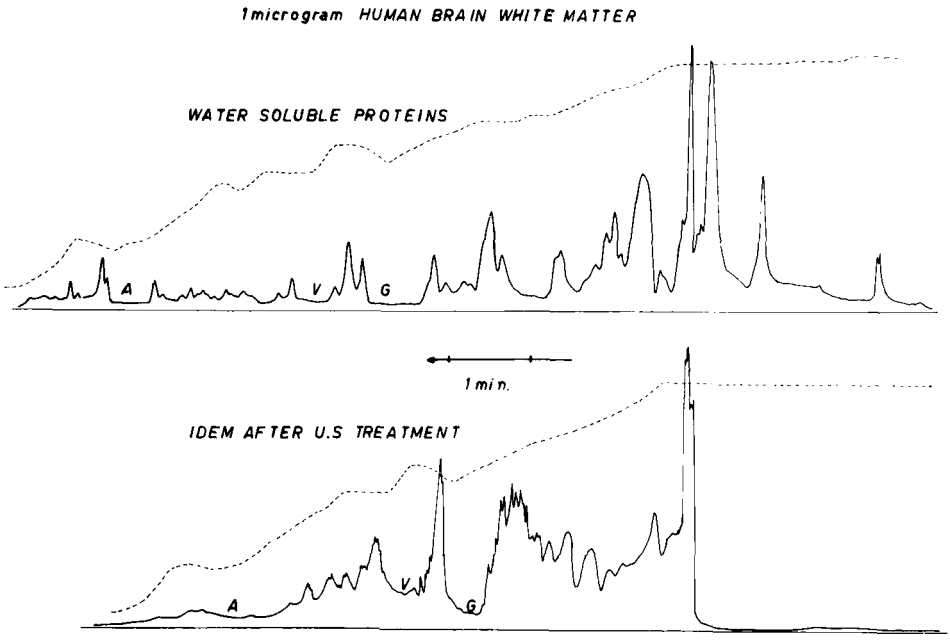

FIGURE 9

Separation patterns of two samples of water soluble proteins obtaines during extraction of human brain white matter. (Spacer mixture same as for human serum).

teresting application of quantitative isotachophoresis lies in the field of enzymology: it provides a fast and easy way to follow the transformation and/or consumption of substrates that are difficult to quantify by other means.

If practically all of the other electrophoretic techniques are for the most part only of use for the analysis of macromolecules, then capillary isotachophoresis is eminently suitable for analysis of all compounds that acquire an electric charge in the pH range from 2,5 to 9,5. Through the choice of appropriate leading and terminating electrolytes and by playing around

with the operating parameters like temperature, working pH, length of capillary, additives to the electrode solutions and strength of electric field, the interested experimenter can tackle many a separation problem.

The wide range of application of capillary isotachophoresis will be very briefly illustrated by the following examples. Figure 10 presents the separation patterns of a biologically active polypeptide during its preparation and purification (16). The first pattern corresponds to the crude mixture after the last step in synthesis. Although in the second pattern there are some hints of plateau formation, there is no dramatic difference with the first sample. The efficacy of the next purification step, an ion exchange experiment, is clear-

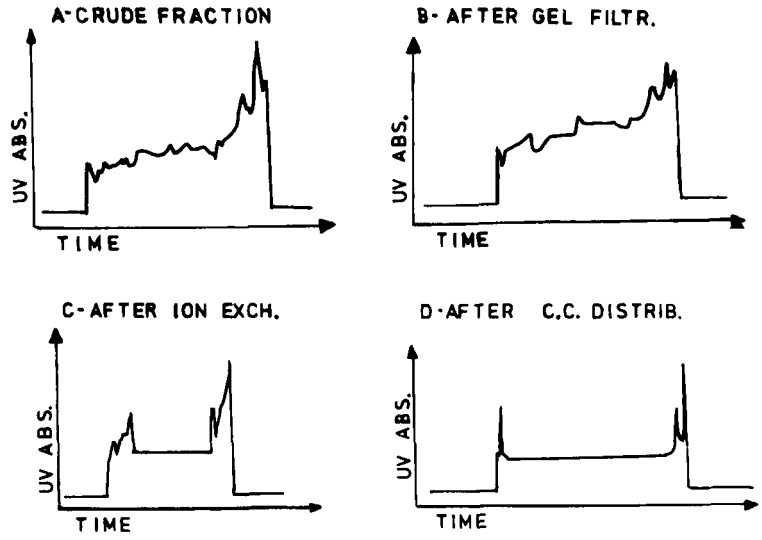

FIGURE 10

Isotachophoretic separation patterns obtaines after injection of samples at four stages during preparative purification of a biologically active polypeptide.

ly illustrated by the third separation pattern. Nevertheless, it is clear that the sample still contains an appreciable amount of impurities. That the last step in purification, a counter current distribution experiment, drastically reduces the amount of impurities is evidenced in the fourth isotachophoretic separation experiment. For compounds like polypeptides, for which very few easy and specific chemical identification reactions exist, isotachophoresis presents a suitable alternative.

The powerful separation possibilities of capillary isotachophoresis are once more illustrated by the separation pattern in figure 11. They were obtained after injection of a crude solution of a pharmaceutical preparation containing 4 chemical varieties of penicillin, carbenicillin, ampicillin, flucloxacillin and amoxicillin (17). Their chemical likeness presents a great challenge to the analytical chemist by any other method.

The last example, presented in figure 12, once again illustrates the excellent separations obtainable with this technique which provides at the same time precious quantitative information. Samples of a soft drink, without any pretreatment, were injected (18). The samples were taken before and after the fluid had been in contact with atmospheric air for several hours.

To conclude, a very incomplete survey of possible applications:

- amino-acids (19)
- polypeptides (16)
- proteines (20, 21, 26)
- amines (22)
- short chain fatty acids (23)
- nucleic acids and derivates (24, 25)
- antibiotics (17)
- hippuric acid metabolites (26)
- inorganic ions (27, 28)

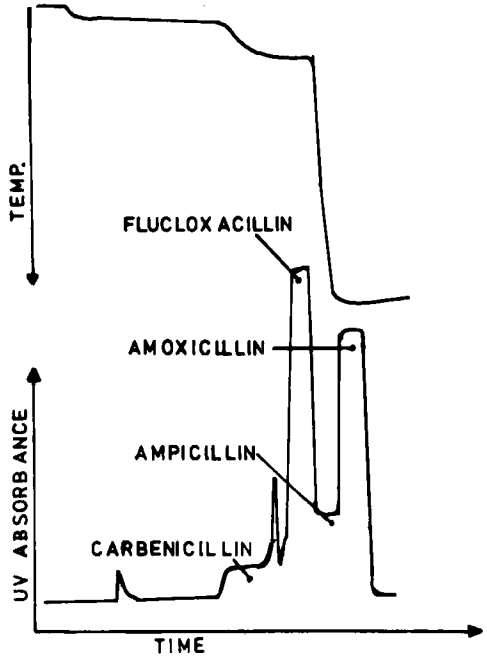

FIGURE 11

Separation pattern from a pharmaceutical preparation containing four different types of penicillins.

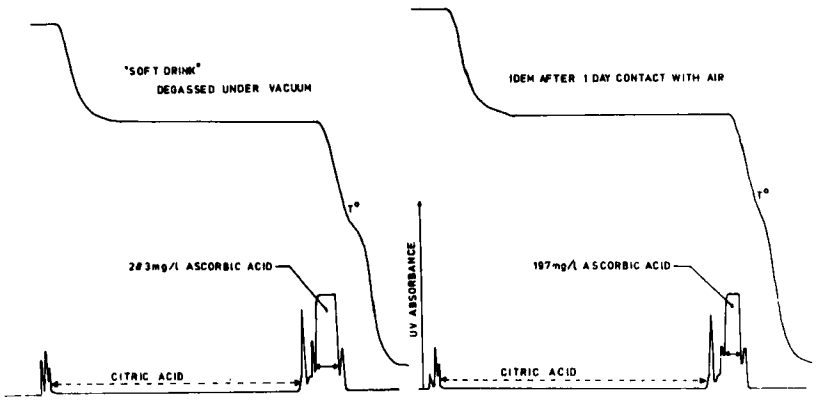

FIGURE 12

Isotachophoretic experiment designed to measure the citric acid and ascorbic acid contents of a soft drink before and after exposure to atmospheric air.

References

1 - A. Tiselius, Nova Acta Regiae Soc. Sci. Upsal., Ser 4, 4, 7, 1931.

2 - L. Michaelis, Biochem. Z., 16, 81, 1909.

3 - H. Svensson, Acta Chem. Scand., 15, 325, 1961.

4 - W.C.D. Whetham, Phil. Trans. Roy. Soc. London, Ser A, 184, 337, 1893.

5 - F. Kohlrausch, Ann. Phys. Leipzig, 62, 209, 1897.

6 - P.J. Svendsen and C. Rose, Science Tools, 17, 13, 1970.

7 - P. Delmotte, Science Tools, 24, 33, 1977.

8 - K.G. Kjellin and U. Moberg and L. Hallander, Science Tools, 22, 3, 1975.

9 - P. Delmotte, in "Humoral Immunology in Neurol. Dis.", Plenum Publ. Cy., 1979, p. 221.

10- F.M. Everaerts, Graduation Rep., University of Technol., Eindhoven, 1964.

11- L. Orstein and B.J. Davis, Ann. N.Y. Acad. Sci., 121, 321, 1964.

12- F.M. Everaerts and J.L. Beckers and Th.P.E.M. Verheggen, in "Isotachophoresis", Elsevier Publ. Cy, 1976.

13- J. Bours and P. Delmotte, in "Biochem. Biol. Applic. Isotachophoresis", Elsevier Publ. Cy., p. 221.

14- P. Delmotte, unpublished results.

15- P.W. Lange, in "Biochem. Biol. Applic. Isotachophoresis", Elsevier Publ. Cy., p. 187.

16- A. Kopwillem and U. Moberg and G. Westin and R. Lumden and H. Sievertsson, Anal. Biochem., 67, 166, 1975.

17- Isotachophoresis News n° 3, LKB, Sweden.

18- J.L. Beckers and F.M. Everaerts and V.J.M. Houtermans, J. Chrom., 76, 277, 1973.

19- D.V. Robinson and M. Rimples, J. Clin. Chem. Clin. Biochem., 16, 1, 1978.
20- P. Delmotte, in "Electrophoresis 1978", Elsevier North Holland Publ. Cy., 1978, p. 115.
21- K.W. Hedlund and R. Wistar and D. Nichelson, J. Immunol. Meth., 25, 43, 1979.
22- A. Sahota and H.A. Simmonds and R.H. Payne, J. Pharm. Meth., 2, 263, 1979.
23- W. Tshoepe and R. Brenner and A. Baldensten and E. Ritz, in "Biochem. Biol. Applic. Isotachophoresis", Elsevier Publ. Cy., 1980, p. 117.
24- D.C. Gower and R.C. Woledge, Science Tools, 24, 2, 1977.
25- B. Sjodin and A. Kopwillem and J. Karlsson, Scand. J. Clin. Lab. Invest., 35, 699, 1975.
26- J. Sollenberg, J. Chrom., 132, 469, 1977.
27- Ph. Gysen and G. Heynen, in "Biochem. Biol. Applic. Isotachophoresis", Elsevier Publ. Cy., 1980, p. 81 and 85.
28- J. Sjodahl and J. Hjalmarson, FEBS Letters, 92, 22, 1978.