Journal of Chromatography, 119 (1976) 9–24

CHROM. 8832

PREPARATIVE CAPILLARY ISOTACHOPHORESIS

PRINCIPLE AND SOME APPLICATIONS

LENNART ARLINGER

Development Department, LKB-Produkter AB, S-161 25 Bromma (Sweden)

(Received September 29th, 1975)

SUMMARY

Capillary isotachophoresis has been developed and modified for use as a preparative method. The sample zones are pushed out of the capillary under voltage by a counter-flow of leading electrolyte through a small T-tube connection immediately after the UV detector. The zones are collected on a moving cellulose acetate strip without loss of high resolution. This permits simple detection and identification by methods new to capillary isotachophoresis, *e.g.*, immunological and radioactive methods. The zymogram technique can be used directly on the strip. The preparative procedure also makes capillary isotachophoresis a powerful purification method.

INTRODUCTION

Analytical isotachophoresis in capillary tubes has proved to be a method of great potential for solving a variety of separation problems[1–8]. Small amounts of sample, high resolution and short separation times are a few of its advantages[9,10]. There has, however, been a restriction in the use of this technique because it has not been possible to investigate highly resolved samples with other than the "ordinary" detectors, *i.e.*, UV absorbance, thermal, conductivity or potentiometric detectors. Nor has it been possible to use capillary separation as an integral step in preparation procedures.

It was therefore considered of great value to use capillary isotachophoresis as a preparative procedure without losing the rapidity and resolving power inherent in the method.

The first successful experiments were made in 1970, but not until several years later were these followed by studies on the method of handling small amounts of sample for further characterization and identification. The work presented here was first concentrated on maintaining resolution during collection of the sample on a moving strip of cellulose acetate. The resolution was determined by densitometric scanning of the strip. Next, the total elution of two proteins from the strip was studied, one by electrophoresis and one by repeated washing to detect possible absorption effects. Finally, some methods for further characterization and identification of the

sample components were studied, mainly utilizing immunological, radioactivity and zymogram techniques. None of these procedures was carried through in a fully systematic manner, but those studied were easily applied and can certainly be modified for different needs.

PRINCIPLE

The capillary elution system

In the isotachophoretic capillary equipment[10] shown in Fig. 1a, the leading electrolyte reservoir is separated from the capillary compartment by a semi-permeable membrane, m_1, to prevent hydrodynamic flows. The potential applied over the electrodes can be very high, up to 30 kV with a capillary length of 25 cm, thereby assuring rapid separation of the sample ions with high resolution. When the electric current is switched off, the ions in the zones will quickly diffuse across the zone boundaries and destroy the resolution within a few seconds, as no gel or other stabilizing medium is present in the liquid phase. The resolution is also immediately decreased by applying a flow along the capillary. Therefore, sample collection has to be performed with an electric field present and without moving the sample zones by a flow along the direction of the capillary.

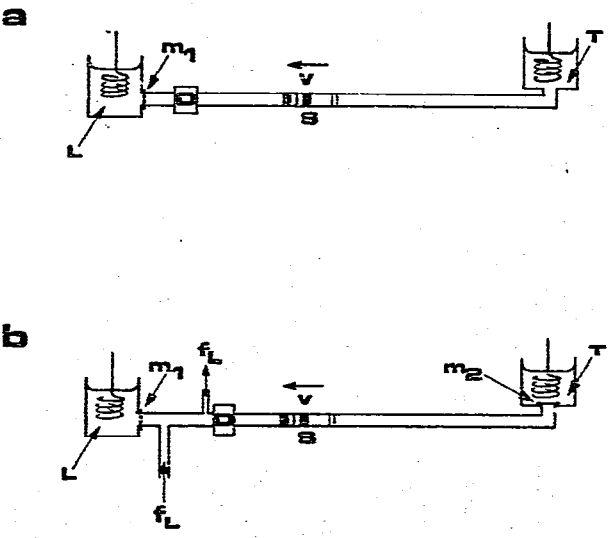

Fig. 1. Principle of preparative capillary isotachophoresis. (a) Schematical diagram of basic analytical equipment; (b) preparative version with an additional flow of leading electrolyte. L = leading electrolyte; T = terminating electrolyte; D = UV detector site; S = sample ions; v = migration velocity; m_1, m_2 = semi-permeable membranes; f_L = flow of L to elute the sample zones.

The technique chosen for the sample collection was to use a T-connection through which the sample is swept by a flow of leading electrolyte. This is shown schematically in Fig. 1b, where m_2 is a membrane inserted in the terminator reservoir to prevent a counter-flow through the whole capillary. After passing the UV detector, the zones will be swept out through the T-tube, provided that the liquid flow-rate

from the pump is greater than the corresponding migration rate of the ions. As the liquid flow will dilute the sample, the flow-rate is set only a few per cent higher than the migration rate. The transport situation for the leading (L), sample (S) and counter ion (C) at the site of the T-tube is shown in Fig. 2. The sample is assumed to have reached the T-tube and started to be pumped out. The flow f from the pump causes ionic flows L_f and C_f in the capillary tube and flows f_L, f_S and f_C through the T-tube, while m denotes the isotachophoretic migration. S_m denotes the electrophoretic flow-rate (the volume through which a set of sample ions is displaced per unit time by the isotachophoretic migration).

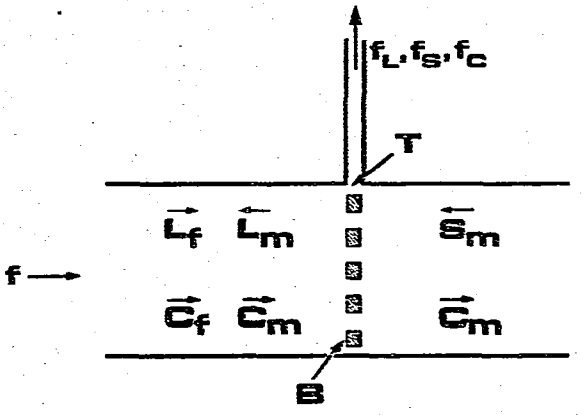

Fig. 2. Transport situation at the site of the T-tube in a capillary column. B is the boundary between the leading ions L and the sample ions S. The leading electrolyte, containing L and the counter ion C, is pumped with the flow-rate f and causes the ionic flow-rates f_L, f_S and f_C out through the T-tube. The subscript m denotes the isotachophoretic migration.

Thus, we have $L_f = C_f$ and $L_m = S_m$. Under stationary conditions, *i.e.*, when the boundary B between L and S is not moving, the sample ions will leave the separation compartment at the same speed as the electrophoretic transport. Consequently:

$$f_S = S_m$$
$$f_L = L_f - L_m$$

and

$$f_C = (C_f + C_m) - C_m = C_f$$

If we assume f to be 5% higher than S_m, we obtain after substitution $f_L = 0.05\,L_m$, $f_S = L_m$ and $f_C = 1.05\,L_m$. Consequently, the ionic composition of the flow in the T-tube will be (for univalent ions): 0.05/2.10 = 2.4% leading ions, 1/2.10 = 47.6% sample ions and 1.05/2.10 = 50% counter ions. The sample ion concentration will therefore be about 5% lower than its isotachophoretic concentration.

If the flow f is set to balance exactly the isotachophoretic migration, $L_f = L_m$, then f_L will be zero, which means that no leading ions would be pushed out and only sample ions and counter ions should be present in the flow out of the T-tube. This is, however, very difficult to achieve in practice.

If $L_f < L_m$, the pump flow-rate being smaller than the corresponding electrophoretic flow, the boundary B is no longer stationary at T but moves along the tube. The velocity of B will be the same as with an ordinary counter-flow in isotachophoresis. The sample zones will be partly pushed out through the T-tube, and partly move along inside the capillary tube. This can easily be seen in practice by using a set of dyes as the sample.

The figures above are calculated for ideal cases, the real cases being subject to diffusion and possible flow disturbances. However, these factors are not likely to change the figures drastically.

The fraction collection device

The flow-rates through the T-tube in the present work were 2.75 μl/min or about 45 nl/sec. Sample zones may have volumes as low as 10–20 nl (ref. 9) and traditional fraction collection is not easily used. It was therefore decided to collect the sample on a strip passing by the outlet of the T-tube. As the high voltage is on during the collection of samples, there is an electric force present with which to transfer liquid from the T-tube outlet to the earthed strip. When the distance from the end of the T-tube to the strip is small, very small drops will pass over to the strip at a frequency of several hertz. This frequency can be increased by diminishing the distance until a continuous stream is obtained, and the voltage will allow proper collection on the strip. One of the reasons for choosing cellulose acetate as the strip material was that it can be obtained as thin sheets with a smooth surface. It is also easy to handle and is a generally well known and acceptable material.

The identification of sample positions on the strip and recorder chart is a vital part of the procedure. By simultaneous, automatic marking of the strip and chart, this is reduced to noting the starting points on each. There is also a constant time difference between the measurement of UV absorbance of an ion and the moment the ion reaches the strip. This constant needs to be established only once for a given leading electrolyte by running a visible compound and is valid for a specific current and the corresponding liquid flow-rate. The time difference in a typical experiment is 3–6 sec.

MATERIALS AND METHODS

Isotachophoresis

The isotachophoretic experiments were performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a Braun Perfusor IV counter-flow pump (Braun, Melsungen, G.F.R.). The pump was used in position "5", giving a flow-rate of 2.75 μl/min with a 500-μl Unimetrics syringe. It was connected to the Tachophor by a PTFE capillary tube and a cannula through the counter-flow septum.

The usual type of capillary plate was substituted with a specially constructed plate with a 43-cm PTFE capillary fitted with a T-connection immediately after the UV detector. The connection was made by inserting a glass capillary tube (O.D. 0.25 mm, I.D. 0.04 mm) through the PTFE wall until it reached the inner surface of the separation tube. The centre of the T-tube was positioned 0.5 mm behind (in the direction of the migration) the centre of the UV beam passing through the separation tube. In order to prevent the flow of leading electrolyte from the syringe pump from

causing a counter-flow in the whole separation tube, a semi-permeable membrane was mounted in the bottom of the terminating electrolyte reservoir and a valve in the terminating electrolyte syringe holder, thereby completely closing the terminator side of the capillary against liquid flow.

The balancing of pump flow against the isotachophoretic migration velocity (the current) was achieved by running an ordinary counter-flow[11] with a visible sample. The pump was set at one position and the current varied until a balance was attained, when the sample zones did not move in the capillary. The balancing current was then decreased by 5% and used for the sample collection phase in the preparative experiments.

The samples were collected as continuous streams on strips of 0.11-mm thick cellulose acetate (Sartorius, Göttingen, G.F.R.) of 3 mm width cut from sheets and connected with tape to make them several metres in length. The strip was transported mechanically past the end of the glass tube and had a constant speed, adjustable between 40 and 55 mm/min with a space between the tube end and the strip surface of 0.05–0.08 mm. This construction eliminates risks of contamination of the sample components collected on the strip. Each turn of the drive wheel (circumference 50 mm) marks the edge of the strip close to the T-tube end and simultaneously closes an electronic circuit, producing a spike on the UV recording (see Fig. 3). Experiments with the sample collection equipment are identical with ordinary runs until 6–8 min before the zones to be collected appear on the UV record. Then the syringe pump with the leading electrolyte is switched on and after 1.5–2 min a switch is closed, activating two small electric motors, one uncovering the T-tube and setting the tube-to-strip distance, the other moving the strip. After the sample zones have passed the detector and have been collected, the pump and sample collector are switched off.

The kit consists of a capillary plate with T-tube, fraction collector and terminating reservoir block with membrane and valve as a preparative accessory to the LKB 2127 Tachophor. It will be available from LKB in the middle of 1976 under the name "Tachofrac".

Tris and β-alanine were purchased from Sigma (St. Louis, Mo., U.S.A.), histidine, barium hydroxide and *p*-nitrophenol from Merck (Darmstadt, G.F.R.), hydroxypropylmethylcellulose (HPMC) from Dow Chemical (Midland, Mich., U.S.A.; Methocel 90 HG, 15,000 cP), and tetrasulphonated indigo from Aldrich, Wolf and Kober (Copenhagen, Denmark).

Densitometry

The laser densitometer used for scanning the strips was a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago, U.S.A.) measuring at a fixed wavelength of 635 nm.

Radioactivity measurements

The radioactive nucleotide samples were purchased from the Radiochemical Centre, Amersham, Great Britain. The [4-^{14}C]uridine-5′-monophosphate, ammonium salt (batch 3), had a specific activity of 24 mCi/mmole and was dissolved in water to make a 5 m*M* solution. A sample of 1 μl therefore contained 5 mmole or 120 nCi.

The [U-^{14}C]adenosine-5′-monophosphate, ammonium salt (batch 12), was supplied as an aqueous solution, containing 2% ethanol, with a specific activity of

570 mCi/mmole and a radioactive concentration of 50 μCi/ml. A sample of 1 μl therefore contained 88 pmole or 50 nCi.

The radioactivity-containing cellulose acetate strips were mounted on a glass plate covered with double adhesive tape and evaluated with an LKB 2105 Radio-Chromatogram camera, set for 20,000 counts, and a polarization of 1. The Polaroid camera was set to $f = 11$ (75 ASA). The sites of activity were measured on the photographs with a special scale-transforming ruler.

The scintillation countings were made in an LKB 81000 liquid scintillation counter. The strips were cut along a ruler edge into 2.5-mm pieces, each piece was put into a vial, 1 ml of distilled water was added and after a few minutes, with slight swirling, 10 ml of Instagel were added and the vial was shaken until the contents became clear. The counting times were 1 or 2 min per vial or 10 min with overnight counting.

Immunoelectrophoresis

The ceruloplasmin sample was a preparation from Kabi AB (Stockholm, Sweden). The transferrin solution was a gift from Dr. P. J. Svendsen, Protein Laboratory, Copenhagen, and was prepared from serum by column isotachophoresis in polyacrylamide[12].

The antibodies were bought from Dakopatts (Copenhagen, Denmark) and were rabbit anti-human serum, type 100 SF. A 1-ml volume was used per 10 ml of agarose gel. The immunoelectrophoretic buffer was Tris–barbital, pH 8.6 (ref. 13), the time required was about 20 h at 2 V/cm and the equipment used was an LKB 2117 Multiphor.

Zymogram technique[14]

The sample used was cholinesterase (Sigma) with 8.6 units/mg protein and 0.70 mg protein/mg solid substance, type IV from horse serum, Lot No. 120C-2240. The zymogram reagent components were the following:

(A) 1% α-naphthyl acetate (Sigma) in acetone.

(B) Buffer, 0.12 *M* Tris and 0.10 *M* HCl, pH 7.5.

(C) Fast Red TR salt (Sigma).

These components were mixed in the proportions A:B:C = 0.25 ml:9 ml:*ca.* 9 mg. The solution obtained is unstable but lasts long enough for the soaking procedures, including the separation with sample collection.

RESULTS

Resolution of collected samples on the strip

The high resolution of capillary isotachophoresis should be maintained during the preparative procedure from the equilibration state in the capillary tube to the stage of collecting the sample on the cellulose acetate strip. There are a number of possible reasons for a loss in resolution, such as pressure variations due to the counter-flow pump and flow irregularities or boundary deformation at the T-tube connection. The collection of a liquid phase also involves possible diffusion and mixing of the sample.

Concerning the pressure variations, the T-tube flow required a slight over-pressure at the start because of the small inner diameter of the T-tube. An appreciable

capillary effect at its opening had to be overcome. This is achieved by starting the pump a few minutes before the T-tube is opened and the collection starts, mainly letting the larger membrane on the leading side (m_1 in Fig. 1) take up the volume expansion (the area of m_1 is nine times that of m_2). This procedure was not found to affect the separation time, or the intensities and resolution of the UV recording. Consequently, no flow occurred in the separation tube. The reproducibility was perfect as judged by both UV recordings and visual inspection of dyes on the strip.

An effect that might decrease the resolution is mixing in the T-tube. This is avoided by choosing a tube of small volume, in this work a tube with an I.D. of 0.04 mm and a length of 8.2 mm, giving a volume of 10 nl. The lower limit of the UV-detectable zone lengths is about 0.1 mm (ref. 9), which corresponds to a zone volume of 16 nl. The ratio between these volumes means that any mixing effects in the T-tube are not significant.

The liquid pushed out through the T-tube (in this work 2.75 μl/min) should be absorbed by the support without the risk of diffusion or flow effects in a liquid phase. The velocity of the strips is therefore determined by the porosity of the cellulose acetate. A strip velocity of 40–55 mm/min was chosen, giving a liquid volume of about 0.5 μl/cm on the strip, one third of the corresponding value for the capillary tube.

Collection of a continuous stream of sample was found to be superior to collecting individual drops, giving even sample distribution on the strip without having to adjust the distance between the T-tube and the strip surface precisely. It is also the most reliable method when using electrolytes that contain methylcellulose in order to prevent electroendosmosis[9].

The usual current settings and concentrations used in capillary isotachophoresis make a zone on the cellulose acetate strip about twice as long as that on the UV recording (chart speed 3 cm/min). The distance between the marking spikes on the recording (m in Fig. 3) always corresponds to 50 mm on the strip. This means that identification and selection of very minor zones on the strip present no difficulties.

It is not easy to demonstrate the reliability of the high resolution with the preparative procedure directly by an experiment because of the minute amounts of sample on the strip. All of the results presented in the remainder of this paper indicate that resolution is maintained during sample collection. The strongest evidence was obtained from some experiments with mixtures of the dyes xylenol orange and tetrasulphonated indigo. The latter gave several blue zones that could be detected by scanning with the red 635-nm laser beam of the Soft Laser Densitometer. Fig. 3 shows parts of two experiments with different sample purities. Only the parts with the blue indigo bands directly after the leading ion are shown. The yellow and orange bands from xylenol orange could not be detected at this wavelength. Scanning at different wavelengths with the Zeiss PMQ II with scanner gave no results, even when the strip was made transparent with Shell Ondina Oil 17. This was probably due to the low light intensity compared with the laser densitometer.

The UV recordings were made at 254 and 278 nm and the absorbance levels are naturally different from those at 635 nm. The shoulder on the first peak in Fig. 3a obviously lacks absorbance at 635 nm. The abscissas do not agree fully, but by measuring the slopes it can easily be seen that the resolution from the capillary is maintained on the cellulose strip.

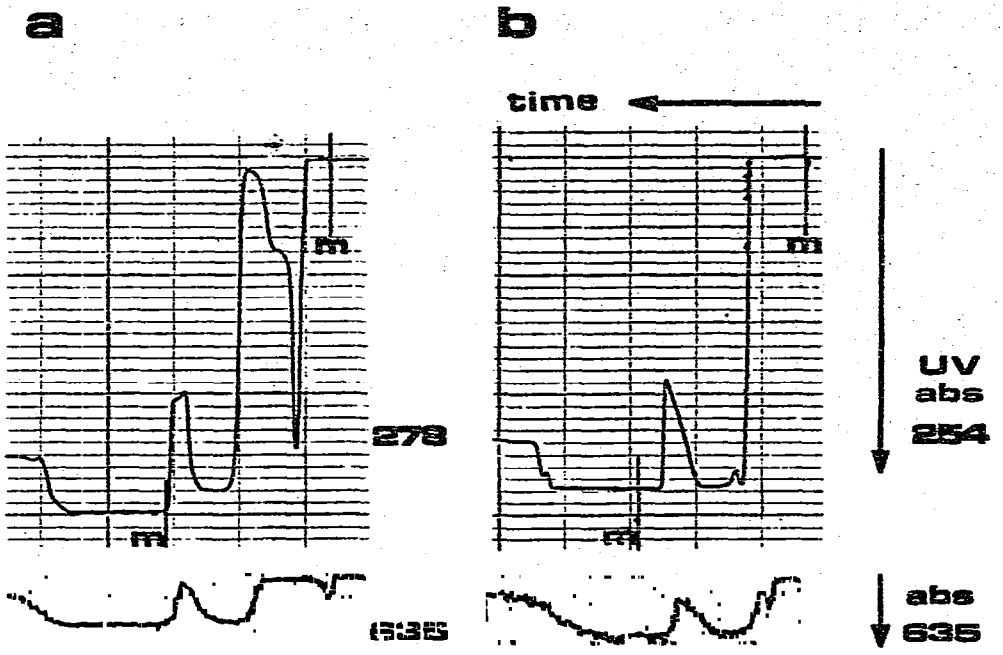

Fig. 3. UV recordings of dye material at 278 and 254 nm compared with soft laser scanning at 635 nm of strips with the collected samples, showing the maintenance of high resolution during the preparative process. The samples were different mixtures of tetrasulphonated indigo and xylenol orange, the figures showing the first blue zones behind the leading ion. The upper traces show the recordings from the UV detector and the lower traces the laser scan results. The time bases differ by about 15%, and intensities are not matched. The spikes m denote the event marker pulses, simultaneously causing a print on the edge of the sample collection strip. In (a), the electrolytes contained other spacing impurities than those in (b). Leading electrolyte: (a) 5 m*M* HCl, 13 m*M* β-alanine with 0.25% HPMC; (b) 10 m*M* HCl, 24 m*M* β-alanine and 0.1% HPMC, all at pH 3.6. Terminating electrolyte: 10 m*M* caproic acid in both. Current: (a) 80 μA and 30 μA during detection and sample collection; (b) 120 and 70 μA, respectively. Temperature, 20°; chart speed, 3 cm/min; capillary length, 43 cm. The slit for the laser beam of the scanner was set to about 0.5 × 0.5 mm.

Adsorption of sample on the strip

A risk with sample collection on a solid support is the possibility of irreversible adsorption of the sample, especially proteins. Two sets of experiments were performed in order to investigate this effect. In the first set, the sample was run out of the strip electrophoretically and detected immunologically, and in the second the sample was eluted by repeated washing with buffer and detected by visible loss of colour.

In the first set of experiments, three different aliquots of a pure transferrin sample were soaked into three pieces of the cellulose acetate strip. After drying for a few minutes at room temperature, the strips were wetted with buffer and placed on an antibody-free agarose gel beside another gel that contained antibodies against total human serum. A further three aliquots of an identical sample were positioned in three holes punched in the former agarose gel and immunoelectrophoresis was performed. After staining, a paper copy was taken of the plate and the areas below the precipitin lines ("rockets") were integrated by the cut-and-weigh technique. The result

TABLE I

IMMUNOELECTROPHORETIC "ROCKET" AREAS (AS MILLIGRAMS OF PAPER) IN THE STUDY OF ADSORPTION OF TRANSFERRIN BY CELLULOSE ACETATE

Method of application	*Relative amounts of transferrin*		
	1	*2*	*4*
Via strip	3.31	4.98	8.70
In hole	3.31	5.14	8.79
Difference (%)	0	3	1

is given in Table I, indicating total migration of the transferrin from the strip by electrophoresis. The numerical differences are small considering the possible experimental errors.

The second set of experiments was carried out in order to study the adsorption of cholinesterase on the cellulose acetate strip. To one strip (3 × 20 mm) 2 μl of water were added as a blank, and to each of the two other strips, 2 μl of an aqueous cholinesterase solution containing 14 μg of protein were added. The strips were dried for 15 min at room temperature, then placed into small glass tubes containing 200 μl of buffer (10 m*M* HCl, 12 m*M* Tris, pH 7.5). The tubes were shaken twice during half an hour, then the strips were transferred to new tubes containing the same amounts of fresh buffer and the procedure was repeated until five tubes with used buffer were obtained from each strip. As soon as a strip was taken out of a tube of buffer, 100 μl of freshly prepared zymogram substrate solution (see Materials and Methods) were added to the tube. The colours were compared visually and, after two washing steps, no colour reaction could be seen in the tubes from the enzyme samples compared with the blank.

Identification by means of radioactive tracer

The use of a radioactive detector has never been possible in capillary isotachophoresis because of the small amounts of sample and other physical conditions encountered. However, the sample strip can easily be investigated by radioactive methods.

The samples used in the experiments were nucleotide mixtures containing the 5′-monophosphates of uridine, cytidine, guanosine and adenosine and adenosine-3′-monophosphate, the A5′MP and U5′MP being labelled with ^{14}C. Fig. 4 shows the UV absorbance pattern (278 nm) from an experiment using a leading electrolyte with a pH of 3.4. The corresponding cellulose acetate was analyzed with the LKB Radio-Chromatogram camera and the result is shown as the line at the top in Fig. 4, marked RCC. Liquid scintillation counting (LSC) of another strip from an identical experiment resulted in the lower part of Fig. 4. The results are in full agreement with each other.

The separation between A5′MP and A3′MP is not absolute, but the overlap is probably due to the normal zone boundary widths. The 88-pmole A5′MP sample will, in this "ordinary" concentration[9] experiment, occupy less than 1 mm in the capillary tube, and the mobilities of the two AMPs will naturally be rather similar.

The LSC values show some spreading, as can be seen in the UMP zone. This is certainly due to the author's inability to cut the strip into pieces of exactly equal

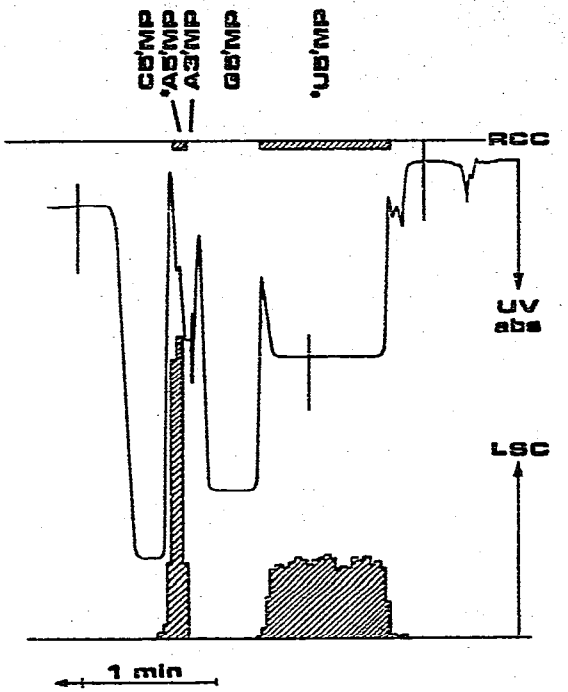

Fig. 4. Capillary isotachophoretic separation of ^{14}C-labelled A5′MP (88 pmole) and U5′MP (5 nmole) together with unlabelled A3′MP (0.77 nmole), G5′MP and C5′MP (each 2.5 nmole). The upper line (RCC) shows the activity as detected by the RadioChromatogram camera. The lower tracing (LSC) shows liquid scintillation counting on pieces of the strip, cut to a length of 2.5 mm. Leading electrolyte: 5 m*M* HCl, 9 m*M* β-alanine, 0.25% HPMC at pH 3.4. Terminating electrolyte: 10 m*M* caproic acid. Capillary length, 43 cm; current, 80 μA and 30 μA during detection and sample collection; total time of experiment, 24 min; wavelength of recording, 278 nm; temperature, 20°.

lengths with a scalpel at right-angles to a ruler placed at the edge of the strip.

The RCC is much simpler than the LSC for this type of radioactivity measurement. The RCC measurements take only few minutes but have no quantitative accuracy. The intensity differences that can be seen on the photographs, however, will probably give sufficient information in most instances to distinguish between radionucleides with different activities.

Immunoelectrophoretic identification

The sample collector strip can be directly used for immunological characterization of sample components when immunoelectrophoresis is used. The strip is preferably wetted with the immunoelectrophoretic buffer, then placed directly on an antibody-free gel beside an antibody-containing gel and the current is applied. All other procedures can be performed as usual[13], except for the punching of the gel holes, which is omitted.

Some experiments were performed with transferrin and ceruloplasmin in order to demonstrate the simplicity of the immunological technique in preparative capillary isotachophoresis. The transferrin pattern in UV and by the "immuno-detector" is shown in Fig. 5. The original immunological pattern shown at the bottom of Fig. 5

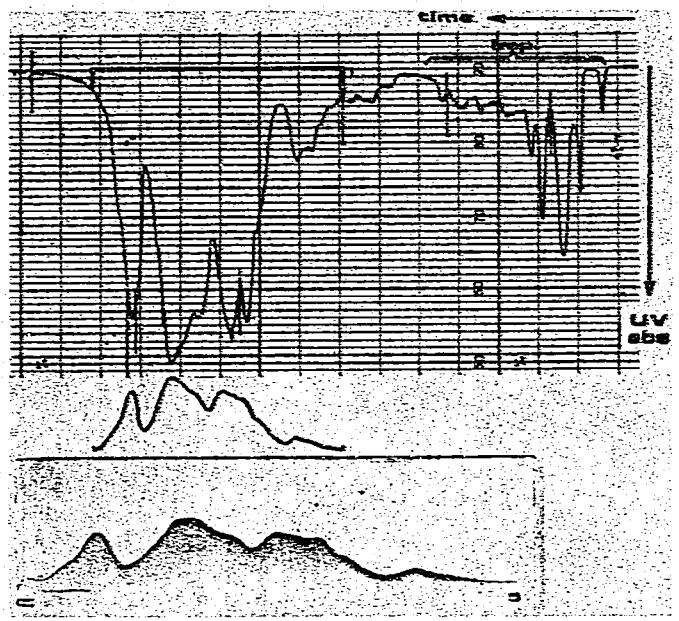

Fig. 5. Capillary isotachophoretic and immunological analysis of a transferrin sample, purified by column isotachophoresis. The sample was 3 μl of a transferrin solution of unknown concentration with 4 μl of ampholine, pH 5–7, diluted 1:50. The part of the cellulose acetate strip corresponding to the line above the UV trace was cut out and placed on top of the antibody-free part of the agarose gel. The final precipitin pattern (total anti-human serum) is shown in the lower part of the figure. The middle trace is identical with the precipitin line with the time axis adjusted to that of the UV trace. Leading electrolyte: 5 m*M* HCl, 6 m*M* Tris, 0.5% HPMC, pH 7.35. Terminating electrolyte: 10 m*M* glycine, $Ba(OH)_2$ added to pH 9.2. Capillary length, 43 cm; current, 90 μA and 45 μA during detection and sample collection; total time of experiment, 22 min; wavelength of recording 254 nm; temperature, 20°; chart speed, 3 cm/min.

is compressed along the time axis to fit the scale of the UV pattern, as illustrated in the middle of the figure. The agreement is perfect; the small decrease in the immunological pattern resolution compared with the UV resolution is certainly due to diffusion during the 20 h of immunoelectrophoresis. It is striking how very pure the isotachophoretically purified transferrin sample was; a very minor component can be seen at the low mobility side on the immunological plate (to the lower left in Fig. 5).

A mixture of the transferrin sample with ceruloplasmin was separated completely in the same electrolyte system. The immunological pattern (Fig. 6, below) shows that there is a region of only ampholine spacing mobility gradient[1–3] between the protein regions. The ceruloplasmin proved to be very heterogeneous; at least six immunologically different components could be seen. They cannot directly be identified with the UV peaks because they overlap with each other and the region also contains impurities which can be seen in Fig. 5. (The UV signal attenuation in Fig. 5 was 40% higher than that in Fig. 6). Experiments such as those shown in Fig. 6, but with no ceruloplasmin, did not show any immunological reaction except for the transferrin bands. The UV picture of transferrin was also identical when run without ceruloplasmin. The transferrin pattern is somewhat changed from Fig. 5 to Fig. 6; there is mainly a quantitative change of the two components in the middle doublet. This is

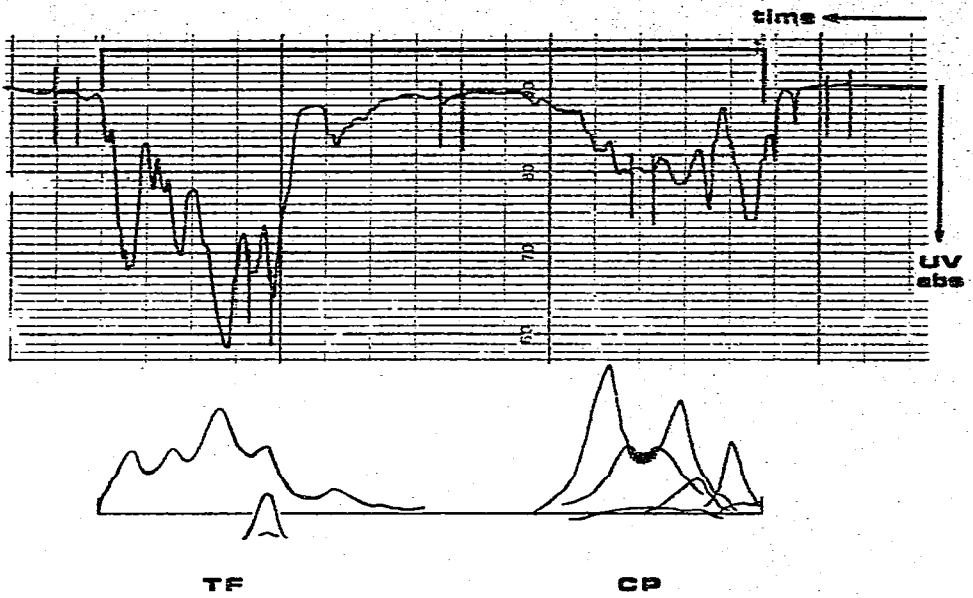

Fig. 6. Capillary isotachophoretic and immunological analysis of a mixture of ceruloplasmin and transferrin. Electrolytes as in Fig. 5, except for 0.35% HPMC concentration. Sample was 0.75 μl of 3% ceruloplasmin, 3 μl of transferrin (unknown concentration) and 3 μl of ampholine, pH 5–7, diluted 1:50. The part of the cellulose acetate strip corresponding to the line above the UV trace was cut out and placed on top of the antibody-free parts of two agarose gels. The precipitin lines (total anti-human serum) are shown in the lower part of the figure with the time axis adjusted to that of the UV trace. Capillary length, 43 cm; current, 40 μA; total time of experiment, 37 min; wavelength of recording, 254 nm; temperature, 20°; chart speed, 3 cm/min. (The marking spikes, noted as m in Fig. 3, were doubled for reasons applicable only to this experiment).

probably due to the fact that the sample in Fig. 6 was run 2 months after that in Fig. 5. The peak below the transferrin precipitation line in Fig. 6 originates from an impurity introduced by the author during the immunological procedures.

The resolution can be seen to decrease somewhat with immunoelectrophoresis. This effect can be diminished by using a higher potential to shorten the migration time. Also, if the protein region is widened on the strip by running at a lower isotachophoretic current or a higher strip speed, the diffusion will become relatively less important. The immunochemical loss of resolution will probably be of importance only when zones are immunologically identical. It is always possible to identify a very narrow UV zone by cutting it from the strip.

Enzyme identification by zymogram

The possibility of performing a specific analysis directly on the strip was demonstrated with the zymogram technique[14]. The cellulose acetate strip was treated with the reagent solution before sample collection. The sample chosen was cholinesterase, which was allowed to hydrolyze α-naphthyl acetate, the product reacting with a diazo salt to form a reddish brown colour. Because of the minute amounts of sample (about 20 μg or 0.17 units of enzyme), the reagent solution was concentrated (see Materials and Methods) more than usual[15]. The amount of reagents in the strip

was further increased by repeated soaking of the strip in the reagent solution and drying with a hair-dryer. This was repeated twice, the total time taken being only a few minutes. The impregnated length of strip was about 40 cm for each experiment.

After sample collection on the pre-treated strip (collection performed as on an untreated strip), it was again quickly dipped into the reagent solution and dried with a hair-dryer. The zymogram developed and was reinforced by one or two more soakings and dryings.

The cholinesterase was run in the same electrolyte system as the previous proteins. In Fig. 7a the electrolytes are shown with no sample (electrolyte impurity pattern) and in Fig. 7b with 20 μg of cholinesterase. The zymogram colour intensities were

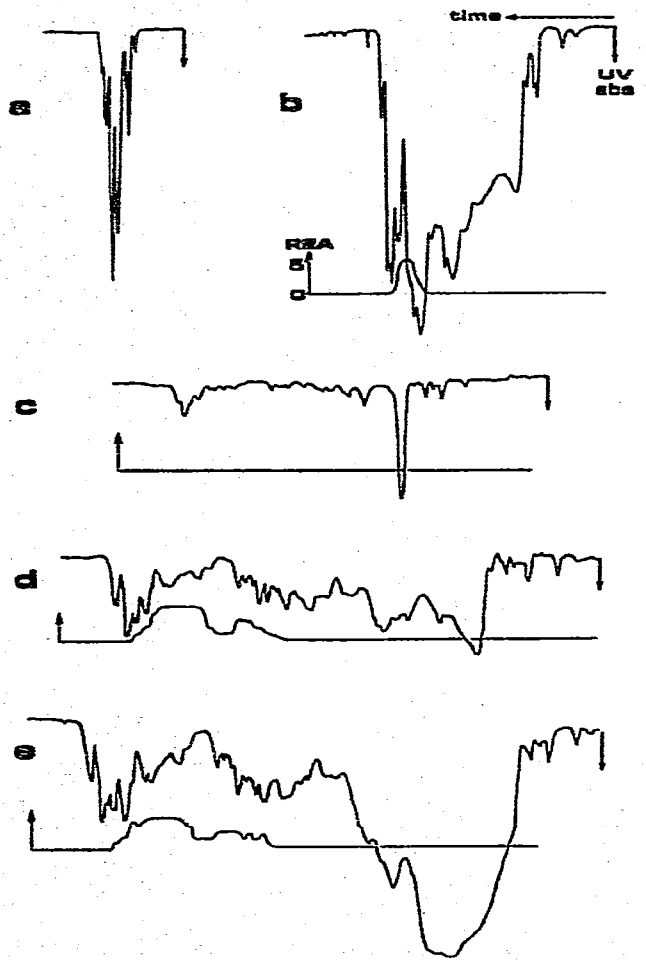

Fig. 7. Separation of a commercial cholinesterase preparation and zymogram detection of activity on pre-treated sample collection strips. The axes are defined in (b), where REA is the relative enzyme activity, estimated from the zymogram colour intensities on a scale of 0–5. (a) No sample, peak pattern is from electrolyte impurities; (b) 20 μg of cholinesterase; (c) 1 μl of ampholine, pH 4–6, diluted 1:50; (d) 20 μg of cholinesterase and ampholine as in (c); (e) 35 μg of cholinesterase and ampholine as in (c). Electrolytes as in Fig. 5. Capillary length 43 cm; current, 45 μA; wavelength of recording, 254 nm; temperature, 20°. The separation time until the first peaks were detected was about 20 min.

estimated visually on a scale from 0 to 5 on a light table. The activity was found in a small part of the UV-absorbing protein region. In Fig. 7c an experiment with 1 μl of ampholine, pH 4–6, diluted 1:50, is shown with the same impurities as in Fig. 7a but spaced apart, giving no zymogram reaction. In Fig. 7d, the enzyme and ampholine samples were combined, giving an excellent separation of the enzymatic components with mobilities lower than those of the main part of the protein. This is more obvious in Fig. 7e, where the amount of cholinesterase is almost doubled to 35 μg. The non-enzymatic protein part of the 35-μg sample was too large to attain isotachophoretic conditions completely, which is the reason for the differences in the shapes of the zones directly after the leading ion. It can easily be seen from the last two figures which zones contain activity. It would thus be an easy task to make another separation as in Fig. 7e, collect the sample on an untreated strip, cut out the desired section and place it in a buffer solution in order to obtain an enzyme solution of high purity with a very high specific activity.

DISCUSSION

The results from the different experiments show that the high resolution achieved with capillary isotachophoresis can be maintained during the preparative procedure. No indications of marked loss of resolution were found in any experiment.

The two proteins that were tested for irreversible adsorption on cellulose acetate did not show any such effect, nor were any adsorption problems found in any of the other experiments carried out so far. This is, of course, a very limited test. However, it is impossible to find a support material suitable for all substances. It may be useful in special cases to change to a more inert material, but it should be hydrophilic for aqueous systems. For non-aqueous systems, the question of inertness of the strip towards the solvent must be considered.

It should also be pointed out that in many instances it is not necessary to have a 100% strip desorption yield. Only when very restricted amounts of sample are present during the preparation steps would this be a necessity. Otherwise, the amount of sample from the strip can be increased with a longer capillary sample zone.

There may be sample components that will denature when the solvent evaporates. In this instance the experiment and sample collection should be interrupted immediately after the zones of interest are collected, the strip removed and placed in a solvent-saturated atmosphere, which takes only a few seconds.

There are, of course, numerous other conceivable ways of utilizing the strip for further analysis or preparation, only a few of which have been used in this work. Preliminary experiments with the LKB 2091 GC–MS system have shown that a piece of the cellulose acetate strip can easily be placed in the direct inlet sample holder of the mass spectrograph. However, the strip contains some phthalates as softeners, which will be included in the spectra. Further, cellulose acetate has an upper temperature limit. These problems can be overcome by eluting the sample from the strip and evaporating the solvent. The sensitivity of mass spectrometers allows the small amounts of sample present in narrow isotachophoretic zones to be detected easily.

One or several parts of a collected sample from a separation may constitute the sample in the next isotachophoretic experiment. The elution of sample from the strip can then be effected "automatically" with the Tachophor. This was done in a

series of experiments where the interesting parts of the strip from one run were cut out and simply pushed into the injection channel and the next experiment started. The liquid flow from the T-tube gives a track on the strip that is less than 1 mm wide and distinguishable from the remainder of the strip by visual inspection because of the differences in light reflection. These tracks were cut out and pushed into the injection channel, sometimes with a thin wire, after the injection septum had been removed. In this way, specific components can easily be removed from a sample, the same sample can then be re-run with different electrolytes or even in different solvents. The procedure is useful when sets of mixing zones occur and sample amounts are restricted.

Methods of sample collection other than that used in this work can also be used. Fine capillary tubes can be passed by the T-tube in an end-to-end manner. The drops could also be dispensed into tiny pits, perhaps partly filled with an appropriate buffer solution. These set-ups, however, would most likely be more complicated to construct and would not have either accessibility to the fraction on the strip or the continuous method of collection to ensure that there would be no decrease in resolution.

The usefulness of capillary isotachophoresis for the separation and analysis of complex biochemical molecules, such as proteins[1–5], will certainly be increasingly utilized. The preparative procedure will widen the applicability of capillary isotachophoresis because it offers completely new and powerful detection and identification possibilities, and also because it makes capillary isotachophoresis a high-resolution purification technique for sample preparation.

The possibility of using specific reactions on the strip reduces the need for new detectors to a certain extent. Today, only the specific UV detector has proved to be fully reliable while the general thermal detector has a lower sensitivity[9]. The general potentiometric detectors are still in the state where they require some maintenance and chemicals can interfere[16].

ACKNOWLEDGEMENT

The author is indebted to Dr. P. J. Svendsen, The Protein Laboratory, Copenhagen, for the generous gift of the purified transferrin sample.

REFERENCES

1 L. Arlinger, *Biochim. Biophys. Acta*, 393 (1975) 396.
2 L. Arlinger, in P. G. Righetti (Editor), *Proceedings of the Electrofocusing and Isotachophoresis Symposium, Milan, 1974*, North Holland, Amsterdam, 1975, p. 331.
3 K. G. Kjellin, U. Moberg and L. Hallander, *Sci. Tools*, 22 (1975) 3.
4 A. Kopwillem, W. G. Merriman, R. M. Cuddeback, A. J. K. Smolka and M. Bier, *J. Chromatogr.*, 118 (1976) 35.
5 K. G. Kjellin, L. Hallander and U. Moberg, *J. Neurol. Sci.*, 26 (1975) 617.
6 A. Kopwillem, U. Moberg, G. Westin-Sjödahl, R. Lundin and H. Sievertsson, *Anal. Biochem.*, 67 (1975) 166.
7 A. Kopwillem, *Acta Chem. Scand.*, 27 (1973) 2426.
8 A. Kopwillem, *J. Chromatogr.*, 82 (1973) 407.
9 L. Arlinger, *J. Chromatogr.*, 91 (1974) 785.
10 L. Arlinger, *Protides Biol. Fluids*, 22 (1974) 661.
11 F. M. Everaerts, J. Vacik, T. P. E. M. Verheggen and J. Zuska, *J. Chromatogr.*, 49 (1970) 262.
12 P. J. Svendsen and C. Rose, *Sci. Tools*, 17 (1970) 13.

13 N. H. Axelsen, J. Krøll and B. Weeke, *Quantitative Immunoelectrophoresis*, Universitetsförlaget, Oslo, 1973.
14 T. Wadström and C. J. Smyth, *Sci. Tools*, 20 (1973) 17.
15 C. W. Young and E. S. Bittar, *Cancer Res.*, 34 (1974) 2675.
16 F. M. Everaerts and P. J. Rommers, *J. Chromatogr.*, 91 (1974) 809.