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VOLUME-COUPLING IN ISOTACHOPHORESIS

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SUMMARY

A new device for isotachophoretic analyses is described in which pre-separation columns with different inner diameters can simply be exchanged (so-called volume-coupling). The most important advantages compared to conventional equipment having a uniform column diameter are: the low end-voltages needed, a ready optimization of analysis time for a particular problem and the possibility of applying terminators with very low effective mobility. The application of Araldite® allows the use of non-aqueous solvents.

INTRODUCTION

Because a scanning detector is not available in present day isotachophoretic equipment, the zones can only be detected during passage by both conductivity (potential gradient) and UV detectors; even though the steady-state is attained long before these detectors are reached. The time of analysis, therefore, often is unnecessarily long. This includes the higher requirements for purity of the electrolyte systems applied[1].

The introduction of separation columns of various lengths[2], *e.g.*, in the commercially available Tachophor (LKB Produkter, Bromma, Sweden) and ITP-2B (Shimadzu, Tokyo, Japan), solves most of above problems. Complex mixtures require adequate separation capacity, but a long separation capillary often results in a high end-voltage. A counterflow of electrolyte is an alternative for elongation of the separation column. However, this results in a disturbance of zone boundaries[2], and increases the complexity of the equipment. An isotachophoretic system with the possibility of volume-coupling[1,3,4], *i.e.*, making use of separation columns with variable inner diameters (which can simply be exchanged), solves these problems.

INSTRUMENTATION

The basic unit, shown schematically in Fig. 1, forms the central block in which the UV-slit, the probe for measuring the conductivity (potential gradient) and the final separation capillary (8 cm × 0.2 mm I.D.) are mounted. It is fixed to the frame

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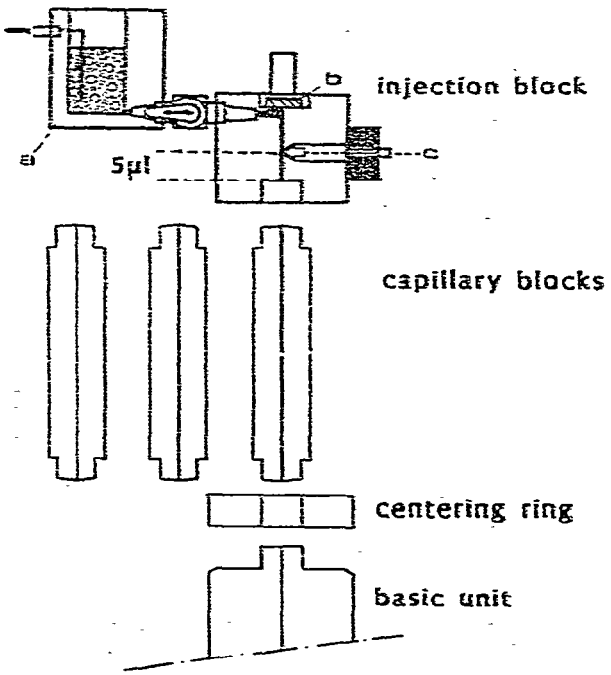

Fig. 1. Equipment suitable for volume-coupling in isotachophoresis. a = Compartment filled with terminating electrolyte; b = septum; c = drain. The inner diameters of the capillary blocks are 0.3, 0.4 and 0.5 mm respectively; all outer diameters are 1.5 cm. The basic unit consists of a separation capillary (0.2 mm I.D.). The lengths of this capillary and of the capillary blocks are 8 cm. In the basic unit the detectors are mounted (see text).

of the instrument. A stable point of attachment is important for the coupling of the other items, *e.g.*, counter-electrode compartment, pre-separation capillary(ies), also called capillary block(s) and injection block. The mode of construction enables direct coupling of an injection block onto the basic unit, which results in the shortest analysis time under the chosen operating conditions. The capillary block has a length of 8 cm. Three different inner diameters (0.3, 0.4 or 0.5 mm) can be selected. The outer diameter is *ca.* 1.5 cm, in order to allow stable coupling and effective temperature control. The load capacity can easily be varied if a vertical alignment is chosen[3]. For a variety of practical problems the analysis time can be optimized. The final voltages remain low, although a high load capacity can be achieved. This is favourable for potential gradient or conductivity detection with sensing electrodes in direct contact with the electrolytes inside the final separation capillary, and for the choice of terminators having very low effective mobilities.

All blocks are made of Araldite (LY 558, hardener HY 32; Ciba-Geigy Plastics and Additives Co., Duxford, Cambridge, Great Britain)[5,6], via injection moulding. This material makes possible the use of non-aqueous solvents and is found to be better for temperature control than PTFE. Although Araldite is coloured, it is sufficiently transparent for accurate machining of crucial parts of the instrument, *e.g.*, the conductivity (potential gradient) probe. For glueing of the Pt/Ir 10% electrodes

(10 μm thick), Araldite AV 138 and hardener HV 998 (Ciba-Geigy, Switzerland) are applied[6].

The end-parts of the capillary blocks are conical (5°). The various blocks are aligned with centering rings. Once the optimal combination has been chosen (calculated), all blocks are clamped with a spring-lever construction. A sufficient thrust is always guaranteed; no O-rings are used. Due to the conical construction of the end-parts of the capillary blocks the contact surface with the central block and injection block is small. Therefore only a small force is required to make a liquid-tight connection. Moreover, during rinsing and refilling of the various compartments, via the counter-electrode compartment and the basic unit, the separation capillary (0.2 mm I.D.) creates a pressure drop due to its resistance. Careful testing of the capillary blocks indicated that the Araldite used was highly resistant to deformation.
A capillary block (0.4 mm I.D.) was dropped 25 times from a height of *ca.* 1 m onto its conical end on a steel plate. After visual inspection under a microscope no damage could be found, not even near the bore of 0.4 mm. Mounting this block again in the ITP equipment for volume coupling and testing it confirmed its liquid-tightness.

The measuring probes, for conductivity or potential gradient detection, are stable and have high resolution. No difference has been found compared with probes in which acrylic is applied.

In volume-coupling the capillary blocks can be exchanged. Both the basic unit (final separation compartment) and the injection block have fixed volumes. It is obvious that these volumes have to be optimized before construction. Fig. 1 shows the injection block, as used in the experiments. The volume below the drain is *ca.* 5 μl, filled with leading electrolyte. The volume above the drain is filled with terminating electrolyte. At the counter-electrode side a semi-permeable membrane is applied. In the compartment filled with terminating electrolyte, no membrane is used in the experiments described. Injecting the sample results in a displacement of the terminator, due to the presence of the semi-permeable membrane. If samples greater than 15 μl are applied, the tap between the compartment filled with terminating electrolyte and the injection block must be closed and the tap behind the counter-electrode compartment is opened. The sample now displaces the leading electrolyte. If the correct volume for pre-separation is chosen, a sample as large as 30 μl can be applied.

EXPERIMENTAL

A series of experiments was performed to test the equipment suitable for volume-coupling. For the investigation of the calibration line (amount *versus* zone length in the isotachopherogram), 2,4-dihydroxybenzoic acid was chosen (Fig. 2), the operating conditions listed in Table I. The volume capacity of the leading electrolyte, determined by the combination of compartments chosen, was approximately 30 μl. The calibration line passes through the origin and has a correlation coefficient of 0.99999 ($n = 16$). This result shows that the various compartments coupled have negligible dead-volumes. It is to be noted that both the samples of 1 μl and 20 μl fit the calibration line (the volume of the separation compartments is just 30 μl). All the samples were injected with a 10-μl syringe (Hamilton, Bonaduz, Switzerland), adjusted under a microscope. The analyses were carried out in duplicate.

In isotachophoretic analyses the temperature increase in a 0.2 mm I.D. tube is

TABLE I

OPERATIONAL SYSTEM AT pH 6, SUITABLE FOR ANIONIC SEPARATIONS

MES = Morpholinoethanesulphonic acid; TRIS = tris(hydroxymethyl)aminomethane; HEC = hydroxyethylcellulose.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	MES
Concentration	0.01 *N*	*ca.* 0.01 *N*
Counter ion	Histidine	TRIS
pH	6	*ca.* 7
Additive	0.3% HEC*	None

* Purified by shaking the 2% solution with a mixed-bed ion exchanger and filtering.

usually negligible[7], Fig. 3. Thus, the electric driving current can be doubled during the pre-separation, providing the terminator migrates in the capillary block (Fig. 1). In Table II some results are given of analyses performed in a 0.2 mm I.D. tube at J = 0.08 A cm^{-2} and 0.16 A cm^{-2}, with acetate as sample ion. The correlation coefficient of the calibration line and the accuracy of the analysis times are excellent at both current densities.

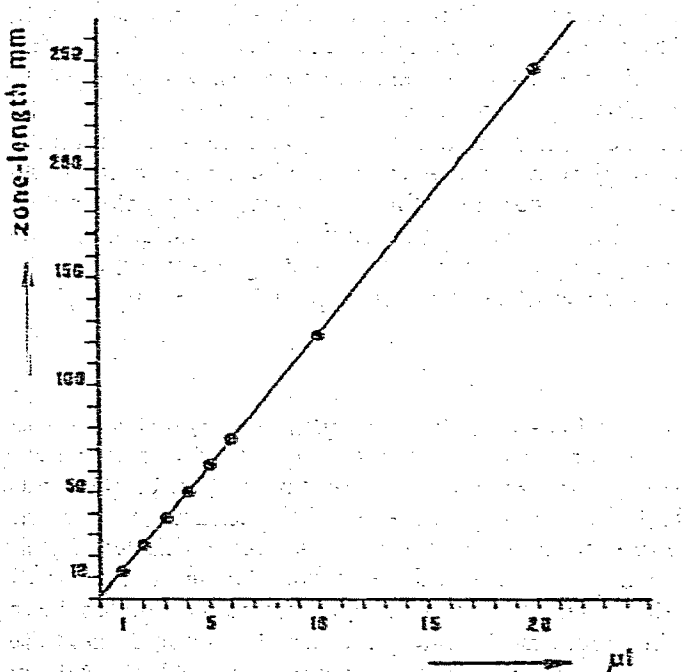

Fig. 2. Calibration line (amount injected in µl *versus* the zone length in mm) obtained for the equipment suitable for volume-coupling. Operating conditions as listed in Table I. The analysis was of 2,4-dihydroxybenzoate: a 1-mm zone length equivalent to *ca.* 90 pmoles. The experiments were carried out in duplicate: correlation coefficient = 0.99999 (n = 16).

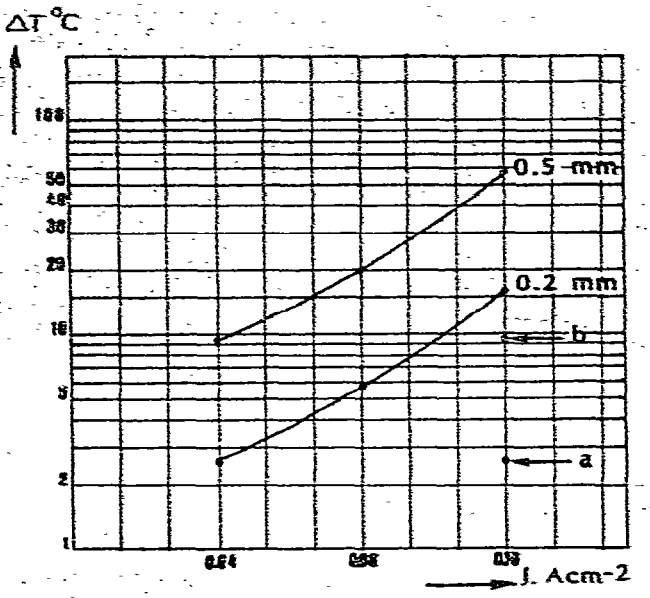

Fig. 3. The increase in temperature for chloride–MES under the operating conditions of Table I in PTFE capillaries with inner diameters of 0.2 and 0.5 mm and outer diameters of *ca.* 0.35 and 0.75 mm, *versus* the current density applied. a, The temperature in the capillary block of 0.5 mm I.D.; b, the temperature in the capillary block of 0.3 mm I.D.

Before a series of analyses, it is always recommended to run an experiment solely with leading and terminating ions, to check the purity of the electrolytes. During this experiment the increase of the voltage as a function of time can be registered (Fig. 4). From this curve the position of the leading/terminating boundary can be deduced. It is then simple to chose the time for pre-separation (double the current). Moreover, this allows automatic handling of the system[8]. The voltage curves, as shown in Fig. 4, were obtained under the operating conditions listed in Table I. Capillary blocks with inner diameters of 0.3, 0.4 and 0.5 mm were used. A

TABLE II

THE INFLUENCE OF THE CURRENT DENSITY ON ISOTACHOPHORETIC PARAMETERS

V_{rec} is chart speed of recorder.

	Injected amount (μl)	*J = 0.08 A cm⁻²* V_{rec} *= 6 cm min⁻¹*		*J = 0.16 A cm⁻²* V_{rec} *= 12 cm min⁻¹*	
		Zone length (mm)	*Time* (min:sec)*	*Zone length (mm)*	*Time* (min:sec)*
	0.5	31.2	13:40	32.0	6:55
	1	59.0	13:51	59.6	6:50
	2	115.0	13:52	115.6	6:55
	3	171.0	13:55	172.3	6:55
Correlation coefficient		0.99999		0.99998	
Slope		55.9		56.1	
Intercept		3.14		3.64	
Average			13:49		6:53
% R.S.D.			0.8		0.6

* Time of arrival of the first zone boundary at the detector.

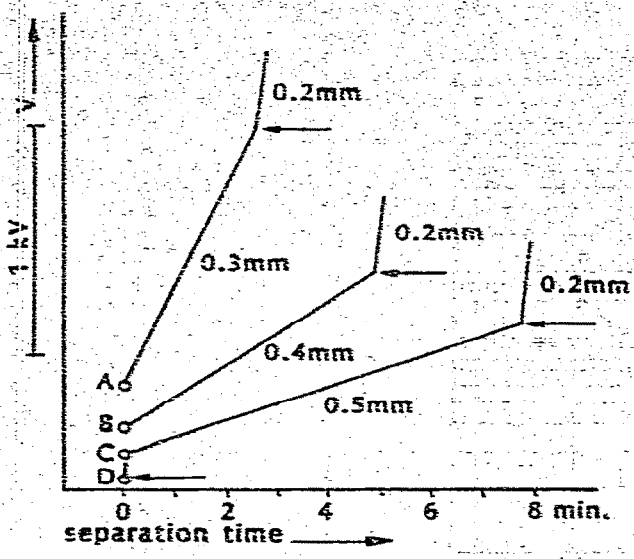

Fig. 4. The voltage increase, V, during an isotachophoretic experiment solely with leading and terminating electrolytes ($I = 0.16$ A cm^{-2}). The capillary blocks used have inner diameters of 0.3 mm (A), 0.4 mm (B) and 0.5 mm (C). In D the injection block is coupled directly to the basic unit having an inner diameter of 0.2 mm. The recording of the curves is started at the moment at which the zone boundary leaves the injection block (O). The arrows indicate the moment at which the leading/terminating ion boundary enters into the basic unit.

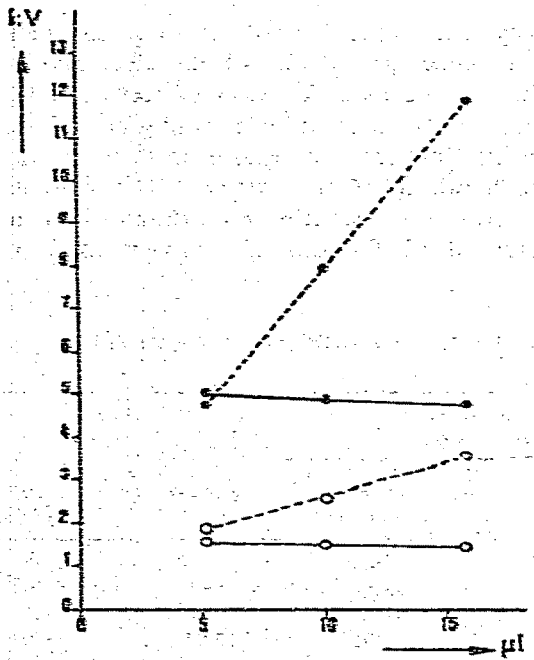

Fig. 5. The comparison of voltages needed in equipment suitable for volume-coupling (straight line) and equipment with a uniform diameter (dotted line). O, Start voltage; ●, end voltage. Operating conditions as in Table I.

curve from an experiment in which the injection block was directly coupled to the basic unit is also shown.

In Fig. 5 start voltages and end voltages, as obtained in the equipment suitable for volume-coupling and the equipment in which a capillary tube of uniform inner diameter is mounted, are compared ($J = 0.08$ A cm^{-2}). It is clear that in equipment suitable for volume-coupling the voltage decreases with increasing separation capacity, because the electrical resistance of a column with greater inner diameter is lower.

Volume-coupling therefore allows the use of terminators with low effective mobilities, as is shown in Fig. 6. The sample consists of sulphate, chlorate, chromate, malonate, pyrazole-3,5-dicarboxylate, adipate, acetate, β-chloropropionate, ben-

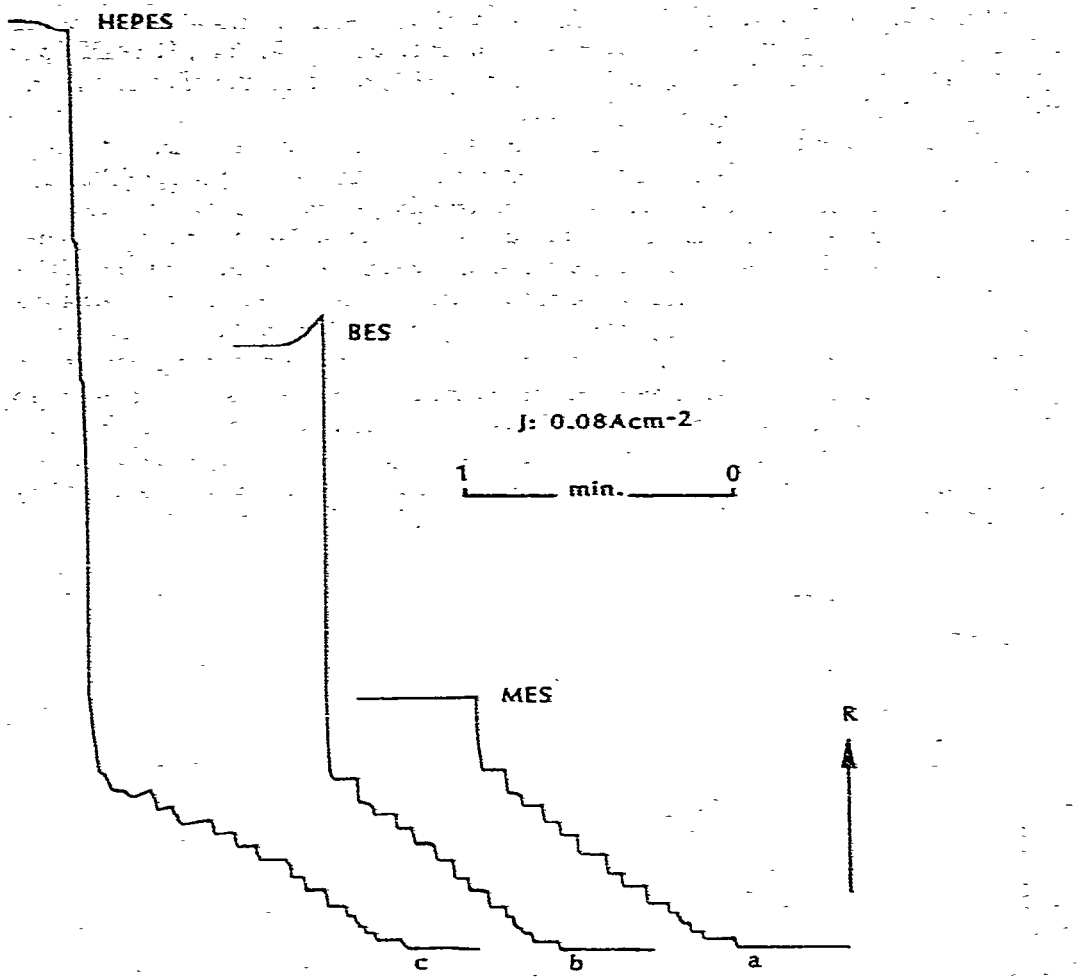

Fig. 6. Isotachopherograms of a test mixture of anions (see text). Terminators with low effective mobilities were applied. MES = 2-Morpholinoethanesulphonic acid; BES = N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid. R = increasing resistance.

zoate, naphthalene-2-sulphonate, glutamate, enanthate and benzyl-DL-aspartate. Different terminators were used: a, MES (end voltage 3.7 kV); b, BES (end voltage 8.8 kV) and c, HEPES (end voltage 13 kV). It is seen that impurities in the terminating electrolyte disturb the "steady-state" zones (zone electrophoresis). All experiments were carried out in a capillary block with 0.4 mm I.D., which is comparable with conventional equipment with a capillary of uniform inner diameter of 0.2 mm and a length of 32 cm. For HEPES in conventional equipment with a uniform diameter of 0.2 mm and $J = 0.08$ A cm^{-2} (25 μA), an end voltage of 24.6 kV would be necessary. Even N,N-bis(2-hydroxyethyl)glycine (BICINE), although very impure, has been used as a terminator at pH = 6. The end voltage in the equipment suitable for column-coupling was 17 kV, which is comparable with *ca.* 32 kV in equipment with uniform diameter (0.2 mm I.D.).

It is obvious that the use of terminators with low effective mobilities increases the possibilities to analyse many more constituents under similar pH conditions (operational system). More attention should be given to the purification of the terminating electrolyte, as is shown in Fig. 6c.

Volume-coupling needs accurate mounting of the various compartments. Therefore experiments were carried out to test the alignment. The top side of the basic unit was found to be the most critical point for coupling (Fig. 1). In order to test this connection, a dead-volume was deliberately created at this point. It is clear that after refilling the system with fresh electrolyte some of the terminating electrolyte remains present. In the following run these "terminating ions" will migrate zone-electrophoretically in the leading electrolyte, present in the capillary of the basic unit. Of course the velocity is lower than the isotachophoretic one under the operating conditions applied. As soon as the isotachophoretic sample stack overruns this zone, there is an effect on the UV detector or on the universal conductivity or potential gradient detector[9]. Thus, the detector traces show a drift (Fig. 7) in the registration of the zone(s). On the other hand, if a dead-volume is present at the coupling of capillary block and basic unit (Fig. 1), this volume will be filled with leading ions while the

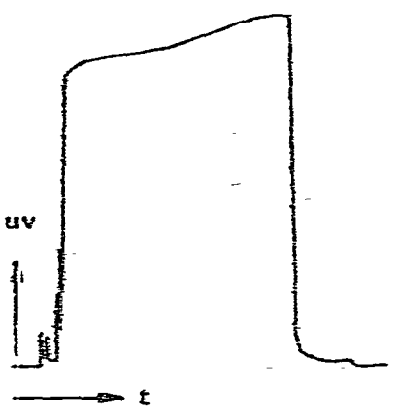

Fig. 7. Deliberate creation of a dead-volume at the point of coupling of the capillary block and basic unit. The figure shows the dilution of the adenosine 5'-monophosphate zone by the zone-electrophoretically migrating MES (terminator) originating from a previous experiment. For further explanation see text.

terminating ions are disappearing, due to the field strength applied. After passing of the isotachophoretic sample stack, leading ions are still present in this dead-volume. This causes a bleading of leading ions (zone-electrophoresis), which may also influence the final registration of the zones. It was found practical to provide the entrance of the basic unit with a conical tip. No further problems with volume-coupling have been found, using the vertical alignment of the separation columns.

In Fig. 8 an isotachopherogram of a series of anions (see Fig. 6) is shown, analysed at pH = 6, see Table I, to indicate the resolution and stability of the conductivity (potential gradient) detector probe made of Araldite. Similar results were obtained in non-aqueous solutions.

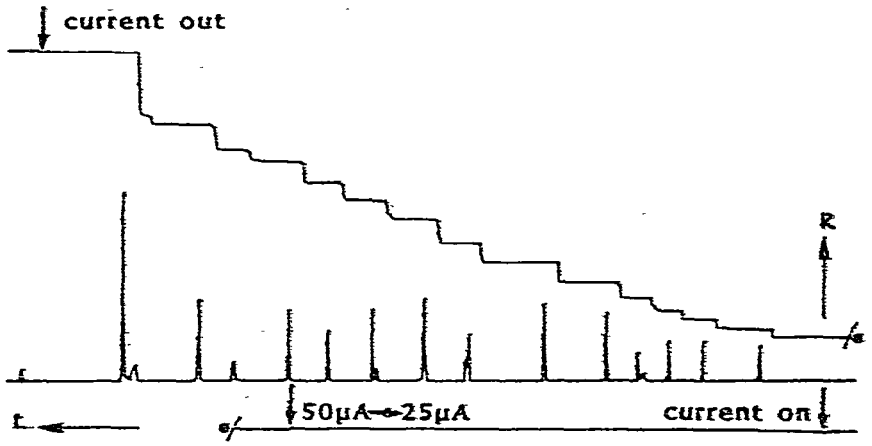

Fig. 8. Isotachopherogram of a series of anions (see text) obtained by use of equipment suitable for volume-coupling to show the resolution and stability of the conductivity (potential gradient) detector at various currents. For the probe, Araldite LY 558 with hardener HY 32 has been used, which enables the use of non-aqueous solvents. Velocity of recorder: 6 cm min^{-1}. J = 0.16 A cm^{-2}/0.08 A cm^{-2} (switch indicated by arrow). R = increasing resistance.

CONCLUSIONS

Equipment suitable for volume-coupling permits the ready optimization of load capacity. The possibility of using high pre-separation currents reduces the analysis time. Low end-voltages permit the use of terminators having low effective mobilities. This increases the range of constituents which can be separated. The modular system allows the use of options such as a bifurcation block[8] or micropreparative compartments if other separation or identification techniques are necessary. The enhanced separation capacity, expected when using volume coupling, was confirmed experimentally. The problems in aligning the various compartments could easily be solved by the use of centering rings and spring-lever devices.

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