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ISOTACHOPHORESIS

ELECTROPHORETIC ANALYSIS IN CAPILLARIES

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SUMMARY

The aim of this paper is to give more details about the equipment used for isotachophoretic analysis. More accurate values, quantitatively as well as qualitatively, can be obtained by thermostating the system where the thermal detector is mounted. Mobilities of the different ion species are also strongly influenced by shifts in temperature. Therefore an electric diagram is given for an aluminum thermostat in which the capillary is mounted. A secondary effect, but also an important one, is that shorter times for analysis can be obtained because higher currents can be used by the increased heat transfer. Some electric diagrams are given for differentiating electronically, which makes balancing of thermocouples[1,2] unnecessary. Detailed designs are given for injection blocs and electrode compartments.

INTRODUCTION

The principles for isotachophoretic analysis (displacement electrophoresis) have been described by EVERAERTS[1] and MARTIN AND EVERAERTS[2].

The apparatus basically consists of a capillary mounted between two electrode compartments, the anode and cathode compartment respectively. For the separation of anions the capillary is filled with a salt of an anion more mobile than any in the sample and a cation with buffering capacity (leading electrolyte). The anode compartment contains a solution of the buffering cation. The cathode compartment contains a solution of an anion less mobile than any in the sample (terminator). The sample must be introduced between the terminator and the leading electrolyte.

A constant current is passed between the electrodes. The anions in the sample move initially at different speeds until they are separated in order of their mobility. Then all anions in the apparatus move down in the capillary tube at the same speed (isotachophoresis), assuming the capillary to be of constant bore. The boundary between each successive pair of ions is more or less sharp[1,2].

Since each zone has a particular potential gradient, it has also a particular rate of heat generation per unit length and a particular temperature. Thus it will be possible

to follow the separation by means of fixed thermocouples on the outside of the capillary tube.

The temperature of each zone gives the information about the ion species in that zone. The length of each zone gives the possibility of calculating the amount of each ion species with the equation of Kohlrausch[1]. The length of each zone is preferably measured from the distance between the peaks of the record provided by a differential thermocouple, measuring the difference in temperature along a short length of the tube, or by differentiating electronically the integral signal provided by the integral thermocouple.

The aim here is to provide experimental details and also to explain the reasons for some particular choices. If a capillary tube, surrounded by air, is freely supported in a thermostat and heat is produced in it by an electric current, it will loose this heat by transferring it to the air and by the low temperatures of the tube a negligible part is lost by radiation. Excessive heat production will result in bubble formation. If the detector is thermal (by a set of thermocouples) no extreme cooling by cold air or thermostated liquid can be used.

Future work in developing other detectors to be used in this field will therefore be very important both for the accuracy of the results and for the speed of the analysis. The current used actually is limited by the dimensions of the capillary, the temperature of the surrounding air and the overall heat-transfer coefficient. During an analysis, a stationary condition will be obtained inside the thermostated compartment, containing the capillary tube. This stationary condition depends on the amount and species of the intermediate ions, the terminator chosen, the current used, the dimensions of the capillary, the overall heat-transfer coefficient, the temperature of the surrounding air and the heat flow to the thermostat. The time for reaching this new steady state is given by the capacity of the thermostated system to dissipate the heat produced in it.

The reference junction of the integral thermocouple (ref. 1), however, will have the temperature of the thermostat. This still means that the temperature of a well-defined zone can differ from experiment to experiment because the air temperature is not constant. The temperature of a zone is used as a characteristic for each ion species. If all steady states now would give the same air temperature inside the thermostat, the zone temperatures should be the same from experiment to experiment. But the temperature is, as said above, also dependent on the terminator used and the amount and species of intermediate ions. Therefore accurate values can not be expected. A new type of thermostat has been developed. The reference junction of the thermocouple must never be mounted free in the air but must be embedded in a heat sink (isolator type) in the aluminum thermostat (see below). The thermal contact of the capillary tube with the metal thermostat must be made as good as possible.

MATERIALS AND METHODS

Description of the apparatus (Fig. 1)

A teflon capillary tube (O.D. 0.75 mm, I.D. 0.45 mm) is embedded in a groove of an aluminum bloc (Fig. 2). The capillary is wound around the aluminum block in the form of a helix. Gaps between the capillary and the aluminum bloc are carefully filled with a heat sink compound (Al_2O_3 powder with silicone oil). Because the heat

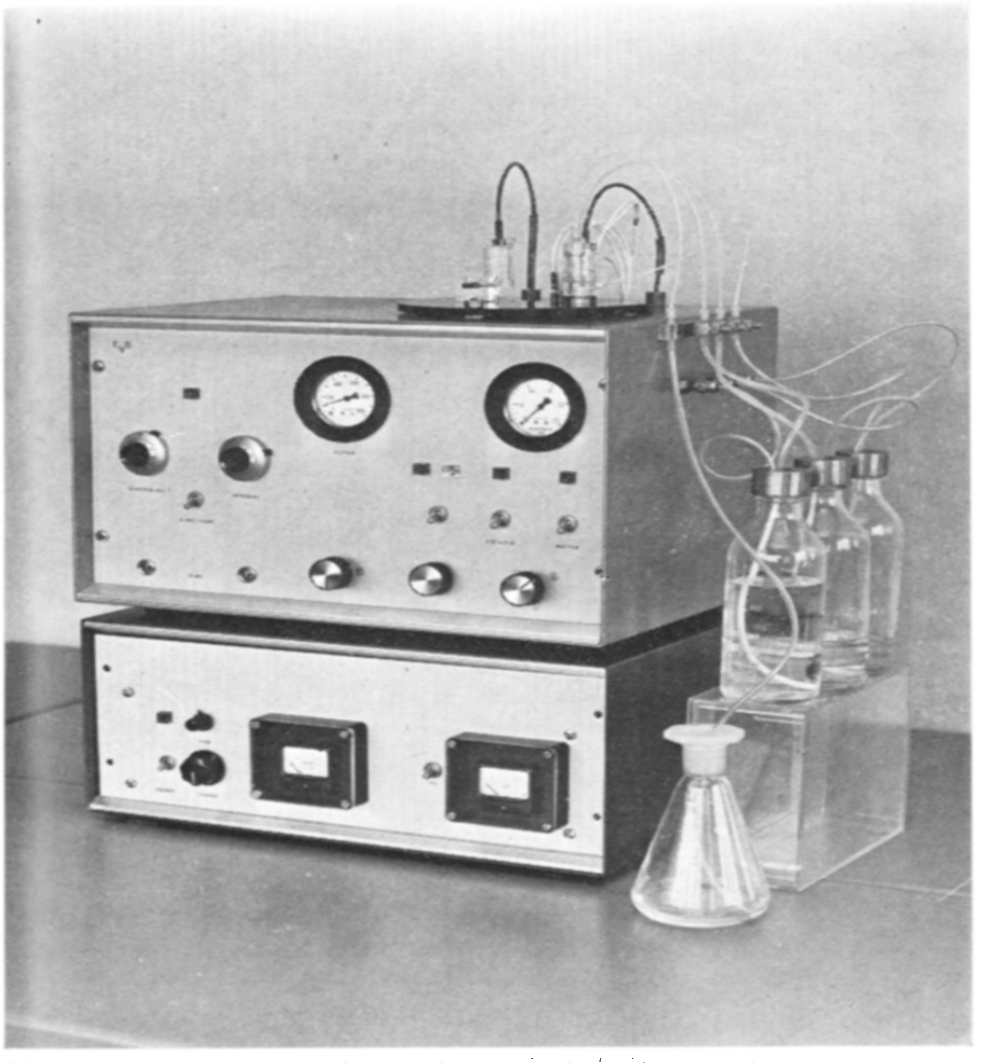

Fig. 1. Apparatus used for the isotachophoretic experiments.

produced in the capillary is so quickly transferred to the aluminum bloc, a compartment (Fig. 2E) is created where the thermocouples are mounted to be sure that there will still be a signal to detect. The reference junctions of the thermocouples have the temperature of the aluminum bloc because a certain amount of heat sink compound is smeared out on the junction, providing for the thermal contact with the aluminum bloc. The teflon capillary tube and the heat sink compound are fixed by a thin layer of shellac (Krylon). For cooling of the aluminum bloc thermostated water (0.1° accurate) is used. A temperature sensor (Pt-resistance 100 Ω) is mounted in the neighborhood of the detector compartment. Here also gaps are filled with the heat sink compound. In the center of the aluminum bloc a load is mounted (60 watt). Pt-resistance and load are connected to the temperature controll unit (see Fig. 11). Rubber O-rings and Devcon material (metal glue) are employed to prevent contact of water, circulating inside the aluminum bloc, with the electric circuits.

Injection bloc and compartment for the terminal electrolyte

The capillary tube protruding from the thermostat is on one side connected

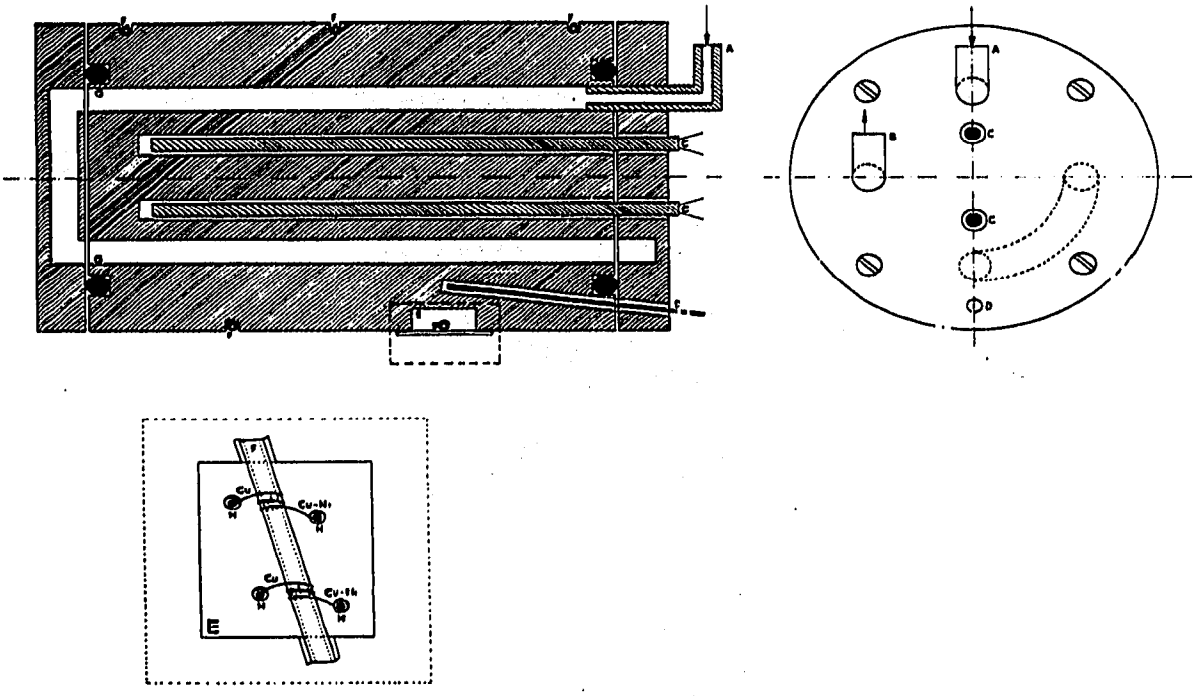

Fig. 2. Aluminum thermostat. A = input thermostated water; B = output thermostated water; C = loads; D = Pt resistance; E = detector compartment; F = capillary tube; G = rubber O-rings; H = thermocouples.

with the injection bloc (Figs. 3 and 4). All parts are made of perspex. Special care must be taken to make all holes in this injection bloc as smooth as possible. Scratches in it can give disturbances during an analysis by adhering impurities, difficult to remove. Holes in the perspex must therefore be made by very sharp tools, used for perspex exclusively. For lubrication no alcohol or glycerine but ordinary petroleum is recommended. Care must be taken too that no dead volumes are present because, in rinsing the system, these are difficult to clean. Thus a new source of errors is prevented. The capillary tube is connected with the injection bloc without the use of a cement. The inside diameter of the piece of perspex (Fig. 3.4) is the same as the outside diameter of the capillary tube (Fig. 3.7).

By stretching the teflon capillary first over a length of 2 cm, it can be brought into the hole of the piece of perspex mentioned above. Then the capillary tube can be brought into this piece such that it fills the hole entirely. The waste part of teflon can be cut off with a sharp knife. So constructed, only fitting the capillary over a length of about 1.5 cm, no leakage occurs even if the rinsing water is pressed through the capillary at 4 atm. A bolt (Fig. 3.6) will press the capillary tube and piece (Fig. 3.4) together in the perspex injection bloc (Fig. 3.1). The surfaces of contact are smooth and by the elasticity of the perspex no leakage occurs. No packing ring has been employed. In the injection bloc (Fig. 3.1) a hole is drilled providing the contact of the plunger compartment with the capillary tube. A septum (Fig. 3.3), fitted in the injection bloc with a bolt (Fig. 3.2), gives the possibility of injection with an ordinary syringe (Hamilton 1-μl syringe). The compartment for the terminal electrolyte (Fig. 3.12) is blocked with a plunger (Fig. 3.15) during rinsing, filling and sampling. To

prevent any leakage the plunger is covered with a piece of teflon. By injecting the sample some of the leading electrolyte will be displaced into the plunger compartment. This will be sucked into the drain (Fig. 3.14) by a pump if the connection is made between the compartment filled with the terminal electrolyte and the capillary hole

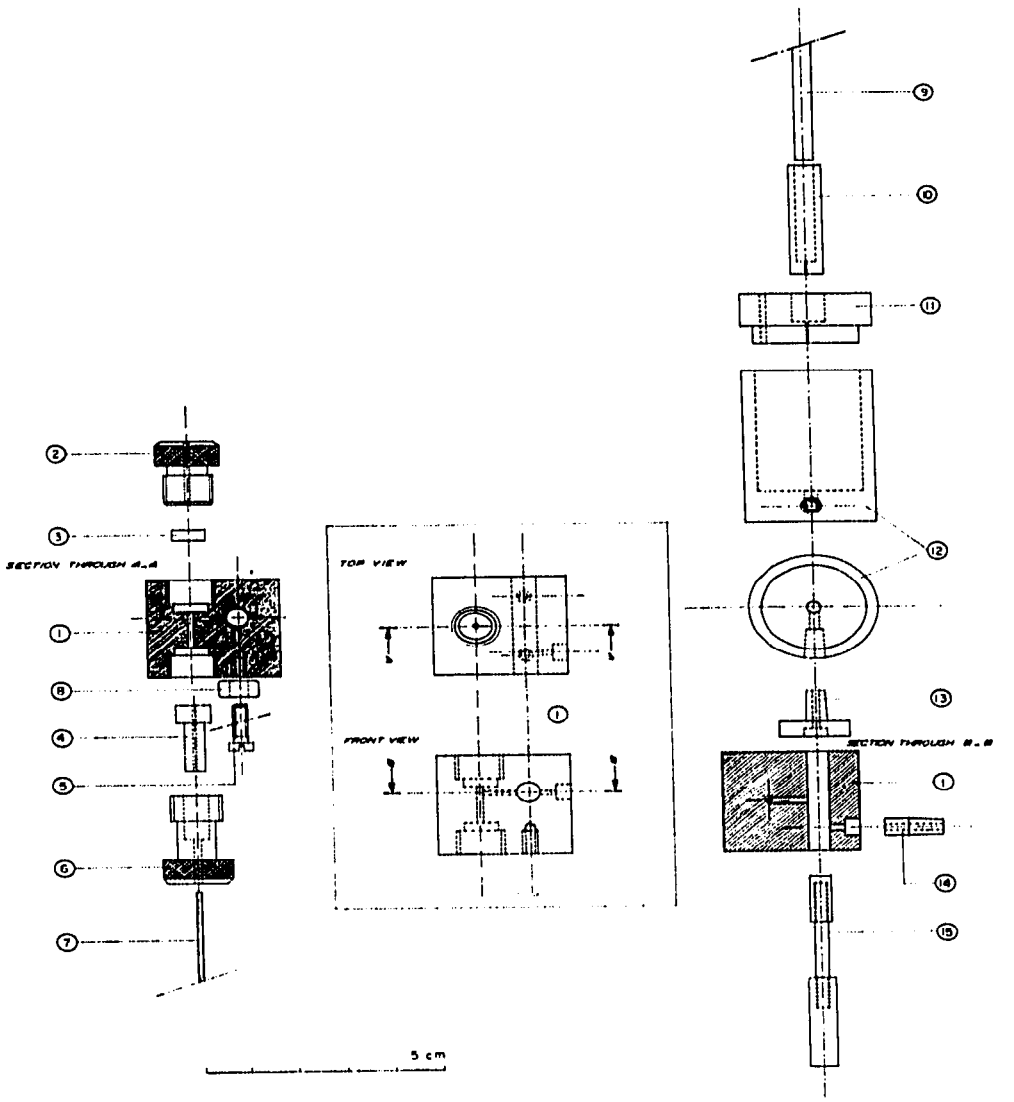

Fig. 3. Injection bloc and compartment for the terminal electrolyte. 1 = injection bloc; 2 = bolt for fitting septum; 3 = septum; 4 = piece of perspex for fitting capillary tube; 5 = screw for mounting injection bloc; 6 = bolt for fitting piece 4 and capillary tube; 7 = capillary tube; 8 = rubber O-ring; 9 = high tension cable; 10 = piece of perspex for mounting high tension cable; 11 = cover of electrode compartment; 12 = electrode compartment; 13 = connection of electrode compartment with plunger compartment; 14 = connection towards drain; 15 = teflon-covered plunger.

in the injection bloc. If the sample should be injected after this connection has been made, some of the leading electrolyte will also be introduced into the plunger compartment, now filled with the terminal electrolyte. The potential gradient in this compartment is very low because the dimensions are large in comparison with the

capillary holes. It will take quite a long time before this leading electrolyte electrically has moved back into the capillary part of the injection bloc. A certain eluting effect will be the result. An electrode, made of Pt wire, is connected to the high tension cable (Fig. 3.9). Piece 13 of Fig. 3 is glued to the injection bloc with chloroform, providing the contact with the compartment of the terminal electrolyte. Thus this compartment can easily be taken off to be rinsed.

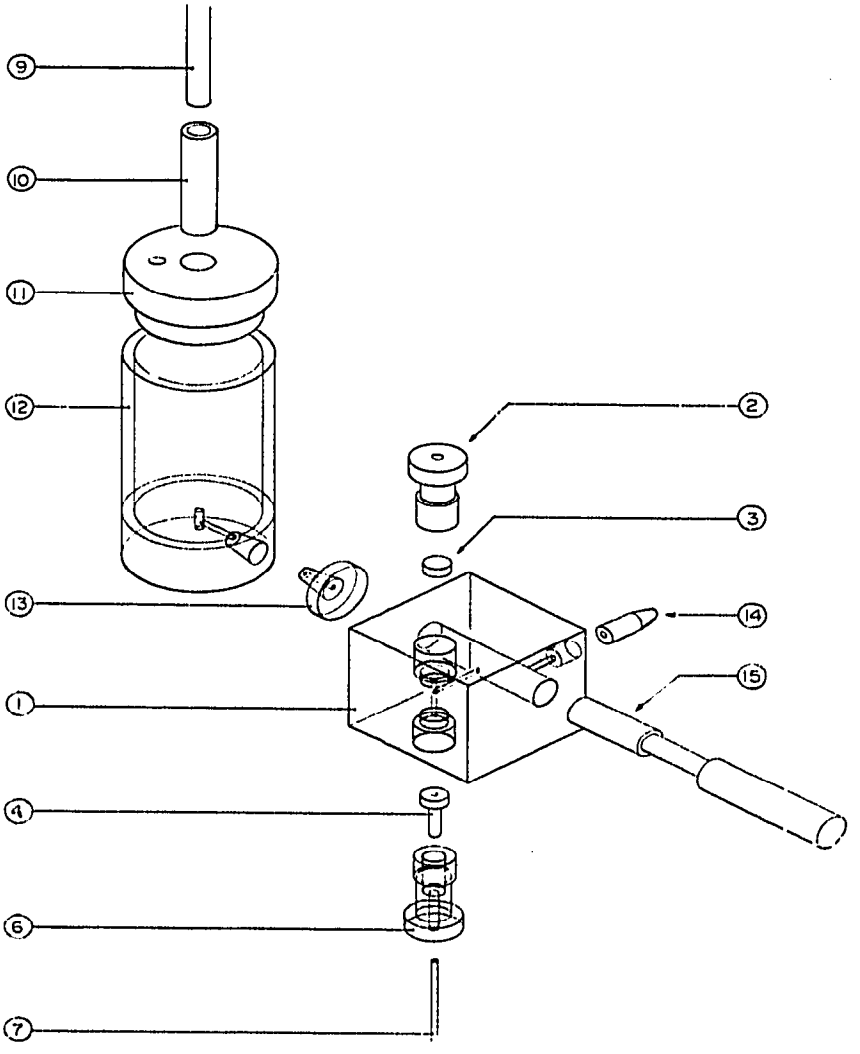

Fig. 4. Injection bloc and compartment for the terminal electrolyte. For further explanation see text and Fig. 3.

Counter-electrode compartment

The capillary protruding from the aluminum thermostat is at the other side connected with an electrode compartment in the same way as described above. The electrode compartment shown in Fig. 5 contains a semi-permeable membrane (Fig. 5.8) made of cellulose acetate. The electrode, made of Pt wire, is separated from the inside of the capillary tube by this membrane.

The use of a semi-permeable membrane has several advantages. First of all the

system is mechanically closed at one side. This decreases the electroendosmotic flow to such an extent that for the separation of small ions no polymer need to be used. For the stabilization of the zones of bigger molecules, such as proteins, a polymer is still needed. Differences in level between the two compartments do not give a hydrostatic flow of liquid. The capillary can be rinsed (Fig. 5.1) or filled with fresh electrolyte without disturbing the solution of the electrode compartment. If during an analysis products are formed at the Pt electrode, the electrode compartment can be continuously

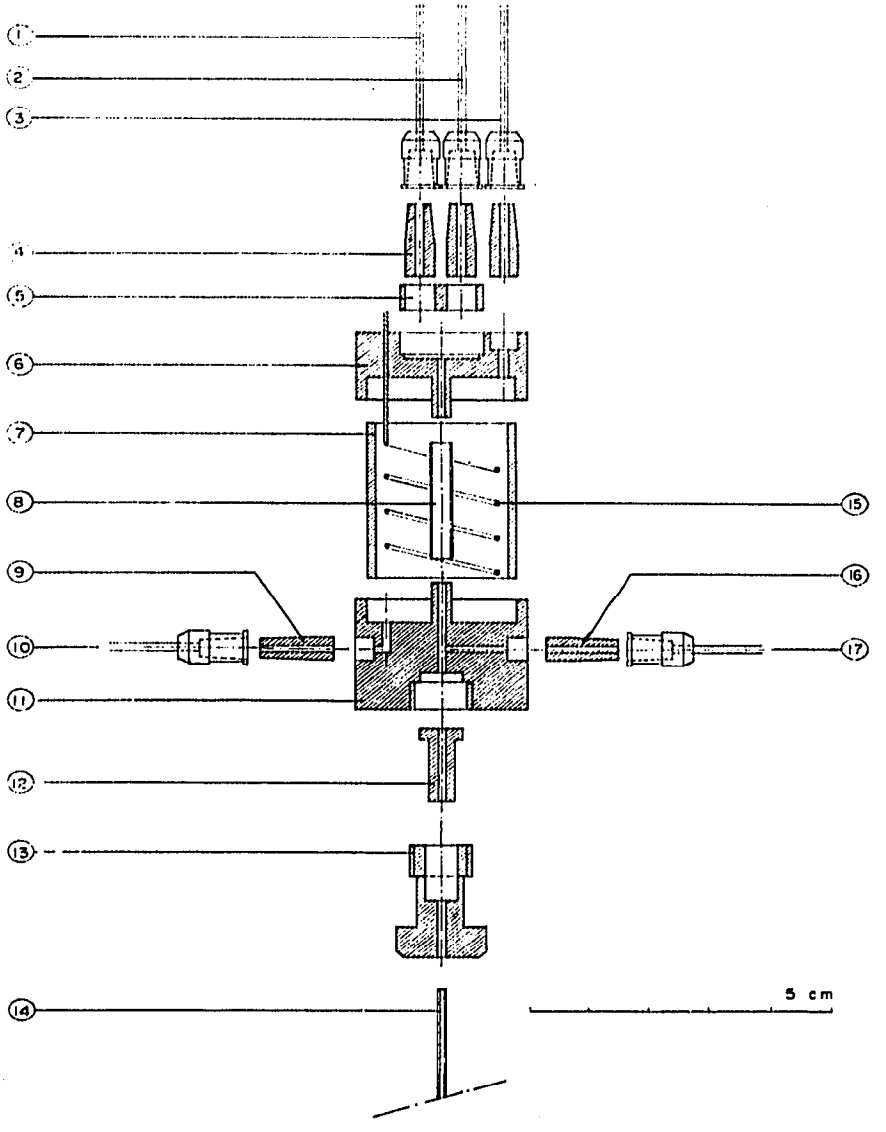

Fig. 5. Counter electrode. 1 = teflon tube, connected with water reservoir; 2 = teflon tube, connected with electrolyte reservoir; 3 = teflon tube, connected with drain; 4 = pieces of perspex used for connection; 5 = pieces of perspex used for connection; 6 = cover of electrode compartment; 7 = electrode compartment; 8 = membrane; 9 = perspex piece used for connection; 10 = teflon tube, connected with the electrolyte reservoir; 11 = bottom of the electrode compartment; 12 = piece of perspex used for fitting the capillary tube; 13 = bolt for fitting piece 12 and capillary tube; 14 = capillary tube; 15 = Pt electrode; 16 = piece of perspex used for connection; 17 = teflon tube, connected with the electrolyte reservoir of the counterflow equipment.

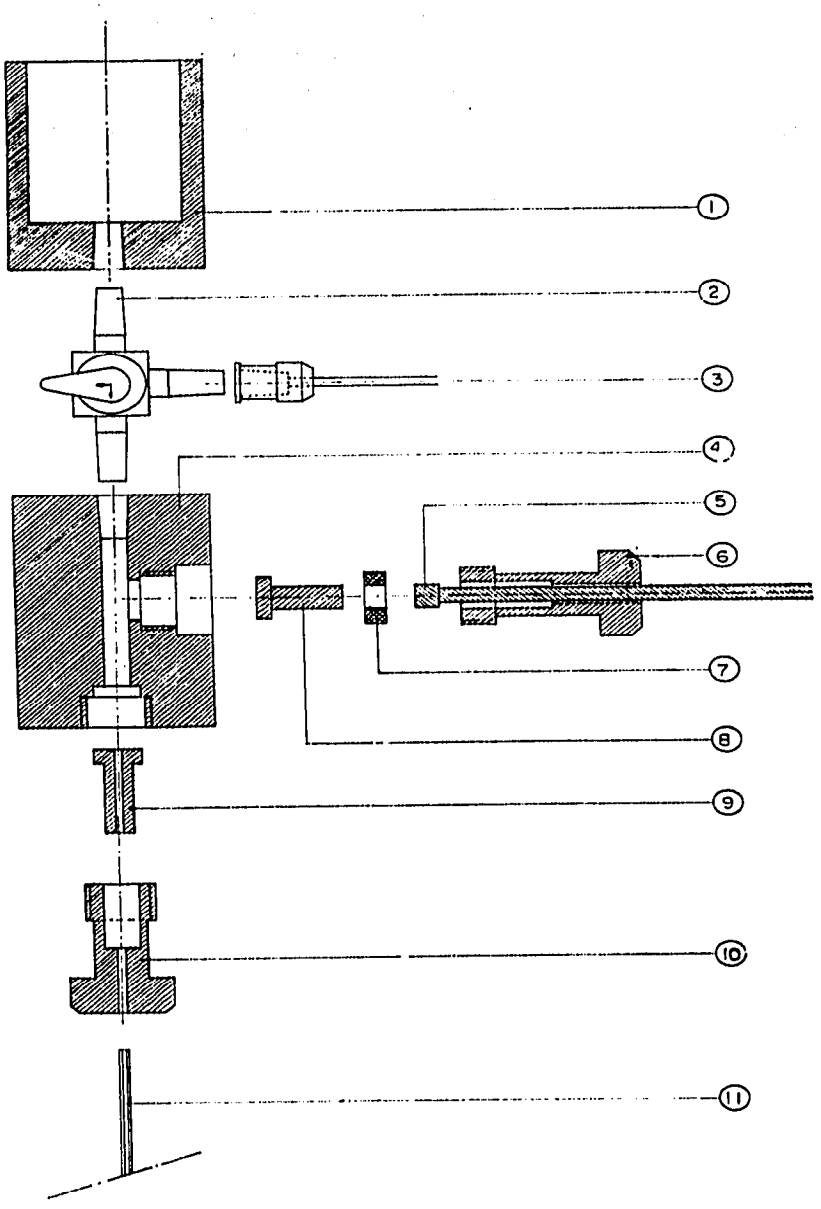

Fig. 6. Counter electrode. 1 = reservoir; 2 = teflon-lined valve; 3 = connection towards the counterflow equipment; 4 = electrode compartment; 5 = high tension cable; 6 = bolt for fitting the electrode; 7 = rubber O-ring; 8 = Ag electrode; 9 = piece of perspex used for fitting the capillary tube; 10 = bolt for fitting piece 9 and the capillary tube; 11 = capillary tube.

rinsed through the connections 3 and 10 of Fig. 5. The pH shift due to this membrane is regarded as a disadvantage[3]. After treatment with alcoholic potassium hydroxide the cellulose acetate membrane can be transformed into a more porous cellulose membrane. The membranes of cellulose acetate were made by rolling a sheet of cellulose acetate (0.2 mm thick) on a glass rod. By dipping it in acetone and washing it with streaming water a membrane is formed. The thickness of the membrane is about 0.5 mm. It was glued on the parts 6 and 11 of Fig. 5 with araldite (CIBA).

Pieces 16 and 17 of Fig. 5 are constructed for the possibility of using the equipment for counterflow analysis. All pieces of Fig. 5, apart from the membrane, are

joined without using cement or chloroform. The membrane is mechanically very strong, so one can work with it for years, if not too acidic (pH 1) solutions are used.

The tubing of Fig. 5.1 and Fig. 5.2 must be made of nonelastic material. This tubing is connected with a teflon-lined Hamilton valve (2 mm 1). After rinsing the capillary tube or filling it with fresh electrolyte a pressure still remains. If the tubing is made of elastic material a counterflow of electrolyte during the analysis will be the result. This causes non-reproducible results and sometimes even leading electrolyte and sample leak into the plunger compartment. Apart from a possible loss of sample it will rise to an error as mentioned under *Injection bloc and compartment for the terminal electrolyte*. If electrode compartments with non-gassing electrodes are wanted, special arrangements can be made. Fig. 6 shows a possibility.

Besides the advantage of the elimination of a pH shift, due to the membrane, and under special conditions the possible prevention or stimulation of electrode reactions, it has also many disadvantages. By rinsing the capillary tube products formed at the electrode must first be removed. The water for rinsing and the electrolyte for filling the capillary tube pass the electrode compartment.

If the electrode compartment is not carefully rinsed after each experiment,

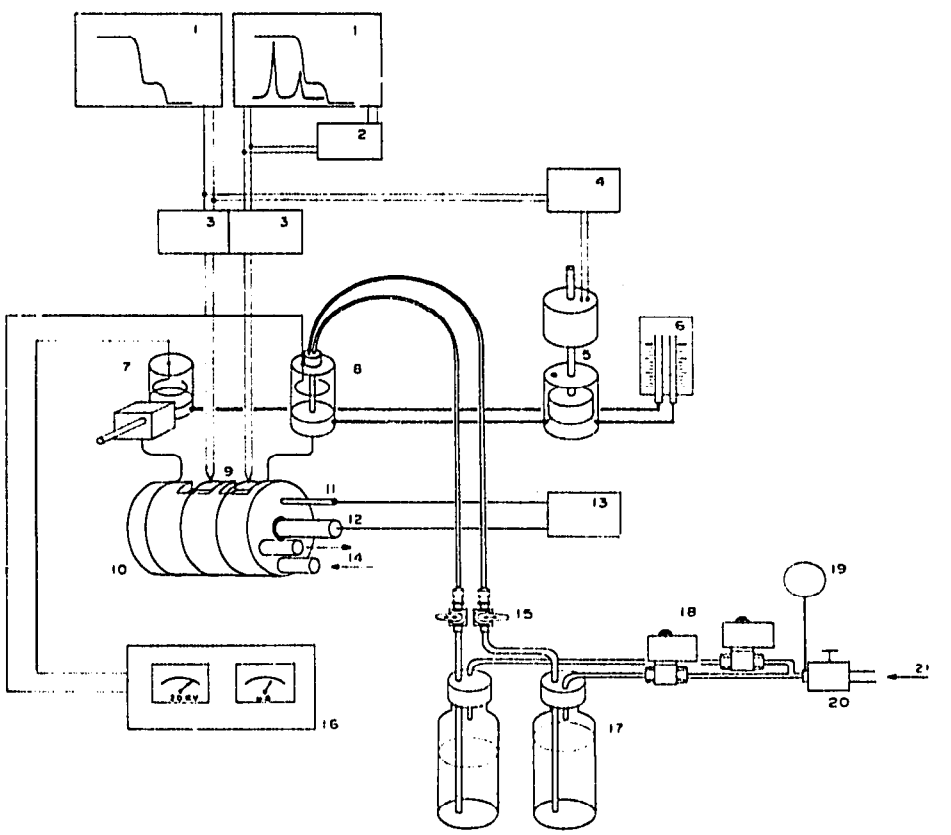

Fig. 7. Bloc diagram of the isotachophoresis equipment. 1 = recorders; 2 = differentiator; 3 = Knick amplifiers, type A; 4 = regulator for the counterflow; 5 = equipment for the counterflow; 6 = level control; 7 = injection bloc; 8 = counter electrode; 9 = thermocouples; 10 = Al bloc with capillary tube; 11 = Pt sensor; 12 = load; 13 = regulator for thermostating; 14 = thermostated water; 15 = teflon-lined valves; 16 = current stabilized power supply; 17 = reservoirs; 18 = magnetic valves; 19 = manometer; 20 = pressure regulator; 21 = air (2 atm).

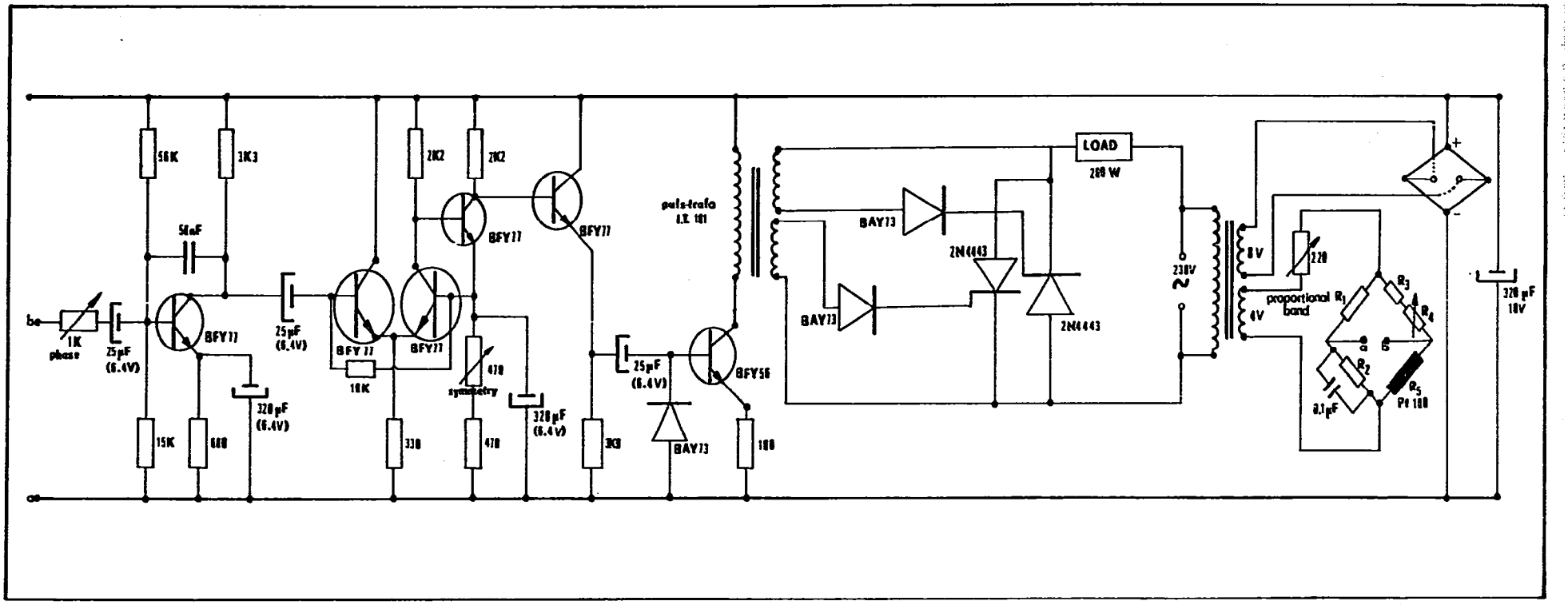

Fig. 8. Proportional temperature regulator. For further explanation see text.

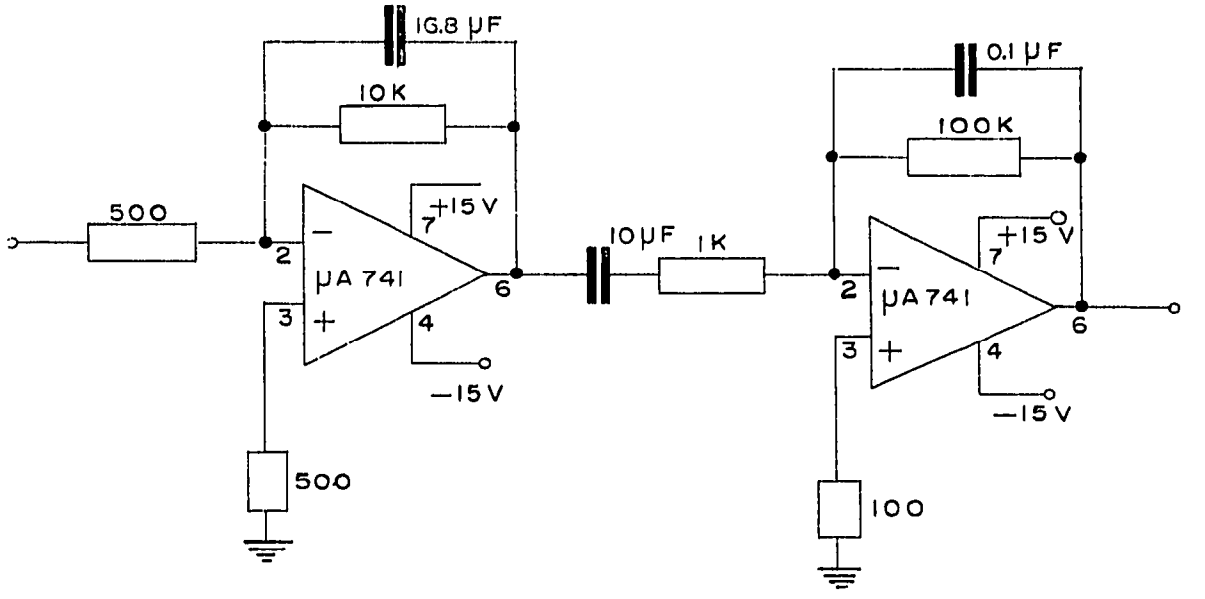

Fig. 9. Differentiator. For further explanation see text.

this too can give disturbances. Another disadvantage is that the electrode must be regenerated after each experiment to prevent formation of gas. Sometimes the pH changes slightly during the analysis, despite all precautions. The perspex reservoir (Fig. 6.1) is constructed because in the tubing (Fig. 6.3) gas bubbles may be present. If these enter the electrode compartment (Fig. 6.4) it will be very difficult to remove them. To make the tubing gas free, the first electrolyte containing the gas bubbles is expelled into this reservoir. The diameter of the boring of this compartment must be as small as possible, otherwise too much electrolyte is needed for each experiment.

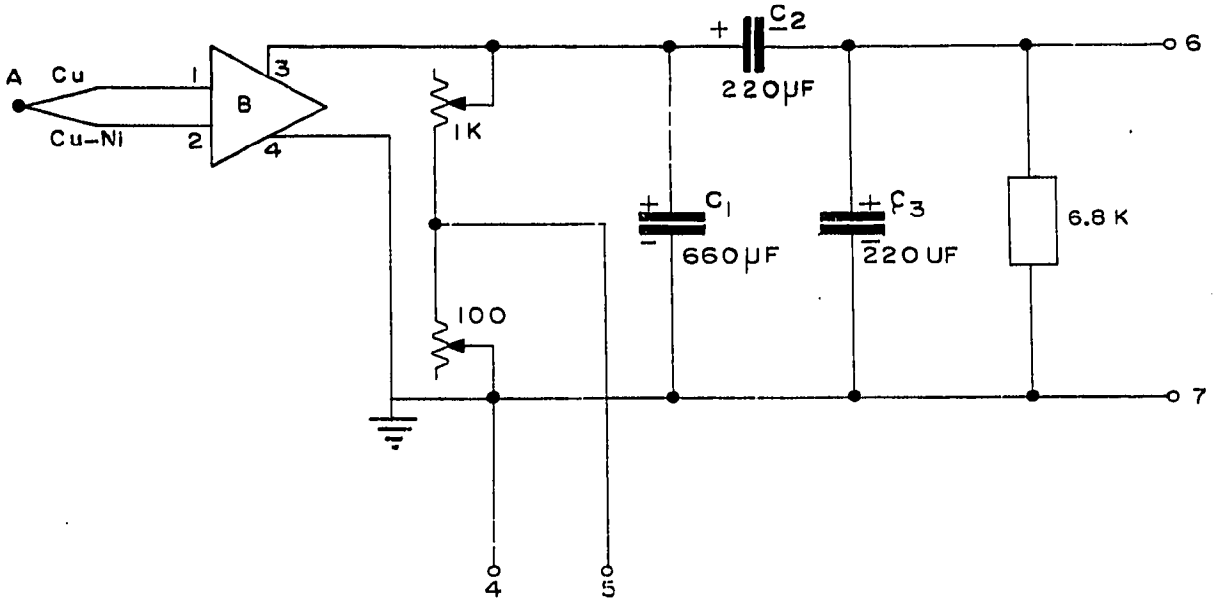

Fig. 10. Differentiator. For further explanation see text.

This diameter must be smaller than the diameter of the non-gassing electrode, to be sure that this electrode fits in the perspex electrode compartment. The diameter of the boring must not be too small; otherwise the contact surface of electrode and liquid is too small. The tap (Fig. 6.2) is also a teflon-lined Hamilton valve with a 90° connection.

Fig. 7 shows a block diagram of the isotachophoresis equipment. The apparatus for counterflow is described[3,6]. The level control unit (Fig. 7.6) must only be used if the analysis is done in an open system such as analysis with counterflow of electrolyte.

More practical data can be found in refs. 7, 8 and 10.

The temperature control unit

The proportional temperature controller[4] used in the thermostat is based on the relatively high temperature coefficient of a Pt resistor. Used is a Pt resistor of 100 Ω (0°). This Pt resistor forms an a.c. bridge (R_5) together with the resistors R_1, R_2, R_3 and R_4. The temperature coefficients of all resistors in the a.c. bridge, apart from the resistor R_5, must be as small as possible. The smaller the values are, the more accurate the thermostat control will be. Of course one of the resistors of the a.c. bridge is a variable one, to allow for balancing the a.c. bridge. If the bridge is unbalanced, a signal, being the result of the unbalanced condition, will be fed to a preamplifier. The phase of this preamplified signal is dependent on the polarity of the unbalance of the bridge. The preamplifier generates a sinusoidal voltage and by a limitor this signal will be transformed into a symmetrical square wave.

By the very high amplification of the preamplifier and the limitor, the amplitude of the bridge voltage is transformed into a phase shifted square wave. The leading edge of this square wave is amplified and triggers two antiparallely connected thyristors. These thyristors control the amount of heat dissipated in a load, mounted in the direct neighborhood of the Pt resistor (Fig. 2).

If the bridge approaches its balanced condition, the output voltage of the bridge decreases. Due to this the phase shift of the thyristor trigger pulse will be reduced. The thyristor will trigger later and the heat produced in the load decreases too. A steady state will be the result.

The temperature can be selected by the variable resistor R_4 of the a.c. bridge, according to the formula:

$$T = 2.59\ \{R_4 - 0.5835\}°C, \text{ (temp. coef. } R_5 = 0.003916)$$

The table in Fig. 8 gives some suggestions. A R–C filter in front of the input of the preamplifier corrects the phase of the trigger pulse. The proportional band (system gain) can be changed by varying the a.c. voltage over the bridge. Uncorrect temperature regulation may result if the temperature coefficients of the resistors of the a.c. bridge are poor, leading to instabilities, if the thermal resistance between the load and the Pt resistor is large, or if the heat capacity of the object to be thermostated is too large.

Differentiator

Qualitative information is obtained from the signal given by the integral thermocouple. The quantitative information however is more easily and with more accuracy obtained from the signal of a differential thermocouple[1,2]. Alternatively the

differential can be taken electronically from the signal of an integral thermocouple. The difficulty in balancing a differential thermocouple as described[1,2] can be anticipated.

The frequency of the signals, however, is very low (5–20 Hz). Therefore a special arrangement must be made. Figs. 9 and 10 show possible electric circuits for differentiating[5]. The use of a Knick amplifier, type A, creates too much noise for using it in combination with the circuit of Fig. 9. Better results with respect to the signal-to-noise ratio are found with the circuit of Fig. 10.

Tantalum capacitors are used because they diminish the signal-to-noise ratio to negligible proportions. Fig. 11 shows two electropherograms of identical mixtures. The electropherogram on the left-hand side (A) is made with the differentiator of Fig. 10. The electropherogram on the right-hand side (B) is made with a differential thermocouple, but this thermocouple is badly balanced[2].

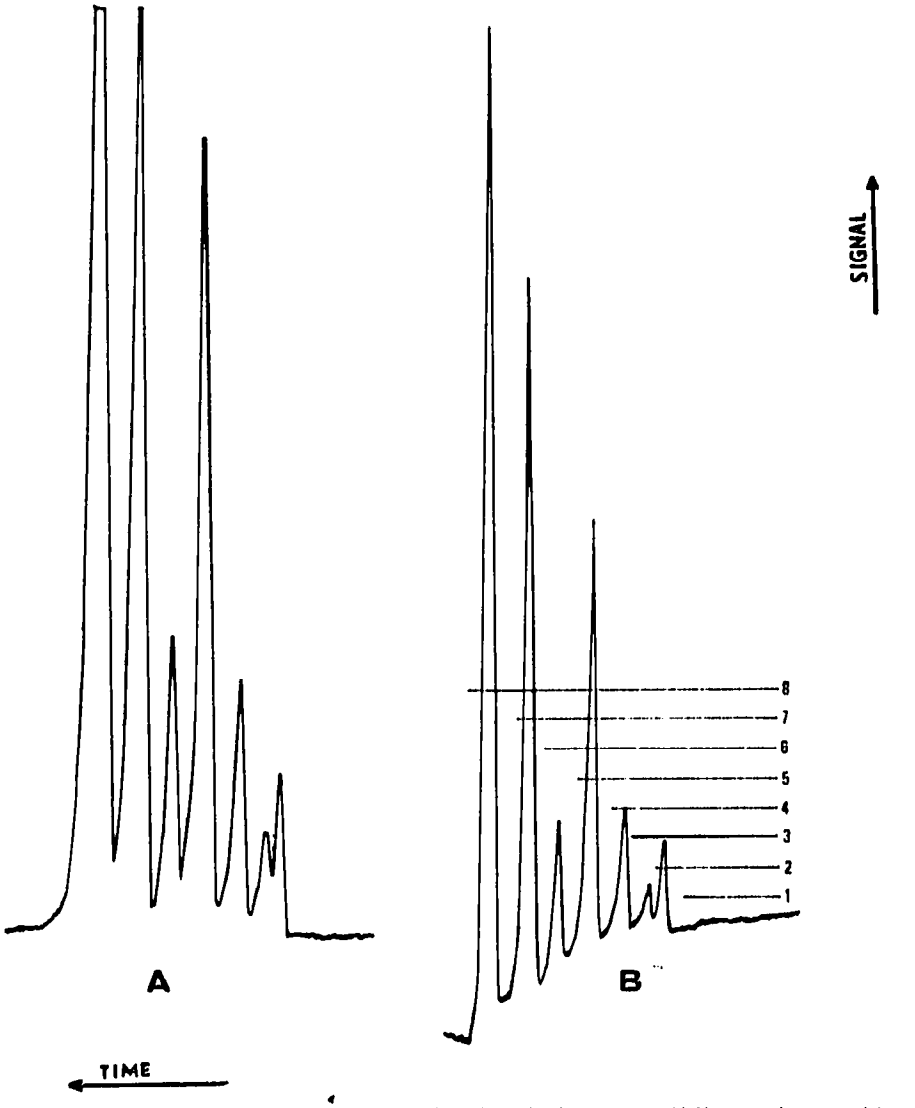

Fig. 11. Electropherogram obtained from a differentiator (A) and from a differential thermocouple (B), badly balanced. 1 = chloride; 2 = sulfate; 3 = oxalate; 4 = formate; 5 = citrate; 6 = adipate; 7 = acetate; 8 = glutamate.

The conditions for the analysis were as follows. The leading electrolyte was histidine (0.01 *M*) and histidine · HCl (0.01 *M*). The terminal electrolyte was glutamic acid (0.01 *M*). Injected was a mixture of sulfate, oxalate, formate, citrate, adipate and acetate. A stabilized current of 70 μA was used. The initial voltage was about 4 kV and the final voltage was 20 kV. The system was thermostated at 18°. The velocity of the recorder paper was 30 cm/h (electropherogram A) and 12 in./h (electropherogram B). Fig. 11 shows clearly that the peaks obtained from the differential thermocouple are smaller than those made by the differentiator. Making the signal quicker by a factor 3 means enlarging the noise by a factor 7 (ref. 5). The use of a differential thermocouple still has an advantage. If a hot zone travels in the opposite direction, the integral thermocouple registers this zone in the normal way and a positive peak is the result by using a differentiator. By the use of a differential thermocouple a negative peak or dip will be seen. The same effect can be obtained if two integral thermocouples, close together, are used.

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