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APPARATUS AND METHODS FOR HORIZONTAL HIGH-VOLTAGE ELECTROPHORESIS ON PAPER WITH UNILATERAL COOLING, OPERATING UP TO 170 $V \cdot cm^{-1}$ AND 0.6 $W \cdot cm^{-2}$

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SUMMARY

The construction is described of an apparatus for horizontal paper electrophoresis with a solid water-cooled heat exchanger. The apparatus is suitable for rapid and safe routine work with paper sheets of larger dimensions at high potential gradients. The temperature of the paper can be measured and the separation process can be followed visually. The method of simultaneous multiple two-dimensional separation and the procedure of sample transfer by the printing technique are described.

INTRODUCTION

Electrophoretic apparatus for high potential gradients with solid heat exchangers are among the conventionally used instruments the most convenient ones if both a high efficiency of heat transfer and also the possibility of a rapid change in experimental conditions, *e.g.*, of the buffer composition, are required.

Experience with existing equipment with solid heat exchangers in heavy-duty operation, which is usual in the chemistry of proteins and peptides, has shown that such apparatus, when complete, satisfies the requirements for the safety of the operator, rapid and simple handling, adequate capacity, and optical control and reliable work at very high potential gradients; however, the individual construction types[1–6] usually do not satisfy all the requirements.

The highest efficiency of heat transfer and versatility in repeated electrophoretic separations have been achieved with the equipment designed by Gross[5], which has a metallic two-sided heat exchanger. The requirements for visual control during the process are satisfied by instruments with unilateral cooling, *e.g.*, the type with a glass heat exchanger described by Wieland and Pfleiderer[2]. However, the high thermal inertia of the latter instrument decreases its suitability for rapid operation. The apparatus designed by Prusík and Keil[4] enables more intensive heat exchange to be achieved and thus a more rapid operation by active circulation of the heat-transferring liquid.

The safety of work at high potential gradients is especially important when the instruments are operated by many workers, including those with only a short training. It is necessary that the individual modifications to the experimental conditions should not lead to extensive damage of the apparatus and that the operation should be controlled automatically as much as possible. Any disturbance of the temperature equilibrium of the paper at potential gradients of 150–200 $V \cdot cm^{-1}$ and current densities of 6–8.5 $mA \cdot cm^{-1}$ will manifest itself by an avalanche process, which is difficult to stop sufficiently rapidly by human action.

An effort has been made in this study to combine the principles found to be dependable by us and other workers and thus to obtain a reliable instrument, which is easy to handle, providing maximal cooling efficiency, the possibility of visual control of the electrophoretic separation and also sufficient space for work with all conventional sizes of paper.

CONSTRUCTION AND FUNCTION OF THE INSTRUMENT

The apparatus consists of a high-voltage supply unit, an electrophoretic chamber and control and safety elements.

High-voltage supply unit

The direct current potential is supplied by a high-voltage source that can be controlled in the range 0–10 kV with three-phase feeding for a maximal load of 500 mA of the high-voltage circuit. The voltage can be controlled continuously by a thyristor circuit. The instrument, a product of the Instrument Development Workshop of the Czechoslovak Academy of Sciences, Prague, is not equipped with a filter circuit in the high-voltage circuit, as filtration of the current is not absolutely necessary for paper electrophoresis. The instrument is therefore convenient from the viewpoint of safe operation; after it has been switched off, even without an outside ohmic load, the residual potential of the high-voltage circuit drops almost instantaneously to zero owing to the negligible capacities. The overcurrent circuit breaker, which can be adjusted stepwise by 100 mA, represents, in combination with a suitable current that is chosen depending on the type of paper and the conductivity of the buffer, a rapidly reacting protection against overloading the temperature of the paper and at the same time also protects the supply against the consequences of a possible short-circuit in the high-voltage circuit.

Electrophoretic chamber

The dimensions of the apparatus were chosen so that the size of the cooled area would correspond to the classical dimensions of chromatographic paper of the Whatman type, *i.e.*, width 46.4 cm and length 57.2 cm. The electric insulation of the apparatus is designed so that it might exclude stress caused by peak pulses occurring with the high-voltage supply at an effective potential gradient of 5 kV and even as high as 11 kV.

The electrophoretic chamber (tray), the longitudinal section of which is shown in Fig. 1, consists of the base plate with the cooling system, the compression cover and the electrode vessels. The functional area of the base plate is made of brass sheet covered by polyethylene foil *ca.* 0.4 mm thick. This foil transfers the heat

from the electrophoretic carrier and electrically insulates the carrier from the base plate. The insulating foil is fixed by a solid packing piece on the anode side whereas on the cathode side it is held in place along its entire width by a special spanning mechanism. The polyethylene insulating foil juts 3 cm over the side walls, which will be described below.

Fig. 1 shows the arrangement of the partitions in the electrode vessels and the differences in the accommodation of the vessels. The anode vessel rests on an insulating plate of Umaplex (polymethyl methacrylate). Fig. 1 also shows the cover, adjusted by hinges and provided with a polyethylene bag filled with air. The bag is used for reproducibly pressing the paper carrier to the insulating foil; it also serves as a protection against the incorrect arrangement of the electrode wicks and membranes. The attachment of the bag to the cover and the arrangement of the electrode wicks and membranes is illustrated in Fig. 2. The cover of the apparatus is made of Umaplex sheets glued together and fixed by metal stiffeners made of stainless steel, which decrease the deformation resulting from the air pressure in the bag. The hinges permit the cover to be lifted to the left-hand side. The

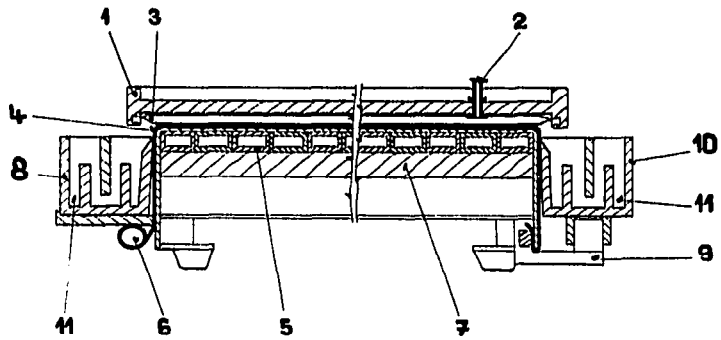

Fig. 1. Longitudinal cross-section of apparatus. 1 = Compression cover; 2 = pressure air inlet of polyethylene bag; 3 = polyethylene bag; 4 = polyethylene insulating foil, 0.4 mm thick; 5 = tray with base plate and cooling system; 6 = mechanism spanning the insulating foil; 7 = thermal insulation; 8 = cathode vessel, made of Plexiglass; 9 = insulator of anode vessel; 10 = anode vessel, made of Plexiglass; 11 = platinum electrode.

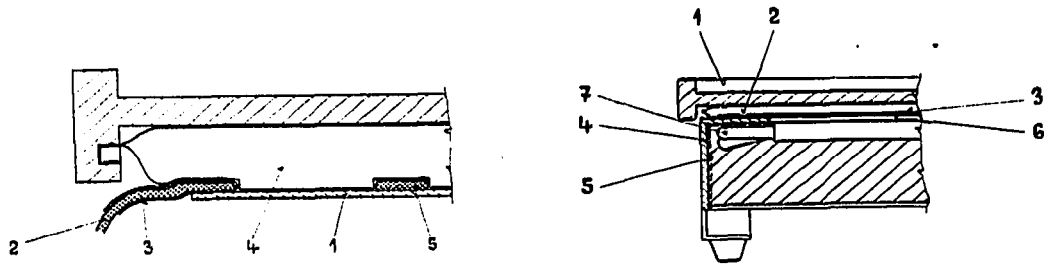

Fig. 2. Arrangement of bag, electrode wicks and membranes. 1 = Electrophoretic carrier (paper); 2 = paper wick conducting electric current from the vessel; 3 = semipermeable membrane; 4 = polyethylene bag; 5 = paper band with sample.

Fig. 3. Partial cross-section of apparatus. 1 = lifted cover; 2 = polyethylene bag filled with air; 3 = polyethylene insulating foil; 4 = insulating side wall (Umaplex); 5 = conductive shading coating; 6 = cooled plate; 7 = connection elements of the cooling system.

cover is protected by two locks against lifting of the bag by the compressed air. The side walls of the electrophoretic tray, shown in Fig. 3, are made of Umaplex. Their inner side is coated with a conductive layer, which is connected conductively with the earthed base plate so that the possibility of an electric discharge occurring between the edge of the paper and the metal parts of the tray is lessened.

The area of the base plate which is in contact with the foil is coated with a thin layer of silicone grease so as to decrease the risk of puncture of the polyethylene foil by the high voltage and to ensure a reliable thermal contact between the foil and the base plate. The gaps along the edges of the polyethylene insulating foil are also filled with silicone grease. The possibility of the leakage of current through sites that have become conductive because of the condensation of vapour during the electrophoretic process is thus prevented.

The removal of thermal energy, *i.e.*, of the Joule heat released, is effected by tap water. The cooling system of copper tubes of rectangular section is brazed to the base plate from below and the direction of the flow of water is perpendicular to the electric field. This arrangement is important for the elimination of the residual thermal gradient. A schematic representation of the arrangement is shown in Fig. 4. The maximal cooling efficiency requires a minimal flow-rate of 8–10 l · min $^{-1}$. The thermal insulation of the bottom part of the cooling system prevents condensation of water from the air in the apparatus.

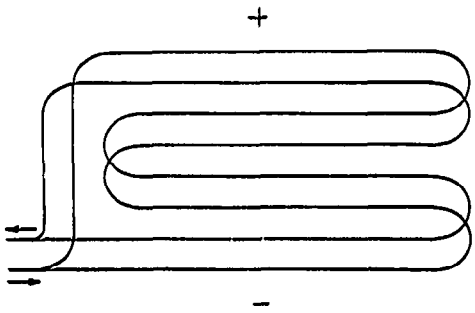

Fig. 4. Scheme of arrangement of cooling channels in the electrophoretic apparatus. The direction of the water flow is marked by arrows.

Control and safety element

The control of the apparatus, except the control of the high-voltage supply unit, is semi-automatic and is effected by a manual four-pole switch (positions 0–3). This switch controls the supply of water, compressed air and of current for the high-voltage supply unit.

The flow of water is controlled by an electromagnetic valve and regulated by a capillary thermostat. When the flow of cooling water decreases during the experiment or when the temperature of the cooling water is higher than that required at the beginning of the experiment, the high-voltage unit is disconnected from operation. The thermostat sensor is placed among the cooling tubes in the electrophoretic tray.

The pressure control disconnects the high-voltage unit until the air pressure in the bag attains the pre-set value, *i.e.*, approximately 0.05 atm. The sparking, which may occur near the membranes if the electrode wicks have been placed incorrectly on the paper carrier, leads to puncture of the thin polyethylene foil of which the bag is made. The pressure drop in the contact mercury manometer then cuts off the high-voltage supply.

A necessary condition for the reliable operation of the bag during electrophoresis is a very low flow-rate of the air through the pressure reducer set at a low pressure. The time necessary to inflate the bag can be reduced at the beginning of the experiment from 4 min to 20 sec by using a bypass valve, which is closed off automatically after the operating pressure has been achieved. The correct locking of the cover is controlled by a ferrite magnet placed in the side wall of the cover and by a reed relay contact inserted into the side wall of the apparatus. After the cover has been lifted from the horizontal position, the circuit that controls the supply of compressed air is interrupted; the polyethylene bag is therefore protected against mechanical stress and also the experiment cannot be started without the correct pressing of the feed wicks to the electrophoretic carrier, *i.e.*, to the paper. A schematic diagram of the electric circuits, the cooling water circuit and the compressed air circuit is shown in Fig. 5.

The feeding of the high-voltage supply unit is controlled by a contactor whose coil is part of the safety circuits, including also the protective metallic net. When the net is elevated, the high-voltage supply is disconnected, and if the net is lifted even higher, it short-circuits the positive and negative poles of the electrode cable inlets. The operator is therefore protected against injury caused by touching live parts of the apparatus. A schematic diagram of the safety circuits is also shown in Fig. 5.

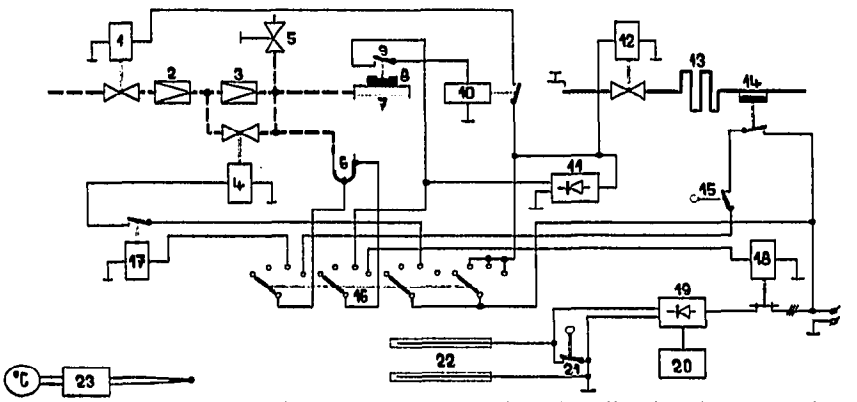

Fig. 5. Scheme of electric, air pressure and hydraulic circuits. 1 = Inlet electromagnetic air valve (solenoid); 2 = reducing valve, 0.1 atm; 3 = reducing valve, 0.05 atm; 4 = electromagnetic air valve (solenoid) for rapid inflation of the polyethylene bag; 5 = hand-operated air valve; 6 = mercury contact manometer, 0–50 torr; 7 = polyethylene bag; 8 = permanent magnet of the cover; 9 = reed contact controlling proper locking of cover; 10 = electromagnetic relay; 11 = rectifier, 24 V; 12 = electromagnetic water valve; 13 = cooling system of the apparatus; 14 = liquid thermostat; 15 = flash microswitch of protection circuit; 16 = function switch; 17 = electromagnetic relay controlling the rapid inflation of the polyethylene bag; 18 = high-voltage supply contactor; 19 = high-voltage supply unit, 0–10 kV; 20 = remote control of high voltage; 21 = short-circuit protection; 22 = electrode vessels; 23 = thermistor thermometer.

For safety reasons, the metallic parts of the apparatus are joined together and earthed; the negative pole of the high voltage is also earthed.

The apparatus is placed in the space formed by an earthed metal construction with metallic net walls; the live part of the apparatus is on the side that is more distant from the operator.

A bridge-connected thermistor temperature indicator is used for the direct measurement of the temperature of the paper carrier. As the galvanic connection between the measuring element and the paper carrier at high voltage must be avoided, a thermistor temperature probe with a glass insulating layer is used as the temperature sensor. After the spring mechanism has been released by a defined force, the thermistor is pressed against the polyethylene foil of the bag through which the temperature is measured. The location of the sensor in the cover (horizontal position) in contact with the paper carrier is shown in Fig. 6.

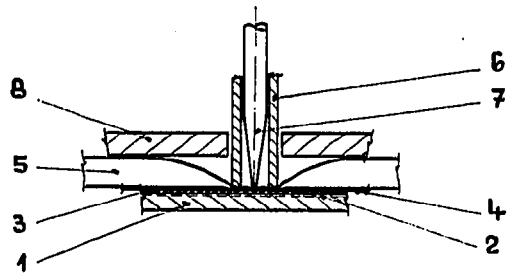

Fig. 6. Detail of thermistor temperature indicator. 1 = Cooled plate; 2 = silicone grease; 3 = polyethylene insulating foil; 4 = electrophoretic carrier (paper); 5 = polyethylene bag filled by air; 6 = thermistor indicator casing with tilting mechanism; 7 = thermistor sensor with its own compressing mechanism; 8 = lifted cover of apparatus.

Operation of the apparatus

The total time necessary for the manipulation of the electrophoretic carrier, including the application of the sample, is comparable with the time required for the electrophoretic separation itself, *e.g.*, in the separation of amino acids and peptides at pH 1.6 it is *ca.* 25–30 min. The entire process, including the manipulation, is completed in 50–60 min when a terminal voltage of 6 kV, *i.e.*, a potential gradient of *ca.* 100 $V \cdot cm^{-1}$, and Whatman No. 3 paper are used. The time necessary for the electrophoretic separation can be reduced to 15–20 min for analytical purposes by using thinner carriers, such as Whatman No. 1, 4 or 52 paper, and a terminal voltage of 10 kV (potential gradient 170 $V \cdot cm^{-1}$). A further reduction in the time necessary for the completion of the experiment depends predominantly on the skill of the operator during the preparation of the experiment.

If a larger number of samples (peptide mixtures) are to be characterized by the combination of electrophoresis and chromatography, the area of the paper carrier is divided into nine 15×15 cm squares that are symmetrical with respect to the centre of the paper. Individual samples are then applied at origins 2.5 cm from the anode side and 2.5 cm from the left-hand side of the square. The electrophoretic separation is carried out for 10 min under the conditions given later in the legend to Fig. 8 and at a terminal voltage of 6 kV. After the electropherogram has been dried, it is cut into bands of three squares each from the anode to the

cathode. The bands are then developed by ascending chromatography (see the legend to Fig. 8 for the composition of the chromatographic system). The coloured indicator, mono(dinitrophenyl)ethylenediamine serves as the internal standard and is mixed with the sample; it does not interfere with the positions of the naturally occurring amino acids. The time necessary for one-dimensional separation is in this instance comparable with the time required for separation by thin-layer techniques.

Procedure

(1) Cool the apparatus — set the function switch at position 1.

(2) Place the electrophoretic carrier and feed wicks and membranes.

(3) Moisten the carrier and feed wicks with buffer.

(4) Absorb the carrier with filter-paper, using a rubber roller carefully pressed against the filter-paper until the paper carrier has become uniformly white.

(5) Apply the sample (a) as a solution directly from a microcapillary tube or (b) by placing a dry paper band cut from a chromatogram or electropherogram (*cf.* Fig. 2); apply at coloured indicator.

(6) Place the blotted feed wicks with membranes in place.

(7) Lower and lock the apparatus cover; turn off the outlet valve in the compressed air line and set the function switch at positon 2 so as to rapidly inflate the bag.

(8) Pull down the protective net.

(9) After operating pressure in the bag has been indicated, set the function switch at position 3 and select the required high voltage.

(10) Allow the separation to proceed for the time required, either according to the path length travelled by the coloured indicator or until the contactor of the high-voltage supply unit has been switched off by the timer; set the function switch at position 0.

(11) Lift the protective net and open the outlet valve on the compressed air line.

(12) Unlock and lift the cover and remove the feed wicks and paper carrier.

RESULTS AND DISCUSSION

The apparatus described has been used intensively for 2 years in the Department of Protein Chemistry in this institute. The maintenance of the apparatus involves occasional replacement of the polyethylene bag, which is made of 0.1 mm thick polyethylene foil. Any leakage through the foil, for example as a result of microscopic perforations caused by sparks, is indicated by a decrease in the air pressure in the bag. Small holes can be repaired temporarily with adhesive tape during the experiment. The replacement of the deflated bag with a new bag, which can be easily prepared by welding polyethylene tubing, takes *ca.* 5 min. If, however, the foil covering the base plate is damaged as a result of mechanical abuse, *e.g.*, by scratching, the foil must be replaced without any temporary repairs. The quality and uniformity of the thickness of the insulating, heat-transferring polyethylene foil is the main factor that governs the uniformity of the separation. In spite of the fact that the thickness of the polyethylene foil used in our laboratory varies to the extent of $\pm$ 5%, the effect of the uneven heat transfer can be limited by correct

orientation of the foil. The foil must be placed so that the direction in which the foil was drawn in the machine during manufacture is perpendicular to the direction of the electric field in the apparatus. Table I shows why polyethylene has been chosen as the most appropriate electrical insulating and heat-transferring material[7–9] for the insulating foil.

TABLE I

THERMAL CONDUCTIVITY AND INSULATING STRENGTH OF DIFFERENT INSULATING MATERIALS

Type of insulating material	*Thermal conductivity* ($kcal \cdot m^{-1} \cdot h^{-1} \cdot °C^{-1}$)	*Insulating strength* ($kV \cdot mm^{-1}$)
Polyethylene	0.26–0.30	100
Polymethylmethacrylate	0.16	40
Polyvinyl chloride	0.14	40
Silicone rubber	0.13	20–30
Glass	0.61–1.19	12–20

The thermal conductivity of polyethylene, although approximately twice as high as those of polymethyl methacrylate, polyvinyl chloride and silicone rubber, is still three to five times lower than that of glass; the high electrical insulating and mechanical strengths, however, are factors that make polyethylene preferable to glass[6]. Likewise, the surface water absorptivity of polyethylene is negligibly low,

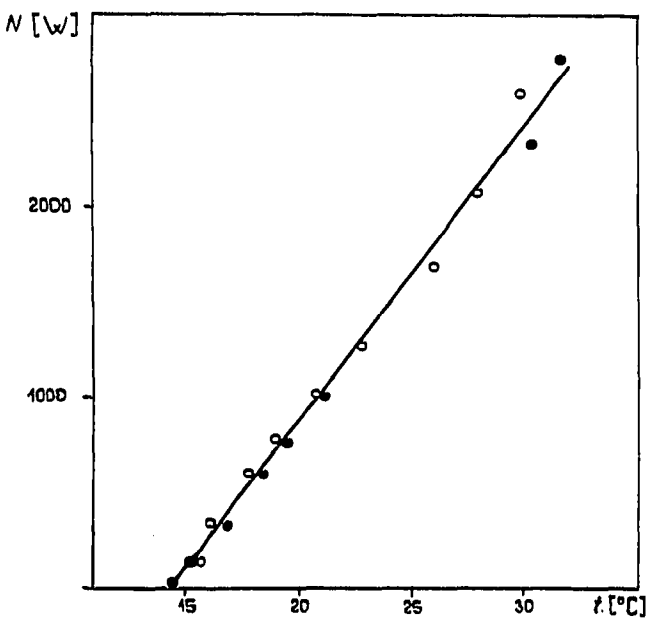

Fig. 7. Temperature (t, °C) of electrophoretic carrier (Whatman No. 3 paper) as a function of the supplied Joule heat (N, W).

similar to that of silicone rubber, whereas that of polyvinyl chloride is about 20 mg per 100 cm^2 per 7 days and that of polymethyl methacrylate is about 75–100 mg per 100 cm^2 per 7 days. The insulating parts of the apparatus, which must be machine-cut, can be made of polyvinyl chloride; polymethyl methacrylate is, however, more convenient for the construction of parts under voltage stress. Surface discharge leads to the charring of the polyvinyl chloride sheets and to an avalanche-like formation of conductive paths; in contrast, polymethyl methacrylate depolymerizes and evaporates during the discharge and no conductive, carbon-rich surface layer is formed instantaneously. For this reason, we have replaced the original polyvinyl chloride vessels with Plexiglass cuvettes.

Loads of the electrophoretic carrier exceeding an output of 2,400 W (over 0.90 $W \cdot cm^{-2}$) lead to softening and to local irreversible deformations of the polyethylene bag. If 25° is chosen as the upper permissible temperature limit of the electrophoretic carrier, then with cooling water at 12–14° and Whatman No. 3 or No. 3MM paper, the total usable output of Joule heat is about 1,500 W, *i.e.*, *ca.*

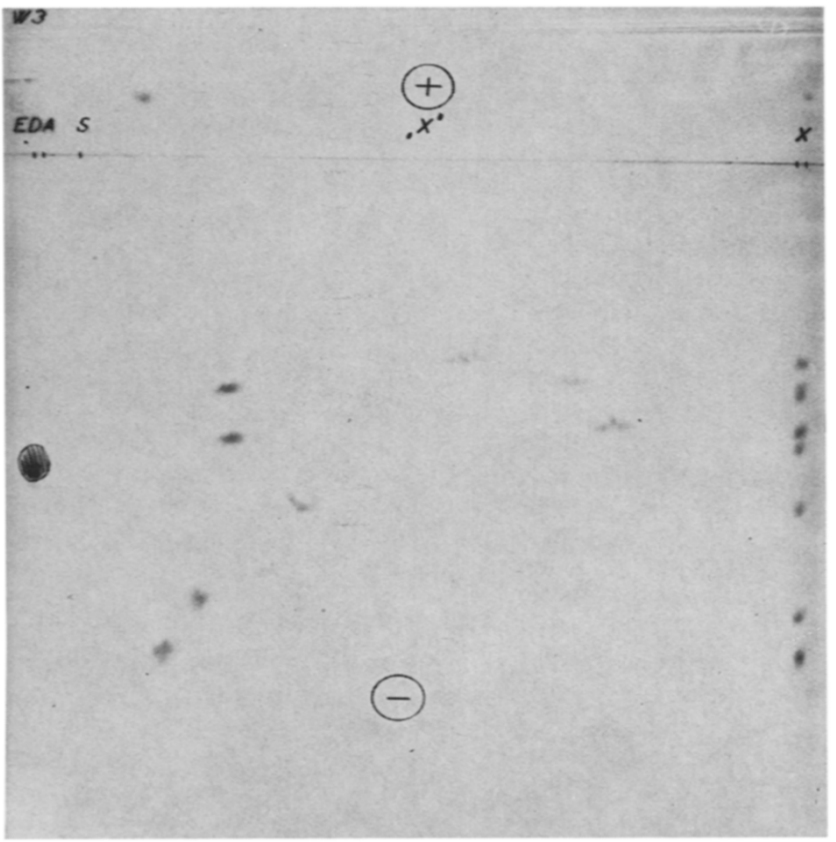

Fig. 8. Two-dimensional separation of amino acid mixture. Sample applied by the printing technique. Horizontally: descending chromatography in the system *n*-butanol–pyridine–acetic acid–water (185:125:40:150, by vol.). Vertically: electrophoresis in the system acetic acid–formic acid–water (15:5:80, by vol.). Amino acid mixture: $CySO_3H$, Lys, Arg, Tyr, Phe, Ala, Glu, Ser, Leu; on the left, mono(dinitrophenyl)ethylenediamine (EDA); S = starting point.

0.57 $W \cdot cm^{-2}$. The value of 0.57 $W \cdot cm^{-2}$ for unilateral cooling is in good agreement with the value of 1 $W \cdot cm^{-2}$ reported by Gross[5] as the limiting value for two-sided cooling. It can be seen from Fig. 7 that a greater variation in the values of the measured temperatures of the carrier can be observed at higher carrier temperatures. This variation can be ascribed both to heat transfer to the air surrounding the thermistor and also to the non-uniform thickness of the paper carrier. Thicker papers, such as Whatman No. 31 Extra Thick, are not convenient for instruments with unilateral cooling because of their high output load. The difference in temperatures in the paper carrier layer is too great, which causes a diffuse broadening of zones and in extreme cases can even affect the mechanical strength of the polyethylene bag.

Coloured indicators were used to advantage to mark the migration. Three indicators, picric acid, ε-dinitrophenyllysine, and mono(dinitrophenyl)ethylenediamine, which cover the required anodic and cathodic range of electrophoretic mobilities, were found to be useful markers for the separation of amino acids and peptides. The distinct yellow colour of these compounds is also easily visible through the polyethylene bag of the locked cover during the experiment.

The application of the sample by transfer from a dry band is effective when the band paper has a thickness comparable with or even thinner than that of the carrier paper. As we have not observed tailing of amino acids or peptides between

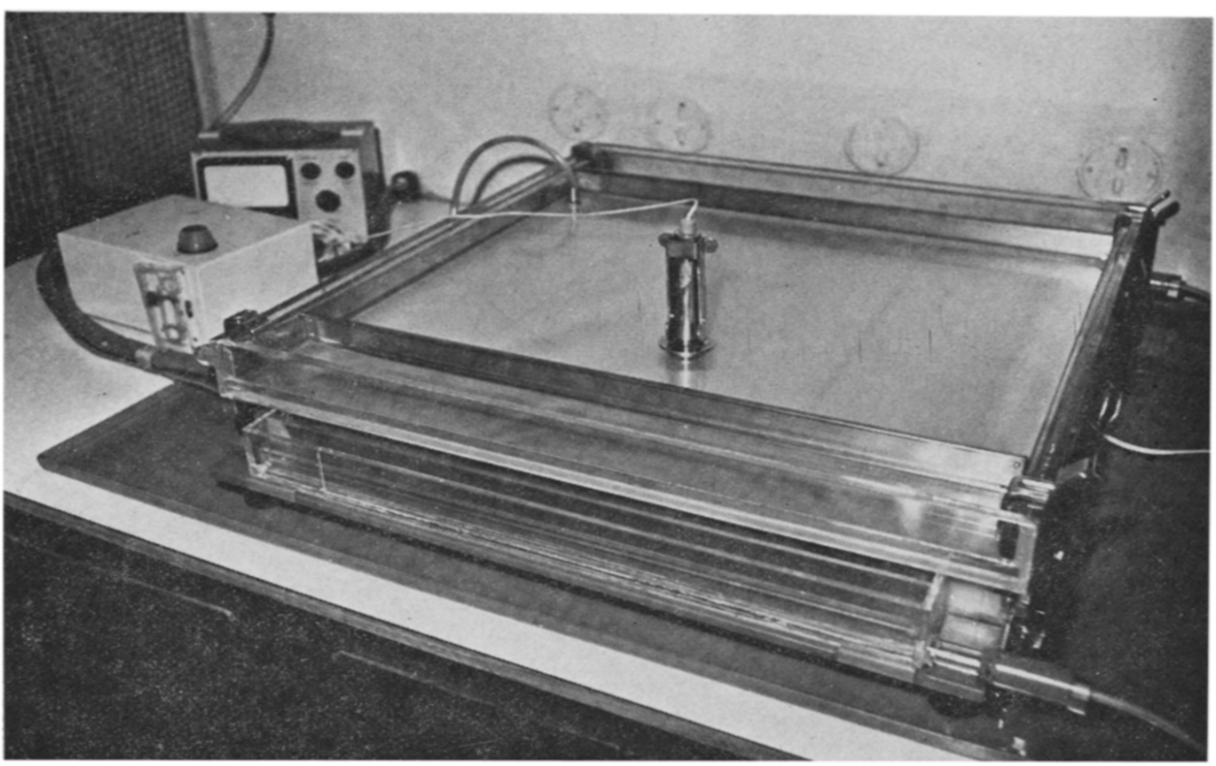

Fig. 9. Apparatus with locked cover. Near left, pressure reducer casing with mercury manometer and hand-controlled air valve; far left, temperature measuring device; middle, thermistor element.

their spots and the site of their application (Fig. 8), and as we have not found ninhydrin-positive material on the paper bands, we assume that any losses of the sample applied in preparative-scale experiments in this manner can be caused exclusively by non-specific sorption to the paper. The transfer of sample from the band is convenient for two-dimensional separations. The process is then more rapid as the entire width of the paper can be used in the first direction either for preparative purposes or for the separation of a series of samples. The samples from separate paper bands are then transferred to the electrophoretic carrier by the printing technique described above. This method was found to be useful for the preparation of comparative peptide maps (fingerprints) and for the determination of disulphide bonds by the diagonal technique.

The apparatus described (see Figs. 9 and 10) has also been used routinely for one-dimensional analytical and preparative separations of mixtures of oligopeptides prepared by enzymatic digestion.

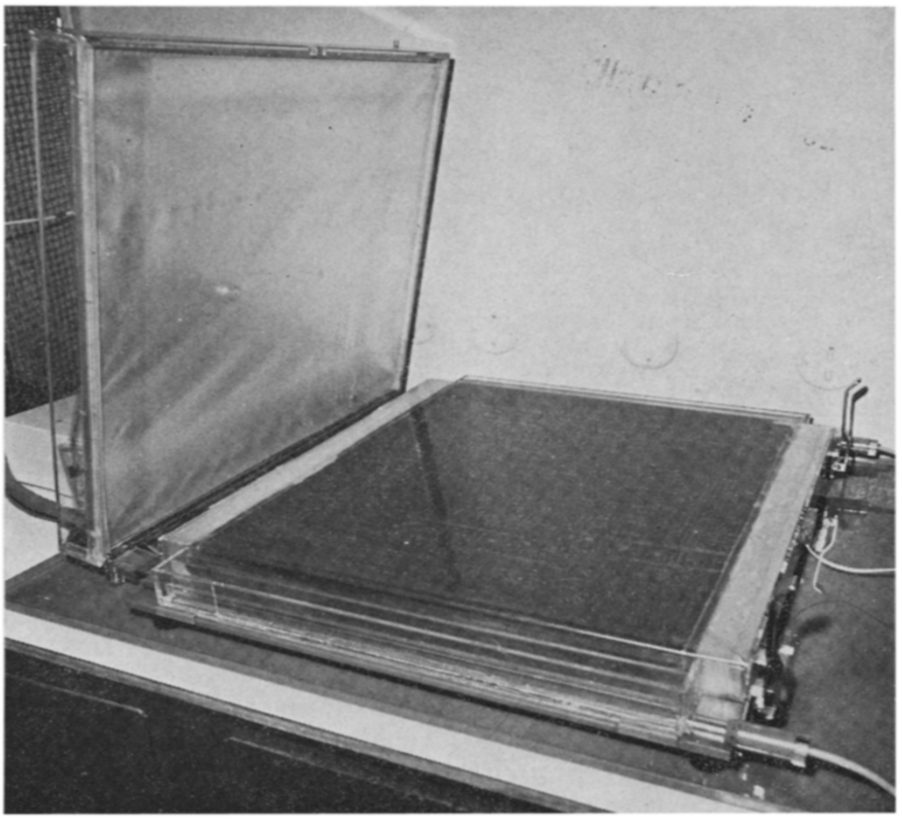

Fig. 10. Apparatus with lifted cover.

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