CHROM. 5577

HIGH-VOLTAGE ELECTROPHORESIS OF ENZYMES

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SUMMARY

Multiple enzymes may be separated by electrophoresis. Increasing the voltage results in a decrease in separation time, but since enzymes are sensitive to heat, the increase in voltage depends on the possibility of removing the heat produced. The isoenzymes of lactic dehydrogenase or malic dehydrogenase can be separated for preparative purposes in starch gel or polyacrylamide gel. If the gel is thoroughly cooled on three sides the temperature will not exceed 25° at 35 V/cm. For analytical separations cellulose acetate foil is very suitable for the electrophoresis. At 60 V/cm the isoenzymes of lactic dehydrogenase are separated by a distance of 8 cm within 75 min. The large surface area of the foil ensures removal of the heat produced, if the electrophoresis chamber is also suitable. An evaluation of the electrophoresis can be carried out by staining or kinetic measurements.

INTRODUCTION

Heat-stable substances of low molecular weight are often separated by high-voltage electrophoresis, but low-voltage electrophoresis is generally used for substances which are unstable to heat, because of the low heat production of this method. However, when high voltage is used most of the time it is kept below 20 V/cm when enzymes are to be separated. ROSALKI[1] separated the isoenzymes of creatinephosphokinase on cellulose acetate foil at a current density of 0.5 mA/cm width. SMITH AND RUTENBERG[2] were able to separate aminopeptidases at 0.4 mA/cm width in 3 h. The same current density was used by AW[3] for the separation of isoamylases. The voltage applied was 200 V. BARKA[4] separated acid phosphatases by disc electrophoresis at 20 V/cm. In 1959 WIELAND *et al.*[5] separated lactic dehydrogenase with a voltage of 30 V/cm and described a clear separation of the five zones.

EXPERIMENTAL AND DISCUSSION

Since the migratory rate depends on the voltage, higher voltages result in a shorter amount of time for the electrophoresis. Fig. 1 shows schematically the separation of malic dehydrogenase (MDH) on cellulose acetate foil in equal amounts of time (75 min) and with currents of increasing voltage. At 100 V, that is 7 V/cm, the distance between the bands is 0.7 cm; at 800 V the distance is 3.2 cm. The bands are

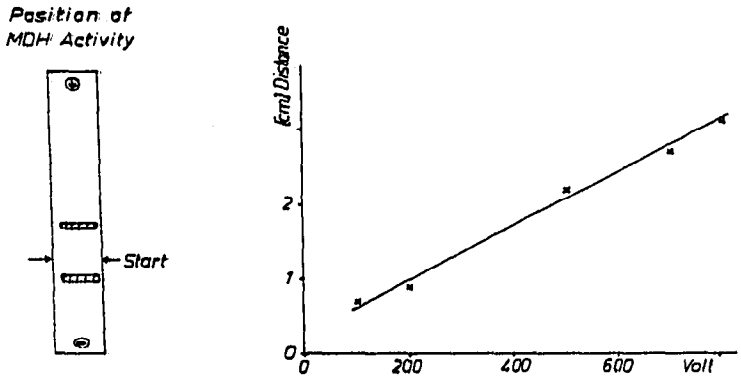


Fig. 1. Electrophoresis of malic dehydrogenase. Dependence of the migratory rate of the isoenzymes on the voltage (period of time, 75 min).

sharply defined without any tailing. Generally the enzymes would migrate only towards the anode under the conditions given, but since the foil has a negative charge which is unable to migrate to the anode the H_3O^+ ions formed migrate towards the cathode. This electroendoosmosis causes the apparent migration of MDH-II towards the cathode.

Any increase in voltage during the electrophoresis is dependent on the feasibility of removing the heat produced. For gel electrophoresis we developed the piece of equipment shown in Fig. 2, which permits good removal of heat. It consists of a PVC

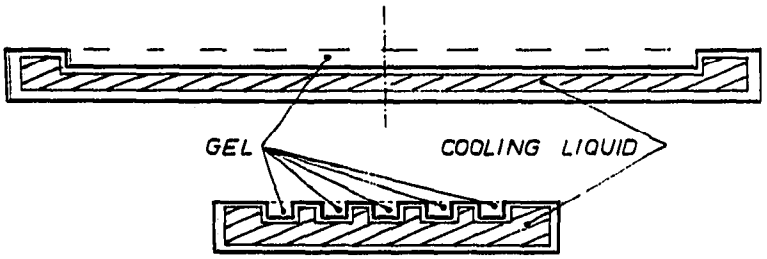
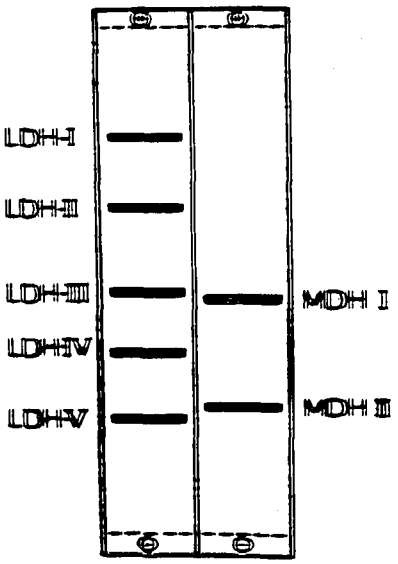


Fig. 2. Diagram of the equipment for gel electrophoresis.

plate with canals 10 mm in width and 7 mm deep for the gel, for example starch or polyacrylamide gel. The canals are thoroughly cooled on three sides. If the cooling agent is −4°, the temperature of the gel will not exceed 25° at 35 V/cm and a current density of 7 mA/cm². The ionic strength of the system was 0.03. The electrode vessels of the Camag chamber (Camag, Muttenz, Switzerland) were used for the electrophoresis, the current being conducted by cotton strips saturated with buffer. The gels were covered by parafilm to prevent evaporation. This equipment is very suitable for preparative electrophoresis. However, the use of this technique for quantitative analysis is not practical because of unsatisfactory elution of the enzyme proteins. This equipment can also be utilized for the electrophoresis of thin tissue slices. The slices are placed between sheets of filter paper 7 × 10 mm, which are inserted in the gel. This produces the same pattern as in normal electrophoresis. The positions and intensities of the enzyme activities are marked by a specific tetrazolium staining in the usual manner. For that purpose, the gel is lifted



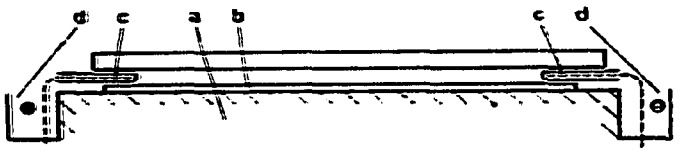


Fig. 3. Positions of the isoenzymes of LDH and MDH after electrophoresis.

Fig. 4. Diagram of the equipment used for electrophoresis on cellulose acetate. a = Cooled plate; b = cellulose acetate foil; c = cotton strips; d = electrode vessels.

up by pushing a wedged PVC plate under it, and then cut by a nylon thread and stained. Fig. 3 shows schematically the position of the bands of lactic dehydrogenase (LDH) and of malic dehydrogenase in the gel. When tissue slices are used instead of extracts it can be shown that the bands are sharply separated without any tailing.

For quantitative analytical purposes electrophoresis on cellulose acetate foil proved valuable. The electrophoresis chamber used was from Camag (Fig. 4). For the experiments in our laboratory we used strips of foil 170 × 25 mm (Cello gel from Chemetron, Milan, Italy) which were saturated with buffer. 10–20 μl of the sample were applied by a sample applicator (Beckman Instruments Inc.) 5 cm from the cathode end. The salts were not first removed by dialysis because salt ions migrate fast in electrophoresis. An advantage of the Cello gel foils is that their lower side is impermeable. Thus the substances applied remain within the gel and do not diffuse into the layer of water condensing on the chamber surface.

The prepared foils are put into the electrophoresis chamber. The ends of the foils are covered by cotton strips which dip into the electrode vessels. In order to prevent electroendoosmosis these cotton strips are placed inside a dialysis tube. The buffer in the electrode vessels is a 0.06 *M* barbiturate buffer of pH 8.6. The cellulose acetate foils are covered by a glass plate lying on the cotton strips. The distance between the electrophoresis foil and the glass plate is about 1 mm. The advantage of using a glass plate instead of a polyethylene sheet for covering is that the gel surface is not touched. Thus, smearing of the bands due to capillary action between the foil and the polyethylene sheet is prevented. The glass plate is pressed to the cotton strips by an inflated polyethylene bag. This ensures even contact between the cotton strips and the cellulose acetate foil. The electrophoresis chamber is cooled by a rapidly circulating mixture of methanol–water (1:1) at 0°. In this way the heat produced by a voltage of 800 V and a current density of 1 mA/cm width is eliminated. We have been using the Camag chamber for over four years without it wearing out, but we are careful to avoid drastic temperature changes by cooling or warming the chamber gradually.

The following should be pointed out, *viz.* that there is no damage of the enzymes due to heat. The isoenzymes of lactic dehydrogenase have different stabilities to heat. LDH-I is stable at 55° for a long period of time, but at this temperature LDH-V loses half its activity within 5 min (ref. 6). Damage by heat would cause a relative increase of LDH-I and a relative decrease of LDH-V. Our results, however, after examining rat tissues correspond exactly with those found by GOODFRIEND AND KAPLAN[7] with rats of the same strain. They obtained their results by a completely different method, namely by kinetic measurements with the help of NADH-analogs. This excludes the possibility of damage to the enzyme by the high-voltage electrophoresis used.

The most suitable period of time for the electrophoresis is 75 min. In this space of time the isoenzymes of lactic dehydrogenase have been separated by a distance of 8 cm and those of malic dehydrogenase by a distance of 3 cm. After electrophoresis the zones of enzymatic activity are made visible. For the qualitative evaluation the foils may be stained with a solution containing substrate, NAD, tetrazolium salt and phenazine methosulphate. A second strip of gel is impregnated with staining solution and pressed to the electrophoresis strip. After incubation for 15 to 120 min at 37° the zones of enzymatic activity are stained a blue colour by formation of insoluble formazan.

A more useful method, however, is that cited by PFLEIDERER AND WACHSMUTH[8], which is applicable to enzymes measurable by an optical test. The method is advantageous because the positions of the enzymatic bands are made visible quickly, after which the enzymatic activity can be measured by the optical test. After electrophoresis, a buffered solution of substrate and NADH is sprayed on the electrophoresis strip. In UV light NADH is highly absorbent and shows a distinct fluorescence. The foil appears light in colour. Where there is enzymatic activity, NADH is transformed to NAD and the fluorescence disappears. The bands of enzymatic activity are visible as dark zones.

This method is very sensitive. Longer times of incubation make it possible to detect zones of little activity. For less stable substrates the time of incubation is limited. If *e.g.* malic dehydrogenase is traced, oxaloacetate is used. Oxaloacetate spontaneously decarboxylates and pyruvate is formed; this now becomes the substrate of lactic dehydrogenase. As a result of this, the zones of lactic dehydrogenase activity become visible after a period of time. Usually this reaction does not disturb the detection of malic dehydrogenase. After marking the positions of the enzymes it is very easy to elute the enzyme protein from the foil. The enzymatic activity is measurable in solution now. The enzyme pattern can be estimated even if very little tissue is available. In these cases the tissue must be ground and the pulp is applied to the cellulose acetate foil by a sample applicator. By this method again we find that after the above-mentioned electrophoresis sharply separated zones without any tailing occur. The enzyme pattern emerges after measuring the zonal activities.

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