IMMUNOISOTACHOPHORESIS ON CELLULOSE ACETATE FILMS

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Cellogel cellulose acetate films are shown to be a suitable supporting medium for isotachophoresis. Antigen in a volume of 0.5 to 20 μl is applied for this purpose to a strip of cellulose acetate film. In front of the antigen zone 1-2 μl of Ampholine is placed. All components on the film are made up in Tris-HCl buffer, pH 6.7, and the electrode buffer consists of Tris-glycine buffer, pH 8.3. A moving Kohlrausch boundary is formed during electrophoresis, at which concentration of the antigen takes place initially in the narrow start zone, and this is followed by separation of the antigens by means of ampholytes. Antigens separated on the Cellogel strip are subjected to cross-electrophoresis on film saturated with the corresponding antiserum, with the formation of precipitation peaks for each individual antigen. With this method it is possible to work with low concentrations of antigens, for they undergo preliminary concentration during electrophoresis and the width of the zones is independent of the duration of fractionation.

KEY WORDS: isotachophoresis; immunoisotachophoresis; cross-immunoelectrophoresis.

Isotachophoresis [2] and isoelectric focusing [4] are methods of electrophoretic fractionation of proteins which have the highest resolving power. In isotachophoresis fractionation of proteins is carried out in a nonhomogeneous buffer system, in the zone of ampholytes migrating in the electrophoretic field between the boundaries of the leading and closing ions.

Proteins with different electrophoretic mobility are separated from each other by "insertions" of amphoteric ions (ampholytes), which have intermediate mobility. Separation of proteins under these circumstances is determined entirely by the concentration of the ampholytes and by their electrophoretic mobility and is independent of the duration of electrophoresis after final distribution of the protein and ampholyte molecules [2]. In isotachophoresis, by contrast with ordinary electrophoresis, the migrating zones are not widened in the course of their separation.

Since isotachophoresis is carried out in a nonhomogeneous buffer medium, it can only take place in systems in which the effect of electroendo-osmosis is absent, for example, in a free solution stabilized by a sucrose density gradient or in polyacrylamide gel, in which electroendo-osmosis in general is absent. The writer showed previously that cellulose acetate films are an excellent supporting medium for electrophoresis in a continuous system of buffers and for concentrating various proteins on a moving Kohlrausch boundary [1]. Hence it was shown that isotachophoresis is possible, in principle, on cellulose acetate films.

In this paper a method of isotachophoretic fractionation of micro quantities of protein on cellulose acetate films, followed by their detection by cross-immunoelectrophoresis, as described by Laurell [3], is described.

In the first stage of electrophoresis the proteins are concentrated in the narrow start zone on the Kohlrausch boundary, after which they are fractionated isotachophoretically in a system of ampholytes (Ampholines). Electrophoresis is then carried out in a direction perpendicular to the first, on film saturated with antiserum against the test antigen. Each antigen forms a peak of precipitate, the area of which is proportional to the quantity of antigen in the sample (Fig. 1).

Gelatinized cellulose acetate films (Cellogel from Serva, West Germany) were used. Fractionation was carried out with the aid of Ampholines with pH ranges of 3.5-10, 4-6, and 5-7 (from LKB, Sweden).

Electrophoresis was carried out under a layer of mineral oil in the apparatus described earlier [1]. Two instruments are necessary for the analysis: one for electrophoresis in a nonhomogeneous buffer system, the

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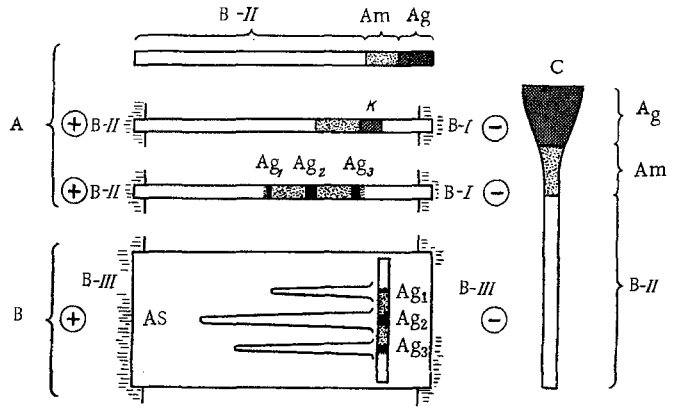

Fig. 1. Principle of the method. A) Charge, concentration, and separation of antigens: Ag) mixture of antigens $AG_{1,2,3}$, Am) mixture of ampholytes; B-I) Tris-glycine buffer, pH 8.3; B-II) Tris-HCl buffer, pH 6.7; K) concentration of antigens on Kohlrausch boundary. B) Cross-electrophoresis of separated antigens; B-III) Tris-HCl buffer, pH 8.3; AS) antiserum against $AG_{1,2,3}$; C) arrangement of components of isotachophoresis in the presence of large volumes of antigen.

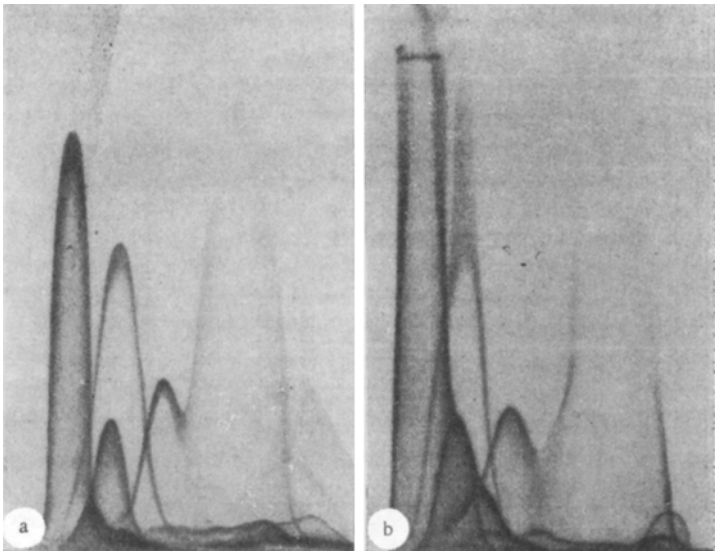

Fig. 2. Immunoisotachophoresis of blood serum proteins: a) normal mouse serum (1:10, 2 μl), Ampholine 3.5-10 (10% solution, 2 μl), antiserum 1:5; b) normal mouse serum (1:10, 2 μl), Ampholine 5-7 (8% solution, 2 μl), antiserum 1:5.

other for a homogeneous system. The cathodal part of the first instrument – the electrode vessel and agarose supports – contain 0.012M Tris-glycine buffer, pH 8.3 (buffer I), the anodal part contains 0.06M Tris-HCl buffer, pH 6.7 (buffer II); the second instrument contains 0.06M Tris-HCl buffer, pH 8.3, in both compartments (buffer III).

Human donors' serum, normal mouse serum, and rabbit immune sera against human (Institute of Forensic Medicine) and mouse serum proteins (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR) were used.

Intensification of the precipitation bands was obtained by treating the films with donkey immune serum against rabbit IgG (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR) or with complement obtained from freshly frozen guinea pig serum.

All dilutions of antigens and Ampholines were made up in buffer II, and of antisera in buffer III.

Isotachophoretic fractionation was carried out on a Cellogel film 2 mm wide and 90 mm long. The film, saturated with buffer II, was placed between strips of filter paper saturated with the same buffer, so that the cathodal edge, about 25 mm long, remained free. This edge was carefully dried with dry filter paper and 2 μl of antigen in a dilution of 1:10, stained with bromphenol blue (BPB) was applied to it. The antigen was allowed to soak into the film, and 2 μl of a 10% solution of Ampholine 3.5-10 or 2 μl of an 8% solution of Ampholine 5-7, diluted in buffer II, was introduced between the zones of antigen and buffer (Fig. 1A). The drops of antigen and Ampholine were spread over the film with a glass rod to ensure their even absorption and distribution in the film. The film was placed in an apparatus for electrophoresis in a continuous buffer system beneath a layer of mineral oil. The agarose feeders were first covered with strips of filter paper saturated with the corresponding buffer solutions. Similar strips were used to cover the edges of the film above in order to fix it and ensure reliable contact with the feeder. The cathodal border of the film, where the boundary was created between buffers II and I, was placed on the feeder with as narrow a zone as possible.

If a large volume of antigen, namely 20 μl or more, had to be applied to the film, should its concentration be low (for example, serum in dilutions of 1:100 or 1:500), the film was cut out so that it was wider at the cathodal end (Fig. 1C), thus increasing its capacity. An additional quantity of antigen could also be introduced into a dry strip of cellulose acetate film placed on the cathodal edge of the Cellogel strip. Electrophoresis was carried out with an initial voltage of 50 V, which was increased during migration of the separating proteins to 100 and 150 V.

During work with a rectifier stabilized for the strength of the current, the increase in voltage in the course of the experiment took place automatically.

At the beginning of the experiment rapid concentration of the proteins took place in the zone of the antigen in the narrow start zone, as shown clearly by the concentration of BPB on the moving Kohlrausch boundary. When the start zone entered the Ampholine zone, separation of the protein zones became clear with respect to the stained albumin and hemoglobin, which separated from the BPB band, which lagged behind the more rapidly migrating proteins on the Cellogel. When the BPB zone was approximately in the middle of the film electrophoresis was stopped and the part of the strip (4 cm) containing the separated protein fractions was transferred to the film with antiserum.

The film measuring 40 × 85 mm was first saturated with antiserum diluted in buffer III. The wet film was immersed vertically in a histological jar with mineral oil to allow excess of liquid to drain from it.

The strip of Cellogel was placed on the film with antiserum up to 20 mm away from its cathodal border (Fig. 1B). The strip was squeezed from below and above respectively by means of plastic and glass slabs, firmly pressed together by two plastic clamps. Under these circumstances the two films were covered with a layer of mineral oil to prevent them from drying during operations with them. The film was transferred to the second instrument for electrophoresis in a homogeneous buffer system. Electrophoresis was carried out overnight (15-18 h) with a voltage gradient of about 2 V/cm. The film was rinsed to remove unbound proteins and mineral oil in physiological saline, with stirring as described earlier [1], and then stained with Amido black or Coomassie R-250 by the usual methods (Fig. 2).

To intensify the precipitation bands the films were treated with antiserum against rabbit γ-globulin [1].

Very good results were given by treatment with complement, namely guinea pig serum diluted 1:7 in solution containing Ca^{++}, for 1 h at 37°C, followed by rinsing of the film. The combination of antiglobulin serum and complement intensified the strips by 4-6 times and made it possible to work in the "invisible" zone of the reaction. Autoradiographic detection of invisible precipitates on the film also is possible [1].

Depending on the concrete conditions, the method described above admits of various modifications. For instance, the Ampholine can be mixed with the antigen, and in that case fractionation will take place simultaneously with concentration of the protein zones. Ampholine in low concentration (0.2-0.5%) can be distributed along the whole length of the film, in which case fractionation is increased during electrophoresis, but without widening of the separated zones. By changing the Ampholine concentration optimal regimes for fractionation of the test antigens can be selected.

Proteins were demonstrated also by simple staining after isotachophoresis on the strip of film. For this purpose the films were first fixed and rinsed to remove ampholytes in a 10% solution of TCA and stained with Amido black or with Coomassie R-250. In this way the optimal regime for fractionation of a test mixture of antigens can be quickly selected.

The variant of immunoisotachophoresis described above has special advantages for the analysis of micro quantities of antigens on account of their preliminary concentration and the absence of "smudging" of the zones during fractionation. It is also useful for the study of electrophoretic microheterogeneity of individual proteins.

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CHEMICAL MYELOTOMY IN GUINEA PIG FETUSES

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A method of immobilizing the fetuses of laboratory animals by chemical myelotomy is suggested: 96% ethanol is injected into the spinal canal.

KEY WORDS: chemical myelotomy; spinal cord.

Investigations on fetuses of animals, when removed by caesarian section into a thermostatically controlled bath, retaining their placental connection with the mother, are often complicated by the considerable mobility of the fetuses. Difficulties also arise during catheterization of the umbilical vessels (separation of the layers of the umbilical vessels, their detachment from the amnion), in the recording of brain electrical activity, in the maintenance of an artificial placental circulation, and during other procedures. Work with young fetuses of animals whose motor activity is continuous is particularly difficult in this respect. Immobilization of the fetuses, on the other hand, by means of curare-like drugs completely prevents observation on their behavior.

To limit the movements of fetuses the writers have used a method of chemical myelotomy (complete blocking of the spinal cord by means of chemical substances injected into the spinal canal). Chemical myelotomy (by the injection of 96% ethanol into the spinal canal) was first used with the aim of immobilization and anesthesia instead of division of the spinal cord in pregnant rabbits, as a more sparing operation [1]. Furthermore, classical anesthesia in very young experimental animals is a difficult procedure because of the limited volume of their subarachnoid space. Schwartze et al. [2], when using this method on pregnant guinea pigs, inject 96% ethanol in a dose of 0.1-0.2 ml intradurally at the level of the 1st and 2nd lumbar vertebrae. The only complication of the method of chemical myelotomy in adult animals, according to these workers, is the possibility of respiratory arrest. However, this complication, associated with the interruption of respiratory movements, is no threat to the fetus, whose gas exchange is maintained by the placental circulation.

EXPERIMENTAL METHOD

Chemical myelotomy was performed on 25 guinea pig fetuses at the 6th-10th weeks of intrauterine life. The pregnant guinea pig likewise underwent chemical myelotomy before the caesarian section operation. For the operation of chemical myelotomy an insulin syringe was used; this is convenient because it enables the

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