COUNTERFLOW ISOTACHOPHORESIS ON CELLULOSE ACETATE MEMBRANES
ROLE OF ELECTROENDOSMOSIS

G. I. Abelev and É. R. Karamova

UDC 612.398-087.4:543.545

A method of counterflow isotachophoresis of proteins on cellulose acetate films is suggested. To produce a counterflow of fluid, an electroendosmotic flow formed in the film during passage of an electric current through it was used. During isotachophoresis of proteins in the presence of ampholytes, the proteins are concentrated on the boundary between the leading and closing ions and, at the same time, they are fractionated by electrophoretic mobility. Microanalysis of protein mixtures in dilute solutions can be undertaken by this method. It is readily combined with immunodiffusion and immunoelectrophoretic detection of protein antigens.

KEY WORDS: counterflow isotachophoresis; electroendosmosis; protein microanalysis.

Isotachophoresis (ITP) is one of the most effective methods of analytical and preparative fractionation of proteins and also of any organic and inorganic ions [3-5]. During ITP proteins are simultaneously concentrated and separated on the moving boundary between solutions of two electrolytes, consisting of anions with different electrophoretic mobility and a common cation. A sharply different potential gradient (E) is created under these circumstances in different parts of the field, and this produces both concentration and electrophoretic separation of the substances. During ITP in supporting media possessing electroendosmosis, an electroendosmotic flow of unequal velocity, proportional to E in its own part of the field, is created in different parts of the field. This acts as an obstacle to ITP, which is usually carried out in nonosmotic systems – in free solution or polyacrylamide gel (PAG) [4, 5]. However, it was shown previously that ITP of cations can be carried out on filter paper [6, 7], and that ITP for concentration and fractionation of proteins can be carried out on cellulose acetate membranes (CAM) [1, 2], despite the presence of electroendosmosis in both systems.

The use of electroendosmosis in CAM for counterflow ITP is suggested in this paper.

EXPERIMENTAL METHODS

Gelatinized CAM (Cellogel, from Serva, West Germany) were used. Electrophoresis was carried out under mineral oil in an apparatus described previously [1]. The anodic part of the apparatus contained 0.06M Tris-HCl, pH 6.7 (B-1) and the cathodic part contained 0.012M Tris-glycine buffer, pH 8.3 (B-2). A block of 30% PAG (12 × 5 × 3 mm), made up in B-1, was used as the "trap." Standard purified preparations of bovine serum albumin (BSA), human α globulin (α-G) (from Koch-Light, England), and of horse (HM) and whale (WM) myoglobins (from Serva, West Germany) were used. Ampholine 3.5-10 (from LKB, Sweden) was used as a 10% solution in B-1.

The Cellogel strip, measuring 85 × 10 mm, was folded to form two gutters K_1 and K_2 (Fig. 1A, B). When strips are cut from standard Cellogel membrane, 3 mm must be left around its borders, in which the properties differ somewhat from those of the remaining area of the membrane. The strip was immersed for 30 min in B-1 solution containing traces of bromphenol blue (BPB), after which the excess of liquid was removed and the strip was transferred to the instrument under mineral oil. The ends of the membrane were placed between strips of ash-free filter paper, saturated on the anodic and cathodic sides with B-1 and B-2 respectively. A "trap" was placed in front of K_2 (Fig. 1A, B). Into K_1 was poured 200 μl of B-1 containing the proteins for analysis (20-50 μg) and 3-5 μl of 10% Ampholine. Six to eight strips of Cellogel can be accommodated in the instrument.

Laboratory of Immunochemistry and Diagnosis of Tumors, Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. A. Éngel'gardt.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 88, No. 9, pp. 334-336, September, 1979. Original article submitted January 16, 1979.

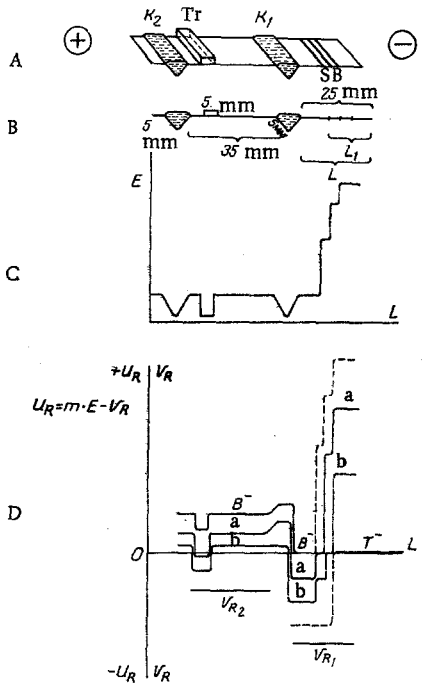

Fig. 1. Diagram showing principles of method. A: general view of film for ITP. B-1 and B-2) Tris-HCl and Tris-glycine buffer solutions respectively; K_1) reservoir filled with protein with Ampholine; K_2) reservoir filled with buffer B-1; SB) Kohlrausch boundary in first stationary position; Tr) "trap:" 30% PAG block; B) section through film in first stationary position of Kohlrausch boundary. L) Distance from reservoir K_1 to cathodic border of film; L_1) distance from boundary to cathodic border of film; C: distribution of voltage (E) in film in first stationary position of Kohlrausch boundary; D: distribution of resultant velocities (U_R) of anions in different parts of film in first stationary position of boundary. U_R) Resultant velocity of anion; V_R) resultant velocity of electro-endosmosis; B^-) leading ion; T^-) closing ion; a and b) protein anions.

During the experiment a constant voltage of 200 V was applied to the instrument. A few minutes after the beginning of the experiment a clear and rapidly migrating boundary appeared, on which the BPB was concentrated. The boundaries slowed down and stopped in front of the K_1 reservoir (first stationary position). Under these circumstances liquid was absorbed from K_1 and concentration of the stained proteins on the boundary could be clearly seen. If necessary, further volumes of the same solution could be added to K_1 to replace the liquid absorbed from it. After all the liquid had been absorbed into the film the boundary again started to move toward the anode, but stopped in front of reservoir K_2, at a certain distance from the trap (the second stationary position). Buffer was now absorbed from K_2, apparently by the stationary boundary (SB) of proteins which had migrated during the first stage from K_1 toward the anode. The experiment ended when 200 μl of buffer had entered the film from K_2. For fixation of the protein zones and removal of the Ampholine the strips were placed overnight in 12.5% TCA solution and the oil carefully removed from their surface. Staining with Coomassie R-250 was carried out by the usual method.

EXPERIMENTAL RESULTS

Migration of proteins located on the two sides of SB took place in opposite directions (Fig. 2). For instance, BAS, HM, WM, and γ-G in region K_1-SB (in front of the boundary) moved toward the cathode, whereas

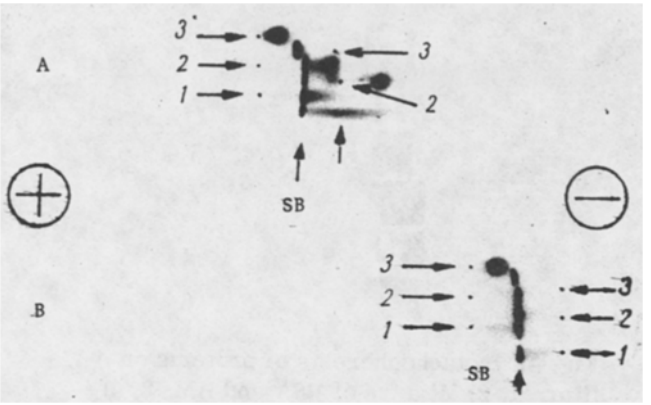

Fig. 2. Concentration of proteins on Kohlrausch boundary: A) closing ion glycine, pH 8.3; B) closing ion β-alanine, pH 8.5. On each strip: 1) γ-G, 2) WM, 3) BSA. Arrows indicate point of application of protein after establishment of SB. In A, much of the WM and γ-G was not held up at the boundary and was carried toward the cathode. Both proteins were concentrated at the boundary in B regardless of whether they were in front of or behind the boundary.

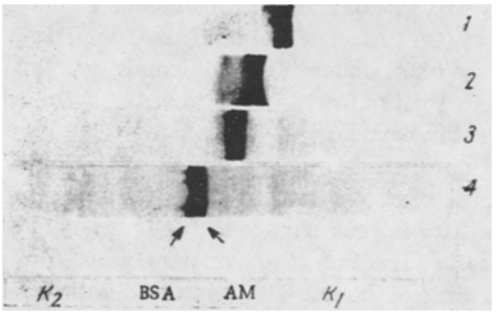

Fig. 3. Concentration of proteins on Kohlrausch boundary during electrophoresis in nonhomogeneous buffer system. Successive stages of electrophoresis for mixture of BSA and HM in nonhomogeneous buffer system. First zone BSA, second zone HM. BSA zone, migrating from K_1 toward anode, is clearly visible on first two strips. During migration the BSA zone is "adsorbed" by the Kohlrausch boundary.

proteins beyond the boundary moved toward the anode. The proteins stopped and were concentrated, each in its own particular zone of SB. It must be emphasized that only those proteins (or protein subfractions) with higher electrophoretic mobility (m) than m of the closing ion in the zone of the film located beyond the boundary possessed anodic mobility. In the case of glycine, these proteins were BSA, α globulins, some HM, WM, and only the fast fractions of γ-G. When β-alanine (0.012M Tris, β-alanine to pH 8.5) or γ-aminobutyric acid (GABA) (0.12M Tris, GABA to pH 8.9) was used as the closing ion, all "slow" proteins were concentrated on the Kohlrausch boundary (Fig. 2).

The dynamics of concentration of BSA and HM during isotachophoresis without Ampholine is shown in Fig. 3. "Absorption" of BSA at the boundary can be seen during final concentration in the second stationary position of the boundary. The results of ITP of various proteins with the use of Ampholines are illustrated in Fig. 4.

The basic assumption for the explanation of the cases examined in this paper is that the liquid in the membrane behaves as a continuous layer, contained in a closed vessel, open only at the inlet and outlet on the

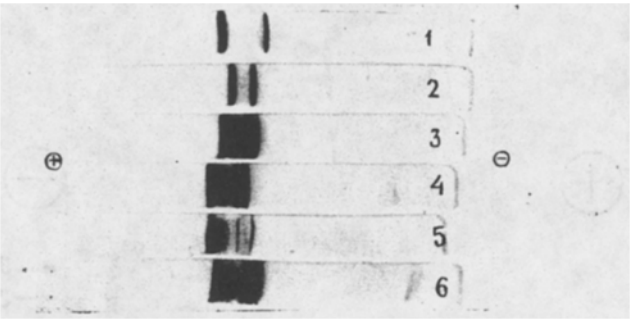

Fig. 4. Isotachophoresis of proteins on film. 1, 2) Mixture of BSA and HM; 3, 4) mixture of BSA, α-G, and HM; 5, 6) mixture of BSA, α-G, and human γ-globulin. Closing ion glycine, Ampholine 3-10, 5 μl 10% solution.

cathodic and anodic sides. Electroosmotic forces, which differ in different parts of the film, undergo summation under these circumstances, so that a resultant velocity of electroendosmosis (V_R) arises, its value being determined by the total electroosmotic force balanced by the force of friction of the whole layer of fluid migrating in the film toward the cathode. If the effect of composition of the buffer of the two sides of the boundary on the velocity of electroendosmosis is disregarded, this velocity will be determined only by the properties of the membrane and the mean value of E, i.e., only by the potential difference on the ends of the standard film. If the voltage is stabilized, V_R will be constant regardless of redistribution of E along the length of the film during migration of the boundary. On the anodic side of the boundary V_R will always be greater than the velocity of electroosmosis which would exist under the same conditions but in a homogeneous buffer system. In the cathodic part, on the other hand, V_R is always lower than the value which would be found during ordinary electrophoresis. During migration of the boundary under constant voltage conditions the resistance in the film rises proportionally to the length of the cathodic region (L_1) [4], the strength of the current falls, and parallel with it the voltage in the anodic part of the film determining migration of the leading ion also falls; under these circumstances V_R in the film is thus changed. When the rate of migration of the leading ion (mE) becomes equal to V_R, but in the opposite direction, the boundary stops, but liquid flows through it continuously, transported by electroosmosis toward the cathode. This situation arises in region K_1-SB of the film (Fig. 1A). In this stationary position all parameters of the system are constant: I, V_R, L_1/L, and the distribution of E.

Because of the conditions of ITP the velocities of movement of the ions with the boundary in the stationary state are equal to one another. In the present case they are also equal to zero relative to the film. The values of $U_R^* = mE - V_R$ for the leading and closing ions are thus 0. Since m for the leading ion, because of the conditions of ITP, is higher than m for any separated ion, all these ions on the anodic side of the boundary will have a resultant velocity $< U_R$ of the leading ion, i.e., < 0. Conversely, on the cathodic side of the boundary, because of the conditions of ITP, their value of m will be larger than m of the closing ion and they will have a positive value of U_R. Only on the boundary, under conditions of stationary equilibrium, when their velocity becomes equal to U_R of the leading and closing ions, do they stop relative to the film (Fig. 1B). In other words, any protein or ampholyte on either side of the boundary will migrate toward its own equilibrium zone, located between the leading and closing ions. This is what is observed in reality (Figs. 2 and 3).

The liquid in K_1 and K_2 creates "gaps" in the continuous layer of liquid contained in the membrane. These "gaps" separate region K_1-K_2 from the region between K_2 and the cathodic boundary. In K_1-K_2 the conditions are those of ordinary electrophoresis in a homogeneous buffer B-1 and with a relatively low value of B_2. Those proteins with a value of a higher than the velocity of electroosmosis in K_2-K_1 will leave reservoir K_1 and migrate toward the anode. A trap is placed along their path, in which the value of E is sharply reduced in this part of the film on account of an increase (about tenfold) in the section of the conductor in this region. The decrease in E leads to a proportional decrease in the velocity of movement of the proteins, but it reduces the results of velocity of electroosmosis due to the region of film between the trap and K_1 only very slightly. The migrating protein moves much more slowly under the trap and, at the same time, it is expelled

*U_R represents the velocity of movement of a protein toward the anode relative to the film (resultant velocity).

from beneath it by the flow of liquid. Protein cannot enter the trap because of the high (30%) concentration of gel. After absorption of the liquid from K_1 the boundary passes across the first reservoir and a second SB is established relative to K_2. Under these circumstances proteins in front of K_2 are "absorbed" at the boundary for the reasons examined in connection with the first stationary position of the boundary.

Protein fractions whose value of m is less than that of the closing ion, i.e., those with $U_R < 0$, under SB conditions will be carried toward the cathode and lost. During work with glycine and the closing ions, some of the "slow" proteins, namely WM and γ-G, are lost in this way (Fig. 2). To keep all these proteins in ITP, β-alanine or GABA, which have much lower values of m, must be used instead of glycine. In that case equilibrium develops at lower values of L_1/L and the distance from K_1 to the cathodic border must be increased.

The technique described above is very simple, because no special equipment is required to create the counterflow [4] or to detect the protein zones. It can be used for work with very dilute solutions of proteins and can readily be combined with immunodiffusion [1] and immunoelectrophoresis [2].

The authors are grateful to laboratory technicians O. A. Muratov, V. V. Gorelov, and T. I. Merkulov for their help with the work. The work was partly subsidized by the immunological division of the World Health Organization.

LITERATURE CITED

1. G. I. Abelev and A. A. Sokolenko, Byull. Éksp. Biol. Med., No. 7, 120 (1977).
2. G. I. Abelev, Byull. Éksp. Biol. Med., No. 7, 110 (1978).
3. B. P. Konstantinov and O. V. Oshurkova, Dokl. Akad. Nauk SSSR, 148, 1110 (1963).
4. F. M. Everaerts, J. L. Beckers, and T. Verheggen, Isotachophoresis, Amsterdam (1976).
5. H. Haglund, Sci. Tools, 17, 2 (1970).

ROLE OF THE ADHESIVE LYMPHOCYTE POPULATION IN AREACTIVITY TO HEPATOMA 22a

I. N. Maiskii, B. A. Botev,
L. Kh. Markholev, M. F. Nikonova,
T. A. Pokrovskaya, T. I. Vatseva,
and I. V. Demeshina

UDC 616.36-006-092.9-07:612.017.1

The role of the adhesive fraction of T lymphocytes in areactivity of mice to hepatoma 22a was studied. Experiments showed that removal of the adhesive fraction from a suspension of spleen cells enriched with T lymphocytes potentiates cellular immunity in mice tolerant to hepatoma.

KEY WORDS: antitumor immunity; tolerance; hepatoma 22a; migration of macrophages; T lymphocytes.

Among the many factors influencing manifestation of immune reactions to tumors an important role is ascribed to immunologic tolerance, development of which is due to the existence of antigens common to embryos and tumors, and also to the appearance of soluble tumor antigens in the circulation. In previous experiments with mouse hepatoma 22a the writers showed that contact with tumor antigens in the neonatal period not only leads to subsequent stimulation of growth of an inoculated tumor, but is also accompanied by depression of antitumor immunity in mice thus rendered tolerant [1].

Research Laboratory of Experimental Immunobiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Zhukov-Verezhnikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 88, No. 9, pp. 336-338, September, 1979. Original article submitted July 3, 1978.

0007-4888/79/8809-1053$07.50