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CONTINUOUS FREE-FLOW ELECTROPHORESIS AS AN ANALYTICAL AND PREPARATIVE METHOD IN BIOLOGY*

KURT HANNIG
Max-Planck-Institut für Biochemie, 8033 Martinsried (G.F.R.)

CONTENTS

1. INTRODUCTION

Within the last few years, continuous free-flow electrophoresis has proved to be a mild, rapid and efficient method for preparative separation problems. It not only permits the separation of soluble substances such as proteins, but also the separation and isolation of viable cells, or cell organelles and membrane systems.

As the principle of electrophoresis uses differences in the surface charge of particles for their separation, its special significance lies in the investigation of those biological processes which are related to a change in the cell surface structure. The outer cell membrane is the communication organ of the cell. Changes which occur at the micro-levels of their molecular structure are mostly the expression of differentiation and transformation events or are diseased abnormalities that can lead to the blocking of information exchange. Most of the components of the cell surface, and of biological membranes in general, represent carriers for different electrically charged groups. They are often the loci of specific functions. Separation techniques such as electrophoresis, which use these different functional properties for the separation of biological particles from mixtures, can be useful tools in membrane and cell research. In this paper, some of the latest results obtained with free-flow electrophoresis are presented.

* Dedicated to the 75th birthday of Professor A. Butenandt.

2. EXPERIMENTAL

2.1. Apparatus for preparative separation

In a narrow separation chamber (55 × 10 cm) a buffer film of thickness 0.5–0.6 mm flows in a laminar manner from top to bottom[1,2]. An electric field is set up perpendicular to the buffer stream.

The samples to be separated are injected continuously into the streaming medium through one of the openings in the upper part of the chamber. Particles with different electrophoretic mobilities move along different paths and can be collected continuously at the bottom end of the chamber by a 90-channel peristaltic pump. The tubes lead to a fraction-collecting container. A very effective cooling system permits the application of high field strengths of 100–120 V/cm, and also a relatively high buffer streaming velocity. The biological material remains in the electric field for only 3–7 min, according to the result required.

With this system, all requirements that are necessary for a careful isolation of biological cell material, including sterile conditions, can be fulfilled, and 200–600 million cells or 20–50 mg of membrane or organelle proteins can be separated per hour. There is no loss of cell vitality as a result of the separation process.

2.2. Apparatus for analytical separation

As a continuously working method, free-flow electrophoresis offers both high throughputs for preparative purposes and a rapid change of difficult samples while maintaining constant conditions for analytical separations. In the latter instance, high sample throughput is unnecessary, but it is essential to increase the resolution by shortening the separation periods. Hence the basis is given for automation of analytical test series, and an analytical separation device based on the principle of free-flow electrophoresis, with the use of optical densitometry by means of slit scanning, has therefore been designed[3]. The detection system permits absorption measurements to be made in the ultraviolet range. The usual analytical separations of serum protein for clinical diagnostics, cell separations and mobility measurements can be carried out in less than 1 min per sample.

The separation chamber gap for analytical purposes has a width of only 3 cm, a height of 18 cm and a depth of 0.3 mm. Because of the high thermal stability and simple procedure for filling the separation chamber, the buffer flows from bottom to top. For the optical detection of the separated sample components through the window of the separation chamber a slit scanning photometer is used. Because of the small transmission differences, a high luminance, a low-noise photomultiplier with high UV sensitivity and special measurement electronics are required. A schematic diagram of the device is shown in Fig. 1.

A 200-W deuterium lamp serves as a light source. Via a monochromator, the light beam is moved continuously back and forth across the separation chamber window by means of mirrors. The output signal from the photomultiplier is amplified and noise is filtered by low passes. For a better determination of the small transmission differences, the bias light intensity is subtracted during each scan. This difference signal is amplified and displayed on-line on a storage monitor, or fed into a data-processing

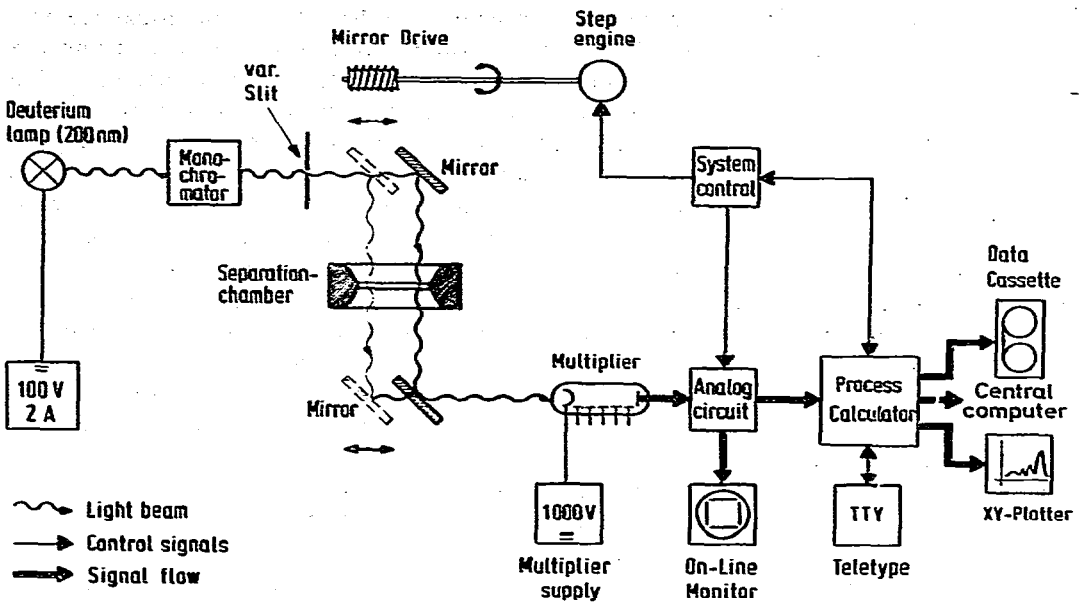

Fig. 1. Schematic diagram of analytical free-flow electrophoresis.

system. A process control computer averages several scans and carries out a background subtraction. The processed distribution curves can be plotted and stored on data cassettes for further processing in central computers. All functions work automatically.

3. RESULTS AND DISCUSSION

3.1. Preparative separation

3.1.1. Separation of cells

In an immune system, it is of great interest to investigate the correlations between the differentiating cells and their environment. The starting point for such investigations was our finding that free-flow electrophoresis permits the separation of immunocompetent peripheral lymphocytes into a population of thymus-dependent T-cells and into another population of thymus-independent B-cells[4–9]. These results, first obtained with rodents, were completed by experiments on baboons and chimpanzees[10–12]. This implies the possibility of studying immune mechanisms on human T- and B-cells.

By combining electrophoretic separation with that of 1-*g* sedimentation, it was possible to isolate five T-cell populations from thymus with high concentrations and to define them in terms of their biological significance[13,14]. These cells are constitutents of a differentiating sequence, which is characterized by increasing maturity of its members. The cell populations thus isolated show functional differences, which permit a useful classification and characterization of its functions[15].

The successful separation and isolation of large numbers of functionally viable

B- and T-cells and their subpopulation or precursor cells[16–19] is of practical medical significance. The understanding of the stage of differentiation during which and the conditions under which immunocompetence or tolerance appear would make possible the systematic manipulation of the immunity tolerance limits, which would be of great significance not only for the treatment of disease but also for transplant surgery.

In addition to the separation of immunocompetent cells, new possibilities arise in the preparation of a homogeneous cell population from a cell suspension from kidney cortex by using free-flow electrophoresis[20,21]. The kidney cells produce a number of physiologically important substances that control metabolic processes. For example, the enzyme renin has been shown to play an important role in the Na^+ and water metabolism of the organism, as it can activate the angiotensin-I–angiotensin-II–aldosterone system. As a consequence, it is involved in certain types of hypertension.

Many questions have been answered concerning the physiology of renin. However, its biosynthesis (its pathway within the cell) and its proliferation from the cell are still unclear. Therefore, it would be an important step to be able to isolate viable renin-active cells. From a cell suspension of kidney cortex cell (rabbit), several homogeneous populations could be isolated[20,21]. Proximal and distal tubule cells have been characterized by their morphology, and another population has been demonstrated to be renin-active (radioimmunoassay). The biochemical characterization of these cells is being investigated, and also the vitality of the cells, which in culture incorporate uridine linearly over a period of 8–12 h. This vitality makes it possible to carry out stimulation and blocking experiments in the isolated cell populations.

3.1.2. Organelles and membranes[22]

Not only the isolation of viable cells, but also the purification of their membrane systems and cell organelles such as lysosomes[23,24] is of importance. Electrophoresis gives experimental evidence of the high purity isolation of these particles and an accurate characterization according to the electrical surface charge.

Great importance is also attached to the achievement of a better knowledge of the other ("interior") side of a membrane. New possibilities arose after it became feasible to turn the membrane surface partly inside out and thus obtain particles the hidden "inside" of which then is located on the outside. The preparative separation of such "inside out" vesicles in a centrifuge according to differences in density is very difficult, if not impossible. However, in most instances the membrane surfaces of the "inside" and "outside" have different electrical charges, the difference being great enough to permit a separation by electrophoresis and isolation in a pure condition.

Such investigations have been carried out successfully with mitochondrial membranes[25,26], erythrocytes[27] and plasma membranes[22]. As an example, experiments with erythrocyte membranes are considered below (Fig. 2).

On the basis of several previous investigations, it is assumed that the erythrocyte membrane can form vesicles with the original inner surface on the outside. As intact erythrocytes carry almost all of their sialic acid on the outside of their cell membranes, the two sides of the membrane should have different electrical surface properties, making electrophoretic separation from each other possible.

After electrophoresis, two fractions of membraneous material were obtained from erythrocyte ghost preparations. The left peak represents vesicles that have the same electrophoretic behaviour as intact erythrocytes and that also have their sialic

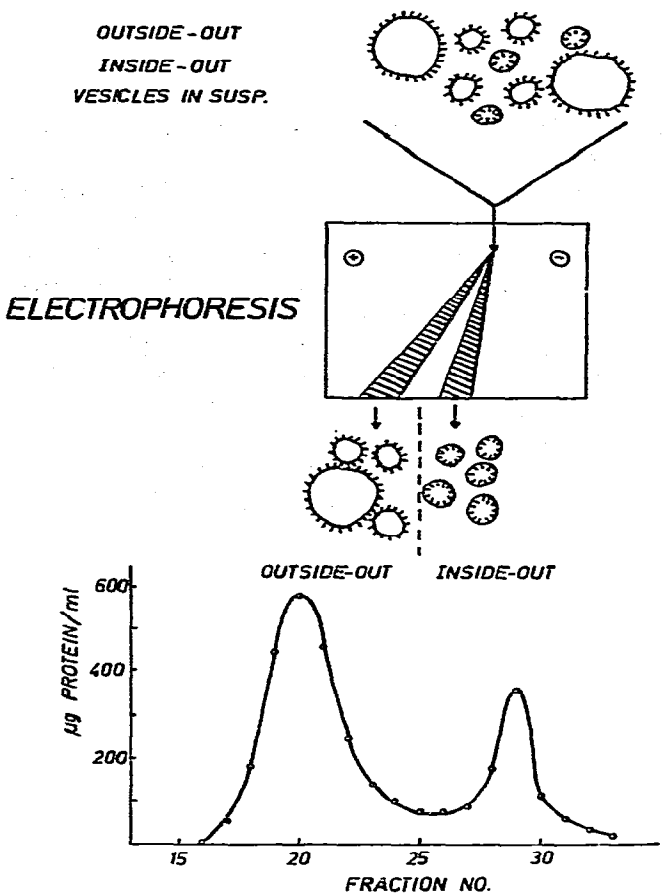

Fig. 2. "Inside-out vesicles"[27]. The upper part of the figure shows a schematic diagram of a separation of "inside-out" from "outside-out" vesicles. The lower part illustrates the original distribution curve of "outside-out" (left peak) and "inside-out" (right peak) vesicles of erythrocytes after a free-flow electrophoresis run. The vesicles in the left peak have the same electrophoretic mobility as intact erythrocytes.

acid on the outside. The right, much more slowly moving vesicle population has their sialic acid hidden on the inside. In contrast to outside-out vesicles they cannot adsorb, for example, influenza virus on their surface. These results demonstrate the usefulness of electrophoresis for solving such problems.

A very promising and interesting example of the application of free-flow electrophoresis is in the isolation of DNA-envelope complexes of *E. coli* and *B. subtilis*. The success of these experiments was due to the additional electrical charge of the DNA units attached to the membrane fragments, which permits the separation of these complexes from the bulk of the envelope particles[28,29].

More examples of the application of the electrophoretic technique to the isolation of membrane systems could be given. In all of these, charges of functionally defined biological particles can be of decisive importance in difficult separation problems.

3.2. Analytical applications

3.2.1. Serum analysis

For the separation of serum proteins by analytical free-flow electrophoresis, a Tris–borate buffer of pH 8.8 was used[3].

Fig. 3 shows the results obtained from normal serum with separation times of 20, 28, 50 and 70 sec. A field strength of 140 V/cm was applied. The total amount of sample injected was 0.1 μl, corresponding 3 μg of protein (picomole range). The protein bands were detected photometrically in the absorption range of the peptide bond (225 nm). A separation of the serum proteins that could be quantitatively evaluated was possible after a separation time of only about 30 sec. Up to 100 samples can be tested per hour. The standard deviation of the reproducibility was proved by separating a normal serum 10 times. The averaged percentage values and the coefficients of variation (CVs) are indicated in Table 1 (column I) and compared with the CVs of membrane electrophoresis[30] (column II) and moving-boundary electrophoresis[31] (column III) taken from the literature. The results show that the analytical free-flow electrophoresis gives a good reproducibility.

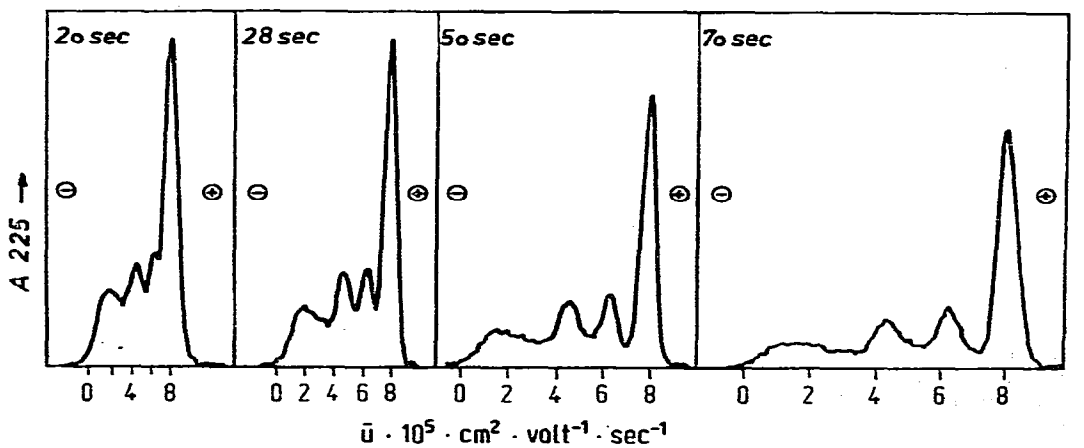

Fig. 3. Separation of serum proteins[3]. Tris–borate buffer (pH 8.8); 480 V, 42 mA.

TABLE 1

COMPARISON OF THE REPRODUCIBILITIES OF 10 SEPARATIONS OF A NORMAL HUMAN SERUM

I = Free-flow electrophoresis; II = membrane electrophoresis[30]; III = moving-boundary electrophoresis[31].

Component	*I*		*II*	*III*
	%	*CV*	*CV*	*CV*
Alb ($+\alpha_1$)	62.1	1.1	3.4	2.0
α_2	7.5	1.9	9.0	4.1
β_{1-3}	9.3	1.7	9.5	4.9
γ_{1-2}	21.2	3.2	9.4	5.0

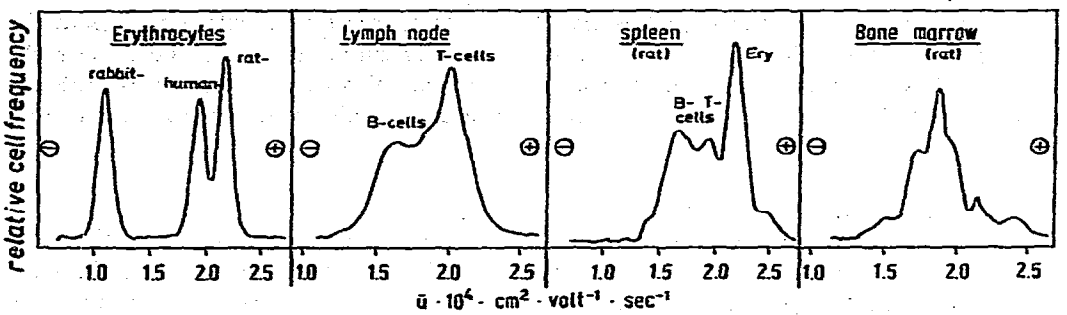

Fig. 4. Separation of diverse cell suspensions[3]. Triethanolamine buffer (pH 7.2); 470 V, 42 mA, residence time 40 sec.

3.2.2. Electrophoretic mobility measurements on cells

Fig. 4 shows separations of different cell materials. For one measurement, only 100,000 cells are required. The distribution pattern of the separated cell populations was achieved by means of nephelometry at 260 nm. In analytical free-flow electrophoresis, a large number of cells (17,000 per scan) is simultaneously monitored, implying a great statistical significance. From systematic experiments, an accuracy and reproducibility of mobility measurements of $\pm 1.4\%$ can be calculated[3].

As an example, an application of analytical free-flow electrophoresis in haematology is shown in Fig. 5. In the Tn syndrome, a rare condition of acquired mixed field polyagglutinability, some of the red cells have a lower sialic acid content and consequently a decreased electrophoretic mobility[32]. In the blood from a patient*, about 50% of the red cells are polyagglutinable erythrocytes, the others showing only a slight, unexplained decrease in electrophoretic mobility compared with normal human erythrocytes. Both cell populations could be separated successfully.

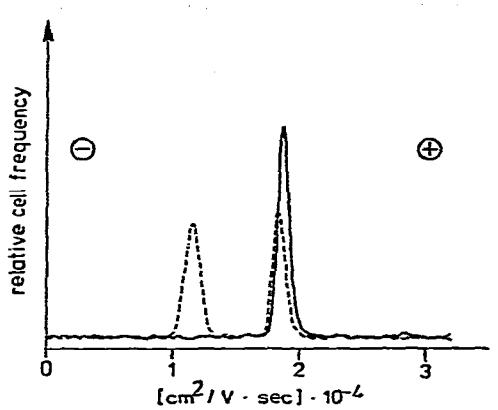

Fig. 5. Electrophoretic mobilities of native human erythrocytes from a patient with Tn syndrome (broken line) and normal native human erythrocytes (solid line). The low-mobility peak indicates the polyagglutinating erythrocyte population.

* The Tn blood sample was a gift from W. Dahr, Medizinische Universität, Köln, G.F.R.

We investigated the applicability of analytical free-flow electrophoresis to the macrophage electrophoretic mobility test for cancer diagnosis[33]. Further, the technique was used for the characterization of the electrophoretic mobilities of different postnatal erythrocyte populations of four different species (rat, sheep, mouse and man)[34].

3.2.3. Isoelectric focusing

In isoelectric focusing experiments, the long separation times required for proteins are incompatible with the principle and the advantages offered by the free-flow system as a fast separation method. The necessary stability is hardly maintained because of thermal convection in a slowly flowing buffer curtain. We avoided these difficulties to a large extent by modifying the separation chamber for the "recycling" procedure. For producing a pH gradient, the top and bottom are connected by means of 30 tubes, using the shortest route through a manifold peristaltic pump for buffer circulation. In this closed loop, an arbitrary number of separation cycles can be performed.

The example in Fig. 6 shows the isofocusing separation of sheep haemoglobin obtained in such a device. After 10 min of cycling, the pH gradient (1 % ampholine buffer, pH 3.5–10) was stabilized, the applied field strength being 140 V/cm. Then 20 μl of a 1 % haemoglobin solution were injected during 60 sec. After 30 cycles, *i.e.*, after an effective residence time of *ca.* 30 min, the final stage of focusing was reached. Continuation of the experiment did not improve the resolution further.

On the other hand, the separation of cells in a pH gradient show different properties. Biological membranes underly reversible and non-reversible changes in electrokinetic behaviour at non-physiological pH values and ionic strengths, owing to the denaturation of cell surface components and adsorption of hydrolysis products. The isoelectric point of cells, organelles or membranes is not a physical constant, as it is with proteins. Therefore, the importance and use of "cell isoelectric focusing" becomes doubtful. From very few experimental results, it can be concluded that neither during continuous operation nor in columns do native cells migrate in the pH gradient to their isoelectric points, which can theoretically be calculated on the basis of mobility measurements. The electrophoretic migration stops at a much

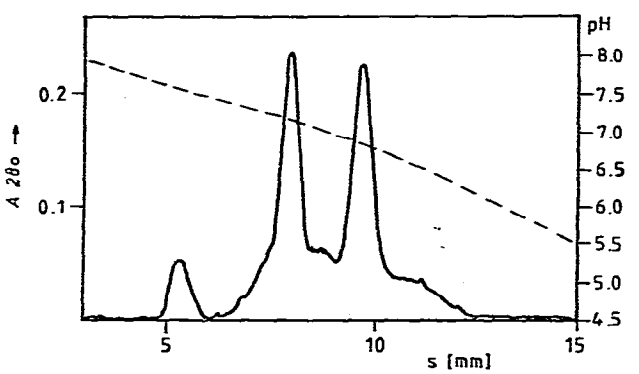

Fig. 6. Isoelectric focusing of sheep haemoglobin[3] with 1 % ampholine solution (pH 3.5–10); recycling method, 30 min.

higher pH level, which also depends on the environmental conditions (apparent isoelectric point), and the cell viability is usually completely lost[35].

4. SUMMARY

Several applications of preparative and analytical free-flow electrophoresis are described and discussed.

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