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Review

Separation of plant membranes by electromigration techniques

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Abstract

The review focuses on the multiple separating regimes that offers the free flow electrophoresis technique: free flow zone electrophoresis, isoelectric focusing, isotachophoresis, free flow step electrophoresis. Also, the feasibility to apply either interval or continuous flow electrophoresis is evaluated. The free flow zone electrophoresis regime is generally selected for the separation of cells, organelles and membranes while the other regimes find their largest fields of applications in the purification of proteins and peptides. The latter regimes present the highest resolution efficiency. Therefore, a large part of this review is devoted to the applicabilities of these different regimes to the purification of organelles and membrane vesicles at the preparative scale. Recent developments, both in instrumentation and procedures, are described. The major achievements in plant membrane fractionation obtained with free flow electrophoresis are outlined. The related procedures are both analytical and preparative: they separate tonoplast and plasma membrane simultaneously from the same homogenate, they discriminate for one type of membrane vesicles of opposite orientation, and process large quantities of membrane material by reason of the continuous flow mode. Recent advances using electromigration techniques that permit confirmation of the dynamic state of membranes, characterisation of complex membrane-dependent functions and discovery of new membrane-localised activities are presented.

Keywords: Reviews; Plant membranes

Contents

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1. Introduction

Electron micrographs of plant cells reveal the abundance of membranous organelles and membrane systems including chloroplasts, mitochondria, vacuoles, endoplasmic reticulum, Golgi apparatus, peroxisomes, glyoxisomes, a variety of vesicles involved in intracellular trafficking, and a nucleus, all bathed in the cytoplasm and surrounded by the plasma membrane. Each of these membranes is specialised in function and therefore has different structural and compositional properties. Membranes are also in close connection to each other with a flow of membrane vesicles between different cell compartments, such as endoplasmic reticulum, Golgi apparatus and plasma membrane. Facing this diversity, biologists want pure membranes in order to study a particular membrane-dependent function. However, all membranes are built on the same scheme: a fluid-like lipid bilayer into which proteins are embedded to varying degrees. As a consequence, extremely different methods for separation and purification of membranes have been developed [1]. In aqueous media, lipid bilayers form vesicles trapping an inside and defining an outside. Polar heads of lipids and proteins adsorbed or anchored into the membrane give to the membrane vesicle a net surface charge. This property was used to separate the complexity of membrane species by electromigration techniques. Placed in an electric field, vesicles bearing different surface charges will migrate different distances during electrophoresis [2].

In this review, we will focus on free flow electrophoresis (FFE) technique for the electromigration of plant membranes and organelles.

2. Separation techniques: the multiple potentialities of free flow electrophoresis

Free flow electrophoresis has been developed for continuous separation of molecular and particular substances according to their electrophoretic mobilities [3]. The basic principle of this type of electrophoresis appeared very promising as particles are processed in a free fluid system, where no matrices forming networks influence their mobilities. Indeed, a lot of very interesting results were obtained with the help of this method regarding analytes originating from plants, animals or bacteria [4,5]. However, a number of studies and projects could not be continued, because limits of resolution prevented progress. These limits were often due to the drawback that free flow zone electrophoresis (FFZE) was the only technique applicable to a FFE device, and the negative surface charge density, the only parameter usable for preparative fractionation of sample components. Within the last few years considerable efforts were made to improve the instruments and to expand their applicabilities towards isoelectric focusing (IEF) and isotachophoresis (ITP) in order to make FFE a method of choice, if organelles, membrane vesicles or lipophilic membrane proteins should be purified on a preparative scale [6–8].

2.1. Free flow electrophoresis instrumentation

Although other instrument configurations have been described, the Octopus instrument (Fig. 1) is a commercially available FFE device (Dr. Weber GmbH, Kirchheim, Germany) which is quite suitable for performing preparative FFZE as well as prepara-

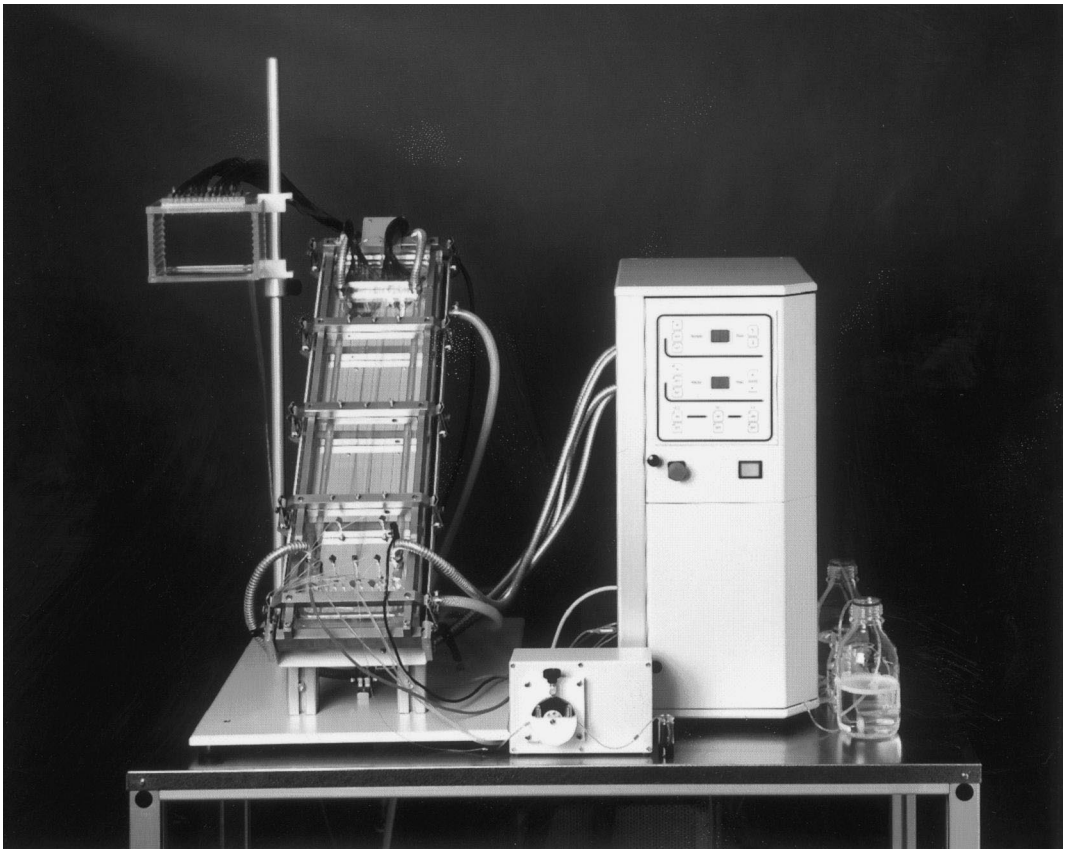

Fig. 1. Photograph of the Octopus FFE equipment suitable for performing FFZE, IEF, ITP and FSE.

tive IEF, ITP and field step electrophoresis (FSE). It is based on a modular concept and offers the choice of manual, semiautomatic or automatic operation. It consists of two independent parts, a process unit and a control unit.

2.1.1. Process unit

The heart of the process unit is the electrophoresis chamber (Fig. 1, left side) with dimensions of 500 mm of length and 100 mm of width. The thickness is normally 0.4 mm and can be reduced to 0.2 mm. Due to the variability of the gap thickness adjustment of heat removal efficiency is possible, if the application of high electric fields is necessary. The inner walls of the separation area are manufactured with the highest accuracy guaranteeing a most precise gap between the walls. The chamber can be tilted at any angle between a horizontal and a vertical direction. Thus a fast switch from the horizontal to vertical FFE is possible. While a vertical chamber position is required for separating particles with high sedimentation velocities, better results are usually obtained in a horizontal chamber, as long as specimen sedimentation does not play a role.

At one end (e.g. at the the lower end as shown in Fig. 1), the chamber has seven chamber fluid inlets and three sample inlets. It is possible to pump various media flowing adjacently to each other through the chamber and to introduce the sample at the optimal site. The central chamber media are used for separating the specimens on preparative scale applying either FFZE in homogeneous or pH-gradient media, or IEF, ITP or FSE techniques to the equipment. The media flowing at both edges cover

the electrode membranes and have to protect the separation media from detrimentous influences of the electrodes. All chamber media are pumped by one pump. However, the inner diameters of the various tubings connecting buffer reservoir and inlet may be different. So the ratio of the various media pumped through the chamber may be varied depending on the specific requirements of the electrophoresis technique applied. The separated sample will be fractionated by means of a pulsefree splitting device which injects counterflow medium from the opposite to the media inlets (Fig. 1: upper chamber end). In this way dead volumes near the fractionation implement are avoided.

2.1.2. Control unit

The control unit is mounted on the white box shown at the right half of Fig. 1. It contains hardware equipped with newly developed software for the automatic control of the total FFE equipment and for the documentation of the electrophoretic process. Electronic control was set up for the pumps driving the flow of the various chambers, electrode and sample solutions, for the high voltage power supply, for the circulation cooler and several other peripheric accessories. Recently, the detection unit OCT-DET-VIS was also included in the electronic control system. OCT-DET-VIS consists of light emitting diodes illuminating an area close to the fractionation device of the separation chamber and of a CCD camera detecting the light scattered by bioparticles (Tyndall effect). Light intensities recorded by the camera are processed to pherograms by the computer. The pherograms may be displayed, but can also be analysed regarding background reduction, calibration of *x*- and *y*-axis peak area integration and zooming into specific peaks.

Automation of the various processes supports precise performance, reproducibility and thorough recording of each step of any electrophoresis experiment. Prior to the experiments, parameters of the various processes may be modified with the help of an editor. During the experiment all actual parameters of the electrophoretic process and the status of the peripheric instruments are displayed on a screen. Every half minute the data displayed are saved so that the protocol of the whole experiment can be stored on a disk and/or printed.

2.2. Buffers suitable for generating pH or ion gradients and for dissolving detergents

2.2.1. Segmentation of chamber medium

The instrumental improvements allow performing preparative carrier free IEF, ITP and FSE in additon to FFZE and thus tremendously enhance the separation efficiency of FFE, because complicated buffer systems can be applied. Using the new chamber with various medium inlets, the principle of segmented chamber fluids called sheath liquids was developed to a routine procedure [8,9]. Various fluid systems were developed which consisted of up to seven different media with chemical and physical properties adjusted suitably to each other [10]. During the preparation of the fluid systems, it was important from a chemical point of view that types and quantities of ions were added to the various media. This procedure guarantees that the total fluid system shows reproducible pH- and conductivity profiles after passing through the chamber. Both laterally flowing media (called anodal) and cathodal margin media, had to be designed to prevent contamination of the membranes with any species from inside sample and to protect the separation process of the sample fluid from the influence of the electrode. They contain types and quantities of ions most similar to the respective electrode media, but are still adjusted to their neighbouring chamber fluids. The central media had to be prepared optimally for the separability and viability/activity of a specimen of interest. From a physical point of view the various media were adjusted regarding specific density and viscosity at temperatures of the experiment [10].

2.2.2. New separation media

For performing FFZE in inhomogeneous media as well as for IEF, fluid systems were developed which form linear or stepwise pH gradients under FFE conditions [11,12]. For establishing stepwise gradients, groups of up to seven different media were prepared following the principle of high capacity medium buffering as described [13,14]. Examples of self-made buffer systems forming a steep stepwise pH gradient within the FFE that ranges from 10.8 at the cathode to 3.2 at the anode or a flat pH gradient ranging from 8.0 at the cathode to 3.2 at the anode

have been described [11]. These systems allow injection of the sample at the best conditions of pH.

Recently, three new media called ''Octolytes-3–5'' ''Octolytes-5–7'' and ''Octolytes-7–9'' were developed and became available on the market (Dr. Weber GmbH, Kirchheim, Germany). These media consist of well-defined non-hazardous chemicals. Immediately after entering the electric field of the FFE chamber, they form linear gradients ranging from 3.5 to 5.5, 5.5 to 7.5 and 7.5 to 9.5. Depending on a separation problem, each of these media can be applied alone or in combination with one of the two others or with both (Weber & Bauer, manuscript in preparation). Since these media contain low quantities of charged compounds (200 to 300 μS/cm), to some degree salts may be added to chamber fluids and samples so that solubility of proteins is far improved at optimal pH conditions as well as under p*I* conditions. Furthermore, no objections are known, which may prevent the use of Octolytes for the purification of proteins for later application in humans.

Alternative sheath fluid systems have been prepared for performing preparative ITP. One of them consisted of imidazole/HCl buffers used as the leader fluid and imidazole/Hepes buffers used as terminator. Spacer media contained glutamic acid, Mes and Mops [8]. For field step gradient electrophoresis, which is most useful for increasing concentrations of rather pure material, a low conductivity buffer was pumped through the chamber adjacent to a high conductivity one [15,16]. The specimen was injected into the low conductivity medium at lower concentration but higher volume rate. On its way through the chamber, analytes were pulled towards the high conductivity medium by the electrical current. Arriving at the conductivity step where voltage decreases, the tip of a probe is retarded while the rest can close up. In this way the total probe is concentrated to a sharp band.

2.2.3. Additives

To each medium developed so far, various substitutes may be added to support solubility, stability or viability of a specimen. The additives may consist of long chain polymers, such as PEG, PVP, dextran, SDS and others, as long as a viscosity of 4.000 cP is not exceeded. If required, complexing agents (anionic, cationic or neutral agent) may also be used for enrichment of cations (e.g. rare earth metals) [17], anions (e.g. polysulphonates), organelles and other bioparticles. If racemic mixtures of analytes should be fractionated into the enantiomers, cyclodextrin and its homologues may be applied [18].

For many FFE separation processes, hydroxypropylmethylcellulose (HPMC) became the most helpful additive, because it breaks electroosmosis as long as the pH is below 9 [8,11]. If electroosmosis has to be prevented at pH above 9, the inside of the chamber walls may be coated with flexible synthetic polymers such as teflon. But these coatings make the glass wall opaque and prevent visual observation of the separation process, which is important and helpful for early detection of precipitation and agglutination events.

2.3. Rationales for choosing the optimal separation regime from FFZE, IEF, ITP or field step electrophoresis

FFZE in a homogeneous medium, which was, for a long time, the only FFE regime applicable for preparative purposes, is a very gentle, however, non-focusing process (Fig. 2). This means that the Gaussian distribution curves of all components, separated during FFZE, are at least as broad as the sample injection stream. In practice they are broader, because the sample stream is widened on passage through an FFE chamber by a number of band deteriorating forces. Mainly dispersion forces such as hydrodynamic, electroosmotic, and diffusion forces, electrohydrodynamic dispersion and Joule heat destroy the band sharpness. They widen the sample streams especially of molecular substances such as proteins and peptides so that those could not be fractionated at a resolution as achieved by other techniques such as chromatography or capillary electrophoresis. Now, several electrophoresis modes are applicable to one machine. Each of them has its inherent advantages and disadvantages (Table 1). Hence the optimal regime may be chosen for each separation problem.

Conventional FFZE in homogeneous media may be applied, if required for processing very sensitive specimens such as whole cells which tolerate only pH ranges between 6.9 and 7.4 and need low ionic

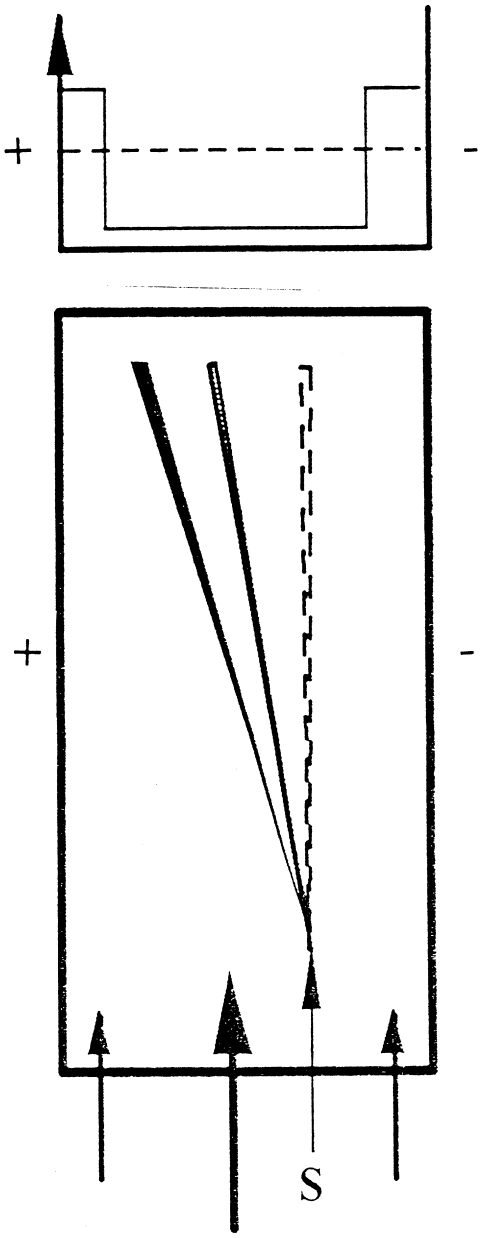

Fig. 2. Scheme of conventional FFZE. Edged by two margin buffers, one homogeneous medium is pumped through the central chamber segment. The sample (S) is introduced as a sharp band, but is diluted on passage through the chamber while separated by the electrical current. The small upper scheme shows the pH (dashed line) and conductivity (solid line) profile obtained by analysing the buffer leaving the chamber.

strength medium for reasonable electrophoretic mobility [9]. Proteins, peptides, and to some degree subcellular particles, were fractionated and purified with much higher resolution, when separated by band focusing techniques such as FFZE in pH gradients, IEF or ITP [7,8,11,12]. During IEF the electric current moves analytes through pH gradients which may be established linearly by ampholytes or Octolytes under the influence of the electrical current (Fig. 3, left panel) or stepwisely by several adjacently flowing media (Fig. 3, right panel). If a protein or peptide reaches a medium, which has a pH identical to their respective p*I* (isoelectric medium), it becomes neutral and does not move under the influence of the electrical current. If macromolecules have different p*I*s, they will focus on different chamber areas and can be harvested to different vials. If band deteriorating forces, active within the FFE chamber, drive a macromolecule out of the isoelectric medium, the electric current will counteract. Thus bands as sharp as the isoelectric areas are formed independently whether a sample was applied via a sample inlet (Fig. 3, left panel) or dissolved in one of the segmented chamber media (Fig. 3, right panel).

Although high resolution was achieved, when IEF was performed within an FFE device, many proteins could not be processed because they had a high tendency to precipitate and/or aggregate when entering isoelectric medium. For these instances FFZE through pH gradients was developed starting from these considerations: if an electrophoresis chamber is filled with a separation medium, whose pH decreases linearly or stepwisely from anode to cathode as indicated in Fig. 3, and a macromolecule like a protein is injected as a sharp band into a medium either more acid or more alkaline as the isoelectric medium, it will move to its counterelectrode. As it approaches isoelectric medium where it would become totally immobile, its mobility already starts to decrease, before it reaches the area of the chamber where the medium has the pH of its p*I*. The closer a macromolecule comes to its isoelectric medium, the stronger it is retarded [19]. So molecules migrating at the front of a band will already be retarded while those at its rear close up. Thus sample components may be concentrated and focused to sharp bands in a similar way as described for field-step electrophoresis [15,16] before they enter their isoelectric medium and start to precipitate or aggregate.

Table 1
Features of the various electrophoresis techniques applicable to the OCTOPUS

FFE-technique	Separation parameter	Resolution	Maximal sample capacity (mg/h)	Minimal sample capacity (μl)	Transit time (min)	Applications
FFZE						
in homogeneous medium	EM	<+3% EM	500	10	1–10	[a]
pH-gradient	(EM)/p*I*	(<+1% EM) +0.01 p*I*	300	10	3–12	[b]
IEF	p*I*	+0.02 p*I*	150	50	7–23	[c]
ITP	EM	<+1% EM	500	10	4–20	[d]
FSE	EM	+30% EM	5000	200	0.5–5	[e]

[a] Cells, organelles, membranes.
[b] Proteins, peptides, viruses, organelles, membranes.
[c] Proteins, peptides, (viruses, organelles, membranes)
[d] Organelles, membranes, viruses, proteins, peptides.
[e] Generally applicable for conditioning of sample and enhancement of component concentrations.

Verifying these considerations, the FFE chamber was equilibrated with a buffer system suitable for IEF through a stepwise pH gradient (Fig. 3). The specimen was introduced as a sharp band near the non-attracting electrode, so that the analytes migrate towards the opposite electrode. In contrast to IEF the speed of the medium flow was enhanced so that the proteins were carried through the chamber before they reached their isoelectric media but were still 0.2 to 0.5 units apart of this site. Thereby protein mixtures were fractionated at high resolution, while the macromolecules remained dissolved in medium with a pH favouring their solubility and biological activity [11]. Furthermore, due to the faster flow of the medium, higher throughput was possible during this advanced type of FFZE than during IEF.

Many organelles and membrane vesicles may tolerate more pH variations than whole cells, but cannot be dissolved in media with pH similar to their p*I*. Still, it is desirable to separate them at high resolution while keeping them at elevated concentration. In these instances ITP may be a method of choice, because it has also band sharpening properties [4,8]. ITP is an efficient but rather complicated electrophoretic technique, which may be applied as anodal as well as cathodal regime. It needs segmented buffer systems, which, for an anodal ITP system consist of anodal margin medium, leader medium, one or more spacers and terminator medium (Fig. 4). It was performed in the Octopus instrument, filling electrophoresis chamber with an ITP buffer system, applying electric current and introducing the sample of organelles dissolved in a spacer medium as indicated by S of Fig. 4[8]. Within the electric field the medium boundaries moved towards the anode at the speed of the leader-anion with the highest mobility (chloride). Simultaneously, the anions of the system lined up between the leader ion and the terminator ion according to their respective mobilities. Since suitable spacers with anions with mobilities faster and slower than the various populations of organelles were applied, three populations were separated so far that preparative fractionation was possible. Two of them were concentrated at boundaries between the various spacers because their electrophoretic mobilities ranged between the mobilities of two spacer anions, the third one was diluted within a spacer medium [8] due to equality of anionic mobilities.

2.4. Rationales for applying either interval or continuous flow electrophoresis

The electrophoresis modes ITP and FFZE through pH gradients proved very helpful in sharpening the bands and increasing resolution while macromolecules or organelles must not be dissolved in media with detrimentous pH values. If these modes were applied for continuous separation, the problem often arose that the band sharpening forces, inherent to the

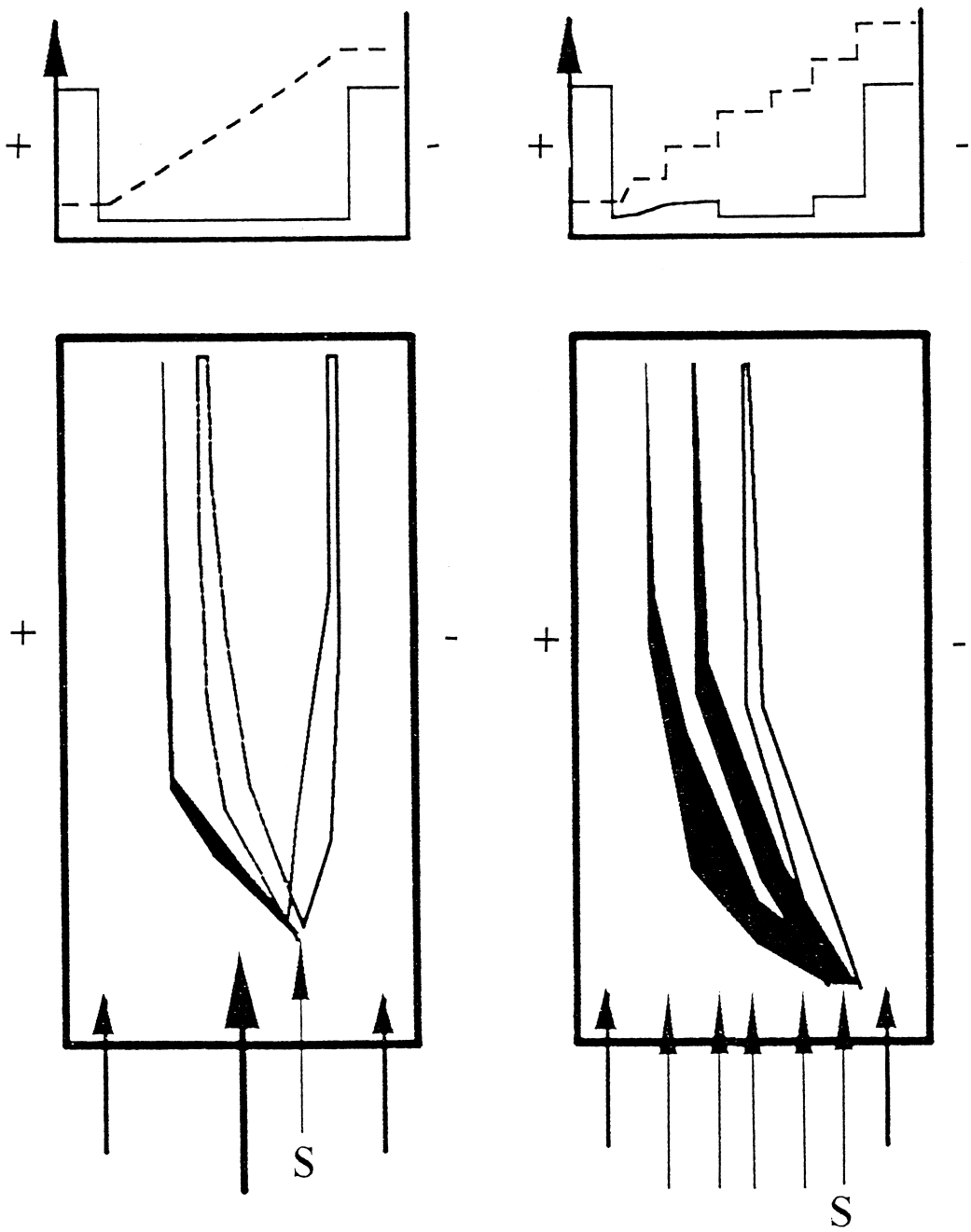

Fig. 3. Scheme of IEF in linear (left panel) and stepwise (right panel) pH gradients. For establishing a linear gradient one medium buffered by Octolytes was introduced together with both margin buffers into the chamber. The pH gradient is formed under the influence of the electrical current within a short distance after the application point. For establishing a stepwise gradient five central media and two margin fluids are pumped simultaneously through the chamber. S indicates the sample introduced either as a sharp band or dissolved in the most cathodal chamber medium. The small upper scheme shows the pH (dashed line) and conductivity (solid line) profile obtained by analysing the buffer leaving the chamber.

Fig. 4. Scheme of ITP. Anodal margin buffer, leader medium (bold arrow) spacer fluids and terminator medium (=cathodal margin buffer) are introduced into the chamber as indicated. S indicates the sample dissolved in the most cathodal spacer medium. The small upper scheme shows the pH (dashed line) and conductivity (solid line) profile obtained by analysing the buffer leaving the chamber.

respective mode, were not strong enough to overcome the hydrodynamic band broadening effects. In these instances a possibility to eliminate hydrodynamic sample distortion forces was to perform interval electrophoresis, i.e. to turn off the medium flow and simulate a static column [20], while samples were electrophoresed in the FFE chamber [21]. For interval electrophoresis the chamber was equilibrated by a medium optimal for dissolving and electrophoresing the specimen. Then, as the medium still was flowing, the sample was injected into the chamber, where it was carried by the flowing chamber medium until its tip reached the farther end of the chamber. When a sample whose volume could vary from 10 to 250 μl had been injected, the fluid flow was turned off and an electric voltage of 1000 to 1300 V was applied for 3 to 7 min. As soon as the components of a sample had been separated so far that fractionation via different outlets was possible, the current was turned off and the fractionated specimens were washed out while new sample material was injected for the next electrophoresis process. Many of these experiments have proved that a tremendous increase of FFZE and ITP resolution is achievable by this so-called interval electrophoresis [11].

However, during interval electrophoresis the continuous sample loading and unloading processes are interrupted for the period of current application and in one experiment, which takes between 8 and 10 min, only 10 to 200 μg can be processed. In order to increase this low throughput, it is necessary to line up several such interval FFE cycles. Lining up 20 and more cycles of FFE experiments became possible after automation of the process (Bocek and Weber, manuscript submitted). The computer control of all parameters accurately ensures that strength and application periods of voltage is equal in each experiment and equal components are repeatedly collected to equal vials.

3. Fractionation of plant membranes: the predominance of zone electrophoresis

Because they define compartments, plant membranes play a vital role in many important aspects of cellular functions. Membranes are effectively respon-

sible for regulating the influx and efflux of ions and metabolites [22], in directing the movement of newly synthesized proteins [23], or in forming the mitochondrial and plastidial electron transfer chains [24]. Membranes also play more specialised roles in cellular communication [25,26], perception and transduction of signals [27], cell–cell recognition and adhesion either to other cells or to cell–wall components [28,29]. All these functions are either vectorised (membranes determine the direction of all communication between the inside and outside through the lipid bilayer) or take place at a specific face of the bilayer (membranes house a series of proteins facilitating interactions for specific reactions). All types of membranes are then crowded pieces of biochemical equipment requiring a complex organisation. So, the demands put on membrane fractionation continually increase. In the first place, they concern:

- the accurate separation of membranes (as well as their subdomains which would exhibit different structural and functional domains),
- the quantity of the isolated membranes (at hand will enable us to isolate membrane proteins of interest, to raise antibodies, or to facilitate the cloning of genes for membrane proteins).

3.1. Electrophoretic procedures applied for fractionation of plant membranes so far

Facing these demands scientists started to apply electrophoretic techniques on purifying plant membranes and membrane components long before the FFE technique has been developed as far as described above (Section 2). Only two reports concern isoelectric focusing [30,31]. The method has allowed the discrimination of membranes from oat root homogenates, etiolated pea stems and *Fucus* embryos. The isoelectric focusing of *Catharanthus roseus* protoplasts was also performed [30]. From pea stems, two subfractions of putative plasma membrane were obtained: the first subfraction was present at a p*I* value of 4.4, the second focused to a p*I* of 4.0. These two populations of vesicles were analysed as the opposite orientations of the plasma membrane, i.e. cytoplasmic side in and cytoplasmic side out vesicles [30]. However, the use of this separating regime has not found large applications in plant laboratories yet. Two types of drawbacks severely impaired the method: (i) once the membranes reached their isoelectric points, they have the high tendency to aggregate; (ii) membranes strongly interact with the commercialised ampholytes. Finally, only small quantities of purified membranes were obtained [31]. In contrast, FFE is the experimental basis for many studies. Two different electrophoretic buffer systems, namely triethanolamine–acetic acid [32] and Tris–borate-based media [33] were designed, and in each case the separating regime was zone electrophoresis. The FFE procedures were also successful for separations involving animal membranes as well [34]: for most cases, the separating regime was again zone electrophoresis, and a triethanolamine–acetic acid medium used as electrophoresis buffer.

The first application of FFE to separate plant membranes utilised a triethanolamine–acetic acid buffer system with a microsomal membrane fraction of soybean hypocotyls as starting material [32]. The electrophoresis buffer contained 0.25 *M* sucrose, 2.0 m*M* KCl, 10 m*M* triethanolamine, 10 m*M* acetic acid, pH 7.4 (adjusted with NaOH). The electrode buffer contained 100 m*M* triethanolamine and 100 m*M* acetic acid, pH 7.4 (adjusted with NaOH). Recently, the same buffers were used to fractionate the microsomes of *Arabidopsis thaliana,* except the concentration of KCl (10 m*M*) [5]. Table 2 shows the optimum electrophoretic conditions for the separations of microsomal membrane from soybean hypocotyls and *Arabidopsis thaliana* seedlings. The parameters shown in Table 2 are those to modify in order to obtain the best separation, together with the concentration of potassium ions which may vary in the range of 1 to 15 m*M* in order to narrow the membrane streams during electrophoresis. If material migrates in an electrophoretic buffer system without any salt present, the material usually separates very broadly with Gaussian curves overlapping. Upon addition of increasing amounts of a suitable salt (e.g. potassium chloride), the material will separate more and more accurately. However, at very high concentration of salts little current is still available for the migration of material, in other words there is no more separation.

Before the run, the glass plates of the electro-

Table 2
Optimum free flow electrophoretic conditions for separations of microsomal membranes from soybean hypocotyls and *Arabidopsis thaliana* seedlings[a]

	Soybean hypocotyls	Arabidopsis seedlings
Membrane sample injection	1.0 ml/h	1.5–2.5 ml/h
Electrophoresis buffer flow	3.0 ml/h/fraction	4.0 ml/h/fraction
Chamber temperature	6°C	4–5°C
Electric field strength	1150 V at 240 mA (~12 V/cm)	900 V at 160 mA (~9 V/cm)

[a] The electrophoretic buffer system was a triethanolamine–acetic acid based medium.

phoresis chamber could be coated with albumin by filling the chamber with a solution of 3% (w/v) bovine serum albumin, allowing it to stand for 1 h and washing the chamber with 2–3 volumes of electrophoresis buffer [34]. Also, the microsomal membranes were washed in electrophoresis buffer as the presence of extraneous ions may interfere with the separation [1]. In this way, any kind of protectant may be introduced during microsomal membrane preparation: however, protectants modifying the electrical (salts) or the viscosity (glycerol) properties of the electrophoresis buffer should be avoided at this stage.

FFE was also applied to separate plant membranes with a Tris–borate-based medium [33]. In this work dealing with a microsomal fraction from *Catharanthus roseus,* the electrophoresis buffer contained 0.25 *M* sucrose, 11 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* Tris and 10 m*M* boric acid (pH 8.3). The electrode buffer contained 100 m*M* Tris and 100 m*M* boric acid (pH 8.3). A modified electrophoresis buffer was used for microsomes prepared from carrot cell suspension cultures [35]: 0.3 *M* sorbitol, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 30 m*M* Tris and 10 m*M* boric acid (pH 8.3). Table 3 shows the optimum conditions for separations of the two plant materials. As before, the concentration of potassium ions may vary depending on the plant material. In addition, in this particular electrophoretic system, the divalent cations (Mg^{2+} or Ca^{2+}) are determinant in establishing the separation: they clearly influence the migration of some vesicle populations during electrophoretic migration. Ca^{2+} and Mg^{2+} certainly act as structural elements in membrane architecture, and at low concentrations function as stabilisers, while at high ones as rigidifying agents. The interactions between phospholipids and metal ions has received considerable attention [36] and it is clear that they bind to the surface of phospholipid vesicles. For instance, plasma membrane vesicles isolated from barley, wheat and oat roots have both Ca^{2+} and Mg^{2+} bound to their apoplastic surface [37,38]. In this way, it is feasible that metal ions form electrostatic bridges either between neighboring anionic phospholipids, or between the latter and membrane proteins, or with cytoskeletal elements [39]. Probably, the divalent cations can also bridge between to closely apposed membrane surfaces and cause membrane aggregation, which is a very undesirable effect in electrophoretic separations.

Each purification procedure of a particular membrane presents unique preparative problems. Apart from the electrophoretic conditions being optimised, one must consider the optimal procedures for cell disruption, i.e. the preparation of a microsomal fraction. Enriched in the microsomal fraction, the

Table 3
Optimum free flow electrophoretic conditions for separations of microsomal membranes from *Catharanthus roseus* and *Daucus carota* cell suspension culture[a]

	Catharanthus roseus	*Daucus carota*
Membrane sample injection	1.5–2.5 ml/h	2 ml/h
Electrophoresis buffer flow	3.7 ml/h/fraction	3.0 ml/h/fraction
Chamber temperature	4–5°C	4°C
Electric field strength	900 V at 90 mA (~9 V/cm)	1200 V at 110 mA (~12 V/cm)

[a] The electrophoretic buffer system was a Tris–borate based medium.

membrane of interest would be easier to purify. A population of vesicles need to be present at a level of 5% of total membrane proteins to be clearly visualised after separation from the bulk of membranes. The pH effect of grinding medium noted for the purification of plasma membrane and tonoplast from *Catharanthus roseus* cells is a relevant example [33]: when the cells were ground in an acidic medium, the relative amounts of plasma membrane and tonoplast were considerably enlarged in the microsomal fraction (up to 30% of total membrane proteins). Grinding the cells in an acidic medium is thought to cause membranes (except plasma membrane and tonoplast) to precipitate during the differential centrifugation steps. Consequently, the time for the electrophoretic purification is shorter.

The two electrophoresis buffer systems described above appear very versatile since, with small modifications of their basic compositions, many types of organelle and membrane have now been successfully isolated and purified from a variety of plant tissues.

3.2. Organelles: intact chloroplasts

Isolated organelles offer some advantages as an experimental system for plant membrane studies, one of which is direct access to the specific proteins and corresponding functions of the outer membrane leaflet. On the other hand, it is often preferable to isolate intact organelles even if these are subsequently ruptured to obtain one of the membrane components.

In this way, FFE allowed the purification of intact chloroplasts from spinach and squash ([40,41], see also [42,43] for preliminary assays). From a crude chloroplast preparation obtained by a simple one-spin procedure, two main green populations have been separated and characterised as intact chloroplasts and free thylakoïds systems [40]. A third fraction free of chlorophyll consisted in cell wall particles and mitochondria [41]. FFE also separates intact from damaged chloroplasts from recalcitrant species such as conifers [44]. For instance, pine chloroplasts isolated by conventional purification procedures (i.e. based on density gradient centrifugations) did not show the Hill reaction [45]. Such a difficulty in obtaining intact chloroplasts from conifers needles was referred to the resins which are essentially composed of monoterpenes [46]. When subjected to FFE a crude chloroplast suspension freshly obtained from spruce *(Picea abies)* needles led to a two-peak distribution. The two populations could not be distinguished according to their proteins (chlorophyll ratios and protein patterns from 2-D gel electrophoresis). The O_2 evolution, however, showed differences: the chloroplast population deflected towards the cathode contained a major part of structurally intact chloroplasts in contrast to the population deflected towards the anode. A high percentage of intact chloroplasts (80%) was calculated for the cathodic fraction [44].

In each case, the separating regime was zone electrophoresis. When the electrophoretic buffer system was triethanolamine–acetic acid, the intact chloroplast fraction migrated toward the cathode [41,44]. In spinach [40], the intact chloroplast fraction deviated towards the anode, probably due to the different buffer conditions (Tris–citrate).

Some of the inherent properties of the FFE technique are enlightened in these studies. The technique is rapid since in all experimental conditions used, the chloroplasts are collected about 5 min after their injection. The separation can be continuously performed starting from a crude fraction, since the distribution of material is constant throughout electrophoresis. The chloroplasts can be kept in the same isoosmotic medium from grinding to collecting in the analysis tube. Because chloroplasts are relatively large and bound by two membranes, they are sensitive to shear, hypo- and hypertonicity. Thus, rapidity and isoosmotic media are crucial requirements for the purification procedures. In addition, the electrophoresis does not alter the physiological properties of chloroplasts: the CO_2 dependent O_2 evolution, which is a criterion of the intactness of chloroplasts, was found similar on preparations obtained after or without FFE [40]. The percentage of intactness of squash chloroplasts as calculated from O_2 evolution was 100% [41].

Because the FFE technique exploits differences in particle surface charges, the separations of chloroplasts permitted the testing of different extraction media or compounds supposed to act on their surfaces. This is well illustrated by the effect of monoterpenes on squash chloroplasts [41]. Detergents and polyamines, which cause differential

solubilisation of membrane proteins or alter surface charges, affect the relative position of fractions during electrophoresis. In contrast, there was no change on the position of the fractions upon addition of monoterpenes. However, the treatment leads to disappearance of the intact chloroplast fraction which is in direct relation to monoterpene concentration. The degradation of chloroplasts by monoterpenes yields thylakoïds (stacked or free) which are combined with the damaged chloroplast fraction, leading to an increase in chlorophyll content of this fraction. The mechanical disruption of the envelope, either by sonification or freezing, yields preparations containing unstacked thylakoïds. The electrophoretic pattern is then similar to that after addition of monoterpenes.

The field of organelles isolation by FFE was not extended to other plant subcellular particles (mitochondria, Golgi,...). However, FFE procedures provided nice animal cell fractionations. Lysosomes and mitochondria [47], mitochondria and their membranes [48] were reported early. Functionally and structurally distinct subpopulations of endosomes can be resolved [49–51]. Also, cisternae of rat liver Golgi apparatus are resolved into a series of fractions by FFE: subfractions nearest the anode are enriched in the *trans*-face elements while fractions nearest the cathode are enriched in the *cis*-face elements [52].

3.3. Membrane vesicles: plasma membrane and tonoplast

The efficiency of FFE in isolating subcellular particles found its largest application field with the purification of plasma membrane and tonoplast. The two membranes occupy unique places in the plant cell, delimiting the cytoplasm from its environment: either the external medium or the vacuolar sap. Therefore, considerable efforts were made to obtain pure preparations of these membranes from numerous plant tissues and species.

A typical distribution profile for membrane vesicles (absorbance, OD 280 nm) is shown in Fig. 5. It concerns an electrophoretic separation of microsomal membranes prepared from maize roots. The membrane suspension was injected into the separation chamber above fraction 5 (near the cathode), since all membrane material was deflected towards the anode. It indicates that most, if not all, membranes carry a net negative charge at neutral pH. Consistent with this observation, the isoelectric point of membrane surfaces (plasma membrane, mitochondria, chloroplast) was found in the range of pH 3.6–5.4 [53,54]. A major peak (fractions 19–26) was revealed with a minor peak on either side, one towards the cathode (fractions 8–14) and one towards the anode (fractions 29–33). As for chloroplasts, the membrane distribution was qualitatively reproducible and constant throughout electrophoresis. Usually, the material eluted from the FFE instrument was gathered in five pools. Pool 1 was identified as plasma membrane and pool 5 as tonoplast. Pool 3 contains the bulk of ER, mitochondria and Golgi membranes. So, the electrophoretic procedure simultaneously yields

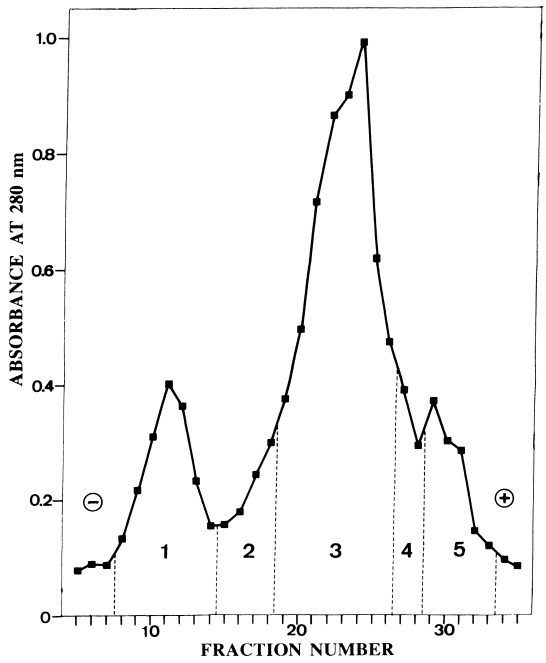

Fig. 5. Absorbance at 280 nm of fractions obtained by free flow electrophoresis separation of total microsomes from maize roots. The microsomes were injected into the separation chamber near the cathode; thus, all membrane material was deflected towards the anode. The regions numbered 1–5 represent pooled fractions. The electrophoretic buffer was a triethanolamine–acetic acid based medium identical to that used for *Arabidopsis thaliana* seedlings (see text). The separation was carried out under conditions of constant amperage 170 mA, 1000±20 V, buffer flow 4.5 ml, fraction^{-1} h^{-1}, sample injection 2 ml h^{-1} (2.4 mg protein ml^{-1}) and a constant temperature of 4°C.

tonoplast and plasma membrane, one resolved from the other, from the same homogenate.

A compilation of the plant tissues and cells where FFE has been used to isolate plasma membrane and tonoplast vesicles are found in Table 4. A general feature of these membrane separations is that whatever the electrophoresis buffer used and whatever the plant species studied, the technique of FFE separates microsomal membranes by providing a three-peak distribution. For all plant species investigated so far, the least electronegative fraction (pool 1) consisted of plasma membrane and the most electronegative fraction (pool 5) consisted of tonoplast.

It is clear that the electrophoretic mobility of tonoplast vesicles is greater than that of plasma membrane vesicles. But, the chemical nature of the group responsible for confering these charge differences is poorly understood. The net electronegative charge is due to the surface-exposed polar groups of intrinsic and extrinsic membrane constituents. The cationic groups such as protein–amino groups, the anionic groups such as the carboxyl groups of glutamic and aspartic acid residues on the proteins, the lipid- and protein-associated phosphate groups, as well as the carbohydrate chains of membrane glycoproteins are all believed to contribute to the surface charges. The plant plasma membrane and tonoplast have different protein [33], as well as different lipid profiles [66,67], but the relevance of these to the individual electrophoretic mobilities of the two membranes remains unknown.

On the other hand, plasma membrane and tonoplast probably possess very large lateral heterogeneities: as a consequence different vesicles may be produced with different electrophoretic mobilities. A vesicle containing different subdomains will migrate at one definite position, i.e. that of an average value of different surface charge densities. A vesicle representative of a specific subdomain will migrate at a different position. Additionally, membranes have a transversal asymmetry: depending upon the leaflet facing the external medium, the membrane will produce vesicles with different electrophoretic mobilities (see Section 4.1). Finally, the net negative charge on a membrane depends on the concentration and type (mono-, di- or trivalent) of

Table 4
Examples of free flow electrophoretic procedures for the purification of plasma membrane and tonoplast vesicles from various plant tissues and cells

Glycine max	– etiolated hypocotyls	[32]
	– etiolated hypocotyls (plasma membrane vesicles of opposite orientation)	[55]
	– etiolated hypocotyls (tonoplast vesicles of opposite orientation)	[56]
Catharanthus roseus	– suspension cultured cells	[33]
Arabidopsis thaliana	– suspension cultured cells and seedlings	[5]
Daucus carota	– suspension cultured cells	[35]
Vitis vinifera	– suspension cultured cells	[57]
Eucalyptus gunnii	– suspension cultured cells	[58]
Bryonia dioica	– internodes	[59]
Nicotiana tabaccum	– pollen tubes	[60]
	– suspension cultured cells	[61]
Triticum aestivum	– green leaves	[62]
Picea abies	– needles	[63]
Fagus sylvatica	– green leaves	[63]
Curcubita pepo	– etiolated hypocotyls	[64]
Lepidum sativum	– roots	[64]
Spinacia oleracea	– green leaves	[65]
Aclya ambisexualis	– fungus mycelium	[66]
Zea mays	– roots	This paper

cations in the medium. By attracting cations and repelling anions, membrane vesicles may have a modified electrophoretic mobility (see Section 3.1). These considerations increase the complexity of separation in electromigration techniques. However, the FFE procedures have showed that the differences in electrophoretic mobilities of the membrane vesicles are well reproducible: after a first electrophoretic run, a recovered population of vesicles will migrate in the same fraction during a second run [5].

During FFE experiments, the mixture of membranes to separate is continuously injected into the chamber (4 to 8 mg protein/h). As the distribution of membrane is fairly constant, long time separation can be run. For instance, a 4 h run will produce about 2 mg membrane proteins of pure plasma membrane and 1 mg membrane proteins of pure tonoplast. Overnight separations (15 h) can be managed [33]. The electrophoretic procedures then reached the preparative scale.

Reasonable amounts of pure membrane fractions are often desirable to start a biochemical description of their components, i.e. either lipid or protein compositions [33,66,68], as well as the characterisation of their functions (transporters, receptors, ...) [64,69–71]. For instance, the identification of a tonoplast protein involved in malate transport [72], which is only a minor component of the tonoplast required 15 mg of purified tonoplast protein, obtained from 500 g of *Catharanthus roseus* cells. The preparative scale is also necessary when antibodies against membrane proteins want to be raised. Very recently, the preparation of antibodies against total membrane proteins was performed from purified *Arabidopsis thaliana* plasma membrane and tonoplast fractions [73]. These antibodies were used for the immunoscreening of a high efficiency expression library of *Arabidopsis thaliana* cDNAs. With the positives clones, two cDNA ordered libraries enriched in genes encoding plasma membrane (522 clones) and tonoplast proteins (594 clones) were constructed. A high proportion (45%) of the clones encoded putative membrane proteins, or membrane-associated proteins. When sequenced, 32% of the cDNAs were not referenced in any databank. The methodology described above can be easily applied to other membranes, provided that they were obtained in sufficient amount and in a pure state. The FFE procedure was effectively decisive in this study.

4. The isolated membrane vesicles: evaluation of membrane purification

Once isolated membrane vesicles should satisfy several criteria to be useful. The demands put on cell fractionation then concern:

- the purity of the isolated membranes (components known to be present uniquely or predominantly in the membrane being purified needs to be assayed),
- the orientation of membrane vesicles (one must consider the potential problem of enzyme latency, if an enzyme is inside of a vesicular membrane, it is unable to interact with its substrate),
- the functional and structural integrity (for the function studied, how close the isolated membrane vesicles from the native status?).

4.1. Purity and orientation of membrane vesicles

For both plasma membrane and tonoplast, fractions purities of around 95% can be obtained with FFE procedures. From the studies reported (see Table 4), most of the assessments of membrane purity was to assay marker enzymes, i.e. the presence of positive markers and the relative absence, compared to the microsomal fraction, of negative markers. However, the markers for plant intracellular membranes are not absolute markers, and a minor contamination is not easy to determine. Other features used to evaluate a membrane preparation include morphology [74] or cytochemical stains [75] as determined by electron microscopy, and antibodies [5]. For instance, the plasma membrane stains positively when exposed to phosphotungstic acid (PTA) at low pH [76]. The stain is highly specific for the plasma membrane, and has the additional great advantage that the actual percentage of plasma membrane in a fraction can be determined by calculating the number of stained versus unstained membranes. Based on these criteria, the purity of plasma membrane was 97% (morphology and PTA staining [32]) or 89% (filipin label [64]) for soybean

hypocotyls, 87% (morphology and PTA staining [65]) for spinach leaves, 84% (filipin label [64]) for cress roots, 88% (PTA staining [62]) for wheat leaves. The reactivity of antibodies directed against tonoplast and plasma membrane proteins confirmed the nature of the isolated membranes, and revealed no cross-contamination between the two membranes [5,56].

However, all the intracellular membranes do not have a clear definite marker [77]: peroxisomes and glyoxisomes membranes as well as that of vesicles involved in intracellular trafficking have never been localised in FFE separations. Membrane lateral heterogeneity may induce a non-uniformly distribution of a marker. A marker may be absent in certain cell types, tissues or species: fucosyltransferase, a reliable marker for Golgi membranes, is lacking from members of the Cruciferae family [5,78]. So, great care should be taken to present the purity assessments and to conclude, for instance, to the location of a particular component.

FFE separates vesicles of opposite orientations for both tonoplast and plasma membrane [55,56,79]. Indeed, FFE yielded two plasma membrane fractions from microsomes of soybean hypocotyls [55]. One, the least electronegative was pure plasma membrane. The other, more electronegative but closed to the first one, was heavily contaminated by various other cellular membranes. When plasma membrane vesicles are first purified by aqueous two-phase partition [80] and then subjected to FFE, two fractions were also yielded. Each fraction exactly migrate, inside the electrophoresis chamber, the same position than the two plasma membrane fractions found from total microsomes. The orientation of both plasma membrane fractions was determined using ATPase activity as a structure-linked latency marker for the inner membrane surface, and Concanavalin A binding (linked to peroxidase) as an imposed electron microscope marker for the outer membrane surface. The least eletronegative subfraction of plasma membrane consisted of cytoplasmic side out vesicles, and the other fraction consisted of cytoplasmic side in vesicles. Similar findings were reported for the tonoplast [56]. So, FFE used in series with either aqueous two-phase partition (first purification step of plasma membrane) or density gradient centrifugation (first purification step of tonoplast) led to the resolution of a total of four different fractions. Each fraction is a highly purified membrane preparation of apparently homogeneous vesicles of opposite known absolute orientations. These results underly the efficiency of FFE for separation of membrane vesicles.

In the previous studies, the membrane material eluted to the electrophoresis chamber in the two fractions containing vesicles of opposite orientation varies according to the grinding procedure of the plant tissue [55,56]. In the two peak distribution, the relative amounts of each type of vesicles was found in the ratio 80–20% or vice versa, but never in the ratio 50–50%. It appeared then that the vesiculisation process after the membrane breakage is not at random. Consequently, the determination of the absolute orientation of vesicles in the minor peak could be difficult. In certain tissues or cell cultures [62,79] or in photoinduced leaves of spinach [65], the plasma membrane is asymmetric as revealed by electron microscopy: the leaflet facing the cell wall is thicker (electron density) than the leaflet facing the cytoplasm. While only few vesicles (5–10% in plasma membrane from wheat leaves [62]) exhibited this natural polarity, those that did, reflected the absolute orientation of the population of membrane vesicles. FFE proved that the charge surface densities of vesicles of opposite orientation are clearly different. While in soybean hypocotyls the least electronegative fraction of plasma membrane consisted of cytoplasmic side out vesicles [55], in wheat leaves the same fraction consisted of cytoplasmic side in vesicles [62]. The authors concluded that the electrophoretic mobility cannot be the only criteria to assign absolute orientation for one membrane type.

4.2. Integrity of membrane vesicles: functional and structural aspects

Once purified the membranes have been submitted to the severe trauma of cell disruption and have spent several hours to centrifugal forces and few minutes to electric fields. The question arises to which extent the vesicles retain their native structures and their functions. One approach to characterise the intactness of the isolated vesicles is to probe their permeability with molecules that selectively penetrate the membrane barrier. As membrane operates dif-

ferentially on the two compartments it separates, vesicles of known orientation is often desirable.

A recognised criterion to assess the degree of sealing of isolated vesicles is their ability to create and maintain a pH gradient across the bilayer [81]. Both the plasma membrane and the tonoplast vesicles obtained by FFE from *Catharanthus roseus* cells presented proton-pumping activities. However, the two membranes have marked differences in the proton permeabilities: the tonoplast is relatively impermeable to protons (the half-time for equilibration of a pH gradient is around 20 min) compared to plasma membrane (half-time of 4 min) [33]. FFE purified tonoplast vesicles from *Catharanthus roseus* cells were also used in malate transport studies. The characteristics of the uptake appeared to be very similar to that of isolated vacuoles, the native organelles [69]. The use of these vesicles revealed that a histidine residue performs an essential function in the malate transporter at the active site of the protein, providing a structural basis for the pH dependence of the malate transport system [70]. Finally, the transporter probably exists in the tonoplast as an homotetramer [82]. Taken as a whole, these elements argue for a certain integrity of membrane vesicles isolated by FFE.

Another example is provided by the water permeability of tobacco membranes [61]. For each FFE purified fraction (tonoplast and plasma membrane), the osmotic water permeability coefficients were determined by stopped-flow light scattering. The tonoplast vesicles were characterised by a 100-fold higher permeability coefficient than that of the plasma membrane vesicles. These results provide functional evidence for channel-mediated water transport in the tonoplast, and suggest a role for the vacuole in buffering osmotic fluctuations occurring in the cytoplasm. Because the two subcellular membranes were simultaneously purified from the same homogenate and retained their biological functions, FFE was a key process in the study. The biophysical properties of FFE isolated membranes are also preserved. Two cell lines of *Eucalyptus gunnii* with different frost tolerances were used to investigate the fluidity of plasma membrane and tonoplast in relation with frost resistance [58]. The lateral and rotational mobilities of lipids in the two membranes were studied by fluorescence recovery after photobleaching and fluorescence polarisation. The results showed that, intrinsically, tonoplast exhibited a higher fluidity than plasma membrane, and that a correlation between membrane fluidity and cold tolerance exists.

Likewise, ligand-binding activities were evidenced after FFE fractionation of *Arabidopsis thaliana* membranes [83]. The study relies on the attachments that exist between the plasma membrane and the cell wall [84,85]. They are thought to be involved in a large variety of plants processes such as developmental events, plant microbe intractions, mechanoperception and adaptation to stress [86]. The purified plasma membrane from *Arabidopsis thaliana* cells was the only one membrane fraction to exhibit specific binding sites for a peptide containing the sequence Arg–Gly–Asp (RGD – the essential structure recognised by animal cells in substrate adhesion molecules). Binding is saturable, reversible and two types of high affinity sites (K_{d1}~1 n*M* and K_{d2}~40 n*M*) can be discerned. These results provide evidence for a functional receptor at the apoplastic face of the plasma membrane vesicles. Closely connected is the purification of actin, a cytoskeletal protein, with the *Arabidopsis thaliana* plasma membrane [5]. Indeed, in animal cells, the adhesion receptors establish transmembrane links between the extracellular matrix and the cytoskeleton. So, the presence of actin with the plasma membrane of *Arabidopsis thaliana* shows that these vesicles are not only a lipid bilayer with integral membrane proteins or directly adsorbed proteins, but rather a complex network of interactive proteins.

5. Concluding remarks

Remarkable achievements with FFZE procedures have been accomplished in the purification of membranes from a large variety of plant species and in the separation of membrane vesicles of opposite orientation. FFZE procedures proved very suitable to manage large quantities of membrane material and to preserve the functions of the isolated membranes. However, some limitations prevent FFZE to reach new fields of investigation. This is essentially due to the very small differences in the electrophoretic mobilities of most cell membranes [2,30,37,38] and

to the intracellular plant membrane system, which is both structurally complex and dynamic with a traffic of membrane vesicles compelling the system to be monitored and repaired.

Given the importance of cell fractionation procedures to start a study on membrane-dependent functions, it is comprehensive and very appropriate to improve these procedures or to define new ones. Introducing immune free flow electrophoresis (IFFE) to plant biochemistry as recently reported for the isolation of peroxisome subpopulations from rat liver [87], may be one way of improvement. The method cleverly combines the advantages of electrophoretic separation (FFZE regime) with the high selectivity of an immune reaction. The expression of different antigens has already been used for the purification of organelles and membrane vesicles [88], and the strategy using antibodies should be widely applicable. But for the fractionation of plant membranes, one of the newly developed FFE techniques such as FFZE and ITP in interval or continuous mode and IEF or FSE may be selected. All are applicable to a modern device and can render its resolution capability comparable to that of capillary electrophoresis or high pressure liquid chromatography. This will surely help to further expand our knowledge in cell membrane biology.

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References

[1] R.B. Gennis, in: R.B. Gennis (Ed.), Biomembranes: Molecular Structure and Function, Springer Verlag, New York, 1989, p. 1.
[2] K. Hannig, H.G. Heidrich, Methods Enzymol. 21 (1974) 746.
[3] K. Hannig, Electrophoresis 3 (1982) 235.
[4] L. Krivankova, P. Bocek, Electrophoresis 19 (1998) 1064.
[5] N. Bardy, A. Carrasco, J.P. Galaud, R. Pont-Lezica, H. Canut, Electrophoresis 19 (1998) 1145.
[6] R. Kuhn, S. Hoffstetter-Kuhn, H. Wagner, Electrophoresis 11 (1990) 942.
[7] V. Kasicka, Z. Prusik, O. Smekal, J. Hlavacek, T. Barth, G. Weber, H. Wagner, J. Chromatogr. B 656 (1994) 99.
[8] G. Weber, P. Bocek, Electrophoresis 17 (1996) 1996.
[9] B. Bondy, J. Bauer, I. Seuffert, G. Weber, Electrophoresis 16 (1995) 92.
[10] J. Bauer, G. Weber, Electrophoresis 17 (1996) 526.
[11] G. Weber, J. Bauer, Electrophoresis 19 (1998) 1104.
[12] G. Weber, P. Bocek, Electrophoresis 19 (1998) 1649.
[13] N.E. Good, S. Izawa, Methods Enzymol. 24 (1972) 53.
[14] M. Bier, J. Ostrem, R.B. Marquez, Electrophoresis. 14 (1993) 1011.
[15] S. Hoffstetter-Kuhn, R. Kuhn, H. Wagner, Electrophoresis 11 (1990) 457.
[16] J.E. Song, T. Liu, X. Shen, G.D. Wu, Q.C. Xia, Electrophoresis 19 (1998) 1097.
[17] U. Keuth, A. Leinenbach, H.P. Beck, H. Wagner, Electrophoresis 19 (1998) 1091.
[18] M. Lanz, J. Caslavska, W. Thormann, Electrophoresis 19 (1998) 1081.
[19] R. Westermeier, in: Electrophoresis in Practice, Verlag Chemie, Weinheim, Germany, 1993, p. 121.
[20] P.M. Bronson, C.J. van Oss, Prep. Biochem. 9 (1979) 61.
[21] J. Bauer, G. Weber, J. Dispersion Sci. Tech. 19 (1998) 937.
[22] W. Tanner, T. Caspari, Ann. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 595.
[23] T.W. Okita, J.C. Rodgers, Ann. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 327.
[24] B.R. Green, D.G. Durnford, Ann. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 685.
[25] S.E. Clark, Trends Plant Sci. 1 (1996) 406.
[26] A.W. Shyjan, R.A. Butow, Curr. Biol. 3 (1993) 398.
[27] H. Barbier-Brygoo, J. Joyard, A. Pugin, R. Ranjeva, Trends Plant Sci. 2 (1997) 214.
[28] R.F. Pont-Lezica, J.G. McNally, B.G. Pickard, Plant Cell Envir. 19 (1993) 111.
[29] C. Reuzeau, R.F. Pont-Lezica, Protoplasma 186 (1995) 113.
[30] L.R. Griffing, R.S. Quatrano, Proc. Natl. Acad. Sci. USA 81 (1984) 4804.
[31] M.E. Steele, R.G. Stout, Plant Physiol. 82 (1986) 327.
[32] A.S. Sandelius, C. Penel, G. Auderset, A. Brightman, M. Millard, D.J. Morré, Plant Physiol. 81 (1986) 177.
[33] H. Canut, S. Baudracco, M. Cabané, A.M. Boudet, G. Marigo, Planta. 184 (1991) 448.
[34] N. Crawford, in: D.J. Morré, K.E. Howell, G.M.W. Cook, W.H. Evans (Eds.), Cell-free Analysis of Membrane Traffic, Alan R. Liss, New York, 1988, p. 51.
[35] P. Thuleau, A. Graziana, H. Canut, R. Ranjeva, Proc. NatI. Acad. Sci. USA. 87 (1990) 10000.
[36] S. McLaughlin, in: A.N. Martonosi (Ed.), Membranes and Transport, Plenum Press, New York, 1982, p. 51.
[37] L.E. Körner, P. Kjellbom, C. Larsson, I.M. Moller, Plant Physiol. 79 (1985) 72.
[38] I.M. Moller, T. Lundborg, Planta. 164 (1985) 354.

[39] Y.Y. Leshem, in: Y.Y. Leshem (Ed.), Plant Membranes: A Biophysical Approach To Structure, Development and Senescence, Kluwer Academic Publishers, Dordrecht, 1992, p. 65.
[40] J.P. Dubacq, J.C. Kader, Plant Physiol. 61 (1978) 465.
[41] H. Klinger, S. Frosch, E. Wagner, Photosynth. Res. 28 (1991) 109.
[42] V.W. Klofat, K. Hannig, Z. Physiol. Chem. 348 (1967) 739.
[43] V.W. Klofat, K. Hannig, Z. Physiol. Chem. 348 (1967) 1332.
[44] D. Afif, H. Canut, P. Dizengremel, Physiol. Plant. 93 (1995) 745.
[45] E. Selstam, G. Oquist, Plant Physiol. Biochem. 28 (1990) 477.
[46] G. Pauly, R. Douce, J.P. Carde, Z. Pflanzenphysiol. 104 (1981) 199.
[47] R. Stahn, K.P. Maier, K. Hannig, J. Cell Biol. 46 (1970) 576.
[48] H.G. Heidrich, R. Stahn, K. Hannig, J. Cell Biol. 46 (1970) 137.
[49] M. Marsh, S. Schmid, H. Kern, E. Harms, P. Male, I. Mellman, A. Helenius, J. Cell Biol. 104 (1987) 875.
[50] S.L. Schmid, R. Fuchs, P. Male, I. Mellman, Cell 52 (1988) 73.
[51] I. Ellinger, H. Klapper, R. Fuchs, Electrophoresis 19 (1998) 1055.
[52] D.J. Morré, K.E. Creek, G.R. Matyas, N. Minnifield, I. Sun, P. Baudoin, D.M. Morré, F.L. Crane, Bio Techniques 2 (1984) 224.
[53] H. Westrin, V.P. Shanbhag, P.A. Albertsson, Biochim. Biophys. Acta. 732 (1983) 83.
[54] P.X. Petit, K.A. Edman, P. Gardeström, I. Ericson, Biochim. Biophys. Acta. 890 (1987) 377.
[55] H. Canut, A.O. Brightman, A.M. Boudet, D.J. Morré, Plant Physiol. 86 (1988) 631.
[56] H. Canut, A.O. Brightman, A.M. Boudet, D.J. Morré, Plant Physiol. 94 (1990) 1149.
[57] C. Deswarte, S. Peyrebrune, H. Canut, J.P. Roustan, J. Fallot, Vitis 33 (1994) 99.
[58] N. Leborgne, L. Dupou-Cézanne, C. Teulières, H. Canut, A.M. Boudet, J.F. Tocanne, Plant Physiol. 100 (1992) 246.
[59] C. Thonat, C. Mathieu, M. Crèvecoeur, C. Penel, T. Gaspar, N. Boyer, Plant Physiol. 114 (1997) 981.
[60] R. Kappler, U. Kristen, D.J. Morré, Protoplasma 132 (1986) 38.
[61] C. Maurel, F. Tacnet, J. Güclü, J. Guern, P. Ripoche, Proc. Natl Acad. Sci. USA 94 (1997) 7103.
[62] A. Egger, D.J. Morré, G. Selldén, A.S. Sandelius, Physiol. Plant. 84 (1992) 121.
[63] E. Toll, F.J. Castillo, P. Crespi, M. Crèvecoeur, H. Greppin, Physiol. Plant. 95 (1995) 399.
[64] G.F.E. Scherer, B. vom Dorp, C. Schöllmann, D. Volkmann, Planta 186 (1992) 483.
[65] G. Auderset, A.S. Sandelius, C. Penel, A. Brightman, H. Greppin, D.J. Morré, Physiol. Plant. 68 (1986) 1.
[66] H. Canut, A.M. Justin, C. Demandre, A. Boudet, P. Mazliak, in: P.J. Quinn, J.L. Harwood (Eds.), Plant Lipid Biochemistry – Structure, Utilization and Function, Portland Press, London and Colchester, 1991, p. 23.
[67] S. Yoshida, M. Uemura, Plant Physiol. 82 (1986) 807.
[68] K.J. Dietz, S. Rudloff, A. Ageorges, C. Eckerskorn, K. Fisher, B. Arbinger, Plant J. 8 (1995) 521.
[69] H. Bouyssou, H. Canut, G. Marigo, FEBS Lett. 275 (1990) 73.
[70] K.J. Dietz, H. Canut, G. Marigo, J. Memb. Biol. 129 (1992) 137.
[71] H. Canut, A. Carrasco, R. Ranjeva, Plant Sci. 90 (1993) 135.
[72] K. Lahjouji, A. Carrasco, H. Bouyssou, L. Cazaux, G. Marigo, H. Canut, Plant J. 9 (1996) 799.
[73] J.P. Galaud, M. Carrière, N. Pauly, H. Canut, P. Chalon, D. Caput, R. Pont-Lezica, Plant J., in press.
[74] D.J. Morré, in: C. Larsson, I.M. Moller (Eds.), The Plant Plasma Membrane: Structure, Function and Molecular Biology, Springer Verlag, Berlin Heidelberg, 1990, p. 76.
[75] J.L. Hall, Methods Enzymol. 148 (1987) 568.
[76] J.C. Roland, C.A. Lembi, D.J. Morré, Stain Technol. 47 (1992) 195.
[77] S. Widell, C. Larsson, in: C. Larsson, I.M. Moller (Eds.), The Plant Plasma Membrane: Structure, Function and Molecular Biology, Springer Verlag, Berlin Heidelberg, 1990, p. 16.
[78] A.C. Fichette-Lainé, V. Gomord, M. Cabanes, J.C. Michalski, M. Saint Macary, B. Foucher, B. Cavelier, C. Hawes, P. Lerouge, L. Faye, Plant J. 12 (1997) 1411.
[79] H. Canut, A.O. Brightman, A.M. Boudet, D.J. Morré, in: C. Leaver, H. Sze (Eds.), Plant Membranes: Structure, Function, Biogenesis, Alan R. Liss, New York, 1987, p. 141.
[80] C. Larsson, in: J.L. Hall, A.L. Moore (Eds.), Isolation of Membranes and Organelles From Plant Cells, Academic Press, London, 1983, p. 277.
[81] H. Sze, Ann. Rev. Plant Physiol. 36 (1985) 175.
[82] K. Lahjouji, H. Canut, Physiol. Plant., in press
[83] H. Canut, A. Carrasco, J.P. Galaud, C. Cassan, H. Bouyssou, N. Vita, P. Ferrara, R. Pont-Lezica, Plant J. 16 (1998) 63.
[84] R.F. Pont-Lezica, J.G. McNally, B.G. Pickard, Plant Cell Environ. 16 (1993) 111.
[85] C. Reuzeau, R.F. Pont-Lezica, Protoplasma 186 (1995) 113.
[86] S.E. Wyatt, N.C. Carpita, Trends Cell Biol. 3 (1993) 413.
[87] A. Völkl, H. Mohr, G. Weber, H.D. Fahimi, Electrophoresis 19 (1998) 1140.
[88] E.M. Bayles, P.J. Richardson, J.P. Luzio, in: J.B.C. Findlay, W.H. Evans (Eds.), Biological Membranes – A Practical Approach, IRL Press, Washington DC, 1987, p. 73.