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RECYCLING ISOELECTRIC FOCUSING AND ISOTACHOPHORESIS

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SUMMARY

A new apparatus for large scale preparative isoelectric focusing in free solution is described. Its main characteristic is rapid fluid recirculation with but short residence times in the electric field. For the first time the recycling principle was also applied to preparative isotachophoresis, by incorporating into the system a computer controlled leader counterflow. The rapid recirculation permits application of unusually high electrical power for rapid resolution, as it suppresses fluid flow instabilities arising from electrohydrodynamic effects.

INTRODUCTION

Isoelectric focusing is a particularly appealing method for scaling-up of protein purification because of its exceptional resolution. Several preparative instruments[1–5] were developed over the last few years based on a novel recycling principle and are gaining increasing acceptance. One of the advantages of recycling is that it permits dissipation of Joule heat outside of the fractionation apparatus. The general usefulness of recycling was also recently demonstrated by Righetti *et al.*[6] in an innovative manner. The diversity of instruments is welcome as it allows the selection of the most appropriate instrument for the problem at hand.

Isotachophoresis has also the potential of high resolution[7], but has received as yet far less attention as a preparative method and no high throughput apparatus was available. Isotachoporesis has an advantage over focusing in lending itself to greater flexibility in the choice of operating pH, thus avoiding the problem of protein precipitation ocasionally seen in focusing.

In this paper we wish to describe our latest recycling apparatus, equally well suited for focusing and isotachophoresis. It provided the first demonstration that the principle of recycling operation is also applicable to separation by isotachophoresis[8,9]. This was not evident *a priori*, as the two methods differ significantly in the characteristics of their final steady state. In isoelectric focusing, the final steady state is stationary, all components becoming virtually immobilized within the pH gradient at their isoelectric point. In such a situation the final state is independent of the route taken, *i.e.*, the recycling of the process fluid does not impede the establishment of the pH gradient or the focusing of the components.

In the steady state of isotachophoresis, to the contrary, all components migrate

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at the same velocity as a train of adjacent components, sorted according to their mobilities[10]. It was not obvious that such an ordering of components would occur also in a recycling mode of operation, where, in effect, only a relatively small part of the total volume (3–10% as a rule) is exposed to the electric current at any one time, the bulk of the fluid being in the external fluid circuitry loops. Moreover, counter-flow of leader ions is essential to provide sufficient migrating distance for the achievement of the steady state.

In common with other researchers interested in scaling up of electrophoretic methodology[11], we use free solutions without any supporting media such as gels or packed granular beds. Gels are essential for analytical separations and granular beds are convenient for micropreparative purposes, but they impose serious problems in scale up. Operating in free solutions, of course, necessitates rigorous control of fluid flow. Drawing analogies with gel electrophoresis, it was axiomatically assumed that best separations will be achieved in nearquiescent fluids. Several "curtain type" continuous flow instruments with fluid confinement to a narrow channel between parallel plates utilize this approach[12].

To our great surprise, we have discovered that in our instrument far better fluid stability is obtained at rapid rates of fluid flow, rather than in near stationary fluids[8]. The fluid instabilities seen at low flow-rates or in stationary fluid were attributed to certain electrohydrodynamic effects not previously associated with electrophoresis[13]. Electrohydrodynamic mixing, droplet deformation and other effects have been extensively studied by Melcher and Taylor[14] and Arp *et al.*[15], but only in two-phase systems, such as formed by immiscible fluids or fluid-air interfaces. The importance of these electrohydrodynamic factors for conventional continuous flow electrophoresis was recently clarified in an elegant manner by Rhodes and co-workers[16,17]. Their discovery merits attention from all workers in free fluid electrophoresis and is fully consonant with our results.

EXPERIMENTAL

Instrumentation

One of the best known methods for the control of fluid flow was pioneered by Hannig[12]. A thin film of flowing buffer is contained between two parallel plates, at least one of which is cooled. Fluid viscosity helps to maintain laminarity of buffer flow and the electric field is applied perpendicularly to the flow direction. The sample is introduced as a narrow stream within this curtain of flowing buffer and its components separate tangentially. Such separating chambers were incorporated into several commercially available instruments and a prototype has also been experimented with in the microgravity of orbiting spacecraft by NASA and McDonnell Douglas. Residence time in such instruments are typically relatively long, of the order of minutes, as separation has to be achieved in a single pass.

These instruments have been mainly used for zone electrophoresis, where separations are obtained as a function of relative mobilities in a background of homogeneous buffer composition. Fawcett[18] and others have also explored their usage for isoelectric focusing, but the results were rather disappointing. Low voltages only could be applied, resulting in residence times of the order of 30 min and correspondingly low throughput. Increase of voltage resulted in "feathering", a breakdown of the flow lines into feather-like structures.

The separation chambers we have used in the present studies of focusing and isotachophoresis use the same principle of a thin buffer film confined between two parallel plates. The chamber design is different, however, as it incorporates a symmetrical arrangement of an (arbitrary) number of inlet and outlet ports at the opposite ends of the chamber. These outlets and inlet ports are connected by closed circuitry loops for process fluid recirculation by means of a multichannel peristaltic pump. The recycling loops can be provided with UV, pH or other sensors, as well as with bubble traps and heat exchanger reservoirs. The latter provide for a changeable volume capacity of the instrument and avoid the necessity of cooling the focusing chamber itself. Recycling loops are also provided for the electrode compartments, separated from the chamber by either ion-exchange membranes for focusing, or by dialyzing membranes for isotachophoresis.

Five different chamber designs were implemented, with various overall length and width, the number of the closely spaced ports varying from 12 to 48. Two instruments had refrigerated chambers, the other three relied only on the heat exchanger reservoirs for Joule heat dissipation. Fig. 1 shows the simplicity of the

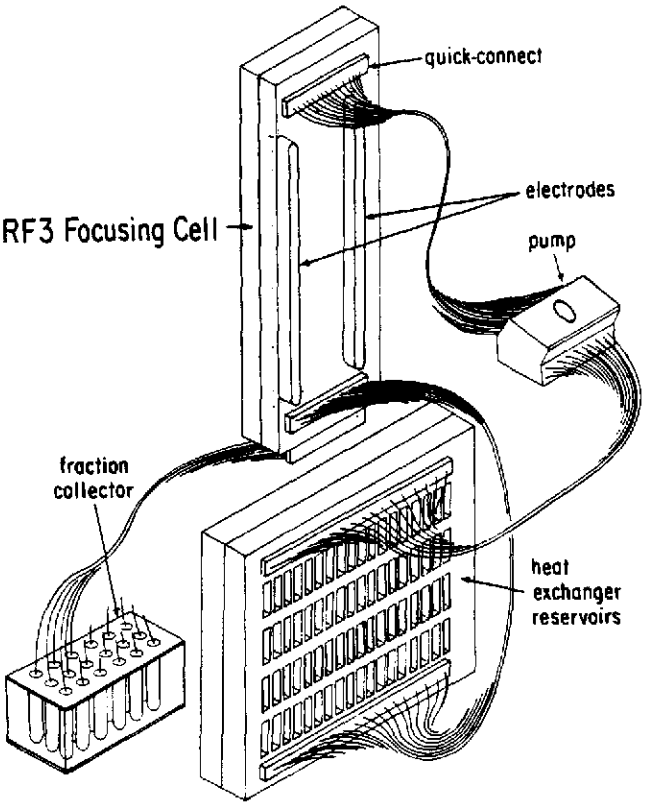

Fig. 1. Schematic presentation of the recycling flow pattern in the RF3 apparatus. Several different designs of the focusing apparatus and the heat exchanger were implemented, all exhibiting the same overall concept. In all of them, connecting the tubing set to the focusing cell, the pump, and the heat exchanger is facilitated by molded quick-connectors, only one of which is identified.

recycling loop in the 48-channel instrument, with a refrigerated focusing cell and heat-exchange reservoirs. All parts of the tubing set terminate in specially developed molded quick-connects, thereby greatly facilitating the apparatus assembly. A transfer of one of the quick-connects to the fraction collector allows for the simultaneous pumping out and collection of the separated fractions.

As in our other recycling instruments, the performance of the apparatus is rather independent of most operational factors, such as apparatus dimension, applied field, etc. Resolution, of course, mainly depends on the pH range of the carrier ampholyte. Two additional factors were found to be critical: the thickness of the fluid film and the rate of recycling. We discovered, indeed, that successful implementation of either focusing or isotachophoresis requires a narrow gap, of the order of 0.5–1 mm, with sufficiently fast flow-rates to give residence times of seconds only in the separation chamber[8]. The higher the applied voltage, the faster must be the recycling rate, if fluid instabilities are to be avoided. The cause of these fluid instabilities were attributed to electrohydrodynamics. Temperature or gravity were eliminated as major factors, as similar instabilities were observed with cooled or uncooled chambers, or with chambers operated in vertical or horizontal direction.

To implement leader counterflow, essential for isotachophoresis, several modifications were necessary. The advancing protein sample boundary was registered by inserting a UV sensor (2138 Uvicord S, LKB, Bromma, Sweden) into an appropriate recycling channel. For anionic isotachophoresis, this channel was somewhere between three and six channels in front of the anode (in a 30-channel apparatus). The counterflow was provided by a Sage Instruments syringe pump, feeding its leader buffer into the recycling channel adjacent to the anode membrane. Excess fluid was allowed to discharge from the last recycling channel, closest to the cathode. For cationic systems, only the polarity of the field needs to be reversed.

The syringe pump was toggled between on and off positions by a microcomputer (Commodore 64) interfaced with the UV sensor. Computer–UV sensor interface comprised an analog-to-digital/digital-to-analog (A-D/D-A) converter, a 12-V d.c. power supply and a relay. The computer was also utilized for data acquisition, treatment and storage. A specially written program provided visual graphic demonstration on the computer screen of the progress of separation, showing the preselected pump-triggering UV absorbance level, the actual absorbance registered by the sensor and the operation of the pump, as a function of time.

Operation

For isoelectric focusing, a batch mode of operation is simplest. The sample to be fractionated is mixed with the desired pH range ampholine or other carrier buffer and the apparatus filled to capacity. Recycling is initiated at a flow-rate of *ca.* 5–15 ml/min per channel. Power is applied to a maximum of 100–200 W, depending on the tolerance of the sample to temperature rise in the apparatus. At the lower power level, the temperature rise is less than 5°C, while at higher levels, the rise may be as much as 15°C. The bulk of the solution is maintained close to 0°C in the heat-exchange reservoir.

Recycling is continued until the amperage decreases to a stable minimum and for a "decent time" interval thereafter. Focusing of colored proteins has demonstrated that with a broadly based pH range, focusing of volumes of the order of 150 ml is completed within 40–60 min. With shallower pH gradients it is advisable to prolong the focusing time.

Three different modes of operation are possible. With a broad distribution of isoelectric points of the sample, the use of a broad pH range of carrier buffer will fractionate the mixture into step-pH fractions with some overlap between fractions. If one knows the isoelectric point of the desired protein, one can also select a narrow pH range carrier buffer, which will expand the resolution in the selected pH range, compressing the other proteins into the side compartments. Highest resolution is obtained in a third mode of operation, where the selected fractions of a first prefocusing, containing the desired protein, are submitted to a second fractionation, without addition of any other carrier buffer. This yields the narrowest pH range and optimizes resolution.

For isotachophoresis, batch or continuous flow operations are possible. The performance of the apparatus was mainly tested with dyes and with colored proteins, hemoglobin and blue-stained albumin. As protein fractions are adjacent in the isotachophoretic train, resolution is greatly enhanced by the choice of appropriate "spacers", non-protein components such as amino acids of intermediate mobilities[7]. Sharply focused protein boundaries were registered, using chloride ions as leader and ε-amino caproic acid as terminator. The choice of leader, terminator and spacers is critical and it is highly advisable that it be optimized by means of preliminary capillary isotachophoresis runs. Several such analytical instruments are available. Our computer programs[19] are also most useful in testing the separability of leader–spacer(s) terminator. Glycine was an effective spacer to separate the two proteins. In either case, the leader is utilized to fill the chamber and one of the electrode compartments, the terminator being used in the other electrode compartment. The sample can be introduced through one of the channels close to the terminator as a single batch or in a continuous manner. A detailed account of this work is in preparation[24].

It is a characteristic of isotachophoresis that sample concentration is self-adjusting to the concentration of the leader, as a function of the Kohlrausch relation. For proteins, this results in their concentration to 1–10%. Thus, isotachophoresis is a convenient method for concentation of large volumes of dilute protein solutions with simultaneous fractionation. In fact, we have managed to fill virtually the whole contents of the apparatus with a desired protein in such a manner.

RESULTS AND DISCUSSION

Isoelectric focusing is gaining attention as a preparative method for protein purification[20,21]. Two instruments are commercially available, the older Rotofor (Bio-Rad Labs., Richmond, CA, U.S.A.)[3,4] and the present apparatus, dubbed recycling free-flow focusing (RF3) (Protein Technologies, Tucson, AZ, U.S.A.)[8] to differentiate it from our earlier recycling isoelectric focusing (RIEF) apparatus[1,2]. In the RIEF apparatus, fluid flow is stabilized by means of screen spacers, subdividing the focusing chamber. In the Rotofor, a combination of rotation around its horizontal axis and the partitioning of the annular space by the screen spacers stabilizes fluid against unwanted convections. The RF3 avoids some of the complications inherent in the presence of screen elements, simplifies the assembly and permits easy *in situ* cleaning. In addition, the rapid recirculation permits application of higher power for faster focusing.

The underlying concept of conventional continuous flow electrophoresis is that

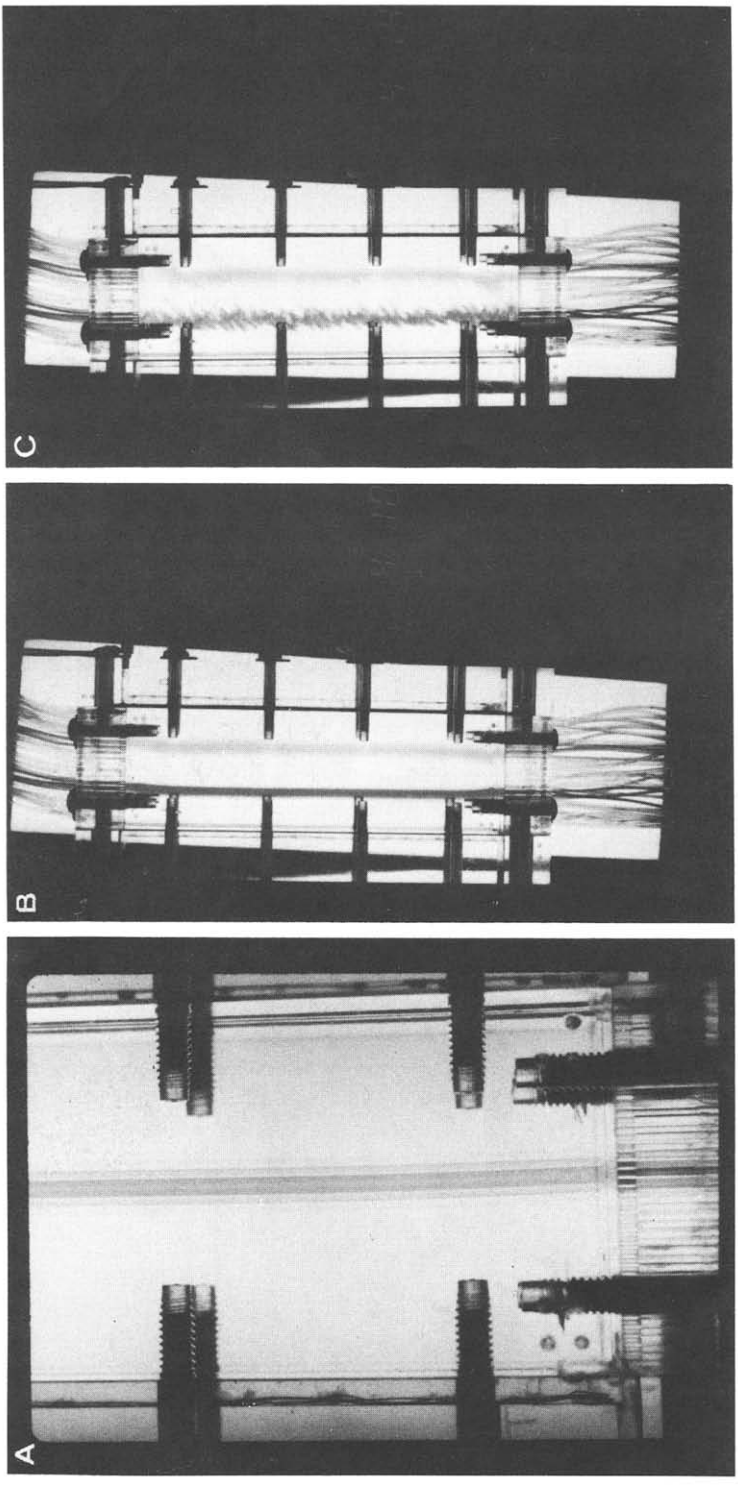

Fig. 2. Photographs of the streamlines of fluid flow in the RF3 apparatus. (A) Close-up of the remarkably sharp streamlines of focused hemoglobin. (B) RF3 cell with focused hemoglobin (left) and blue-stained albumin, rapid flow. (C) Dramatic disruption of the flow lines obtained within 4 s of flow stoppage.

a thin film of fluid, contained between two parallel plates, will provide the best medium for electrophoretic separations. At first appearance, this arrangement bears similarity to fractionation in a thin gel film. Unfortunately, this analogy is incorrect, as operation of such devices is subject to well known distortions of fluid flow caused by: the parabolic nature of flow through narrow gaps, the effect of Joule heating, gravitational slumping, if any, and electroosmosis[22]. Mathematical models seem to predict reasonably well the behavior of these instruments for zone electrophoresis[23].

In our type of recycling operation, none of the above factors seem to be of importance, the key consideration being the high rate of recycling. The parabolic flow of fluid is obviously of no concern, and there is no noticeable electroosmosis within the short residence times. Fig. 2 shows the remarkable sharpness of fluid flow lines obtained at fast recycling rates and the near explosive breakdown caused by electrohydrodynamic effects.

The only electrically driven fluid convection previously recognized was electroosmosis, resulting from the zeta potential of the walls of the vessel. In a homogeneous buffer, such as present in zone electrophoresis, electroosmosis is predictable and measurable by various methods. Rhodes and co-workers[16,17] have recently demonstrated that even in zone electrophoresis there can be a second kind of electrically driven convection, provided there is a significant conductivity or dielectric constant gradient within the otherwise uniform buffer system. Such gradients can arise through the use of high concentration (or conductivity) sample solutions.

These electrohydrodynamic effects have been previously observed[14,15] only when electric fields were imposed across immiscible water–solvent phase boundaries, giving rise there to feather-like disturbances as seen in our apparatus or to distortion of droplets. It is easy to differentiate between electroosmosis and the socalled electrohydrodynamic effect: electroosmosis is proportional to the field strength E, and thus requires d.c. currents. The electrohydrodynamic effect is proportional to E^2 and is equally responsive to direct or alternating currents. Thus, as a fringe benefit of our research, we have provided a confirmation of this effect in aqueous systems. In isoelectric focusing, of course, conductivity gradients are more prominent than in most other electrophoretic systems and the effect is more pronounced. As with gravity driven natural convection, a certain time element is necessary for the instabilities to develop and the rapid recycling is sufficient to avoid this in our case.

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