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Continuous Separation of Proteins in Electrical Split-Flow Thin (SPLITT) Cell with Equilibrium Operation

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Abstract

The continuous separation of charged species in an electrical split-flow thin (SPLITT) cell is described. By operating the electrical SPLITT system in an equilibrium mode at a solution pH lying between the isoelectric points of two proteins, a mechanism is available for the rapid and complete separation of the proteins. The theoretical conditions necessary for such a separation are established. The apparatus constructed to test this concept is described. Preliminary experimental results are reported for several proteins, and the complete resolution of a binary mixture of ferritin and cytochrome *C* is demonstrated.

INTRODUCTION

The split-flow thin (SPLITT) cell continuous separation method, designed for small-scale preparative work (*1–3*), evolved from field-flow fractionation (FFF), a versatile family of analytical macromolecular separation techniques (*4*, *5*). Both methods utilize a separation channel whose geometry is thin and ribbonlike. However, for SPLITT cells, one or both ends of the channel must have stream splitters. The outlet stream splitter provides a means for splitting the film of liquid flowing through

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the channel into two or more thin laminae. At the inlet, the splitter allows two independent laminae to smoothly join. For FFF, both flow splitters are optional and, in practice, infrequently used.

The difference in splitter requirements for FFF and SPLITT systems reflects their unlike separation mechanisms: in FFF components are separated from a narrow sample zone along the axial (flow) coordinate and in SPLITT cells species are fractionated along the short transverse axis. The latter feature makes continuous separation possible in SPLITT cells (*1*). In general, both channel systems must be subject to a transverse driving force capable of impelling species toward one of the major channel walls.

In the case studied here, an electrical field is applied perpendicular to the flow axis in a manner similar to that of electrical FFF (*6*, *7*). However, the electrical field induces separation across the thin (submillimeter) dimension of the channel. The outlet splitter then divides the differently enriched laminae into two (or more) fractions of different composition. With continuous sample introduction, these fractions can be continuously collected.

SPLITT cells are generally capable of operation either in a dynamic transport mode or in an equilibrium mode (*1*). In the transport mode, different components must be introduced, using an inlet splitter, on one side of the channel. The components are driven in the same direction, usually twoard the same wall or streamplane; enrichment in different laminae occurs because of different transport rates. For electrical fields, transport-induced separation is based on differences in electrophoretic mobilities (*1*).

In the equilibrium mode, different components are driven to different equilibrium positions. The enriched laminae are then converted into enriched fractions by the outlet stream splitter. The inlet splitter is optional since each component will be driven to its equilibrium plane no matter where it starts or how it is introduced.

In the case of electrical fields, two species can be driven to equilibrium positions at opposing walls if they are oppositely charged. Proteins should thus be continuously separable providing the operating pH lies between their isoelectric points. This hypothesis is tested and verified here.

The principles of the separation (in a system with an inlet splitter) are illustrated in more detail in Fig. 1. The figure shows the sample solution entering inlet a′ at a volumetric flow rate of $\dot{V}(a')$. Sample-free carrier enters inlet b′ at a flow rate of $\dot{V}(b')$. As the two inlet streams are swept past the inlet splitter, they conjoin to form a single stream. The joining

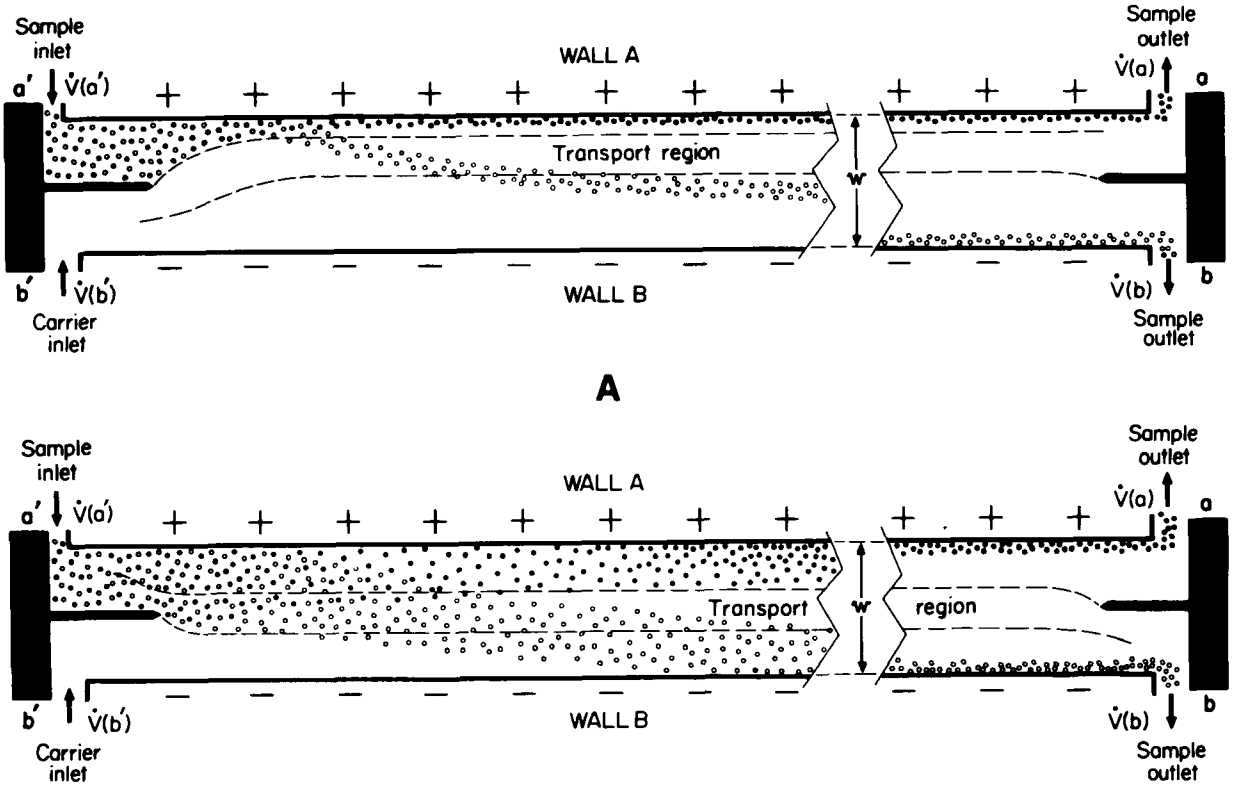

FIG. 1. Electrical SPLITT cell operation under different flow conditions. In A the inlet splitting plane (upper dashed line) lies above the outlet splitting plane (lower dashed line). In B these are reversed. Positively charged species are represented by open circles; negative species by solid circles.

plane between the two streams, which we call the *inlet splitting plane* (ISP), extends down the length of the channel. This splitting plane separates fluid elements entering in stream a′ from those entering in b′. If the flow rate through b′ is greater than that through a′ ($\dot{V}$(b′) > $\dot{V}$(a′)), then the splitting plane will swerve up toward wall A immediately beyond the splitter (Fig. 1A). In the opposite case, $\dot{V}$(a′) > $\dot{V}$(b′), the inlet splitting plane will be deflected down toward wall B (Fig. 1B). In both cases the splitting plane reaches a constant equilibrium position just a few channel thicknesses beyond the physical splitter. Only when $\dot{V}$(a′) = $\dot{V}$(b′) does the splitting plane extend straight down the channel, bisecting the liquid stream into two equal laminae.

Analogously, the *outlet splitting plane* (OSP), shown in Fig. 1 as the broken line terminating at the outlet splitter, serves to separate those fluid elements destined to elute from outlet a from those that will emerge from outlet b. The steady position of this splitting plane may also lie above or below the center plane depending on the relative outlet flow rates $\dot{V}$(a) and $\dot{V}$(b). Clearly, the inlet splitting plane (ISP) may lie above or below the outlet splitting plane (OSP). These two cases, illustrated in Figs. 1(A) and 1(B), respectively, were both used in our experiments.

With the polarity of the channel shown in Fig. 1 (designated as A+, B− to show the positive charge at wall A and the negative charge at wall B), the object of the experiment is to recover all negatively charged species from outlet a and all positive species from outlet b. For the case shown in Fig. 1(A), where the ISP lies above (closer to wall A than) the OSP, all negatively charged species will egress outlet a even without electrically induced transport. However, the positively charged species must all be transported across the OSP. The positive charges entering the cell next to wall A must therefore be transported the entire distance from wall A to the OSP for experimental success.

In the case of Fig. 1(B) the negative charges would emerge from both outlets if there were no transverse transport due to the electrical field. The latter transport must be sufficient to carry negative charges from the OSP to a point above the ISP. Positive charges at a maximum must migrate from wall A to the OSP as before, but now this distance tends to be shorter.

THEORY

The purpose of this section is to develop the criteria that must be met for all negative species to emerge from outlet a and all positive species

from outlet b. Transverse diffusion effects will be ignored in this treatment although they have been thoroughly examined for a diffusion-based SPLITT cell (*3*).

The splitting planes divide the volume of the channel into distinct regions or laminae. Each of the distinguishable laminae carries a calculable fraction of the total volumetric flow rate $\dot{V}$. It has been shown (*2*) that to transport a species across a lamina of flow rate $\dot{V}_i$, the inequality of Eq. (1) must apply:

$$bLU \geq \dot{V}_i \tag{1}$$

where b is the channel breadth, L is the channel length, and U is the velocity of the induced transverse transport. For electrically induced transport, U may be written as the product of electrophoretic mobility μ and electric field strength E. Thus Eq. (1) becomes

$$bL\mu E \geq \dot{V}_i \tag{2}$$

where μ becomes μ_+ for positively charged species and μ_- for negative charges.

As indicated above, positive charges must be able to traverse the distance from wall A to the OSP in order to be fully collected at outlet b. As shown in Fig. 1, the lamina enclosed by the planes of wall A and the OSP has a volumetric flow rate of $\dot{V}(a)$. Consequently, for either of the cases shown in Fig. 1 the positive species must be subject to the condition

$$bL\mu_+ E \geq \dot{V}(a) \tag{3}$$

As indicated before, the negative species requires no transport in case a, Fig. 1(A). However, in case b, Fig. 1(B), the species must be capable of a displacement from the ISP to the OSP. The volumetric flow rate of the lamina enclosed between these two splitting planes can be seen to equal $\dot{V}(a') - \dot{V}(a)$. Consequently, the condition for the adequate transport of the negative species is expressed by

$$bL\mu E > \dot{V}(a') - \dot{V}(a) \tag{4}$$

The operating conditions specified by Eqs. (2)–(4) are valid in the absence of diffusion. In most cases diffusive effects are expected to be

slight; they can be offset by increasing the $bL\mu E$ values slightly above the minimum criteria levels indicated in Eqs. (2)–(4).

EXPERIMENTAL

Separation Cell

A diagram of the component layers comprising the SPLITT cell and electrode compartment are shown in Fig. 2. This multilayered structure is designed in large part to prevent perturbations from electrolytic gases; such gases could interfere with the free passage of current-carrying ions through the frit and membrane layers and thus cause nonuniformity in the electrical field. These gases and the Joule heat are eliminated from the fluids in the electrode compartments by circulating these fluids through a cooling loop where heat is dissipated and bubbles are expelled. A similar loop was used to treat the fluids adjacent to the frit.

As shown in Fig. 2, both channel walls consist of membranes (made of dialysis tubing) backed by frits. Another membrane serves to partition each frit from the electrode chamber. The latter consists of Lucite blocks inside which large grooves were cut, and coiled platinum wires were placed to serve as the electrodes.

The field was applied by connecting the two electrodes to a power supply. The distance (2.5 cm) between the two electrodes is determined by the thickness of the two frits and the outer intervening layers. The channel thickness, w = 635 μm, is fixed by the two thin Teflon spacers (254 μm) and the Mylar splitter (127 μm). Channel length (measured between splitting edges) and breadth were 28.8 and 2 cm, respectively. The calculated void volume V° of the cell equals 3.66 mL.

Spectra/por (Spectrum Medical Industries, Inc., Los Angeles, California) dialysis membrane tubing (molecular weight cutoff = 6000–8000) was used both for the channel wall and the membrane partitioning between the electrode and the frit chambers. Channel inlet and outlet tubes (Teflon) were inserted between the splitter and the membrane. Finally, all layers of the system were clamped together by bolts.

Ancillary Instrumentation

A schematic of the entire system is shown in Fig. 3. Two pumps delivered carrier solution independently into the two inlets of the

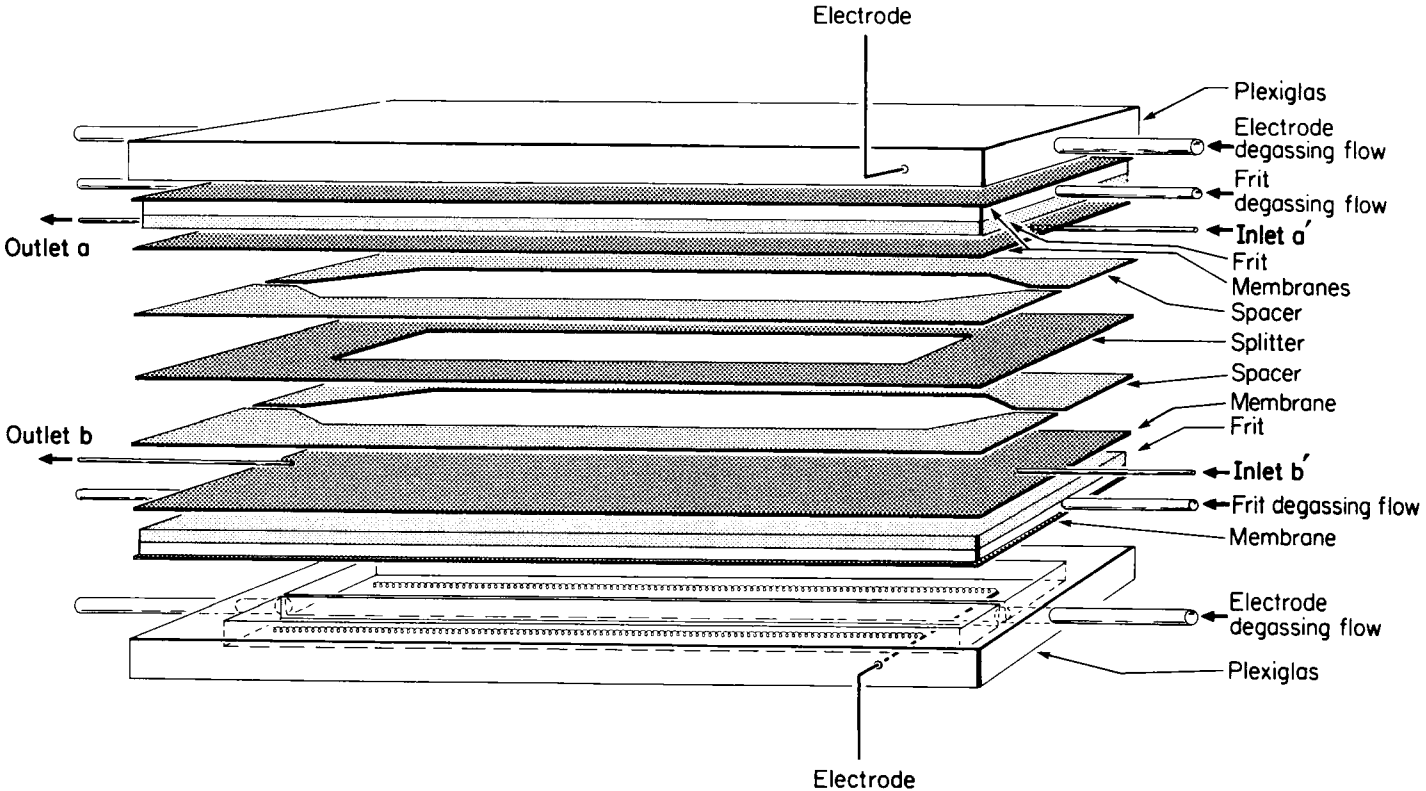

FIG. 2. Component layers of the prototype electrical SPLITT cell.

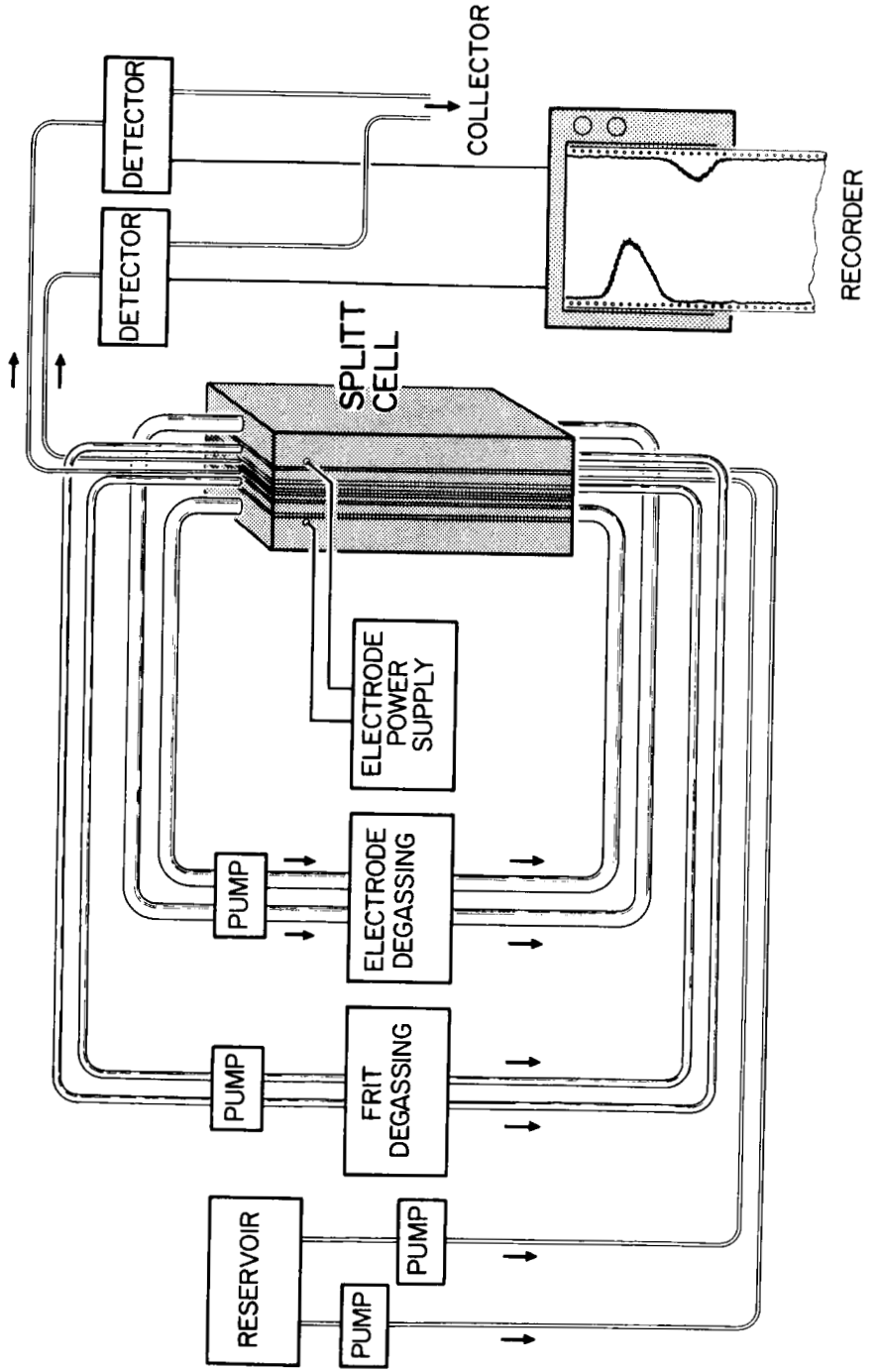

FIG. 3. Experimental system for electrical SPLITT cell operation.

separation channel. A Minipuls 2 peristaltic pump (Gilson, Middleton, Wisconsin) provided the flow to feed inlet a′ and a laboratory built syringe pump served carrier inlet b′; another Minipuls 2 pump was used for the frit circulation. A Masterflex peristaltic pump (Cole-Parmer, Chicago, Illinois) circulated the buffer solution through two reservoirs of 2 L each at a flow rate high enough (~1 mL/min) to degas and cool the electrode chamber. Outlet flows were controlled by lengths of small diameter tubing.

A detector was connected to each of the two outlets in all experiments. In the experiments run at pH 4.5 (see later), the detectors were a model 153 UV detector (Beckman Instruments, Fullerton, California) and a Spectroflow SF769 variable wavelength detector (Applied Biosystems, Ramsey, New Jersey), both operated at 254 nm. In the pH 5.4 experiments a Spectroflow SF770 variable wavelength detector (Applied Biosystems, Ramsey, New Jersey) was operated at the cathode side at 440 nm, a wavelength selective to cytochrome *C* in the presence of ferritin. The above Spectroflow SF769 detector was operated at the anode side at 280 nm to monitor ferritin. A strip chart recorder equipped with two pens, Series SS-250F (Esterline Angus, Indianapolis, Indiana), was used to record the peaks.

The electrical field was maintained using a laboratory-built dc power supply operated at 40 V. The pH of the solution in the electrode compartment was frequently monitored using a model 609 digital pH meter (Extech, Boston, Massachusetts). Sample injections were made with a metal-free Type 50 Teflon Rotary Valve 5025 (Rheodyne, Cotaty, California) equipped with a 90-μL loop.

Materials

Tris acetate buffers of pH 4.5 and 5.4 were used as the carrier fluids in the separation channel as well as for the fluids circulating through both frit and electrode chambers. The buffers were composed of 0.02 *M* Trizma Base (tris(hydroxymethyl)-aminomethane) from Sigma Chemicals (St. Louis, Missouri). A stock solution of 1 *M* glacial acetic acid (J. T. Baker, Phillipsburg, New Jersey) was used to adjust the pH to the desired levels (requiring 40 mL/L for pH 4.5 and 22.5 mL/L for pH 5.4). Sodium azide (Sigma Chemicals, St. Louis, Missouri) was also added (0.0017% w/v) to prevent bacterial growth inside the channel. The solutions were filtered with a 0.22-μm filter (Millipore Co., Bedford, Massachusetts) after preparation.

All protein samples were purchased from Sigma Chemicals, St. Louis, Missouri. The sample solutions were prepared by dissolving the proteins in the buffer used as a carrier. The sample solutions were all filtered before use. Five proteins were used at pH 4.5: cytochrome *C* (horse heart, 0.28 mg/mL), bovine serum albumin (BSAj) (0.7 mg/mL), hemoglobin (human, 0.4 mg/mL), α-chymotrypsinogen (bovine pancreas, 0.5 mg/mL), and pepsin (Porcine Stomach Mucosa, 0.5 mg/mL). Two proteins were used at pH 5.4: cytochrome *C* (horse heart) and ferritin (horse spleen). In these cases stock solutions of both cytochrome *C* (1 mg/mL) and ferritin (0.5 mg/mL) were prepared. Three samples were obtained by diluting and mixing stock solutions. One sample contained 0.5 mg/mL cytochrome *C*, another contained 0.03 mg/mL ferritin, and the third contained both proteins at the above concentrations. All dilutions were made with the standard carrier buffer.

Procedure

The field was turned on at least one hour before the beginning of a run. The electrode degassing and cooling fluids (see Fig. 1) were turned on at the same time to keep the system at room temperature. The frit degassing circulation fluid was run constantly; the carrier was circulated through the channel continuously between runs. When the baselines and the flow rates at the inlets and outlets were stable, the sample was injected and detected at both outlets. The wavelength was then changed when necessary for a selective detection of cytochrome *C*. Peak areas were calculated by multiplying the heights (absorbance units) by the widths (volume units) at half the peak height.

RESULTS AND DISCUSSION

Although the SPLITT system is designed for continuous separation, the evaluation of system performance is most conveniently carried out with small discrete samples. In the present case the injected samples were 90 μL in volume. The relative recovery from the two outlets was obtained from the detected peak areas.

The experiments in this study were carried out in four sets. In the first set (set I) the pH 4.5 buffer was used. The inlet flow rates were $\dot{V}(a') = 0.16$ mL/min and $\dot{V}(b') = 0.49$ mL/min; the flow rates at the outlets were $\dot{V}(a)$

= 0.26 mL/min and $\dot{V}(b)$ = 0.39 mL/min. Because the outlet flow ratio, $\dot{V}(a)/\dot{V}$ = 0.4, exceeds the corresponding inlet flow ratio, $\dot{V}(a')/\dot{V}$ = 0.25, the flow conditions correspond to case a, Fig. 1(A). The isoelectric point pI (*8*) and the estimated electrophoretic mobilities of the five proteins used in this and subsequent studies are summarized in Table 1. The table shows that at pH 4.5, the first four proteins are positively charged and the last, pepsin, is negatively charged.

The collection of reliable mobility (μ) data for the calculation of $bL\mu E$ values was a difficult task. Literature values could not be found corresponding to our experimental conditions. In order to make a crude estimation of μ from the scattered literature values (e.g., *9*, *10*), we assumed that μ was proportional to the absolute value of the difference between the experimental pH and the protein's pI value. The μ values reported in Table 1 reflect this approximation and also reflect errors in the original measurements, which are often inconsistent with one another. However, in most cases the resulting $bL\mu E$ values are so large in relationship to the inequalities of Eqs. (2)-(4) that accurate values are not critically important.

Equation (3) was used to test the suitability of the experimental conditions used in set I for the complete recovery of proteins from their respective outlets. With a voltage drop between electrodes of 40 V and with the mobility values listed in Table 1, values of $bL\mu E$ were calculated for each protein. These, along with the minimum required values from Eq. (3), are shown in Table 1. We observe that the calculated values for set I are in all cases much higher than those required; therefore, in theory, 100% recovery should be realized from the corresponding outlet.

The experiments carried out under the above conditions verified the calculations. The detector signal for pepsin showed a peak emerging from outlet a but not from outlet b, indicating the full recovery of this protein from the expected outlet. Similarly, the four proteins other than pepsin all displayed signals only at outlet b, showing complete recovery at that outlet.

In the second set of experiments (set II) all flow rates remained the same but the polarity was reversed. The pH remained at 4.5. In this case the role of the positive and negative species is reversed; the positive ions are no longer subject to a transport requirement and the negative ions are subject to Eq. (3) with μ_- replacing μ_+. Thus for pepsin the required $bL\mu E$ value was 0.26 mL/min. No experimental value of μ was found for pepsin to allow a comparison of $bL\mu E$ values. However, the recovery of pepsin from outlet b was found to be 100%. Similarly, the four positively charged proteins were found only to elute from outlet a.

TABLE 1
Estimated and Required Values of $\dot{V}$ for Various Protein Species in the Electrical SPLITT Channel

Protein	pI	$\mu \times 10^5$ $cm^2/V \cdot s$	$bL\mu E$ calculated (mL/min)	$bL\mu E$ required (mL/min)	
pH 4.5:				Set I	Set II
Pepsin	2.9			0.00	0.26
BSA	4.9	1.3	0.72	0.26	0.00
Hemoglobin	6.9	3.1	1.7	0.26	0.00
Chymotrypsin A	9.0			0.26	0.00
Cytochrome *C*	10.6	1.0	0.56	0.26	0.00
pH 5.4:				Set III	Set IV
Cytochrome *C*	10.6	0.86	0.48	0.48	0.26
Ferritin	4.4	3.3	1.8	0.26	0.48

The third set of experiments was carried out to demonstrate that the separation of a simple protein mixture could be realized in the SPLITT system. For this purpose the two proteins cytochrome *C* and ferritin were used. At the experimental pH of 5.4, ferritin has a net negative charge and cytochrome *C* has a net positive charge (see pI values in Table 1). The flow rates in this set of experiments were changed in such a way that the inlet splitting plane dropped below the outlet splitting plane as shown in Fig. 1B. For this purpose $\dot{V}(a')$ and $\dot{V}(b)$ were 0.74 mL/min while $\dot{V}(b')$ and $\dot{V}(a)$ were 0.48 mL/min.

For the above conditions the required $bL\mu E$ values were 0.48 mL/min for cytochrome *C* (see Eq. 3) and 0.26 mL/min for ferritin (see Eq. 4). As shown in Table 1, the calculated $bL\mu E$ values equaled or exceeded these requirements, suggesting that these two proteins should be fully resolved.

Experiments were first done using the two individual proteins as shown in the two top panels of Fig. 4(A). The detector signals show clearly that the two proteins were fully recovered from their respective outlets. A sample consisting of a mixture of the two proteins at the same concentration levels as above was then run through the system. The results, presented in the third panel of Fig. 4(A), show that the mixture separated into two peaks, one eluted from each of the outlet ports, with each peak having a profile virtually identical to that found for the two individual protein injections (shown in the top two panels of Fig. 4A). The experiments of Fig. 4(A) were repeated twice with the same results. These results confirm that the electrical SPLITT cell is capable of carrying out protein separation.

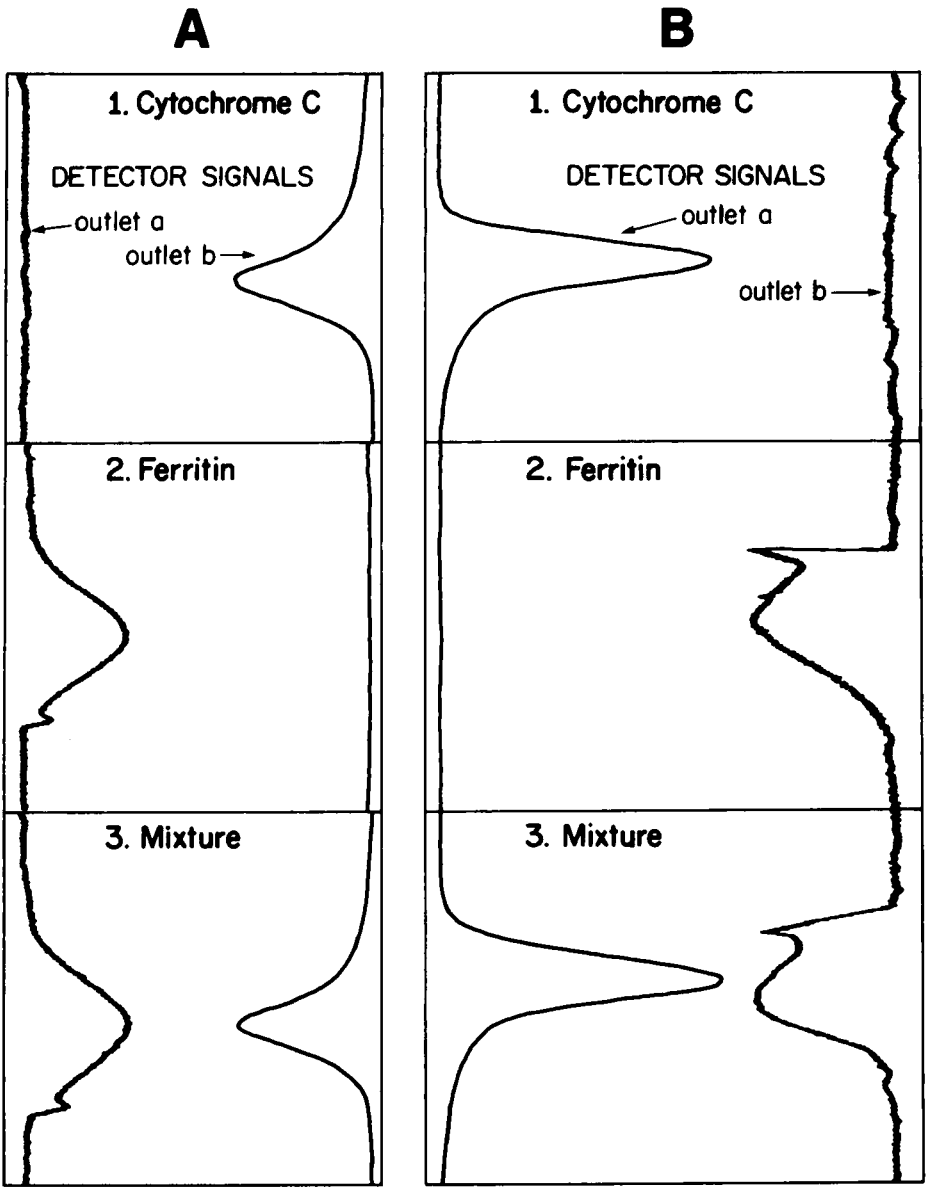

FIG. 4. Detector signals from outlets a and b for discrete samples of cytochrome *C*, ferritin, and a mixture of the two. In A the polarity is that shown in Figure 1 (A+, B−); in B the polarity is reversed (A−, B+).

In the final set of experiments (set IV) the polarity was again reversed with all flow rates and the pH remaining as they were in set III. The required $bL\mu E$ values are reversed with respect to cytochrome *C* and ferritin as shown in Table 1. These required values were substantially exceeded by the calculated values.

The experimental work demonstrated that cytochrome *C* emerged solely from outlet a as shown in the top panel of Fig. 4(B). The entire content of Ferritin emerged at outlet b. Once again a mixture of the two was clearly separated into the constituent proteins, each yielding almost identical elution profiles as they did when injected singly.

The assumption that the proteins are fully separated from the binary mixture is further supported by the very close agreement of peak areas for the single and combined injections of Fig. 4. Thus the two "cytochrome *C*" peaks in Fig. 4(A) do not measurably differ from one another while the "ferritin" peak emerging from the mixture has 5% less area than that of ferritin injected alone. The latter small difference can be explained by variations in injection volume or in ferritin concentration in the two solutions. For Fig. 4(B) the "cytochrome *C*" peak arising from the mixture has an area less than 4% under that of the pure cytochrome *C* injection. In the same figure the "ferritin" peak area from the mixture is within 3% of that of the singly injected ferritin peak.

Several experiments were also done to determine if the proteins were fully recovered from the SPLITT cell. For this case the area of single peaks was compared upon emergence from the SPLITT cell and from a short length of impermeable tubing. The results indicate that the ferritin yield was 100% while the cytochrome *C* yield was 86%.

CONCLUSIONS

The experiments performed in this paper confirm the workability of the electrical SPLITT cell for simple mixtures of oppositely charged proteins. Obviously the work must be expanded to examine more complex and realistic protein mixtures, particularly those having closer isoelectric points. Also, such factors as the durability of the system under continuous operation must be established. The maximum practical concentration of protein in the incoming stream also needs investigating.

Despite the many questions that must still be resolved about the system, these initial experiments are highly promising. According to the calculations presented for set I in Table 1, the throughput could be increased almost threefold without adverse effects. Under these condi-

tions the proteins would pass through the channel and be completely separated in about two minutes. Thus the exposure of the protein mixture to the separation system would be minimal. While closer isoelectric points would slow down the separation, increases in the moderate voltage would lead to substantial gains.

While the SPLITT system described here yields only two fractions, it is in principle a simple matter to link such cells together and by changing the pH in succeeding cells, expand the separation from its binary form to a multifraction form (*1*). By carefully adjusting the pH in two cells working in sequence so that in one case the pH is slightly higher than that of a desired protein and in the other case lower, it should be possible to isolate the chosen protein from impurities as long as the contaminant isoelectric points did not fall in the bracketed range.

Investigations are currently underway to explore these additional aspects of electrical SPLITT cell operation.

Acknowledgments

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