
**Separation Science and Technology**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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**To cite this Article** Raymond, Samuel and Jordan, E. M.(1966) 'Separation and Recovery of Proteins by Elution-Convection', Separation Science and Technology, 1: 1, 95 — 111
**To link to this Article: DOI:** 10.1080/01496396608049437
**URL:** http://dx.doi.org/10.1080/01496396608049437

SEPARATION SCIENCE, 1(1), 95-111 (1966)

# Separation and Recovery of Proteins by Elution-Convection

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## Summary

The design, construction, and operation of an apparatus for preparative electrophoresis is described. This apparatus employs high-resolution gel electrophoresis to separate protein components with subsequent electroconvection to effect their elution and concentration.

## INTRODUCTION

The separation and recovery of proteins on a laboratory scale is a problem which engages the attention of many biochemists. Pure proteins in quantities of a few milligrams up to a gram may be required in studies of enzymes, genetic variants, protein structure, and so forth. The classical methods of protein separation and purification—salt precipitation, adsorption, organic solvent solubility, pH variation, and so forth—often produce low yields of chemically altered proteins after many laborious processing steps. New techniques of processing have been introduced, for example, gel filtration and countercurrent distribution [Tiselius et al. (*17*)]. These techniques provide additional parameters of separation, such as molecular size (gel filtration) or phase partition (countercurrent distribution).

In all these techniques the practical problems can be regarded as twofold: (1) to separate the desired proteins from each other and

from the natural mixture, and (2) to recover them in a suitable form. The first part usually requires the successive applications of more than one separating parameter because of the similarity in chemical properties of proteins generally; the second usually requires reconcentrating or recrystallizing the products, because most of the practical techniques yield dilute solution of the desired proteins.

In protein separations, gel electrophoresis offers a resolving power which is unmatched by any of the techniques previously used, including other forms of electrophoretic separation. In both starch gel [Smithies (*15*)] and acrylamide gel [Raymond and Weintraub (*13*)] the proteins migrating through the gel matrix are subject to separation by a combination of electrophoretic mobility (which depends on the electrical charge of the molecule and the particular ionic environment) and molecular size (because of the so-called "sieve effect" in a matrix of comparable molecular dimensions). It is, furthermore, comparatively easy to design gel electrophoresis equipment of laboratory size capable of handling up to 500 mg of a protein mixture as a single charge (and not too difficult to scale this up 10- or 100-fold if required).

The chief practical problem in applying gel electrophoresis on a preparative scale has been the means for eluting the protein components from the gel after resolution has been achieved. The designs that have been proposed [Altschul et al. (*1*), Avrameus (*2*), Lewis and Clark (*4*), Racusen and Calvanico (*7*), and Roos and Gemzell (*14*)] comprise concentrating the entire initial protein charge in a single zone on the gel, spreading the proteins out in a spectrum or pattern by forward migration through a column of the gel (so that separated components occupy separate zones arranged in a linear or serial order along the gel column), continuing the forward migration through the end surface of the column (so that the zones emerge serially from the end surface of the gel column), sweeping each zone aside in a transverse flow of buffer solution as it emerges, and finally collecting it separately as a series of fractions in a standard fraction collector. The movement of protein through apparatus of this kind takes place entirely within a volume of buffer solution (both external to and within the gel-support medium) which is continuous with the buffer solution in the electrode compartments, in order to provide and maintain the electric field which causes electromigration of the proteins. A mass flow of

buffer transverse to the direction of electrophoretic migration is required (1) to carry the protein out of the migration path (and so prevent it from reaching the electrode region, where electrode reactions might cause unwanted chemical changes in the proteins), and (2) to keep the emergent zones separated until they reach the fraction collector. The transverse mass flow of buffer must be sufficiently rapid to ensure complete hydrodynamic sweeping of the complete volume of the elution chamber, to ensure total removal of one protein zone before the following one has reached the chamber.

Although it is not the purpose of this paper to offer a complete analysis of the design factors involved in these designs, some general remarks may be in order which will elucidate the rationale of the new design we shall present herein. These remarks will be arranged under the headings: heat, time, dilution, sample size, and continuous-flow operation.



### Heat

It can be demonstrated theoretically that the proper figure of merit for a preparative electrophoretic apparatus is the power capacity of the cell, i.e., the maximum allowable heat input into the cell calculated as Joule heat generated by the current and voltage passing through the cell. The theoretical basis of this statement has been presented by Raymond (*9,10*) for two types of electrophoresis cells and can be generalized for any electrophoretic method. It is, therefore, advisable to maximize the cooling capacity of a practical design. In most of the previously mentioned designs, the gel column has the shape of a cylinder. This geometry has the least surface area for a given contained volume of specified length and, hence, has the least surface through which heat can be removed from the gel matrix. The design can be improved considerably in this respect by inserting a "cold-finger" type of heat exchanger axially through the center of the gel cylinder, in addition to providing a coolant around the external cylindrical surface. The cold-finger design thus converts the gel cylinder into a flat sheet or slab of gel matrix which is bent around the circumference of a circle. This introduces some complexities in the practical realization of the equipment, especially in the coolant connections and in the provision of a uniform electric field around the circumference of the cylinder. Nevertheless, the cold-finger design

represents a significant improvement over the simple solid cylinder of gel matrix in terms of power capacity.

### Time

The time required to effect recovery of protein in usable form following the electrophoretic separation is an important factor in the design of a preparative cell, if for no other reason than that many proteins are chemically unstable in solution, although the economic value of the research worker's time and the optimum utilization of laboratory facilities ought also to be considered. The achievement of a given degree of separation of two components is directly related to the total energy input into the electrophoresis cell, and the time required is therefore a direct function of the power-handling capacity of the cell. This time is fixed by the properties of the protein system being separated. Two additional operational times have to be considered: (1) the time required for elution and (2) the time required to process the protein solution after elution.

1. In the designs described above, the protein zones are eluted one at a time by successive migration through the end of the gel column. To obtain an adequate separation of successive zones it is often necessary to make the over-all length of the column much larger than the diameter (or the thickness, if the column is regarded as a slab). But the time required for elution of any specified zone increases linearly with the length of the column, and may be of excessively long duration if the protein has a low rate of migration. One solution to this dilemma is to make the gel column sufficiently long to achieve the required degree of separation, and then to elute each zone via the shortest path through the face of the gel matrix. A practical apparatus design for this is presented in Fig. 1.

2. An important element of the over-all procedure is the time required to process the protein after recovery from the elution cell. Evidently, if the protein is recovered in a dilute form, the necessity for and difficulties involved in reconcentrating the protein must be considered in evaluating the over-all design. For the reasons presented above, the eluting volume of individual protein zones may be comparatively large; a dilution of $10^2$ or $10^3$ is not uncommon in the practical application of these techniques, and it is larger for slower-moving proteins than for faster-moving ones.

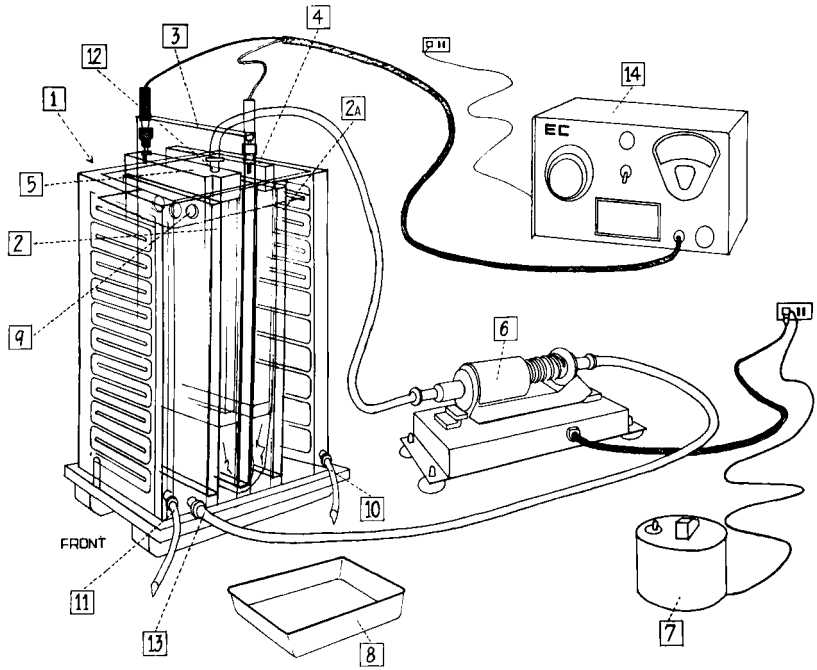


**FIG. 1.** Elution–convection apparatus as operated, showing: 1, buffer tank; 2, front support plate, 2A, rear support plate; 3, elution grid; 4, positive electrode; 5, negative electrode; 6, buffer pump; 7, powerstat; 8, Teflon pan; 9, dummy connection; 10, cooling inlet; 11, cooling outlet; 12, buffer inlet; 13, buffer outlet; 14, power supply.

The design principle we shall present herein attempts to meet this problem by a unique combination of simultaneous eluting and reconcentrating effects.

### Dilution

In addition to the time required for processing a desired protein, dilution in itself may have specific effects on the integrity and stability of a protein. Some proteins are intrinsically unstable at high dilution, even at the optimum pH and in the presence of a favorable supporting electrolyte. Others may become altered through loss of the stabilizing substances normally associated with them in more concentrated solution, which act as protective colloids, dissociation inhibitors, antioxidants, and the like. A special

case of dilution comprises the effect of dialysis against a large volume of buffer solution, during which the nominal concentration of the protein itself may remain within the range of stability, but the concentration of protective substances of low molecular weight may be reduced below the danger point. It will be seen later how far our design is not successful in meeting this specific requirement. A final aspect of dilution involves the reduction of the ionic constituents of the solvent system, i.e., the buffer itself, as by dilution with deionized water. Obviously this does not constitute any particular problem in preparative electrophoresis work.

### Sample Size

A consideration of sample size involves the total weight of protein which can be processed in a single charge (and this in itself is affected to some extent by the composition of the charge) and the volume of solution in which it is applied at the start of the separating process. It is an observable fact that different gel matrices offer different carrying capacities, and that the carrying capacity may be greater if the charge is relatively heterogeneous compared with one containing only a single component. In the latter case, the high local concentration of the single protein often affects the local pH and buffer conditions within the gel in a way adverse to good separation. Thus, for example, the albumin component of the serum protein system usually occupies a zone much larger in physical volume than can be accounted for by ordinary diffusion effects during the electrophoresis.

This effect is significantly reduced by choosing a gel and buffer combination with high "carrying capacity" suitable for the specific protein system under study. The term "carrying capacity" cannot at present be defined in general terms, as it relates to many specific chemical and physical interactions within any given system. Although the choice may well vary in accordance with specific requirements and is to some extent a matter of opinion, in common with most other workers in this field we believe that acrylamide gel usually proves superior to other gels. In addition to certain desirable properties of strength, flexibility, and ease of preparation, it exhibits zero endosmotic effect and therefore eliminates any disturbance of the electrophoretic pattern resulting from this cause. This is especially important in the elution-convection

apparatus, which depends, as will be seen, on controlled convection for satisfactory operation.

The other aspect of sample size, i.e., the total volume of the charge (as a solution), comes into play in many problems where the protein starting material is obtained as a dilute solution, as, for example, in extracts of a solid tissue or in press juice from a plant source. In such cases the application of initial concentrating conditions such as ionic strength [Hjerten et al. (3)] or discontinuous buffer systems [Poulik (6); Ornstein (5)] within the cell itself may well prove highly advantageous by eliminating a preliminary concentration step. We have not heretofore found it necessary to use a concentrating step, although in principle it should apply in a straightforward way to our procedure. On the other hand, it has not seemed to us very sensible to dilute an initially concentrated protein source deliberately in order to apply such a technique.



### Continuous Operation

For the sake of completeness in this discussion, brief mention may be made of continuous flow preparative methods. The above treatment is not easily adaptable to continuous flow processing of the type illustrated by the well-known paper-curtain type of electrophoresis apparatus or the newer free-flow electrophoresis (e.g., the Brinkmann Elphor). It is in principle possible to devise gel electrophoresis apparatus in which, after suitable initial conditions are established, a continuous feed of protein solution is introduced and continuous product streams are taken off. The critical points, however, are (1) the difficulty of establishing, maintaining, and controlling initial conditions, including flow rates, on a laboratory scale, and (2) the comparative rarity of requirement for processing large enough volumes of initial sample to make such controls economically advantageous. In short, by the time one has used up several liters of solution in adjusting a continuous-flow type of apparatus, a batch-type apparatus could well have processed sufficient material for the problem at hand.

## PRINCIPLES OF ELUTION-CONVECTION

With this preliminary discussion as background, we now introduce the principle of elution-convection, and thence proceed to

describe a realization of the apparatus required to effect the principle. A preliminary description of this technique has already been published [Raymond (*12*)]; what follows will provide greater detail and offer improvements resulting from experience over the past year.

The elution-convection technique comprises two essential steps: (1) preparation of a gel pattern in which protein components of a mixture are resolved into parallel zones separated on a gel matrix in the form of a flat slab, and (2) elution of all zones simultaneously through the face of the slab into parallel, vertically positioned electroconvection channels [Raymond (*8*)] which concentrate the eluted protein zones into separate reservoirs or collection tubules. The standard design to be described will accept up to 100 mg of protein and will collect 31 fractions of 1.0-ml volume each.



Because of the way in which the elution-convection cell operates, its application is limited to those proteins which demonstrate downward convection when concentrated by electrophoretic migration at a vertical dialysis membrane. It is therefore inapplicable to the recovery of low-density lipoproteins which float rather than sink, small polypeptides which may pass through the dialysis membrane, and materials which precipitate upon or adhere to the dialysis membrane used. During the application of the technique, the eluted proteins are subjected to strong electrodialysis effects, which may cause loss of small cofactors or prosthetic groups. In the present state of technology, the apparatus is necessarily constructed principally of Plexiglas, a methyl methacrylate sheet polymer incorporating certain plasticizers and UV-absorbing materials, which are by no means completely inert toward protein and buffer solutions. Finally, the supporting matrix is acrylamide gel, which ordinarily contains small amounts of unreacted monomer and short-chain-length soluble polymers as well as catalyst residues, which contaminate product. (It is, however, possible to eliminate these by suitable pretreatment of the gel slab.)

Many of these limitations are common to most protein processing techniques. Any individual one, and most of them together, could be eliminated by suitable design of a cell at the cost of considerable additional effort and expense. What follows is a design which meets the requirements of most practical problems.

## DESCRIPTION OF APPARATUS

The elution-convection technique as a whole includes both the preparation of the gel pattern and the subsequent elution of the proteins. Each of these steps is normally carried out in a separate piece of apparatus: the electrophoresis cell and the elution cell, respectively. The following description applies to one specific design of apparatus, which is commercially available, although obviously departures from this specific design could also be effective in applying the general principle. That to be described represents the optimum result of many individual designs.

### The Electrophoresis Cell

This is nearly identical in functional design to that previously described [Raymond (*11*)], differing principally in the dimensions of the gel slab. For preparative work, the optimum compromise between a thick gel (with capacity to accommodate a larger amount of sample) and a thin gel (with a more uniform temperature throughout the gel) seems to fall at about 6 mm. This is twice the thickness of the standard analytical vertical gel electrophoresis cell previously described. The slot form used with this cell produces slots 4 mm by 25 mm, 10 mm deep, accommodating optimally up to 50 $\mu$l of 10% protein solution in each slot. Four slots are used to ensure uniform distribution of the sample across the width of the gel slab. With additional care, a single continuous slot can be utilized across the entire width of the gel slab to gain a minor increase in sample carrying capacity. Since the total area of the slots is 4 $cm^2$, compared to over 6 $cm^2$ total cross section of the gel slab, an increase of 50% in sample size could be obtained by applying the sample to the completely flat top of a gel slab. Unfortunately, difficulties in forming such a top and in preventing leakage of protein down the face of the slab appear insurmountable. Improvements over the original design include (1) a more convenient locking clamp, (2) a better electrical-contact assembly, (3) a more rigid cell, and (4) continuous recirculation of buffer to prevent change in the buffer compartments.

Although the cell described provides maximal control of experimental variables, other designs can also be used if they provide a gel slab 6 mm thick containing the separated proteins in a pattern

of parallel zones, over an area 10 × 10 cm to fit the elution grid described below.

The starch gel apparatus of Smithies (*16*) could be used (at lower power input), or any of the available designs of horizontal gel electrophoresis. The essential requirements are adequate resolution and dimensions to fit the elution grid.

### The Elution Cell

This cell consists of four major components, plus power supply.

**The Elution Grid.** This is a flat plate of Plexiglas, 12 mm thick (Fig. 2). On one face (the "gel face") there is a rectangular depres-

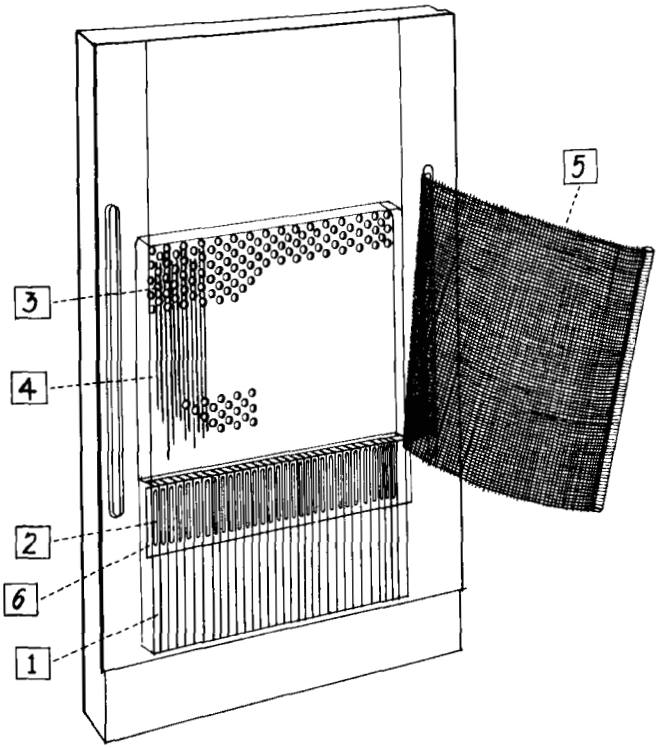


**FIG. 2.** Elution grid (gel face forward), showing: 1, collecting tubules; 2, vertical ports; 3, grid holes; 4, electroconvection channels; 5, screen; 6, dialysis tubing over ports.

sion 10 cm × 10 cm, 6 mm deep, to receive the gel slab to be eluted. Covering the gel space is a sheet of plastic screening, held in place by a bar at each side, to retain the gel slab in position during operation. A grid of small holes is pierced through the thin wall of the gel space to the opposite face (the "grid face"). This grid of holes conducts the eluted protein to the electroconvection channels, which are vertical grooves in the grid face, aligned precisely with vertical rows of holes in the grid. The spacing of these grooves is 2 mm, allowing theoretically for the separate collection of two narrow zones 3 mm apart. However, in practice, a mismatch, nonalignment, or lack of parallelism of the zones may increase this figure to 6 mm.

Each electroconvection channel is bounded on four vertical sides by the two walls of the groove, the face of the gel slab itself, and a dialysis membrane on the opposite wall which is held in place by external hydrostatic pressure. The four-sided channel thus formed leads downward to a separate collecting tubule at the bottom of the elution grid. Connection between each channel and its corresponding tubule is made by a passage slanting downward from channel to tubule. The tubules are arranged in a single row across the bottom of the grid. Adjacent tubules are separated by a thin partition, with spacing of 3 mm corresponding to the spacing of the electroconvection channels. The volume of each collecting tubule is 1.0 ml. Through the front and back face of each, near the top, there are aligned rectangular ports, 1.5 mm × 25 mm. These ports provide additional electroconvective concentration above that available in the main electroconvection channels. Across the face of the row of ports on each side of the grid is cemented a sheet of dialysis tubing to permit passage of the electric field through the ports, while retaining the nondialyzable protein within the collecting tubules.

The edges of the elution grid are keyed for insertion into the buffer chamber (described below), so that the grid can be assembled only in the correct orientation with respect to the electrodes and the buffer chamber.

**The Electrodes.** There are two electrodes, one for each side of the elution grid. The frame of the negative electrode carries a buffer inlet channel, with a connection for recirculation of the buffer. The electrode itself is a sheet of platinum gauze which completely covers the area of the gel and also the exposed area of the ports.

In certain applications, where trace amounts of heavy metal contamination are not important, certain grades of stainless steel can be used for the electrodes. Stainless steel cannot be used at all, however, if the buffer contains chloride ions, as very rapid corrosion of the steel takes place, with liberation of a copious brown colloid of ferric hydroxide, even at the low current densities normally used in the elution convection cell.

The electrodes (Fig. 3) are used in conjunction with a sleeve of

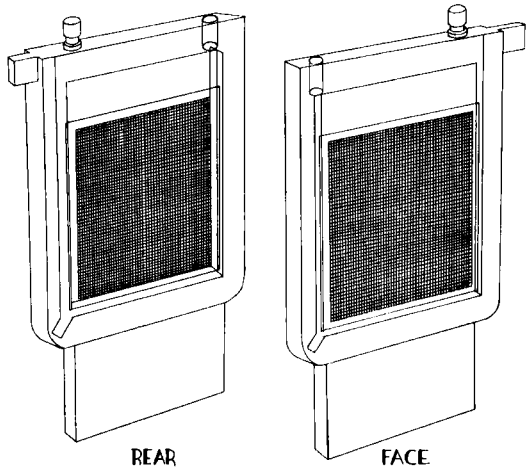


**FIG. 3.** Negative electrode, face and rear views.

Visking dialysis tubing of standard size 16 cm wide (flat width) and 58 cm long. One electrode is inserted into each end of the sleeve so that when it is folded at the center, the electrodes face each other in proper orientation to provide a horizontal uniform electric field through the elution grid which is supported between and parallel to them. Like the elution grid, the electrodes are keyed so that they can be inserted into the buffer chamber in only one way to provide the proper orientation with respect to the elution grid.

The dialysis sleeve, meanwhile, when folded, as mentioned above, forms a continuous U-shaped bag enclosing the two faces of the elution grid. During operation of the elution cell, buffer is pumped continuously into the buffer inlet connection previously mentioned, passing down the inlet arm and up the other arm, then overflowing the outlet arm of the bag. The buffer flow serves to

continuously remix the anode and cathode buffer, preventing any significant pH or buffer composition changes from developing, and at the same time expands the dialysis tubing to provide the hydrostatic pressure head necessary to hold the membrane wall of the electroconvection channels in place, which wall is itself formed by this same dialysis bag.

In operating the elution cell the polarity of the field and the charge on the protein ions must bear a definite relationship to each other, in such a way that the protein migrates electrophoretically out of the gel slab toward the elution grid and through the grid toward the dialysis membrane wall of the electroconvection channel, where it accumulates and concentrates, forming a descending convection current. In normal operation with, for example, serum proteins in alkaline pH buffers, this requires that the positive electrode must be placed on the grid face and the negative electrode on the gel face of the eluting grid. The electrodes are keyed so that in these conditions the red electrode is to be connected to the red (positive) output of the power supply.

**The Buffer Tank.** The buffer tank is a rectangular Plexiglas box, open at the top, with cooling channels in the walls (see Fig. 1). Inlet and outlet connections are provided for coolant; also a buffer recirculation connection is provided through the wall near the bottom of the chamber, and another, dummy connection, near the top (the function of the latter will be described below). In the two side walls are placed three sets of grooves, each set consisting of a groove in one wall aligned with a groove in the opposite wall. The center groove set comprises one wide groove and one narrow one, for keying the orientation of the elution grid. This set is aligned at a slight angle to the vertical and permits the insertion of the elution grid in a slightly tilted position. The orientation of the elution grid is such that the grid face leans away from the vertical, so that when a heavy protein solution emerges from the gel and enters the corresponding hole in the grid, it will have a tendency to run down the hole toward the membrane face of the electroconvection channel.

The other two pairs of grooves each receive one of the two support plates, whose function will be described later.

**The Buffer Pump.** The buffer pump is a small, oscillating diaphragm type of pump in which the pumped fluid is completely insulated chemically and electrically from the metal parts of the

pump (see Fig. 1). This prevents contamination and electrolytic action within the pump. The pump intake is connected to the buffer connection near the bottom of the buffer chamber by tygon tubing, and similar tubing leads from the pump to the buffer inlet at the top of the negative electrode. Circulation is continuous through both sides of the buffer sac in sequence, thence into the buffer chamber external to the sac (by overflowing the top of the sac), where it is cooled by the refrigerant circulating in the walls, and thence through the pump to complete the circulation.

The rate of pumping is controlled to produce a steady but not excessive overflow of buffer from the top of the sac.

**The Power Supply.** The power supply used with the elution-convection cell (see Fig. 1) differs from that used with the vertical gel electrophoresis cell in that it furnishes a low voltage (20 to 50 volts) at a moderately high current (500 ma). The power supply recommended furnishes a completely isolated output (for safety), unregulated (for economy and reliability).



## Synopsis of Operating Procedure

The prior separation of the mixture takes place in the EC470* Vertical Gel Electrophoresis Cell on a 6-mm gel slab [Raymond (*11*)] or an equivalent unit. The account which follows deals only with steps occurring after a suitable gel has been prepared and run. It is useful to excise a guide strip and stain it routinely to facilitate the location of the eluted material.

**Connection of Circulating Systems.** The cooling system is connected with tygon tubing to carry the coolant (ethylene glycol and water at 5°) through the cooling channels of the buffer tank.

Similarly, tubing from the buffer pump is connected to the buffer-tank outlet and buffer-tank inlet dummy connection. This dummy connection serves to hold the buffer inlet temporarily above the level in the tank. The permanent connection is made later. Figure 1 shows the general arrangement employed.

At this stage the tank is filled with 4 liters of the appropriate buffer. Experience to date indicates that an ionic strength of 0.02 is optimal, but the precise formulation must depend on the nature of the sample being eluted.

* E-C Apparatus Corp., Philadelphia.

**Electrode Assembly.** The electrodes are inserted into a sleeve of dialysis tubing (16 × 58 mm). It is important that they be inserted face up, that is, with the less-recessed side up (Fig. 3).

**Insertion of Gel Slab.** This operation is completed with the collecting tubules submerged in the buffer. The pre-cut gel slab is then positioned on the grid. Compared with its original orientation in the Vertical Gel Cell, the gel slab is rotated through 90°. In this way the individual fractions are arranged serially from left to right.

**Final Assembly.** The electrode assembly, elution grid, and support plates are positioned in the buffer tank as shown in Fig. 1. It must be appreciated that due regard be given to the direction of movement of the protein fractions during elution. (For anionic species the grid face should be toward the cathode.)

At this point it is necessary to transfer the buffer inlet from its dummy connection to its proper socket; the electrodes should also be connected to the power supply.

**Elution Conditions.** A constant potential in the range 15 to 20 volts is applied for a period of up to 6 hours. The current should drop from 500 ma to approximately 200 ma over this interval. The precise conditions should be determined empirically by staining the gel slab after elution to ensure that no material is retained therein.

**Collecting the Fractions.** After elution the fractions are found in the collecting tubules. They are conveniently removed with a small hypodermic syringe fitted with polyethylene tubing. In this way the material is contained within 1.0 to 1.5 ml.

## RESULTS AND CONCLUSIONS

Owing to the recent development of this equipment, it is not possible to give a full account of its scope and performance at this stage. To date, particular attention has been given to achieving high recovery values for some well-characterized proteins. For example, figures relating to albumin and hemoglobin are shown in Table 1. By any standards the recoveries are excellent. No denaturation during elution was observed.

Current work in this laboratory is orientated toward achieving similar recoveries of proteins with low mobilities in the system employed. With present techniques, such material is eluted too slowly to give the density gradient at the membrane necessary to

effect collection. It is possible to minimize this limitation by extending the time of elution, but for extreme examples (e.g., slow $\gamma$ = globulins at pH 8.4) this maneuver is not profitable. Even when recoveries are low, there appears to be no loss of resolution.

**TABLE 1**

Typical Recoveries of Pure Proteins with the Elution-Convection Technique[a]

| | Quantity, mg | Sample volume, $\mu$l | Recovery, % |
|---|---|---|---|
| Cyanmethemoglobin | 0.5 | 5 | 70 |
| Cyanmethemoglobin | 10 | 2 × 50 | 89 |
| Cyanmethemoglobin | 50 | 1000 | 62 |
| Human serum albumin | 9 | 3 × 60 | 72 |
| Human serum albumin | 18 | 3 × 100 | 59 |

[a] All the samples were separated and eluted in a pH 8.4 tris-EDTA-borate buffer ($I = 0.02$) except that the elution of albumin was conducted in a tris-glycine buffer of identical pH and ionic strength. This enabled the biuret reaction to be employed for the estimation of this protein. Hemoglobin was determined by a standard colorimetric procedure.

It is worth noting that the technique of elution convection relies for its resolution on the ultimate analytical technique—vertical gel electrophoresis. Unless a new and fundamentally different approach to the separation of proteins is conceived, it is difficult to see how any other single-stage fractionation method can hold more promise as a high-resolution preparative technique.



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*Received by editor December 24, 1965*
*Submitted for publication January 19, 1966*