# United States Patent

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[54] **ELECTROPHORETIC SYSTEM**
**6 Claims, 6 Drawing Figs.**


[52] **U.S. Cl.** ..... **204/180 G**
[51] **Int. Cl.** ..... **B01k 5/00**
[50] **Field of Search** ..... 204/180 R, 180 G, 299



[56] **References Cited**

UNITED STATES PATENTS

| | | | |
|---|---|---|---|
| 3,539,494 | 11/1970 | Pretorius et al. | 204/299 |
| 3,384,564 | 5/1968 | Ornstein et al. | 204/180 |
| 3,470,080 | 9/1969 | Raymond et al. | 204/180 |
| 3,506,554 | 4/1970 | Broome | 204/180 |

FOREIGN PATENTS

| | | | |
|---|---|---|---|
| 833,364 | 2/1969 | Italy | 204/180 |

OTHER REFERENCES

Schwalbe, " Pulsed Field Electrophoresis," Medical Electronics, Internat. Conf. on Med. Electronics, Paris, 1959, QT34 I64m (1959)

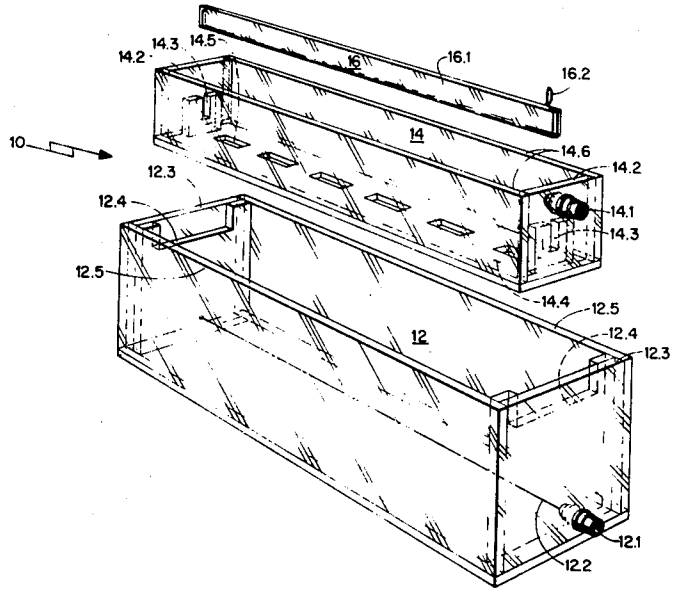
Davis, " Method and Appt. to Human Serum Proteins." Annals of the N.Y Academy of Sciences, Vol. 121, Article 2. Page 424 Dec. 28, 1964.



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**ABSTRACT:** An electrophoretic system for concentrating and separating charged particles and for improving the resolution of the separated particle laminae that utilizes a buffer composition which will provide a ratio of leading-trailing ion boundary to the fastest particle mobility that approaches unity, during the period of free electrophoresis.

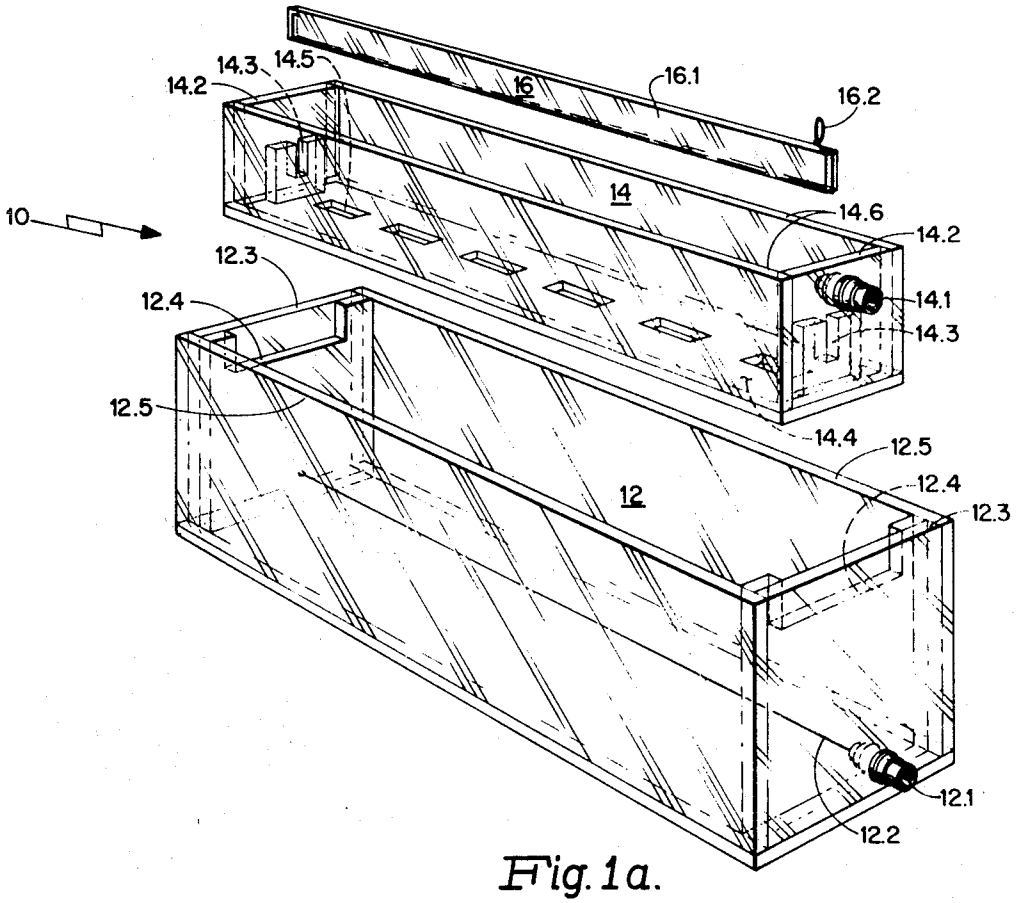



Fig. 1a.

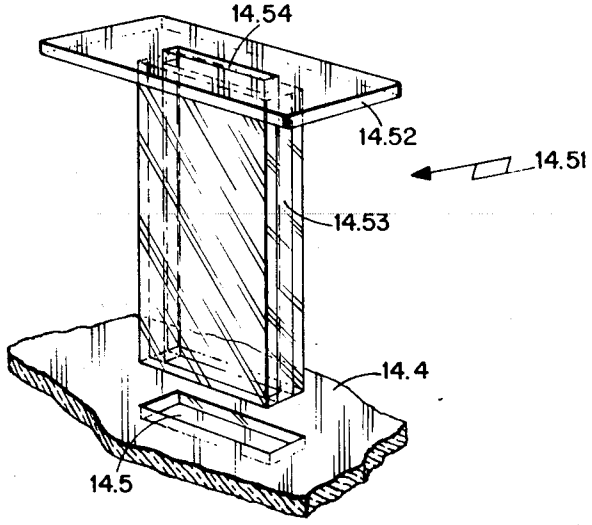



Fig. 1b.

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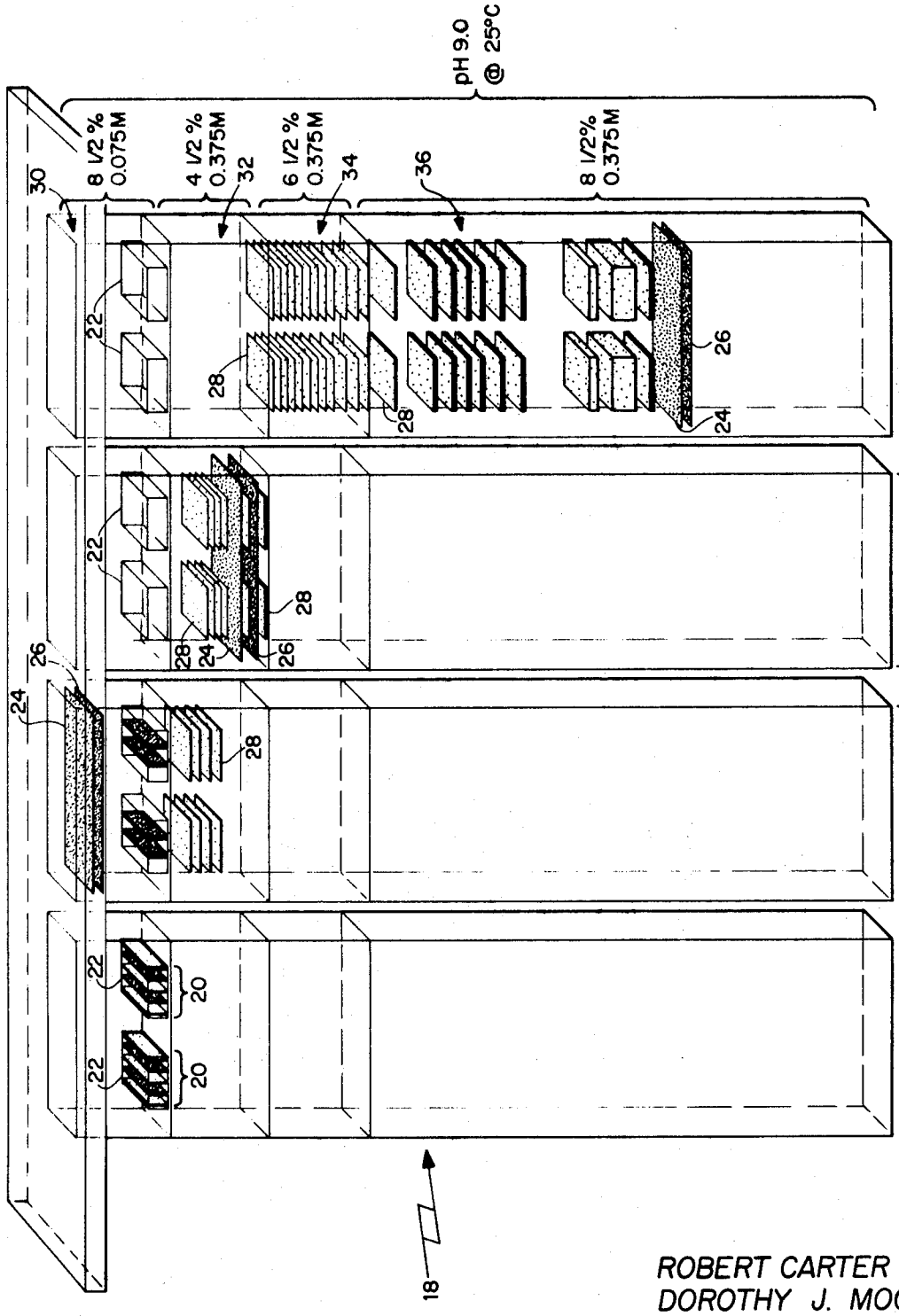



Fig. 5.

Fig. 4.

Fig. 3.

Fig. 2.

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# ELECTROPHORETIC SYSTEM

## BACKGROUND OF THE INVENTION

This invention relates to an electrophoretic system and more particularly to an improved electrophoretic system for producing greater resolution of the separated particle laminae.

Electrophoresis, particularly that carried out in a molecularly sieving medium such as starch gel and more importantly in acrylamide gel, represents one of the most effective procedures for the separation of charged particles. However, a major problem in such gel electrophoresis, whether carried out with either single ion buffer systems or discontinuous buffer ion systems or in discontinuous pH and buffer ion combination systems, has been in the separation and consequent quantification of isoenzymes, i.e., multiple molecular forms of the same enzyme. (Early qualitative or quantitative changes in a particular isoenzyme profile of an individual may have great significance in the early detection of physiological changes associated with a disease such as cancer, etc., prior to the onset of the present commonly recognized clinical signs.)

The drawback of a continuous buffer system resides in the fact that one must use low current densities and voltage gradients to limit joule heating to levels below that which may destroy enzyme activity. This serves to lengthen separation time with a resultant loss in zone resolution. In addition, excessively long (10 cm.) separations must be performed to obtain adequate resolution.

The use of a discontinuous pH and buffer ion systems, wherein samples to be separated are first polymerized in an anticonvection large pore gel medium, may lead to enzyme inactivation due to free radical formation in the polymerization procedure. In this situation, up to 65 percent of some types of enzyme activity may be lost in such a procedure. While current densities utilized in protein separations with this technique may be employed at levels sufficient to perform separations up to 2½ cm. in length in 30 to 40 minutes, current and voltage must, nevertheless be markedly reduced for enzyme separations of even the more heat stable isoenzymes such as esterases. Thus, separations of 75 to 90 minutes are required for acceptable resolution.

With discontinuous ion and pH systems, which require trailing ion migration to be programmed by means of pH differences, and where the counter ion is Tris (hydroxy methyl amino methane), lowering the temperature of the system to separate heat labile enzymes increases the pH of the system approximately 0.03 pH units per degree Centigrade of temperature decrease. Thus, a buffer made up at the proper pH at room temperature will be about 0.7 pH unit higher at 1° C. Since the resolution in the discontinuous pH and ion system depends in major part on the trailing ion mobility being programmed by appropriate pH to be less than that of the slowest particle to be separated during the stacking phase, lowering the temperature sufficiently to protect many enzymes will raise the pH and thus cause the trailing ion to migrate faster than the slower particles, thereby degrading separation quality. The necessity to carefully adjust buffer ion concentrations for the different temperature conditions utilized with this technique for protein and isoenzyme separations severely limits the versatility of this systems.

When it is necessary to separate isoenzymes and proteins of both plasma and tissues, under identical conditions for accurate qualitative and quantitative comparison, a multiple sample gel slab rather than individual separations is most desirable and may, in certain instances, be required.

A thin gel slab is desirable also for the following additional reasons: (1) temperature and voltage are identical across the entire slab, and (2) slabs, due to their flat surfaces, are considerably more suitable for densitometric analysis due to the absence of combinations of vertical alignment usually encountered with cylindrical shapes.



## SUMMARY OF THE INVENTION

The present invention provides an electrophoretic system for concentrating and separating charged particles resulting in an improved resolution of the separated particle laminae by utilizing a buffer combination which provides a ratio of leading-trailing ion boundaries to fastest particle mobility that approaches unity, during the period of free electrophoresis. In our system, the buffer combination and pH range, together with the gel buffering concentration is carefully chosen so that a discontinuous buffer ion and continuous gel pH system is provided to produce the enhanced resolution of separated particle laminae.

It is, therefore, one object of the present invention to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system, wherein charged components are separated from a starting medium to produce thin laminae in a similar pore separating gel by utilizing a conductivity shift.

Another object of the present invention is to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system wherein two sequential, rapidly moving ionic boundaries pass through the components that had been previously separated, resulting in a sharpened zone as the boundaries formed at the leading and trailing ions pass through each zone.

Another object of the present invention is to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system wherein an enhanced resolution of various, similar size particles, in a mixture of many sizes of particles is achieved by providing gradient pore size gels so that particles differing only slightly in size with similar charges, may be maximally resolved.

Still another object of the present invention is to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system utilizing a continuous pH gel and a discontinuous buffer ion solution to enhance the resolution of the separated particle laminae.

Yet another object of the present invention is to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system capable of producing increased resolution by selection of leading-trailing buffer ion and gel combinations such that conductivity differences may be used to effect changes in separation time and resolution in various areas, as may be desired by the operator.

A further object of the present invention is to provide a two-step, continuous pH, discontinuous buffer ion electrophoresis system which allows simultaneous separation of various types of particles on the same gel slab under a set of conditions for maximal resolution for each sample, to allow for the simultaneous analysis of proteins and enzymes from plasma together with tissue extracts from various species.

The features of our invention which we believe to be novel are set forth with particularity in the appended claims. The invention itself, however, both as to its organization and method of operation, together with further objects and advantages thereof, may best be understood by reference to the following description taken in combination with the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1*a* is an exploded view of the buffer tanks and electrodes for our flatbed, vertical, continuous pH, discontinuous buffer ion electrophoresis system;

FIG. 1*b* is the typical cell configuration for use with the tank of FIG. 1*a*;

FIG. 2 is an isometric representation of the gel portion of our electrophoretic system after insertion of a sample but before application of the appropriate electrical potential;

FIG. 3 is an isometric representation of the gel portion of the same system after power has been applied for a given duration and shall hereinafter be referred to as "phase 1";

FIG. 4 is an isometric representation of the system of FIG. 3 after electrical power has been applied for a longer given period than in FIG. 3 and is hereinafter referred to as "phase 2"; and

FIG. 5 is an isometric representation of the system of FIG. 3 and 4 after the separation process has been completed.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Referring now to FIGS. 1*a* and 1*b*, there is shown the tank system **10** for performing our novel electrophoresis process. Lower tank **12** is provided with sidepieces **12.5**, end walls **12.3** and an electrode **12.2**. End walls **12.3** are further provided with shoulders **12.4** so that when the lower tank **12** is filled with an appropriate buffer solution, (not shown) upper tank **14** will be properly positioned therein. Electrode **12.2** is preferably made of platinum or some other inert material that is not affected by either the buffer solution nor the passage of current therethrough and terminates in and is connected to a jack **12.1** which passes through one wall **12.3** so that an appropriate source of power may be applied thereto.

Upper tank **14** is also provided with sidewalls **14.6** as well as end walls **14.2** which end walls are also provided with appropriate slotted portions **14.3** for accepting and holding the upper electrode or cathode wire **16.2**. The bottom or floor member **14.4** is provided with plurality of apertures **14.5** so that cell members **14.51** may be placed therein. To complete the device, upper electrode **16** is provided and consists of a platinum wire **16.2** supported by dielectric member **16.1**. The upper electrode or cathode wire **16.2** is provided with a loop or some other appropriate means for connection to jack **14.1** so that power may be applied thereto by any of the many well known conventional methods. One example of such source of power is disclosed in the copending U.S. Pat. Application Ser. No. 736,642 of Robert H. Dilworth, III, entitled "Process and Apparatus for Particle Separation," on June 13, 1968 and assigned to the assignee of the subject application.

To operate our device, cell **14.51** is provided with an upper flange portion **14.52** and a body portion **14.53** having a slotted tapered portion **14.54** extending the length thereof.

A sample, as will hereinafter be described with regard to Figs. **2–5** is prepared and placed in the slotted portion **14.54** of cell **14.51**. Thereafter, the underside of flange portion **14.52** is provided with a nonconductive fluid seal and cell **14.51** is inserted into aperture **14.5** of upper tank **14**. It should be apparent, to those skilled in the art, that the unused sample cell apertures in the bottom wall **14.4** of tank **14** are blocked or otherwise covered if not in use.

Thereafter, lower tank **12** is filled with the selected buffer solution, to the desired level, and upper tank **14**, with the inserted cells **14.51**, is placed into tank **12** to rest on shoulders **12.4**. Upper tank **14** is then provided with a sufficient amount of buffer solution, electrode support **16** is placed in notches **14.3** and the looped portion of cathode **16.2** is connected to jack **14.1**. The system is now ready for operation upon the application of a suitable potential across jacks **12.1** and **14.1**.

Referring now to FIGS. **2–5**, there is shown an isometric representation of a rectangular cell as it would be when completely immersed in cold buffer in the lower tank **12** (FIG. **1**) and containing a typical vertical gel slab **18** capable of separating from one to twelve samples FIG. **2** denotes the typical electrochemical setup of the system after appropriate amounts of the sample **20** have been placed in well portion **22** and prior to the application of electrical energy. FIG. **3** illustrates the initial separation and concentration, beginning with the application of power and due to the conductivity shift phase of the separation. The particles have all been initially partially concentrated and separated prior to the trailing ion boundary **24** and leading ion boundary **26** overtaking the particles **28**, even though the ionic boundaries are moving (during the conductivity shift phase) at five to **10** times the velocity of the particles to be separated. At this point, the power is gradually increased by increasing the pulse rate at which the power is applied.

Following this step in our procedure the moving boundary formed by the trailing and leading anion layers **24** and **26** passes sequentially through each lamina of protein or particles **28** separated and molecularly sieved in phase 1 of our procedure.

Each lamina **28** is sequentially caught in a zone **32**, **34** or **36** that has been depleted of trailing and leading ions and thus having high-voltage gradient. As this high-voltage gradient region moves through the lamina, the rear portion of the lamina, which the high-voltage gradient region encounters first, is speeded up and moves faster than the front portion of the lamina which has not yet been reached by the high-voltage gradient region. This process effectively compresses each successive lamina into an ultrathin layer as the high-voltage gradient region of the moving boundary passes through.

After the boundary produced by the trailing and leading ion **24** and **26** has passed through all the protein zones **30**, **32**, **34** and **36**, the ultrathin protein laminae **28** travel at their own mobilities as in conventional acrylamide electrophoresis.

FIGS. **4** and **5** show the leading **26** and trailing **24** ion boundaries after passing sequentially through a portion of the laminae initially separated in FIG. **3**, where a resharpening of laminae has taken place along with a greatly increased lamina mobility. At this time a maximum pulse rate of constant power consistent with maximal resolution is employed and FIG. **5** illustrates a completed plasma protein separation.

The system illustrated takes particular advantage of both the conductivity shift procedure and the moving ion boundary, along with the molecular sieving effects of the gradient gel, as shown in this illustration, for maximum resolution in regions of both high and low molecular size particles. Thus, band widths as small as 25 to 50 microns by actual measurement are found to be present following separation. With the technique, 25 or more protein bands can be resolved from human plasma over a distance of 3.5 to 4 centimeters in 40 to 60 minutes under conditions sufficiently mild so that easily inactivated substances such as mouse plasma esterases and tissue extract isoenzymes suitable for quantitative studies can be separated simultaneously on the same gel.

In comparing the electrochemistry of this disclosure with the method described by Ornstein and Davis in U.S. Pat. No. 3,384,564, particular attention must be paid to their FIGS. **1**, **2**, and **3** and our FIGS. **2–5**. It is evident that, at all times, as shown in their FIGS. **1** and **2** that the totality of the protein sample or particles to be separated, is located or sandwiched between the trailing glycine ion and the leading chloride ion, in a zone relatively depleted of leading and trailing ions; thus all particles or proteins are in a region of high-voltage gradient. However, this is in a supporting medium with a pore size that is large in relation to the diameters of the various proteins so the proteins will separate in this zone on the basis of their charge density alone with no separation based on size. Once this whole region, i.e., the sandwich of glycine, protein and chloride, reaches the separating gel region of pH 8.9 the proteins are slowed down by the sieving action of the small pore size gel and the glycine mobility increases due to the increase in pH. The glycine then passes through the entire stack of proteins to migrate directly behind the chloride ion. From this point on, separation is carried out by conventional electrophoresis. However, an ultrathin starting zone consisting of all the proteins or particles was produced at that point in time when the gylcine started to pass through the proteins.

Also it should be noted in their procedure (FIG **1**) that prior to the application of electrical energy to the system the trailing glycine ion (located in the upper buffer reservoir) is in direct physical contact with a part of the protein or particles to be separated. Thus the "steady state stacking," the basis of the thin starting zones and resolving power of their technique, takes place in a sandwich bounded by the leading and trailing ions between the two ion boundaries.

## BUFFER SELECTION

It has been determined that the resolution of these techniques depends on the selection of the discontinuous buffer ions. As indicated in table 1, the mobility of the leading

and trailing ion boundaries at a given power level is dependent on the combined transport numbers of the ions involved, rather than on the free mobility of the leading ion alone. Thus, under identical power conditions the chloride-glycine front mobility is greater than sulfate-glycine, which in turn, is greater than citrate-glycine, while chloride-borate is greater than sulfate-borate which is greater than citrate-borate, even through the mobilities of the leading ions alone are in the reverse order of mobility. As indicated in table 1, the greater the mobility of the ion fronts, the greater is the ratio of front mobility to the mobility of the particles to be separated. From the two extreme cases stated, i.e., chloride-glycine where the fronts are farthest separated and citrate-borate where the fastest particle (a prealbumin) travels directly on the trailing ion front, overall resolution was found to be best when the ratio of the ion fronts' mobility to the fastest particle's mobility was closest to one. The most striking example appears in some esterase isoenzyme separations of tissue extracts. The choice of the best leading and trailing ions for maximal resolution in our system can be determined to be related to the ratio of the mobility of the ion boundary to that of the fastest particle to be separated after such particle has migrated a unit distance. Thus the lowest ratio tends to produce the best resolution.

TABLE 1

Boundary and plasma albumin from mobility at 75 ma. constant current, pH 9.35, and 1 to 5 conductivity shift for various leading-trailing ion combinations

| Anions | Boundary mobility, phase 1, mm./min. | Protein mobility, phase 1, mm./min. | Boundary mobility, phase 2, mm./min. | Protein mobility, phase 2, mm./min. | Overall boundary mobility, mm./min. |
|---|---|---|---|---|---|
| Chloride-glycine | 3.56 | 0.25 | 1.91 | 0.88 | 1.81 |
| Sulfate-glycine | 3.40 | 0.24 | 1.08 | 0.62 | 1.28 |
| Citrate-glycine | 1.87 | 0.22 | 0.74 | 0.53 | 0.86 |
| Chloride-borate | 2.28 | 0.25 | 1.07 | 0.71 | 1.39 |
| Sulfate-borate | 2.28 | 0.23 | 0.95 | 0.62 | 1.11 |
| Citrate-borate | 1.78 | 0.20 | 0.66 | 0.50 | 0.79 |

A study of the conductance of these various systems suggests only slight differences, if any, in electrochemical behavior between systems utilizing a conductivity shift and discontinuous buffer ions at a continuous pH for at least 20–30 minutes. However, in the case of chloride-glycine, sulfate-glycine and sulfate-borate, the distance between the ion fronts increases with time. There is then a decrease in conductance, i.e., an increase in cell impedance. This is most marked in the chloride-glycine system where the ion fronts are separated by about 3 millimeters by the time the albumin front has migrated about 3.5 millimeters with a resultant drop in conductance of approximately 33 percent. On the other hand, the sulfate-borate fronts separated by only 0.25 to 0.35 millimeters during the same time the albumin migrates a similar distance, with a drop in conductance of less than 5 percent. Thus, the sulfate-borate system appears to be more desirable as it produces a higher field strength across the boundary, throughout the separation process. There is, however, a marked difference between those systems utilizing discontinuities in both the buffer ions and pH and in those not utilizing a conductivity shift.

The effect of the conductivity shift in the initial phase of separation indicates that this is a necessary part of the total electrochemistry of the system for optimal resolution of various particle laminae, particularly in the high molecular weight macroglobulin and haptoglobin regions and in the lower molecular weight alpha-1-globulin region.

Thus, for optimal resolution in a minimal running time, either the sulfate-borate or citrate-borate leading-trailing ion combination is preferred, with the sulfate-borate system being somewhat faster. Furthermore, borate, sulfate and citrate ions do not produce an initial pink background in the gel following reactions for esterase isoenzymes as does glycine. Thus, borate-sulfate or borate-citrate are also preferable not only in resolution but because they produce less background for subsequent densitometric studies. However, citrate, like Tris, has a pH dependency on temperature which makes sulfate a somewhat better choice for some applications.

## SLAB PREPARATION

The polyacrylamide slab (FIGS. 2–5) in cell **14.51** (FIG. 1*a*) is prepared in two or more layers of recrystallized acrylamide at the desired gel concentration and at the same pH, with five layers typically being used in plasma protein and isoenzyme separations. As shown in FIGS. 2–5, for plasma separations a lower separating layer **36** of between 8 — 8 ½ percent gel is followed by a layer **34** of between 6 — 6 ½ percent gel which is overlayered directly on layer **36**. Layer **34** is then waterlayered to produce an optically flat surface and a 4 ½ percent gel layer **32** is cast on top, waterlayered and allowed to polymerize. Wells **22** are then cast on top of this layer, with the aid of a well former (not shown), of between 8 — 8 ½ percent gel, at an ionic strength 1/5 to 1/10 of the previously casts gels. Samples **20** in 50 percent sucrose buffered to the same pH and ionic strength as the gel columns separating each well are then placed in the wells. Following application of the sample, each well is then filled to the top level of the cell with between 8 — 8 ½ percent acrylamide gel, at the lower ionic strength and consists of the gel cap **30**. The interface between the 4 ½ percent gel and the 6 — 6 ½ percent gel should be an optically flat surface for optimal resolution.

Since acrylamide monomer in the standard commercially available form contains numerous impurities injurious to several enzyme systems, optimal separations and reproducibility require the use of recrystallized monomer.

## RECRYSTALLIZATION PROCEDURE

Dissolve 90 grams of acrylamide monomer in a mixture of 300 ml. ethyl acetate and 300 ml. of benzene and bring to 70° C. in a water bath.

Filter hot into a heated 1,000 ml. florence flask through Whatman No. 1 paper in a jacket heated funnel to prevent crystallization.

Following filtration, the monomer should be recrystallized in an ice water bath. The temperature of solution in the flask should not be allowed to drop below 20° C. The crystals are then removed by vacuum filtration in a Buchner funnel with No. 1 Whatman paper. The crystals are dried until there is no further odor of benzene. They are then redissolved in 540 ml. of chloroform and heated to 60° C. in a water bath. The solution is again filtered hot and then cooled to no lower than 22° C. and the crystals removed again by vacuum filtration.

Dropping the temperature in the second (chloroform) step below 22° C. has proven to produce an unsuitable monomer for use in esterase isoenzyme studies.

Crystals may be stored in the cold by placing them in a dark bottle and adding just enough chloroform to cover the crystals. Monomer stored using this procedure has been found to produce suitable isoenzyme separations after 5 months storage at 8° C. Since acrylamide is very reactive monomer, several useful hints and observations may be used to indicate the shelf life of each recrystallized batch.

A pH change of 0.4 units from the 4.9–5.2 pH of the freshly recrystallized monomer and problems of removal of gel from the cells are both early signs which indicate that the monomer is deteriorating and which can be determined before changes

in isoenzyme resolution become apparent.

To cast gels utilizing any of the systems of TABLE 1, the following gel buffer stock solutions are prepared:

1a. Tris citrate pH 9.0 1.5M:
36.3 gm. tris (hydroxy methyl amino methane)
28,0 ml. 1 M citric acid
0.48 ml. N,N,N',N'-tetramethylethylenediamine (TEMED) $H_2O$ to 200 ml.
Resulting pH of 9.0 at room temperature

1b. Tris citrate pH 9.0 0.30M:
20.0 ml. tris citrate 1.5M pH 9.0 from above 0.19 ml. TEMED
$H_2O$ to 100 ml.

1c. 50% sucrose tris citrate pH 9 0.075M buffered:
6.25 ml. tris citrate pH 9.0 0.30M from above
12.5 gm. sucrose
0.1 ml. 0.1% aqueous bromphenol blue
11.0 ml. $H_2O$
Final volume 25 ml.

2a. Tris sulfate pH 9.0 1.5M:
36.3 gm. tris (hydroxy methyl amino methane)
62.0 ml. N/1 $H_2SO_4$
0.48 ml. TEMED (N,N,N',N'-tetramethylethylenediamine)
$H_2O$ to 200 ml.

2b. Tris sulfate pH 9.0 0.30M:
20.0 ml. tris sulfate pH 9.0 1.5M from above
0.19 ml. TEMED (tetramethylethylenediamine)
$H_2O$ to 100 ml.

2c. 50% sucrose tris sulfate pH 9.0 0.075M buffered:
6.25 ml. tris sulfate pH 9,0 0.30M from above
12.5 gm. sucrose
0.1 ml. 0.1% aqueous bromphenol blue
11.0 ml. $H_2O$
Final volume 25 ml.

3a. Tris chloride pH 9.0 1.5M:
36.3 gm. tris (hydroxy methyl amino methane)
40.0 ml. N/1 HCl
0.48 ml. TEMED (N,N,N',N'-tetramethylethylenediamine)
$H_2O$ to 200 ml.

3b. Tris chloride pH 9.0 0.30M:
20.0 ml. tris chloride pH 9.0 1.5M from above
0.19 ml. TEMED (tetramethylethylenediamine)
$H_2O$ to 100 ml.

3c. 50% sucrose tris chloride pH 9.0 0.75M buffered
6.25 ml. tris chloride pH 9.0 0.30M from above
12.5 gm. sucrose
0.1 ml. 0.1% aqueous bromphenol blue
11.0 ml. $H_2O$
Final volume 25 ml.

The following buffers are applicable:

4a. Tris borate pH 9.0 0.65M:
61.3 gm. tris (hydroxy methyl amino methane)
395 ml. 0.35M boric acid ($H_3BO_3$)
0.78 gm. merthiolate
$H_2O$ to final volume of 7.8 liters

4b. Tris borate upper tank buffer pH 9.0 0.065M:
3.9 liters tris borate pH 9.0 0.065M from above
1.2 ml. 0.1% aqueous bromphenol blue

5a. Tris glycine pH 9.0 0.0625M:
58.95 gm. tris
56.95 gm. glycine
0.78 gm. merthiolate
$H_2O$ to 7.8 liters

5b. Tris glycine upper tank buffer pH 9.0 0.0625M:
3.9 liters tris glycine pH 9.0 0.0625M from above
1.2 ml. 0.1% aqueous bromphenol blue

All molarities refer to molarity of tris.
All pH refer to pH at 25°C.

Stock solution 6*a* is a stock persulfate catalyst solution prepared fresh daily and consists of 0.105 g. ammonium persulfate diluted to 100 ml. Stock 6*b* is a similar stock persulfate catalyst at double the concentration of 6*a*, i.e., 0.21 g./100 ml.

Stock solution 7
32 percent acrylamide (if final lower separating layer of 8 percent gel is desired)
4.8 g. of twice recrystallized monomer
0.12 g. methylene bis acrylamide
$H_2O$ to a final volume of 15 ml.
This stock solution is prepared fresh daily.

The cell is clamped in an appropriate gelling stand and the stand leveled. A gasket is usually provided at the base of the cell to prevent leakage during addition of reagents.

In the upright position approximately 20 ml. of 8 percent gel consisting of 5 ml. of stock gel buffers 1*a*, 2*a* or 3*a*, plus 5.0 ml. of stock solution 7, plus 10 ml. of stock solution 6*a* is mixed in a beaker at room temperature and poured into the gel. Immediately 3.4 ml. of 6 percent monomer concentration consisting of 1.2 ml. stock solution 1*a*, 2*a* or 3*a*, 0.90 ml. of stock solution 7, 0.30 ml. $H_2O$ and 2.4 ml. of stock solution 6*a* is carefully overlayered on top of the first layer. This layer is then carefully waterlayered from one or both edges of the cell and the two layers of monomer allowed to polymerize. Two milliliters of a 4 ½ percent gel consisting of 0.80 ml. of stock solution 1*a*, 2*a* or 3*a*, 0.45 ml. of stock solution 7, 0.35 ml. of $H_2O$ and 1.6 ml. of stock solution 6*b* is added and waterlayered. Following polymerization, 3.6 ml. of 0.075 molar 8 percent gel consisting of 0.90 ml. of stock solution 7, 0.90 ml. of stock solution 1*b*, 2*b* or 3*b* and 1.8 ml. of stock solution 6*b*, is added, a well former inserted with the bottom edges of the well former resting on top of the 4 ½ percent layer, and the gel allowed to polymerize for at least 20 minutes. The well former is then removed and the wells rinsed with distilled $H_2O$ and then drained carefully by inverting the cell. The cell is then placed in the upright position and the sample (200 to 1,000 μg. of protein per wall depending on the test) in 20 μl. of 0.075 molar 50 percent sucrose from stock solution 1*c*, 2*c* or 3*c* is placed in the well.

Next, 0.075 molar 8 percent sealing gel is added carefully on top of each sample layer. This sealing gel consists of 1.0 ml. of stock solution 7, 1.0 ml. of stock solution 1*b*, 2*b* or 3*b*, and 2.0 ml. of stock solution 6*a*. This is allowed to gel and the cell is then ready for electrophoretic separation.

Solutions **1**, **2** or **3** must not be intermixed in the same gel, as each gel slab contains either all Tris citrate (solutions 1*a*, 1*b* and 1*c*) or all Tris sulfate (solutions 2*a*, 2*b* and 2*c*) or all Tris chloride (solutions 3*a*, 3*b* and 3*c*).

Should one wish to perform ascending electrophoresis, the cell is cast in the inverted position and the polarities at jacks 12.1, 14.1 (FIG. 1*a*) reversed.

Electrophoresis is usually performed in the vertical position, preferably with the negatively charged electrode 16.2 (FIG. **1**) in the upper tank 14 and the positively charged electrode 12.2 the the lower tank **12**. The lower tank is filled with Tris-borate buffer at 40° C., giving a pH of 9.35 to 9.40, to a level even with the bottom of the upper buffer tank **14**. The cell or cells **14.51** containing the gel slab **18** and sample **20** are inserted through slots **14.5** in the upper tank **14**. To prevent leakage between the upper and lower tank, the cell end plates are sealed to the floor of the upper tank by pressing the cell and plate into a bead of silicone grease or a special sealing gasket (not shown). Then sufficient Tris-borate is poured into the upper tank to cover the platinum electrode wire 16.2. Thus, the gel serves as a bridge between the two electrodes and the cell is completely immersed in a large volume of cold buffer for additional cooling during electrophoresis.

An electromotive force is then applied across the cell, preferably in the form of constant power pulses, such as described in the aforementioned copending patent application of Robert Dillwoth, III.

The pulse rate is initially set at a level so that a power level at about 45 milliwatts per mm.² gel surface is achieved, e.g., for a standard cell, 3.5 mm. width, 100 mm. length and 90 mm. depth, a peak voltage of about 700 v. is discharged through a 1.0 microfarad capacitor at a rate of about 45pulses per second. The power level is then raised to 70 milliwatts/mm.² (125 p.p.s.) after 5 minutes. Following completion of the initial conductivity shift step in the separation, 12–15 minutes, the power is raised in two steps to 85 and 100 milliwatts/mm.² at 15 and 20 minutes, respectively (200 and 300 p.p.s, respectively). The second phase of separation is carried out for both protein and isoenzyme separations typically at power levels of 100 milliwatts/mm.² gel surface, although higher power levels with shorter run times may be used. Voltage level is kept constant throughout a given run.

For two-cell operation, power setting of about 400 volts and similar pulse rates are used during the first stage conductivity shift, then the power level is raised by bringing the pulse rate to about 225 p.p.s. and 350 p.p.s. at 15 and 20 minutes, respectively.

Pulsed power electrophoresis is carried on until the albumin front migrates a distance of 3.5 to 4.0 cm., a matter of 40 to 60 minutes depending on whether the divalent sulfate or trivalent citrate ions are used as the leading ion.

Following electrophoresis the upper buffer tank is removed, the buffer discarded and the cell removed. Excess sealing grease is removed from the end plate and the gel is ready for

removal from the cell. A 23 gauge 3-inch long cannula, through which water is flowing under slight pressure, is inserted down both side edges of the cell and the gel loosened. The cannula is then moved across both remaining surfaces to loosen the gel glass seal. The gel can the be teased out of the cell with the cannula or gently pushed out from bottom to top with a special plate of slightly smaller dimensions than the inside cell dimension. A rocking motion imparted to the plate provides the best results. Thus a gel may be readily removed from a cell in one to two minutes.

Following removal, the gel may be stained in a common protein dye (such as Amido Black) in 10 percent acetic acid overnight or for 2 hours at 65° C. and then destained by electrophoresis or repeated washing in 10 percent acetic acid with about five acid changes in two days.

Alternatively, the gels may be placed in a pH 6.6, 0.04 M Tris-chloride buffer at 37° C. for 5 minutes and then into a dye substrate system of fast blue RR$\alpha$-napthyl butyrate at pH 6.6 for 15 minutes for the quantitative development of nonspecific esterase isoenzymes. Some fifty other isoenzyme staining procedures previously described are amenable to these techniques of separation.

While the conditions described in detail here are applicable to plasma and tissue extract proteins and isoenzymes, it will be obvious to those skilled in the art of electrophoresis that with the system described herein, ion selection and pH conditions and gel pore sizes can be varied widely, making the system suitable for separations of a wide variety of charged particles. Thus, for example, positive charged particles will behave in a like manner if appropriate changes are made in buffer and pH, or separations may be carried out with negative ions at low or lower pH levels than those specifically described for plasma and certain tissue extracts above.

While there has been described what is presently considered the preferred embodiments of the invention, it should now be obvious to those skilled in the art that various other changes and modifications may be made therein without departing from the inventive concept contained herein, and it is, therefore, aimed to cover all such other changes and modifications that may fall within the true spirit and scope of the invention.

What is claimed is:

1. In an electrophoretic process for concentrating and then separating certain components of a mixture of like sign-charged particles through a sieving gel medium, the gel medium having a plurality of contiguous zones; the particles suspended in solution adjacent the gel medium; having a pair of electrodes of opposite polarity, each electrode positioned at opposite end of the gel medium; a source of power for connection to the electrodes; and a buffer solution surrounding the gel medium and the electrodes, the improvement comprising:
   initially positioning the particle solution between two gel zones;
   providing a source of ions of like sign nd of different mobilities in both the buffer solution and the gel medium;
   providing the faster ion in the gel medium and the slower ion in the buffer solution; and
   applying a source of constant, pulsed DC field across the gel medium during the concentrating and separating process.

2. The process of claim 1 further comprising:
   providing a single gel zone between the particle solution and the buffer solution;
   lowering the conductance of both the single gel zone and particle solution to raise the voltage gradient appearing thereacross; and
   maintaining all gel zones, the particle solution and buffer solution at the same pH.

3. The process of claim 2 wherein the buffer solution is a member of the group consisting of citrate-borate, sulfate-borate and chloride-borate.

4. The process of claim 3 wherein the buffer solution is citrate-borate.

5. The process of claim 3 wherein the buffer solution is sulfate-borate.

6. The process of claim 3 wherein the buffer solution is chloride-borate.

* * * * *

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# UNITED STATES PATENT OFFICE
# CERTIFICATE OF CORRECTION

Patent No. 3,620,947 Dated November 16, 1971

Inventor(s) Robert Carter Allen and Dorothy J. Moore

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 3, line 33, before "on" insert --filed--.

Column 8, line 33, delete "40°" and insert --4°--.

Column 8, line 53, delete "45" and insert --75--.

Column 10, line 15, delete "nd" and insert --and--.

Signed and sealed this 8th day of May 1973.

(SEAL)
Attest:

EDWARD M.FLETCHER,JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents