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IONOGRAPHY

ELECTROPHORESIS IN STABILIZED MEDIA

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by

HUGH J. McDONALD, D.Sc.

Professor of Biochemistry
Stritch School of Medicine of Loyola University, Chicago

In collaboration with

ROBERT J. LAPPE, M.S.
Research Assistant, Department of Biochemistry, Loyola University

EDWARD P. MARBACH, Ph.D.
Associate Chemist, American Meat Institute Foundation and Research Associate (Instructor), Department of Biochemistry, University of Chicago

ROBERT H. SPITZER, M.S.
Research Assistant, Department of Biochemistry, Loyola University

MATTHEW C. URBIN, Ph.D.
Associate Chemist, Chemical Division
Corn Products Refining Company, Argo, Ill.

1955

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CHAPTER 1

Historical Introduction

IN THE PAST, two general methods have been available for studying the electrophoretic behavior of charged particles in a liquid. The microscopic method, which depends on direct observation of the motion of the particles with a microscope, is adapted to the study of mobilities of fairly large particles. Its usefulness was extended somewhat by finding that various finely subdivided inert materials such as glass, oil and collodion can in some instances be so completely covered with absorbed protein that they behave as if they were large protein particles.

In the moving-boundary method the displacement of the particles in an electric field is recorded photographically as the movement of a boundary between a dispersed phase and the dispersion medium. The method has found particular usefulness in the study of protein systems. The heart of the apparatus is essentially a U-tube in which buffer solution can be layered carefully over the protein-buffer dispersion. Sharp boundaries can be maintained if thermal convection currents are minimized by careful temperature control and if the protein buffer has been previously dialyzed against the buffer itself so that the electrolyte concentration does not change appreciably across the boundary. An adequate description of the microscopic and moving-boundary techniques has been given by Briggs (79), and the general theoretical background of electrophoresis has been reviewed by Alberty (4, 5, 7).

During the past few years a third method of achieving electrophoretic separations and of determining electrophoretic mobilities has come to the fore. This technique, whose origin dates back about seven decades, depends on the electromigration of charged particles, either ions or colloidally dispersed substances, through conducting solutions which have been stabilized with agar, gelatin, filter paper, starch, glass-fiber ribbon and other materials. The extent of movement is determined by such procedures as developing colored derivatives, incorporating radioactive tracers,

using light-absorptive characteristics or determining changes in other physical chemical properties of the stabilized media. The method appears to be more flexible than the microscopic and moving-boundary techniques. It lends itself equally well to the study of electromigration of ions and colloidal materials and has scored notable success particularly in the fractionation, absolute separation and identification of microamounts of high molecular weight substances which are difficult to separate by chromatography.

The idea of stabilizing solutions of electrolytes, in which ionic migration experiments were being conducted, goes back many decades. Lodge (406), in 1886, used gelatin-stabilized media in glass tubes in determining the electromigration velocities of a number of inorganic ions by measuring the movement of colored boundaries or the formation of rings of precipitates. Arrhenius (29), also in 1886, reported on a study of the relationship between the fluidity of electrolytes stabilized with gelatin and the conductivity of the same solutions of inorganic salts. Whetham (716), in 1894, showed that the addition of sufficient agar to just cause a dilute solution of an inorganic electrolyte to set reduced velocities of the ions by less than 10 per cent, and he consequently used it to stabilize the boundaries of solutions in which ionic migration velocities were being determined (716–7). Masson (437), in 1899, used gelatin as a stabilizer in his studies of the relative migration velocities of inorganic ions.

The next group of publications on electromigration in stabilized media is that of Teague and co-workers. In 1907, Field and Teague studied the electromigration characteristics of diphtheria toxin and antitoxin (183) as well as of tetanolysin and antitetanolysin (184) in agar-stabilized electrolytes. Teague and Buxton (669), also in 1907, studied the electromigration characteristics of hemolytic amboceptors, hemagglutinins and complement in agar jelly.

Kendall and his associates (322–8) from 1923 to 1928 applied the method of electromigration in agar gels to the separation of isotopes, of ions of rare earth metals, of radium from barium and of hafnium from zirconium, besides doing some work on the separation of mixtures of alkaloids and of organic isomers.

In 1934, Veil (689h–i) reported on the effect of an electric field on the diffusion of alkali carbonates in gelatin. Her use of radioactive substances as tracers (689j) is illustrated by the experiment in which she impregnated a strip of gelatin with barium chloride containing radium chloride, electrolyzed it and then placed it in contact with a photographic plate. The details

of her technique are not far removed from those in use for a similar purpose today.

The year 1939 was a banner one as regards publications on the technique of electroseparatory processes. Strain (643) described an arrangement in which two electrodes were inserted in an alumina column of the type used in chromatographic adsorption analysis. By using the combined influences of chromatography and electromigration, he separated a number of mixtures of colored substances.

Coolidge (123), also in 1939, described a technique in which a column packed with ground-glass wool was used to fractionate serum proteins. The techniques described by Strain and Coolidge embodied many of the principles employed today in the column technique.

It was also in 1939 that von Klobusitzky and König (698), in Brazil, published a procedure in which they employed a strip of medium-hard filter paper, 1 × 5 cm, the ends of which were connected by means of clamps to a 110 volt d-c source in series with an adjustable resistance and a milliammeter. The paper was moistened with a dilute solution of an electrolyte, and 1 drop of the solution under study, containing yellow-colored snake venom, from Bothrops jararaca, was placed on the moistened paper at a point midway between the ends. During some experiments the paper strip was opened to the air, while during others it was placed between two glass microscope slides in order to retard evaporation. On allowing the current to flow for 20 min at pH 6.0, the spot of colored snake venom was found to migrate toward the positive electrode. Starting at the center, the paper strip was cut transversely into several narrow sections, from each of which the material was extracted and suitably tested in various ways. The authors refer to an earlier paper by König (351) in which the experimental technique and some preliminary results were described. In the earlier work, the moistened strips of $BaSO_4$-free filter paper (4 × 1.5 cm) were placed on a glass surface. Several drops of the migrant substance, dissolved in a diluted solution of a neutral salt, were placed on the center of the strip, the ends of which were connected by means of clamps to a 100 volt d-c source. After several hours, the strips were cut into several pieces, each piece was extracted with the same quantity of the same solvent and the extract examined. As an example, a 1 per cent solution of the venom of Bothrops jararaca was subjected to electromigration, and at the end of the run the coagulant power of the material in the various sections of the paper strip was determined on oxalated horse blood. When

the 4 cm strip was cut transversely into six sections of equal width, after a 2-hr period of electrophoretic separation, the following results were obtained (351).

Section	Coagulation Time
−3	several hours
−2	19 min
−1	9 min
+1	4 min, 30 sec
+2	2 min
+3	61 min

The section designated "−3" refers to the section closest to the negative end of the strip, while the section designated "+1" refers to the first section, measured from the midpoint of the strip, toward the positive end of the paper sheet.

In 1943, Berraz (52–3), in Argentina, described a procedure for the separation of inorganic ions by the action of an electric current along a narrow strip of filter paper previously wetted with a conducting buffer solution. The paper strip was held in a vertical position in a tall glass cylinder of the type used in paper chromatography. The potential was impressed along its length by placing one electrode in the trough holding the upper end of the strip and the other in the bottom of the cylinder. After the current flowed for a certain period of time, the paper strip was dried and sprayed with the characterizing reagent, resulting in an "electrocapillogram." In some cases, selective precipitation of a specific species of ions was resorted to, when their presence could not be made readily identifiable by spray reagents. In this selective precipitation procedure, a suitable reagent was added to the paper strip which captured and immobilized the desired ions, as they migrated past, through the formation of insoluble colored complexes. Comparison of electrocapillograms with those obtained with small known samples, and observation in ultraviolet light before and after development with suitable reagents, helped in interpreting the results. Berraz also observed that the velocity of displacement varied with the nature of the ions and that it increased with increase in potential gradient and temperature. It is interesting to note that he suggested the use of the term "electrochromatography" in the conclusions to his second paper (53).

In 1944, Lecoq (383) described a technique quite similar to that developed earlier by Strain but apparently representing an independent development on his part. He investigated the influence of an electric potential when applied to the top and bottom

of a column in which the chromatographic separation of inorganic salts was being conducted and named the technique "electrochromatography."

In 1946, Consden, Gordon and Martin (117) published a paper on the ionophoretic separation of amino acids in media stabilized with silica gel. They used the method in their study of the amino acids and peptides of wool, insulin and other proteins and described a procedure whereby the separate bands representing the various components of a mixture could be localized by placing a sheet of filter paper in contact with the gel surface. This "print" was then treated with ninhydrin to develop a pattern of the separated amino acids.

Butler and Stephen (96), in 1947, described the use of asbestos fiber as a stabilizer in a tube partitioned with filter paper and used the procedure to obtain a separation in 24 hours of glycine from glycylglycine at pH 9.3 and a potential gradient of 2 volts/cm.

In 1948, Haugaard and Kroner (275) announced the results of some experiments in which electromigration was combined with chromatography to separate amino acid mixtures. A vertical sheet of paper was threaded with electrodes along the two vertical edges. As the migrant mixture separated, chromatographically, on its downward course from the center of the top edge of the sheet, the presence of the electric field resulted in a simultaneous lateral separation. The combination of these two forces gave a reasonably good separation of the amino acid mixtures which were investigated.

Wieland and Fischer (725), in 1948, published the first account of their work in which paper strips were used to stabilize the electrolytes. In 1949, Garrison, Haymond and Maxwell (209) reported the separation of some radioactive elements from other inorganic compounds based on electromigration through a stack of 30 circular filter paper sheets which had been placed between two platinum electrodes. Also in 1949, Gordon, Keil and Sebesta (225) utilized agar-stabilized media to carry out an electrophoretic separation of proteins.

In 1950, reports on the subject of electromigration in paper-stabilized electrolytes were published by Cremer and Tiselius (126) in Sweden; by Durrum (150), by McDonald, Urbin and Williamson (456) and by Kraus and Smith (353) in the United States; by Biserte (55) in France; by Grassmann and Hannig (236–8), by Körver (352) and by Turba and Enenkel (684) in Germany. In 1951, an ever larger number of papers on the technique appeared, and the number of reports on various appli-

cations, modifications and limitations of the technique has grown phenomenally since that time, so that this simple procedure has moved into the front ranks of important tools in biochemical and clinical chemical research.

Next to paper itself, one of the most promising stabilizers for media in which electromigration experiments are to be carried out is starch. The pioneering work in determining the characteristics and potentialities of starch as a stabilizer has been done principally by Kunkel and his associates. Kunkel has recently published a full review in which the merits and limitations of starch are compared to those of paper and other stabilizing materials (357), and the details of carrying out electrophoretic fractionations using the starch-block or -column technique are presented in some detail. One of the principal arguments put forward in its favor, by the proponents of the starch technique, is based on the lowered adsorption of serum proteins and some other complex materials such as lipoproteins, as compared to paper. It is claimed that very little adsorption of serum proteins, for example, takes place with potato starch as the stabilizer, and they shift with the liquid volume without tracing. The technique has found greater application in qualitative separations and small-scale preparative procedures than in mobility determinations (11, 193, 195, 356, 357, 368, 399, 611).

By the starch-block procedure as much as 5 ml of human serum can be separated into the five main components observed in free-solution electrophoresis and the fractions isolated. Nitrogen analyses have indicated up to 97 per cent recovery by displacement filtration of the starch segments in short ground-glass filter columns. Certain peptides, particularly the basic type, showed reversible adsorption to the starch in aqueous buffers, and other supporting media such as glass beads and purified cellulose proved more suitable for preparation experiments in the block form (356). A column procedure with collection of fractions in a mechanical fraction collector after electrophoretic separation proved useful for a few specific purposes, but theoretical as well as experimental disadvantages over the block procedure were apparent (193).

Although this account of the historical development of the technique of electromigration in stabilized media has been presented in a chronological fashion, a closer look at the literature will quickly reveal that the ideas in most cases up to about 1951 appear to have arisen independently in widely scattered laboratories throughout the world, and the various investigators usually were quite unaware of the previous work in the field at the time of their initial publications.

Any account of electromigration in stabilized media would be incomplete without a reference to the work of Hermance and Clarke (109, 282) on the use of paper as a medium in which to conduct microchemical reactions, including electrolysis (282), to the excellent monograph by Butler (96a) on the electrical phenomena at interfaces in chemistry, physics and biology and a brief mention of the *electrographic* method of analysis, which also involves electromigration of ions through paper-stabilized media. The principle of the technique is that the test substance is used as anode with aluminum foil as cathode, and filter paper moistened with a suitable reagent is placed between the poles. The reagent paper is not laid directly on the aluminum foil, but on another filter paper moistened with potassium chloride or sulfate as electrolyte. This arrangement allows the current, usually provided by a small battery, to flow more easily. When the circuit is closed, the metal whose analysis is sought is dissolved anodically and can react directly with the reagent in the paper, to produce characteristic color stains. The method has found considerable application in the non-destructive testing of metals and alloys. Among the principal contributors to the development of the electrographic method of analysis are: Glazanov (217–9), Jirkovsky (308), Dyachkovskii and Isaenko (158), Arnold (28) and Evans and Lewis (174). A general review of the technique has been written by Lingane (403).

From the foregoing account it is evident that despite the development of the moving-boundary type of electrophoresis apparatus to the point where it became a practical tool for studying high molecular weight charged substances, and their naturally occurring mixtures such as blood plasma proteins (397, 674), it failed to satisfy all needs. The absolute separation of the constituents of a mixture, and the handling of ions, for instance, were not very practicable on the moving-boundary apparatus, and fairly large quantities of material were required. Some classes of substances, such as lipoproteins, have always presented difficulties when submitted to electrophoretic analysis in non-stabilized media (680). The microscopic method for studying the electrophoretic behavior of charged particles in a liquid is even more limited in application (79).

Because of this situation, an interest was maintained in electromigration through electrolytes stabilized with substances such as agar (134, 225, 262a, 322–8, 390, 527–8, 663), ground-glass wool (123), glass powder (261), cotton (643), cotton gauze (707), cotton string (33), asbestos fiber (96), silica gel (117, 225), starch (11, 193, 195, 356, 357, 368, 399, 611), silk (161), cation exchange resins (633–5) and other materials. In these

techniques (except for the last-named, which is rather specialized), the preparation of the stabilized column or trough is usually time-consuming, the removal of separated fractions of such materials as protein hydrolysates is not always easy, and with the exception of starch when used with certain substances, a large element of chromatography is inseparably bound up with the electrophoresis. Ingenious combinations of chromatography and electrophoresis have, of course, been developed (3c, 76, 154, 235, 275, 317, 543a, 651, 658), and are often very useful in the resolution of complex mixtures. This follows from the fact that many substances may be similar as regards adsorption or partition behavior but may be quite dissimilar as regards electrokinetic properties. These combinations of chromatography and electrophoresis, which are nicely described by the term "electrochromatography," are, of course, of little value in determining such electrokinetic properties of substances as, for example, the isoelectric point of proteins or amino acids (360–1, 444, 446, 458, 603, 687, 741), which are determined from mobility data. In fact, it might be said that if electromigration through stabilized electrolytes is to be utilized for any purpose involving mobilities of the migrant, every effort should be made to reduce chromatographic interference to a minimum. How this objective is accomplished, using paper and similar materials in sheet or strip form to stabilize the electrolyte, will be made evident in later chapters.

CHAPTER 2

Types of Apparatus and Nomenclature

AS MENTIONED EARLIER, many variations in the technique of carrying out separation processes in stabilized media due to differences in electromigration rates have been developed in attempts to control variables inherent in the process or to meet the contingency of some specific problem. The separation of charged particles has been carried out in tubes (134, 183, 184, 322, 406, 437, 527, 717), or columns (96, 123, 193, 383, 643) filled or packed with various substances, in slabs of gel or starch (225, 227, 356-8, 390), in sheets or strips of filter paper and similar materials (33, 53, 55, 126, 150, 263, 363, 456, 471, 684, 698, 725). Paper has been used dry (595), it has been compressed between glass or plastic plates (126, 361, 651, 698), hung over a rod or suspended vertically (52, 53, 150, 194, 471), stretched horizontally in a closed vessel (221, 234, 237, 241, 444, 456, 458, 474), or immersed in a non-miscible, non-polar solvent (122, 433). Many ingenious arrangements have been suggested in attempts to control the temperature, evaporation and solvent movement through the paper. Continuous evaporation of the solvent has been promoted (416, 418), high-frequency power sources have been used (414) and truly microtechniques, employing combined histological and photometric procedures, have been developed (161).

All these variations in technique might be included as separate items in a scheme of classification, but it was decided, for the sake of simplicity, to base the classification (where a single potential gradient served as the driving force) on the manner in which the paper strips were held in place in the apparatus and to discuss in each case mainly the earlier known papers and principal modifications in design. Those procedures in which more than one principal driving force is involved, and the more

unusual modifications of the basic techniques, are then considered separately. The main procedures utilizing paper as a stabilizer in use today include: the sandwich technique (126, 361, 651, 698), the solvent-immersion technique (122, 433), the ridgepole technique (150, 194) and the gas-enclosed horizontal-strip technique (221, 234, 237, 241, 458, 474). Each of these procedures has been applied successfully in simple ionographic fractionations, but some modifications possess inherent advantages when mobility determinations are to be made. In a few instances, combinations of the above techniques have been utilized simultaneously, e.g., glass plates, enclosing a wetted paper sheet, are immersed in a non-miscible, non-polar solvent (126).

SINGLE POTENTIAL GRADIENT SERVES AS DRIVING FORCE

Paper Strips Clamped between Glass Plates: Sandwich Technique

As noted in Chapter 1, von Klobusitzky and König (698) appear to have been the first to utilize the sandwich technique. Later, Cremer and Tiselius (126) described an arrangement in which the paper strip, immersed in buffer and containing the specimen to be analyzed, was placed between glass plates, immersed in a solution of chlorobenzene and connected to electrode vessels. The chlorobenzene retarded evaporation from the paper and acted as a partial cooling agent. While reasonably good separations could be made, the apparatus was certainly not convenient to use.

Kunkel and Tiselius (361) have reported on a modification of the apparatus in which the use of chlorobenzene was discontinued. The paper sheet or sheets were clamped between glass plates and the edges sealed with silicone grease, thus retarding evaporation. The two ends of the filter paper dipped into buffer solution contained in the electrode vessels, on which the glass plates rested. To reduce pH changes at the ends of the filter paper due to the products of the electrode reactions, the principal measure taken was simply to enlarge the electrode vessels themselves until they held sufficient buffer solution to offset pH changes in a normal run. A rubber tube connecting the electrode vessels could be opened to balance the liquid levels. The polysaccharide, dextran, which has a very low electromigration rate in free-solution electrophoresis, was used in an effort to approximate the extent of electroosmotic flow. These authors also apply

another correction factor to their observed electromigration rates to correct them for "increased migration path length." Experimental evidence is cited in Chapters 4 and 5 which raises a question as to the general validity of these correction factors. The apparatus was used to fractionate several artificial mixtures of purified proteins, and serum proteins. Curves were obtained showing the same five major peaks for normal serum as found by the classic moving-boundary method. With proper precautions, the apparatus can be used both for separation processes and for mobility determinations (357, 361).

In some of their experiments, Strain and associates (578, 646, 650) have used a modification of the sandwich technique which

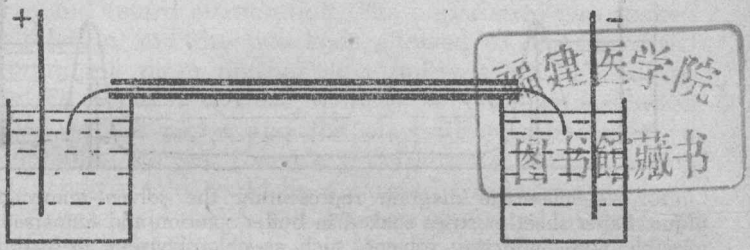

FIG. 1.—Schematic diagram of the sandwich-type apparatus. One or more sheets or strips of filter paper clamped between glass or plastic sheets.

resembles that originally described by von Klobusitzky (698) in that the electrodes were attached directly to paper sheets sandwiched between glass or plastic plates or film. With this arrangement they have observed that electroosmotic effects are reduced to a minimum. Although the technique is satisfactory for separations of some inorganic ions, it can hardly be considered a suitable choice for the separation of ampholytes such as plasma proteins, amino acids, and enzymes, whose electromigratory properties are sensitive to slight pH changes along the paper strip and which may even be denatured due to gross changes in the pH of the medium. That pH changes of serious magnitude can occur when the electrode products are not separated adequately from the strip can be shown readily by incorporating a series of indicators into the buffer mixture used to saturate the paper strips and observing the color changes that occur when a potential is impressed across the ends of the strip.

While the technique of clamping the paper sheets between glass plates would seem to offer certain advantages over other methods to be described later, as far as evaporation from the

paper is concerned, complicating factors are introduced which in many cases more than offset the gains. For example, the factor of electroosmosis between the glass and paper surfaces, the interfering surface tension and capillary effects and the difficulty of preventing a loss of the migrant on the glass surface may be mentioned. Flynn and de Mayo (194) tried, then discarded, the technique of holding the paper between glass sheets. They found that suspending the strips in a moist atmosphere was preferable from the standpoint of experimental results.

More recently, Robbins and Rall (561) described a modifica-

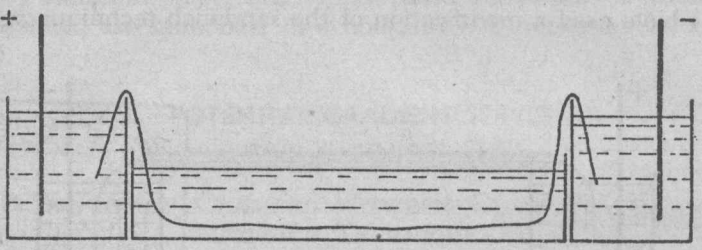

FIG. 2.—Schematic diagram representing the solvent-immersion technique. Paper sheet or strips soaked in buffer solution and immersed in non-miscible non-conducting solvent, such as chlorobenzene or carbon tetrachloride.

tion of the Kunkel and Tiselius apparatus. They raised the upper glass plates about 0.5 cm above the paper and sealed the sides of the glass plates with masking tape. The paper sheet itself was pressed flat on the lower glass with a roller.

Actually, the greatest weakness of the sandwich technique, as ordinarily applied, lies in the uneven ratio of buffer to paper which one finds unless elaborate precautions are taken to offset the effect. This problem is treated in more detail later.

DIRECT SOLVENT-IMMERSION TECHNIQUE

Markham and Smith (433) dispensed with the glass plates altogether and immersed the wet filter paper, in the form of a V-fold, directly in a tall-form rectangular glass jar containing carbon tetrachloride to cool the strip and to prevent evaporation of water. Later, Consden and Stanier (122) described a similar technique except that a long shallow glass tray was used to hold the solvent and the paper strip was arranged so that the portion throughout which electromigration was being studied remained in a horizontal plane. The non-miscible, non-polar liquid, chloro-

benzene was chosen because its specific gravity is similar to that of wet paper and thus allows the immersed strip to remain horizontal in the trough. This helps to prevent secondary flow of electrolyte through the paper strip. The technique has certain limitations where labile materials such as enzymes might be affected by the solvent (741).

Simple Catenary Suspension

The apparatus of Wieland and Fischer (725) consisted of a strip of filter paper, 1 × 20 cm, which was suspended between two glass plates, 5 × 20 cm, so as to close off the strip from the atmosphere and retard evaporation. The paper strip was soaked in buffer solution and the two ends allowed to dip into Petri dishes, containing more of the same buffer solution, and the electrodes. To begin operations, a drop of amino acid solution was applied to the paper and the electrodes connected to a 110 volt potential source. Using a 0.1M acetate buffer solution at pH 3.7, aspartic acid could be separated from glutamic acid; alanine, glutamic acid and histidine were separated in 1 hr. At pH 7.0, histidine could be separated from lysine. The strips were dried at 110°C, treated with acetone containing 10 per cent formamide, dried again and then dipped into a solution of copper acetylacetonate in chloroform. On rising through the strip, this solution converted the amino acids into copper acetylacetone complexes. The excess reagent was removed by washing with chloroform. The amino acid spots were then made visible by spraying with an acetone solution of rubeanic acid which converted the areas with copper present into dark spots. While suspension of the paper strip or sheet as a simple catenary is satisfactory for some empirical separations of mixtures, it does not lead to linear movement of the migrant with time (444) and hence does not lend itself to mobility determinations.

Ridgepole Suspension

Durrum (150) constructed an apparatus consisting of two 150 ml tumblers covered with Lucite plates which sealed their tops and supported an inverted L-shaped glass rod. The horizontal portion of the glass rod served to support the apex of the filter paper strips, which were draped symmetrically over it so as to form an inverted V, with the ends of the strip passing through slots in the Lucite covers and extending into the buffer