

The CLINICAL

Chemist

***** IN THIS ISSUE *****

- Electromigration in Stabilized Electrolytes Part III:
Applications Of The Techniques
H.J. McDonald, R.J. Lappe, E.P. Marbach, R.H. Spitzer, and
M.C. Urbin..... page 51
- A Standardized Plasma Preparation Suitable For Use As A
Control In The Determination Of Prothrombin Time
M.E. Hodes page 59
- Fifth Annual Meeting Program, Chicago, Ill.
September 9-11 page 48

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STATED ANNUAL MEETING

September 10, 1953

Chicago, Ill.

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VOL. 5, NO. 4

AUGUST 1953

THE SECRETARY REPORTS

The fifth annual convention of the AACC is scheduled to take place in Chicago on September 9-11, 1953 during the 124th ACS National Meetings. It may be pointed out that each of the former national conventions was held during an ACS national meeting.

A short summary of the previous annual meetings of the AACC might be in order at this time. On September 15-17, 1949 the Association, then a tottling of less than one year of age, held its first national meetings in Atlantic City. This was attended by clinical chemists from all parts of the country, and a thrilling one for those of us fortunate to be there. The program consisted of a symposium, general session, dinner, and business meetings at which a great deal was accomplished.

The second annual convention was held in Philadelphia on April 11-12, 1950. In addition to the full program of the previous year, the AACC inducted Professor Walter R. Bloor as the first honorary member.

The third annual convention was held in Boston on April 2-3, 1951. At that time Dr. Donald D. Van Slyke was inducted as honorary member. Also a symposium on instrumentation in clinical chemistry was held with the collaboration of the Division of Analyti-

Fifth Annual Association Dinner will be held at the Conrad Hilton Hotel, Room 2, on Thursday Evening, Sept. 10 at 6:30 pm. Feature of the evening will be the presentation of the Ernst Bischoff Award to Michael Somogyi. Dr. Somogyi will deliver the Second Ernst Bischoff Lecture. Tickets to the dinner will be on sale at the ACS registration areas of the Conrad Hilton, Palmer House and the Hotel Sherman during the registration hours and until 12:30 of the day of the dinner.

* * * * *

Stated Annual Meeting will be held following the afternoon scientific session Thursday, Sept. 10, 4:30 in the Normandy Lounge, Conrad Hilton.

cal Chemistry of the ACS. This is of interest, since all the other scientific programs of the Association have been held with the Division of Biological Chemistry.

Although not an official annual convention, the AACC took an active part in the Diamond Jubilee of the ACS during September, 1951. The international aspect of those meetings presented the opportunity to lay the foundation for the International Association of Clinical Biochemistry. At that time also honorary memberships were awarded to Dr. Norman R. Blatherwick, Professor E. J. King of Great Britain, and Professor E. C. Noyons of the Netherlands.

The fourth annual convention of the AACC was held in Milwaukee on April 1-2, 1952. At that time Professor P. A. Shaffer was inducted as an honorary member.

The fifth annual convention promises to be as fruitful as the previous ones. The AACC, in the relatively short period of its existence, has already written some exciting footnotes. Yet no historical review would be complete without noting the cordial collaboration given to us at all times by the American Chemical Society.

Max M. Friedman, National Secretary

SCIENTIFIC SESSIONS

THURSDAY MORNING - Sept. 10

Conrad Hilton, Normandy Lounge

Symposium on Electromigration in Stabilized Electrolytes

In Participation with the Division of Biological Chemistry, American Chemical Society

Hugh J. McDonald, *Presiding*

- 9:00- Hugh J. McDonald. Introductory Remarks.
- 9:05- Quintin P. Peniston. Separation by Electromigration in Agar Gels.
- 9:35- John G. Reinhold and C. A. J. Von Frijtag Drabbe. Some Clinical Chemical Applications of Electromigration in Paper.
- 10:05- E. L. Durrum. The Separation of Serum Proteins by Zone Electrophoresis.
- 10:35- W. F. White and J. W. Giffey, Jr. Paper Electrophoresis of the Pituitary Hormones.
- 11:05- Henry G. Kunkel. Zone Electrophoresis in a Starch Supporting Medium.
- 11:35- T. R. Sato, W. P. Norris, and H. H. Strain. Studies of Phosphorus Compounds and of Bone by Neutron Activation Plus Electrical Migration.

THURSDAY AFTERNOON

Symposium on Electromigration in Stabilized Electrolytes

In Participation with the Division of Biological Chemistry, American Chemical Society

H. H. Strain, *Presiding*

- 2:00- H. H. Strain. Introductory Remarks.
- 2:05- Y. T. Oester and Edward P. O'Malley. Re-Evaluation of Iontophoresis in the Field of Medicine.
- 2:35- Harold H. Strain and T. R. Sato. The Separation of Inorganic Ions by Electrochromatography.
- 3:05- Edward P. Marbach, Hugh J. McDonald, and Robert H. Spitzer. Determination of Electromigration Mobility in Paper-Stabilized Electrolytes.
- 3:35- Scott E. Wood and John L. Engelke. Electroosmosis in Electrochromatography in Paper.
- 4:05- Matthew C. Urbin and Hugh J. McDonald. Molecular Weight Determinations from Electroacceleration in Paper-Stabilized Electrolytes.
- 4:30- STATED ANNUAL MEETING

Conrad Hilton, Normandy Lounge

Clinical Chemistry

In Participation with the Division of Biological Chemistry, American Chemical Society

Max M. Friedman, *Presiding*

- 2:00— Max M. Friedman. Introductory Remarks.
- 2:05— Arthur W. Devor. The Brönsted Theory Applied to Acid-Base Balance and Respiration.
- 2:20— Lewis Danziger and George L. Elmergreen. Mathematical Theory of Periodic Relapsing Catatonia.
- 2:35— G. Podio and B. Rovatti. Influence of Parenteral Injections of High Molecular Polyvinylpyrrolidone on Survival of Rats Receiving a Lethal Dose of X-Rays.
- 2:50— Florence B. Seibert, Eva Soto Figueroa, Elizabeth E. Miller, Mabel V. Seibert, and Margaret Reed Lewis. Chemical Analyses of Oncolytic and Immunizing Extracts of Rat Sarcomas.
- 3:05— Julius Schultz, Harry Shay, Anne Turtle, and Margot Gruenstein. Chemistry of Experimental Chloroma.
- 3:20— Alfred D. Winer and Kenneth F. Emst. A Rapid Quantitative Flame Spectrophotometric Method for Urine Calcium with a Study of Normal and Pathologic Values.
- 3:35— Samuel Natelson, Ralph Penniall, and Jean Secard. Colorimetric Determination of Calcium with Alizarin and Alizarin Sulfonic Acid.
- 3:50— Hertha H. Taussky. A Micro Method for the Determination of Creatine in Urine by the Jaffe Reaction. (With the Technical Assistance of Gloria Kurzmann).
- 4:05— Daniel Sanshuk and Monroe E. Freeman. Pontacyl Standards for the Biuret Method for Total Serum Protein.
- 4:20— Albert Hanok and Albert Edward Sobel. Composition of Bone and Teeth. I. Relation to Blood and Diet.
- 4:35— Albert E. Sobel, Albert Hanok, and James H. Shaw. Composition of Bones and Teeth. II. Relation to Caries Susceptibility. (BY TITLE). L. P. Munan and E. Munan. Hypoxia and Vitamins. A Preliminary Study with Pantothenic Acid.

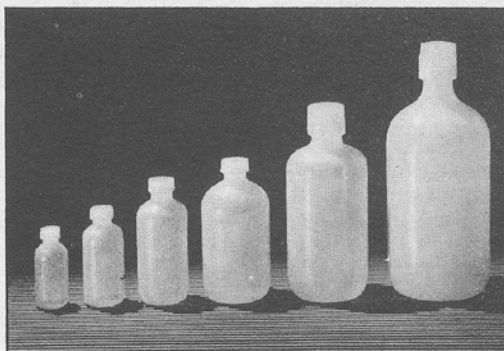
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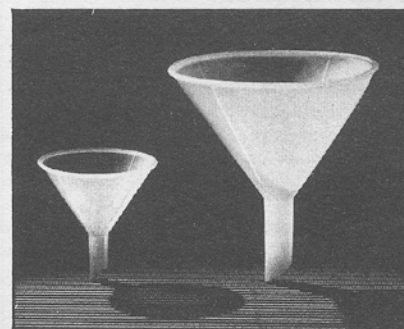
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The American Board of Clinical, Inc., since its formal organization in April 1950, has received requests for applications from 689 individuals. Of these 409 were completed and filed with the necessary data and supporting information. The Board during the past three years has met on several occasions at which time 379 of the filed applications were considered, were granted certification.

Applications were received from nearly every state as well as Canada, Hawaii, the Phillipines, Puerto Rico and an occasional one from countries where there was stationed United States Army or Navy personnel. The greater number of applications originated from areas of concentrated population and centers of scientific or medical training: New York, Pennsylvania, Illinois, California and Louisiana being outstanding. Many of those applying conduct private laboratories, the majority however, being connected with institutional work.

The Board carefully weighed the facts submitted in many instances found it desirable to seek additional information of those in the same field and areas as the applicant.

Those certified to date include the names of many who will be recognized by their numerous contributions toward the advancement of the profession, others who are younger will, it is expected, advance themselves to the same position of respect as the years pass.

The names of those certified up to and including the Annual 1953 Spring Meeting of the Board, have been published in the CHEMICAL AND ENGINEERING NEWS, Vol. 31, No. 31, page 3203, August 3, 1953.

POSITION OPEN

CLINICAL CHEMIST, Fed. Civil Service GS-7. To supervise Analytical and Analytical Research unit. Personal interview required. Write Capt. Bernard Balikov, Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

NEW UNIVERSAL BALANCE

A manual explaining the new Universal Balance has recently been published by the manufacturer, Voland & Sons, Inc., 32 Relyea Place, New Rochelle, N. Y.

This new instrument, which the manufacturer claims is a completely new concept in balances, is fully illustrated in the manual. Comprehensive diagrams and photographs describe the many unusual features of the Universal Balance, including the beam and projection systems, weight operation, compensating stirrups.

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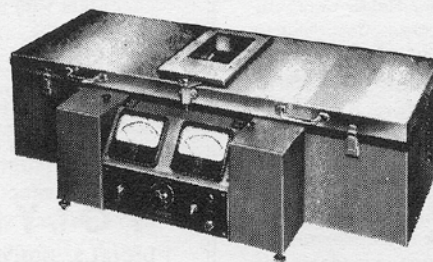
ing situations leading to identification and measurement of components in a mixture and assignment of structural formulas; the publication includes further specific data on the spectrometer and suitable magnet system. Data is included on basic operating characteristics; general arrangement of sub-units encompassing the probe, the radio-frequency transmitter and receiver, sweep device, power supply, and oscillograph. The folder is identified as publication No. 40. Varian Associates - 522 Hansen Way, Palo Alto, Calif.

RESEARCH PAPERS

The Editorial and Advisory Boards of THE CLINICAL CHEMIST invites for publication papers on original research. Authors should submit two copies of their manuscript and the format should follow that suggested in "Instructions To Authors" published in the JOURNAL OF BIOLOGICAL CHEMISTRY.

THE CLINICAL CHEMIST is now being distributed to the major libraries and papers will be abstracted in CHEMICAL ABSTRACTS.

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ELECTROMIGRATION IN STABILIZED ELECTROLYTES

PART III: APPLICATIONS OF THE TECHNIQUES

by

H. J. McDonald, R.J. Lappe, E.P. Marbach, R.H. Spitzer, and M.C. Urbin

Department of Biochemistry, The Graduate School, and Stritch School of Medicine, Loyola University, Chicago.

The general fields of application of electromigration in mechanically stabilized electrolytes may be listed as: (1) simple fractionation and empirical resolution of mixtures of compounds, including ionic and colloidal substances as well as materials which are normally thought of as being relatively uncharged in solution, such as carbohydrates. (2) mobility determinations and related quantities, or derived relationships such as isoelectric point determinations, "pH versus mobility" curves for ampholytes, ionic strength effects, studies of factors influencing electroosmosis, interrelationships between mobility of a migrant and its molecular weight and volume, chemical kinetic quantities such as the energy of activation, and order of a reaction, etc., (3) reaction-site studies, which include investigations of the effect of ionic strength on the dissociation constants of complex ions, the determination of equilibrium constants and such related thermodynamic quantities as the change in free energy, in heat content and entropy, etc.

For the simple fractionation of complex mixtures, and this constitutes the largest fraction of the papers published on ionography, variations in the design of the apparatus employed and the conditions under which the experiments are carried out seem not to be very significant, when used by experienced workers. In fact, while for mobility determinations the buffer ionic strengths are generally 0.05 or less, much higher ionic strength buffers may be used for empirical separations of mixtures and in some instances seem to yield sharper separations of the individual zones on the ionograms. In simple fractionation procedures, it is also unnecessary to go to any great length to eliminate the role of chromatography in the separation process, and indeed, the judicious simultaneous or consecutive combination of electromigration and chromatography may yield better results than when either technique is used alone. From the stand-point of simplicity of operation and the chances of getting usable separations (where mobilities are not involved) with the minimum of experience, it is evident from work in the authors' laboratory that any variation of the apparatus other than the compressed-sandwich type is preferable.

When mobility determinations are to be made, the many factors discussed in Part II of this series must be given consideration. The ridgepole suspension or hanging-strip technique (17) would seem to lend itself less readily to work on mobilities than the horizontal suspension technique (52, 53, 59)

or the sandwich technique (42, 153), although Macheboeuf and co-workers (173, 175) have suggested an analytical treatment of results obtained by means of the hanging strip method (17). Although both the sandwich technique and the horizontal suspension technique present certain difficulties when used in mobility studies, they both have been used successfully for such purposes (42, 52, 59, 171). The better control of evaporation in the compressed sandwich technique is offset to a large extent by such factors as electroosmosis between the surfaces of the glass sheets and the paper filler, the interfering surface tension and capillary effects, by the difficulty of preventing a smudge of the zones on the ionogram when removing the covering plates, and perhaps most importantly, by the difficulty of maintaining a uniform and reproducible ratio of "buffer solution" to "paper". If the pressure exerted on the glass plates varies from point to point throughout its length, so also will the potential gradient and the weight ratio of "buffer" to "paper", and consequently so will the thermodynamic activity of the migrant. Such factors have been pointed out in Part II of this series of articles under the section dealing with the determination of so-called free-solution mobilities from those obtained in paper-stabilized electrolytes. To reiterate, at points where the covering plates are pressed together with greater force, there will be less water per given weight of paper, and a lower mobility of a migrant, than in a zone where the pressure is less and the water content of the paper higher. This same factor makes it difficult to attain complete agreement of results, as regards mobility, from one laboratory to another or between successive workers in the same laboratory using an identical sandwich-type apparatus. With such equipment it is not sufficient to state the usual conditions under which the experiments were run, such as buffer-type, ionic strength, pH, potential gradient, temperature, etc., but the pressure exerted on the paper filler by the covering plates should also be given, or in lieu of this information, the weight ratio of "buffer solution" to "paper filler".

It is of interest to note that much of the early work on electromigration in stabilized media was slanted toward applications of a biochemical or clinical chemical nature. For example, the studies of Field and Teague in 1907, concerned with electromigration characteristics of diphtheria toxin and antitoxin (122) as well as of tetanolysin and antitetanolysin (123) in agar-stabilized electrolytes, and the work

of Teague and Buxton (204), also in 1907, on the electromigration behavior of hemolytic amoebocytes, hemoglobulins and complement in agar jelly, may be mentioned. The earliest investigations on electromigration in filter-paper sheets wetted with electrolytes deal with biochemical studies on snake venom (153).

In Part I of this series, the article by Wieland and Fischer, (102) published in 1948, was mentioned as the earliest known publication on this particular technique. Since then the paper by von Klobusitzky and König (153), published in 1939, has come to the authors' attention. These investigators employed a strip of medium-hard filter paper, 1 x 5 cm., the ends of which were connected to a 110 volt direct current source in series with an adjustable resistance and a milliammeter. The paper was moistened with a dilute solution of an electrolyte and one 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 a period of 20 minutes at a pH of 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 transversally into several narrow sections, from each of which the material was extracted and suitably tested in various ways. The authors refer to an even earlier paper by König (156), in which the apparatus itself is described in more detail.

The material in the present paper will deal with the ionographic separation and some of the electromigration characteristics of the following substances: proteins, amino acids, carbohydrates, lipids, and lipoproteins, enzymes and hormones, vitamins, inorganic ions, and some miscellaneous applications to less familiar compounds. No attempt will be made to cover all the minor variations of the technique, such as automatic scanning devices, apparatus for continuous separations, etc., since papers covering these aspects of the subject are listed in a separate bibliography (169).

PROTEINS

Introduction

A large fraction of the papers concerned with the technique of electromigration in mechanically stabilized electrolytes de-

ELECTROMIGRATION IN STABILIZED ELECTROLYTES - PART III

scribe the use of proteins as migrants. Davis et al. (15) and Gordon et al. (26) have investigated these compounds by using agar to stabilize the buffer solutions. The former group of workers carried out the electromigration in agar-filled glass capillaries, while the Gordon group employed a rectangular slab of agar jelly as the stabilizer in their separations of hemoglobin from ferritin, of the resolution of the proteins of egg white, and the investigation of a pepsin preparation. For a more thorough discussion of Gordon's apparatus and the pertinent factors affecting the resolution of mixtures of proteins, an earlier paper (11) should be consulted.

By far the majority of workers employ filter paper to stabilize the electrolyte solution during protein studies. The references that appear to have definite value in describing and elaborating on the general apparatus and procedure accompanying protein investigations include: (6; 14, 17, 21, 23, 24, 28, 29, 36, 41a, 42, 72, 73, 77, 80, 81, 92, 95, 98, 104, 107, 115, 120, 133, 134, 139, 141, 159, 162, 166, 167, 179, 197, 202, 204).

Previous to the introduction of these methods, the separation and the quantitative estimation of proteins by free solution electrophoresis was not very practical. This traditional technique of electrophoresis required access to an expensive instrument which demanded the use of large volumes containing high concentrations of protein solutions, followed by difficult manipulations for the estimation and for the isolation of the protein constituents after their electrophoretic separation. retic separation.

Two papers which illustrate the advantages of the new technique of ionography in protein studies are those by Levin and Oberholzer (166, 167). These workers estimated the absolute amounts of plasma proteins in blood by both micro-Kjeldahl and dye-elution analyses, and compared the results of their separations obtained by free solution electrophoresis and salt fractionation with those obtained via electromigration in paper-stabilized electrolytes. They concluded that the absolute amounts of plasma proteins in a blood sample measured by means of nitrogen analyses of the proteins on the paper were in agreement with those obtained from free solution electrophoresis using optical methods of analyses. They also maintained that the method of separation is preferable to that of salt fractionation.

I. Techniques and Experimental Conditions Applicable to Studies of Proteins

In any extensive discussion of the experimental conditions utilized during protein experiments it will be found that there is a variance of opinion as to the optimum choice of buffer, to the optimum choice of pH, ionic strength, temperature, filter paper to be used, etc. In this section no attempt will be made to evaluate the argument for or against a particular variable,

but rather a presentation of the conditions maintained in certain successful experiments will be made.

(a) Pretreatment of the proteins: concentration and dialysis.

Flynn and de Mayo (21) state that with their procedure a concentration of migrant solution containing 2-3 gms. of protein per 100 mls. was satisfactory. In more dilute solutions a preliminary concentrating was necessary by some suitable means, as for example, by ultrafiltration. Further, for routine blood analyses serum was preferable to plasma since the extra fibrinogen band made the interpretation more difficult. These workers indicated that the serum specimens should be fresh and unhemolyzed.

When working with urinary proteins, Slater and Kunkel (195) took 1-15 mls. of urine and concentrated it to 0.2-0.3 mls. by dialysis in 25% aqueous polyvinylpyrrolidone. Earlier, Schneider and Wallenius (78) performed a similar manipulation but used dextran as the colloid. Aside from the need, in some cases, to obtain an adequate protein concentration, the question arises as to the necessity of using the protein solution at the identical pH of the buffer solution. A number of sources indicate that preliminary dialysis is unnecessary when qualitative fractionations are desired (21, 42, 167); however, it would appear from work in the authors' laboratory that for clear-cut separations and mobility determinations, the protein solutions and the buffer solutions should be at the same pH values.

(b) Buffer solution: system, pH, ionic strength

Obviously the choosing of an appropriate buffer system is dependent on the characteristic proteins being studied. It would seem that the veronal buffer system at pH 8.6 and ionic strength 0.05-0.10 is applicable to the separation of the serum proteins. The veronal buffer at pH 8.6 may be formulated by using 0.05 M sodium diethyl barbiturate (10.3 gms./liter) and 0.01 M diethyl barbituric acid (1.84 gms./liter) (21, 73, 167, 134). When the protein is to be subsequently determined by the nitrogen analysis via a micro-Kjeldahl technique, a reagent blank is necessary, since nitrogen is a constituent of the barbituric molecule (167). The Michaelis' veronal-buffer system is convenient for varying the buffer strength and/or pH. (180)

(c) Filter paper

The choice of an appropriate filter paper is also a function of the protein characteristics being investigated. Apparently the selection must also be based on the type of apparatus used. For example, the compressed sandwich type demands a thick hard paper.

(d) Identification and Quantitative Analysis of Protein Fractions.

The proteins have been generally stained

with the bromphenol blue reagent (1% bromphenol blue in ethyl alcohol saturated with mercuric chloride) (21, 42, 167). Addition of an acid, such as acetic acid to this solution or to the washing solution, will enhance adsorption of the proteins on the filter paper and minimize their loss on developing. The blue color may be intensified by passing the ionograms over ammonia vapor. Flynn and de Mayo (21) used a saturated solution of Naphthalene Black 12B.200 in methyl alcohol containing 10% acetic acid to stain the serum proteins. The strips are then washed with methyl alcohol alone. This represented a qualitative technique, but they described a quantitative technique also, in which bromphenol blue was used. A large number of dyes have been examined and discussed by Griffiths (134). He preferred to use the brom phenol blue reagent for general use but instead of using methanol as a wash suggests a different technique. The strip is washed with water until the background is almost white and then immersed in Dioxan (commercial grade dioxane) and then ether. Dioxan has the property of bleaching unadsorbed dye while the ether removes the Dioxan and hastens drying. Quastel and Van Straten (73) stained the proteins with a hot aqueous solution of Solvay purple (0.05%) containing 0.5% sulfuric acid. After five minutes in the solution the strips are washed with warm water to remove the excess dye and are dried. A saturated solution of the dye, amidoschwarz 10B in methanol, containing 10% glacial acetic acid was used by Knedel (35). The above color tests for the proteins are not only able to qualitatively localize a protein fraction but in most cases may be extended to give a quantitative indication of the concentration. There are at least three general methods by which quantitative evaluations may be made. The first may be called the "dye elution method". This involves staining the proteins on the filter paper, cutting the paper up into smaller segments, eluting the dye from the segments and reading the color in a spectrophotometer (21, 42, 95). A variation of this technique involves the omission of the staining procedure, but nevertheless cutting the paper into sections and ultimately determining the proteins by nitrogen analyses (106, 166, 167). The third method of quantitative protein analysis renders the ionograms (in this case the stained proteins on filter paper) transparent by means of paraffin oil, etc., and then passing this filter paper through a light beam, thus obtaining a direct photometric estimation of the adsorbed dye (115, 134, 160). For further details the original articles should be consulted. In other interesting methods to identify the proteins Sternberg (199) rendered them fluorescent by coupling with a few drops of riboflavin solution. Thus no fixing and staining is required. Homolka (140) after separating the proteins made a polarographic examination of the fractions.

II. Protein Studies of Special Clinical Interest

Fischer (124) in a recent review article lists many of the contributions of electrophoresis to clinical pathology. Both the work on free solution electrophoresis and electrophoresis in paper stabilized electrolytes is included in this survey of the literature from 1948 to 1952. The technique of ionography has been found by Slater and Kunkel (195) to be applicable to the differential diagnosis of proteinuria especially in cases of multiple myeloma with Bence-Jones urinary protein. In the process they simultaneously analyzed under identical conditions specimens of serum and urine from the same patient. The clinical use of ionography as applied to liver disease is described by Knedel (35). Kautzsch et al. (146), Gras (132) and Kanzow (145) have determined clinical changes in the serum protein components. The separation of methalbumin and hemoglobin has been demonstrated by Hensley and Blackburn (137). These compounds are identified with the benzidine reagent and subsequently a quantitative determination is attained. Brante (111) after achieving protein fractionation correlated the results with other laboratory findings to diagnose diseases of the liver and biliary tract. In their studies on the hepatic and extra-hepatic regulation of serum protein metabolism, Roberts and Brunish (189) injected C^{14} -labeled amino acids into normal and hepatectomized rats. The serum proteins from the respective animals were separated and the radioactivity of the components determined. Using alanine- $2C^{14}$ they concluded that the liver is directly involved in the formation of albumin and α -globulin, and that the gamma globulin is not immediately dependent on liver activities for its formation. Köiw, et al. (37, 155) find that the values for protein concentration obtained when using paper as the stabilizer compare well with those from the Tiselius apparatus and are as suitable for clinical purposes. Application of the technique to the separation of the various hemoglobins has been attempted by Spaet (198). He separated the adult normal hemoglobin, sickle cell hemoglobin and c hemoglobin but not type F hemoglobin. Macheboeuf et al. (174) use the method to obtain information from pathological serums, especially from cases of lipide nephrosis, multiple myeloma, and Laennec's cirrhosis. A discussion on the separation of protein fractions, lipides, and carbohydrates bound to protein was undertaken by Grönwall (135). Methods for the visualization of protein-bound lipides and carbohydrates, and radioautographic detection of isotopes is included. The determination of albumin-globulin ratios has been put on a routine basis by Natelson (67). Sternberg (199) has described a technique that he has found applicable to clinical work.

III. Cerebrospinal Fluid Proteins

A logical consequence of the many papers devoted to the investigation of the

serum proteins is the extension of the studies to other protein solutions, as for example, the cerebrospinal fluid proteins. And there are, in this case, several reports on this topic (8, 78, 114, 119, 121, 312, 205, 181).

Schneider and Wallenius (78) have devised a procedure for the dialysis and ionographic separation of this fluid. They concluded that the albumin-globulin ratio is similar to that of blood serum and it possessed corresponding components with similar electrophoretic behavior.

Preliminary concentration of the cerebrospinal fluid in a dessicator while dialyzing against a buffer solution previous to the electrophoretic separation was performed by Esser, et al. (121). Esser and Heinzler (119) utilized an ultrafiltration method to concentrate the proteins.

Bücher et al. (8), however, concentrated the fluid by precipitation with acetone at low temperatures. In contrast to Schneider and Wallenius, they found that on fractionation, there appeared to be two extra bands, in addition to the usual zones observed in serum protein patterns.

A number of conclusions were made by Wallenius in his studies (205). He found the cerebrospinal fluid proteins are normally identical to the serum proteins and possessed an albumin-globulin quotient near that of serum.

IV. Iodine-Protein Relationships

The versatility of the ionographic technique was further expressed in a series of papers dealing with the localization of protein-bound radioactive iodine after the administration of I^{131} into the circulatory system (3, 17, 25, 43, 75).

In 1950 Durrum (17) injected I^{131} intraperitoneally into rats. An extract of the thyroid was obtained and utilized as the migrant. Autoradiographs were then made of the strips to indicate the distribution of radioactive material.

Gordon et al. (25) using the veronal buffer system at pH 8.5, fractionated the serum collected from patients who had been given doses of iodine-131. They found the maximum radioactivity in a band immediately following the albumin and a smaller amount associated with the albumin fraction. In vitro experiments showed that radiothyroxine has a mobility near that of the α_1 -globulin.

In similar work Larson et al. (43) procured the serum of euthyroid patients with a Hurthle cell carcinoma of the thyroid with metastases. Iodine-131 had been previously administered to the patient. After protein fractionation they found that the main concentration of radioactivity was near the α_2 -globulin area with an indication of some in the albumin.

Robbins and Rall (75) likewise are in close agreement with the distribution of radioiodide in serum.

V. Immunological and Related Considerations

Contained in this section are six papers

which are unusual due to the substances the authors chose to investigate by the technique of electromigration in stabilized electrolytes. The substances include, among others, antibodies, antigens, hemolysins, agglutinins, toxins, and antitoxins. Several decades ago, in the year 1907, three papers appeared in the literature utilizing the technique of electromigration in agar-jelly (122, 123, 203). The authors, Field, Teague, and Buxton expressed an awareness of certain difficulties that have concerned contemporary workers. For example, they were cognizant of the fact that the migrant may be adsorbed by the stabilizer and that the products of electrolysis could affect the direction of electromigration and that due to the heat generated in the agar as the current passes through it, the migrants may be modified or deviated from their native state. They studied the electrical charge of diphtheria and tetanus toxins and antitoxins, of certain agglutinins, and hemolysins.

In 1953 Grabar and Williams (131) described a method permitting the simultaneous study of electrophoretic and immunological properties of a protein mixture using agar to stabilize the electrolyte.

Gros et al. (30) used paper as the stabilizer in their work on the speed of renewal of antibodies and other protein fractions of blood serum through the medium of marking valine with C^{13} in the isopropyl group.

Le Beau (45) in his studies on the allergic fractions of *Alternaria* sp. has utilized ionography to indicate association of allergenic activity with a carbohydrate fraction of an extract and antigenic activity with the protein portion.

Purified diphtheria toxin was separated by Poulik (187) into four fractions, one of which showed flocculating activity.

VI. Miscellaneous Ionographic Studies of Proteins

Included in this section are topics which in some cases could have conceivably been described elsewhere in this paper but due to the specificity of their subject nature were included here. All papers pertain to protein studies even though in certain cases only indirectly.

In the first group are a series of investigations concerned with peptides and the hydrolytic products of the proteins. Conden and Gordon (12) in their study of the peptides of cystine in partial hydrolyzates of wool, separated the acidic peptides into groups with similar electrical mobilities using silica jelly as the stabilizer. Boulanger and Biserte (110) in their review paper of chromatographic techniques for separating small and large peptides and proteins discussed electromigration on filter paper for fractionation. A crude polyglutanyl peptide was chemically fractionated by Strange and Harkness (201) from *B. anthracis* capsule and the components of the peptide mixture were separated using ionography. An agent to identify the peptides is described.

ELECTROMIGRATION IN STABILIZED ELECTROLYTES - PART III

Kutzeim (157,158) in his study of blood proteins has indicated that generally the sulfamidides move independently of the proteins.

In an interesting experiment Cholsy et al. (10) have separated the protein components of beef and human lens. Two components are isolated from the beef lens while a single constituent was present in the human lens extract.

Zweig and Block (215) have resolved the α -, β -, and gamma-caseins using paper as the stabilizer. It is interesting to note that they found the relative mobilities of the caseins of the same order as those determined by the free solution electrophoretic technique.

A comparison of the serum proteins of man, monkey, horse, rabbit, guinea pig, and rat has been made by Ganzin et al. (22). It was demonstrated that the patterns differed in the spacing of the globulin spots.

Latner and Ungley (163) separated human gastric juice on filter paper. Several protein staining peaks were found. Besides all possessing vitamin B₁₂-binding activity one peak contained either a mucoprotein or mucopolysaccharide.

Wunderly (105) separated the serum proteins on filter paper, segmented the strips eluted the proteins with physiological saline and studied the lyophilic properties of the fractions.

AMINO ACIDS

Wieland and Fischer (102) utilized electromigration in filter paper to study the complexing of amino acids with cupric ion. In further work Wieland et al. (103) incorporated radioactive copper into the complexes to attempt a quantitative determination of the amino acids. Wieland and Bauer (101) made separations of amino acids, and purine and pyrimidine derivatives. Haugaard and Kroner (31) used paper chromatography and electromigration simultaneously to achieve good separations of amino acids.

As described in Part I of this series of papers, Durrum (17) studied among other compounds, the separation of amino acids and peptides. He found the ridge-pole technique to be satisfactory for simple separation work but stated that it could not be employed for mobility measurements. As mentioned previously Macheboeuf and associates (173,175) have recently shown how it is possible to surmount some of the obstacles inherent in the hanging-strip method. In later work Durrum (16,18,117) used two-dimensional electrophoresis in studying amino acids and mixtures. He found in these experiments that the amino acids reached an equilibrium position on the paper strips and cites these as possible characteristics of the various amino acids. These equilibrium points, however, can be shown to be characteristic only of the hanging-strip method of paper electrophoresis. The author states that the equilibrium point changes with a change in the

angle of the apex at which the strips are hung.

Biserte (4) separated amino acids and polypeptides using filter paper as the stabilizing agent. Suzuki and Maekowa (89) also have used electromigration in paper to separate amino acids and biogenic amines.

McDonald, and co-workers (52,58,59,60) have used amino acids as migrants in the study of some of the fundamentals of the technique of ionography. They found that amino acids possessed specific mobilities because in an electric field they moved linearly with time and potential gradient. They studied the effect of pH and ionic strength on the rates of migration of amino acids. In further work they found the method to be satisfactory for the determination of isoelectric points of amino acids (55,60).

McDonald and Urbin (172) have used square sheets of filter paper in simultaneous crossed-current ionography and have described how the method may be used for the determination of molecular weights. This method differs from two-dimensional paper electrophoresis experiments performed by other authors since in the case of two-dimensional experiments the sheets are run first in one direction and then the electrodes are changed and the run is set up in the other direction, much the same as in two-dimensional paper chromatography. In simultaneous crossed-current experiments McDonald and Urbin found that the molecular weight of the migrant was inversely proportional to the acceleration as derived from Newton's second law of motion. It is of interest to note here that Markham and Smith (178) have calculated theoretical mobilities of ribonucleic acids from the charge and molecular volume. They based their theoretical mobilities on the fact that mobility is proportional to the net charge and inversely proportional to the cube root of the molecular volume. So it would seem that, with certain restrictions, from unidirectional electrophoresis on paper the molecular volume of a migrant may be calculated, while from experiments involving simultaneous crossed-currents the molecular weights could be computed.

CARBOHYDRATES AND RELATED COMPOUNDS

The ionographic separation of carbohydrates and related compounds can be divided into two sections. In the first part there is considered the separation of compounds which are usually thought of as being electrically "neutral" substances, but which are made ionic by methods involving the formation of "complexes". In this group can be included carbohydrates and nucleosides. In the second part, there is considered the separation of carbohydrate-containing materials by methods not requiring "ionic complex formation". This group includes the nucleotides, nucleic acids, mucopolysaccharides, and protein containing carbohydrates.

Carbohydrates and Nucleosides

Ionography has been used for the separation of carbohydrates as well as for the identification of the carbohydrate constituents of a mixture. In general, the procedure employed is to saturate the paper with a borax, a borax-HCl, or a boric acid-NaOH solution of requisite ionic strength and pH, and then to apply the migrant solution to the paper. Under the influence of a potential gradient applied for the proper period of time, the carbohydrates will separate one from the other according to the amount of borate with which they have complexed. The amount of borate that carbohydrates will bind depends: (1) upon the steric configuration of the monosaccharides. An excellent review concerned with the configurational aspects of the binding of borate with carbohydrates has been provided by Böeseken (109), and (2) upon the number and type of carbohydrate units composing a carbohydrate polymer.

In the separation of polyols as borate complexes the differences in technique lie less in the particular apparatus used to effect separation than in the procedure used to stain and locate the carbohydrate materials on the paper strips or sheets which themselves are composed of carbohydrate. In general, the staining techniques which are employed in chromatography are utilized in this method.

After a period of electromigration of 6-9 hours, Jaenicke (143) found that the sequence in which the sugars arranged themselves, from the starting point to the anode, was in the order: maltose, xylose, ribose, glucose with fructose, arabinose, and galactose. He employed a 0.1 M borate buffer at pH 9.2 and 0.2 M AgNO₃ as the developing agent in a modification of Tollen's aldehyde test.

Michl (65) separated not only compounds classically known as sugars but also non-carbohydrate substances which combine with borate. For example, he separated the poly-alcohols, glycol, glycerol, and mannitol from a mixture containing all three. He separated ortho-phenols such as pyrocatechol, methyl gallate, protocatechuic aldehyde, pyrogallol, tannin, etc. He noted that carbohydrates moved as single bands above a borate concentration of 0.08 M and above pH 9. Otherwise it was observed that there was one moveable component and one stationary component owing to the presence of alpha- and beta-forms of the carbohydrates. He maintained that these differences could not be due to different combining ratios of the hydroxyl compound to borate (this was always 1:1), nor to different molecular size, but rather to different dissociation constants of the borate complexes. Dissociation constants calculated from observed mobilities agreed with those predicted on the basis of steric considerations.

In a borate buffer at pH 9.2 Jaenicke and Vollbrechtshausen (144) separated the ribosides from ribonucleic acid and found the successive groups to be adenosine,

cytidine with guanosine, and uridine. In glycine buffer of the same pH, the ribosides do not migrate. In acetate buffer at pH 3.2, guanosine and uridine can be separated not because their constituent sugars are complexed with borate ion, but instead because of the ionic charge of the carboxylic and amino groups of the guanine and uracil. That is to say, under the conditions employed for the electrophoretic fractionation of these two substances, the separation depends on their inherent ionic charge. They detected the ribosides by ultraviolet photography or by spraying the paper with a fluorescent dye. Adenosine-3' -phosphoric acid and adenosine-5' -phosphoric acid can be separated by addition of boric acid or borate buffer to their solution.

Micheel and van de Kamp (182) combined electromigration in filter paper with chromatography for the separation of sugar mixtures. The mixtures were used as boric acid complexes and the electrophoretic separation was carried out simultaneously with the downflow of the solution through the filter paper. Fractions were collected and then chromatographed separately.

By choosing conditions of pH and time such that mobilities differ by 20% or more, Consden and Stanier (12a) were able to separate fructose, sorbose, glucose, galactose, mannose, arabinose, ribose, rhamnose, raffinose, and cellobiose using borate buffer. The same sheet of paper which they used for the electrophoretic separation was subsequently employed for a further chromatographic resolution. By this procedure a group A hemolytic streptococcus hydrolyzate showed the presence of 4 sugars which had the mobilities of hexosamine, rhamnose, ribose, and glucose. A human fibrin hydrolyzate showed 3 sugars migrating at the rate of hexosamine, mannose, and galactose.

Hashimoto et al. (136) separated flavanoids by ionography employing a 1-3% borax or boric acid solution as the electrolyte. The flavanoids migrate to the cathode, the distance being related to the number of adjacent *cis*-hydroxyl groups of the sugar and the number of *vic*-hydroxyl groups of the aglycone. The flavanoid spots were made visible with ethereal LiAlH_4 and subsequent treatment with water or dilute HCl. The sugars were developed with aniline hydrogen phosphate (186).

Foster (125) found that 2,3-, 2,4-, and 3,4-dimethyl-L-rhamnopyranoses chromatographically are separable, the latter two only difficultly so. Of the three dimethyl rhamnopyranoses only the 3,4-dimethyl derivative had a structure suitable for complex formation with boric acid since it has *cis*-hydroxyl groups at carbon atom 1 and carbon atom 2 in the beta-form. In his work Foster (126) employed the technique in which a sheet of filter paper is sandwiched between two glass plates. The lower plate was cooled by means of a

copper coil through which ice water flowed.

In borate buffers at pH 10 and from the migration of numerous methyl sugars via ionography, Foster (127) ascertained that the evidence suggests that the *aldehyde*-forms, in addition to ring structures of the derivatives, interact with borate ion.

Foster and Stacey (128) attempted to derive a value, " M_g ", relating to the movement of migrants in an electric field, analogous to the " R_f " values used in chromatography. They defined this factor as

$$M_g = \frac{\text{True distance of migration of the substance}}{\text{True distance of migration of D-glucose}}$$

By the so-called "true distances of migration" they understood those values of migration (mobilities) that have been corrected for electroosmotic flow by the use of a non-borate-complexing derivative (2,3,4,6-tetramethyl-D-glucose). The choice of this electroosmotic indicator was arbitrary because several other "inert" indicators did not "migrate" at the same velocity.

These authors discuss complexing in relation to sugar structures and point out that carbon atom one and carbon atom two are paramount in complex formation.

Nucleotides, Nucleic Acids, Mucopolysaccharides and Protein-Bound Carbohydrates

Protein-bound carbohydrates of blood serum were studied by Köiw and Grönwall (154) in connection with clinical alterations of blood-serum carbohydrate values. After suitable electromigration in non-complexing buffers, the proteins on the ionograms were stained for their carbohydrates content by a dye specific for carbohydrate but not for the paper (cellulose) which was used as the stabilizing medium. The dye was a modified fuchsin-sulfite solution.

Since no stains for acid-mucopolysaccharides are known which will not also stain the paper, Rienits (188) after suitable ionographing in a non-complexing buffer, i.e., phosphate or acetate, cut up the ionograms, eluted the polysaccharides, and analyzed the eluates for hexosamine or hexuronic acid. Employing a phosphate buffer, he studied the electrophoretic migration of hyaluronic acid, chondroitin sulfate, and heparin. The positions of these on the ionograms were detected either by staining with toluidine blue or more satisfactorily by elution followed by a suitable spectrophotometric test.

By incubation with trypsin Woodin (211) was able to prepare corneal acid mucopolysaccharide free of protein. Using ionophoresis on filter paper as one of several analytical tools including occasional prior chromatography of the muco-

polysaccharide hydrolyzate, he was able to fractionate and to estimate the chemical composition of the corneal extracts.

Although Kannigieser (32) also did not use a non-carbohydrate-complexing buffer like borate, he was able to separate nucleic acids in 4-6 hours by electrophoresis on filter paper at pH 2-3. The nucleic acids migrate in an electric field due to the charges on the carboxylic acid and amino residues of the constituent purines and pyrimidines. He stained the ionograms for non-cellulose carbohydrates using fuchsin-acetic acid solution and nucleic acids by the method of Zinzade (214).

In an attempt at elucidating the structure of polynucleotide anhydrides by correlating mobilities of the nucleotides with their possible structures, Markham and Smith (178) employed a 0.05 M ammonium formate buffer at pH 3.5. According to these workers the charge on the anhydride derivatives of the nucleotides is entirely due to that on the -OH groups of the phosphoric acid radicals and the amino groups of the bases.

By means of a moderately successful electrophoretic isolation using agar gels as the supporting matrix for ionographic separation, followed by the more auspicious use of filter paper instead of the gel, Davidson and Smellie (116) were able to separate, analyze for, and recover in high percentages the nucleotides in a hydrolyzate of ribonucleic acid. These authors using a 0.02 M citrate buffer at pH 3.5 found that adequate separation required 18 hours at a potential gradient of 11 volts/cm. It is interesting to note that they dried their ionograms not in an oven as so many other workers report but more conveniently under an infrared lamp. The separated bands of the nucleotides were ascertained and photographed under ultraviolet light. These workers found that all the known ribonucleotides can be separated by ionophoresis on filter paper, that the method is most convenient to employ, and that it is suitable for small quantities.

In a study of the nucleoproteins of cytoplasm and the nuclei of the cells of rat liver and other tissues, Irvin and Irvin (142) found the average mobility of yeast nuclei acid to be 13.5×10^{-5} cm/sec per volt/cm at pH 6.5 (no buffer stated) and ionic strength 0.02. No electroosmotic correction is given. The bands of nucleoproteins or nucleic acids were located in the dried paper strips by direct measurement of optical densities at 260 millimicrons in a Beckman spectrophotometer equipped with a device which permitted continuous passage of the ruled strip past the exit slit of the monochromator.

Employing both ionophoresis on filter paper and chromatography, Gordon and Reichard (130) investigated the mixture of oligonucleotides formed by the action of pancreatic bovine deoxyribonuclease on deoxyribonucleic acid. The electrophoretic separation was carried out in

ELECTROMIGRATION IN STABILIZED ELECTROLYTES - PART III

agar jelly first at a potential gradient of 2.3 volts/cm for two hours followed by an increase to 3.7 volts/cm and an additional 15 hours.

In order to separate chondroitin sulfuric acid from hyaluronic acid, Gardell, et al. (129) utilized Hyflo Super-Cel (a commercial kieselguhr) as the stabilizing agent for the electrophoretic separation of these two substances. After suitable electromigration the slab was cut up into strips which were extracted with water in order to elute the polysaccharides, and these were ascertained by a suitable carbohydrate test. Three different buffers (citrate - 0.1 M and pH 3.7, acetate - 0.1 M and pH 4.7, and phosphate - 0.1 M and pH 6.7) were each individually used in the electrophoretic separation at a potential gradient of 6-7 volts/cm. The time for which the electrophoresis was conducted was estimated from the visible movement of a dye which these authors had incorporated into the agar, and amounted to a distance of 30-35 cm. from the agar inlay containing the migrants which traveled toward the anode.

LIPIDS AND LIPOPROTEINS

Several analyses of lipids and lipoproteins by the method of electromigration on wet filter-paper surfaces have been reported. The chief difficulties encountered in the study of these substances are in the detection of the bands or zones on the ionogram, in the adsorption of the lipid or lipoprotein on the surface of the paper, and in the retention of the lipid stain by the paper itself. The usual technique is to run at least two strips, one of which is stained for lipids and lipoproteins while the other is stained for proteins. In this manner the positions of the lipoprotein bands can be given relative to the protein bands.

Swahn (91) used a semi-saturated solution of Sudan black B in 50% ethanol for the detection of lipids and lipoproteins. Since Sudan black B is not a dye in the technical sense, it does not stain, but colors lipids black by dissolving in them. The coefficient of distribution for Sudan black B between lipids and 50% ethanol is very favorable to the former. The coloring appears to be quite specific in that at present the list of substances known to be colorable with Sudan black B include nothing but lipids and lipid-complexes such as lipoproteins. Swahn stained the paper strips for 30-45 minutes and then rinsed them with 3-4 changes of 50% ethanol until only a faint blue tone remained on those parts of the paper where there were no lipids. He also reported that with Munktell's No. 20 paper there was more adsorption of the Sudan black B than with Whatman's No. 1 paper. At room temperature a good separation of the α and β -lipoproteins was obtained using a 0.05 molar barbital buffer solution at pH 8.8.

Employing an apparatus similar to that which was used by K iw, Wallenius and Gr nwall (38), he studied the distribution and relative amounts of the serum lipids from normal and from pathological patients. Fasoli (20a) obtained rather sharply differentiated bands of the α_1 - and the β_1 -lipoprotein fractions using an instrument similar to that employed by Flynn and de Mayo (21). He stained the lipids with a saturated solution of Sudan III in 50% ethanol for 30 minutes at 40°C., then washed them thoroughly in 50% ethanol and subsequently in distilled water.

The conditions for carrying out his separations were: barbital buffer, pH 8.6, ionic strength 0.05, S. and S. No. 598 paper; a potential gradient of about 3 volts/cm for a duration of 12 hours. Rosenberg (190) in his studies of human serum has confirmed the observations of Swahn (91) and of Fasoli (20a).

Durum, et al. (19) separated the α_1 - and β_1 -lipoproteins using Whatman 3MM filter paper employing a veronal buffer of pH 8.6 and ionic strength 0.05. The lipids and lipoproteins were stained for 16 hours in a bath comprising a saturated solution of oil red 0 in 60% ethanol. The strips were then rinsed with tap water, blotted, and dried. The resulting strips show a red pattern against a pink background. The use of oil red 0 as a lipid or lipoprotein stain is not too practical because the α_1 -lipoproteins do not show up clearly. Later, Smith, Crawford, Jetton and Durum (196) state that studies by paper electrophoresis of top fractions of serum prepared by the Goiman technique have proved unsatisfactory due to adsorption of the material by the paper. It has been shown by Marbach (51) that if the lipoprotein fraction is denatured or partially denatured, the adsorption on the paper is greatly increased. Kunkel and Slater (40) used both Whatman 3MM paper and potato starch to obtain lipoprotein patterns of serum with a barbital buffer of pH 8.6 and ionic strength 0.10.

Marbach and McDonald (51) determined the electrophoretic properties of bovine serum lipoprotein using the technique of ionography. They determined the electromigration rate of bovine serum β -lipoprotein over the pH range from 2.7 to 8.6. The pH versus mobility plot they obtained is similar to that of a protein, except that the beta-lipoprotein is a much slower moving substance; its isoelectric point was determined to be approximately 5.2. The conditions for the experiment were: veronal buffer pH 2.7 to 8.6; ionic strength 0.015; potential gradient 10 volts per cm.; temperature 10°C; time 2-4 hours; atmosphere, helium saturated with water vapor.

Nikkila (183), using Munktell 20 filter paper, fractionated 0.25 ml of serum in 4 hours. His analyses on 10 normal and 2

atherosclerotic human serums and one hypercholesterolemic rabbit serum show that all beta-globulin is richest in lipid material. A high gamma-globulin lipid value is found but it is believed to be caused by some technical difficulty. In patients with active atherogenesis there seems to be a shift of lipids from alpha-globulins to slower fractions.

ENZYMES, HORMONES AND VITAMINS

The technique of electromigration on wet surfaces has been applied to the fractionation of several enzymes, hormones, and vitamins.

Schwimmer (82b) was able to distinguish between a purified and a partially purified preparation of Schardinger dextrinogenase using this technique. Paleus (185) using a 100 mg sample of lyophilized cytochrome c from beef or chicken hearts, in 1 ml. of pH 9.87 glycine buffer (and similarly using a phosphate buffer at pH 7) was able to distinguish between ferro- and ferricytochrome. Nikkila et al. (184) showed that partially purified pancreatic amylase contained 3 components of which the middle fraction had amylase activity, with a mobility of 2.9×10^{-5} cm/sec per volt/cm. at pH 8.6 and ionic strength 0.05. He also showed that under similar conditions crude trypsin appeared to contain 2 fractions; the larger and more active fraction had zero mobility (isoelectric point) under the conditions of the experiment, while the other fraction exhibited a mobility of 7.0×10^{-5} cm/sec per volt/cm.

Mills and Smith (66) used a slight modification of the apparatus of Cremer and Tiselius (14) but without any special cooling arrangement, to study certain enzyme preparations. The filter paper used was Whatman 3 MM in strips 3 or 4 cm wide, the other conditions being similar to those employed by Cremer and Tiselius. Enzymic activity was localized by means of the chromogenic substrates, phenolphthalein glucuronide for glucuronidase, phenolphthalein phosphate for phosphatase and p-nitro phenyl butyrate for esterase. A strip of filter paper soaked in substrate and buffer was placed in contact with the ionogram, the two were clamped between glass plates, incubated, and the color developed after a suitable time. Wallenfels and Von Pechmann (99) separated amylase, proteinase, lipase, and phosphatase from mushroom culture.

Simonart et al. (194) showed the presence of pepsin in commercial rennet by three methods, including the technique of electromigration on filter paper using a modification of the apparatus described by Markham and Smith (178). Michl, et al. (52) separated two components from a chorionic gonadotropin preparation, by means of electromigration in paper-stabilized electrolytes. McDonald and Marbach (170) fractionated ionographically a low-potency ACTH preparation from pig pituitaries, with the result that the active material was separated out, largely into one well-

defined zone. Approximately one milligram of the ACTH preparation was required for a single run. The experimental conditions were: a veronal buffer of ionic strength 0.015 at pH 5.5 to saturate the filter paper strips across which was applied a potential gradient of 6 volts per cm. for 3 hours. The strips were held on a horizontal position in a water-saturated helium atmosphere, at a temperature of 24-26° C. The ACTH preparation separated into 3 fractions. One of these contained 80-98% of the biological activity and 30% of the starting material. It bore a net positive charge and had an isoelectric point range of 6.0-6.6. A second fraction which contained 2-20% of the activity and 13% of the starting material, failed to undergo electromigration under the conditions of the experiment. The third fraction, which bore a net negative charge, exhibited no biological activity although it contained 40% of the starting material.

Several authors have used the technique of ionography to the study or fractionation of vitamins. Ericson, et al. (118) used the sandwich-type of electromigration apparatus (42,153) to separate out vitamin B₁₂ in the fermenting liquor of *Streptomyces griseus*.

Schweitzer and Wittern (82) used the technique of ionography for the purpose of microbiological assay. Liver extracts, in a mixture of 0.05 M borate and 0.067 M KH₂PO₄ buffer of pH 8.6 were submitted to electrophoresis on filter paper. Vitamin B₁₂ formed a narrow stationary zone whereas other active compounds migrated both to the anode and to the cathode. The electrophoresis was run for 5 hours. The various zones were easily observed by their fluorescence in ultraviolet light.

In order to identify vitamin B₁₂-like compounds of the cobalamin series derived from various animal excrements, Holdsworth (138) used the electromigration technique. Latner et al. (161) used ionography in the study of vitamin B₁₂ - binding in serum.

INORGANIC IONS

The earliest known investigations on electromigration in stabilized media deal with inorganic ions. Lodge (168) in 1886 filled a tube with gelatin, to which phenolphthalein was added, colored red by a trace of alkali. One end was in contact with a dilute acid, and on passing a current through the tube, the color was progressively bleached. From the rate of motion of the boundary the velocity of hydrogen ion was computed to be 0.0025 cm./sec. per volt/cm. Lodge's method was improved by Whetham (207,208,209) who used two solutions with a common ion, with the same concentrations and nearly the same specific conductances, but different in color and density and stratified in a vertical tube. In one case, 0.1 N potassium dichromate and potassium carbonate were used. The colored ion moved and the rate

of motion of the boundary could be followed. The addition of agar did not appear to affect the results, so it was used to stabilize the boundaries. Since the potential gradient was constant through the tube, the mobilities could be calculated from the measured current and the motion of the boundaries. Arrhenius (108) studied the relationship between the fluidity of electrolytes stabilized with various amounts of jelly and their conductivity.

Kendall and co-workers (33,147,148, 149,150,151) from 1923-28, applied the method of electromigration in agar gels to the separation of isotopes, of rare earth metals, of radium from barium, and of hafnium from zirconium besides doing some work on the separation of mixtures of alkaloïds and of organic isomers. At present the most active investigators working on the ionophoretic separation of inorganic substances include (1) Strain, Sato, and co-workers (75a,75b,86,86a,87,88,191,200) who have used, principally, paper-stabilized media and have pioneered in electrochromatographic procedures, and (2) Lederer and co-workers (46,47,48,49,164,165) who have utilized both paper-stabilized and agar-stabilized media for the ionophoresis of inorganic ions. Other investigators whose work has dealt with special aspects of inorganic chemistry include: McDonald, Urbin and Williamson (57), transference numbers; Kraus and Smith (39), complex ions; Brewer, Madorsky, Straus, and co-workers (112,113,176,177) countercurrent electromigration in media stabilized with a wide variety of substances; achieved a concentration of the isotopes of potassium (112,113), chlorine (176), and copper (177); Spiegel and Coryell (84), use of ion-exchange resins.

MISCELLANEOUS APPLICATIONS

King and Doery (152) employed the technique of ionography to determine whether unknown antibiotics in culture fluids were acidic, basic, or amphoteric, and to approximate their isoelectric points. It is interesting to note that they used chloromycetin, which shows an electroosmotic movement in filter-paper close to that of dextran, as an electroosmotic indicator. They found that the contraction of the whole paper sheet, on drying, was a factor to be considered when comparing electrophoretic measurements made in "non-stabilized" and in "paper-stabilized" buffer solutions.

Schild and Maurer (192) utilized P³² to make an *in vitro* study of the phosphorus metabolism of red blood cells. The phosphorus turnover of the erythrocytes, as inorganic phosphorus, was found to be 3% per hour, of the total phosphorus content. The phosphorus esters were then extracted with trichloroacetic acid, separated electrophoretically on paper strips, and their concentration determined by their radioactivity. Three maxima were identified as representing adenosine triphosphate, creatine phosphate, and inorganic phosphate.

McDonald and Spitzer (171) have determined the electromigration characteristics of the plasma expander polyvinylpyrrolidone (PVP) on paper. They describe a color test for locating the substance on paper and outline the necessary conditions for separation of PVP from the albumin, and α - and β -globulin fractions of blood plasma. Weber (206) has described the separation of organic amines on filter paper by migration under the influence of an electric field. The sensitivity was found to be about 10⁻⁹ mole. Human urine was shown to contain methylamine, dimethylamine, and another amine not yet identified.

Wieland and Feld (210) have described the ionophoretic separation and determination of hydroxycarboxylic acids. Wunderly (212) has described the separation of 4-dimethylaminoazobenzene (butter yellow) in serum.

Peniston, Agar, and McCarthy (71) utilized the technique of electromigration in agar-stabilized electrolytes to fractionate sulfonated aromatic substances comprising dialyzable lignin sulfonic acids and phenolic substances obtained by the alkaline oxidative degradation of lignin. The agar was contained in Vycor glass tubing, which permitted analysis after migration by direct scanning of the tube for absorption of ultraviolet radiation by use of a spectrophotometer. In some cases as little as 10 micrograms of a component was found to suffice for analysis. Ion mobilities for vanillin, ferubic acid and vanillic acid were determined. Acetone and benzophenone were used as electroosmotic indicators. Ionophoretic separations were made of known mixtures of four phenols, - guaiacol, acetovanillone, vanillin, and vanillic acid, in a phosphate buffer system, made up to a pH of 7.

ELECTROMIGRATION IN PAPER-STABILIZED NON-AQUEOUS SOLVENTS

The technique of ionography is not limited to compounds which are water soluble. It is only necessary that the migrant be dispersed or soluble in the solvent, that it acquire a net charge and that the medium conduct an electric current. Paul and Durum (70) separated dye mixtures on filter paper using non-aqueous solvents such as nitromethane, glacial acetic acid, ethyl alcohol, methanol and pyridine-glacial acetic acid. In the authors' laboratory aqueous solutions containing up to 50% dioxane have been used successfully by Marbach and McDonald in measuring the effect of dielectric constant on the mobility of bromophenol blue.

ACKNOWLEDGMENT

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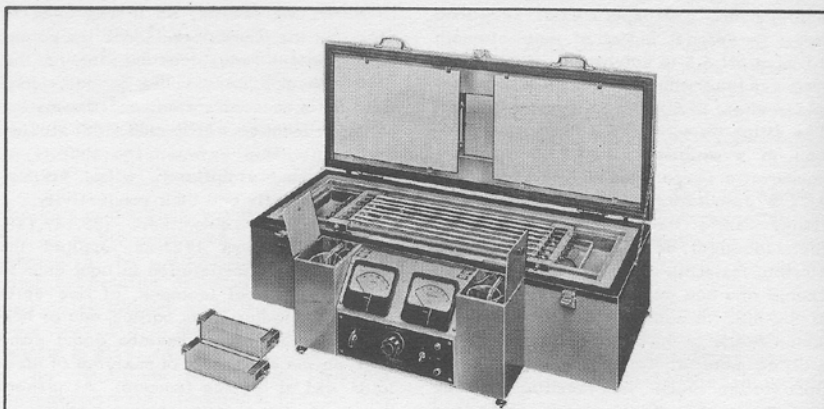
ELECTROMIGRATION IN STABILIZED ELECTROLYTES - PART III

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An ionograph in compact self-contained form with line-operated rectifier, water-jacketed chamber, levelling screws in base, adjustable frame shown with 7 filter paper strips in place; window in cover through which migrant may be applied and anti-condensation pads on each side of window. At left, in front, shown separately, feeder vessels for use in certain experiments where special precautions are necessary to maintain constant the water content of the strips (52).

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A STANDARDIZED PLASMA PREPARATION SUITABLE FOR USE AS A CONTROL IN THE DETERMINATION OF PROTHROMBIN TIME

by

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To the chemist who has to do an occasional prothrombin time, as well as to him who has many determinations each day, the problem of securing a satisfactory control plasma is well known. There is always uncertainty as to whether a random specimen is really normal, and a technician often finds himself the unwilling donor of a control sample. To overcome these objections, various attempts have been made to provide stable control preparations of plasma. The present paper describes our results with a preparation known as Diagnostic Plasma Warner-Chilcott[®], which we tested under the designation CH-90, and an essentially similar precursor known as CH-70.

METHODS AND MATERIALS

Thromboplastin: Either Simplastin[®] or Maltine Thromboplastin[®] was used.

Lyophilized Control Plasmas, CH-70 and CH-90: These were reconstituted by addition of distilled water to volume. To produce the 12.5 per cent plasma, 0.5 cc. of the reconstituted material was diluted to 4.0 cc. with normal saline.

Some of the stability experiments required pooling of several ampules of the reconstituted material, as there was not sufficient plasma in a single ampule for all the determinations required. Pooled material was stored in the refrigerator at about 4° C., except for the room-temperature experiments. Several ampules were stored at room temperature for stability studies on the dry material.

Determination of Prothrombin Time: The method used was the Link-Shapiro modification of the Quick one-stage technic, as described by Sudduth (1). Tests were done in the routine laboratory by one technician. Questionable determinations were repeated.

RESULTS

Comparison of CH-70 and laboratory normals.

A condensation of the results of a two-month's comparison of laboratory normals with CH-70 is presented in Table I. Most values for CH-70 are 13 and 14 seconds for whole plasma, and between 30 and 40 seconds for the dilute plasma. These figures are for plasmas run using Simplastin[®].

When Maltine Thromboplastin[®] was used, the prothrombin times were somewhat shorter.

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A STANDARDIZED PLASMA PREPARATION

Thromboplastin*	Range of Laboratory Normals		Range of CH-70 Determinations	
	whole plasma	dilute plasma	"whole" plasma	"dilute" plasma
Simplastin (16)	13-15	33-37	13-14	33-39
Thromboplastin (9)	12-15	30-35	12-13	27-42
Simplastin (6)	13-16	33-40	12-14	31-40

* The number in parentheses indicates the number of individual determinations in each series.

Comparison of CH-70 and CH-90

CH-90 prothrombin time values were compared to CH-70 values. Results were essentially the same with both preparations.

Stability of CH-70 and CH-90

A. Before reconstitution

(1) *Under refrigeration:* The material appeared to be stable indefinitely in the refrigerator.

(2) *At room temperature:* The material was stable for about 3 months during a hot Spring and Summer. A sample of CH-70 58 days at room temperature, and one of CH-90 28 days away from refrigeration, gave the same results as identical ampules kept under refrigeration.

B. After reconstitution

(1) *Under refrigeration:* Experiments on several batches of CH-70 indicate that the material is stable for at least 4 days if kept in the refrigerator after preparation. During this period there is a slight increase in the dilute prothrombin time. The results of several experiments are summarized in Table II.

CH-90 also showed essentially no deterioration over a 4-day period.

(2) *At room temperature:* The material as stable 5-8 hours when kept at room temperature of about 25° C. At 40° C. it deteriorates in less than 2 hours, as does normal plasma.

Reproducibility of results

A dilution error of ± 10 per cent in the 1 cc. size ampule produced no significant change in the whole or dilute prothrombin times. Quadruplicate determinations on CH-90 showed the results to be reproducible within a few tenths of a second in both the whole and dilute ranges (Table III). The results were very similar even with different batches of Simplastin of the same or different lot numbers, and with different batches of CH-90. Experiment 5 was performed on CH-90 left unrefrigerated for 28 days.

SUMMARY AND CONCLUSIONS

Comparisons have been made between the prothrombin time values determined using lyophilized normal plasma preparations (CH-90 and CH-70) and routine laboratory (patient) normal plasmas. These indicate that the lyophilized preparations,

Expt.	Hours Old	Patient Normal*		CH-70		Lot No.
		whole minutes	dilute minutes	whole minutes	dilute minutes	
I	0	13	36	13	38	79611
	24	14	41	15	41	
	48	13	35	13	36	
	72	13	37	15	42	
	96	13	33	15	44	
	130	13	34	15	—	
	190	15	40	19	—	
II	0	13	36	13	37	90221
	24	14	41	15	42	
	48	13	35	13	35	
	72	13	37	14	40	
	96	13	33	14	42	
	130	13	34	15	—	
	190	15	40	19	—	

* Refers to a fresh normal drawn the day of the determination.

Expt. No.	Range of Prothrombin Time		CH-90 Lot No.	Simplastin Lot No.
	whole	dilute		
I	15.0-15.4	47.2-47.5	94421	28121
II	14.8-15.3	42.0-42.4	94421	16321
III	14.4-14.6	43.1-44.9	94421	16321
IV	15.0-15.2	45.0-45.3	94421	28121
V*	15.1-15.5	43.8-45.0	93821	28121

* This experiment was performed on CH-90 stored 28 days at room temperature.

when used in conjunction with Simplastin[®], give constantly reproducible prothrombin time values in both the whole and dilute (12.5 per cent) plasma ranges.

CH-70 has been shown to be stable at least 58 days in the dry state at room temperature, 4 days after reconstitution and preservation at ice-box temperatures, and 5-8 hours after reconstitution and storage at 25° C. CH-90 has similar keeping powers.

With use of these lyophilized plasma preparations and Simplastin[®], a reasonably-skilled technician should obtain normal plasma prothrombin times of 13-15

seconds, and thus obtain a simple daily check on his technique and preparation of his thromboplastin.

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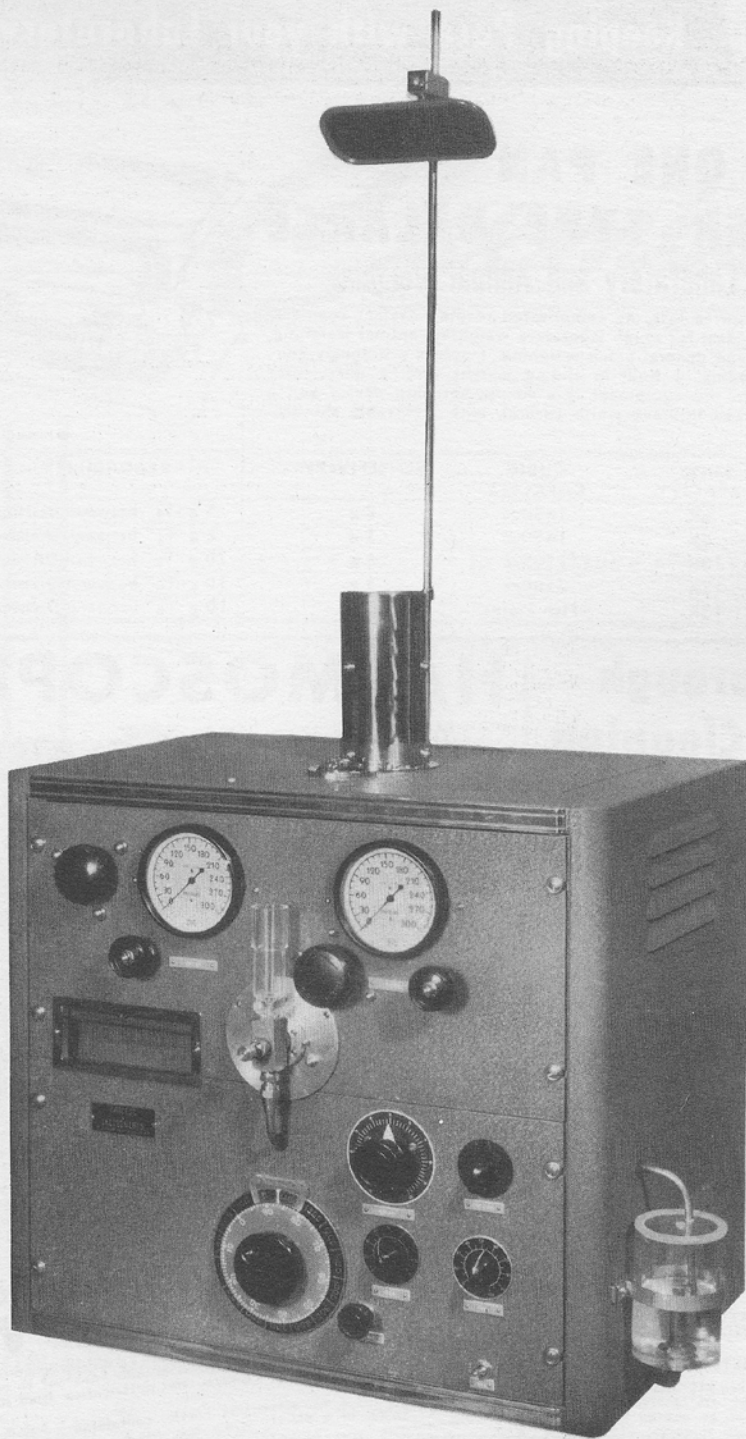
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[®]Warner-Chilcott Laboratories

The CH-70 and CH-90 used in this work were supplied through the courtesy of the Warner-Chilcott Laboratories. The manufacturer states that these two forms of the experimental reagent are essentially the same in performance, and that CH-90 was developed in an effort to increase the long-term stability and uniformity of the reagent.

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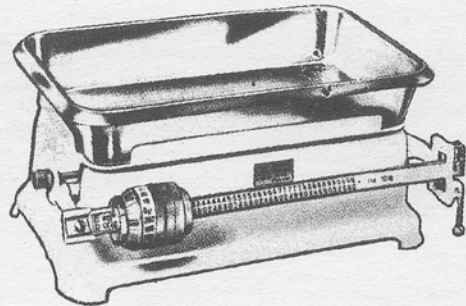
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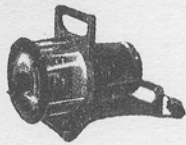


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