Electrophoresis in Biochemical Studies

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MICRO-DETERMINATION techniques in electrophoresis are among the most powerful tools in the hands of the biochemist. A variety of substances of biochemical interest have been examined in this manner : proteins, peptides and amino acids, carbohydrates, enzymes, hormones and others. Such studies have resulted in the accumulation of newer facts about the functions of known molecules or existence of newer molecules which hitherto had eluded detection due to the lack of a more powerful analytical tool to complement those already available.

The fractionation of a material into components of different electrical mobilities—has been achieved both in free solution or in manner that stabilizes against convection by a supporting medium. The latter technique is known as *zone electrophoresis* and has advantages over the *free electrophoresis*[1] method. Thus, only minute quantities of material to be separated are required. There is complete separation and not merely a boundary separation into zones of different migration. Apparatus needed is less expensive. Determinations on several samples could be carried out simultaneously and there is less interference by the so-called boundary anamolies thus making possible separation of low molecular weight substances such as amino acids, peptides and nucleotides. However, owing to the greater accuracy involved, developments in free electrophoresis continue to be made, and a micromethod employing small volumes and a somewhat simpler technique has been developed.[2]

Of the various supporting media used such as filter paper, starch, agar, silica and other gels, glass powder, cellulose columns, and ion-exchange resins, filter paper has come to stay as the medium of choice owing to its simple practicability.

The techniques[1, 3, 4] adopted in each case continue to be refined and made more quantitative. For example, the two dimensional versions, including the one using paper chromatography as one of the dimensions of separation, has proved of value in separating moleculese of closely related physico-chemical properties.

An important application of electrophoresis is for the separation of serum or plasma proteins[5]. Analyses of these proteins in most clinical laboratories is still confined to determination of total protein concentration and of the albumin-globulin ratio by the Howe technique. However, the necessity for subfractionation of plasma globulin has long been recognized. The data already available indicate the practicability and usefulness of paper electrophoresis in clinical diagnosis.[6, 7] General protein disturbances in pathologic conditions such as myeloma, nephrotic syndrome, agamma globulinemia, portal and biliary cirrhosis and Kala-azar are readily revealed.[6, 8] Although the method has its limitations[9, 13] and continued investigation is necessary, there are already many indications that its use in the clinical laboratory will be greatly extended.

Plasma protein changes accompanying nutritional deficiency states such as protein and vitamin deficiences are being keenly followed.[14-19] There is every indication that electrophoretic analysis may go a long way to provide valuable information regarding the specificity of

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functions and metabolism of these proteins.

The importance of vitamin B_{12}—binding substances in body fluids and tissues has received widespread recognition ever since the identification of Castle's intrinsic factor, but has aroused augmented interest only recently because of the developments in paper electrophoretic techniques and in improved methods of vitamin B_{12} estimation. Studies concerning the binding of this vitamin and intrinsic factor activity have been carried out in serum,[20] gastric juice,[22] extracts of gastric mucosa,[23] liver,[21] and in milk of a various species[24],[25] and an impressive background of information now exists.

Of special interest is the reverse-flow zone electrophoresis technique employed to the study of thyroxine-binding by human serum proteins.[25] The movement of albumin towards anode is just balanced by a flow of buffer in the opposite direction. The serum globulins do not migrate in the path of albumin and artefacts due to adsorption of albumin-bound thyroxine on the filter-paper medium are thus eliminated. Preliminary observations by the author show, that the technique may be extended to studies on the binding of vitamin B_{12} by serum proteins wherein tailing of protein components is, at present, a serious handicap.

The nature of the lipid-protein complex in the sera has been investigated by utilizing a differential staining procedure[27],[28] in which two simultaneously run electrophoretic patterns of the same serum are stained one with a lipid staining dye such as oil red *O* or Sudan black and other with a protein staining dye such as bromophenol blue or naphthalene black 12*B*. Chemical analyses of cholesterol and phospholipid in the separated fractions also yield information relating to the lipoprotein complex.[29] The knowledge of the distribution of lipids in normal and pathological sera has provided a valuable diagnostic clue for diseases such as lipid nephrosis and coma hepaticum[30]. In atherosclerosis[31], for example, the ratio between β and α lipoproteins is increased, and is considered a measure of severity, which, together with the determination of heparin-type lipide clearing factors in blood which show a clear reduction in this disease, provides a valuable diagnostic test.

The interrelationship between hemoglobin structure and the anemias is an extremely active field of investigation at present. Recent communications [32-3] have pointed to the indispensability of electrophoresis in characterization of hemoglobins. All the five variants, viz. *F*, *C*, *D*, *S*, and *E* of adult human hemoglobin A, accompanying a variety of heriditary hemolytic anemias, have been studied and characterized electrophoretically in free solution as well as on paper. Any characterization besides helping accurate diagnosis makes possible genetic interpretation of this disease.

Radioactive tracer studies could be coupled with zone electrophoresis[1]; the components can be located; analytically determined and investigated for radioactivity using the same strip. Both counting techniques and radioautographs have been used for the purpose. Thus, on *in vivo* incorporation of ^{131}I into blood, the radioactivity has been located in the α_1-globulin fraction in rabbit[36] and in man[37]. These and other similar studies[26] have shown that the thyrotropic activity of human serum is associated with the α_1-globulin fraction. The rate of formation of serum proteins has been studied by incorporation *in vivo* of valine $^{13}C^{38}$, methionine $^{35}S^{39}$ and $^{35}S^{40}$ tagged albumin. The latter work, in particular, suggests the possibility that albumin may serve as precursor of globulins in the intact rat. Studies with ^{198}Au, ^{52}Mn, ^{57}Co and ^{131}I have been published[41].

When dealing with proteins, enzymes, etc. one has often to cope with the

problem of purity or homogeneity of a given preparation. The separation and purification of enzymes has been successfully achieved by paper electrophoresis using specific colour reactions and elution techniques[4]. Preparations of amylase, trypsin, pepsin, cholinesterase, intestinal and alkaline phosphatases, cytochromes and the enzymes of *Aspergillus Oryzæ* have all been purified in this way. Several proteins such as bovine serum albumin, chymotrypsin and chymotrypsinogen, lysozyme and ribonuclease have been studied for heterogeneity by electrophoresis on starch[42]. Protein adrenocorticotropic hormone preparations from sheep have been purified from inactive principles by starch electrophoresis[43].

The complexity of mixtures in which amino acids and peptides are found, has called for modifications in electrophoretic techniques. Striking separation of mixtures of chemically and electrically closely related amino acids and peptides could be effected by the use of very high voltages, even up to 200 volts/cm.[44] Often, two dimensional electrophoresis has yielded fruitful results[4].

A few biologically important peptides including commercial vasopressin, oxytocin, *ACTH* preparations and extracts of pituitary have been studied for purity ; the peptides of Gramicidin *S* are separated on silica gel strengthened with paper pulp[45].

Nucleic acids and their hydrolysis products, the nucleotides, nucleosides and nucleobases have been studied by paper and agar electrophoresis[4]. The adenosine phosphotates, which are vital to energy transfer reactions, have also been separated and characterized ; thus, fractionation of muscle extracts[46] yields separate zones of phosphocreatine, adenosine mono-, di-, and tri-phosphates, creatine, anserine and carnosine.

The electrophoretic behaviour of the *B*-group of vitamins may be utilized for the separation on paper and agar of the derivatives of thiamine, riboflavin, pyridoxine and nicotinic acid[47,48]. In a modification of the technique[49], the growth stimulus provided by the separated components is observed in a layer of agar jelly which constitutes a medium containing all the substances required by the test organism seeded into the agar except that to be determined. The method enables the use of larger volumes of test substances which may even be in a solid state such as feces or minced organs.

A similar technique[50], using agar as the supporting medium, has been used for the separation and comparison of antibiotics produced by different strains of *E. coli*. The zones of inhibition are revealed after electrophoresis by using the indicator *Shigella sonnei* strain. Paper electrophoresis has also been used in the search for unknown antibiotics.[4] In these studies, the rates of migration of unknown antibiotics (as revealed by zones of inhibition) are compared with those of known antibiotics, such as chloromycetin and streptomycin. Thus a number of antibiotics related to streptomycin have been isolated.

Carbohydrates, from simple sugar to complex polysaccharides, are separated as borate complexes[4,51,52] ; the mobilities of these complexes suggest the structural details of the original carbohydrate. The The effectiveness of separation of a mixture of ^{32}P-labelled hexose phosphates would make possible the use of this technique in following many enzymic reactions and in obtaining rapid quantitative analysis of complex mixtures.

The follow-up of a biochemical reaction may sometimes result in the identification of new end-products or some product accumulating at intermediate stages of the reaction. This possibility has been suggested by the isolation of a strongly fluorescing intermediate con-

densation compound formed both from pyridoxal + alanine or from pyridoxamine + pyruvate systems in a study of chemical transmination reaction by paper and cellulose—column electrophoresis.[53]

As other illustrations of the application of electrophoresis can be cited, the study and isolation of plant viruses[54] which helps in differentiating between the proteins of healthy and infected plants; the separation of steroids from normal and pathological cases[55]; the separation of porphyrin pigments on agar tubes[56]; and the studies of electrokinetically effective surface structure of bacterial cells[57] and spores.[58]

Electrophoresis has thus helped the biochemist in making great strides of progress and in his contributions to our better understanding of life process.

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