Electrophoresis of proteins and nucleic acids: II—Techniques and applications

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Following from our discussion of the theory of electrophoresis,[1] we will present in more detail the methods for peforming two of the more powerful techniques—namely, sodium dodecyl polyacrimide gel electrophopresis (SDS/PAGE) and isoelectric focusing. We will then discuss clinical applications of electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

This procedure separates poplypeptides according to their size; as it is most commonly used proteins are resolved as a consequence of differences in their rates of migration. SDS/PAGE systems use buffers that contain a detergent, sodium dodecyl sulphate (SDS), which denatures proteins.[2] In a solution of SDS most multi-subunit proteins are denatured and the individual subunits dissociate.

Each molecule of SDS carries a negative charge. At neutral pH most proteins become coated uniformly with SDS, which eliminates the intrinsic charge of the protein, so that the total charge of the protein-SDS complex is determined by the SDS. As a consequence, protein-SDS complexes all have a rod-like shape and possess essentially identical charge densities. The electrophoretic mobility of the complex therefore depends on the molecular weight of the protein.

During SDS/PAGE protein samples are applied at the cathodal end of a slab of acrylamide gel. Under the influence of the electric field the protein-SDS complexes migrate towards the anode and enter the polyacrylamide gel as a narrow uniform band or zone. Within the gel the migration rate of large proteins is slower than that of small proteins owing to the sieving properties of polyacrylamide (fig 1). To determine the molecular weight of a sample protein standard proteins of known molecular weight are run in parallel with it and their mobility compared.[3]

Isoelectric focusing of proteins

Proteins are amphoteric molecules, and their net charge depends on the pH of the environment. Their charge is determined mainly by the amino acid side chains on their surface, in particular the ionisable amino and carboxyl side chains. At very low pH most proteins are positively charged, and as the pH is gradually increased the net charge progressively becomes negative. For each protein there is a pH at which the net charge is equal to zero. This pH value is called the isoelectric point (pI). For most proteins the value of pI lies between pH 4 and pH 7.

Isoelectric focusing is an electrophoretic technique that separates proteins on the basis of their isoelectric points.[4] The special feature of isoelectric focusing is that electrophoresis is performed in a stationary pH gradient and proteins migrate to an equilibrium position. As electrophoresis proceeds the sample components are concentrated and focused as sharp bands at their respective isoelectric points (fig 2). Exceptionally high resolution can be obtained of proteins that differ in pI by as little as 0·001 of a pH unit. Isoelectric focusing provides information about the number of components in a mixture of proteins and also permits the isoelectric points of the proteins to be determined.

Electrophoretic separation with isoelectric focusing is carried out in a continuous pH gradient that increases from the anode to the cathode. When a protein is introduced into such a gradient it will be repelled electrostatically by one of the electrodes according to its charge in the electric field and will migrate to the position in the pH gradient at which it has no overall charge. For example, when a protein is placed close to the anode where the pH is lower than its pI value the protein becomes positively charged and will migrate towards the negative cathode. As it migrates, the protein moves "up" the pH gradient and progressively loses its positive charge. Eventually, when the net charge on the molecule becomes zero, migration stops; this point in the pH gradient is the pI of the protein. If the protein should move by diffusion from the pI position it will become charged and be forced back to the pI. Overall, this focusing effect tends to concentrate proteins into very narrow bands.

As previously stated isoelectric focusing requires the formation of stable and continuous pH gradients between the electrodes. Such gradients cannot be formed by conventional biochemical buffer solutions of different pH. Suitable buffers were first developed by Vesterberg[5]; these contain a series of specially synthesised compounds that contain polyamino and polycarboxyl groups and have been given the generic name of ampholytes. Like proteins they are amphoteric and each will migrate and focus at its pI in an electric field. These compounds have a high buffering capacity at their pI and create a constant localised pH zone where they focus. Blends of ampholytes that buffer over different pH ranges are produced commercially; they allow the production of either broad pH gradients (such as pH 3·5-9·5) or narrow, high resolution gradients which buffer over only a limited pH interval.

Focusing of protein samples occurs more slowly than focusing of the ampholytes (fig 2), and the time taken to reach the pI position increases with the size of the molecule. Isoelectric focusing of proteins is carried out in thin horizontal layers of agarose or polyacrylamide

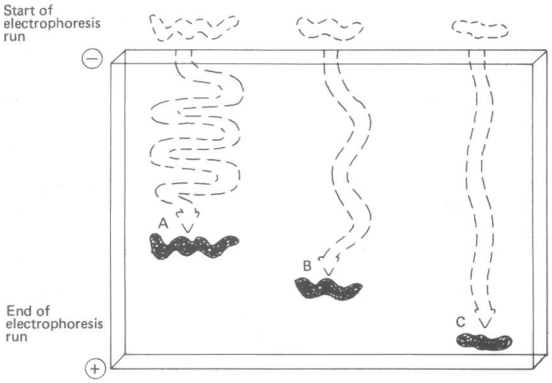

FIG 1—*In SDS-PAGE the gel matrix acts as molecular sieve so that proteins of higher molecular weight migrate most slowly*

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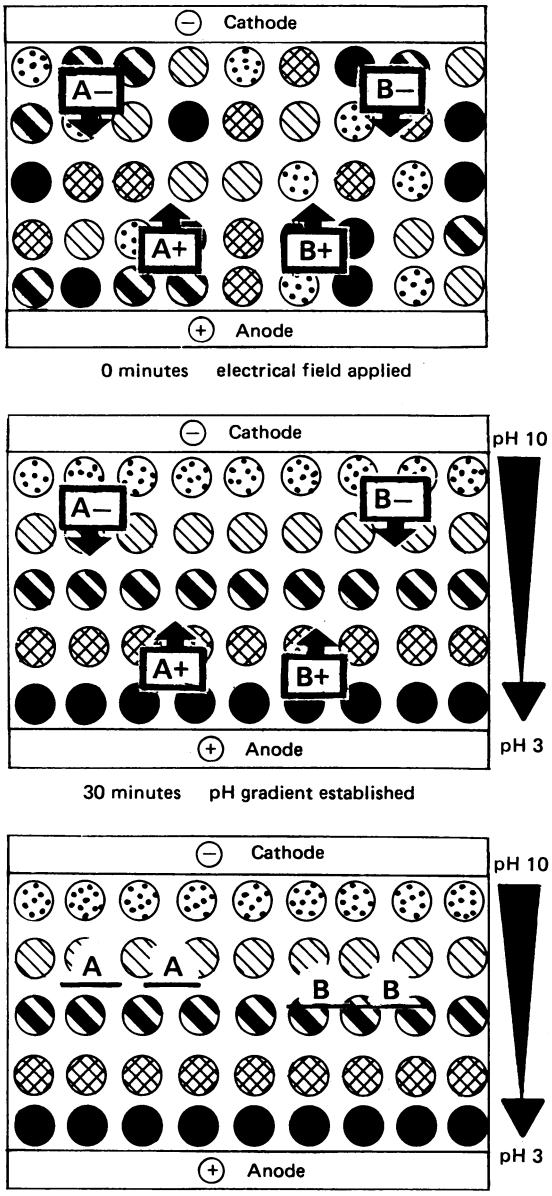

FIG 2—*In isoelectric focusing protein samples (A and B) migrate in pH gradient created by ampholyte molecules (◯). Rate and direction of protein migration depend on pH value at each point in gradient*

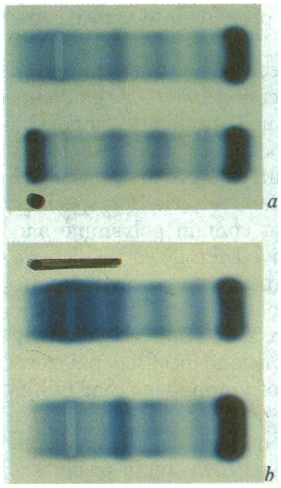

FIG 3—*Serum electrophoresis with agarose gel (stained with Amido black 10B). (a) Normal serum and serum from patient with myeloma; (b) normal serum and serum from patient with oligoclonal increase in IgG*

gel. High electrical field strengths are used to improve sample resolution and to minimise running times. Isoelectric focusing in the presence of urea can be used to determine the pI of subunit polypeptides.

Two dimensional gel electrophoresis

As described above, proteins are separated by electrophoresis on the basis of size or charge. When either method is used on its own to analyse complex biological samples it is probable that two or more proteins will co-migrate and be visualised as a single band. Such a lack of resolution is undesirable and, by masking the complexity of the proteins present in the sample, may lead to the misinterpretation of results. To help overcome this problem two dimensional electrophoretic separations have been developed.[6] Clearly, best resolution is achieved if the two electrophoresis runs separate proteins by entirely different principles. Usually isoelectric focusing is used to separate proteins in the first dimension and is followed by a second separation with SDS/PAGE at right angles to the first. This electrophoretic method is sometimes referred to as IsoDalt as proteins are resolved by *iso*electric point in the X axis and by their subunit *dal*ton value (molecular mass) in the Y axis.

Agarose gel electrophoresis of nucleic acids

Agarose gel electrophoresis is of central importance in DNA sequencing[7] and is therefore an indispensable tool in clinical research.

It uses molecular sieving to separate nucleic acids on the basis of their size. Because nucleic acids are generally large molecules (>500 kDa) an electrophoresis gel of large pore size is required. Agarose gels are most suitable.

The action of restriction endonucleases on double stranded DNA generates a series of fragments. Separation and analysis of these fragments is a common requirement in recombinant DNA manipulations and is readily achieved with agarose gel electrophoresis.

At neutral pH nucleic acids carry a negative charge that is proportional to their length. This charge is carried by the phosphate groups of the polynucleotide backbone, and all nucleic acid fragments have virtually the same ratio of charge to mass. When mixtures of nucleic acid fragments are subjected to agarose gel electrophoresis the rate of migration of individual fragments is determined by their size, small fragments migrating faster than large fragments. As with SDS/PAGE samples are applied to the cathodal end of a thin vertical gel and the nucleic acids enter the gel in a narrow zone and then migrate in zones of equal mobility. The mobility of nucleic acids is inversely proportional to the logarithm of their molecular weight.

Clinical applications

ZONE ELECTROPHORESIS OF PROTEINS IN SERUM, CEREBROSPINAL FLUID, AND URINE

Measurement of proteins in body fluids is widely used as a screening technique to look for either abnormal proteins or changes in the concentrations of normal proteins, which may reflect pathological processes or genetic defects. Serum electrophoresis is most commonly carried out on cellulose acetate with continuous buffer systems under non-dissociating conditions. Zone electrophoresis on cellulose acetate yields only low resolution separation but this is sufficient to show abnormalities in serum such as a paraprotein in myeloma or the variant proteins that occur in bisalbuminaemia. Better resolution of serum proteins can be obtained with agarose as a support matrix.[8] Agarose gel electrophoresis makes it easier to recognise more subtle changes in the concentration of proteins (fig 3), such as increases in the acute phase proteins that are associated with tissue damage due to trauma or are found in acute and chronic inflammation; oligoclonal increases in IgG, which can occur in patients with chronic aggressive hepatitis; and decreases in albumin and transferrin concentrations in patients with nephrotic syndrome. Higher resolution of proteins in serum can be obtained with polyacrylamide gels rather than agarose gels, but when cost is considered the additional information provided by polyacrylamide gel electrophoresis (PAGE), gradient PAGE, or SDS/PAGE is probably not needed for a screening procedure.

Electrophoresis of cerebrospinal fluid is not commonly performed. As cerebrospinal fluid contains only about 0·3% of the protein concentration found in serum its proteins are best detected with silver staining.[9] Isoelectric focusing can be used to identify oligoclonal IgG bands in the cerebrospinal fluid of patients with multiple sclerosis; agarose gel is used and IgG is identified by immunoblotting.

Electrophoresis of urine may be performed to study glomerular proteinuria. SDS/PAGE is useful in this instance as it separates proteins by their size and the

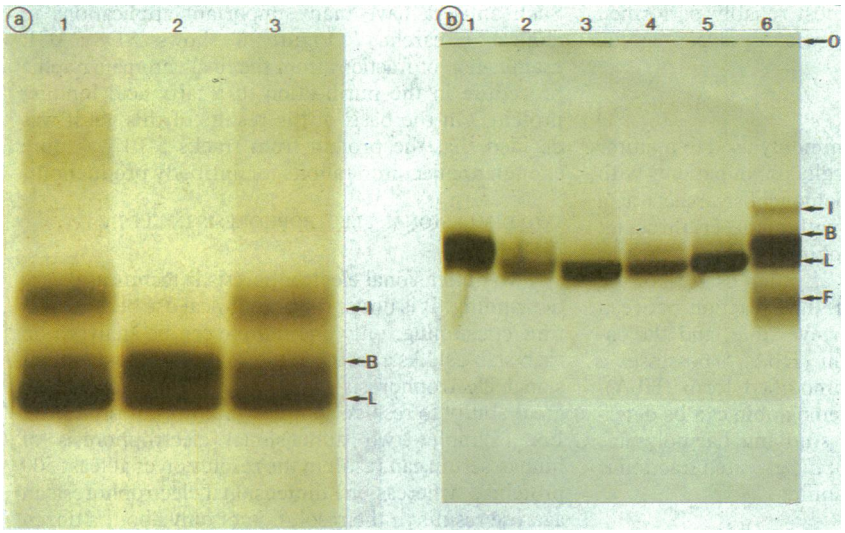

FIG 4—*Alkaline phosphatases in human serum were resolved in polyacrylamide gels and visualised by using α naphthylphosphate as substrate. Position of liver (L), bone (B), and intestinal (I) alkaline phosphatases are shown. Samples 1 and 3 in panel (a) contain liver, bone, and intestinal forms; sample 2 contains liver and bone alkaline phosphatase only. Sample 1 in panel (b) is from an adolescent boy and contains predominantly bone alkaline phosphatase; samples 2-5 contain predominantly liver form. Sample 6 also contains fast migrating form (F) associated with neoplasms*

size of urinary proteins reflects the extent of glomerular damage. Tubular damage can be detected by the appearance of α_2 microglobulins and β_2 microglobulin, proteins that are not readily detected in serum with electrophoresis but can be detected by electrophoresis in the urine of patients.

ZONE ELECTROPHORESIS TO IDENTIFY ISOENZYMES IN SERUM

Many enzymes in man exist in multiple forms either because distinct genes encode several proteins with similar catalytic properties or a single gene product is subject to modification after synthesis. The enzyme activity measured in serum then represents the sum of the activity of several distinct isoforms. If different organs contain different isoenzymes the identification of the forms in serum can provide a clue about the origin of the enzyme activity. In practice it is not worth undertaking such an identification unless the enzyme activity in serum is raised, but such information can then be of great diagnostic value.

Zone electrophoresis performed under non-denaturing conditions is often used to "split" enzymes into several bands; this allows the relative proportions of the different forms to be estimated by the use of special activity stains. This procedure is called zymogram analysis and is usually performed in polyacrylamide gels to optimise the resolution of the isoenzymes. Although only semi-quantitative, zymogram analysis has the great advantage of being able to identify abnormal enzymes such as those associated with tumours, enzyme immunoglobulin complexes, and enzyme polymorphisms.

Polyacrylamide gel electrophoresis is widely used to identify the alkaline phosphatase isoenzymes—that is, enzymes of liver, bone, or intestinal origin—in serum. Figure 4 shows the common isoenzymes and the fast electrophoretic mobility of an alkaline phosphatase associated with cancer. It also shows the biliary alkaline phosphatase, which has such a high molecular weight that it cannot penetrate the 7% polyacrylamide gels used in the analysis and is characteristically found at the origin.

The isoenzymes of alkaline phosphatase could be resolved by column chromatography techniques such as gel filtration, which separates proteins by size, or ion exchange, which separates proteins by charge, but conventional column chromatography has considerable disadvantages as a routine clinical tool for isoenzyme analysis when compared with zymogram electrophoresis. Chromatography is considerably slower and allows only one sample to be analysed at a time, and the materials are much more expensive than those used for electrophoresis. Moreover, each fraction obtained from a chromatographic run (which may yield as many as 50 fractions) would have to be assayed for enzyme activity, whereas with zymogram analysis the localisation of activity is achieved in a single step, several samples being studied simultaneously.

The use of reagents for detecting a wide variety of enzymes in starch gels has been described,[10] and a list of methods for the detection of enzymes in polyacrylamide gels has been compiled.[11]

PHENOTYPING α_1 ANTITRYPSIN

Pronounced population polymorphisms are associated with α_1 antitrypsin. As certain phenotypes give rise to pulmonary emphysema there is clinical interest in identifying genetic variants and phenotyping people at risk. Most clinical interest has focused on the reduced plasma concentrations of α_1 antitrypsin. Different degrees of deficiency exist owing to allelic variation, and these result in decreases in the production of α_1 antitrypsin by the liver. These differences range from the normal PiM gene with a production of 100% through the PiW gene with 80%, the PiS gene with 60%, the PiP gene with 25%, and the PiZ gene with 15% of normal levels to the Pi null gene which fails to produce α_1 antitrypsin. Severe deficiency can result in progressive lower lobe panacinar emphysema and, less commonly, micronodular cirrhosis in adults. The fact that the reduction in plasma α_1 antitrypsin concentrations produced by certain Pi genes does not correlate with the risk of emphysema or cirrhosis has emphasised the need to phenotype people. In the rare inherited condition of α_1 antitrypsin deficiency the homozygous state is readily diagnosed by the almost total absence of the α_1 globulin band on routine serum electrophoresis. Apart from this null phenotype other genetic variants, of which there are over 30, are difficult to show by zone electrophoresis. The identification of people with PiZZ and PiSZ genes, who are

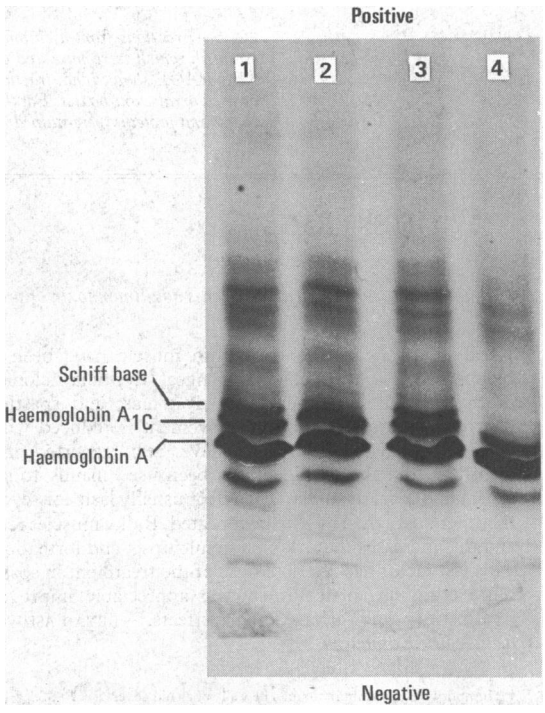

FIG 5—*Isoelectric focusing in the pH 5·0-6·5 range can be used to separate haemoglobin from glycated haemoglobin and intermediate Schiff base, which is unstable and disappears if left in saline overnight*

likely to develop disease, is most reliably performed with isoelectric focusing in agarose or polyacrylamide gels.

GLYCATED HAEMOGLOBIN

Glycated haemoglobin is commonly used to monitor the long term control of blood glucose in patients with diabetes mellitus. Glucose spontaneously reacts at a slow rate with haemoglobin within the erythrocyte. Normally 3-6% of the haemoglobin is glycated, whereas in poorly controlled diabetics as much as 20% of the haemoglobin may be glycated. The reaction between glucose and haemoglobin is irreversible, and the covalently modified haemoglobin (HbA_{1C}) possesses a different charge from the unmodified form (HbA). The proportion of glycated haemoglobin can be determined electrophoretically by studying haemolysates with isoelectric focusing (fig 5); the glycated fraction is measured by densitometric scanning.

MONITORING PROTEIN PURIFICATION

SDS/PAGE is often used to monitor the purification of proteins, the presence of a single band in the preparation being used as one criterion of purity. This criterion needs to be met if the purified protein is to be used as an immunogen to raise polyclonal antibodies.

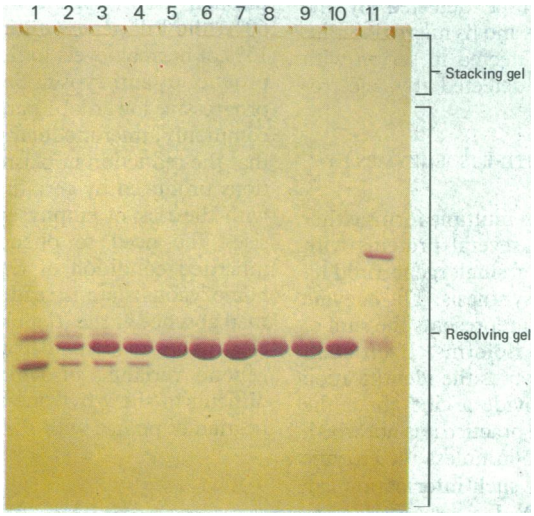

FIG 6—*Fractions from a chromatography column were examined to establish which were pure and could be used as specific immunogen. SDS/PAGE showed that fractions 5-10 were homogeneous and could be used to raise antibodies. For the photograph the stacking gel was left intact and proteins were stained with Coomassie brilliant blue R-250*

Such antisera have many important applications in clinical research.[12-14] Figure 6 shows SDS/PAGE examination of fractions from the final chromatographic procedure in the purification of a fatty acid binding protein. On the basis of the results of this gel it was decided that the protein from tracks 5-10 was pure enough to inject into rabbits for antibody production.

TWO DIMENSIONAL ELECTROPHORESIS IN CLINICAL RESEARCH

Two dimensional electrophoresis is technically very demanding. It is not in routine clinical use because it is time consuming: only one sample can be analysed on each slab gel. As a research tool, however, two dimensional electrophoresis is widely used because of its great ability to resolve complex mixtures of proteins.[15] For example, two dimensional electrophoresis of human serum can result in the resolution of at least 500 proteins,[16] whereas one dimensional electrophoresis in agarose results in the resolution of only about a dozen protein bands.[8]

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ANY QUESTIONS

In what chronic neurological disorder is botulinum toxin of proved value?

Local injections of the toxin into muscle have been used to relieve blepharospasm, torticollis, largyngeal dystonia, clonic facial spasm, oromandibular and other facial dystonias, and spasticity. By careful control of the dose abnormal movement is reduced without producing weakness. Favourable results have been reported in all the above disorders, but the treatment has been used mainly to relieve blepharospasm and focal dystonia. The effect usually lasts for several months, and treatment may then have to be repeated. Bulky muscles require large doses and this increases the risk of systemic upset and formation of antibodies.[1] This last factor has restricted use of the treatment in spasticity. A double blind study in torticollis showed appreciable improvement with the treatment and no serious adverse effects.[2]—BRYAN ASHWORTH, *consultant neurologist, Edinburgh*

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Are there any essential differences between chlorine and iodine based water purification tablets for use when travelling overseas?

The principal difference between chlorine and iodine water purification is that iodine disinfects water more rapidly and is more effective against cysts, the commonly encountered ones being giardial and amoebic. Although iodine in a concentration suitable for sterilisation seems to have little in the way of toxic effects on the water consumer, it can have an unpleasant taste. It is perhaps more important that there is no standardised commercially available preparation prepared for this purpose in Britain. The traveller can use tincture of iodine (2% solution) and add 5-10 drops (0·25-0·5 ml) to one litre of water and then leave the solution for 30 minutes. Of the easily available methods of purifying water, boiling or controlled filtration is probably the best for travellers. Chlorine releasing tablets available from chemists and used carefully according to instructions are a good second best option.—E WALKER, *consultant epidemiologist, Glasgow*

Jarroll EL Jr, Bingham AK, Meyer EA. Giardia cysts destruction: effectiveness of six small quantity disinfection methods. *Am J Med Hyg* 1980;**29**:8-11.