APPLICATIONS OF TWO-DIMENSIONAL GEL ELECTROPHORESIS IN THE CLINICAL LABORATORY

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The purpose of this article is to familiarize the reader with a technique that might very well have a major impact upon the clinical laboratory and the practice of clinical pathology: high-resolution, two-dimensional gel electrophoresis. We will cover several areas, including: (1) a general discussion of two-dimensional electrophoretic techniques and a history of the particular method in which we are most interested, the semiautomated Iso-Dalt variant; (2) a detailed method section, needed for a full understanding of the information obtained by Iso-Dalt electrophoresis; and (3) a review of the current uses of two-dimensional gel electrophoresis and a look toward future applications.

Electrophoresis has been defined as "the motion of dissolved or suspended material under the influence of an applied electric field".[1] The continuing study of the electrophoretic properties of biologic molecules was begun by Tiselius in the late 1930s with the moving boundary Tiselius apparatus.[2] Although this apparatus is cumbersome by current standards, the results obtained by examination of plasma proteins would be familiar to most pathologists. The offspring of moving boundary electrophoresis, zone electrophoresis, requires a solid or semisolid support to counteract diffusion. This method has been used with many different media, including starch blocks, paper, agarose and agar, powered cellulose, cellulose acetate, and, most recently, polyacrylamide.[3] Currently, cellulose acetate and agarose are the most popular choices in the clinical laboratory. Figure 1 compares the resolving power of these two techniques, using serum (plasma) as the sample. Two characteristic features of this type of electrophoresis are as follows. First, the proteins under examination more or less retain their "native" structure. For example, the structure of haptoglobin, which is made up of two alpha- and two beta-chains, is virtually the same in the electrical field as it is in serum. Second, the rate of migration, at a given field strength, is for each protein a function of its molecular weight, shape, and overall charge. Hence, alpha-2-macroglobulin, much larger than IgG and

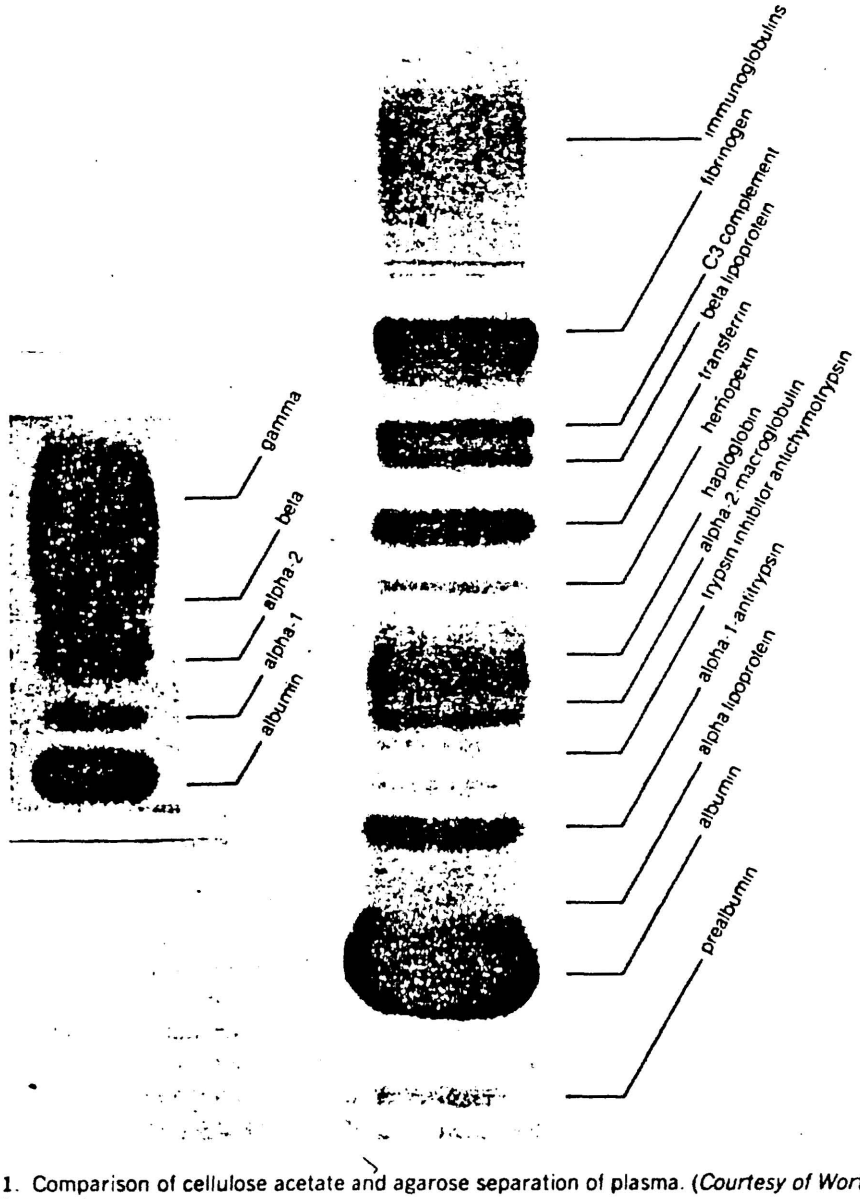

FIG. 1. Comparison of cellulose acetate and agarose separation of plasma. (*Courtesy of Worthington Diagnostics.*)

therefore subject to much greater sieving effects from the support medium, nonetheless migrates faster due to charge considerations.

These two characteristics, however, may be altered by the use of the detergent sodium dodecyl sulfate (SDS), which is used mainly in polyacrylamide gel electrophoresis (PAGE) and binds very tightly to proteins. SDS tends to negate the intrinsic charge of the protein, imparting a uniformly negative charge per unit mass.

Also, SDS will "unfold" proteins, thereby forcing all proteins to assume a similar, rod-like shape.[4] These two effects allow SDS-PAGE to separate proteins, for the most part, on the bases of their molecular weights alone.[5] If a disulfide reducing agent is also used, such as 2-mercaptoethanol, then the separating species will be single-chain polypeptides, such as the heavy and light chains of immunoglobulins.

There are other dissociating agents besides SDS (e.g., urea and guanidine-HCl), but these are either uncharged or do not bind tightly to proteins. One area in which urea is useful, however, is in a specialized type of electrophoresis, isoelectric focusing. In isoelectric focusing (IEF), also most commonly done in polyacrylamide gels, an artificial pH gradient is set up in the gel by the use of small polycarboxylic acid, polyamine molecules called ampholytes.[6] These gradients generally extend from a

TABLE 1. Major Steps in the Development of High-resolution Two-dimensional Electrophoresis

Proposed that higher resolution in electrophoretic separations may be achieved by "sieve" or "molecular filtration" effects using gels as supporting media	Smithies, 1955[47]
First two-dimensional electrophoretic separation	Smithies and Poulik, 1956[48]
Use of acrylamide gel as an electrophoretic support medium	Raymond and Weintraub, 1959[3]
Development of stacking gel concept and suitable buffers	Ornstein, 1964[49]
Use of two unassociated parameters for separation—mobility and molecular weight	Margolis and Kendrick, 1969[50]
Isoelectric focusing followed by electrophoresis	Dale and Latner, 1969[51]
Mapping of tissue proteins for genetic studies—IEF followed by PAGE	Macko and Stegemann, 1969[52]
Relationship of SDS electrophoretic mobility and molecular weight	Weber and Osborn, 1969[5]
Use of concentrated urea in gels and development of multiple-slab-gel system	Kaltschmidt and Wittman, 1970[53]
Introduction of SDS stacking gels	Laemmli, 1970[29]
Combination of IEF with SDS-PAGE	Stegemann, 1971[54]
Electrophoresis followed by SDS-PAGE	Martini and Gould, 1971[55]
IEF-SDS-PAGE of nonhistone nuclear proteins	Barrett and Gould, 1973[56]
Acid urea electrophoresis—SDS-PAGE of nuclear proteins	Orrick, Olson, and Busch, 1973[57]
Beginning of automation—system for casting centrifugally and simultaneously 500 tube gels	Neel, Tiffany, and Anderson, 1973[58]
Discovery that SDS reacts rapidly with proteins in urea without heating	Mets and Bogorad, 1974[59]
High resolution mapping: IEF followed by SDS-PAGE	O'Farrell, 1975;[8] Klose, 1975;[9] Scheele, 1975;[10] Iborra and Buhler, 1976[11]
Optimization of the system, using very small samples and autoradiography	O'Farrell, 1975[8]
High-resolution analysis of human serum	Anderson and Anderson, 1977[7]
Development of semiautomated Iso-Dalt system	Anderson and Anderson, 1978[12,13]

From Anderson NG, Anderson NL: Behring Inst Mitt 63:169, 1978

pH of 4 to 8. When proteins are introduced into the gradient they migrate electrophoretically until they find the pH at which they are uncharged, i.e., the isoelectric point or pI. All proteins have unique pI values due to their unique primary amino acid sequences. Urea, which is uncharged, is often used in the gels to free proteins that are particularly "sticky" such as albumin, from small charged molecules which had been adsorbed and also to separate non-covalently bound protein subunits. Concentrated urea also counteracts the tendency of many proteins to precipitate at their

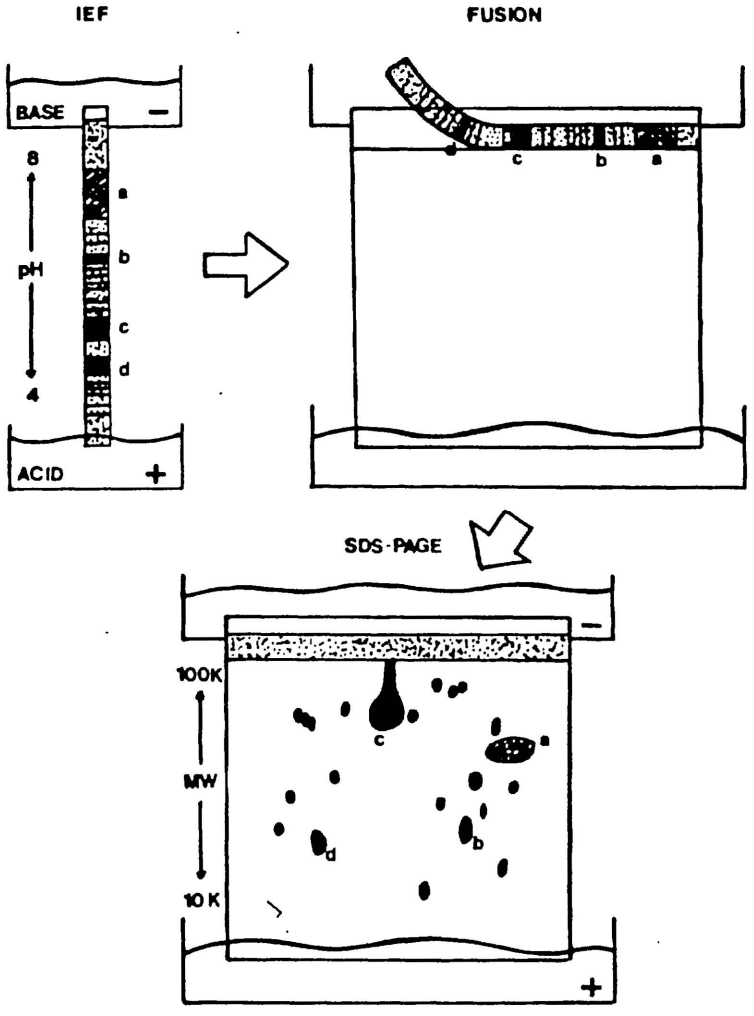

FIG. 2. The process of two-dimensional gel electrophoresis. The upper-left portion of this figure depicts isoelectric focusing (IEF) in polyacrylamide tubes; in the Iso-Dalt system, 9 moles urea/liter and 2 percent detergent would also be present. Several "proteins" are labelled (a-d) so that they may be followed through the process. After focusing is complete, the gel tube is equilibrated in a buffer containing SDS and then attached to a slab gel (FUSION), acid end to the left. Electrophoresis then takes place top to bottom (SDS-PAGE), and proteins are separated by their molecular weights (MW). Abundant proteins (e.g., protein C) may show streaking.

pI. To help achieve both of these ends, many IEF systems also incorporate a non-charged detergent, such as Triton-x-100, into the gels. As in SDS-PAGE above, if 2-mercaptoethanol is used in the original sample preparation, individual single chain polypeptides will be focused.

These two techniques, IEF and SDS-PAGE, are the two methods used in what is currently called high resolution two-dimensional gel electrophoresis (2DGel). There is a long history of achievement in electrophoresis leading to the current semi-automated Iso-Dalt method of 2DGel,[7] as is outlined in Table 1. In 1975-1976, a

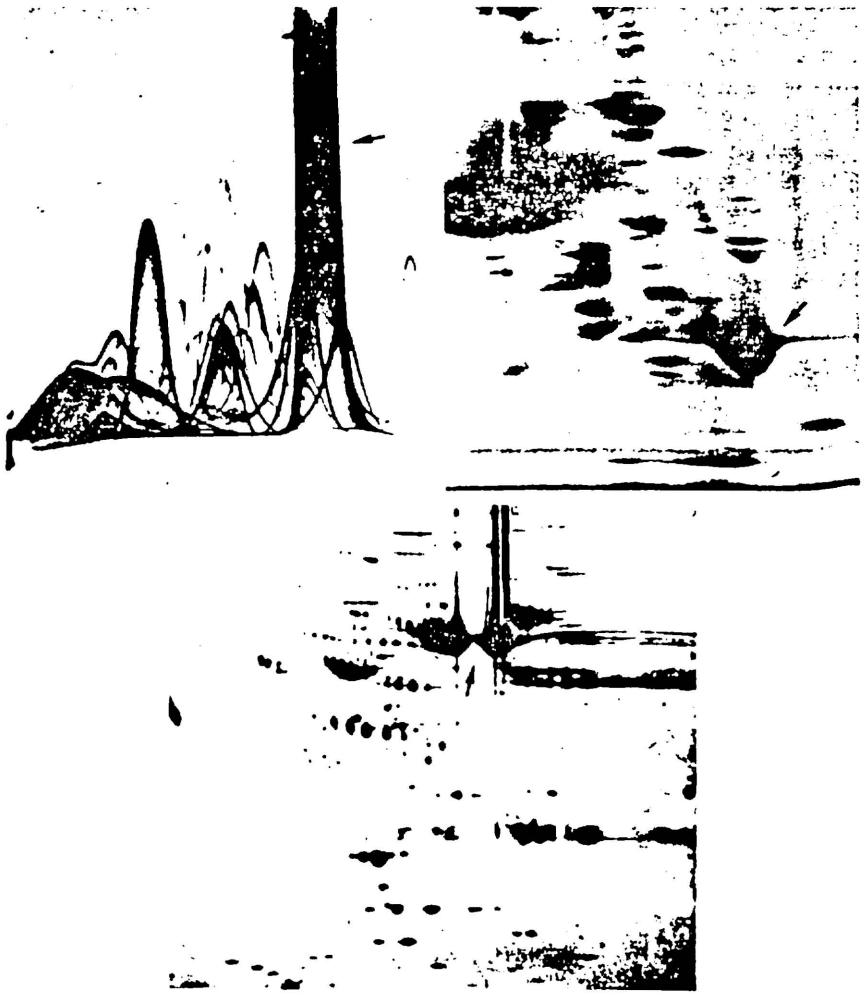

FIG. 3. Comparison of two-dimensional techniques. In all three pictures the arrow points to the albumin of these plasma samples. Upper left: two-dimensional immunoelectrophoresis (by permission of LKB Instruments, Inc.). Upper right: nondenaturing two-dimensional electrophoresis (*Courtesy of Dr. Karl Lonberg-Holm, by permission of the American Association for Clinical Chemistry*). Bottom: Iso-Dalt two-dimensional gel electrophoresis (*From Anderson NL, Anderson, NG: Proc Natl Acad Sci USA 74:5421-25, 1977, with permission*). Gels stained with Coomassie Blue.

number of individuals came to the conclusion that an isoelectric focusing step followed by SDS-PAGE offered the best possibility for very high resolution.[8-11] The adoption of IEF in urea and non-ionic detergents as a first dimension proved particularly valuable. O'Farrell's version of this system was optimized to the point of demonstrating actual separation of more than a thousand different proteins,[8] and with this advance the problem of the feasibility of very high resolution protein separations was essentially solved. In fact, since each of the individual dimensions can be made to yield a resolution of about 100 protein species, a theoretic resolution of 10,000 proteins is predicted. Recently, systems have been constructed capable of performing many analyses in parallel,[12-14] solving the problem of throughput.

In current two-dimensional gel techniques, a protein mixture such as serum is separated in a thin cylindrical rod of polyacrylamide in an IEF step, as shown in the first part of Figure 2. This rod is then equilibrated for a short time in a small volume of buffer containing SDS and then "fused" with hot agarose to the top of a rectangular slab gel, also containing SDS. Electrophoresis is performed, top to bottom, and the slab gel is stained and photographed. Each spot on the gel represents an in-

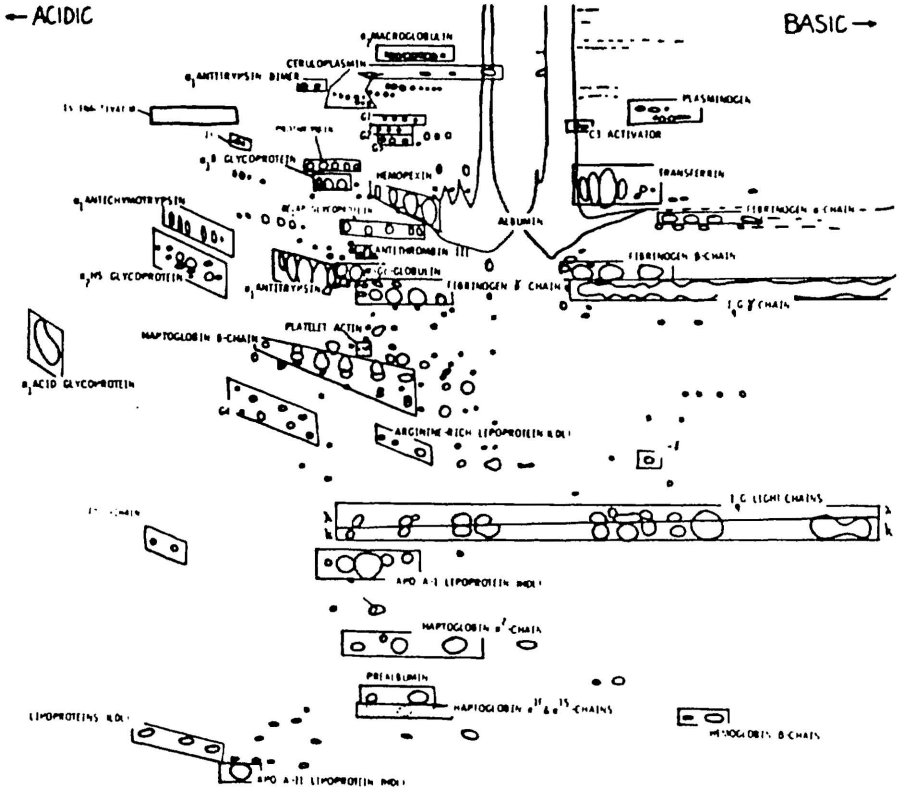

FIG. 4. Schematic drawing of the Iso-Dalt two-dimensional gel of plasma. This drawing illustrates the known protein spots present in the bottom picture of Fig. 3. (*After Anderson NL, Anderson NG: Proc Natl Acad Sci USA 74:5421-25, 1977, with permission.*)

dividual gene product. Microheterogeneity is commonly seen in the IEF dimension due to the addition of one or more charged sugar residues. This manifests itself as the common "string" of spots, all at the same or slightly different molecular weight values.

During the course of the development of 2DGel, several other "two-dimensional" techniques have been introduced. The two-dimensional crossed immunoelectrophoresis technique is the most familiar to the clinical scientist.[15]

Of the others, electrophoresis in agarose followed by SDS-PAGE, both without reducing agents, is the most popular.[16] Figure 3 compares the Iso-Dalt, crossed immunoelectrophoretic, and nondenaturing two-dimensional techniques; Figure 4 is a schematic drawing of the Iso-Dalt gel in Figure 3.

One final note about nomenclature: the term "high-resolution electrophoresis" has recently come into use to denote non denaturing electrophoresis done in agarose,[17] as is illustrated in Figure 1. This method should not be confused with high-resolution two-dimensional techniques. Serum is generally resolved into approximately 20 bands by agarose electrophoresis, while over 300 spots may be seen on an Iso-Dalt gel.

ISO-DALT TWO-DIMENSIONAL GEL ELECTROPHORESIS

Necessity for a Very High-resolution Separations Technique for Proteins

Several hundred human proteins have been isolated, and their properties have been studied in some detail.[18] While this represents a substantial achievement and has allowed the development of meaningful clinical assays for a number of proteins, it falls far short of a complete analysis of the human proteins, even of human blood proteins. It is estimated that there are perhaps 30,000 to 50,000 protein-coding (structural) genes in the human genome, and that 5,000 to 8,000 of these may be expressed in a given tissue or cell type. Thus a technique capable of resolving something in the neighborhood of 10,000 different proteins is required in order to contemplate the comprehensive analysis of human cellular proteins.[19] As already noted, high-resolution 2DGel meets this requirement. Such a comprehensive approach will almost certainly be required to solve some of the major problems in human disease, since there is every reason to expect that the majority of proteins not yet discovered will be as important in some way as those which have been thoroughly studied. In particular, a genetic defect occurring in any structural gene may give rise to a genetic disease or predisposition to disease.

For clinical application, however, it is important for virtually any technique to have a reasonably high throughput. As described by O'Farrell and others, high-resolution two-dimensional gels may be run only at the rate of 5 to 8/week/person.[8-10] It was not until Anderson, Anderson, and coworkers described methods for running multiple gels that clinical evaluation on a large scale could truly begin. Using their Iso-Dalt system, Tracy et al. found that two people may easily run 50 to 60 gels/week in a clinical laboratory with excellent reproducibility.[20] The technique, therefore, is available for clinical investigation and implementation in the clinical laboratory.

Techincal Aspects of High-resolution Two-dimensional Electrophoresis

Sample Preparation. The essential features required in a sample preparation procedure are completeness of solubilization and absence of artifacts (principally those due to proteolysis). Because of the high resolving power of the separation technique, it is usually unnecessary to perform a "prefractionation" even on very complex starting samples; such procedures tend to generate substantial proteolytic and deamidation (conversion of asparagine and glutamine to aspartic and glutamic acid) artifacts and should be avoided when possible. The following two general schemes of preparation have found substantial use, but it should be recognized that special types of samples may require novel approaches, both in the area of solubilization and concerning proteolysis inhibitors.

Human plasma[7] and other body fluid proteins may be effectively prepared for analysis by mixing one volume of sample with three volumes of a solution of 2 percent SDS, 1 percent dithiothreitol or 2-mercaptoethanol (as a disulphide reducer), 10 percent glycerol, and 0.05 cyclohexylaminoethanesulfonic acid (CHES) moles buffer/liter. This has been done at both pH 6.5 and pH 9.5. This method of preparation makes sure that all proteins have a high negative charge at the beginning of the focusing step and therefore that they move rapidly into the focusing gel with a minimum of precipitation in the sample loading zone, particularly at pH 9.5. It also effectively prevents most proteolytic damage by denaturing the proteases.

Cellular samples may not be electrophoresed immediately following solubilization because of the viscosity resulting when the cell nuclei are disrupted and DNA is released into solution. In general, the presence of high molecular weight nucleic acids results in poor resolution separations in the two-dimensional system. One answer to this problem is to use a different sample preparation procedure, following the general approach described by O'Farrell.[8] The sample material is dissolved directly in a solution of 9 moles urea/liter, 2 percent Nonidet P-40 (NP-40; a nonionic detergent), 2 percent 2-mercaptoethanol, 1 moles pepstatin/liter, and 2 percent 9 to 11 pH range ampholyte at pH 9.5. While the urea and NP-40 quickly dissolve the cells and denature most proteolytic activities, the positively charged basic ampholytes complex with the DNA (in place of the solubilized histones), keeping it condensed and the sample nonviscous. High pH and the presence of pepstatin effectively eliminate the proteolytic activities present in some otherwise difficult samples, such as blood leukocytes (tissues containing little proteolytic activity can be prepared in a similar solution at pH 7). Alternatively, tissue samples may be centrifuged to eliminate the nucleic acids. Human urinary proteins are usually prepared in such a solution without NP-40 in order to avoid streaking,[22] but they may also be prepared effectively by the first method mentioned above.

Isoelectric Focusing. Current two-dimensional procedures are almost universally based on isoelectric focusing in glass tubes followed by SDS electrophoresis in rectangular slab gels. In general, focusing tubes are 1.0 to 3.0 mm in diameter, with 1.5 mm diameter as the standard size. Length can vary from 5 to 90 cm, depending on the resolution required, with 18 to 25 cm the usual size. Focusing gels are prepared by polymerizing (in the tube) an acrylamide gel made from a solution containing (in 15

ml): 8.25 gm urea; 0.75 ml ampholytes (40 percent solution) of various composition, depending upon the range of the pH gradient desired; 2 ml of a 30 percent acrylamide/1.8 percent bisacrylamide stock solution; and 6 ml water. This mixture is made to polymerize by the addition of ammonium persulfate (70 μl of a 10 percent solution in water) and tetramethylethylenediamine (TEMED, 10 μl). The amounts of persulfate and TEMED must generally be optimized in each laboratory to give a polymerization time of approximately 10 minutes (or sufficient to load the gel solution into the gel tubes before polymerization). The quality of reagents (including water) used in isoelectric focusing gels is critical, since the presence of extraneous charged compounds can have very detrimental effects on the shape of the pH gradient established during the run.

While it is possible to fill 1.5 mm diameter focusing tubes individually after sealing the bottom ends with parafilm, it is more convenient to load a number to the same height simultaneously. The ISO apparatus shown in Figure 5 allows 20 to 40 tubes to be loaded at once by hydrostatic displacement of the dense, urea-containing gel solution out of a "boat" into which the bottoms of the gel tubes extend.[12] The device consists of an upper buffer chamber with an electrode, through the bottom of which the tubes are mounted; a back plate which holds the upper buffer chamber and carries the lower electrode; a removable gel solution boat and retainer strip for casting the gels; and a larger lower buffer chamber into which the other components fit. While the original design calls for 20 tubes/apparatus, a recent modification designed specifically for the clinical laboratory uses units of 12—10 for samples and 2 for quality control.[20] If the upper chamber, gel solution boat and retainer, and backplate are assembled and the gel solution is loaded into the boat, then gradually lowering this assembly into the water-filled lower buffer chamber will result in gel solution being displaced upwards into the tubes to a point approximately 2 cm from the tops of the tubes. After polymerization is complete (generally 1 hour if gelation is apparent in 5 to 10 minutes), the internal assembly is removed, the gel solution boat and retainer are taken off, and the upper buffer chamber/backplate are placed in the lower buffer chamber, now filled with 1 liter of 0.02 Eq/liter H_3PO_4 in double-distilled water. In most methods, the upper buffer chamber is filled with 200 ml of 0.02 mol/liter NaOH (freshly degassed and in double-distilled water) and the air remaining in the top 2 cm of the gel tubes is displaced by filling this space with the NaOH top buffer. Following prefocusing for 200 V Hr, the dense samples can be loaded under the catholyte into each tube with a 100 μl Hamilton syringe and focusing can be carried out for the required period (usually 10,000 to 14,000 V Hr for cellular protein samples on 16 cm gels). After focusing, gels are removed from the tubes by water pressure exerted via a 1 ml syringe fitted with the pointed end of a yellow Eppendorf disposable micropipette tip, which fits snugly into the 1.5 mm tube ends. Gels are not usually placed directly on the SDS-PAGE slab gels, but are extruded individually into vials containing 2 ml of equilibration buffer (10 percent glycerol, 2 percent SDS, 1 percent 2-mercaptoethanol [this may be left out for most samples, as it causes streaking with silver-stained gels; see below], and 0.1 mol/liter tris at pH 6.8 with a trace of bromophenol blue as a tracking dye) and shaken for 10 to 45 minutes, depending upon the sample. At this point, the focusing gels can be frozen or placed on slabs for the second dimension separation. The equilibration step is necessary to remove most of the ampholytes and to infiltrate the gel with SDS. Some loss of pro-

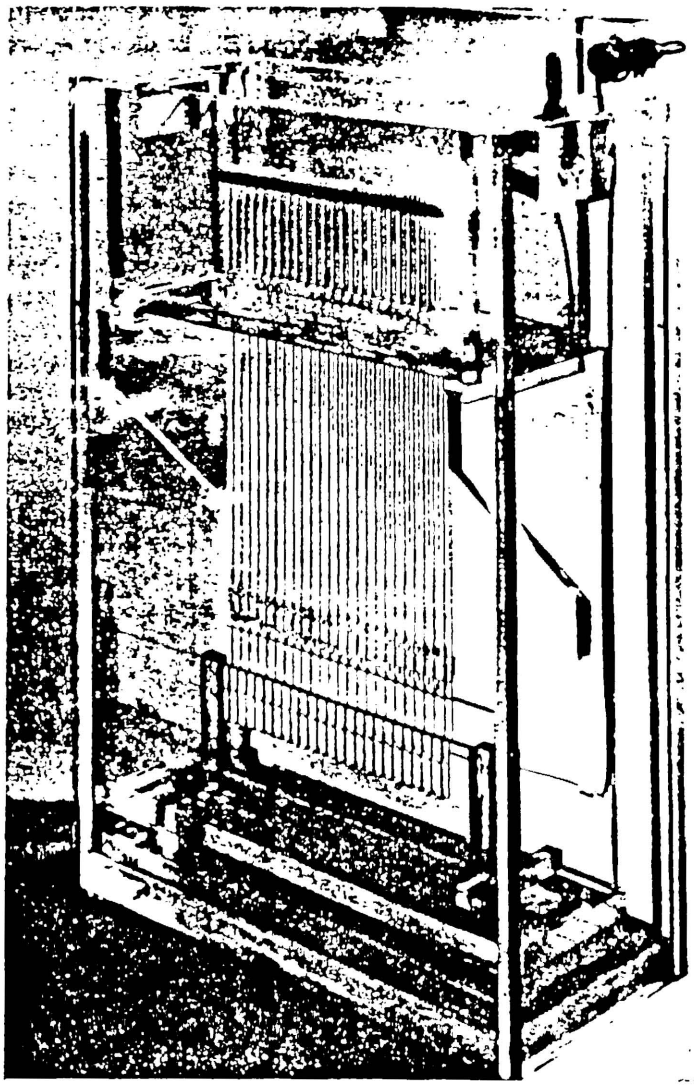

FIG. 5. "ISO" apparatus for the isoelectric focusing step (first dimension) of two-dimensional gel electrophoresis.

tein may occur at this time via diffusion. Glass tubes are cleaned by immersion in hot chromic acid solution and thorough rinsing.

Using wide-range ampholytes, the conventional (ISO) procedure described here can generally resolve proteins with isoelectric points between pH 4 and pH 8.5. More basic proteins can be resolved using O'Farrell's nonequilibrium pH gradient electrophoresis (NEPHGE) technique,[23] the BASO modification of it,[24] or the wide-range modification of Tracy et al.[25] The first two methods use the same apparatus and gels as the conventional method, but the electrode buffers are reversed and the

prefocusing step is eliminated. Thus the sample is applied at the acid end of the focusing gel (now at the top) and the proteins run into the pH gradient as it forms. Since the pH gradient in the region of pH 8.5 to 10.5 is still unstable, the basic proteins cannot be allowed to reach their isoelectric points (these would not fall in the gel). Therefore, the run is terminated before equilibrium is reached, with the proteins separated on the basis of their mobilities in a pH gradient. These mobilities are closely related to the isoelectric points of the proteins, but are also influenced by molecular weight as well; hence the pattern of proteins on a NEPHGE or BASO gel in the region of overlap with the conventional ISO will be similar but not identical to the ISO pattern. The wide-range modification makes use of basic amino acids to act as basic ampholytes and a very strong catholyte, 1 moles/NaOH liter.[27]

A nonequilibrium approach may also be taken in the analysis of very acid proteins, such as the most acid urinary protein.[22] In this case, gels are set up and run as for the conventional ISO procedure (sample loaded at basic end), except that the run is terminated before equilibrium is achieved (i.e., when the very acid proteins are still in the gel). Proteins with isoelectric points in the range of pH 2.5 to 4.0 can be resolved with this procedure.

In general, the isoelectric focusing dimension is the most difficult part of the two-dimensional technique. This results partly from our lack of knowledge about the detailed physical chemistry of the ampholyte pH gradient, why it drifts cathodically with time, and why the properties of various commercial batches of ampholyte differ as they do.[28] A great deal of trouble can be avoided, however, by following a few general rules:

1. Use the best grades of chemicals available for the focusing gels.
2. Use double-distilled water for gels and buffers (house-distilled is often not good enough).
3. Optimize the focusing time for the type of sample to be run.
4. Evaluate the commercially available ampholytes for the sample type in question and then order as much of the optimal ampholyte batch as is feasible (or likely to be used over the following several years) and keep it frozen until needed.
5. Make certain the focusing tubes are extremely clean.
6. Where possible, attempt to see that gels are always polymerized at a constant, cool temperature (18° to 20°C).
7. Always try to run samples for detailed comparison in the same batch of gels.

SDS-Slab Gel Electrophoresis. SDS-electrophoresis separates proteins primarily on the basis of the size of their SDS-denatured forms.[5]

Numerous slab gel devices have been devised[13] around the general principle of a rectangular gel between glass plates set up to contact separate electrode-containing buffer reservoirs at opposite edges of the slab (the other two edges being bounded by spacers defining the gap between the glass plates). Since in the two-dimensional technique, one slab is required per sample, it is important to be able to run as many slabs simultaneously as will be required for a batch of samples. Most commercially available slab apparatuses run only one or two slabs, and therefore a new device (called the DALT apparatus) was designed for the purpose of running 10 to 20 slabs

in parallel (Fig. 6).[13] The standard DALT system is configurated to prepare and run 16.5 cm x 16.5 cm gradient slab gels of 1.5 mm thickness (i.e., the same thickness as the diameter of the standard focusing gel). Size matching of the first and second dimensions allows the focusing gel rod to be applied directly to the top of the second dimension gel between the glass plates. The polyacrylamide used in these slabs as the sieving medium is poured in an exponential concentration gradient to allow resolution of as wide a range of SDS molecular weights as possible (approximately 6,000 to 250,000 on 10 to 20 percent gradients), and stacking gels are not generally used since

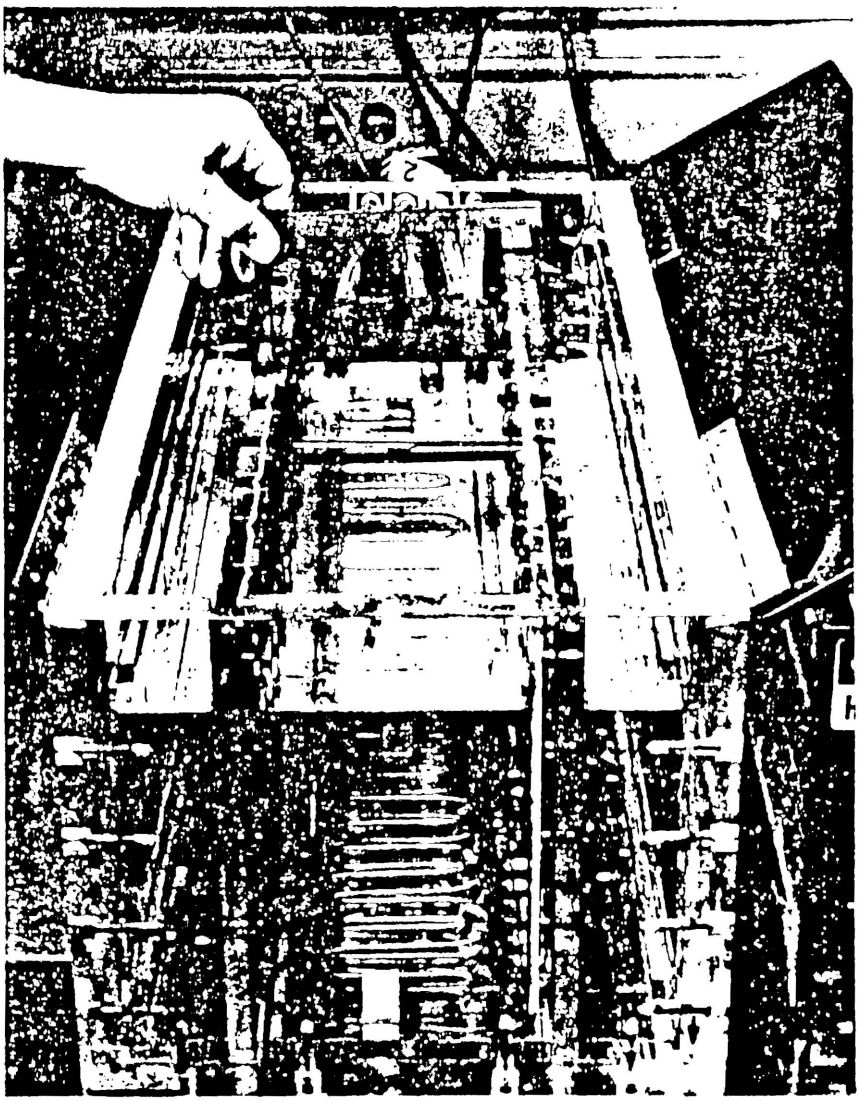

FIG. 6. "Dalt" apparatus for the SDS-PAGE step (second dimension) of two-dimensional gel electrophoresis. (*From Anderson NL, Anderson NG: Anal Biochem 85:341–54, 1978, with permission.*)

they do not produce increased resolution for a starting zone as small as 1.5 mm diameter tube gel.

In order to prepare a number of uniform gradient slab gels, the DALT system employs a conventional gravity-driven gradient maker feeding a plastic box containing sets of glass plates and spacers to define the gel volumes. Gel solution from the 10 and 20 percent sides of the gradient maker meet and pass through a magnetically stirred mixer and then through a distribution manifold and into the casting box, where flat funnels evenly distribute the inflowing gradient. Generally, 1 cm is left at the top for placement of the focusing gel.

The amounts of polymerization initiator (ammonium persulfate) and catalyst should be optimized carefully to yield approximately 7 to 10 minutes working time before the top of the gels polymerize and 30 to 60 minutes before gelation occurs at the bottom of the gradient. A typical recipe for making 10 gels with 10 to 20 percent gradients uses 250 ml of 10 percent acrylamide, 0.25 percent bisacrylamide, 0.1 percent SDS, 0.3 moles tris buffer/liter at pH 8.5, and 360 ml of 20 percent acrylamide, 0.53 percent bisacrylamide, 0.1 percent SDS, 0.3 moles tris buffer/liter at pH 8.5. Just before loading into the gradient maker, 4 ml of 10 percent ammonium persulfate and 80 μl TEMED are added to the 10 percent mix, and 2 ml ammonium persulfate and 10 μl TEMED are added to the 20 percent mix. Temperature and variations in the reagents can have major effects on the polymerization rate, and substantially different amounts of TEMED (the principal reaction rate controller) may be required.

During the casting process (before polymerization), serial numbers typed on small pieces of filter paper with a carbon ribbon are dropped into each gel. These small labels are polymerized into the gel and thus identify each gel throughout subsequent processing. Also, before polymerization, a shallow layer of water-saturated sec-butanol is gently introduced over each gel by carefully pipetting the liquid over the top of the casting box. The butanol forms a sharp interface over the gel solution, resulting in a very flat upper surface to the gel slab. The flatness of the gel top is a major determinant of resolution in this system.

Following complete polymerization, the casting box can be opened by removal of its front face (held on by bolts and sealed with a gasket during casting) and the gel-containing pairs of glass plates can be removed individually. The conventional DALT system employs pairs of square glass plates 7 inches by 7 inches separated by two 1.5 mm glass spacer strips. Devices that employ 8 inch by 10 inch and 12 inch by 12 inch plates have also been built. The spacers are glued in place on one of the plates with silicon rubber cement, and the two plates are held together with a strip of silicon rubber sheet glued along their edges on one side like the spine of a book. Thus each pair of gel plates with spacers is a single piece (a "cassette") capable of opening like a book for removal of the gel. Following removal from the casting box, the cassettes are washed to remove excess gel adhering to the outer surfaces and drained.

Isoelectric focusing gels are dumped from their equilibration vials onto plastic tea strainers (to remove excess liquid) and then transferred to a loading easel where they are rolled into the gap between the slab gel plates. Using a small spatula, the focusing gel is manuevered to lie flat and unstretched on the flat top of the slab gel and is then "fused" in place with 1 to 2 ml of melted 0.5 percent agarose in SDS electrode buffer.

Once all the focusing gels are sealed in position and ready for SDS elec-

trophoresis in the second dimension, the slab cassettes are placed in a DALT electrophoresis tank. This tank is a rectangular chamber filled with SDS electrode buffer[29] and divided into three compartments by two internal walls each containing 10 or 20 slots. The outer two compartments are the electrode chambers, and the center compartment is used for gel cooling. Gel cassettes are inserted into a corresponding pair of slot seals in the internal walls, so that the open ends of the slab protrude into each of the electrode compartments. Note that contrary to how it is depicted in Figure 2, electrophoresis proceeds horizontally, with proteins migrating out of the now vertical focusing rod through the slab. Bromophenol blue from the equilibration buffer serves as a tracking dye; when this blue line reaches the anodic end of the slab, electrophoresis is complete. Gel cassettes are then removed from the tank and opened on an unloading lectern, and the gels are carefully removed and placed in stain/fix.

If the pH gradient in the Iso gel (IEF) is measured routinely and plotted against gel length, the slope of this line may be used as a quality control parameter. Also, the protein log molecular weight versus relative migration curve for the Dalt dimension (SDS–PAGE) may also be used in this manner. The between run coefficients of variation for both of these parameters are approximately 5 percent or less.[21]

Visualization of Proteins. There are four principal methods in use for detecting proteins resolved on two-dimensional gels: Coomassie Brilliant Blue (CBB) and "silver" staining, to reveal proteins generally, and autoradiography and fluorography, to detect radioactively labeled molecules.[30] CBB staining is the most widely used method and allows detection of spots containing as little as 50 to 100 ng of protein, although staining intensity depends upon the individual protein. Ten gels can be stained by gentle shaking overnight in a plastic box containing 1 liter of 0.2 percent CBB, 50 percent ethanol, 5 percent acetic acid, and 44.8 percent water. Destaining is accomplished by shaking in four 1 liter changes of 20 percent ethanol, 5 percent acetic acid, and 75 percent water. CBB bound to protein has an absorption maximum near 550 nm, and thus the gels are best photographed through an interference filter with bandpass centered at this wavelength.

The "silver" stain is the subject of numerous recent publications, which may be consulted for detailed recipes.[31-33] It is our experience that the proportions of the different reagents used must be varied from laboratory to laboratory, as the technique is extremely reagent-dependent. It is generally recognized that this procedure can detect many proteins with about 100 times the sensitivity of CBB. Figure 7 illustrates the pattern that may be obtained with 0.5 μl of serum analyzed using silver stain. A possible disadvantage of the procedure is the occasional difficulty in attaining reproducible results.

For detection of radiolabeled proteins, it is generally necessary to dry the gel in order to reduce self-absorption. The pliability of the dried gel can be satisfactorily increased by the addition of 2 percent glycerol to the last two water rinses. Gels prepared in this way can be dried in several hours on any of several commercially available drying devices.

Fluorography is a procedure for impregnating gels with a scintillant in order to allow detection of ^{3}H (whose β-particle is too weak to be seen by autoradiography) and to enhance imaging of ^{35}S and ^{14}C approximately 10-fold.[30,34] Low-temperature

FIG. 7. Two-dimensional Iso-Dalt gel of serum, stained with silver nitrate. Approximately 0.5 μl of serum was analyzed by the method of Tracy et al.[19] and stained with an ammoniacal silver solution.

exposure is necessitated by the fact that the fluorographic procedure exposes film with light photons (a number are required to expose each grain, and partially exposed grains are unstable at room temperature), whereas in autoradiography, each β-particle can completely and irreversibly expose a single grain. Films are developed conventionally.

Ancillary Techniques Enhancing the Usefulness of Two-dimensional Electrophoresis Analysis.

Two-dimensional electrophoresis, done either by Iso-Dalt or by other techniques, is a very powerful tool for the analysis of complex mixtures of proteins. The Iso-Dalt system described above represents an attempt to standardize the production and running of two-dimensional gels. In addition, however, it is necessary to internally stan-

dardize the gel coordinate system and to identify useful landmark proteins in the pattern.

Internal Standards. It is possible to produce a set of pI standards by progressively modifying a single protein by single charge changes.[35] A mixture of these modified forms can be added to original samples to appear on the gels as a countable row of spots stretching across the gel from acid to basic end at a constant SDS-molecular weight (Fig. 8). Carbamylation of basic proteins (thereby blocking the positive charges of lysine ϵ-amino groups) produces "charge trains" of spots extending toward the acid end of the gel. Choosing the unmodified form as the zero position, the modified spots are given progressively more negative integral values (indicating increasingly more negative net molecular charge). Using rabbit muscle creatine kinase standards of this type, pI positions accurate to 0.1 charge (or approximately 0.01 pH unit) can be determined.

Internal standards for SDS-molecular weight can be placed at the top of the SDS-slab gel just before second dimension electrophoresis and do not need to go through the focusing step. These standards can be placed at the sides of the slab or all the way across (when mixed into the agarose used to seal the focusing gel in place). Convenient standards of this type can be made easily from rat heart muscle proteins.[36]

Identification of Known Proteins. In establishing the use of the two-dimensional system with each new type of sample or species, it is important to identify as many of the known protein spots as possible. If pure proteins are available, coelectrophoresis of each with the sample mixture usually reveals its location with respect to the pattern as a whole. Alternatively, if specific antisera are available, proteins may be immunoprecipitated or immunosubtracted from the sample mixture,[37] or they may be

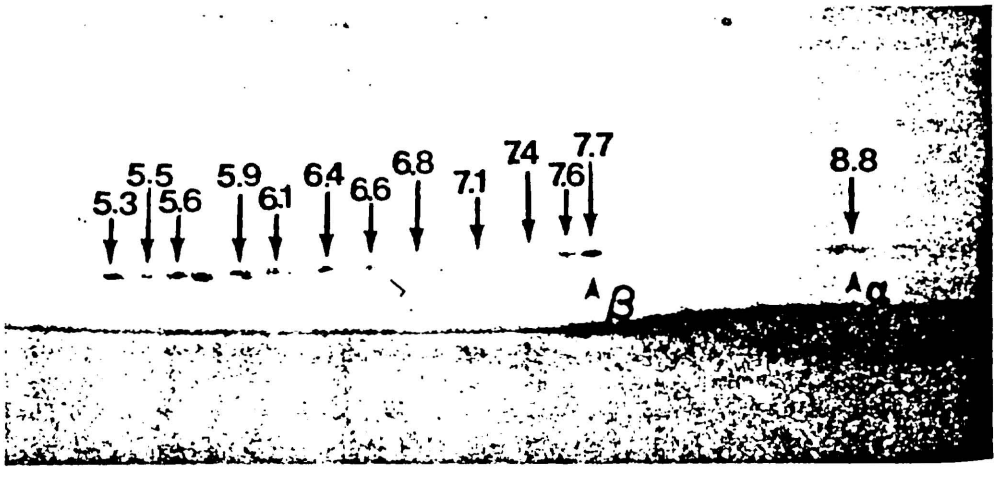

FIG. 8. Internal charge standards for two-dimensional gel electrophoresis. Carbamylated hemoglobin alpha (α) and beta (β) chains, and the iso electric forms of the various chains (*From Tracy RP, et al.: Clin Chem. 28:900, 1982, with permission.*)

identified by immunochemical staining of two-dimensional protein patterns electrophoretically transferred to nitrocellulose or diazobenzyloxymethyl cellulose paper.[38,39] Although most proteins lose functional activity following two-dimensional electrophoresis under the denaturing conditions described, most still retain the ability to react specifically with the appropriate antiserum after further transfer to nitrocellulose.

Combinations of the above methods have been used to identify more than 150 proteins in the two-dimensional pattern of the bacterium *E. coli*,[40] 35 proteins in human plasma,[7] 15 proteins in rabbit and human muscle,[41] 10 proteins in human leukocytes,[42] and a few proteins in human saliva,[43] seminal plasma,[44] cerebrospinal fluid,[45] urine,[22] and red cell lysates.[46]

CLINICAL POTENTIAL OF HIGH-RESOLUTION 2DGEL ELECTROPHORESIS

In this section we will attempt to review the uses to which two-dimensional electrophoresis has been put which either have clinical significance or point toward some future clinical application. In truth, most references fall into the latter category at this point in time. This section is not intended to be an exhaustive catalog of all reports in which the technique has been used. Even though high-resolution 2DGel is barely 6 years old, our off-line Medlars II search, which forms the basis for this review, returned well over 1000 references, using "two-dimensional gel electrophoresis" as the major key word. We hope to simply highlight those reports which may be of interest to clinical laboratorians.

Long Range Concepts

Two areas previously beyond practical experimentation are opened to investigation by high throughput, reproducible 2DGel: (1) the large-scale screening of individuals for genetic variation, mutation rate, toxic effects (both chemically induced and radiation-induced), and inborn errors; and (2) the development of a catalog of all human proteins.[19] Work in these areas will require the analysis of many gels by computer-assisted methods.[60-63] These methods are just becoming available and will open the door for the development of the appropriate data bases needed to answer these fundamental questions. Much interesting work has already been done, however without the aid of computer analysis.

Nucleated Cells

Fibroblasts and Lymphocytes: Model Systems. The various forms of 2DGel have been used extensively to study genetic expression in lymphocytes and fibroblasts. These cell types offer the opportunity to obtain large, homogenous cell populations and for that reason are a logical starting place for examining cellular peptide gene

products. Recently, Anderson et al. published a comprehensive 2DGel map of human peripheral lymphocytes as part of the development of their TYCHO computer system (Fig. 9).[60] Lester et al. have also analyzed human peripheral lymphocytes and analyzed the results by computer-assisted image analysis,[63,64] as have Merril and Goldman.[65] In several of these groups, over 1000 spots are routinely visualized on 2DGel maps of lymphocytes. As discussed below, correlations have been made between normal and diseased (or otherwise perturbed) lymphocytes.

Comings and Cohen have published 2DGel maps of ^{125}I-labeled fibroblast membrane proteins,[66] and Walton et al. and Celis et al. have extensively studied the radioactively labeled proteins of fibroblast cell lines,[67,68] with an emphasis in one case on determining the amount of genetic polymorphism in the human population.[65] Klose has developed large-sized gels for fibroblast analysis which resolve close to 4500 separate peptide gene products.[69]

Monitoring Induced Changes. As pointed out by Garrells,[61] one advantage of 2DGel is that many of the components of a cell or tissue may be examined simultaneously to determine the molecular change caused by a specific perturbation (e.g., growth, drug, heat, etc.). This type of analysis has been started by several

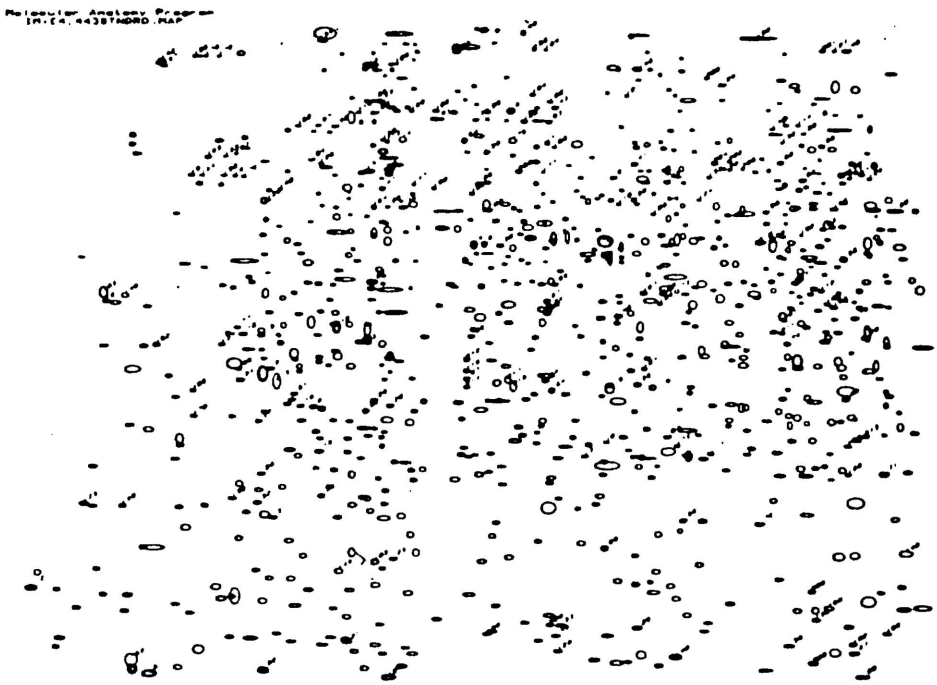

FIG. 9. Computer plot of lymphocyte proteins. A two-dimensional gel was analyzed by computer and then the individual spots were numbered and plotted; for the sake of clarity, not all numbers are shown. The thickness of the line used to draw the spot indicates the relative density. (*From Anderson NL, et al.: Clin Chem 27:1807-20, 1981, with permission.*)

workers in the field. Anderson, working with cells in culture, has defined about 20 "sets" of proteins that may be turned on or turned off by various additions to the short-term labeling culture media (e.g., Concanavalin A,[71] human urine,[72] etc.).[70] In addition, disease-specific peptides have been seen in lymphocytes from patients with leukemia and infectious mononucleosis.[73,74]

As might be expected, endocrine research has been a major area in which workers have attempted to monitor induced changes with 2DGel. Ivarie et al. examined the effect of thyroid and glucocorticoid hormones on rat pituitary tumor cells and identified rat growth hormone and prolactin on their 2DGel maps by immune precipitation.[75] Using calf thyroid slices as their sample, See et al. have shown that thyrotropin stimulated the phosphorylation of several sets of nonhistone thyroid chromosomal proteins beyond the previously recognized histone H_1.[76]

Several groups have looked at the modulation of uterine proteins by estrogens. Skipper et al., utilizing an initial step of nondenaturing gel electrophoresis, have shown that the "single band" of estrogen-induced protein previously noted by several workers in the rat uterus is actually three separate peptide gene products, and that these proteins are also synthesized in liver and muscle cells.[77,78] Korach et al. examined the mouse system and pointed out that estrogen stimulation causes several proteins to redistribute into the different cell fractions, possibly as a method of eliciting estrogen action.[79]

Landfeld et al., using rat ovary cells grown in culture, have shown observable changes in the synthesis rate of several proteins when animals were exposed to human choriogonadotropin.[80] These changes occurred early, prior to the morphologic changes that accompany luteinization and cell differentiation.

Contractile and Structural Components of the Cell. Because they make up a large part of the protein mass of the cell, structural proteins and contractile proteins in muscle cells have been extensively analyzed by 2DGel. In 1976, Whalen et al. and Garrells and Gibson used 2DGel to clearly demonstrate the presence of multiple forms of actin in most, if not all, eukaryotic cell types.[81,82] The muscle form of actin, α-actin, was present only in differentiated muscle cells, while β- and γ-actin were present in all cell types examined. This has been extended to chick embryo fibroblasts.[83] It has been shown recently that γ-actin is absent in the mouse fibroblast cell line, L.[84] Also, Anderson has shown, using 2DGel, that γ-actin binds MgADP more strongly than β-actin.[85] The physiologic implications of these findings are unclear. Actin has been shown to be a major component of lymphocytes,[86] and there is some evidence linking actin to the Ly-8.2 murine lymphocyte alloantigen.[87]

Devlin and Emerson examined contractile protein synthesis during quail skeletal muscle myoblast differentiation.[88] They analyzed myosin heavy chain, two myosin light chains, two subunits of tropomyosin, and α-actin and found their synthesis to be highly coordinated when compared to the synthesis of 30 other cellular proteins picked as controls.

Brevit and Whalen reported that there are several unique tryptic peptides present in fetal calf myosin that are not found in myosin from beef heart.[89] Price et al. examined the light chains of human adult and fetal cardiac myosin and reported a "fetal" form, which appears to be present in the adult, but only in the atria and not in

the ventricles.[90] Giometti et al. electrophoresed skeletal muscle samples and established a tentative map with over 20 major proteins identified.[91] They also developed methods to do this analysis from frozen sections, allowing correlation between histology and the 2DGel map.[92]

Cell Surface Markers. The study of cell surface genetic markers was begun in 1977 by Jones, who analyzed the H-2 and Ia molecules of mouse spleen cells by 2DGel.[93] She clearly demonstrated, using immunoprecipitation coupled with 2DGel, that these molecules exhibited considerable molecular complexity, and, using kinetic studies, she indicated potential precursor molecules on the 2DGel maps. More recently there has been a report by Delovitch and Barber indicating the presence of two structurally identical molecules in the I-EC region in the mouse system.[94] As a first step toward determining the degree of polymorphism present in the rat MHC, Frelinger et al. ran a 2DGel analysis on the immunoprecipitated RT1 products of this system.[95] Their studies showed that all strains examined have unique 2DGel maps except for the strains AUG and MNR.

Recently, several studies concerned with the human MHC (the HLA complex) have been reported. The concept of molecular genotyping by 2DGel was introduced by Charron and McDevitt.[96] This procedure utilizes monoclonal antibodies for immunoisolation and 2DGel for analysis of the isolated proteins. Charron et al. used 2DGel with a monoclonal antibody directed against a nonpolymorphic HLA-D/DR antigenic determinant to show that normal resting T-cells don't express Ia antigens, but that alloreactive T-cells do express HLA-D/DR molecules demonstrable on 2DGel.[97] When expressed, these molecules are similar to those expressed on B-cells from the same donor. Similar data have also been reported by Altevogt et al.[98] These findings are of interest in light of recent studies indicating that expression of Ia on activated T-cells is accompanied by large functional changes.[99] It is presently not known whether the Ia antigens are involved in these changes.

Erythrocytes and Platelets

Erythrocytes. The individual cell type most commonly subjected to electrophoretic analysis over the last decade is the erythrocyte. In particular, erythrocyte membrane preparations, or ghosts, have been and continue to be examined in great detail. The current nomenclature is based upon the work of Fairbanks et al. in 1971,[100] although ever-increasing complexity has been revealed in the last few years as 2D techniques have been applied.

Many versions of 2D electrophoresis have been used which differ from the Iso-Dalt type described earlier. All use SDS in the first dimension to effect complete solubilization. Briefly, SDS gel electrophoresis followed by nondetergent electrophoresis has proved useful,[101] especially in the evaluation of erythrocytes from patients suffering from congenital dyserythropoetic anemia types I and II.[102] Substituting phenol-aqueous urea-acetic acid gel electrophoresis as the second dimension, Conrad and Penniston demonstrated increased resolution of certain proteins, especially the 95,000 dalton membrane glycoprotein.[103] Also, increased resolu-

tion has been claimed by Liljas, whose system uses deoxycholate, a bile detergent, in the first dimension, followed by SDS gel electrophoresis.[104]

The most popular nonIso-Dalt 2D technique involves the use of SDS gel electrophoresis in both dimensions. Conditions are varied either by adding or removing 2-mercaptoethanol or by using different SDS gel methods in the two dimensions. Using these methods, Liu, Palek, and coworkers determined the effect of pH,[105] ATP depletion,[106] and ionic strength,[107] on intramembrane protein complex formation. The topic of membrane protein complexes in erythrocytes has also been addressed by Lux et al. and Koch and Haustein, using 2D SDS gel electrophoresis.[108,109] Possibly the highest resolution in a system of this type has been achieved by Thompson et al., who utilized two very different SDS gel methods in the two dimensions.[110]

The first 2D gel electrophoresis of the Iso-Dalt type was done in 1975 by Bhakdi et al., who electrofocused Triton-X-100 (a nonionic detergent) or EDTA extracts of erythrocytes in 8 mol/liter urea, 1 percent Triton-X-100.[111] They followed this with SDS gel electrophoresis. Although the resolution was not good, they were able to demonstrate reproducible patterns consisting of at least 30 components from 40 healthy donors. Using the same system, Bienzle and Pjura examined "Triton extracts" from fresh and aging erythrocytes.[112] They demonstrated the disappearance within a few hours of a Mr ≈ 75,000 protein, as well as quantitative changes in several other proteins with inceasing "aging" times. Harell and Morrison published an improved technique, with which one can easily visualize over 90 polypeptides in a reproducible manner,[113] while the method of Rubin and Milikowski resolves over 200 polypeptides in human erythrocyte membrane preparations.[114] Rubin et al. used this high-resolution method to examine erythrocytes from patients with sickle cell anemia.[115] While failing to demonstrate any unique membrane constituents in these cells, they were able to illustrate significant differences in cross-linking patterns, which implies alterations in the organization of the membrane proteins in this disease.

Recently, Rosenblum et al. reported a 2DGel technique that resolves over 500 polypeptides from human erythrocyte membrane preparations.[116] They can eliminate high molecular weight components from the gels and improve resolution by adjusting the concentration of urea in the original solubilization mix.

It is clear that currently, the highest resolution method is 2DGel as described in this paper. Figure 10 illustrates our current Iso-Dalt pattern, which includes staining with silver using the erythrocyte membrane solubilization technique of Rosenblum et al.[116] Over 600 spots are visible on a gel of this sort.

Platelets. The work done with platelets has been done universally with human cells, presumably due to ease of procurement and direct clinical relevance. Clemetson, McGregor, and coworkers have published several papers describing the 2DGel patterns seen with normal human platelets.[117,118] Their work indicates clearly that the apparently homogeneous major membrane proteins are in fact quite heterogeneous. The patterns they have generated, using both staining and autoradiographic visualization,[117,118] are reproducible and have led them to propose a revision of the standard platelet membrane nomenclature to include the increased heterogeneity.[119]

Sixma and Schiphorst have also examined normal human platelets, under reduced and nonreduced conditions, with various staining and autoradiographic

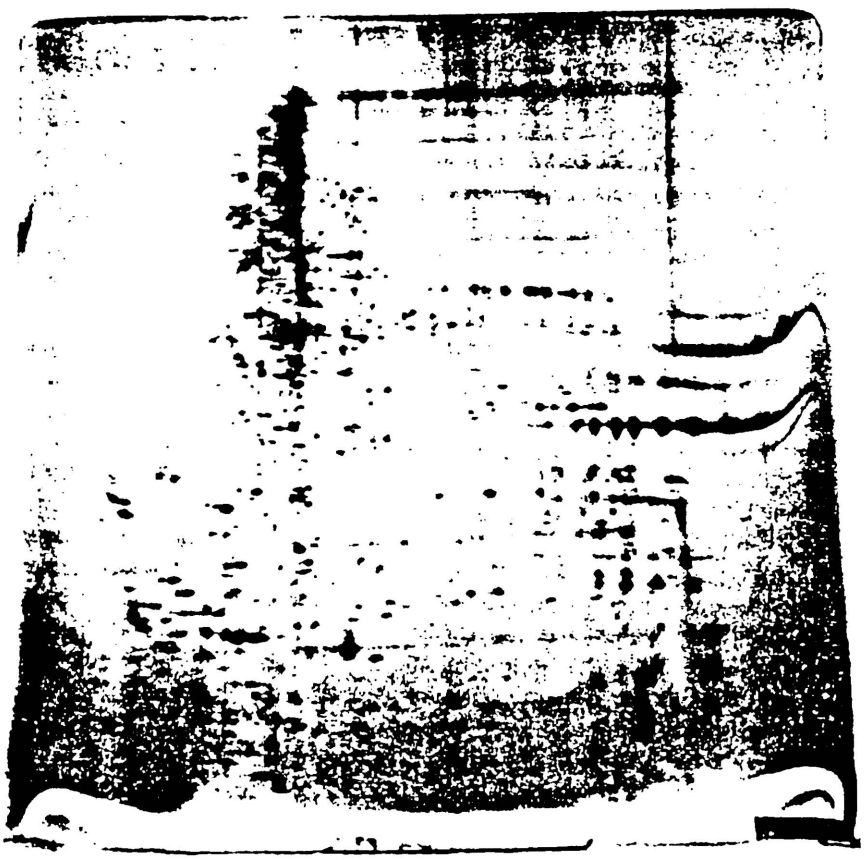

FIG. 10. Two-dimensional Iso-Dalt gel of erythrocyte membranes, stained with silver nitrate.

techniques.[120] They too have observed more discrete proteins than were previously assumed to exist. They have also proposed a new numbering system for the platelet membrane proteins they have observed. Future work with improved gel techniques will likely allow the various workers in this field to arrive at a universally accepted platelet membrane protein map and numbering system.

As a first attempt at clinical application in this area, several groups have compared the patterns seen with normal platelets to those seen with platelets from patients having Glanzmann's thrombasthemia, an inherited bleeding disorder in which platelets fail to aggregate. Clemetson et al. having examined 16 patients suffering from this disorder, have concluded that, along with the well documented decreases in membrane proteins IIb, IIIa, and fibrinogen, there are also changes in the pI values of proteins Ib and IIIb as well as increased glycosylation of several other proteins.[121] These results indicate that there may be more general perturbations in this disease as well as in the specific protein deficiencies.[121]

Holahan and White have also looked at platelets from thrombasthemic patients and report that another membrane protein is also deficient (Mr 93,000).[122] Moreover, they present evidence that, along with the work of others,[123] indicates the disease may be heterogeneous, the various forms being the result of genetic variability.

Transformed Cells

Most of the work done with 2DGel in the field of cancer research has utilized the ability of this system to separate and compare large numbers of proteins at once by comparing transformed and nontransformed cells. This has led to the identification of a large number of "transformation-specific" peptide gene products. The exact relationship of most of these proteins to the transformation process, however, remains obscure. Also, since whole tissue is composed of several cell types, research has in most cases been done in tissue culture, where the patterns are more easily attributed to one or another type of cell. This means, however, that it will be some time before the clinical utility of these findings becomes clear.

One of the earliest findings was that of Milman et al., who demonstrated that the spot on the 2DGel map of HeLa cells that corresponded to hypoxanthine phosphoribosyl transferase (HPRT) was seen to alter or disappear in HPRT mutants.[124] Moreover, revertants displayed both types of HPRT, indicating expression from a previously "silent" gene.

Steinberg et al. examined 549 mouse lymphoma cells and identified the spot on the 2DGel maps that corresponds to the regulatory subunit of the cAMP-dependent kinase.[125] Mutant cells exhibited changes in the pI of this peptide that appear to represent single amino acid substitutions. This study gave direct evidence for mutation of a structural gene in this culture system. Wada et al. demonstrated the presence of placental alkaline phosphatase in human breast carcinoma, using 2DGel.[126]

Strand and August initiated the work comparing normal and transformed cells with their study, reported in 1977.[127] They compared BALB/3T3 cells with their counterparts following transformation with either a DNA or an RNA virus. They showed that an extensive number of proteins were altered, up to 30 percent of the spots seen on the gels.[127,128] The patterns from the two types of transformed cells, though very different from those of the normal cells, were quite similar. In contrast to this, however, is the work of Leavitt and Moyzis.[129] These authors compared the chemically transformed, highly tumorigenic Syrian hamster cell line BP6T with the parental embryo cells and saw very limited differences. Only seven peptides could be seen to be altered, a number constituting less than 1 percent of the total number of peptide gene products seen on the gels. Litin and Grimes, comparing BALB/c mouse fibroblasts with their virally and spontaneously transformed descendants, observed changes similar in magnitude to those seen by Strand and August—i.e., approximately 30 percent.[130] In their hands, RNA and DNA transformation also yielded similar 2DGel patterns. In contrast, Brzeski and Ege demonstrated a difference in only 5 percent of the proteins seen in 2DGel patterns of normal rat kidney cells and those cells transformed with an avian sarcoma virus mutant.[131] Also, Ogata et al.,

when comparing glycoproteins of human kidney cancer cell lines and short-term kidney epithelium cultures, could find only a single consistent polypeptide difference.[132] Wu et al. compared the nuclear proteins of several human tumor cells and several normal cells.[133] They report two peptides found in all the tumor cells but in none of the normal cells. Recently, Tracy et al. and Thorsrud and Jellum compared normal colonic mucosa and colonic adenocarcinoma.[134,135] The differences seen were small. Finally, Scheele compared the peptides present in the pancreatic juice of patients with pancreatic cancer to those present in normal pancreatic juice and observed extensive differences.[136]

The discrepancy that exists in the literature concerning the number of peptides affected by transformation is not easily explained. There are several possible reasons—e.g., different cell types, different viruses, inattention to such details as proteolysis, chemical versus viral transformation, etc. More experimentation will be required to answer these questions definitively.

Specific tumor products have also been characterized by 2DGel. Gottesman and Cabral examined the major excreted protein (MEP) of transformed fibroblasts and found that its synthesis rate is transformation-specific.[137] Moreover, in a cell-free translation system that is incapable of post-translational glycosylation, MEP continued to exhibit charge heterogeneity, implying that there may be multiple MEP mRNAs or multiple ways to translate a single MEP mRNA. Kobayashi et al. reported the phosphorylation of an Mr = 36,000 protein due to Rous sarcoma virus transformation of chicken embryo fibroblasts.[138] They suggest that, in this system, this protein is at least one of the targets of the "SRC" gene product, a known protein kinase. Raju et al. examined the cytosol fractions from Novikoff hepatoma as well as from several Morris hepatomas.[139] They identified a protein of Mr = 64,000, pI = 7.2 (64/7.2) in all of these, which is not present in normal liver cytosol or 18 hour regenerating liver. Also, the concentration of 64/7.2 seems to correlate with the growth rate of the tumors. Eisinger and Kennel used 2DGel to demonstrate that a tumor surface protein (TSP-180) of line 1 mouse alveolar carcinoma cells exhibits charge heterogeneity.[140]

Viral Proteins

Direct viral analysis by 2DGel has also been undertaken. Leavitt et al. isolated various strains of human influenza A virus and subjected them to 2DGel analysis.[141] They showed clear differences between the different strains, especially in the hemagglutin subunits, even in the case of serologically indistinguishable strains. Churchill and Radloff resolved all the capsid proteins and all the known virus-specific proteins in encephalomyocarditis virus-infected cells using 2DGel.[142] Finally, Brackmann et al. have done an extensive 2DGel study of human adenovirus type 2-induced early peptides.[143] They mapped the genetic products produced in a cell-free system and identified many of them as products of specific map positions. Tryptic peptide maps were used for identification.

Clinical Chemistry: Tissue and Fluid Proteins and Alteration with Disease

Tissue and bodily fluid maps produced by 2DGel and the determination of changes in these patterns brought about by disease might be considered an area of clinical chemistry research. Results in this area might be expected to translate more rapidly into clinically useful tests than results in some of the other areas already covered. Much preliminary work has been done, and, in fact, several findings may well have clinical utility in the near future.

Peptides of the Central Nervous System (CNS). Interestingly, CNS tissues and fluids have been extensively studied by 2DGel, more so, in fact, than other, more easily obtained tissues. Rosenberg et al. examined the 2DGel maps of the frontal cerebral cortex, putamen, and cerebellum from normal cadaver donors and from a patient with Joseph disease.[144] Significant differences were noted. The question still remains whether these differences represented gliosis or the expression of the disease-producing genetic mutation. Comings and Peters examined nearly 200 brains from normal cadavers and from patients with various neurologic and psychologic disorders.[145,146] They detected 11 putative mutants from among nearly 50,000 alleles tested (approximately 300 spots per gel), including a polymorphism of glial fibrillary acidic protein present in many neurologic diseases, a possible mutation of protein 17:1 in Joseph disease, and a mutation of myelin basic protein in one schizophrenic patient.[146] Jackson and Thompson examined human brain and 12 other human tissues by 2DGel.[147,148] They located 8 peptides present in large quantity in brain that are absent or present in trace amounts in other tissues. Four of these have been identified as 14-3-2 protein, creatine kinase-BB, aldolase C_4, and 14-3-3 protein.

Marotta et al. recently reported a 2DGel study of human brain proteins translated in vitro from purified postmortem polysomes.[149] Proteins produced included actin, tubulin, and over 300 other proteins, indicating that postmortem polysomes contain many active mRNAs.

Goldman et al. examined cerebral spinal fluid (CSF) in detail and published a map in which many of the spots were identified.[150] Using a sensitive silver staining technique, Merril et al. demonstrated differences in the protein make-up of cisternal, lumbar, and ventricular fluid in primates, including differences in nonplasma proteins.[151]

Serum, Plasma, and Urine. The initial work with plasma using 2DGel was done by Anderson and Anderson, who identified 35 plasma proteins on their gel maps.[7] Until recently, little has been done beyond this. Clark et al. examined the protein matrix of quality control sera,[152] and Goldman et al. used plasma maps for the identification of CSF proteins.[150] Zannis et al. used 2DGel to classify Apo-E lipoprotein subclasses.[153]

Tracy and co-workers have recently embarked upon a large scale evaluation of serum and plasma specimens using 2DGel.[21,154,155] Many of the analytic variables have been examined and a large base of normal serum samples has been analyzed. This data base has led to the development of a standardized plasma protein map used to evaluate any pathologic serum or plasma specimen.[155] Also, a 2DGel system has been

FIG. 11. Two-dimensional Iso-Dalt gel of serum from a patient with macroglobulinemia. Large arrow, albumin; medium arrows, IgM heavy and light chains (monoclonal); small arrow, transferrin. Stained with Coomassie Blue.

optimized for serum specimens from patients with monoclonal gammopathies. The serum protein changes associated with these disorders are easily visualized by 2DGel, and the determination of the light chain pI may have prognostic implications.[154] Figure 11 illustrates the serum pattern seen in a patient with macroglobulinemia. It may be compared with Figure 4 for spot identification.

Anderson and co-workers studied urinary protein patterns,[156-158] and a standardized urine protein map has recently been reported by Edwards et al.[157] Clark et al. examined the patterns of urinary peptides from patients with rheumatoid arthritis and demonstrated specific proteins not present in urine samples from a control group.[160] Frearson recently demonstrated a unique protein in the urine of patients with muscular dystrophy.[161]

Other Human Tissues and Fluids. Many other human tissues have been examined, including saliva,[162] hair follicles,[19] placental brush border membranes,[163] nail,[164] skin,[165] milk,[166,167] pancreatic fluid,[168] semen,[169] and muscle.[171] Most of these studies represent attempts to establish the normal condition, although some[170] have already demonstrated differences between normal and diseased tissues.

SUMMARY

This article is an attempt to familiarize the clinical laboratorian with 2DGel and its potential for clinical application. While 2DGel has already been used in a widespread manner to answer questions in basic biomedical research, the effective exploitation

of this technique in the clinical laboratory will require unprecedented cooperation between physicians and laboratory scientists.

Those among the readers with computer experience may recognize the term GIGO, standing for "garbage in, garbage out." This term is particularly applicable to 2DGel. Tremendous care must be taken to avoid artifacts caused by proteolysis, deamidation, carbamylation, etc. The laboratory scientist wishing to examine human tissue or fluid must acquire the samples from clinical colleagues, with whom he or she works in a collaborative manner. A sample of colonic adenocarcinoma may not be suitable for analysis if it has sat in the surgical pathology suite for several hours prior to homogenization, and the same may be said for virtually all tissue and fluid specimens.

High-resolution 2DGel offers to the medical scientist the opportunity to elaborate the "working parts" of a cell and, potentially, to determine which "part" has malfunctioned in a particular disease. Taking advantage of this opportunity will require the establishment of a close working relationship between clinician and laboratorian, an arrangement that can only benefit both parties.

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