

Analytical Techniques for Cell Fractions

XXI. Two-Dimensional Analysis of Serum and Tissue Proteins: Multiple Isoelectric Focusing^{1,2,3}

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Two-dimensional electrophoresis, using isoelectric focusing in one dimension and electrophoresis in sodium dodecyl sulfate in the second, yields the highest resolution separation of proteins and protein subunits thus far obtained. For general research use, for genetic screening, and for clinical studies, it is important to be able to make these analyses easily, relatively rapidly, and reproducibly. This paper describes a device and methods for casting up to 20 isoelectric focusing gels in parallel, for running them in the same apparatus, and for recovering them in preparation for the second-dimension separations.

The highest resolution separations of proteins and protein subunits thus far reported utilized isoelectric focusing in urea (usually with added neutral detergent) in the first dimension, followed by slab gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) in the second (1–4). The separation is dependent on two independent characteristics, namely, charge as reflected in the isoelectric point and molecular weight as reflected in the mobility of SDS–protein complexes migrating through acrylamide gels (5). We have chosen to call the two-dimensional systems we are developing ISO–DALT systems since they are based on ISOelectric focusing and on molecular weight separations (expressed in DALTons). The DALT system is described in the following paper (6).

From studies initiated by one of us (N.L.A.) and reported elsewhere (7,8), it now appears that a single two-dimensional analysis using this system may yield more clinically useful information concerning serum proteins than can now be obtained from almost any combination of tests

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currently available. If this method is to find general application in protein chemistry and in the clinical laboratory, four groups of problems appear to require solution. This first is mechanization, to be followed by the automation essential for routine clinical use and for screening studies.

The second class of problems is concerned with the identification of the individual spots on the patterns produced. Because many serum and tissue proteins are composed of subunits (9) which are separated by this method, we are faced with the problem of discerning which subunits were originally associated and discovering the identity and function of the undissociated proteins. If pure proteins are available at the outset, this problem is easily solved. However, the development of general systematic methods for the identification of subunits in complex mixtures is a much more difficult problem, to be considered in detail in a subsequent paper.

The third class of problems is concerned with the detection and quantitation of the separated proteins or protein subunits. As shown by O'Farrell (1), the size of the separated spots, and hence the resolution of the system, is very dependent on the amount of sample initially applied. To obtain very high resolution, he used protein mixtures radioactively labeled during their synthesis and detected them by autoradiography. This is not routinely feasible in clinical studies. Postsynthesis labeling which may also be employed has the disadvantage that the electrophoretic properties of proteins may be altered, or that all proteins are not uniformly labeled. Hence methods using stains are required, and very sensitive methods for locating stained spots and for integrating the absorbance of each spot are needed if the potential high resolution of the system is to be obtained in practice.

The last group of problems is concerned with standardization and with data reduction. When very large numbers of spots are to be dealt with, internal standards for isoelectric values and for molecular weights are required, together with precise determination of the coordinates and densities (or radioactivity or fluorescence) of spots. Computer programs to deal with overlapping spots and for the normalization and intercomparison of spot patterns will be required.

In this paper we are concerned with the problem of developing a system and methods that will allow a number of isoelectric focusing gels to be cast reproducibly in parallel, to be isoelectrically focused together in a convenient manner, and to be recovered without loss of either sample identity or orientation. A method for casting up to 500 gels simultaneously for disc electrophoresis has been previously described (10), but is not easily adapted to the solution of the present problem. In previous studies (1), narrow glass tubes have been sealed at the lower end with Parafilm, and the acrylamide solution was introduced into each tube using a long narrow piece of polyethylene tubing attached to a syringe. Great care is required to eliminate bubbles, and only a limited number of gels may be cast before

polymerization occurs. The Parafilm is then removed and replaced with a cellulose membrane held in place by a small ring of silicone rubber. Since polymerization generally does not occur all the way to the bottom of the tube, there is a small space that may become filled with air when the Parafilm is replaced by the cellulose membrane.

For the present purposes, our requirements are that: (a) a series of uniform-bore tubes be filled with a polymerizing solution rapidly and to the same level, (b) polymerization extend all the way to the bottom of the tubes, (c) provision be made to close the bottom of the tubes with a porous closure in instances where the gels tend to slide out of the tubes, (d) the tubes be completely immersed in liquid during electrophoresis to insure adequate cooling, (e) the ends of the tubes be electrically isolated, (f) the tubes be permanently numbered, (g) the gels be recovered easily and in order, and (h) the tubes be easily and completely cleaned in preparation for the next run. In addition, the system should be adaptable to automatic sample loading and gel unloading. Ideally all events should occur in one device or apparatus. In our earlier versions of this system, the tubes were immersed in a three-phase liquid system consisting of a dense solution containing sucrose on the bottom, a Freon-Varsol mixture of intermediate density in the middle to insure cooling and electrical isolation, and a less dense upper electrode solution on top. In practice, this technique was found to be less convenient than the one described here.

THE ISO SYSTEM

Principles. The loading principle chosen is simple displacement of the dense urea-containing acrylamide solution into a row of identical tubes by water. Since there is excess acrylamide solution around the base of the tubes, polymerization all the way to the ends of the tubes is insured. A metal retaining strip is used to prevent gel from being withdrawn inadvertently from the tubes when they are pulled out of the acrylamide reservoir after polymerization is complete. The gel is then cut along the lower ends of the tubes, and the retainer strip is removed. Preisoelectric focusing to form a pH gradient of ampholyte, loading of samples, isoelectric focusing, and unloading are then done all in the same apparatus.

Apparatus. The ISO system is shown diagrammatically in Fig. 1, and a photograph of the apparatus is shown in Fig. 2. The large Lucite box (Fig. 1A), which serves both for displacement loading of the acrylamide gels and for isoelectric focusing, is closed with top B during electrophoresis. A removable rack C holds one electrode (the anode) and also supports the upper electrode reservoir D with attached glass electrophoresis tubes. Reservoir D, which contains the cathode, also has attached to its under surface two supports for the gel retainer F. The gel casting trough E holds the unpolymerized acrylamide solution during loading.

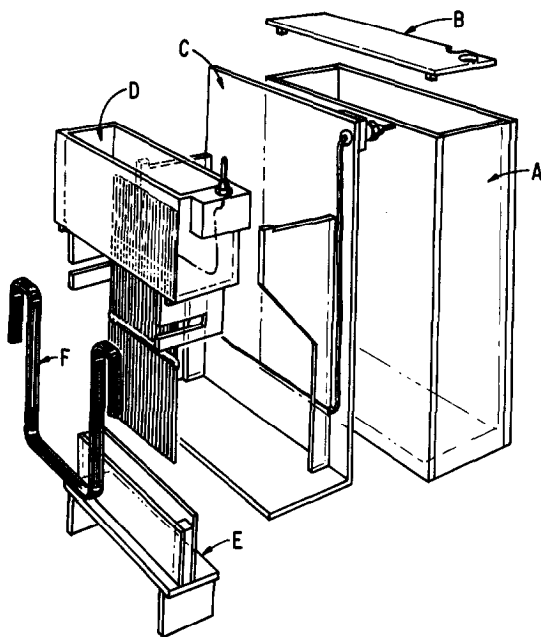


FIG. 1. Diagrammatic presentation of the ISO apparatus used for multiple-parallel isoelectric focusing in acrylamide gels. Components are: lower buffer box, A; top, B; removable rack supporting one electrode and upper buffer reservoir, C; upper reservoir with upper electrode and attached glass gel-holding tubes, D; gel casting trough, E; and gel retainer, F.

Two sizes of glass tubes have been used. For work with very small protein samples, especially where autoradiography is used for detection, 1.51-mm-i.d. tubing is employed.⁴ The pipets from which the tubes are prepared are supplied fire polished at the upper end. Only pipets with square ends and with no decrease in internal diameter due to fire polishing are chosen. These are cut off square 7.5 in. from the upper end, and the cut ends are ground briefly to remove sharp edges. The pipet calibration marks are removed by soaking in hot chromic acid. The tubes are then attached to the upper buffer reservoir in such a manner that no electrical leakage around the outside of the tubes can occur. This has been done in two ways. In the first, the tubes are attached together in rows of 20 with two strips of RTV silicone adhesive,⁵ and care is taken to insure that no pinholes remain between the tubes. When the adhesive has set, the strip of tubes is

⁴ The 1.51-mm-i.d. tubes were made from Corning disposable glass pipet, 2/10 in 1/100 ml, Catalog No. 7090, while the 2.67-mm-i.d. tubing was made from the 1-ml pipets, Catalog No. 7078, Corning Glass Works, Corning, N. Y. 14803.

⁵ Room temperature vulcanizing (RTV 108) translucent silicone rubber adhesive/sealant and SS-4124 silicone plastics primer are available from the Silicone Products Department, General Electric Co., Waterford, N. Y. 12188.

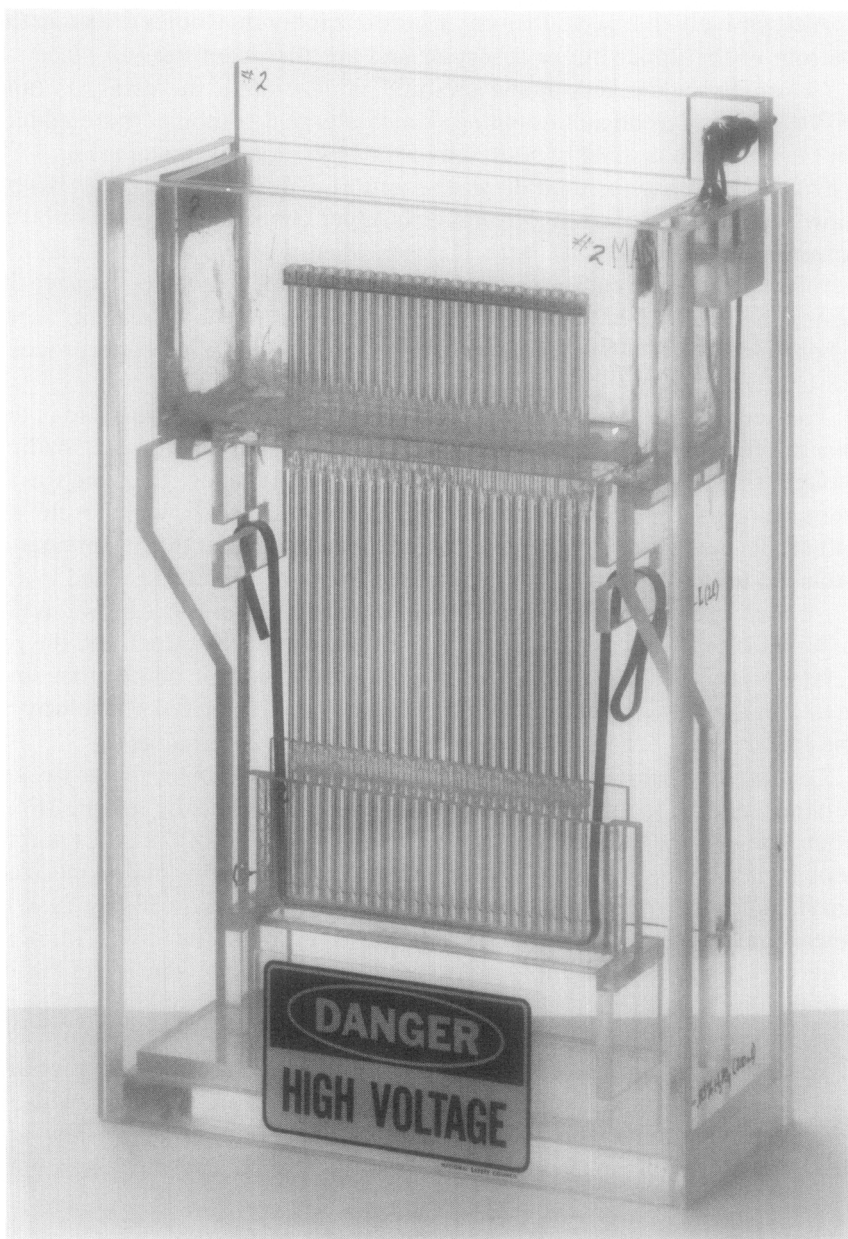


FIG. 2. Complete ISO apparatus in configuration for gel casting.

positioned in a slot in the bottom of the upper buffer reservoir and is sealed in place with more adhesive. The bond to Lucite is enhanced by the use of a silicone plastic primer.⁵ A numbered paper strip embedded in transparent silicone rubber attached to the tops of the tubes serves to identify them.

Alternatively the glass tubes are inserted in individual holes drilled in the bottom of the upper buffer reservoir and are then cemented in place.

Gel casting. The acrylamide mixture⁶ is deaerated, the catalysts (and NP-40,⁷ if used as an additional nonionic detergent solubilizer) are added, and the mixture is quickly poured into the lower loading trough (Fig. 3A). The gelling solution is then displaced up to the desired level (Fig. 3B) by slowly lowering the loading trough and upper chamber assembly into the lower reservoir which has been partially filled with water. Note that the acrylamide-urea solution is denser than the water used to displace it; hence the gel level in the tubes is below the level of the displacing water when casting is complete (Fig. 3C). Polymerization is allowed to proceed for approximately 60 min.

The acrylamide gels all the way to the bottom in the tubes and also in the loading trough. If the tubes are now pulled out of the gel in the loading trough, small lengths of gel may be pulled out of the tubes. To avoid this, a bent stainless steel strip is used as a gel retainer (Fig. 3D), which is pulled out of the loading trough *with* the loaded tubes. Note that the gel retainer is attached to the upper reservoir and not to the rack used to raise and lower the upper reservoir, tubes, and loading trough. The gel is cut between the tubes and the retainer with a fresh razor blade or a sharp scalpel, and the gel retainer is removed (Fig. 3E). The objective of this portion of the procedure is to produce gels of identical length which have polymerized all the way to the lower ends, but which do not connect to any external gel.

Loading and running. The apparatus is now reassembled, minus the gel retainer and the loading trough, as shown diagrammatically in Fig. 3F. A Hamilton syringe⁸ is used to remove the air in the tops of the tubes and to replace it with dilute NaOH (overlying a shallow layer of unpolymerized acrylamide-urea solution). The upper reservoir contains 30 mM NaOH (bicarbonate free), while 10 mM H₃PO₄ is used in the lower (anode) reservoir. Prefocusing is then carried out for 1 hr at 200 V. The lower buffer volume is a sufficiently large heat sink to make auxiliary cooling unnecessary.

Power is then turned off, the cover is removed, upper gel surfaces are rinsed with 30 mM NaOH, and the samples are applied with a microsyringe.⁸ Since the samples contain concentrated urea, they are denser than the upper electrode solution and hence stay in their proper

⁶ The following solution has been employed to form isoelectric focusing gels for the analysis of human serum proteins: urea, 8.25 g; pH 3.5–10 ampholyte (LKB), 750 μ l; 30% acrylamide + 1.8% bisacrylamide, 2.0 ml; water, 6.0 ml. Mix and degas thoroughly. Then add NP-40, 300 μ l; 10% ammonium persulfate, 30 μ l; and TEMED (*N,N,N',N'*-tetramethylethylenediamine), 20 μ l. This yields a volume of 15 ml which is more than adequate for a battery of 25 isoelectric focusing gels.

⁷ Available from Particle Data Laboratories, Ltd., 115 Hahn Street, Elmhurst, Ill 60126.

⁸ Hamilton 100 λ -syringe, Hamilton Co., Reno, Nev. 89510.

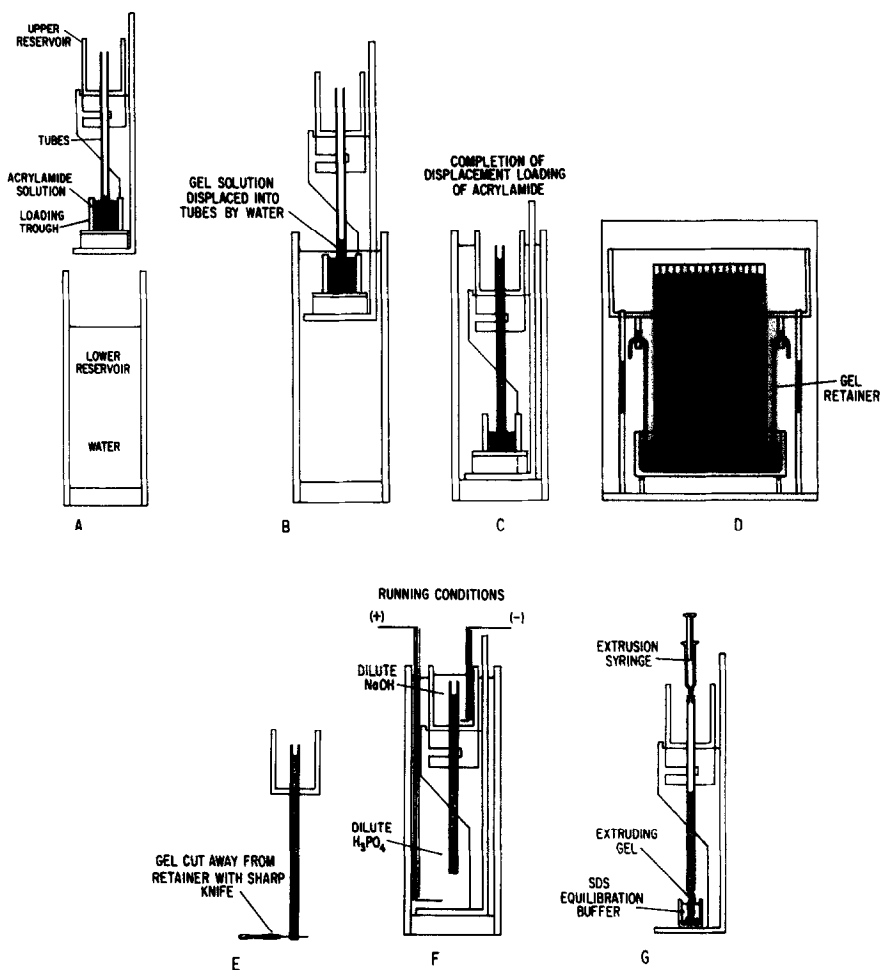


FIG. 3. Schematic drawings of operation of ISO system. (A) Acrylamide solution is placed in loading trough in position above water-filled lower reservoir. (B) Gel loading trough and entire upper assembly is lowered into water, displacing acrylamide solution up into glass tubes. (C) Completion of loading step: acrylamide solution displaced to final position in glass tubes. System left undisturbed until polymerization is complete. (D) Front view of system after removal from lower buffer box showing position of the gel retainer. (E) The gel is cut away from the retainer with a sharp blade. (F) Running conditions for preelectrofocusing or, after sample addition, electrofocusing. Anode is shown diagrammatically to the left for clarity. In practice, it is incorporated in the removable rack shown at right. (G) Recovery of gel by extrusion into SDS-containing equilibrium buffer.

positions atop the column. Samples may be overlaid with ampholyte if desired, and focusing is then carried out at 500–700 V for 20 hr using the arrangement shown in Fig. 3F.

When gels containing 4% acrylamide and 6% of that quantity of bisacrylamide (4% T, 6% C) are used, and when the tubes have been

properly cleaned, the gels adhere to the tube walls and do not slide out during the run. However, with lower concentrations of acrylamide, or when polymerization is incomplete, the gels may slide slowly out of the tubes. To prevent this, several layers of thick filter paper may be placed on a small Lucite block and positioned in such a manner that the ends of the glass tubes rest on the filter paper during the run, effectively preventing gel extrusion while allowing free passage of electric current.

Unloading. To unload the gels, the upper reservoir and attached tubes are positioned over a row of 5-ml (1.5-dram) collecting vials each containing 2 ml of an SDS equilibration buffer.⁹ The gels are extruded manually (see Fig. 3G) using a water-filled 1-ml plastic syringe fitted with a small Teflon adapter¹⁰-attached to a blunt-end 20-gauge needle.¹¹ The gels are extruded slowly since rapid or uneven extrusion frequently yields broken or shredded gels.

As soon as the gels are extruded, the vials are capped and placed on a rocking table¹² for 15 min and then frozen at -80°C until used for second-dimension runs as described in the subsequent paper (6).

Cleaning. After the gels are extruded, the upper reservoir and the tubes are rinsed with distilled water. The lower ends of the tubes are then placed in contact with a strip of thick filter paper to absorb water remaining at the ends of the tubes. The tubes are then carefully cleaned in a chromic acid cleaning solution by immersing the lower ends of the tubes in a deep rectangular Teflon vessel filled with warm cleaning solution. The tubes are then rinsed in distilled water followed by acetone and are dried in a stream of clean air.

Preparation of samples. Our initial studies have been concerned with human serum (7,8). An aliquot of fresh serum is added to a tube containing sufficient lyophilized urea and dithiothreitol (DTT) to make the final sample 9 M in urea and 100 mM in DTT. Since the urea occupies a large fraction of the solution volume, the correct proportions are 10 μl of serum added to the lyophilizate of 17 μl of 9 M urea-100 mM DTT. Alternatively, the proteins may be solubilized initially in SDS to which urea is then added. In this case, 10 μl of serum is mixed with 20 μl of 2% SDS, 5% mercaptoethanol, 20% glycerol, and the mixture is heated to 95°C for 5 min. After cooling, urea is added to saturation. During isoelectric focusing the SDS is gradually displaced and migrates to the bottom of the gels, forming a zone that swells

⁹ The SDS equilibration buffer contains: 10% glycerol, 5% mercaptoethanol, and 2.1% SDS, made up in 0.125 M Tris-HCl, pH 6.8.

¹⁰ The Teflon adapters were made by heat shrinking 2-mm Teflon tubing to form a small cone and attaching this to a 20-gauge stainless steel hypodermic needle having a squared end which had been polished to remove burrs.

¹¹ Available as Luer Stab Adapter, 20 gauge, Catalog No. 7564 from Clay Adams, Division of Becton Dickinson, Parsippany, N. J. 07054.

¹² Ames Aliquot Mixer, Ames Co., Division of Miles Laboratories, Elkhart, Ind. 46514.

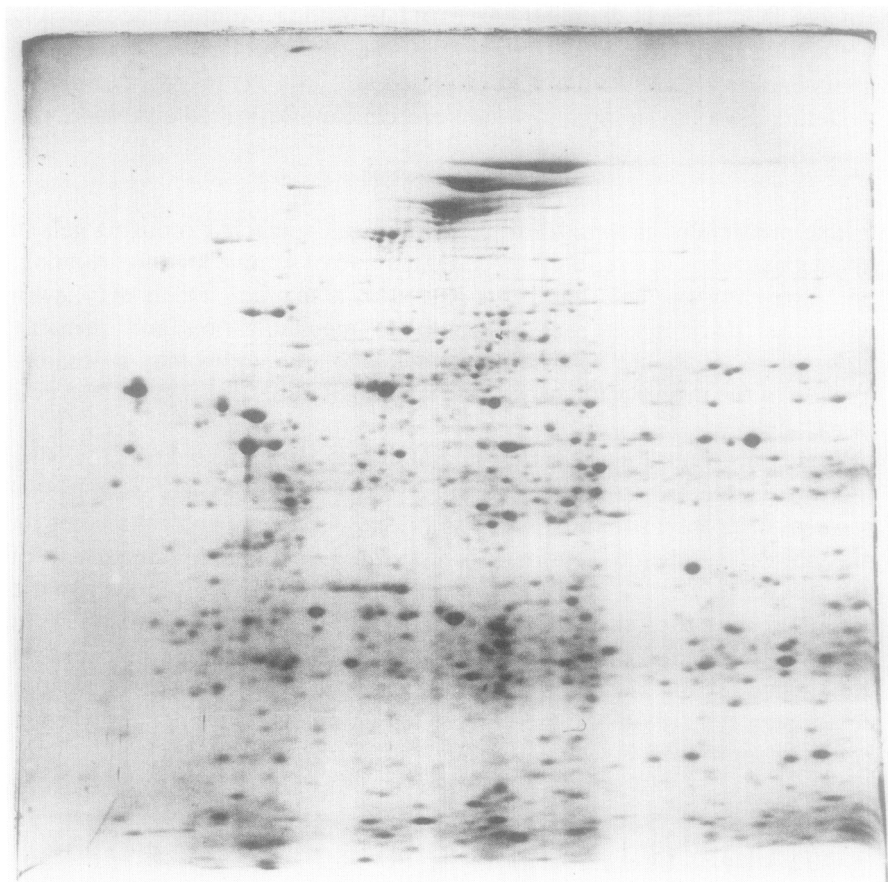


FIG. 4. Two-dimensional separation of proteins from crude rat liver mitochondria preparation. Acid side of isoelectric focusing gel is to the left.

during treatment with equilibration buffer and conveniently marks the acid end of the gel. Similar sample preparation techniques are used with subcellular fractions.

RESULTS

The resolution obtained using the combination of isoelectric focusing (as described here) and slab gel electrophoresis in SDS [as described in the subsequent paper (6)] is extremely high. The problems associated with measuring and evaluating resolution and with quantitation will be discussed in subsequent studies. However, an example of the resolution obtainable with a crude rat liver mitochondrial preparation stained with Coomassie blue and photographed at high contrasts with monochromatic light is shown in Fig. 4. A total of 677 spots are observed, many of which are

smaller than 1 mm in diameter. An estimate of the resolution theoretically obtainable may be gained from noting that approximately 29,000 1-mm spots can be fitted into the area of gel used.

Detailed studies on human serum proteins are described elsewhere (8).

DISCUSSION

The problem of casting 20 or more identical isoelectric focusing gels in small glass tubes in a reproducible manner and of sample loading, running, and extruding the gels has been solved in a manner which may, with additional development, allow complete mechanization and possibly automation. It should be noted that the same techniques may be readily applied to the problem of casting and running numbers of regular disc gels in parallel.

Since the theoretical resolution obtainable with isoelectric focusing followed by electrophoresis in sodium dodecyl sulfate is in excess of 20,000 proteins or protein subunits, the system is applicable to clinical studies on plasma, cell lysates, and urinary proteins and for the characterization of subcellular fractions. A central objective of our work is the systematic exploration of the differences between normal and tumor cells (11). In addition, the method has wide application in the evaluation of chemical mutagens (12) and in human genetic screening (10), hence our interest in developing semiautomatic methods to allow large numbers of analyses to be made reproducibly.

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