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Analytical Techniques for Cell Fractions

XXII. Two-Dimensional Analysis of Serum and Tissue Proteins: Multiple Gradient-Slab Gel Electrophoresis[1,2]

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Two-dimensional electrophoresis of proteins and protein subunits, employing isoelectric focusing in the first dimension and electrophoresis in the presence of sodium dodecyl sulfate in the second, yields the highest resolutions currently available. In this paper separations in the second dimension are considered (the so called DALT system). Methods for multiple-parallel casting of gradient gels in slab gel holders are described. The problem of electrical isolation of the ends of the slabs together with continuous cooling of both surfaces of the slab gel holder along their entire length has been achieved by running the gels in a horizontal direction in a three-compartment tank with the holders inserted in insulating septa. In the system described, 10 slabs are run simultaneously. This, however, is not the upper limit of the number of slabs which can be conveniently run in parallel.

In this series of papers, systems and techniques have been systematically developed for the fractionation of cells and tissues down to the molecular level with the ultimate aim of writing a molecular anatomy of human cells and tissues. The number of structural genes in man is not known with certainty, but has been estimated to be approximately 30,000 (1). Any serious attempt to develop a molecular anatomy must deal with this order of complexity. Two-dimensional electrophoresis using isoelectric focusing in the first dimension and SDS electrophoresis in the second (2–6) now begins to fulfill this requirement. In the previous paper, the development of a method for multiple-parallel isoelectric focusing (the first part of the ISO–DALT system[3]) was described (7). The present paper is concerned

[1] The United States Government's right to retain a nonexclusive royalty-free license in and to any copyright covering this paper is acknowledged.

[2] Work at Argonne was supported by the U. S. Energy Research and Development Administration.

[3] The term ISO–DALT is used to indicate briefly that the separation is based on ISOelectric focusing in one dimension and is proportional to molecular weight in the second, molecular weight being expressed in DALTons.

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with SDS electrophoresis in the second dimension for separation by molecular weight (the DALT part of the system). The problem is to perform, in parallel: (a) preparation of both the identical gradient acrylamide slab gels containing SDS and the stacking gels; (b) loading of the first-dimension gels onto them; (c) electrophoresis in buffer containing SDS, with both cooling and electrical isolation of the ends of the slabs; and (d) recovery of the gels for fixing and staining, or for autoradiography.

Since this two-dimensional electrophoretic method has wide applications in clinical chemistry and in genetic screening, it is important to be able to perform large numbers of analyses with a minimum expenditure of time and energy. The problems associated with the identification of the separated entities and with data reduction are considered elsewhere (8) and in later papers in this series.

THE DALT SYSTEM

Slab gel plates. Multiple clamps or fasteners to hold plates together have been inconvenient and, together with devices used to seal the bottom aperture between the plates, a source of leakage during gel casting. Loading, electrophoresis, unloading, washing, and drying of the components of the gel-holding apparatus are greatly facilitated if the glass plates and spacers are all in one piece, and if no clamps are required to hold them together. The holders we have developed (Fig. 1) are made of Pyrex plates,

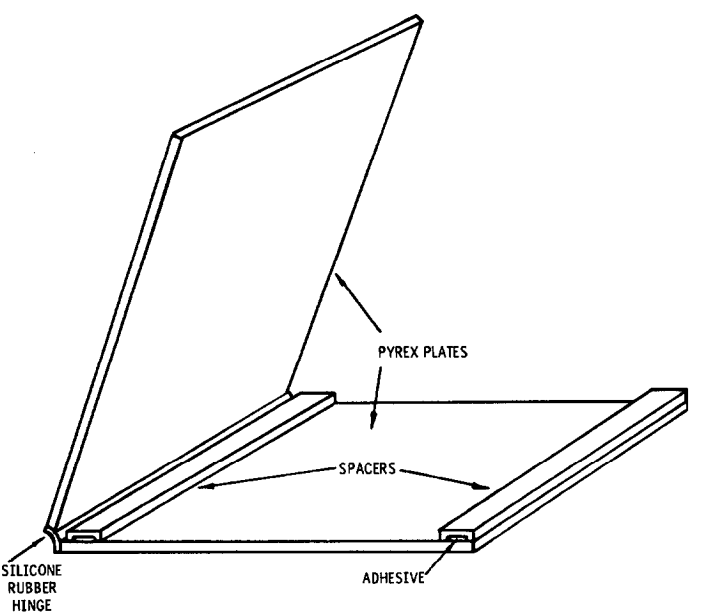


FIG. 1. Drawing of gel holder used in the DALT system.

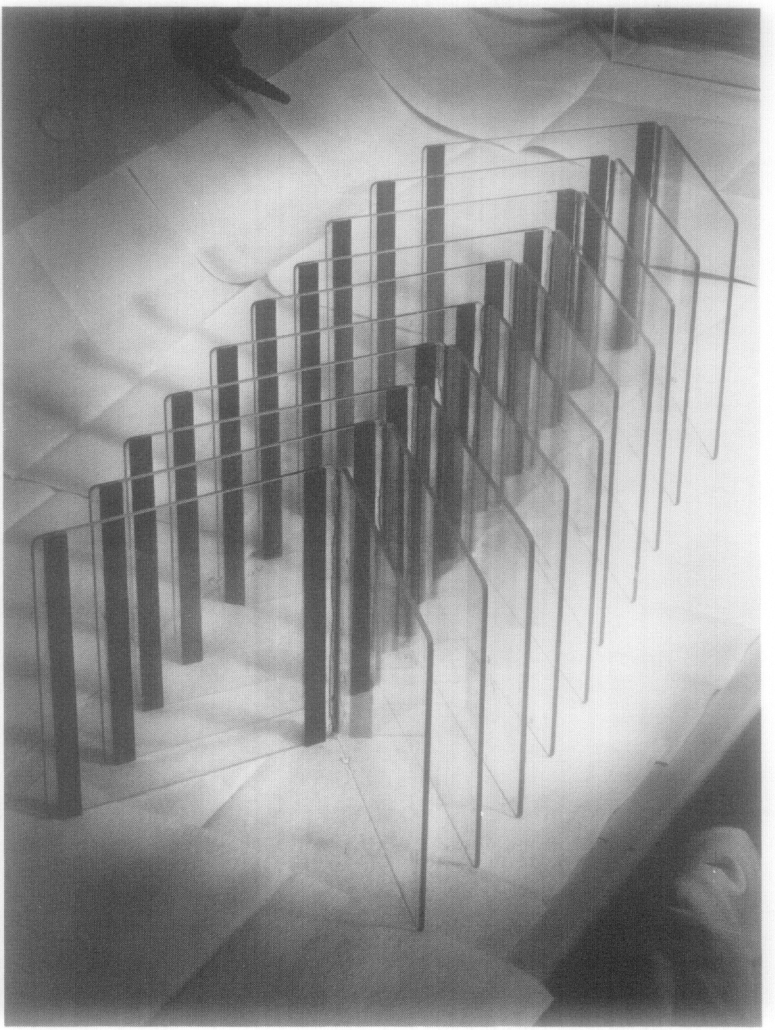


FIG. 2. Set of DALT gel holders drying after being washed in preparation for loading.

7 in.^{2} and ⅛ in. thick. Spacers of polyvinyl chloride or Lucite, either 1.5 or 3.0 mm thick, are glued in place with RTV silicone adhesive.[4] The spacers are grooved so that the adhesive is only along the center and therefore does not interfere either with the dimensions of the spacing or with polymerization of the acrylamide through the release of oxygen dissolved in the silicone rubber. The edges of the glass plates are ground and the corners are slightly rounded to minimize breakage. Each pair of plates is bound together by a silicone rubber sheet binding hinge, 1/16 in. thick,

[4] Room temperature vulcanizing (RTV 108) translucent silicone rubber adhesive/sealant, Silicone Products Department, General Electric Co., Waterford, N. Y. 12188.

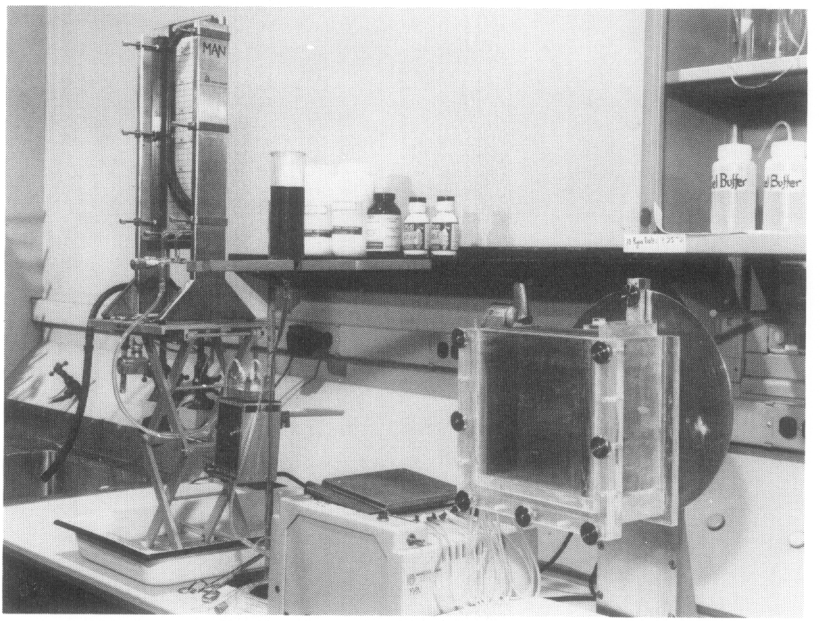


FIG. 3. Apparatus for simultaneously casting 10 gradient slab gels. Gradient former is on left, connected to magnetic mixer, manifolds, and peristaltic pump, and to the gel casting chamber, on right.

attached with RTV silicone adhesive. For drying, the holders may be arranged as shown in Fig. 2. Experimental holders have also been fabricated with glass spacers cemented in position with epoxy cement.

Gradient loading. We have initially designed systems for loading and running 10 gels in parallel. The principles described here apply also to larger numbers of gels, however. A gradient loading apparatus is shown in Figs. 3 and 4 and consists of a gradient forming device[5] attached to a small magnetic mixer. This, in turn, is attached to a first Tygon manifold (9) which connects the gradient line, vacuum line, dense displacing fluid line, and the line to the distributor manifold as shown diagrammatically in Fig. 4. The second Tygon manifold, in turn, connects to 10 peristaltic pump lines,[6] a peristaltic pump,[7] and to a gel casting chamber which can be rotated about one axis.[8] A level is incorporated into the chamber so that it can be

[5] Reeve Angel gradient former, available from ISOLAB, Inc., Drawer 4350, Akron, Ohio 44321.

[6] Peristaltic pump lines were 2.06-mm i.d., 2.5-ml/min flow rate, code color purple, Catalog No. 14-190-113. Tygon tubing, available from Fisher Scientific Co., 711 Forbes Avenue, Pittsburgh, Pa. 15219.

[7] Pump II, available from Technicon Corp., Tarrytown, N. Y. 10591.

[8] Two versions of the gel casting rotator have been fabricated. The second version is adapted to tilt the gel-casting chamber back before it is opened to prevent the gel holders from tilting forward and falling out when the chamber is opened.

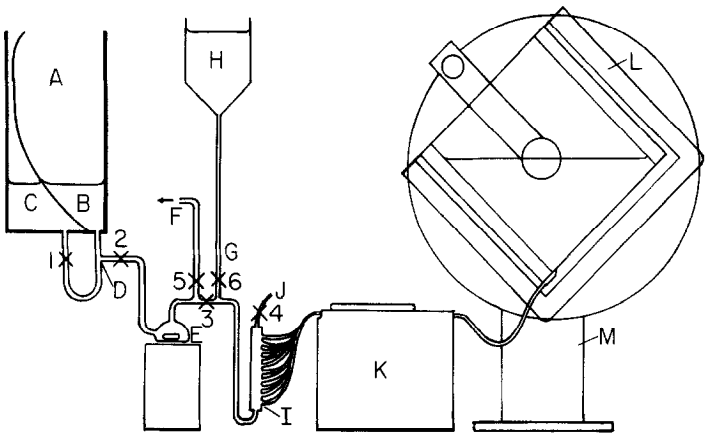
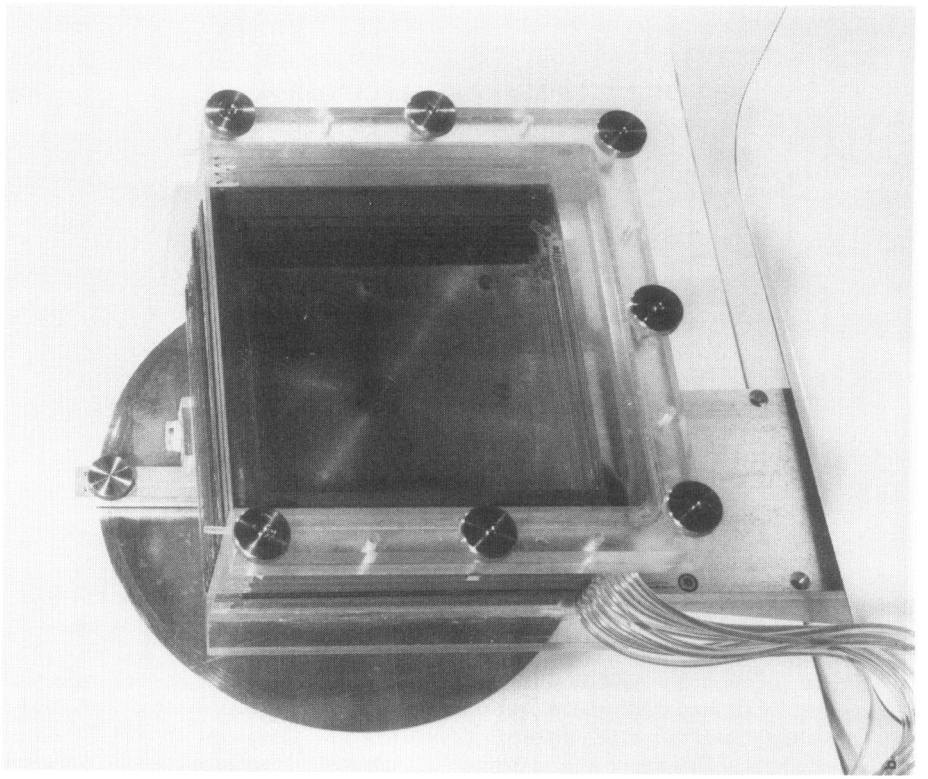
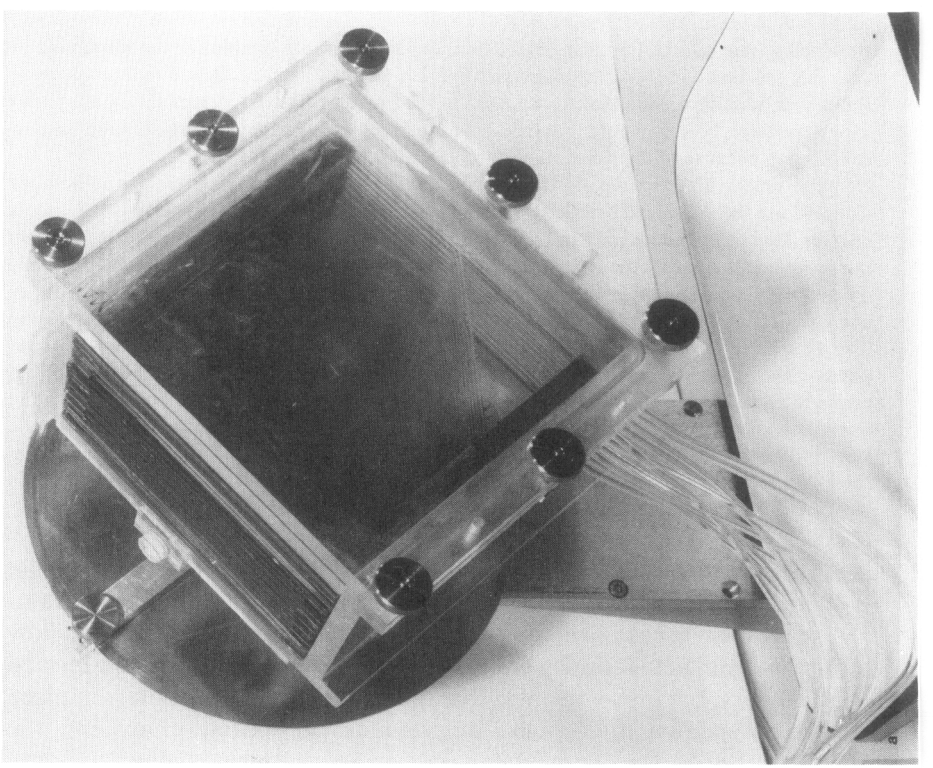


FIG. 4. Schematic drawing of gel casting apparatus. A, Gradient former; B, light acrylamide solution; C, dense acrylamide solution; D, junction where light and dense solutions meet; E, magnetic mixer with input at edge and exit at top to pass air bubbles easily; F, line to vacuum; G, inlet line for dense underlay solution; H, reservoir for underlay; I, 10-exit manifold; J, opening for air bubble removal; K, peristaltic pump; L, rotatable gel casting chamber; M, apparatus for holding and rotating gel casting chamber; X, clamping points: Initially all are closed except 3. Clamp point (C.p.) 1 is opened very slightly after dense solution is in place and fluid is allowed to flow as far as junction D. Line is then closed. C.p. 2 is then opened after light solution is in place, and light solution is allowed to fill magnetic mixer (which is started only after filling) and 10-exit line manifold. There should be no air bubbles in the displacing or underlay solution line G. At this point position 2 is clamped, and additional light acrylamide solution is added to reach the proper level in the gradient former. Proper levels are found experimentally; however note that light solution level is higher than dense solution level at equilibrium because of their density differences. The platten on the peristaltic pump is now clamped down with the pump off, thus closing the 10 pump lines. Any air bubbles in the large manifold are then removed by briefly opening C.p. 2 and 4. Flow to the gel casting chamber is now initiated by opening C.p. 1 and 2 and starting the peristaltic pump. When all lines through the pump and the line all the way to the chamber are clear of air bubbles, the pump platten is opened and the gradient is allowed to flow by gravity. Note relative heights of gradient former and gel casting chamber in Fig. 3. As the level of fluid in the casting chamber approaches the level at which it would overflow on the left side, the chamber is slowly rotated to a horizontal position and filling is completed. At this point, the pump platten is closed (with the pump off) to clamp the lines, C.p. 3 is closed, and C.p. 5 and 6 are opened. The pump is then started while water is poured into the gradient former, flushing out it and the magnetic mixer through the vacuum line to an aspirator pump. When the blue underlay solution has filled the small space in the lower left corner of the gel casting chamber, the pump is turned off, and after C.p. 6 is closed the platten may be opened. The gel is then allowed to polymerize for 1 hr.

accurately returned to a horizontal position. The chamber holds 10 gel holders and is closed via a front cover which seals against a narrow gasket.

The problem in casting identical gels is to arrange for the gradients to flow into them from below slowly enough so that identical gradients are formed in all holders, but fast enough so that the gradients are completely in place before polymerization occurs. In previous gel casting chambers, the chamber itself has been positioned above a large integral funnel of square





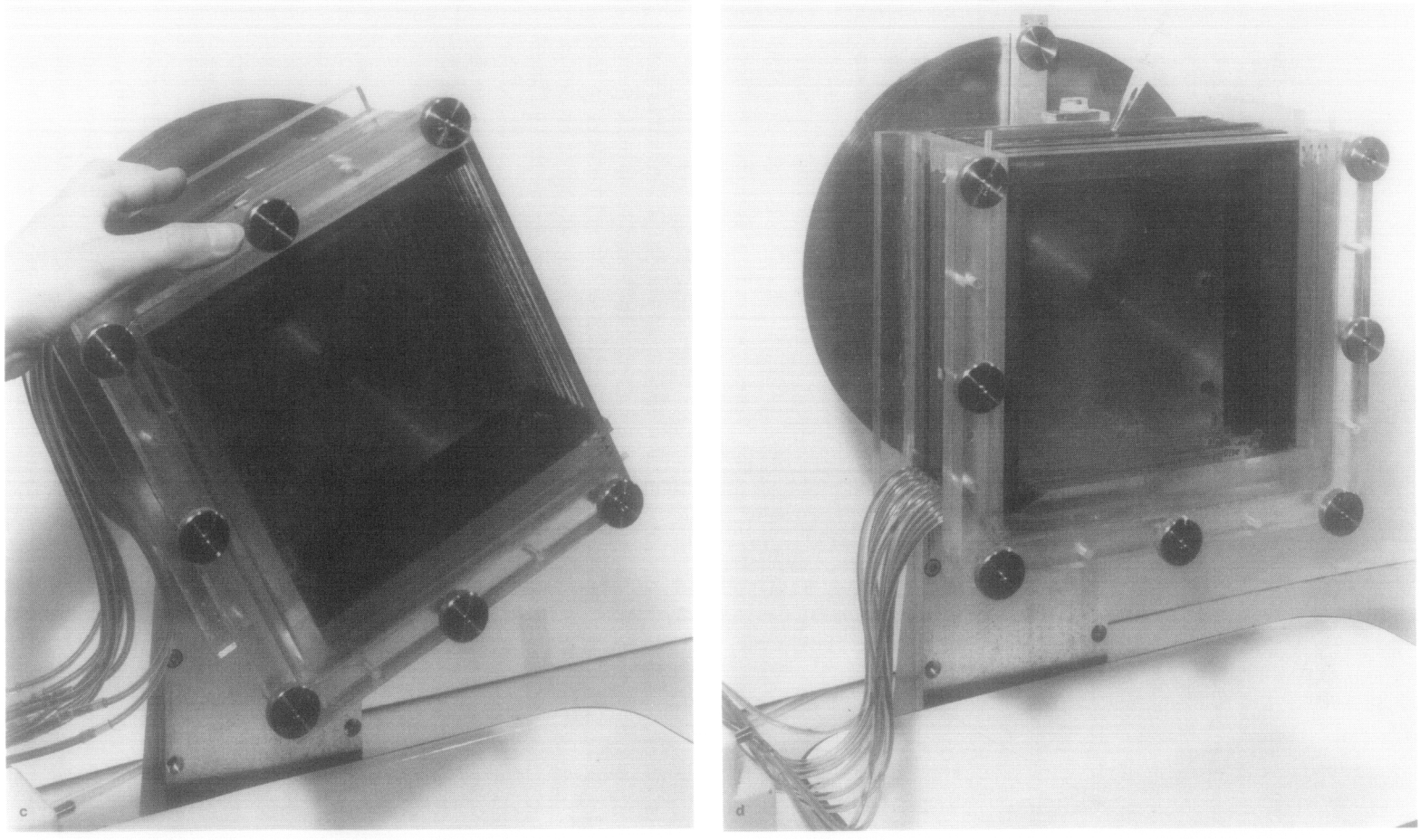


FIG. 5. Operation of rotatable gradient slab gel loading chamber. (a) Chamber tilted so that inflowing gradient enters a V-shaped space. (b) Horizontal position at completion of gradient loading. Identifying number strips are in position in lower righthand corner of gels. (c) Position for draining butanol or wash buffer. (d) Addition of stacking gel or butanol.

cross section which must be completely filled before any solution flows into the gel holders (10). The velocity of fluid flowing into the bottom of the funnel must be limited or mixing will occur. The function of the funnel is to provide a mechanism for decelerating the liquid gradient and for expanding the horizontal cross section to equal that of the bottom of the gel holders. We have solved the problem of flowing gradients rapidly into 10 gel holders simultaneously in the following way.

Our previous studies have shown that liquid gradients may be reoriented in a wide variety of physical configurations with little change in the shape of the gradient (11,12). We have therefore used the holders themselves to form the velocity deceleration funnel by tilting the chamber holding them to 45°. To insure even flow along one edge, the gradient is initially pumped in through 10 identical peristaltic pump tubes to the small corner chamber created by the bottom spacer (Fig. 5a). This provides even inflow along the entire length of one side. As mentioned, the chamber is tilted so that one corner forms the lower end of a Y-shaped funnel. Thus the inflowing gradient flows into all gel holders equally and, while flowing up in this orientation, decelerates. As filling proceeds, the chamber is slowly rotated back to an upright position (Fig. 5b). When the gradient is in this position, the gradient former, mixer, and vacuum line are isolated from the downstream portion of the system and a dense solution (generally 50% glycerol containing bromophenol blue for identification) is allowed to flow into the first manifold, through the second manifold, and on into the pump lines, finally filling the small corner space below the gel holders in the chamber and thus preventing clogging of the lines as polymerization proceeds. In practice the pump may be used initially to insure that all lines are filled and flowing, after which the pump platten may be raised and the liquid allowed to flow freely under gravity from the gradient former into the gel casting chamber. The pump is again used to insure even inflow of the glycerol solution. The preparation of solutions for 10 1.5-mm gels is described in Table 1.

As soon as the glycerol underlay is in position, the vacuum line attached to the first manifold is opened, and the gradient former and the magnetic mixer are thoroughly washed with distilled water and allowed to air dry. Note that by using flexible manifolds (9) made from Tygon tubing and clamps (hemostats) instead of valves, no blind spaces occur, and washing is simplified. In addition, if gelation occurs by accident, the gel can usually be extruded from the manifold and associated tubing.

When large numbers of samples are to be run, a positive method for gel identification is required. This is achieved by typing or stamping identifying run numbers on a strip of filter paper, cutting these into small squares, and dropping them in order into the right edge of the gel holders before polymerization occurs. These markers are thus an integral part of the gel and also appear on the gel photographs (e.g., Fig. 5b).

Before polymerization of the gel in the gel holders occurs, approximately

TABLE 1

MATERIALS USED IN PREPARATIONS OF GELS AND BUFFERS

Running gel buffer (stock solution)		
1.5 M Tris		
0.4% SDS		
HCl to pH 8.8		
Dilute 1:4 to rinse top of running gel		
Stacking gel buffer (stock solution)		
0.25 M Tris		
0.2% SDS		
HCl to pH 6.8		
Dilute 1:2 to rinse top of stacking gel		
Solutions for gradient gels		
10% gel	20%	
400 ml total	200 ml total	
130 ml	130 ml	30% acrylamide, 0.8% bisacrylamide in water
100 ml	50 ml	Running gel buffer, undiluted
160 ml	—	Water
—	17 ml	Glycerol
4 ml	2 ml	10% ammonium persulfate
130 μl	80 μl	TEMED
DALT tank buffer		
Tris base	111.0 g	(0.025 M)
Glycine	528.5 g	(.192 M)
SDS	36.7 g	
Water to 36.7 liters		

1 ml of water-saturated sec-butanol[9] is very slowly added to the top of each gel. This forms a sharp, level interface with the acrylamide solution. Polymerization is allowed to proceed for an hour, after which the gel casting chamber is rotated to allow the sec-butanol and any unpolymerized gel to drain out (Fig. 5c). The upper surface of the gel is then briefly washed with the buffer used to make the running gel, and this buffer is in turn drained off by rotation. After return of the chamber to a horizontal position, 3 ml of stacking gel (composition given in Table 1) is pipetted into each gel holder, followed again by 1 ml of water-saturated sec-butanol (Fig. 5d). When polymerization of the stacking gel is complete, the butanol is removed by rotation and draining, and the upper surface of the gel is washed with stacking gel buffer and again drained. The front of the gel casting chamber is then removed, the gel holders are separated, their

[9] Water saturated sec-butanol is prepared by mixing 500 ml of the alcohol with 250 ml of water at room temperature and shaking. The water phase remaining is about 60 ml.

exterior surfaces are rinsed with distilled water, and any external gel is removed. The holders are then placed in a suitable tray ready for sample loading. Note that the gel holders are not held together by clamps. Some care in handling is therefore indicated.

Loading with first-dimension gels. If the first-dimension gels (7) have been frozen, they are thawed by placing the vials containing them on a small heating block at 37°C until thawed. The gels are then poured out on a small sieve to allow the fluid to drain off, and the drained gel is placed on the loading platform shown in Fig. 6. The slab gel in its holder is placed in position as shown, and the isoelectric focusing gel is stretched out on the

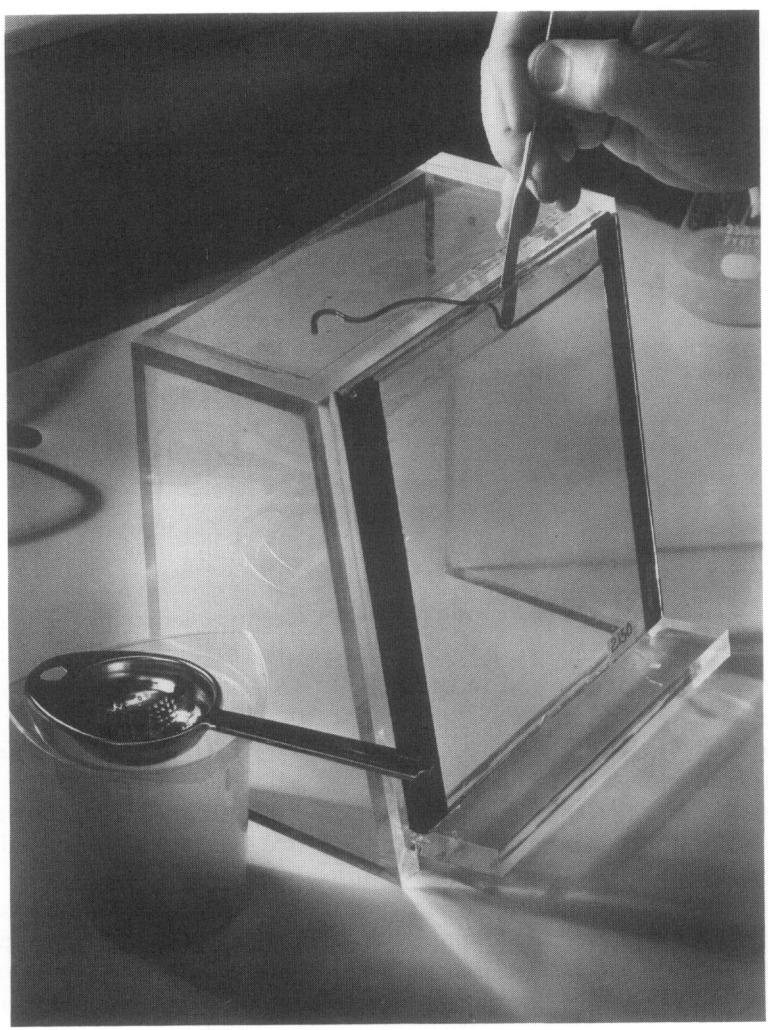


FIG. 6. Platform for loading isoelectric focusing gels onto slab gel. Sieve at left is used to drain equilibration buffer from sample gel.

loading platform, swollen end to the left.[10] The sample gel is then slid across into the slot between the slab gel plates and carefully smoothed into place with a small blunt spatula, taking care to remove air bubbles. The sample gel is held in position by an overlay of melted agarose prepared as described in Table 1. Note that the equilibration buffer used to partially soak out the ampholytes in the isoelectric focusing gel and to soak in the buffer used for SDS slab gel electrophoresis contains bromophenol blue. Hence the sample gel is easily visible. For convenience, the agarose solution is stored at 4°C in solid form and is quickly melted using a microwave oven immediately before use.

Slab gel electrophoresis. Since the sample gel from the isoelectric focusing step is imprisoned in position by an agarose gel overlay, the orientation of the slab gel during electrophoresis is of little consequence. (This is not the case when samples are in liquid form.) Hence the slab may be run in a horizontal (or even inverted) position. The important points are that the ends of the gel be electrically isolated and that the plates be cooled along their entire length. Both requirements are solved in the DALT tank shown in Fig. 7. The slab gels are simply slid into position in the septa which divide the tanks lengthwise into three chambers. Silicone rubber flaps close the 10 slots on each side when the slab gel holders are not in place, open sufficiently to accept the holders as they are inserted, and seal against the glass of the holders to prevent appreciable electrical leakage. Because the tanks are made of Lucite and are transparent, the movement of the dye front can readily be observed in all 10 gels, and the run is terminated when the dye reaches the end of the plate. Microswitches attached to the hinged tops of the tanks interrupt the current when the tops are opened. It is a simple matter therefore to remove and insert holders as required. When a 1-A power supply is used,[11] the run time is approximately 3 hr. At the completion of the run, the slabs are withdrawn manually and placed in a plastic rack to drain in preparation for unloading.

Two circulation systems are used in the center buffer chamber. The first[12] circulates chilled ethylene glycol through glass tubes in the bottom of the tank (Fig. 7). The second withdraws buffer from one side of the center compartment along its entire length and pumps[13] it back into the other side of the same chamber. A pair of plastic covers prevents evaporation and also activates the safety microswitches previously mentioned. Vertical

[10] The acid end of the gel generally swells, providing a useful polarity marker. In many instances sodium dodecyl sulfate is used to solubilize the protein before it is applied to the ISO gel. It is electrophoresed away during isoelectric focusing in urea and forms an identifying band at the acid end, again providing a useful marker.

[11] Direct current power supply, 0–600 V, 0–1.5 A, No. 6448B, available from Hewlett–Packard Inc., 195 Page Mill Road, Palo Alto, Calif. 04306.

[12] Masterline refrigerated bath and circulator, No. 2095, available from Forma Scientific Co., Marietta, Ohio 45750.

[13] Model MDX magnetic drive pump, with 5/8-in. inlet and outlet, available from March Manufacturing Co., 1819 Pickwick Avenue, Glenview, Ill. 60025.

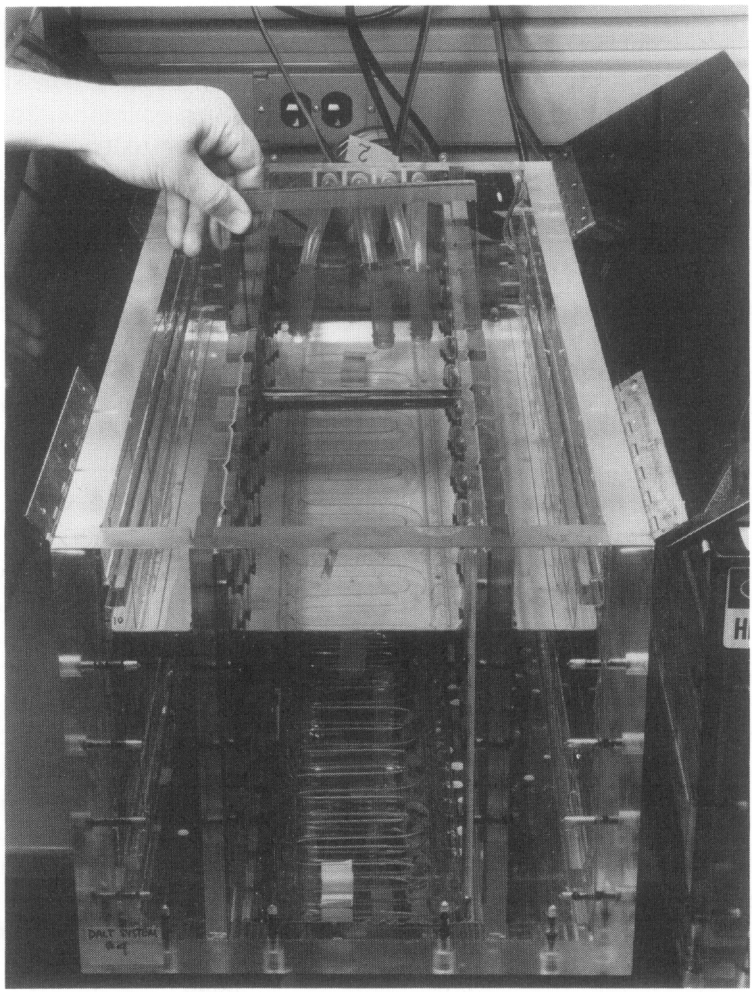


FIG. 7. DALT electrophoresis tank. Serpentine cooling coil is visible in the bottom of the tank. Above it on either side are visible buffer recirculation inlets and outlets. Two lengthwise dividers hold silicone rubber septa and divide the tank into three longitudinal compartments. Loaded slab gel is shown being inserted into position.

platinum wire electrodes are positioned along both side walls midway between the positions of the slab gel holders, thus insuring a uniform voltage gradient through all gels.

Gel recovery, fixing, and staining. For unloading, the gel holders are placed on a plastic holder as shown in Fig. 8, and the plates are opened using a narrow screwdriver. The holders ought not to be opened to lie flat, but can easily be opened 90°. The stacking gel is cut away with a small knife, which is also used to separate the gel from the spacers. The remaining gels

FIG. 8. Stand for unloading slab gels. These are carefully split open using screwdriver on left. Stacking gel is peeled away. Gel is cut along spacer and then carefully lifted out. In practice, two hands are used. Gel is placed directly in fixing and staining solution.

are gently lifted out and placed in groups of 10 in a plastic tray[14] where they are fixed and stained while undergoing constant slow shaking.[15] Alternatively, the gels may be placed on nylon or stainless steel screen holders which are then stacked and fastened together with metal or plastic screws, and the entire assembly is placed in a plastic fixing and staining box. The box may be adapted for electrophoretic destaining if desired, provided nonmetallic screens are used.

Photography. Destained gels are swollen in distilled water and are then placed on a sheet of flashed opal glass,[16] to which has been cemented a photographic step tablet under a strip of protective Pyrex glass, and then are positioned above a light box[17] for photography. For gels stained with

[14] Fixing, staining, and destaining, when done manually, are done in 4-qt plastic food keepers, No. 3034, available from Rubbermaid, Inc., Wooster, Ohio 44691.

[15] Trays are shaken on an Eberbach reciprocal shaker, No. 5850, Eberbach Corp., 505 South Maple Road, Ann Arbor, Mich. 48106.

[16] Flashed opal glass, 10 × 12 in., Catalog No. 140-4508, and photographic step tablets, No. 2, calibrated in 21 steps with a density range of approximately 0.05 to 3.05, Catalog No. 152 3406, are available from Eastman Kodak Co., 1186 Ridge Road West, Rochester, N. Y. 14650.

[17] Fluoroline Slimline Illuminator, Catalog No. SME 5003, available from Medical Systems Division, General Electric Co., 1120 Jorie Boulevard, Oak Brook, Ill. 60523.

Coomassie brilliant blue, an interference filter having a transmission maximum at 550.4 nm is used,[18] and photographs are taken through it using a 215-mm f 6.3 lens. For reproduction and where very small and often faint spots are to be visualized, high contrast film and processing are used.[19] For automatic densitometry of patterns, to be described in a subsequent paper, wide contrast films and developers are employed, with careful monitoring of exposure and processing and incorporation of the step tablets in each photograph.

Results obtained with this system using human serum proteins are described in detail elsewhere (8).

DISCUSSION

The technique and system for multiple-parallel slab gel electrophoresis described allows 10 gels to be run in parallel with a minimum of effort. For initial development, we have standardized on 10 gels cast and run together. However much higher capacity systems using the same principles can be constructed. The problems associated with reproducible staining, calibration, photography for quantitation, and precision automated densitometry are discussed in subsequent papers. We conclude that the ISO–DALT system is now sufficiently well developed to allow exploratory studies to be done on human patients to ascertain its usefulness in clinical chemistry and genetic screening.

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[18] Interference filter, 550.4-nm transmission maximum, 10.4-nm bandwidth at 50% T max, 50 mm², available from Baird Atomic, Inc., 125 Middlesex Turnpike, Bedford, Mass. 01730.

[19] Contrast Process Panchromatic Film 4155, 4 × 5 in., Catalog No. 168 7656, is developed for 5 min at 68°C in D-11 developer, Catalog No. 146 4569, diluted 1:1 with water, available from Eastman Kodak Co., 1186 Ridge Road West, Rochester N. Y. 14650. This is a very high contrast film and the exposure must be carefully controlled.