Illustrated Handbook for High Resolution of IAA Oxidase-Peroxidase Isoenzymes by Isoelectric Focusing in Slabs of Polyacrylamide Gel

by Merrill C. Hoyle

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ABSTRACT

Specific techniques are presented for high resolution of peroxidase-IAA oxidase isoenzymes in polyacrylamide gel slabs by isoelectric focusing in pH gradients. Banding patterns are entirely reproducible. pH gradients are linear and there is no "cathode shift." The enzyme stains are very sensitive to low catalytic activity. Since the extreme cathodic or anodic isoenzymes of peroxidase- IAA oxidase enzyme have isoelectric points beyond the limits of wide-range carrier ampholytes, it was necessary to adopt a more realistic definition of run-time. Also, a labeling scheme similar to that used in electrophoresis was adapted for isoelectric focusing by use of a standard "marker isoenzyme." The number of peroxidase-IAA oxidase isoenzymes revealed by this technique is more than twice that previously reported in the literature.

CONTENTS

INTRODUCTION

THE EXISTENCE of multiple molecular forms of proteins, which exhibit the same enzymatic activity, has been known since the early fifties (Shannon 1968). The term "isozymes" was first introduced by Markert and Møeller (1959) to describe these forms. Now, however, the term "isoenzymes" is preferred by the International Union of Biochemistry.

Isoenzymes are amphoteric molecules and will migrate in an electric field. Isoenzymes with the greatest net charge will move fastest toward the electrode of opposite charge, and vice versa. This fact is the basis for separation by the widely used method known as electrophoresis. Support media for electrophoresis are starch gels or polyacrylamide gels.

Improvements to this technique for separating isoenzymes came about with the development of carrier ampholytes by Vesterberg and Svensson (1966). The first commercially available carrier ampholytes were mixtures of polyamino-polycarboxylic acids that would establish a pH gradient (pH approximately 3 to 10) when mixed in gels to which voltage was subsequently applied. The gel is needed to prevent convective mixing of the carrier ampholytes, which are lined up in order of their isoelectric points, i.e., the points at which their electric mobility is zero (Haglund 1971).

The separation of isoenzymes by application of a voltage to carrier ampholytes in a gel is known as isoelectric focusing (IEF). An enzyme preparation is applied to a gel containing wide-range (i.e., pH 3 to 10) carrier ampholytes, a voltage is applied, a pH gradient forms, and each isoenzyme migrates to the pH in the gradient at which its net charge is effectively zero. Each isoenzyme collects or comes to "focus" at its isoelectric point (abbreviated as pI). This process, theoretically, results in a sharp banding pattern in which each isoenzyme is clearly resolved from its neighboring isoenzymes. The method can have a very high resolving power, i.e., be capable of separating isoenzymes with isoelectric points differing by only 0.02 pH unit (Fawcett 1968).

In practice, however, such excellent results have been difficult to obtain. Delincée and Radola (1970) reported a maximum of 20 horseradish peroxidase (HRP) isoenzymes (from a commercial preparation) resolved by IEF in flat beds of Sephadex[1] gel. More recently, Gove and Hoyle (1975) also found a maximum of 20 HRP peroxidase isoenzymes (from a different commercial preparation of slightly higher purity) by IEF in polyacrylamide gel cylinders. The former workers had applied constant voltage to drive their system, and had stained for peroxidase activity with *O*-tolidine.[2] The latter workers used constant pulsed power (watts) and stained with benzidine.[2]

Continuing studies in my laboratory of all the factors affecting the results of IEF culminated in the development of a method that yields much greater resolution of HRP isoenzymes than any previously reported.[3] More than 40 peroxidase isoenzymes of commercial HRP were resolved by use of the improved method, more than twice the number of peroxidase isoenzymes reported to date. In addition, all the isoenzymes were found to exhibit IAA oxidase activity in direct proportion to their peroxidase activity. This evidence, together with earlier work (Hoyle 1972; Gove and Hoyle 1975), provided rather conclusive evidence about the dual catalytic nature of this enzyme.

This handbook seeks to provide complete details of all the procedures used in this improved method of high-resolution IEF, for the benefit of those not familiar with this relatively new technique. Since this method utilizes inexpensive, easily acquired materials to construct the gel former, electrode assembly, and IEF chamber, the costs are much lower than those of commercially available equipment. The main expense is for a suitable power supply.

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[1] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

[2] Use of these compounds is now discouraged, for they are known to be strongly carcinogenic.

[3] High Resolution of Peroxidase-IAA Oxidase Isoenzymes from Horseradish by Isoelectric Focusing, (in press). Plant Physiology.

AN OVERVIEW

My earlier comments were intended to give the uninitiated, but potential, user of IEF a brief idea of how the technique developed, how it has been used, and what kinds of results one may expect.

Before getting into the step-by-step details that follow, I suggest that you, the user, read through the entire paper and make an inventory of the electronic equipment, various materials, and reagents needed. Check off those items you already have in your laboratory, then proceed to acquire the rest.

Prepare the necessary stock solutions, and make masters of the standard data sheets, with numerous copies. It would also be worthwhile to practice making a few gels in the gel former. (Note: It is best to use a new piece of tubing for each new gel slab.) Actual test runs should then be made with commercially purified enzyme to complete your check-out of all phases of the method. (Note: The method should work equally well for other enzymes and globular proteins, so your commercial enzyme or protein may be other than peroxidase. However, as discussed in the section on labeling isoenzymes, it is always necessary to use commercial HRP of RZ-1 purity to establish the location of the critical "marker isoenzyme" on your gel. I recommend Sigma HRP.)

Feel free to write or call the author if you wish to discuss any point.

PREPARATION OF STOCK SOLUTIONS

20 Percent acrylamide stock

Weigh out 20 g of acrylamide monomer (Fig. 1), taking all safety precautions—i.e., use of mouth and nose masks, rubber gloves, safety glasses, and lab coat—because acrylamide in monomeric form is a *neurotoxin*. Place in a 400-ml beaker. Then, weigh out 0.8 g of Bis (N, N′ — methylene-Bis-acrylamide, which is a cross-linking co-monomer) and add to the 400-ml beaker. Add about 75 ml of distilled-deionized (d-d) water, stir, and allow to set in the hood (a further safety precaution). Clean thoroughly area around balance. Stir acrylamide solution for a few minutes, then pour it into a 100-ml volumetric flask with three small rinses of the beaker. Build to volume with d-d water and invert 25 times. No particles should be visible. Pour solution into a plastic bottle, label, date, and store at 4° C. This stock solution is good for 6 months. When removing this solution from the cold, let it warm to room temperature before use—any visible particles should disappear with warming. Wash off gloves and bare hands.

Summary: 20 Percent acrylamide stock.

a. 20 g acrylamide
b. 0.8 g Bis
c. Bring to 100 ml final volume with d-d water.

2 Percent ammonium persulfate

This solution is made fresh on the day it is to be used. Weigh out 200 mg, place in a 10-ml volumetric flask, bring up to volume with d-d water, and invert the flask 25 times. Place flask in the hood. The bottle of ammonium persulfate (AP) should be kept at 4° C in a plastic bag half filled with a desiccant, e.g., silica gel. Replace gel when color changes from blue (dry) to pink (moist).

TEMED

This is N,N,N′,N′,-tetramethylethylene-diamine. It is used to catalyze the polymerization of acrylamide, and AP is an initiator for this reaction. TEMED comes as a solution ready for use. Caution—it is highly flammable and toxic to the skin. Use it in the hood and wear your rubber gloves, safety glasses (regular eyeglasses are satisfactory) and a lab coat. Store at 4° C.

Carrier ampholytes

These reagents come ready for use as 40 percent (w/v) solutions. At present, there are three manufacturers (refer to Appendix) of ampholytes. They can be purchased in wide (approximately pH 3 to 10, depending on the commercial source) or narrow ranges, e.g., pH 2 to 4, pH 6 to 8, pH 9 to 11. All work described in this handbook utilized wide-range carrier ampholytes. The bottles are sealed against contamination. When needed, a quantity of ampholyte is extracted by inserting the needle of a sterile, disposable hypodermic syringe through the rubber stopper, inverting the bottle, and withdrawing the required volume. Ampholytes are stored at 4° C.

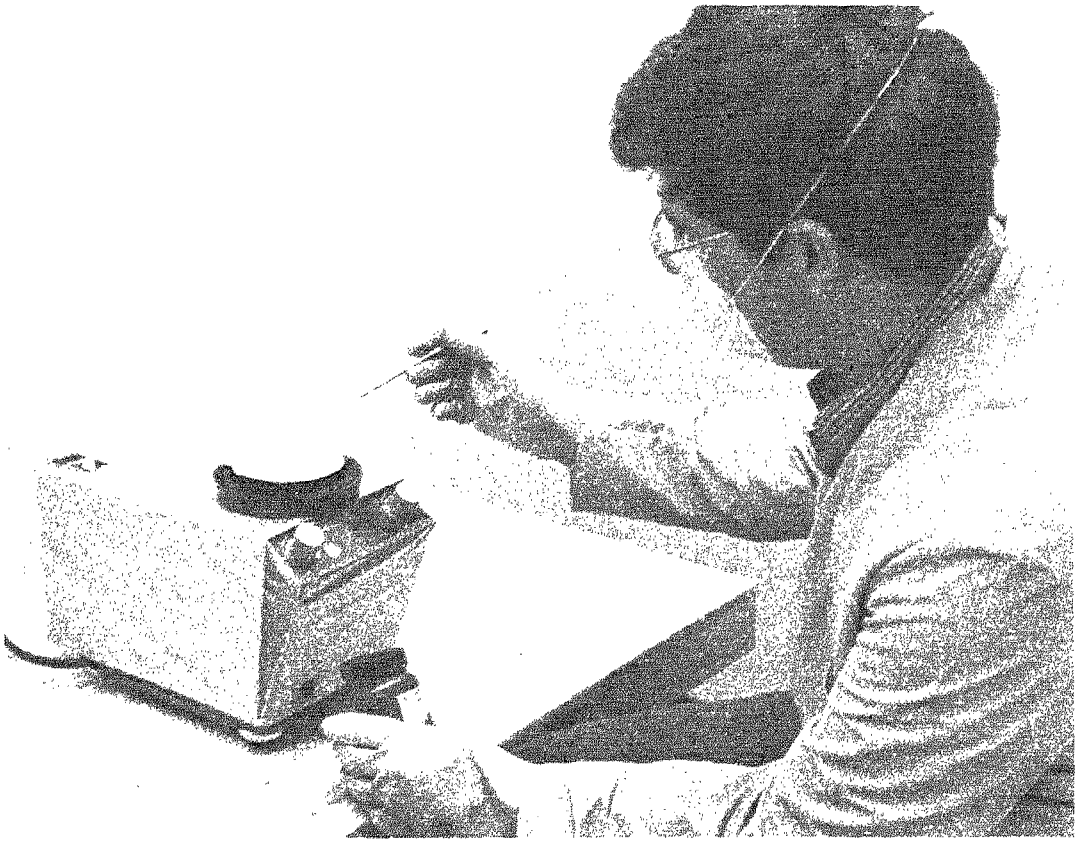


Figure 1—Weighing acrylamide monomer, using safety clothing (lab coat and rubber gloves) and safety devices (mouth and nose mask and eyeglasses).

Electrode solutions

The anolyte (for + electrode) is a 0.1 *M* solution of hydrogen chloride (HCl). Prepare a liter by adding 8.3 ml of 37 percent HCl to d-d water in a volumetric flask, mixing and bringing to final volume with d-d water. Do this in a hood. Do not breathe fumes or get acid on your hands.

The catholyte (for − electrode) is a 0.5 *M* solution of sodium hydroxide (NaOH). Prepare a liter by adding 20 g of 98.7 percent NaOH pellets slowly to a volumetric flask containing a few hundred ml of d-d water. Be careful of heat of dissolution; it is wise to run cool water over the flask until all NaOH pellets have dissolved. Build to volume with d-d water. Be careful in handling. NaOH is *very caustic.*

Peroxidase activity stain

The recipe given here is a modification of that given by Shaw and Prasad (1970). Buffer strength was increased, calcium chloride was omitted, development was carried out at room temperature, and glycerine as a fixative was omitted.

Dissolve 50 mg of 3-amino-9-ethyl carbazole in 5 ml of dimethylformamide (DMF) (Caution — work in a hood, because DMF is highly irritating to skin, eyes, and mucous membranes), and add the solution to 100 ml of 2 *M* sodium acetate buffer, pH 5.0. Prepare the buffer by mixing 29.6 ml of 2 *M* acetic acid with 70.4 ml of 2 *M* sodium acetate. Prepare a separate solution of 3 percent hydrogen peroxide (H_2O_2) by pipetting 1 ml of 30 percent H_2O_2 into a 10-ml volumetric flask and

bringing to volume with d-d water. Invert to insure good mixing, and store at 4° C. When a gel is ready for peroxidase staining, add 0.5 ml of this 3 percent H_2O_2 solution to 100 ml of the carbazole solution, stir, and pour gently over the gel slab.

IAA oxidase activity stain

This recipe was greatly modified from that given by Endo (1968), and is essentially a new formulation. As Endo has stated, the Fast Blue BB (FBBB) dye forms an insoluble complex with the IAA oxidase reaction products and stains the gel at the isoenzyme location. Three stock solutions are required:

Solution A — Dissolve FBBB in ethanol at a concentration of 4 mg/ml and store at 4° C. This solution is stable for many weeks.

Solution B — Add 16.4 mg of *p*-coumaric acid and 17.6 g of indoleacetic acid (IAA) to a 50-ml volumetric flask, and dissolve both compounds with a few ml of ethanol. Both compounds will be at 2 m*M* concentration when brought to final volume. Prior to this, however, prepare a 0.1 percent solution of H_2O_2 by diluting 0.3 ml of 30 percent H_2O_2 to 90 ml with d-d water. Pipet 2.4 ml of the 0.1 percent H_2O_2 into the volumetric flask and bring to final volume with d-d water.

Solution C — prepare 100 ml of 2 *M* sodium acetate buffer, pH 4.2, by mixing 73.6 ml of 2 *M* acetic acid with 26.4 ml of 2 *M* sodium acetate. When a gel is ready for IAA oxidase staining, prepare the staining solution by mixing 25 ml of A + 50 ml of B + 25 ml of C. This final solution contains 1 m*M* IAA, 1 m*M* H_2O_2, 0.5*M* sodium acetate buffer, pH 4.2, and FBBB, 1 mg/ml.

PREPARATION OF GEL SLAB

Assembling the gel former

Two glass plates, 24 cm x 16 cm x 3 mm, are needed. (Chemicals for making the gel can be warming up while the gel former is being prepared.) Plates should be *thoroughly clean* and should be handled by the edges only. Dab 12 spots of silicone stopcock grease on one plate and smear it around evenly with your fingers. With a Kimwipe,[1] smooth out the grease by brushing it gently in the direction of the long axis. With a fresh Kimwipe, repeat the brushing in the direction of the short axis. Repeat each wiping with lighter strokes. The grease should now be spread, thinly and evenly, over the glass surface. Set the plate aside for a few minutes to allow the grease to flow, creating a smoother, more uniform coating. The grease prevents the polymerized acrylamide gel from sticking to the plate.

Take a 65-cm length of polyethylene Intramedic[1] tubing (I.D.-1.14 mm, O.D.-1.57 mm) and lay it along the edge, 1 cm from the long side of the greased plate (Fig. 2). Cover this plate with a clean plate and clamp their edges together with binder paper clips, as illustrated. Continue laying in the tubing in this fashion along the bottom and up the remaining long side of the glass plates. Attach binder paper clips as you proceed, being careful to make the corners as square as possible without kinking the tubing. Upper end is left open. (Tubing can be re-used two or three times, but the risk of leaking gel solution increases with each use.)

Place the completed gel former in the hood, *standing upright* on a paper towel and wedged between two heavy blocks (or some thick books). Turn on the exhaust fan of the hood.

Mixing and pouring the gel

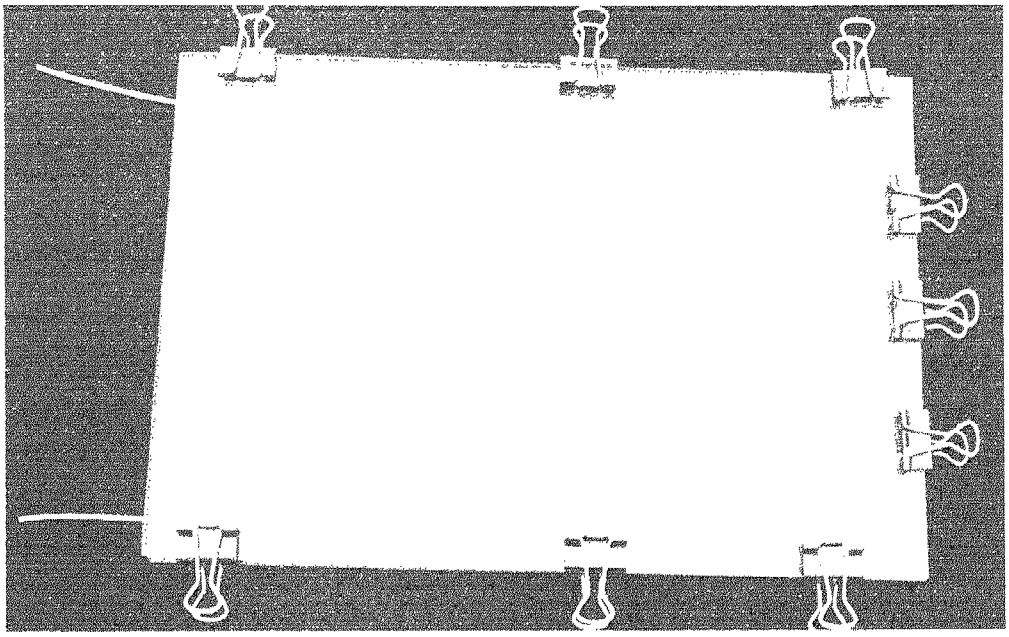
Put on rubber (or plastic) gloves and lab coat. Assemble the following items in the hood: a beaker and rinse water, a 50-ml Erlenmeyer flask, microcapillary tubing or Hamilton[1] syringe, two 10-ml T.D. serological pipets, one 1-ml T.D. serological pipet, a rubber pipet filler (to avoid mouth pipetting), and one 3-ml sterilized, disposable syringe.

Pipet 23.3 ml of d-d water into the 50-ml flask. Add 8.9 ml of 20 percent acrylamide stock, 30µl (0.03 ml) of TEMED, and 2.6 ml of wide-range carrier ampholytes (40 percent w/v); degas the mixture, then add 0.6 ml of freshly prepared 2 percent AP and swirl the flask to mix all components well.

Introduce the acrylamide solution, with a 9-in Pasteur pipet and squeeze bulb, into the gel former so that the solution runs down the tubing at the side (Fig. 3). It will be 5 min before the acrylamide starts to polymerize. Fill the gel former up to 1 cm from the top. Dispose of the Pasteur pipet and 3-ml plastic syringe. Rinse off gloves and wash hands.

Gel should be well polymerized after ½ h. Verify polymerization by tilting the gel former

Figure 2—Completed assembly of gel former. Note position of tubing between glass plates held with binder paper clips.



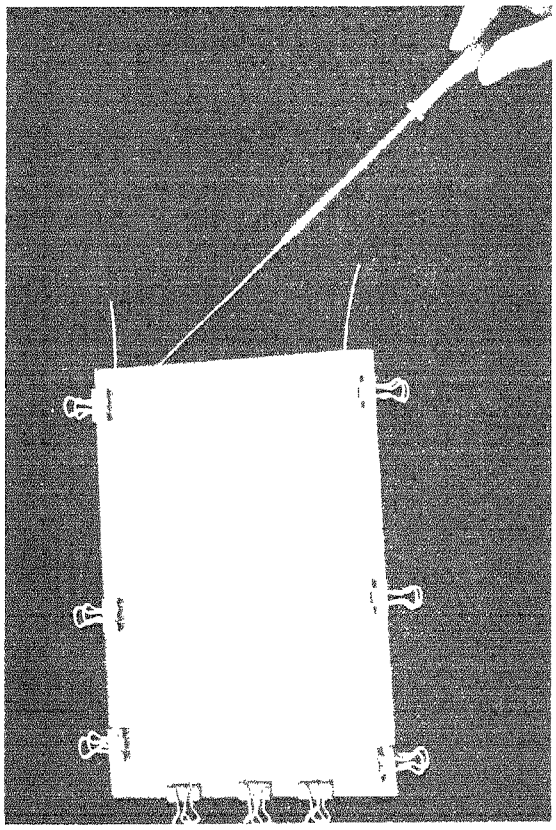


Figure 3—Using a Pasteur pipet to fill gel former with polyacrylamide solution.

back and forth edgewise to see if a thin layer of water moves back and forth over the top edge of the polymerized gel. When gel is fully polymerized (approximately 1 h), remove the binder clips, greased plate, and tubing. The gel slab will stick to the other plate. It is now ready for sample application.

PREPARATION OF DATA SHEETS

The following outline is suggestive only, but we have found it to be very satisfactory.

Date:

Title: Brief and clear.

Apparatus: Isoelectric focusing on a polyacrylamide gel slab using a _____power supply.

Gel: 5% polyacrylamide gel, persulfate-catalyzed, with 3 percent carrier ampholytes (state brand name).

Samples:

Slit #	*Sample*	*Concentration or Activity*	*Position Load*
1 to 6 or 1 to 8	Give name, extract No. date, etc.	1 mg/ml for commercial HRP. Variable for fresh extracts (express in units/ml).	Usually 4 μl, but can use 8, 12, or 16 μl, as needed.

Sample preparation: 1 mg/ml for commercial HRP. For fresh extracts, tell how they were prepared and to what degree they were purified.

Sample application: Direct injection into the gel or into slits.

Gel size and preparation: 16.5 cm x 13 cm x 1 mm (NOTE: thicker gels could be made with larger diameter tubing) gel slab made with 23.3 ml of d-d water, 8.9 ml of 20 percent acrylamide with Bis, 30 μl of TEMED, and 2.6 ml of ampholytes (mixture degassed) then 0.6 ml of 2 percent AP.

Activity stain: Whatever is pertinent.

pH gradient: Record as taken on the gel and plot on graph paper.

Power: _____W for _____h of continuous constant power maintained manually or automatically.

APPLICATION OF ENZYME SAMPLE

Template for sample placement

Draw to scale on paper a template of the glass plate and the outlines of the gel, as given in the data sheet (Fig. 4). Show on it the locations and polarity of the electrodes, the locations of paper wicks at the ends, and the intended location for sample applications. We use replications of a sample applied diagonally across the gel, as shown, or side-by-side applications (usually only six) located 7.4 cm above the anode end of the gel.

Applying samples to the gel

Samples (4 μl at a time) are taken up in a Hamilton syringe and applied into slits cut in the gel with a razor blade (Fig. 5), or are injected directly into the gel (Fig. 6). If more than 4 μl are required, due to low enzyme activity, another 4 μl can be injected along a 1-cm line directly below the first line of samples. We have applied as much as 16 μl total by this technique, with excellent results.

SETTING UP THE ISOELECTRIC FOCUSING APPARATUS

Preparation of electrode assembly

Two carbon electrodes (of the kind used in welding), about 8mm in diameter and cut to 16.5 cm length, are glued (epoxy resin with catalyst) to the ends of 20-cm glass stirring rods (Fig. 7). The distance between the inner edges of the carbon electrodes should be exactly 16.5 cm. Each carbon electrode should extend 1 cm beyond the glass rod, so that battery clips can be attached. Arbitrarily label one electrode (+) and the other electrode (−), as illustrated. A third glass rod is glued across the other two glass rods to give more ridigity to the assembly.

The wick-electrode-gel connection

After enzyme samples have been applied, place filter paper wicks, 3.5 cm x 15.0 cm (Whatman[1] 3 MM), across each end of the gel so that their inner edges are 16.5 cm apart; this will be indicated by the paper template beneath the gel plate (Fig. 8). Then, place the electrode assembly on top of the inner edge of the paper wicks, as illustrated (Fig. 8). Tape each glass rod, in two locations, to the underside of the glass plate (Fig. 9), thus bringing the electrodes to bear firmly on the paper wicks and gel.

Place the entire assembly in a glass baking dish slightly larger than the assembly (Fig. 9). The glass dish is then set on an aluminum cookie sheet. Use a Pasteur pipet to moisten the (+) wick with 0.1 *M* HCl and the (−) wick with 0.5 *M* NaOH. *Do not* moisten paper wicks to the point that free solution runs out on the plate! Attach battery clip of the red cable to the (+) electrode and the battery clip of the black cable to (−) electrode (Fig. 9).

Press a plastic cover, about 10 to 13 cm high, down over the glass baking dish and lead the electrodes out through their respective holes in the top (Fig. 10). Tape the electrode cables to the plastic cover, so they are held firmly in place. Place weights on the plastic cover to hold it down. Plug the electrodes into the proper receptacles on the power supply (Fig. 10), then place all this apparatus in a cold room at 4° C.

MONITORING THE ISOELECTRIC FOCUSING RUN

The next crucial issue is electricity. What electrical parameters are important, and how should electricity be supplied to the electrodes of the IEF apparatus? Various types of power supplies are available (Fig. 11). Those providing constant voltage or constant current were widely used in electrophoresis, and were also tried with IEF in the early stages of its development. Heating of the gel proved to be a problem, and isoenzyme separations were not as good as predicted by theory. When pulsed power (i.e., watts) was used, the results were not much better than with other types of power supplies (Righetti and Drysdale 1974). The determination by Allington et al. (1975) that continuous constant power should produce the best resolution of isoenzymes for IEF in polyacrylamide gels has been confirmed by results obtained in my laboratory.

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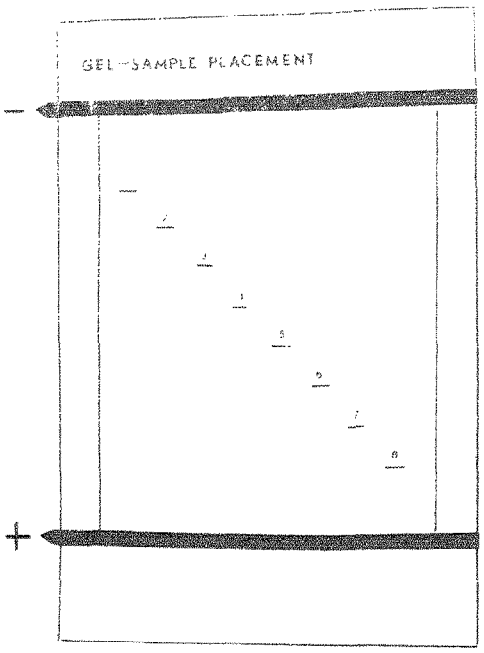
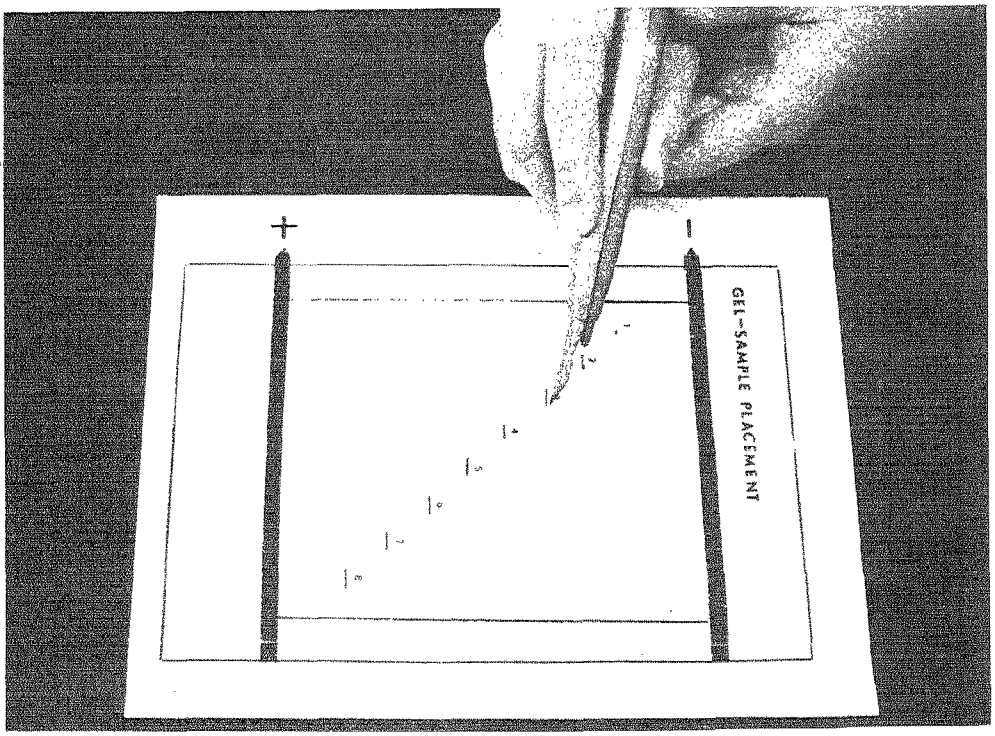


Figure 4—Paper template used in making 1-cm slits for enzyme placement for a diagonal run.

Figure 5—Cutting sample slits in the gel, with a template beneath the glass plate.



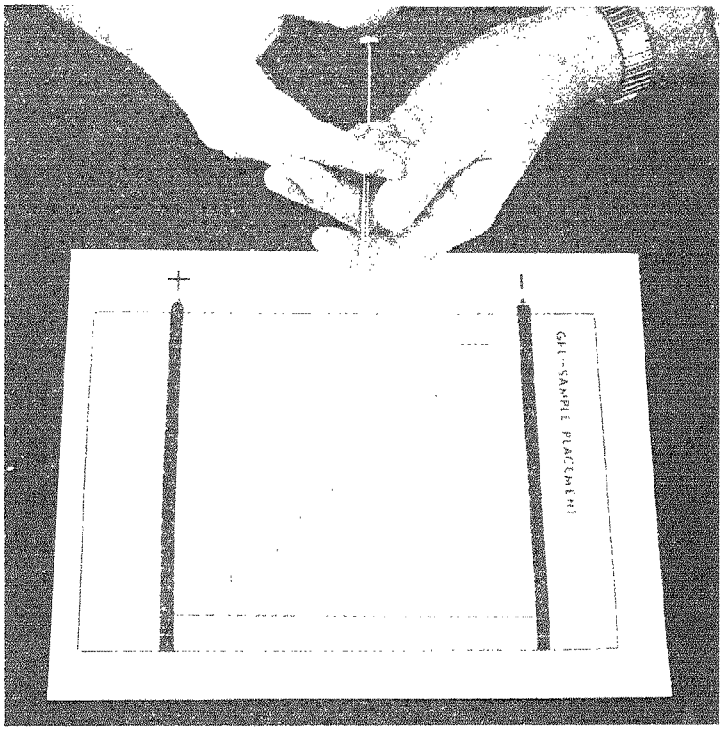
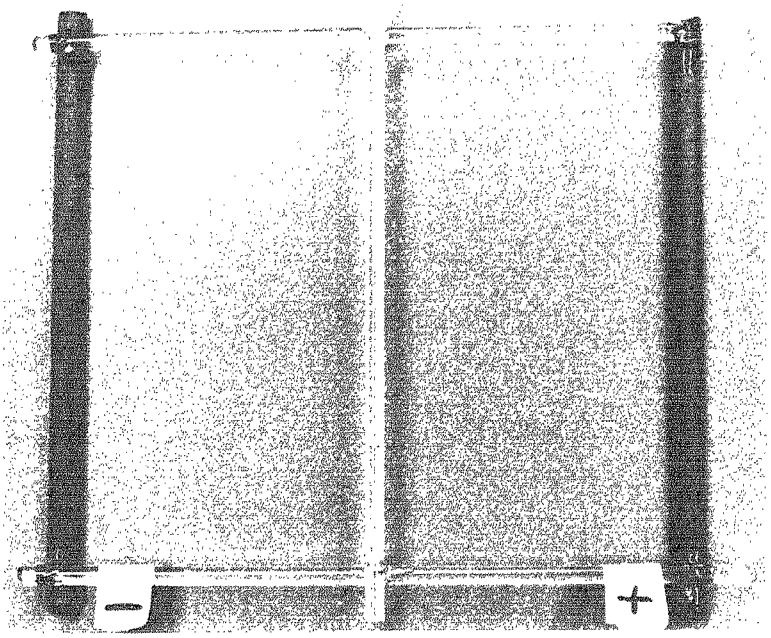


Figure 6—Applying enzyme samples into slits, or by direct injection, with a microliter syringe.

Figure 7—Completed electrode assembly.



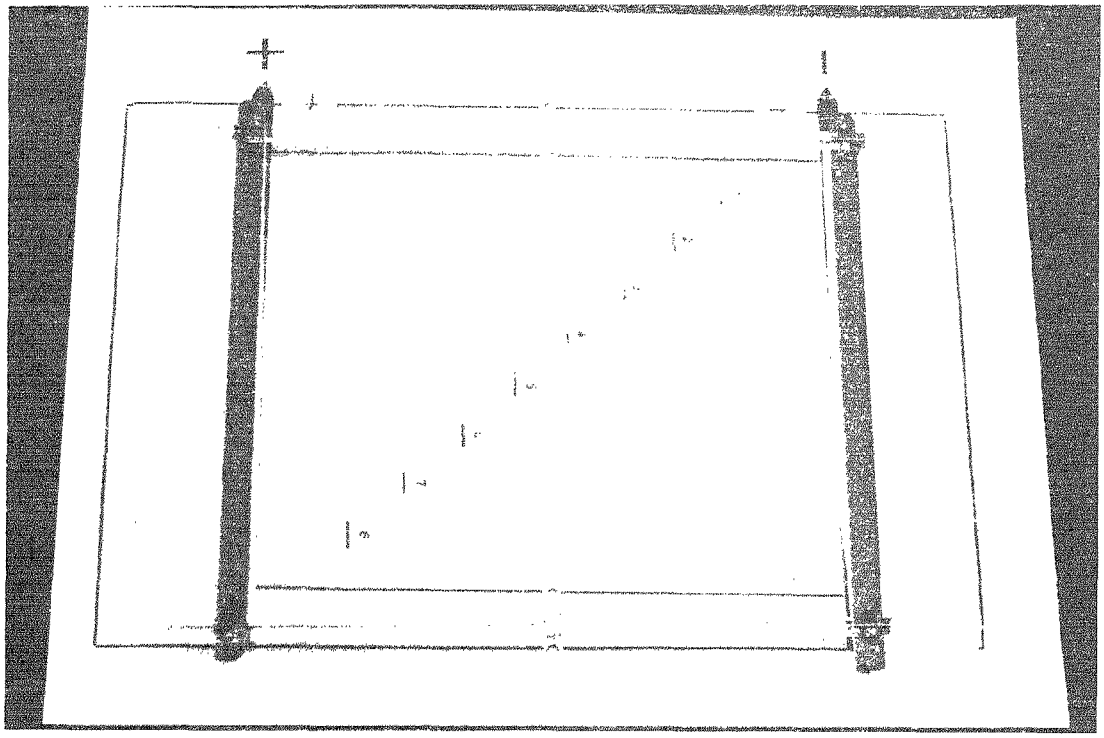


Figure 8—Electrode assembly in place on the gel.

Figure 9—Electrode assembly is taped to the glass plate (to insure close contact between gel and electrodes) and set in the baking dish; cables are clamped to their respective electrodes.

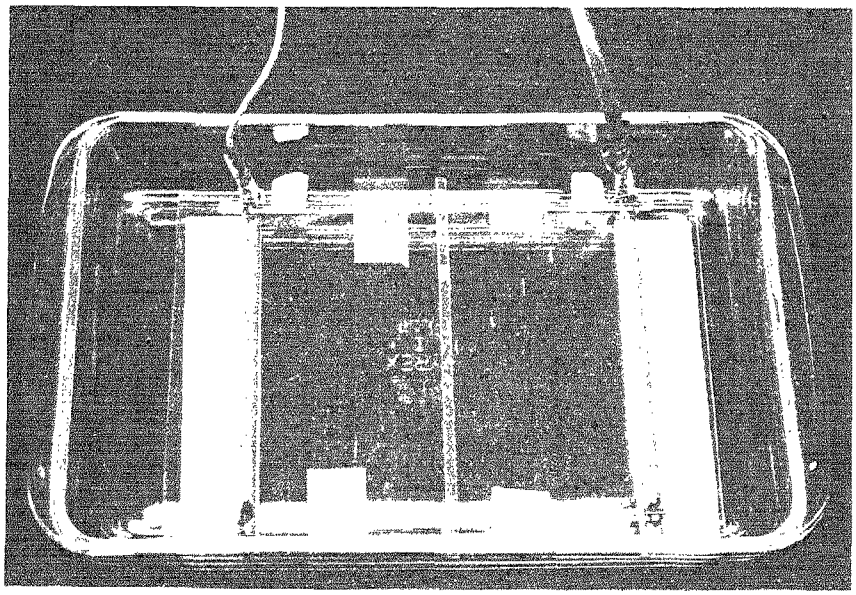


Figure 10—Entire IEF chamber is assembled and cables are plugged into a power supply.

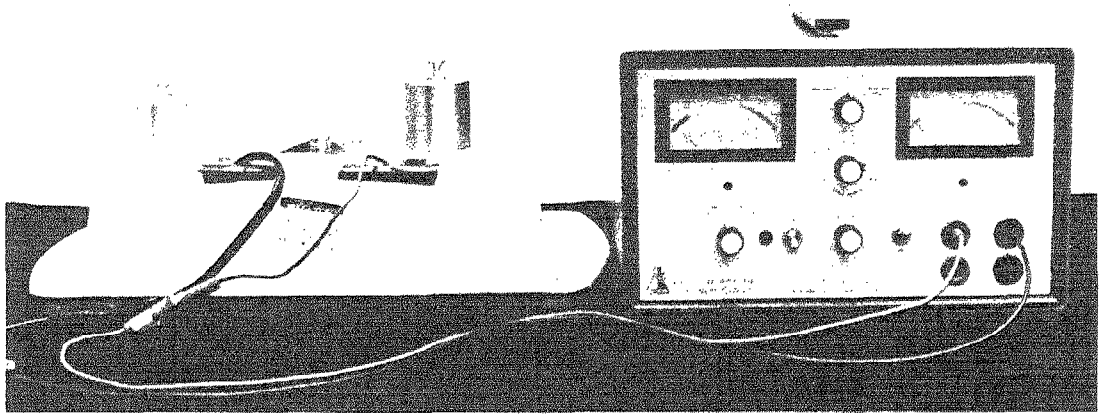
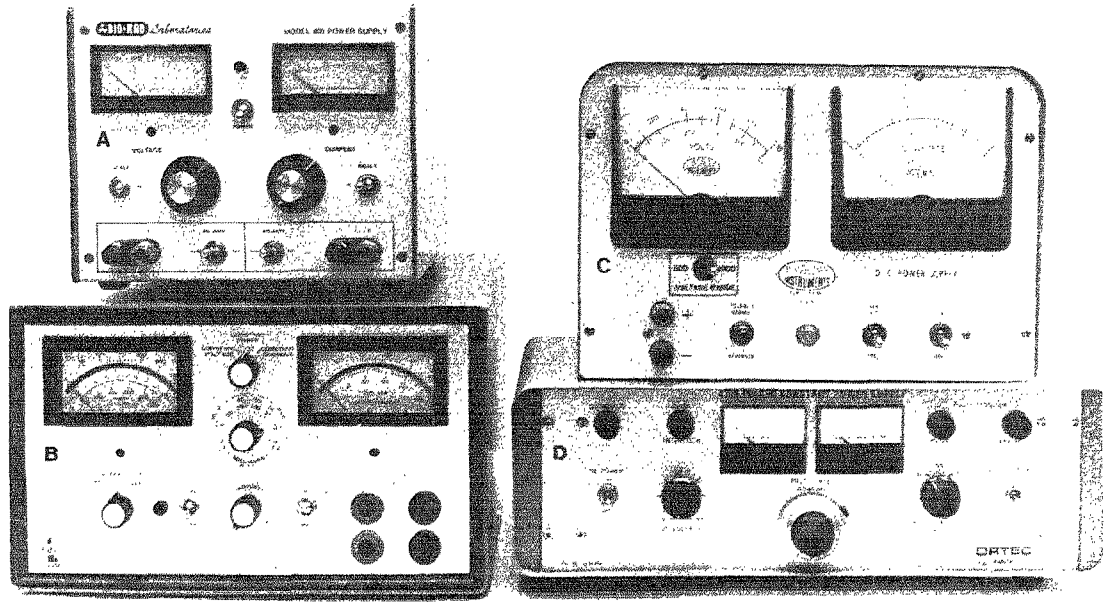


Figure 11—Examples of power supplies: A—constant voltage plus constant current; B—constant power; C—nonregulated; D—pulsed power.



Manual regulation of constant power

Continuous constant power can be supplied to the IEF apparatus by an unregulated power supply (e.g., C in Fig. 11) that is monitored by a technician and readjusted, as necessary, to maintain constant power. In practice, the technician reads the voltage and current,[4] multiplies the two to calculate power level, and then manually readjusts the power supply to maintain the desired power output. In our laboratory, we use a prepared worksheet in taking readings and making calculations at 15-min intervals for the duration of the run (see Table 1). The power cannot be kept absolutely constant; it fluctuates over a narrow range. The technique is very effective despite these small fluctuations in power.

[4] Current is recorded from the meter in milliamps. Multiplication by 10^{-3} gives amps (I). See Table 1.

Automatic regulation of constant power

More recently, power supplies have been developed that can supply continuous constant power by automatic regulation (e.g., B in Fig. 11). This type of power supply does not require an attendant during a run, and gives excellent results.

Power, run-time choices

With an unregulated power supply requiring constant monitoring and readjustment, we have found that a 6.5-h run, with power maintained between 1.5 and 3.0 W (Table 1), will give good separations for HRP when wide-range carrier ampholytes are used. Use of a power supply that is automatically regulated for continuous constant power, employed on a run of 6.5 h at 2.5 W, yields similarly good results.

In these two cases, the gel slab is prepared in the afternoon of the day before the run and is stored in a plastic bag at 4° C. The following day,

Table 1.—*Sample worksheet for regulating power (W) during a 6.5-h IEF run with a manually regulated power supply*

Time	Settings changed?	Volts (E) $E = \frac{P}{I}$	mA $\times 10^{-3} = (I)$	Power (W) $W = E \times I$	Resistance (R) $R = \frac{E}{I}$
8:30 a.m.	Yes	100	19.8	1.98	5,051
8:45	No	105	13.2	1.39	7,954
8:46	Yes	200	23.4	4.68	8,547
8:47	Yes	160	18.5	2.96	8,649
9:00	No	165	14.8	2.44	11,149
9:15	No	170	11.8	2.01	14,407
9:30	No	170	10.0	1.70	17,000
9:45	No	175	9.3	1.63	18,817
10:00	No	180	8.5	1.53	21,176
10:01	Yes	260	12.5	3.25	20,800
10:02	Yes	240	11.7	2.81	20,513
11:00	No	250	8.1	2.03	30,864
11:15	No	250	7.5	1.88	33,333
11:30	No	255	7.0	1.79	36,429
11:45	No	255	6.3	1.61	40,476
12:00 p.m.	No	260	5.6	1.46	46,429
12:01	Yes	320	7.1	2.27	45,070
12:02	Yes	340	7.6	2.58	44,737
12:03	Yes	360	8.3	2.99	43,373
12:30	No	360	6.9	2.48	52,174
1:00	No	360	4.5	1.62	80,000
1:15	No	365	4.0	1.46	91,250
1:16	Yes	460	5.0	2.30	92,000
1:17	Yes	500	5.5	2.75	90,909
1:30	No	500	5.5	2.75	90,909
1:45	No	505	5.0	2.53	101,000
2:00	No	510	4.5	2.30	113,333
2:15	No	510	4.1	2.09	124,390
2:30	No	515	3.8	1.96	135,326
2:45	No	515	3.5	1.80	147,143
2:55	No	515	3.4	1.75	151,471
3:00	STOP RUN —				

samples are applied, the IEF run is made, the pH gradient is measured, the gel is stained, and pictures are taken of the results.

We have obtained optimal resolution of HRP isoenzymes with a 17-h run at 1 W. On a given day, the gel slab is prepared and the samples are applied. The entire apparatus is put in the cold room and power is applied[5] at the end of the work day. The following morning (17 h later), the run is stopped and processing of the slab is carried out as described above. This schedule has now become our standard method of operation. It utilizes time efficiently, gives optimal results (runs with high power for shorter times are always inferior), and does not crowd too much work into one day.

DETERMINING THE pH GRADIENT IN THE GEL SLAB

Preparing the microelectrode

Allow the pH meter to warm up for ½ h before use. Calibrate the microelectrode (Fig. 12), first in pH 7.0 standard buffer, then in pH 4.0 standard buffer. Be sure to remove the rubber cap over the potassium chloride filler hole (or slide the sleeve down, whichever is appropriate) before calibration and actual measurement.

Making pH measurements on the gel slab

Place the glass plate (with gel slab and paper wicks) on a flat surface. Insert a 30-cm (1-ft) ruler under the edge, so that the 0 cm mark is at the edge of the anode (+) wick, and the 16.5 cm mark is at the edge of the cathode (−) wick (Fig. 13).

Slip the microelectrode down (in its holder) until the glass membrane electrode (the center electrode in Fig. 12) makes contact with the gel at the edge of the anode (+) wick. This is your zero reading. The reference electrode (side-arm in Fig. 12) should touch the gel at the same position. Continue readings at 1-cm intervals and record the distance *vs* pH in a lab notebook. The final pH reading is taken at 16.5 cm, i.e., right at the edge of the cathode (−) wick. Plot your pH gradient curve (e.g., see left half of Fig. 16).

[5] We use lineman's rubber gloves—capable of insulating against 10,000 V as a safety precaution when operating the power supply, and we have a rubber mat on the cold-room floor.

STAINING THE GEL SLAB FOR ISOENZYME ACTIVITY

Peroxidase activity

After pH measurements have been made, place the glass plate with gel and paper wicks into the baking dish again (Fig. 14). Pipet 0.5 ml of 3 percent H_2O_2 solution into 100 ml of the aminocarbazole solution, mix them, and gently pour the mixture over the gel. Reddish-purple bands will appear in 10 to 15 min. Although development time depends on the activity of the enzyme preparation, do not let straining proceed too long, lest the most active bands become too dark and blur around the edges. Less active bands will appear later.

With plastic gloves on, pick up the glass plate by the edges and use a wash bottle of d-d water to rinse off the residual straining solution: Caution—*do not* let the stream of water hit the edge of the gel at any angle less than 90°, because water may get under the gel and break the adhesive forces that hold the gel to the plate. It is desirable to keep the gel stuck firmly to the glass plate until the conclusion of an IEF experiment. Insure that the paper wicks remain in their initial positions, because the edge of the anode (+) wick marks the zero point along the gel (refer to Fig. 13). If the cathode (−) wick should slip, replace its edge at exactly 16.5 cm from the zero edge of the anode (+) wick.

A deposit of carbon from the (+) electrode distinguishes the anode wick (see this in Fig. 13). The carbon anode will eventually have to be replaced, but only after numerous runs.

IAA oxidase activity

Mix 25 ml of solution A plus 50 ml of solution B plus 25 ml of solution C and pour the mixture gently over the IEF gel adhered to the glass plate and resting in the glass baking dish (i.e., same process as used for the peroxidase stain).

Again, the duration of developing time will depend on your personal evaluation; the bands should stain dark gray. Rinse the gel with d-d water, as described for the peroxidase stain, and take the same precautions in handling.

Text Continues on Page 16.

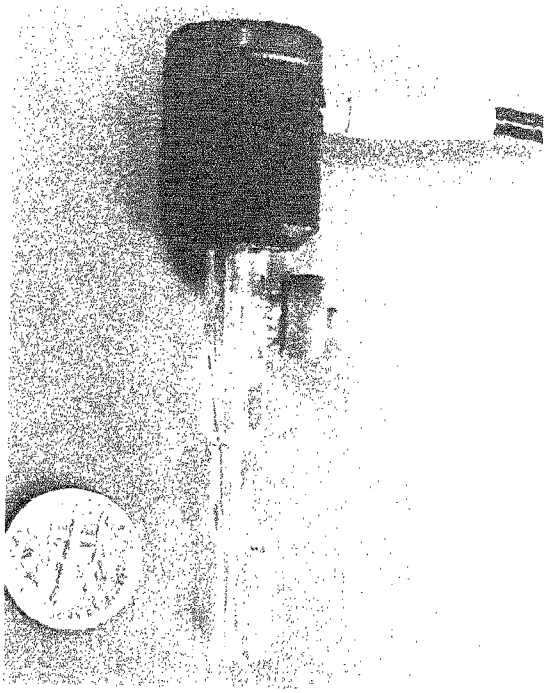
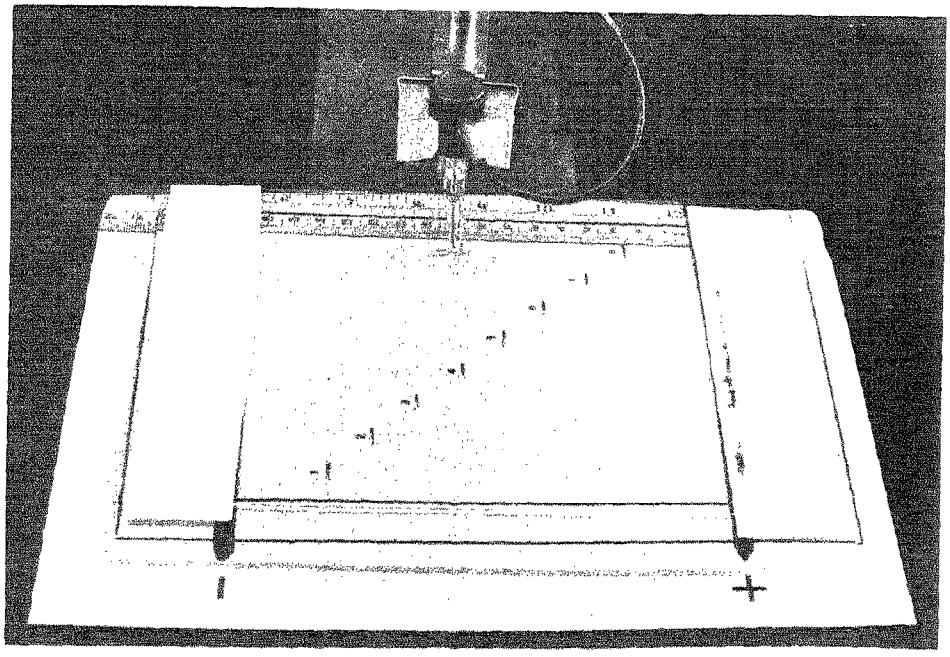


Figure 12—Enlargement of the combination micro-pH electrode. Coin is 18 mm diam.

Figure 13—Arrangement of gel, micro-pH electrode, and metric ruler for measuring the pH gradient at 1-cm intervals.



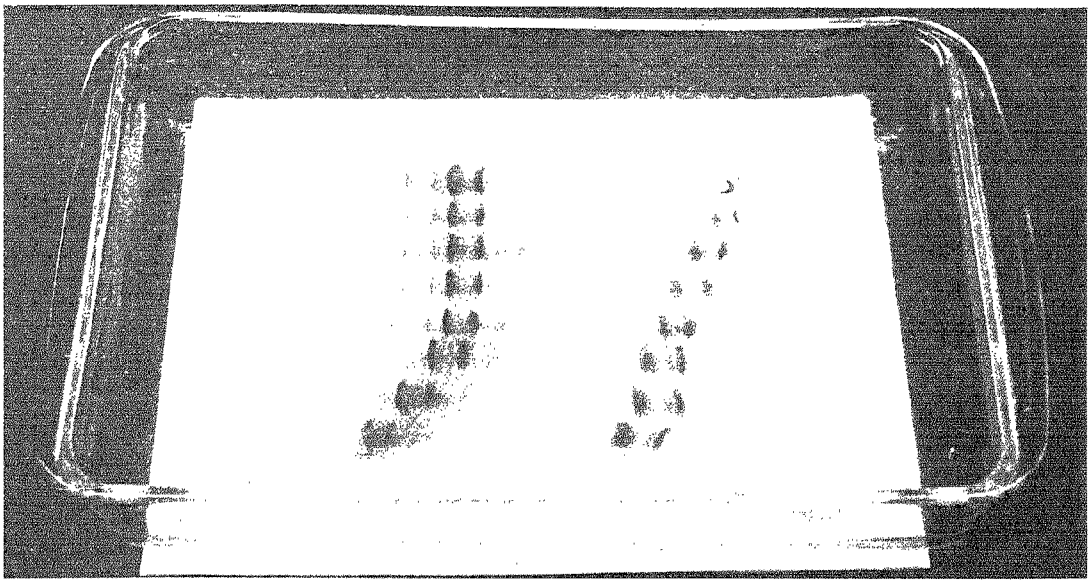


Figure 14—Use of the baking dish as a container for developing a gel slab for peroxidase activity.

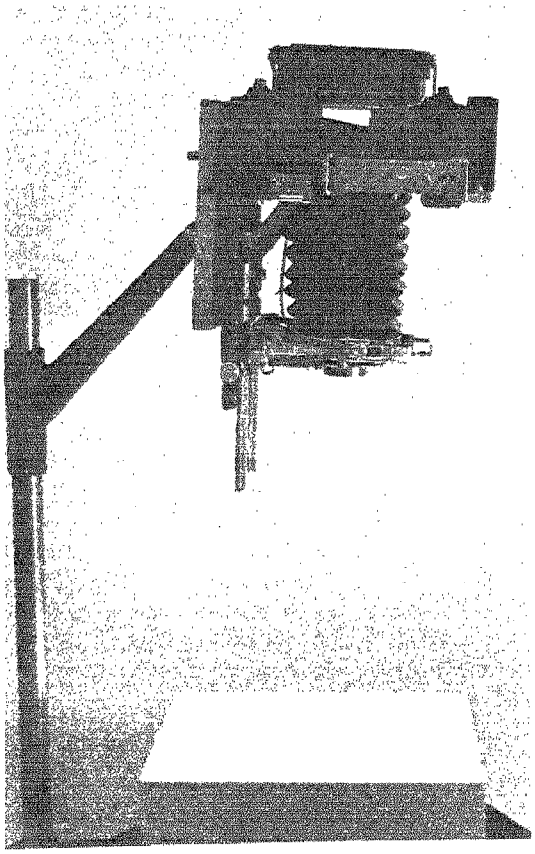


Figure 15—Arrangement of camera, developed gel slab, and light box for taking B & W and color photographs.

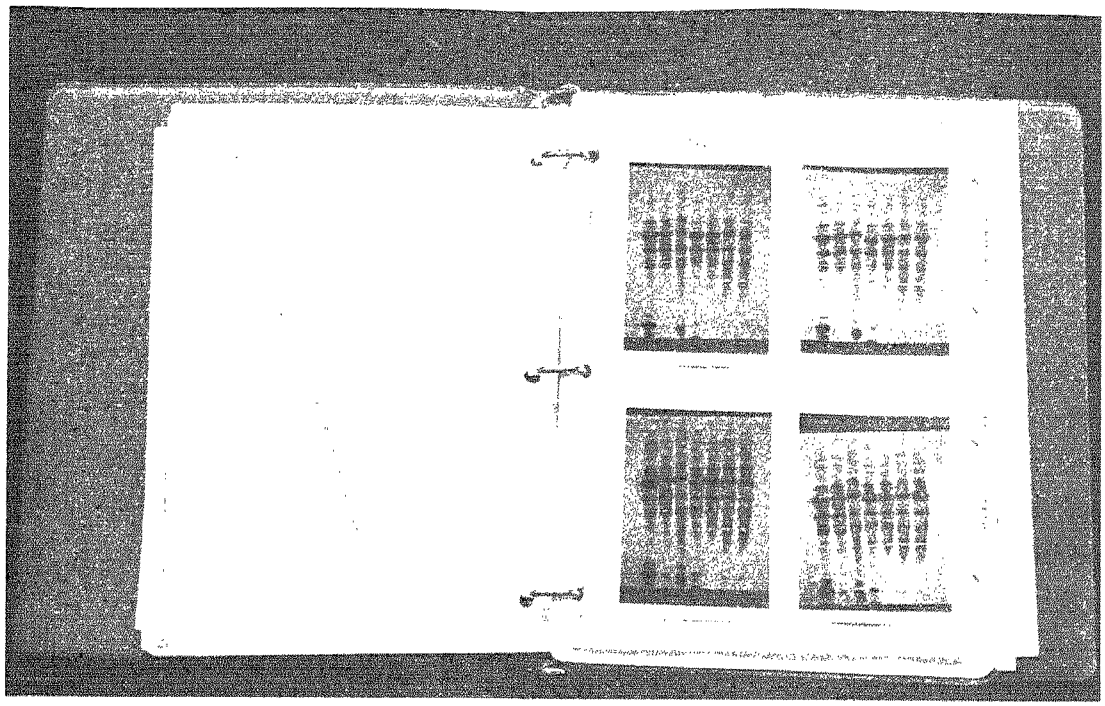


Figure 16—Notebook pages showing a plotted pH gradient (left), and photographs taken the day of development (top right) and day after development (bottom right) for both B & W and color.

PHOTOGRAPHING THE STAINED GEL

Camera and film

We use both black-and-white and color photographs for a permanent record of all IEF runs. Also, 35-mm color slides are taken for use in presenting our results at meetings and seminars. The complete arrangement of equipment is illustrated in figure 15. Pictures and slides are taken routinely (1) on the day of staining, and (2) on the day after staining (i.e., about 24 h later). Medium bands are darker a day later, and light bands that were invisible or barely visible on the first day will have become visible or more visible, respectively. More about this later.

Description and use of camera

We use an old Crown Graphic[3] camera with extension bellows and ground-glass focusing. The lens is a f/4.7, 125 mm. Any camera that can accept a 4 x 5 film pack holder would probably suffice.

In practice, the stained gel and glass plate are placed on a light box (this can be home-made), the camera back and shutter are opened and the gel image is focused on the ground glass. Be sure to place a clear plastic metric ruler along the edge of gel (as in Fig. 13), so that band locations will be measurable in the pictures. It is usually easiest to focus on the numbers along the ruler. Finally, the bellows is locked in position, the shutter is closed, and the camera back is closed. Room lights are turned off.

Film pack and film — black and white

We have found that Polaroid[4] film is the easiest and most expedient to use for obtaining a permanent record of IEF results. Polaroid Black and White, Land Pack Film, Type 105, Positive/Negative, is inserted into a Polaroid Land Pack Film Holder, Model 405, and the holder is inserted into the camera back. The pack contains

eight 3¼ x 4¼-in prints and negatives. Next, pull out the film-slide protector and adjust the camera for f-stop and time of exposure. The following values work well for us:

1. Dark-stained gel: f/11 @ 1/10 to 1/25 s
2. Medium-stained gel: f/11 @ 1/25 s
3. Light-stained gel: f/11 @ 1/25 to 1/50 s

Expose the film, and extract it from the film pack, according to the manufacturer's instructions. Develop for 30 s, peel apart, coat the positive (print) with the applicator supplied, and place the negative into 68° F water. Soak the negative for a while, remove extraneous material by rubbing it gently under a stream of warm water, dip the clean negative in properly diluted Photoflow solution, and hang it up to drip-dry.

Film pack and film — color

Color prints are obtained with Polaroid's Polacolor 2 Land Film, Type 108. The number and size of prints are the same as those for black-and-white film. Place the color film in a separate film pack, insert the latter into the back of the camera, as before, and pull out the film-slide protector. Focusing remains unchanged. Suggested f-stops and exposure times are:

1. Dark-stained gel: f/11 @ 1/5 to 1/10 s
2. Medium-stained gel: f/11 @ 1/10 s
3. Light-stained gel: f/11 @ 1/10 to 1/25 s

Expose the color film and extract it from the holder. Develop the film for 60 s, then peel positive and negative apart. No coating is needed. Let all pictures dry for a few hours, then paste them in the laboratory notebook for a permanent record of your IEF results (Fig. 16).

Color slides — 35 mm

We use Kodak[1] Kodachrome 64 (ASA 64), KR 135 film in a single-lens reflex camera with an f/1.4, 50 mm lens. The minimum focus is 17.7 in (45 cm). In practice, we set the lens at its minimum focus and move the camera to bring the gel slab into focus. Under these conditions, the gel slab and glass plate nearly fill the field of view, which is satisfactory (a typical setting might be 1/60 s @ f/8). The background of the slides comes out light green because of the cool-white fluorescent tube (circular) in the light box (Fig. 15), which emits strongly in the green (i.e., around 500 nm) wavelengths. A blue background can be obtained by taping the gel-plate to a north-facing window before making exposures, but a sunny day without clouds is required for this.

Follow-up procedure

Place 2 mm thick spacers around the edge of the developed gel and cover it with another glass plate. Do not let the second plate touch the gel. Wrap the entire two-glass-plate assembly in clear plastic (to prevent moisture loss) and store at 4° C in a cold room overnight. On the following morning, remove the clear plastic and re-photograph the gel with black and white, color, and 35 mm film.

DRAWING A FIGURE OF THE STAINED GEL

Procedure

Prop up the light box (used in photographing gels--Fig. 15) at a 45° angle and set the gel-plate against the milky-white plastic cover. The gel plate may be supported by the bottom edge of the light box (this is possible with our light box), or it may have to be taped in place. Once again, the 30-cm clear plastic ruler is placed along the right-hand edge of the gel with zero positioned at the inside edge of the lower (anode) wick (i.e., similar to the arrangement shown in Fig. 13). Illuminate the box.

Next, prepare your drawing on a piece of metric chart paper, one with light horizontal lines at 1-mm spacing and dark horizontal lines at 1-cm spacing, to the same dimensions as the actual stained gel, by drawing six 1-cm-wide columns that are 16.5 cm long and spaced 1 cm apart.

Use the metric ruler as a guide for marking the bands on your graph paper. We generally mark-in the darkest bands first, the intermediates next, and the lightest bands last. Make the first gel drawing on the day of staining, and revise it on the following day, when intermediate and light bands will be more easily detected. We have arbitrarily chosen six different marks to draw-in bands varying in intensity from high, major bands to low, minor bands (see key in Fig. 21). Label your drawing with a minus symbol at the top and a positive symbol at the bottom, to denote polarity. Number the centimeter scale on each side of your drawing.

Finally, count the number of bands in each column and write this total beneath the column.

Numbering the bands

There is no universally accepted scheme for numbering (or otherwise labeling) the isoenzyme bands resolved in an IEF gel. Through experience, we learned the probable isoelectric points (pI's) of several easily recognizable peroxidase bands from HRP that usually come to focus near the middle of the gel.

Foremost among these is a darkly stained band that invariably occurs at pH 6.9. We decided to use this band as a standard reference and to number all other bands in relation to it. This reference band is labeled A1 (see Fig. 19 and 21) because it is the band closest to neutrality (pH 7.0) that migrates toward the anode. The remaining bands, in descending order, are numbered A2, A3, A4, etc. The first band above A1 is labeled C1, because it is the band closest to neutrality that migrates toward the cathode. (C1 always occurs at a pH greater than 7.0.) In the same way, the remaining bands, in ascending order, are labeled C2, C3, C4, etc.

Labeling peroxidase-IAA oxidase isoenzymes from other plant species

Obviously, other plant species will produce different (at least partially so) isoenzyme banding patterns.

In labeling these, we have used essentially the same procedure as described above. It works this way. Run a standard load of HRP (i.e., 4 μl of 1 mg/ml stock) in the first and last columns and your other enzyme extracts in the remaining four columns. After staining, locate the A1 band in the HRP columns and label it. Then place a ruler's edge across the drawing at the level of the HRP—A1 band. Use the scales on each side of your drawing to get the ruler truly horizontal. In the other columns, label as A1 only other bands at that level. Move the ruler down (keeping it horizontal) the paper until the next band is encountered. It makes no difference which column the band is in—mark it and any other bands at the same level A2. Continue down the page with the ruler held horizontally and mark each succeeding band, i.e., A3, A4, A5, etc.

The same procedure, in reverse, is used to mark the bands that have migrated toward the cathode. Move the ruler *up* from the A1 band of HRP and label the first band encountered as C1, no matter what column it is in. Continue labeling bands in sequence as you move the ruler up the paper, i.e., C2, C3, C4, etc.

When band numbering has been completed, you can determine which bands your extracts and HRP have in common and which bands are unique to a particular extract. Also, when writing reports of your work, you will be able to refer to a particular band by extract label and band label. (Fig. 21 illustrates how this labeling scheme is actually used.)

We have used this scheme for IEF examination of peroxidase-IAA oxidase isoenzymes in leaf fractions of two birch *(Betula)* species and tobacco *(Nicotiana tabacum)*.[6] It works well.

RESULTS OBTAINABLE WITH THIS IEF TECHNIQUE

In our early work with the IEF technique and HRP, we discovered that the extreme anionic and cationic isoenzymes would run off the ends of the gel into the paper wick. All of this early work was done with wide-range carrier ampholytes produced by LKB Instruments Inc.[7] We generally obtained pH gradients of 4.0 to 9.5 with LKB wide-range ampholytes.[7] With shortened run times, we kept all the isoenzymes confined to the space between the two electrodes, but well-resolved from each other. Although all isoenzymes were retained on the gel, those at the ends of each column were not focused at their isoelectric points. It was obvious from these results that the extreme cathodic or anodic isoenzymes had isoelectric points beyond the range of the pH gradient. Finding the isoelectric points of these extreme isoenzymes would require the use of narrow-range ampholytes, e.g., pH 9 to 11 and pH 2 to 4.

In our work, we are principally interested in isoenzyme patterns (i.e., their relative distribution one to the other, their relative activity, and the total number present) as a way to determine the

[6] Unpublished work of the author.

[7] After numerous tests with carrier ampholytes from each commercial source, it was apparent to us that Bio-Lytes gave the sharpest resolution of isoenzymes. In addition, Bio-Lytes produced a lower pH gradient (at the acid end) than did Ampholines, and they were free of the inhibitors of gelation found in some lots of pHisolytes.

effects of treatment in various physiological experiments. We used commercial preparations of HRP solely to work out the methodology and techniques presented in this handbook.

Diagonal runs

We know, from personal experience, that the peroxidase-IAA oxidase isoenzymes of horseradish roots, birch leaves, and tobacco leaves are numerous and extend over a wide pH gradient. This fact presented a problem in resolving visually two or more bands with only slightly different isoelectric points. High resolving power is necessary in order to determine the maximum number of isoenzymes present in an extract.

We solved this problem (without resorting to longer gel slabs) by applying our enzyme samples in a diagonal from upper left (cathode end) to lower right (anode end). The left column permitted maximum spreading of the anodic bands, and the right column permitted maximum spreading of the cathodic bands (Fig. 17). Intermediate columns gave intermediate degrees of spreading between these two extremes. The number of bands in a particular pH range is counted in the column giving the best resolution in that range. This practice is continued until every segment of the columns has been evaluated. The drawing that results is a *composite* of the best separations obtained for each segment of the total length. Therefore, the number of isoenzymes in the composite will be greater than in any individual column on the gel plate.

The composite drawing is revised on the day after staining because additional minor isoenzymes that were not visible on the day of staining may have appeared, and the medium isoenzymes may be more visible (compare Fig. 18 with Fig. 17). Dark isoenzymes, by comparison, are less compact and somewhat faded on the second day.

Side-by-side runs

With different commercial sources of HRP, we wanted to know which bands were common and which bands were uncommon. To obtain this information, we made IEF runs with HRP from various sources all placed (in duplicate) at the same distance from the bottom (anode end)—usually 7.4 cm (Fig. 19). We found that common bands in each source lined up across the gel, but uncommon bands in one source were not matched by bands at the same location in other sources. The same methods can be used with enzyme extracts from different species, different organs, or different experimental treatments of the same species, etc.

With this technique, it is also possible to demonstrate the equivalency of IAA oxidase activity and peroxidase activity in this dual catalytic enzyme (compare Fig. 20 with Fig. 19).

Runs with various concentrations

In this technique, the enzyme extract is run in six (or eight) different concentrations in a side-by-side placement. The results of such a run will answer two questions: (1) Is enough enzyme being applied to permit visualization of all the minor bands? (2) Do the major bands really consist of only one isoenzyme, or are there several isoenzymes with isoelectric points very close to each other?

Summing up results

Composite drawings from each type of run are used to make an overall final composite. In our example (Fig. 21), a summary composite was made for HRP from a given commercial source; it had been tested in diagonal runs made with carrier ampholytes from three commercial sources. Similarities between banding patterns of the HRP from all three commercial sources were also determined, and a final overall composite for HRP showed 42 isoenzymes. As stated in an earlier report,[3] this was *more than twice* the number of HRP isoenzymes previously reported.

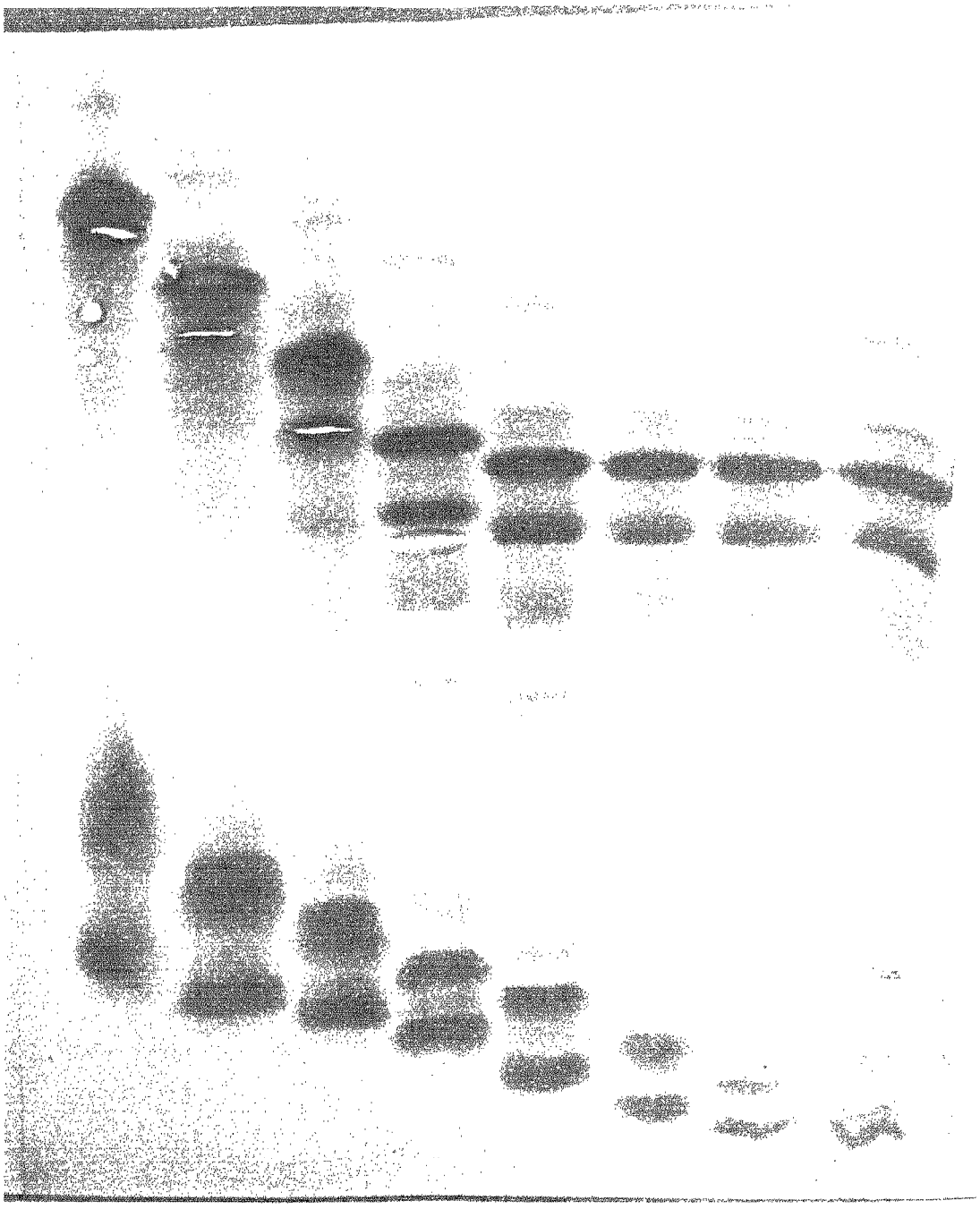


Figure 17—Diagonal run with Sigma HRP applied in slits; gel stained for peroxidase activity on *day of* IEF run with constant power (manual) for 6.5 h; 3% Bio-Lytes carrier ampholytes.

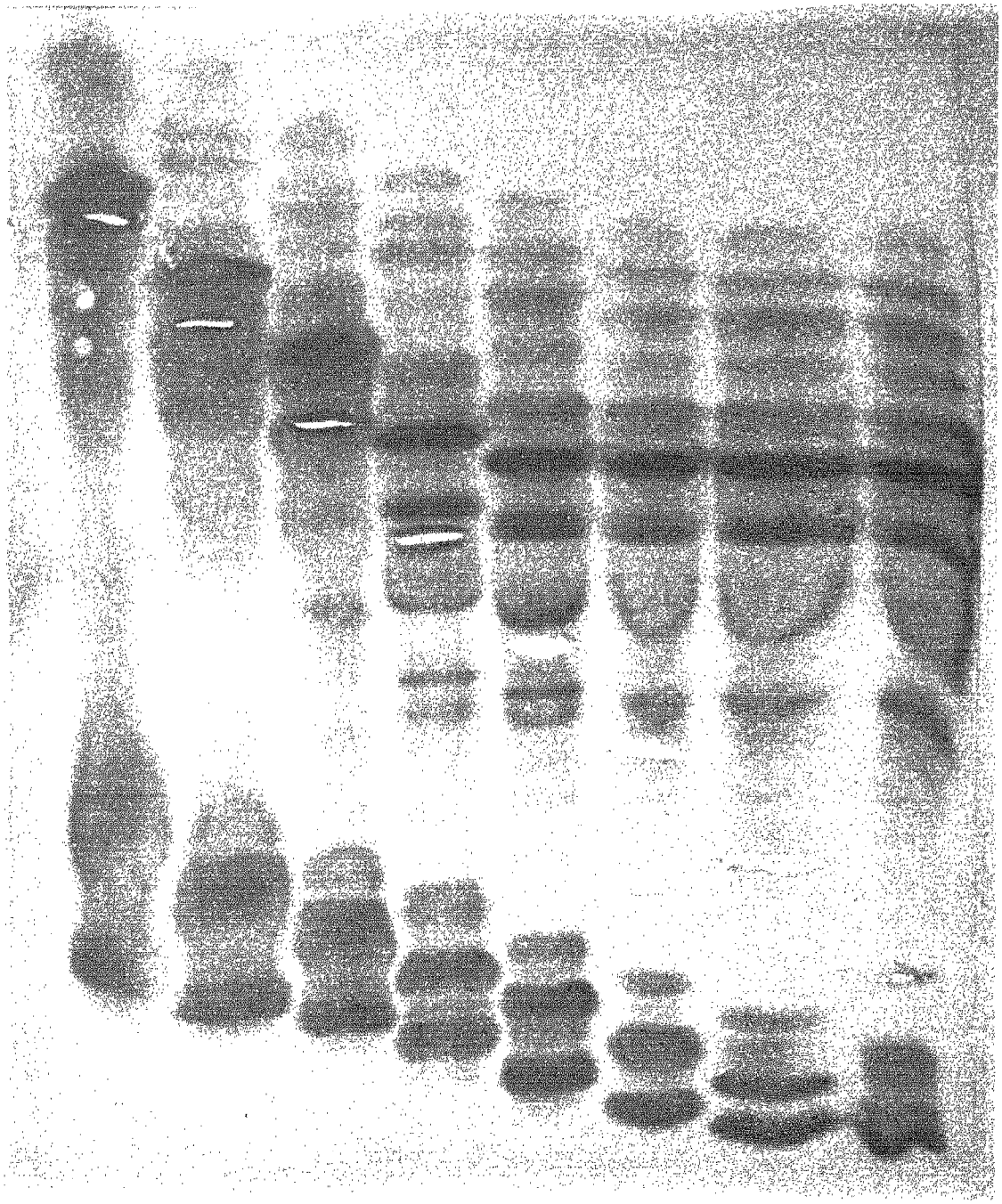


Figure 18—Same as Figure 17, but taken the day *after* IEF run.

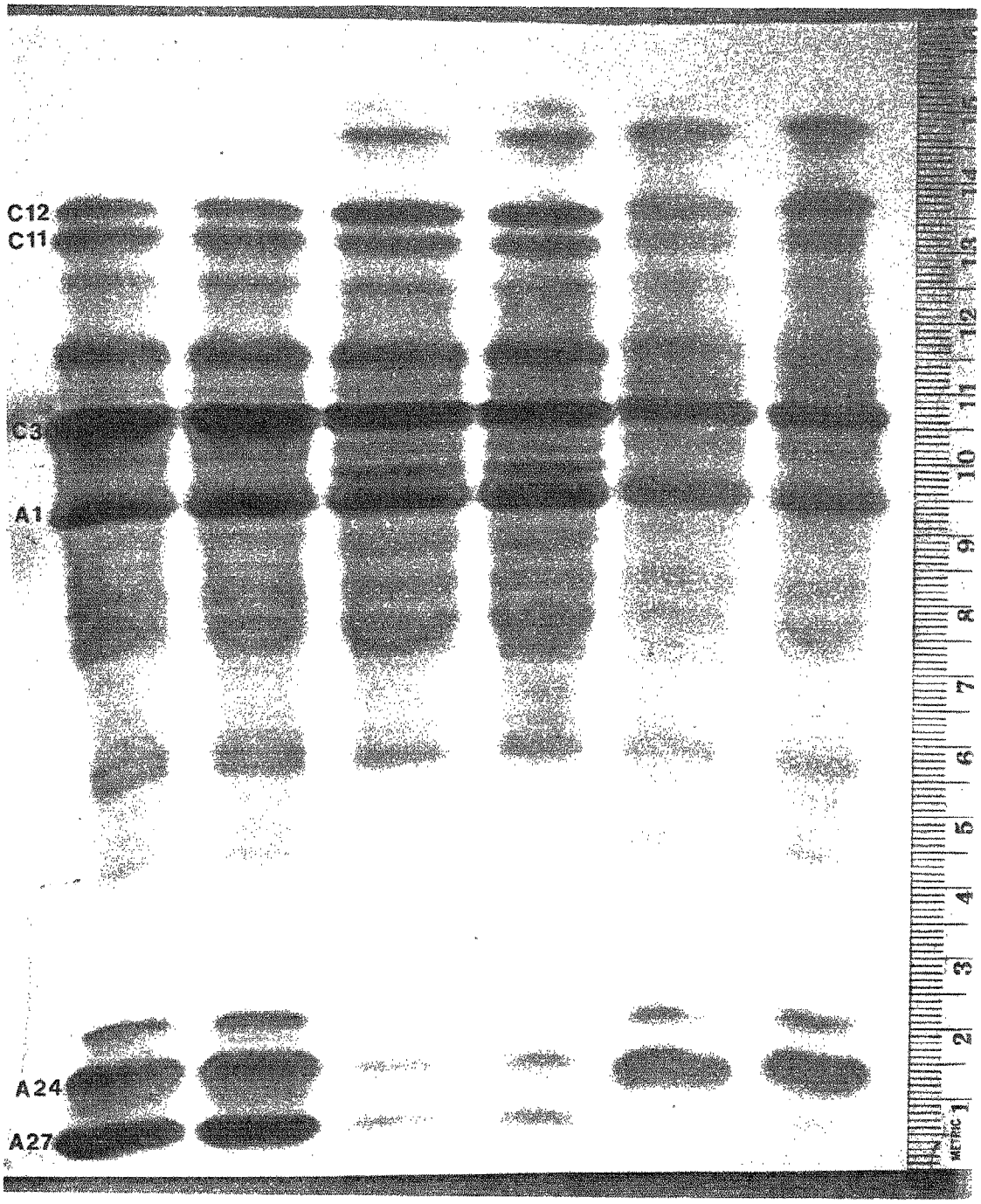


Figure 19—Side-by-side runs, in duplicate, of Sigma HRP (left), Worthington HRP (center), and Nutritional (right), stained for peroxidase activity. Photo taken *day after* IEF run with constant power of 1 W for 17.5 h, 3% Bio-Lytes carrier ampholytes.

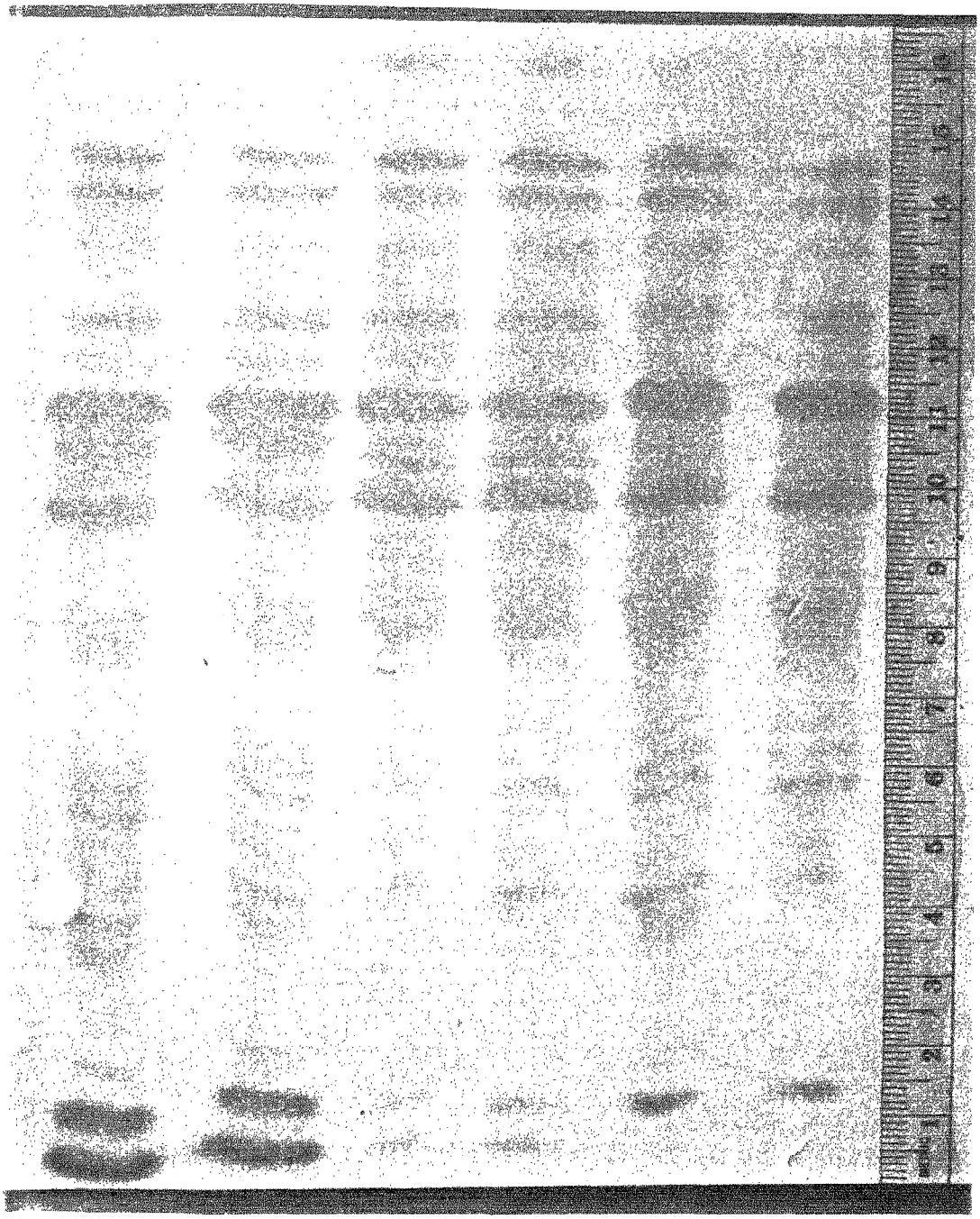
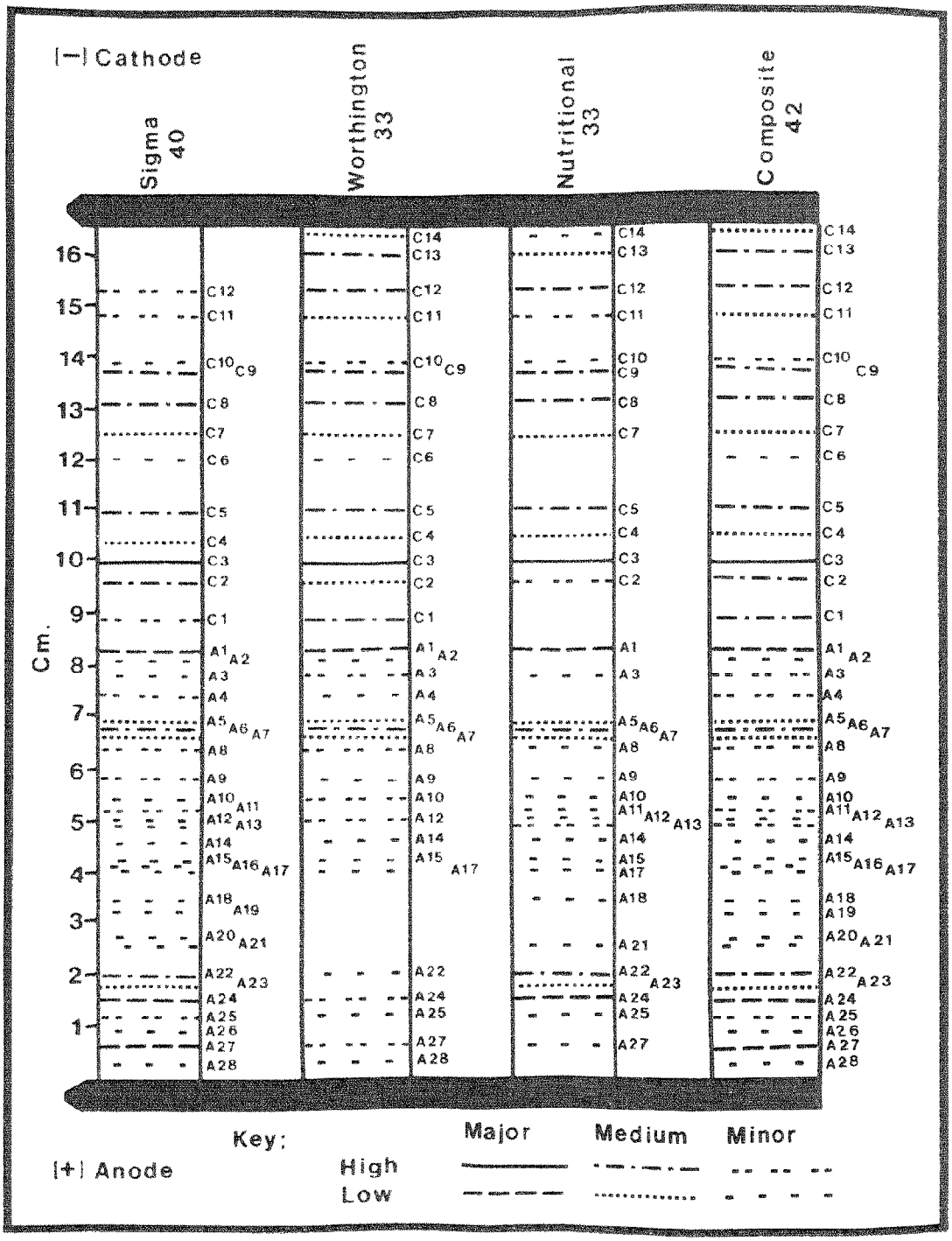


Figure 20—Same as Figure 19, but stained for IAA oxidase activity.

Figure 21—Composite drawing of IEF banding patterns of peroxidase activity for HRP from three commercial sources, each tested with carrier ampholytes from three commercial sources, plus an overall composite. Note the numbering system and six-part key for illustrating band intensities.



APPENDIX

Manufacturers and Sources of Reagents, Materials, and Equipment

1. Reagents
   a. Acrylamide kit—including acrylamide monomer, BIS, TEMED, ammonium persulfate. BIO-RAD Laboratories, 220 Maple Ave., Rockville Centre, N.Y. 11570.
   b. Wide-range carrier ampholytes
      Ampholines, pH 3.5-10, LKB Instruments, Inc., 12221 Parklawn Drive, Rockville, Md. 20852.

      Bio-Lytes, pH 3-10, BIO-RAD Laboratories, 220 Maple Ave., Rockville Centre, N.Y. 11570.

      pHisolytes,[8] pH 2-10, Brinkman Instruments, Inc., Cantiague Road, Westbury, N.Y. 11590.
   c. Commercial horseradish peroxidase, purified
      RZ 1.0, Nutritional Biochemicals Corp., 26201 Miles Road, Cleveland, Ohio 44128

      RZ 1.0, HPOD, Worthington Biochemical Corp., Freehold, N.J. 07728.

      RZ 1.0 to 1.5, Type II, Sigma Chemical Co., P.O.B. 14508, St. Louis, Mo. 63178.
   d. Peroxidase stain
      3-amino-9-ethyl-carbazole, K & K Laboratories, Inc., 121 Express St., Engineers Hill, Plainview, N.Y. 11803.

      dimethylformamide, reagent grade, J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, N.J. 08865.
   e. IAA oxidase stain
      Indoleacetic acid—J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, N.J. 08865

      p-coumaric acid—Nutritional Biochemicals Corp., 26201 Miles Road, Cleveland, Ohio 44128.

      fast blue BB base—Sigma Chemical Co., P.O.B. 14508, St. Louis, MO. 63178.
   f. Miscellaneous reagents
      Acetic acid, sodium acetate, hydrochloric acid, sodium hydroxide (pellets), 30% hydrogen peroxide—any supplier of reagent-grade chemicals.

2. Materials
   a. Epoxy glue—tube of resin plus tube of hardener, from local hardware store (many manufacturers of same product).
   b. Intramedic tubing—Fisher Scientific Co., 461 Riverside Ave., Medford, Mass. 02155.
   c. Glass plates—made to order by Anderson Glass Co., Old Turnpike Road, Fitzwilliam, N.H. 03447.
   d. Binder paper clips—The Mattauck Mfg. Co., Waterbury, Conn. 06705.
   e. Carbon electrodes—purchased from a local welding-supply house.
   f. Glass stirring rods—any supplier of scientific equipment.
   g. Pyrex[1] baking dish—local hardware store.
   h. Plastic cover for baking dish—local hardware store.
   i. Aluminum cookie tray—local hardware store.
   j. Metric chart paper (rolls)—Cat. No. S-72165, 0–250 mm, from Graphic Controls Corp., Recording Chart Division, 189 Van Rensselaer St., Buffalo, NY 14210.
3. Equipment.
   a. Pasteur pipet—any supplier of scientific supplies.
   b. Hamilton syringe, 50 µl—Hamilton Co., P.O. Box 10030, Reno, Nev. 89510.

[8] Note: Some lot numbers of pHisolytes prevent gelation, whereas others work fine. The reasons for this disparity have not been determined.

c. Safety pipetor, rubber—any supplier of scientific supplies.

d. Combination pH microelectrode, range 0-11, Cat. No. ES47310-2, Ingold Electrodes Inc., 113 Hartwell Ave., Lexington, Mass. 02173.

e. Light viewing box, 115 volt, Model No. 4205-1—ORTEC, Inc., 100 Midland Road, Oak Ridge, Tenn. 37830.

f. Power supplies:

Nonregulated, 0–1000 VDC and 0–200 mA— Buchler Instruments, 1327 Sixteenth St., Fort Lee, NJ 07024.

Constant voltage 10–400 VDC, constant current 3–80 mA, 110 V, 60 Hz, Cat. No. 165-0510, BIO- RAD Laboratories, 220 Maple Ave., Rockville Centre, N.Y. 11570.

Pulsed Power, 115 V, Model No. 4100-1-ORTEC, Inc., 100 Midland Road, Oak Ridge, Tenn. 37830.

Constant power, Model 493, Instrumentation Specialties Co. (ISCO), P.O. Box 5347, Lincoln Neb. 68505.

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ACKNOWLEDGMENTS

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Headquarters of the Northeastern Forest Experiment Station are in Broomall, Pa. Field laboratories and research units are maintained at:

- Beltsville, Maryland.
- Berea, Kentucky, in cooperation with Berea College.
- Burlington, Vermont, in cooperation with the University of Vermont.
- Delaware, Ohio.
- Durham, New Hampshire, in cooperation with the University of New Hampshire.
- Hamden, Connecticut, in cooperation with Yale University.
- Kingston, Pennsylvania.
- Morgantown, West Virginia, in cooperation with West Virginia University, Morgantown.
- Orono, Maine, in cooperation with the University of Maine, Orono.
- Parsons, West Virginia.
- Pennington, New Jersey.
- Princeton, West Virginia.
- Syracuse, New York, in cooperation with the State University of New York College of Environmental Sciences and Forestry at Syracuse University, Syracuse.
- Warren, Pennsylvania.

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