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Analytical Techniques for Cell Fractions

XXVII. Use of Heart Proteins as Reference Standards in Two-Dimensional Electrophoresis[1,2]

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The preparation and use of rat heart whole homogenate as a standard for the sodium dodecyl sulfate (SDS) electrophoresis dimension of two-dimensional electrophoresis is described. By including the rat heart homogenate in the agarose overlay used to hold an isofocusing gel (first dimension) in contact with a slab gel (second dimension), 80 horizontal lines can be superimposed on a two-dimensional electrophoresis pattern. Such an internal standard is useful as a reference marker for the intercomparison of many gels and also, when calibrated, can be used to determine the approximate molecular weight of proteins.

Two-dimensional electrophoresis, based on the methods described by O'Farrell (1) and others (2–4), separates large numbers of proteins or protein subunits according to isoelectric point in the first dimension and molecular weight in the second dimension. Using the ISO-DALT version of this system (2,3), this laboratory has begun the systematic mapping of an estimated 30,000 to 50,000 human protein gene products (4,5). At the present time, 100 analyses may be made per day, yielding large amounts of data for analysis by computerized image analysis systems. Standardization of the first-dimension separation, isoelectric focusing, has been described by Anderson and Hickman (6). Still to be dealt with, however, is the standardization of the sodium dodecyl sulfate (SDS)[3] electrophoresis used in the second dimension. Such standardization is necessary if the full potential of two-dimensional electrophoresis is to be realized, for only with standardization will intercomparison of patterns from different complex protein mixtures or data from different laboratories be possible.

When gels containing many hundreds of protein spots are to be intercompared to determine how many spots are identical and how many are different, closely spaced molecular weight standards could serve as vertical reference points on each gel in the SDS dimension. These standards could be prepared from mixtures of pure proteins of known molecular weight, but these are expensive in the quantities required for routine use, do not cover a wide range of molecular weights evenly, and deteriorate with time (usually in the hands of the supplier). Another possibility as a standard for molecular weight is a series of crosslinked polymers of one protein of known molecular weight (7). However, these may not be exactly comparable to linear chains of the same molecular weight as suggested by the observation of marked differences in SDS

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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mobility when disulfide bridges are present (8). Phage proteins are a reasonable source of useful standards, but these are not widely available and do not cover the spectrum of protein molecular weights found in tissues.

In an effort to find a molecular weight standard with the required pattern of evenly spaced bands which could be obtained economically from a source available to most laboratories, we have examined serum, red cell lysates, urinary proteins, seed proteins, and a variety of rabbit, rat, and mouse tissues. Of these only two, skeletal and heart muscle, yielded promising results. In this paper, we describe the use of rat heart whole homogenate as a standard for the SDS electrophoresis dimension of two-dimensional electrophoresis.

EXPERIMENTAL

Preparation of Rat Heart Protein Samples

Sprague–Dawley rats (male or female, 4–6 months old) were sacrificed by decapitation. The heart was quickly perfused by introducing phosphate-buffered saline (PBS) directly into the chambers. Perfused or unperfused hearts were removed while still beating, dropped into tared beakers containing cold PBS, and weighed. The hearts were then immediately blotted, placed in SDS homogenization buffer (1% SDS, 0.5% dithiothreitol, and 0.125 M Tris–HCl, pH 6.8), and minced with scissors. The minced tissue was then mixed with additional SDS homogenization buffer to yield a 5% homogenate (5 g of tissue per 100 ml homogenate) and then homogenized at 4°C for 30 s at full speed in a chilled Waring blender. The homogenate was filtered through two layers of cheese cloth and mixed with a stock agarose solution (1.4% agarose in the SDS homogenization buffer) to yield mixtures containing from 2.5 to 20 mg tissue/ml of 0.7% agarose. These dilute preparations were frozen in 5-ml aliquots and stored at −20 or −70°C.

For the preparation of myofibrils and cell sap, perfused rat hearts were homogenized in the relaxation buffer described by Zak and co-workers (9). Myofibrils were isolated according to Zak *et al.* (9) without the deoxycholate treatment. The cell sap was prepared by centrifuging the initial 5% homogenate for 1 h at 100,000*g* at 0°C. Both the isolated myofibrils and cell sap preparations were made up in the agarose–SDS homogenization buffer mixture to yield mixtures containing approximately 20 mg tissue material/ml 0.7% agarose, based on the original homogenate concentration.

Preparation of Commercial Protein Samples

Bovine serum albumin (BSA), ovalbumin, trypsinogen, β-lactoglobulin, and lysozyme were purchased from Sigma Chemicals. Rabbit phosphorylase b and human carbonic anhydrase B were obtained from Worthington Biochemicals, soybean trypsin inhibitor from Calbiochem, and human transferrin from Behring Diagnostics. Each purified protein was made up in 0.7% agarose with SDS homogenization buffer so the final concentration was 0.05–0.1 mg protein/ml.

SDS molecular weight standard kits were purchased from Bio-Rad, Sigma, and Boehringer-Mannheim Biochemicals. These protein mixtures were mixed with 0.7% agarose to obtain final protein concentrations of 0.5, 0.6, and 0.2 mg protein/ml, respectively.

Preparation of Rabbit Psoas Muscle Proteins

Rabbit psoas muscle whole homogenate was prepared as described previously (10). For rat heart whole homogenate calibration experiments, the psoas preparation (a 5% homogenate) was mixed with agarose in SDS homogenization buffer to obtain 5 mg muscle tissue protein/ml of 0.7% agarose.

Electrophoresis

Sodium dodecyl sulfate electrophoresis was performed on 1.5-mm-thick gels cast

between 7 × 7 in. glass plates using the gel casting system previously described (3). Unless otherwise specified, the gels used were composed of 10–20% acrylamide in a linear gradient. Standard protein mixtures (rat heart whole homogenate, myofibrils, cell sap) and purified proteins in 0.7% agarose were applied to the top of the slab gels in a 1- to 2-mm-thick layer. Horizontal electrophoresis in DALT tanks, gel fixing, staining, and destaining were all done as previously described (4). A slab gel with no sample applied was included with each set of gels during the electrophoresis as a tank buffer control. Such a control was necessary because bacterial contamination of the DALT tank buffer has been shown to result in a pattern of protein bands similar to that seen when muscle proteins are applied to a gel (K. Willard, manuscript in preparation). All of the gels shown in this publication were in groups that showed no bands on the DALT tank buffer control gel.

Two-dimensional electrophoresis was done as described (4). The rat heart standard protein mixture in agarose was applied over the isoelectric focusing gel on top of the slab gel used for the second-dimension separation (0.5 ml of the standard was used routinely, yielding an agarose layer approximately 2 mm deep). This served to seal the first-dimension gel in place. The electrophoresis and handling of the gels were performed as described previously.

Photography

Slab gels were placed on either clear or flashed opal glass plates with a calibrated absorbance step tablet to one side. The gels were illuminated from below with even diffuse white light. An interference filter with peak transmission at 590 nm (the absorption maximum of Coomassie blue) was used. Eastman Contrast Process Pan film, 4 × 5 in., was used for photographic prints. For computer analyses that included densitometer scans Eastman Plus-X Pan Professional film, 4 × 5 in., was used. Prints or negatives were scanned using an Optronics P-1000 rotary drum scanner at 50-μm increments, and the data were stored on a disk attached to a PDP 11/60 dedicated computer. The system was calibrated for reflective density with a calibrated step tablet, and the results were printed out on a Printronix 300-dot matrix printer.

RESULTS

Choice of Standard Protein Mixture

A variety of protein mixtures were examined by this laboratory in order to find one source that would give a large number of evenly spaced lines in SDS electrophoresis patterns, with some lines more accentuated than others in order to facilitate orientation and line identification. The first finding was that, for reasons thus far unexplained, many highly purified proteins available commercially do not, in our hands, give lines as well resolved as those in freshly prepared tissue extracts (Fig. 1). Extracts from several tissues (heart, skeletal muscle, and liver) in addition to seed proteins, saliva, serum, urinary proteins, and milk were examined as possible standard protein mixtures. Of these, skeletal muscle and heart muscle produced the most useful line patterns. Although rabbit skeletal muscle has the advantage of containing a larger number of proteins of known molecular weight than either rabbit or rat heart muscle, the skeletal muscle does not give the evenly spaced major lines interspersed with minor ones that heart tissue does. Therefore, even though few of the protein bands in heart muscle have been identified, that tissue has been selected for more extensive examination as a molecular weight standard mixture. Rat heart was chosen over rabbit heart because the smaller animal is less expensive, more easily sacrificed, and more widely available. Having chosen the rat heart as our standard protein mixture, we have experimentally explored which type of heart extract yields the most useful information, how stable the protein mixture is, how to

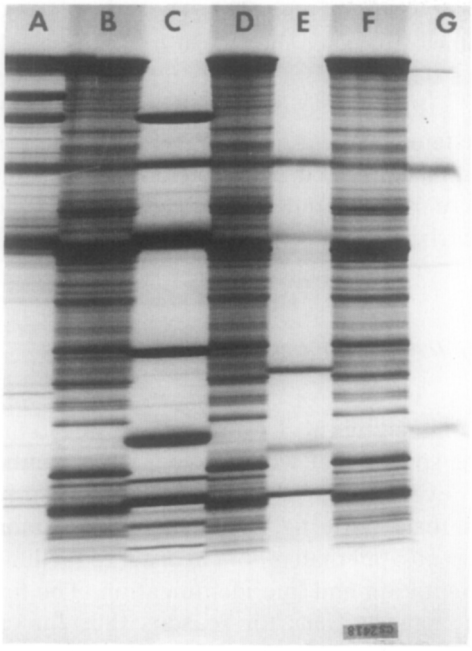

FIG. 1. Comparison of rat heart whole homogenate with commercial SDS molecular weight standard proteins. (A) Bio-Rad high molecular weight protein standards containing myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin (0.5 mg protein/ml agarose); (B), (D), and (F) rat heart whole homogenate (10 mg tissue/ml agarose); (C) Bio-Rad low molecular weight protein standards containing bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (0.5 mg protein/ml agarose); (E) Sigma Dalton Mark VI containing bovine serum albumin, ovalbumin, pepsin, trypsinogen, β-lactoglobulin, and lysozyme (0.6 mg protein/ml agarose); (G) Boehringer-Mannheim Biochemicals Combithek containing RNA polymerase (α, β, and β′ chains), bovine serum albumin, and soybean trypsin inhibitor (0.2 mg protein/ml agarose).

quantitate objectively the number of bands the rat heart preparations produce, and how to calibrate the heart preparations with respect to proteins whose molecular weights are known.

Choice of Rat Heart Standard Preparation Method

Several possibilities exist for the preparation of a standard mixture from rat heart tissue. Whole homogenates from unperfused hearts or perfused hearts provide the entire spectrum of protein lines. A myofibril preparation provides major lines at evenly spaced intervals without the many minor lines which complicate the whole homogenate pattern. The cell sap pattern is also simpler than the whole homogenate pattern, but the major lines are not as evenly spaced as those in the myofibril pattern. For purposes of spot identification within a two-dimensional electrophoretic pattern, we have found that the whole homogenate pattern provides the most lines at intervals spaced evenly from the top to the bottom of the slab gel. The perfused heart pattern is more useful in two-dimensional electrophoretic work than the unperfused since the hemoglobin band of the latter is so dense that it covers protein spots in two-dimensional patterns at that molecular weight [approximately 15,500 (11,12)].

Figure 2 shows patterns from perfused rat heart when the homogenate is made up in

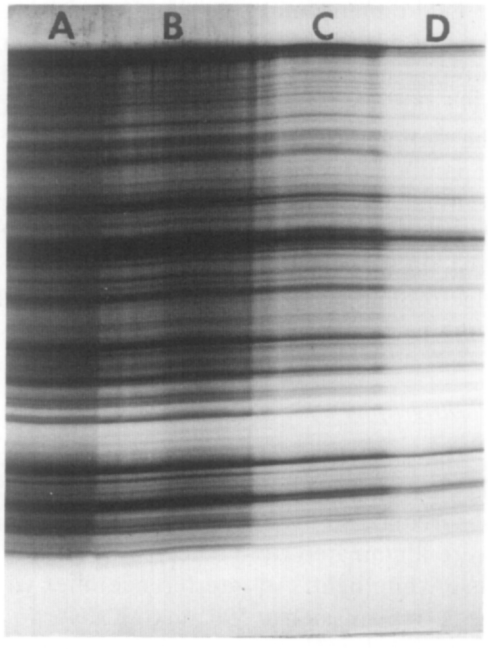

FIG. 2. A comparison of the perfused rat heart whole homogenate pattern at different homogenate concentrations. Aliquots of rat heart whole homogenate were mixed with agarose to obtain final concentrations of (A), 20, (B), 10, (C), 5, and (D), 2.5 mg tissue/ml agarose.

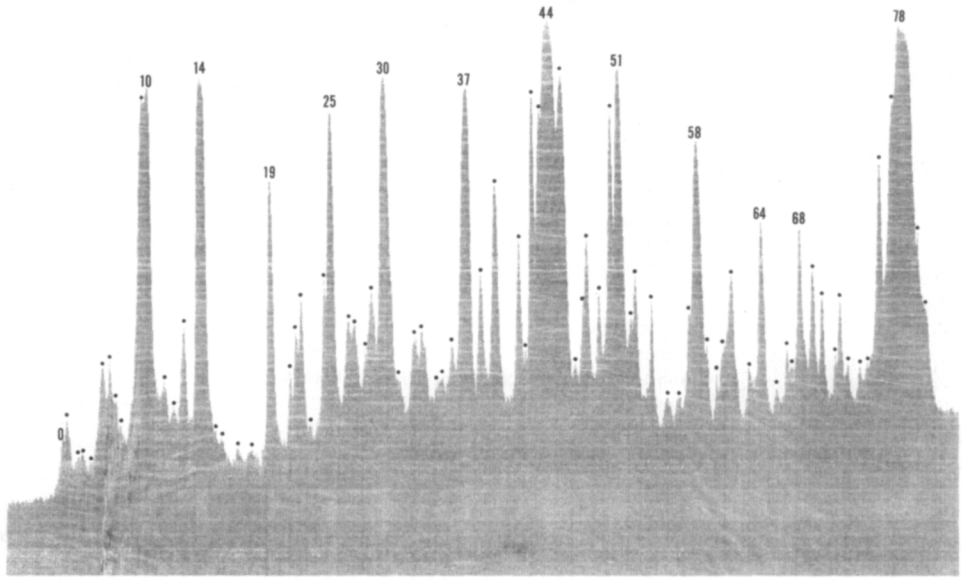

FIG. 3. Digitized image of rat heart whole homogenate sodium dodecyl sulfate electrophoresis pattern. A black and white photograph of the rat heart whole homogenate pattern was scanned using an Optronics P-1000 rotary drum scanner. The data shown here were plotted by a Printronix 300-dot matrix printer. Zero marks the peak corresponding to the lowest molecular weight protein in the homogenate pattern. Peaks corresponding to the 12 major lines seen reproducibly in the rat heart homogenate pattern are marked with the line number. The remaining 67 peaks resolved by this system are marked with closed circles.

agarose at various tissue concentrations. This photograph illustrates the ease with which the line pattern can be made simple or complex depending on the uses anticipated. For routine use, we selected the perfused rat heart whole homogenate at a concentration of 10 mg tissue/ml agarose.

Stability of Rat Heart Whole Homogenate Standard

The rat heart whole homogenate protein pattern remains unchanged for over 12 months when the proteins are in 0.7% agarose with SDS and stored at either −20 or −70°C. However, changes occur in the pattern if whole homogenate is stored frozen without the addition of SDS and then thawed, solubilized, and mixed with agarose.

Identification of Rat Heart Whole Homogenate Protein Lines

A photograph of the rat heart homogenate pattern was scanned in the Optronics scanner and the results printed out as shown in Fig. 3. For mapping or comparison purposes, it is necessary to identify each of the lines in the whole homogenate pattern numerically. The computer scan provides the most objective method for numbering the lines, some of which are just barely visible to the eye. As higher resolution systems are developed, more lines may be resolved than those seen in these figures. Those new lines can be identified using decimals for the original numbers assigned in this paper. In a previous report (13), 86 lines were found when a mixture of 75% rat heart myofibrils and 25% rat heart whole homogenate was analyzed. As illustrated by the scan shown in Fig. 3, 80 lines are resolved by the present technique when rat heart whole homogenate (10 mg/ml) is analyzed. The source of this discrepancy is bacterial contamination in the buffer used for electrophoresis in the earlier experiments. This contamination is now controlled and blank gels are included with each experiment to demonstrate the absence of protein

TABLE 1

PROTEINS USED TO CALIBRATE THE RAT HEART WHOLE HOMOGENATE PATTERN

Protein	Source	Molecular weight[a]	Reference	Line No.[b]
α-Actinin	Rabbit skeletal muscle	(106,000)	14	68
Actin	Rabbit skeletal muscle	41,719	15	44
Aldolase	Rabbit skeletal muscle	38,994	16	42
Carbonic anhydrase (B)	Human red blood cell	28,739	17	29
α-Hemoglobin	Rat	15,196	11	9
β-Lactoglobulin	Bovine milk	18,363	18	16.5
Lysozyme	Chicken	14,314	19	11
Myosin heavy chain	Rabbit skeletal muscle	(210,000)	20	78
Myosin light chain A2	Rabbit skeletal muscle	15,792	21	11.5
Ovalbumin	Chicken egg	(43,000)	22	45
Phosphorylase b	Rabbit skeletal muscle	97,412	23	66
Pyruvate kinase	Rabbit skeletal muscle	(56,000)	24	54
Serum albumin	Bovine	66,296	25	58
Transferrin	Human	(76,600)	26	62.5
Triosephosphate isomerase	Rabbit skeletal muscle	26,626	27	23.5
α-Tropomyosin	Rabbit skeletal muscle	32,682	28	37
Trypsin inhibitor	Soybean	20,095	29	17.5
Trypsinogen	Bovine pancreas	23,990	30	25.5

[a] Molecular weights enclosed in parentheses were based on sedimentation equilibrium measurements; all others were based on amino acid sequences.

[b] Numbers denote which lines in the rat heart whole homogenate pattern migrated to the same point as the corresponding calibration protein. Decimal numbers indicate that the calibration protein migrated to a position in between two lines in the rat heart homogenate pattern.

in the buffer. Of the 80 lines resolved by our current technique, there are 12 major lines that are distributed at convenient intervals along the gel pattern. The 12 major lines are 10, 14, 19, 25, 30, 37, 44, 51, 58, 64, 68, and 78.

Calibration of the Rat Heart Whole Homogenate Standard

As mentioned previously, few of the heart muscle proteins have been purified and characterized. In order to use the rat heart homogenate as a molecular weight standard as well as a reference marker, it was necessary to use proteins for which molecular weights have been determined to calibrate the rat heart homogenate pattern. A list of the proteins used for calibration, together with their respective molecular weights, is given in Table 1. Whenever possible, proteins with molecular weights obtained by amino acid sequencing were used. Use of purified proteins obtained through commercial sources for the calibration of the rat heart whole homogenate pattern is demonstrated in Fig. 4. In addition to these purified proteins, rabbit skeletal muscle whole homogenate was also used for the calibration. The molecular weights of many rabbit skeletal muscle proteins have been obtained by amino acid sequencing. Many of these same proteins have been identified on the ISO-DALT patterns by Giometti *et al.* (10), thus facilitating the assignment of molecular weights to specific protein bands in the rabbit skeletal muscle line pattern when rabbit psoas whole homogenate in agarose is used in the second dimension. Positions of the reference proteins (purified or in rabbit skeletal muscle whole homogenate) with respect to lines in the rat heart

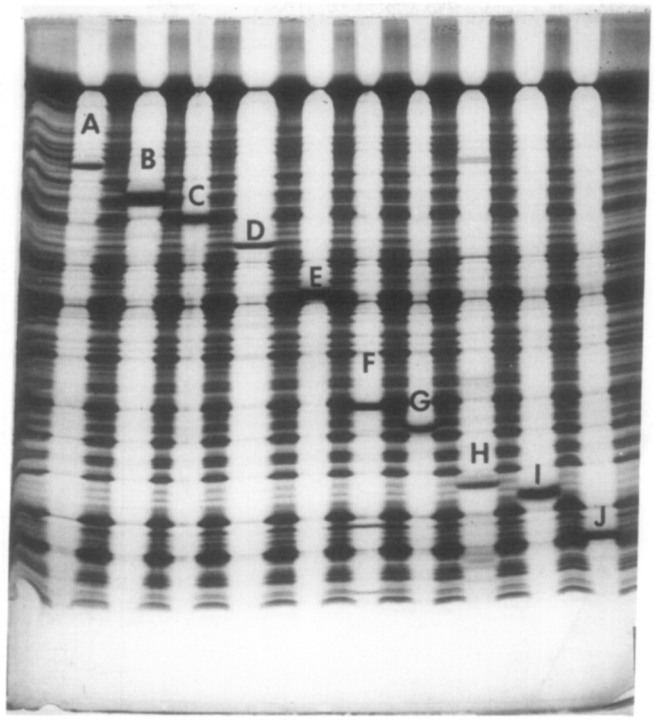

FIG. 4. Calibration of rat heart whole homogenate pattern using purified proteins. Rat heart whole homogenate in agarose was applied to the top of the slab gel. Slots (1 cm) were made in the agarose after it solidified and one purified protein in agarose was put into each slot. (A) Rabbit phosphorylase b (100 μg/ml); (D) rabbit skeletal muscle pyruvate kinase (50 μg/ml); (E) ovalbumin (100 μg/ml); (F) human carbonic anhydrase B (50 μg/ml); (G) bovine pancreas trypsinogen (100 μg/ml); (H) soybean trypsin inhibitor (250 μg/ml); (I) β-lactoglobulin (100 μg/ml); (J) lysozyme (50 μg/ml).

whole homogenate pattern are given in Table 1 and the combined data are shown graphically in Fig. 5. Line numbers were assigned based on the scan shown in Fig. 3. The SDS molecular weights of the major lines in the rat heart whole homogenate pattern were determined from this graph and are listed in Table 2.

Variation of Rat Heart Whole Homogenate Line Pattern with Gel Concentration

The work described thus far was done using gradient slab gels made of 10 to 20% acrylamide. In order to determine how the rat heart whole homogenate pattern changes with gel concentration, linear gels of 10% and 15% acrylamide were used. The whole homogenate standard was used over a two-dimensional pattern of human red blood cell lysate proteins. Figure 6 shows the comparison of a portion of the pattern from a 10% gel and a 15% gel. Note that the 20 lowest molecular weight proteins seen on the 15% gel are compressed into a single line on the 10% gel. These results illustrate the usefulness of the heart standard mixture in making comparisons between results from non-identical gels.

DISCUSSION

As two-dimensional electrophoresis becomes a more widely used technique, the need for standardization becomes more crucial to the intercomparison of results. Standardization of the isoelectric focusing dimension with carbamylated charge standards has already been described (6). An exactly analogous standard for the molecular

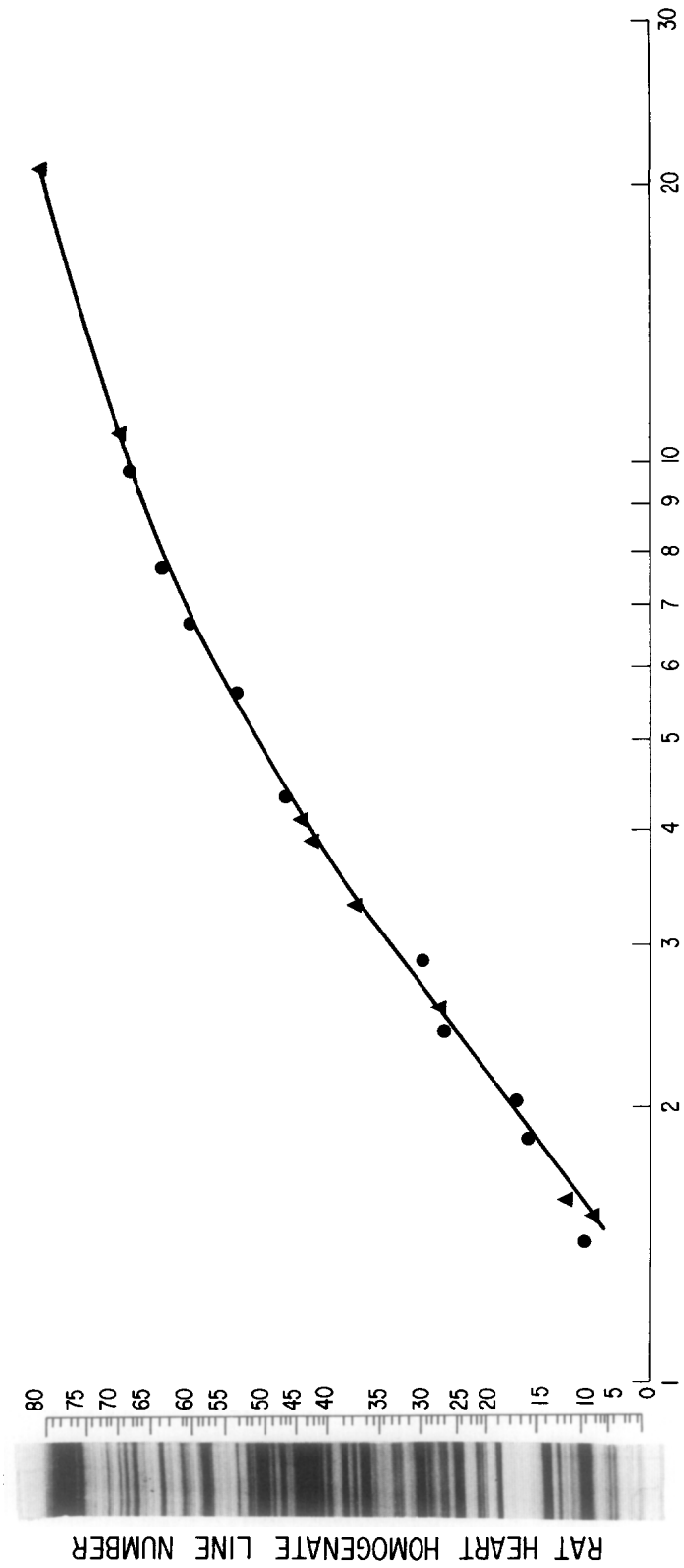

FIG. 5. Molecular weight standard curve. The molecular weights of the calibration proteins used (Table I) were plotted against the lines in the rat heart whole homogenate which migrated to the same place after SDS electrophoresis. Note that this curve is for a gel composed of 10–20% acrylamide in a linear gradient. A photograph of the rat heart homogenate pattern is shown to the left of the curve. Line numbers were taken from the density scan shown in Fig. 3. ●, Purified calibration proteins; ▲, rat or rabbit proteins.

weight separation dimension, i.e., having even, small increments in molecular weight, remains to be found (although bacterial polyphosphates labeled with ^{32}P show some promise; N. L. Anderson, unpublished results). Ideal molecular weight standards should be a mixture of proteins ranging in molecular weight from approximately 10,000 to 200,000 in narrow, but evenly spaced, increments. In addition, the protein mixtures of choice should be (a) economical, (b) readily available in fresh form, and (c) capable of producing very sharp lines in the high resolution SDS electrophoresis system currently available. Commercially prepared purified proteins which could be combined to produce standard mixtures are unsatisfactory on all three points. The SDS molecular weight standards available as kits range in price from $20 to $60 for 1–10 mg of protein and individual purified proteins are equally expensive. Figure 1 shows that the commercially prepared standards result in much broader protein lines than those seen with fresh tissue preparations. In addition, several commercial preparations yield more lines than can be accounted for by the listed protein content of the standard (e.g., Figs. 1A and C where each preparation supposedly contained just five proteins). The extra lines are probably the result of protein degradation in the samples used. Whatever the source, a standard containing unexpected proteins is less than ideal. On the other hand, a survey of seeds, body fluids, and tissues has shown that tissue homogenates fulfill all three of the qualifications listed above. Of the tissues examined thus far, we have found rat heart to be the most useful with its evenly spaced lines of differing intensities covering the full range of molecular weights resolved using available methods.

The heart homogenate may be fractionated into myofibrils and cell sap, but such preparations are less useful as standards than the whole homogenate because they produce fewer lines after SDS electrophoresis. Use of the whole homogenate decreases the number of sample manipulations necessary before addition of SDS and agarose, thereby decreasing the possibility of protein degradation during preparation. At a concentration of 10 mg/ml, the rat heart whole homogenate produces 80 lines. When lower concentrations of the whole homogenate are used (e.g., 2.5 mg/ml), only the 12 major lines are seen. The concentration of the homogenate can be adjusted so that there are enough lines visible to make useful comparisons, but not so many lines that the two-dimensional pattern being studied is obscured. We have numbered the lines in the rat heart whole homogenate pattern in order to have coordinates to use when patterns are compared or mapped. As the resolution of SDS electrophoresis improves and more lines are resolved in the whole homogenate pattern, the lines can be identified in the future using decimal divisions of these original numbers.

TABLE 2

SDS MOLECULAR WEIGHTS OF MAJOR LINES IN RAT HEART WHOLE HOMOGENATE PATTERN[a]

Line No.	SDS Molecular Weight
10	15,200
14	17,500
19	20,600
25	23,700
30	27,500
37	32,500
44	41,700
51	51,500
58	68,000
64	86,000
68	106,000
78	220,000

[a] Molecular weights were determined using the graph shown in Fig. 5.

Since few of the rat heart muscle proteins have yet been purified and characterized, it was necessary to calibrate the whole homogenate SDS electrophoresis pattern using proteins from other sources for which molecular weights have been determined. Although the primary purpose of the rat

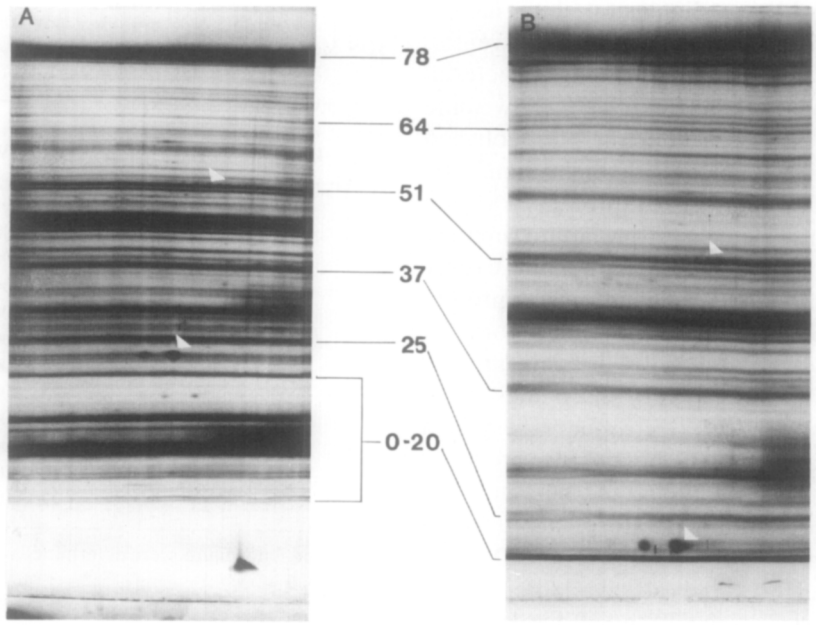

FIG. 6. Comparison of human red blood cell lysate two-dimensional electrophoresis patterns from 15 and 10% slab gels. Human red blood cell lysates were prepared and separated in the first dimension as described by Edwards *et al.* (5). The first dimension gels were sealed onto the second dimension gel using agarose containing 10 mg rat heart whole homogenate/ml. The second dimension separation was done using (A) 15% acrylamide slab or (B) 10% acrylamide slab. The white arrows mark the positions of the same proteins in both patterns and numbers refer to the lines in the rat heart homogenate pattern. Both gels were run until the bromophenol blue tracking dye reached the edge of the separating gel.

heart standards is to serve as reference for the intercomparison of two-dimensional electrophoresis patterns, some information about approximate molecular weights is desirable in order to describe different protein spots. Using SDS electrophoresis, purified proteins and rabbit psoas muscle whole homogenate were separated along with rat heart whole homogenate. The migration positions of the proteins with known molecular weights were correlated with the positions of lines in the rat heart pattern. These data were used to plot the graph shown in Fig. 5, a plot which can be used to determine approximate molecular weights for protein spots which fall within the range of rat heart whole homogenate patterns. Based on these data, interline SDS molecular weight differences in the rat heart whole homogenate pattern range from approximately 300 (approximately equal to three amino acids) at the lower end of the pattern to 10,000 (approximately equal to 100 amino acids) at the upper end when a 10–20% acrylamide gradient gel is used. This conclusion is made with the assumption that all of the rat heart proteins bind equal amounts of SDS. This remains to be proven because certain types of proteins demonstrate anomalous electrophoretic behavior (31–33) that is related to disproportionate binding of SDS to the proteins (34,35).

The rat heart whole homogenate standard is now sufficiently well characterized for use with two-dimensional electrophoresis systems. By including the rat heart preparation in the agarose overlay used to hold the isofocusing gel (first dimension) in contact with the slab gel (second dimension), horizontal lines will be superimposed on

the two-dimensional electrophoresis pattern. These lines are useful in the comparison of two gel patterns on the same or on different types of slab gels (e.g., gradient versus linear), thus permitting intercomparisons of gel patterns from different labs using different equipment and/or techniques. At the present time, such intercomparisons of gel patterns appearing in the literature are difficult because investigators use different ampholine ranges in the first-dimension separation and different acrylamide concentrations in the second dimension. If each investigator were to include charge standards (6) for the first dimension and use rat heart whole homogenate standards in the second dimension, reference points independent of the ampholines and acrylamide concentration would make intercomparisons straightforward.

Charge and molecular weight standards can be useful as coordinates for the mapping of protein gene products by two-dimensional electrophoresis in addition to their use for the intercomparison of gels. Both intercomparisons and mapping will be facilitated by the development of computerized image analysis systems (36). The charge standards and rat heart protein standard will be used in the data reduction systems being developed in this laboratory.

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