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High Resolution Two-Dimensional Electrophoresis of Basic as Well as Acidic Proteins

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Summary

A previously described two-dimensional electrophoresis procedure (O'Farrell, 1975) combined isoelectric focusing and sodium dodecylsulfate slab gel electrophoresis to give high resolution of proteins with isoelectric points in the range of pH 4–7. This paper describes an alternate procedure for the first dimension which, unlike isoelectric focusing, resolves basic as well as acidic proteins. This method, referred to as nonequilibrium pH gradient electrophoresis (NEPHGE), involves a short time of electrophoresis toward the cathode and separates most proteins according to their isoelectric points. Ampholines of different pH ranges are used to optimize separation of proteins with different isoelectric points. The method is applied to the resolution of basic proteins with pH 7–10 Ampholines, and to the resolution of total cellular proteins with pH 3.5–10 Ampholines. Histones and ribosomal proteins can be readily resolved even though most have isoelectric points beyond the maximum pH attained in these gels. The separation obtained by NEPHGE with pH 3.5–10 Ampholines was compared to that obtained when isoelectric focusing was used in the first dimension. The protein spot size and resolution are similar (each method resolving more than 1000 proteins), but there is less resolution of acidic proteins in this NEPHGE gel due to compression of the pattern. On the other hand, NEPHGE gels extend the range of analysis to include the 15–30% of the proteins which are excluded from isoelectric focusing gels. The distribution of cell proteins according to isoelectric point and molecular weight for a procaryote (E. coli) was compared to that of a eucaryote (African green monkey kidney); the eucaryotic cell proteins are, on the average, larger and more basic.

Introduction

A high resolution two-dimensional gel electrophoresis procedure has recently been developed for the analysis of complex mixtures of proteins (O'Farrell, 1975). By combining isoelectric focusing (IEF) in the first dimension with sodium dodecylsulfate (SDS) gel electrophoresis in the second dimension, this procedure (IEF-SDS) can resolve over 1000 proteins with isoelectric points in the pH range 4–7. When the pH gradient is extended to higher pH, however, the few slightly basic proteins which enter the gel are not well resolved (Piperno, Huang and Luck, 1977), and the pH gradient cannot be further extended to include very basic proteins. Two-dimensional electrophoretic techniques designed for the separation of basic proteins have been successfully applied to ribosomal proteins (Kaltschmidt and Wittman, 1970; Howard and Traut, 1973; Metz and Bogorad, 1974; Warner and Gorenstein, 1977), but these procedures give little resolution of moderately basic and acidic proteins. Furthermore, the protein spot areas are large relative to the gel area used for separation.

Here we describe an alternative electrophoresis procedure which was developed to resolve basic proteins, but which also gives high resolution of proteins with isoelectric points across the entire pH range. This method, referred to as nonequilibrium pH gradient electrophoresis (NEPHGE), can be combined with SDS gel electrophoresis to give a high resolution two-dimensional procedure, NEPHGE-SDS. The method is described, and the separation achieved with NEPHGE-SDS is compared with the separation achieved with IEF-SDS. Most of the components, both chemical solutions and equipment, are the same as in IEF-SDS (O'Farrell, 1975). NEPHGE-SDS is highly adaptable, and we describe one set of conditions designed for the resolution of basic proteins, and a second capable of simultaneously separating acidic and basic proteins. In addition, differences in the overall distribution of cell proteins with respect to isoelectric point and molecular weight for a procaryote (E. coli) and a eucaryote (African green monkey kidney) are described.

Results and Discussion

We tested several approaches for the resolution of basic proteins involving modifications of the first dimension, while the second dimension remains the same as that originally developed by Laemmli (1970). Modifying IEF to include more basic Ampholines instead of the usual pH 5–7 and pH 3–10 mixture was not successful since in the presence of urea, the basic region of the pH gradient was unstable. Although including basic Ampholines in the gel resulted in a small extension of the pH gradient, the few moderately basic proteins which entered the gel always produced streaks. Attempts to develop an acceptable isotachophoresis method (Haglund, 1970) for the first dimension were also unsuccessful, but led to the current empirical procedure which we refer to as nonequilibrium pH gradient electrophoresis (NEPHGE).

The present studies describe an empirical method whereby proteins are separated in the presence of a pH gradient according to both their electrophoretic mobility and isoelectric point. The first dimension gel is cylindrical and contains urea, the nonionic detergent NP-40 and Ampholines as before. The pH range of the Ampholines is varied depending upon the range of proteins to be resolved. Electrophoresis is toward the cathode with the acidic reservoir on top and the basic reservoir on the bottom. Thus basic proteins lead in the separation. Results of comparable quality were not obtained when proteins were electrophoresed in the opposite direction. Electrophoresis is for short times so that equilibrium is not attained, and as a consequence, the time of electrophoresis determines the protein distribution. The effects of different variables are described below and are used to suggest the basis of the separation.

Time of Electrophoresis

Figure 1 shows the changes in pH gradients in the first dimension as a function of time of electrophoresis for two Ampholine ranges, pH 7–10 (Figure 1A) and pH 3.5–10 (Figure 1B). It can be seen that the pH gradient is formed rapidly for both pH ranges, and that after 500 V hr, the pH gradients are relatively stable in the more acidic pH range but not at the basic ends of the gels. The collapse of the basic end of the pH gradient is not understood, but may be partly due to cathodic migration (Chrambach et al., 1973), which is especially severe in the presence of urea. This is why equilibrium IEF, which requires long periods of electrophoresis, is unsatisfactory for resolving basic proteins in urea.

The resolution of proteins by NEPHGE is optimal after relatively short times of electrophoresis. Figure 2 shows the two-dimensional patterns of ^{35}S-methionine-labeled whole cell extracts from African green monkey kidney cells resulting from electrophoresis in the first dimension for 1, 2, 4 and 10 hr at 500 V. The first dimension gels containing pH 7–10 Ampholines resolve proteins with isoelectric points greater than pH 7; none of the proteins shown in Figure 2 enters an IEF gel (see Figure 5 below). Basic proteins lead the separation from right to left, and the right sides of the gel patterns represent the tops of the first dimension gels. Both IEF and NEPHGE patterns are oriented with the more basic proteins to the left. Increasing the time of electrophoresis in the first dimension does not increase the number of proteins entering the gel,

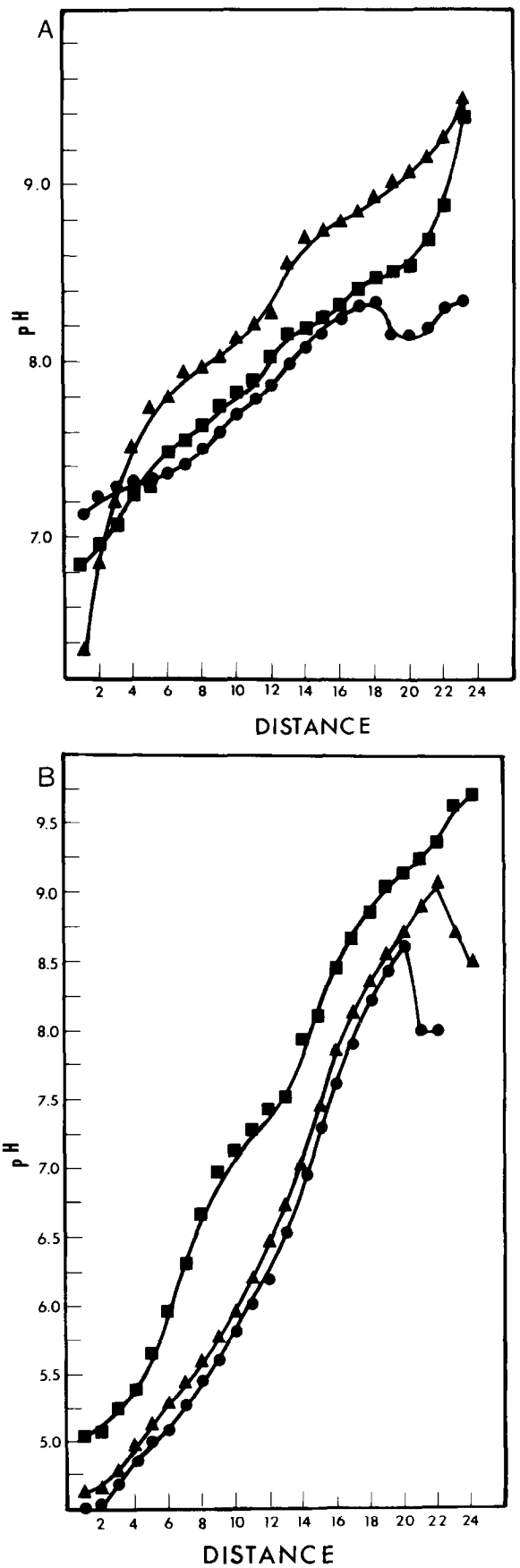


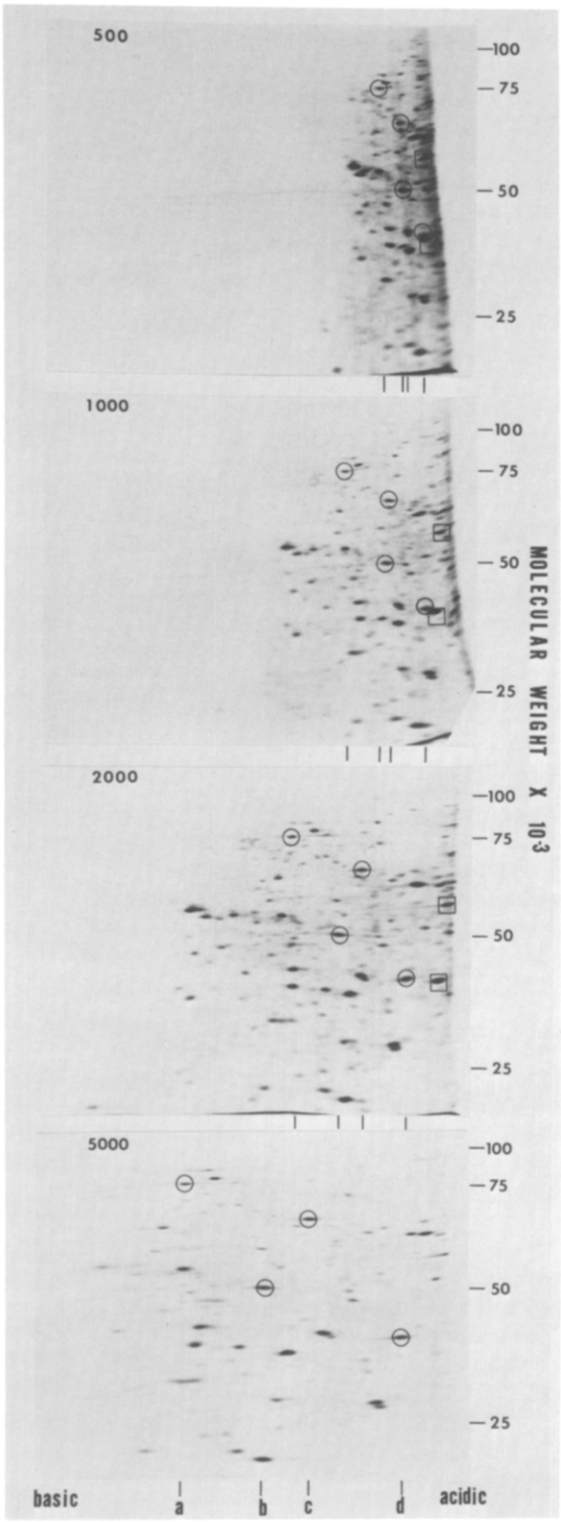
Figure 1. Formation of the pH Gradient in First Dimension Gels as a Function of Time of Electrophoresis.

(A) shows pH 7–10 gels after 500 (▲), 2000 (■) and 4000 (●) V hr. (B) shows pH 3.5–10 gels after 500 (■), 1500 (▲) and 3000 (●) V hr. 24 pH measurements were taken at half cm intervals.

but does increase the separation of the protein spots. Optimal resolution occurs, however, after relatively short periods of electrophoresis; by 5000 V hr, the protein spots have become broad and some are lost from the ends of the gels—for example, the spots enclosed in squares (Figure 2).

Figure 3 graphically displays the migration of the protein spots a-d (circled in Figure 2) as a function of the time of electrophoresis. While the mobility of all proteins decreases with time, the more acidic proteins slow down earlier than the basic proteins. In fact, protein spot d appears to reach equilibrium at about 2000 V hr.

A more striking illustration of changes in mobility of proteins with different isoelectric points is shown in Figure 4 for pH 3.5–10 NEPHGE gels electrophoresed for between 1000 and 5000 V hr. Small sections of several of these final two-dimensional patterns are shown in Figure 4A. Figure 4B



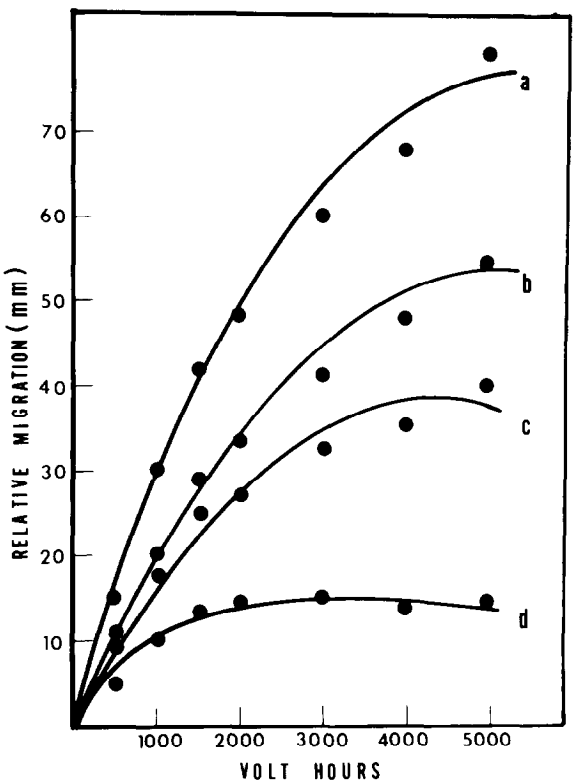


Figure 3. Relative Migration of Proteins as a Function of Time of Electrophoresis

The distances migrated by proteins enclosed in circles in Figure 2 (protein spots a, b, c and d) are plotted as a function of time of electrophoresis in V hr.

Figure 2. Migration of Proteins as a Function of Time of Electrophoresis on pH 7–10 Gels

^{35}S-methionine-labeled whole cell extracts from African green monkey cells were subjected to electrophoresis in the first dimension from 1–10 hr at 500 V on a pH 7–10 gradient. These gels were electrophoresed in the second dimension on 10% acrylamide SDS gels. The patterns resulting from 500, 1000, 2000 and 5000 V hr of electrophoresis in the first dimension are shown. The tops of each first dimension gel, which is at approximately pH 7, is oriented toward the right. The more basic proteins are to the left. Those protein spots enclosed within circles or squares are discussed in the text.

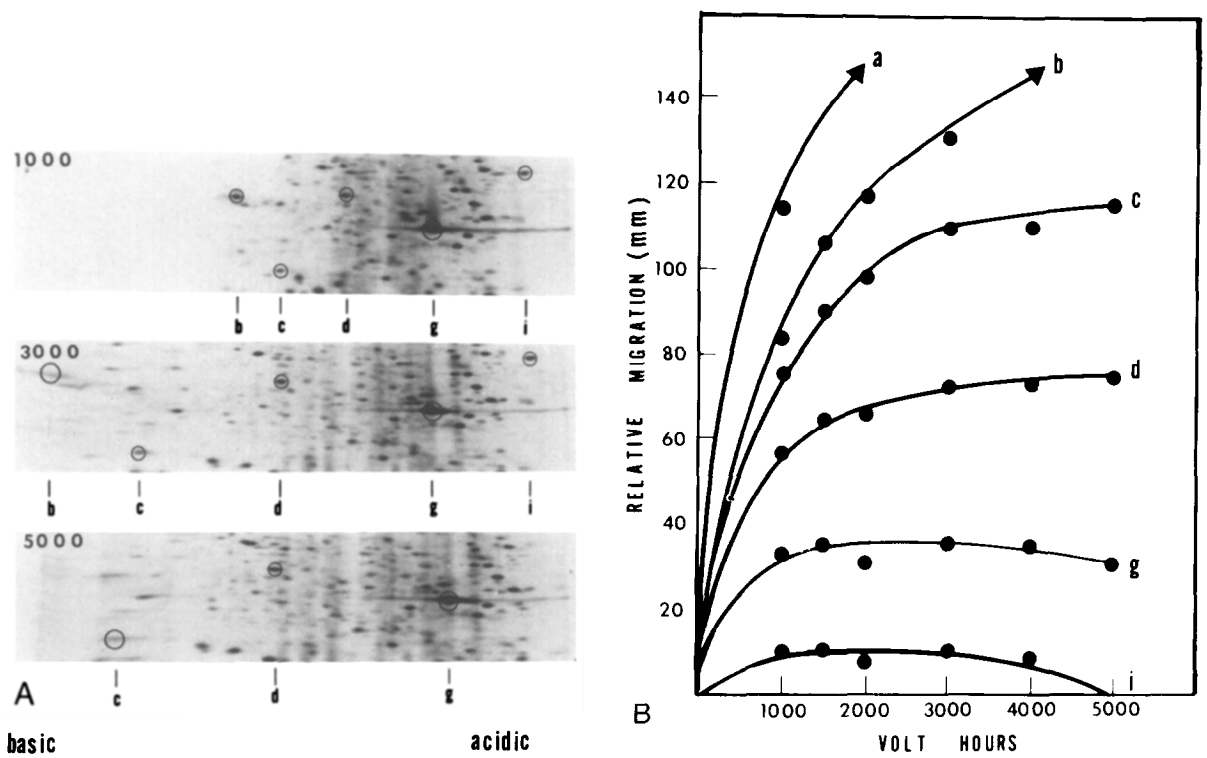


Figure 4. Migration of Proteins as a Function of Time of Electrophoresis on pH 3.5–10 Gels

Electrophoresis of radioactively labeled monkey cell extracts was carried out as described in Figure 2, except that the first dimension gels contained pH 3.5–10 Ampholines. (A) shows portions of the gels resulting from electrophoresis for 1000, 3000 and 5000 V hr. The distances migrated by the circled protein spots b, c, d, g and i (also indicated in Figure 5A) as well as spot a (seen only in Figure 5A) are plotted as a function of time of electrophoresis. The arrows in the curves for protein spots a and b indicate that these protein spots have run off the end of the gel.

graphically displays the rate of migration of protein spots a, b, c, d, g and i (circled in Figures 4A and/or 5). The region of the gel around protein spot d represents approximately pH 7 in the gradient. The majority of the protein spots from the acidic end to slightly before the d region (roughly 70% of the number of proteins resolved) have reached apparent equilibrium after 1000 V hr since most of these proteins do not migrate further with continued electrophoresis. After very long times (5000 V hr), a few of the very acidic proteins usch as spot i migrate backwards off the gel. The mobilities of the moderately basic proteins (such as spots c and d) gradually decrease, and the mobilities of the most basic proteins (such as spots a and b) hardly decrease, so that these proteins eventually migrate off the basic end of the gel.

To summarize, in NEPHGE the proteins are separated in the presence of a rapidly formed pH gradient. The mechanism of the separation is not understood but involves both isoelectric focusing for acidic proteins and nonequilibrium electrophoresis for more basic proteins. Most importantly, the protein spots in all pH ranges are sharp except after prolonged periods of electrophoresis. This sharpness is probably due to several forces. A transient stacking force analogous to isotachophoresis occurs naturally as a consequence of the differences in mobilities between the different ion species; since there is no leading or terminating electrolyte, however, this effect probably has an influence only at very short times. In addition, a sharpening force occurs due to the continually decreasing mobility of each protein as it moves through the pH gradient (decreased mobility is the result of titration of the proteins). Finally, the more acidic proteins are focused at their isoelectric points.

The optimal time of electrophoresis depends upon the particular proteins being examined. The acidic proteins rapidly reach their isoelectric points and retain their sharpness even after long periods of electrophoresis. The more basic proteins never reach their isoelectric points, and if electrophoresis is continued after the pH gradient begins to collapse at the basic end, these protein spots will become broader. Thus for overall quality, we recommend 2000 V hr and standardly use 1600 V hr.

As is discussed below, however, for very basic proteins even shorter times of electrophoresis are optimal.

Distribution of Proteins as a Function of Ampholine pH Range

Although the present system was developed for the resolution of proteins with basic isoelectric points, it is useful as a general method to resolve proteins within all pH ranges. As shown in Figure 5A, both acidic and basic proteins from monkey cell extracts are resolved by electrophoresis through a pH 3.5–10 gradient. Figures 5B and 5C show the same cell extract following electrophoresis in a pH 7–10 NEPHGE system or in an IEF system (O'Farrell, 1975). It can be seen that Figure 5A includes the total distribution of proteins resolved in both Figures 5B and 5C. Protein spots a–i are circled in Figures 5A–5C so that the three patterns can be directly compared. The line drawn through Figure 5A indicates the approximate position of pH 7, which also corresponds to the tops of both the isoelectric focusing gel (more basic end) and the pH 7–10 NEPHGE gel (more acidic end). Even though there is little overlap between the proteins displayed in Figures 5B and 5C, comparison with Figure 5A reveals that few if any proteins are not represented in one of these two patterns.

The separations in Figure 5 show the distribution of monkey cell proteins as a function of relative isoelectric point and molecular weight. For comparison, we analyzed the relative distribution of proteins for a procaryote, E. coli, in the same manner (Figure 6). Once again, the separation shown in Figure 6A includes the total range of proteins seen in Figures 6B and 6C.

Comparison of Figure 5 with Figure 6 shows that E. coli and monkey cells differ substantially in their distribution of proteins relative to isoelectric point and molecular weight. There are very few moderately basic proteins in E. coli. Most of the very basic proteins are ribosomal, since they comigrate with total proteins from purified ribosomes (stained patterns not shown). Most of the basic proteins in E. coli are present in trace amounts except for the abundant ribosomal proteins. Roughly 15% of the E. coli proteins are basic—that is, not present on the IEF gel. On the other hand, the eucaryotic proteins are less clustered, forming a more continuous distribution with respect to isoelectric point and abundance. Roughly 30% of the eucaryotic proteins are basic. The higher number of basic proteins in a eucaryotic cell may reflect those proteins involved in chromatin structure and function. It should be emphasized that these percentages are calculated on the basis of number of individual proteins and not on the basis of mass.

Using the procedure described in the Experimental Procedures, we determined the average of the molecular weights of the detected proteins; this average does not formally correspond to either weight average molecular weight or number average molecular weight since the relative abundance of the different species is ignored. The average of the molecular weights of E. coli proteins is 49,000 daltons, and the average of the molecular weights of monkey cell proteins is 58,000 daltons. The increased average molecular weight in the eucaryotic cell is due to the larger number of very high molecular weight proteins (>100,000 daltons). (These estimates rely on the accuracy of SDS gel electrophoretic mobility as a measure of molecular weight, and are an overestimate of the average because the sensitivity of detection of low molecular weight proteins is less than that for high molecular weight proteins, and smaller proteins are more likely to be lacking methionine.) For both E. coli and monkey cells, basic proteins tend to be of lower molecular weight.

Reproducibility and Resolution

The reproducibility of the separation is excellent when gels are run under identical conditions; it is usually possible to locate the same protein spot on two different separations by using surrounding spots for orientation. In addition, most proteins retain their mobilities relative to one another after different times of electrophoresis, as can be seen from Figures 2 and 4A. There are exceptions, however; for example, the relative mobilities of the two spots enclosed in squares in Figure 5A are reversed in Figure 5B. This variation presumably reflects some changing condition during electrophoresis in the NEPHGE system. Because of such variations, exact reproduction of a separation requires a constant experimental protocol.

In addition to time of electrophoresis, length of the gel and pH of the Ampholines, minor changes in the sample mixture can change the resolution and distribution of proteins. To illustrate this point, Figure 7 shows one-dimensional NEPHGE separations of protein samples containing salts, buffers or detergent. In general, salt decreases the electrophoretic mobility of proteins nonuniformly; moreover, salt at very high concentrations reduces the sharpness of the protein bands in the basic region of the gel (at <0.25 M, the effects are not too severe, but 1.0 M has drastic effects). Obviously, if two or more samples are to be compared, all should contain equivalent amounts of any additives which will alter the mobility of the proteins.

The presence of SDS affects neither the sharpness nor the mobility of the proteins, but severely

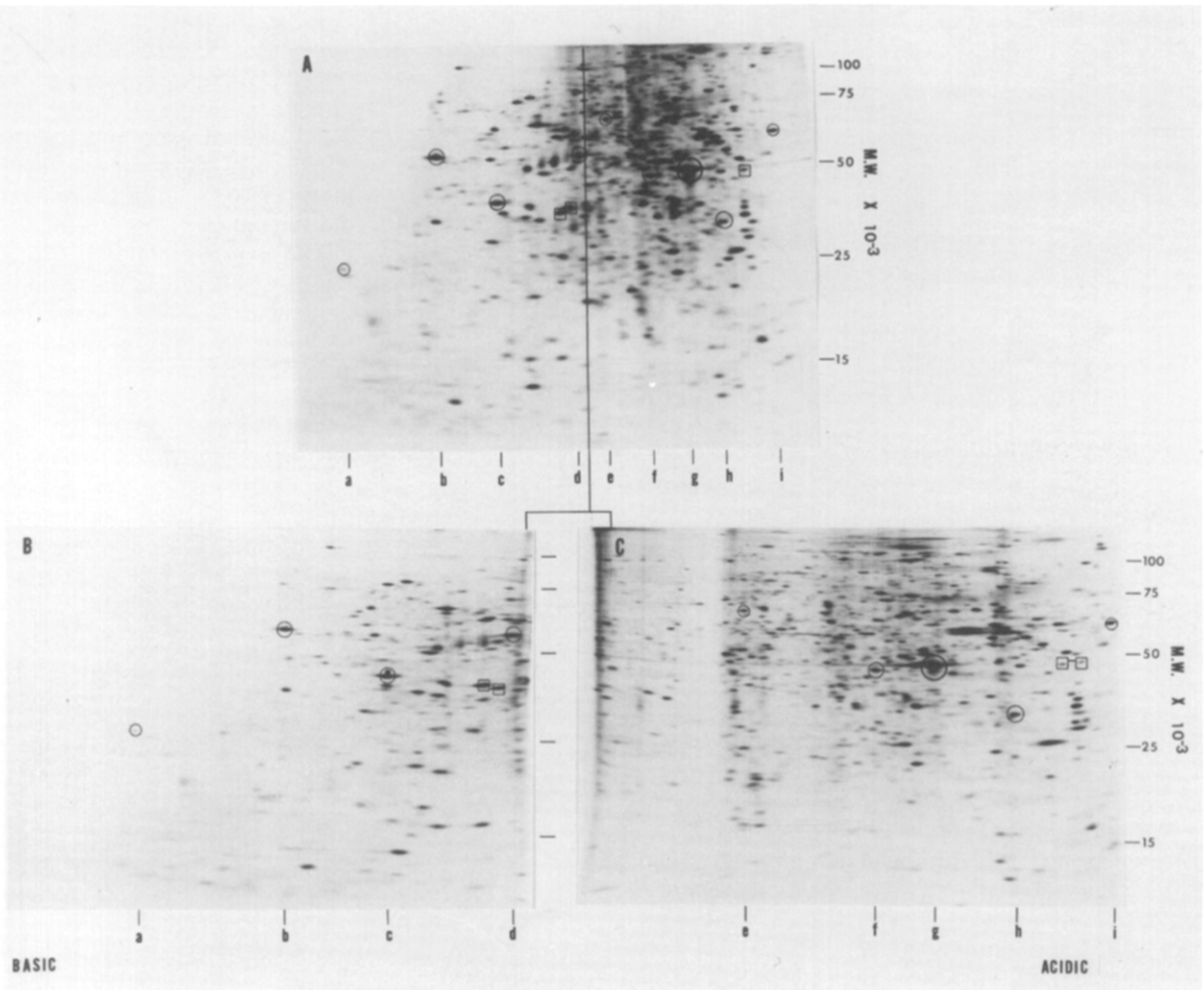


Figure 5. The Distribution of Total Cell Proteins from Monkey Cells as a Function of Molecular Weight and Relative Isoelectric Point

^{35}S-methionine-labeled monkey cells were subjected to three different types of electrophoresis in the first dimension, either by NEPHGE with a gradient of pH 3.5–10 (A), by NEPHGE with a gradient of pH 7–10 (B), or by equilibrium isoelectric focusing (C). The NEPHGE gels were electrophoresed for 1600 V hr and the IEF gels for 6800 V hr. Each first dimension gel was run in the second dimension on gels containing an exponential gradient of acrylamide from 10–18% with 9 ml per gel initially in the constant volume chamber. The line drawn through the gel pattern in (A) represents approximately pH 7. The protein spots a–i circled in (A) are also circled in (B) and (C) for comparison. The proteins enclosed in squares are described in the text.

affects the proportion of proteins which enter the gel. Presumably the SDS remains bound to a fraction of the protein and due to the negative charge, causes proteins to migrate into the upper reservoir. Thus if there is SDS in samples, excess NP-40 should be added to help dissociate the SDS protein complexes (O'Farrell, 1975). The following protocol was devised to take advantage of the properties of SDS to aid in protein solubilization. We recommend that samples be dissolved in 0.5% SDS, 9.5 M urea, 5% β-mercaptoethanol and 0.2% Ampholines pH 3.5–10. After 10 min at room temperature, the samples are mixed with an equal volume of the NP-40 containing lysis buffer. To avoid any loss of protein during electrophoresis, the sample is overlayed with 25 μl of 5% NP-40, 8 M urea and 1% Ampholines (0.8% pH 5–7 and 0.2% pH 3.5–10).

When different methods of preparation of whole cell extracts for electrophoresis are used, the relative positions of the proteins in the resulting separations do not change, but the presence of a few specific protein spots is dependent upon the method of sample preparation. Thus the method of sample preparation should be carefully standardized.

By the criteria of spot size and number of spots detected across the area of the gel, the resolutions of the pH 3.5–10 NEPHGE gels are comparable to IEF. For example, we have been able to count up to 1400 protein spots from monkey cells on either a pH 3.5–10 NEPHGE gel or an IEF gel. A pH 3.5–10 NEPHGE gel, however, only resolves 70% of the

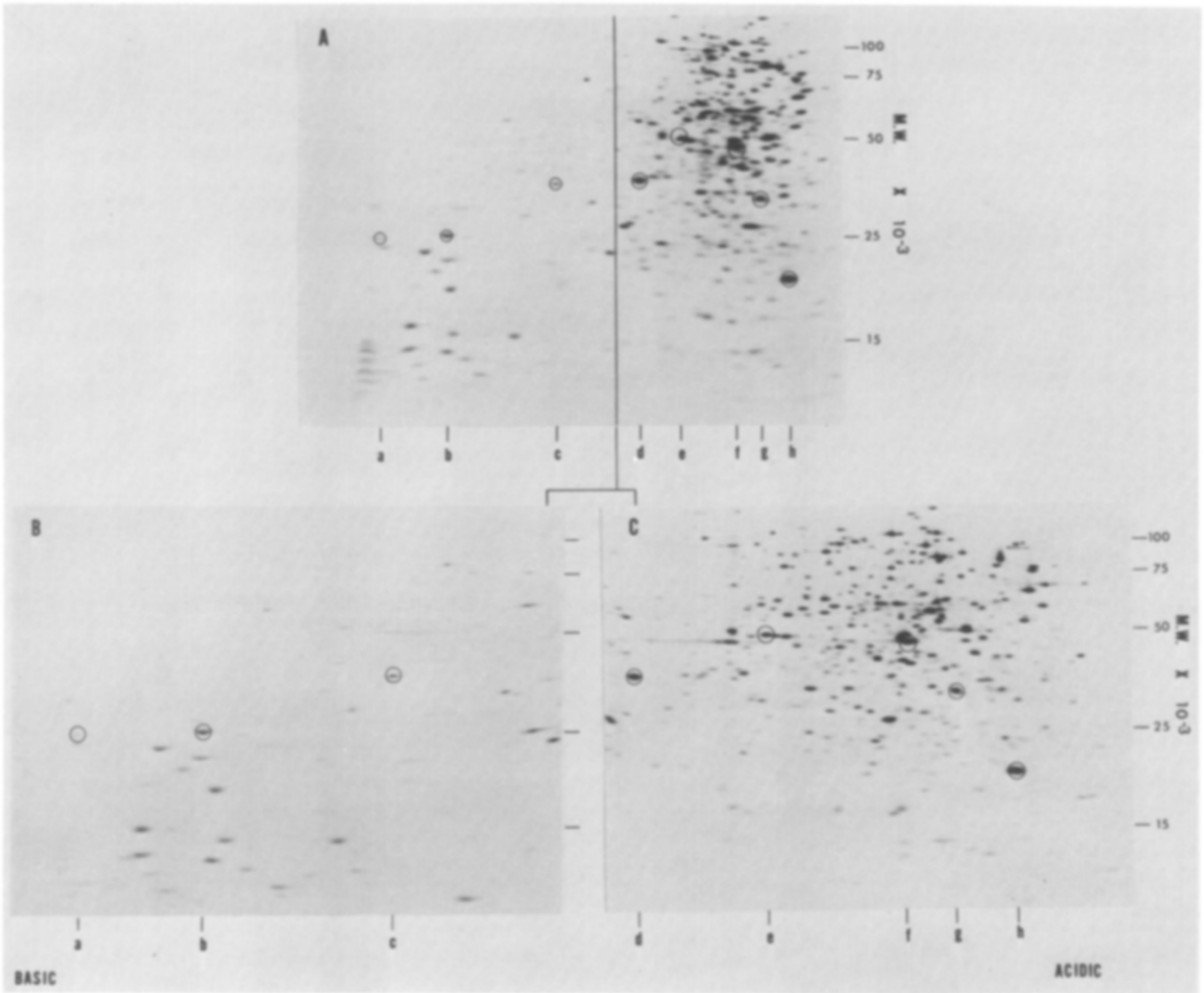


Figure 6. The Distribution of Total Cell Proteins from E. coli as a Function of Molecular Weight and Relative Isoelectric Point

^{35}S-methionine-labeled E. coli was subjected to electrophoresis, and the resulting protein patterns were compiled as described in Figure 5.

total number of monkey cell proteins detectable on a pH 7–10 NEPHGE gel (650 protein spots) plus an IEF gel (1400 protein spots); this loss of resolution is due to crowding of proteins with isoelectric points below pH 7. For example, the two protein spots enclosed in squares in Figure 5C appear as a single spot in Figure 5A. Thus for the highest resolution of proteins across all pH ranges, both an IEF gel and a pH 7–10 NEPHGE gel should be run.

Applications and Recommendations

The pH 3.5–10 NEPHGE gel is most useful for surveying overall patterns of proteins, while narrower pH ranges can be used for more detailed studies of particular proteins. The best resolution of acidic proteins is obtained with equilibrium IEF since the region of the gels containing acidic proteins is compressed in NEPHGE. The best resolution of basic proteins is with a pH 7–10 NEPHGE gel. If greater overlap between a NEPHGE gel, resolving predominantly basic proteins and an IEF gel, is required, a gel mixture containing equal parts of Ampholines pH 6–8 and pH 8–10 (1% each) can be use in place of Ampholines pH 7–10 (2%). For the highest resolution of the entire range of proteins, two gels are required—an IEF gel for acidic proteins and a NEPHGE gel for basic proteins.

The time of electrophoresis can be varied. For most investigations, we recommend a relatively short period of electrophoresis between 1500 and 2000 V hr; this time provides a good balance between the sharpness and the resolution of the protein spots. For very basic proteins, we recommend 1200 V hr or less. For example, many of the E. coli ribosomal proteins in Figures 6A and 6B have electrophoresed off the basic end of the gel

after 1600 V hr. In addition, we have noted that at 1200 V hr, the protein spots corresponding to the histones have become broad and are close to the end of a pH 3.5–10 NEPHGE gel (data not shown). In our hands, these very basic proteins are best resolved at 1000 V hr on a pH 3.5–10 NEPHGE gel and 1200 V hr on a pH 7–10 NEPHGE gel.

In all the figures of gels presented, we have deliberately omitted any specification of the pH at any location in the gel since NEPHGE gels cannot be used for the determination of absolute isoelectric points of proteins. The isoelectric point of a protein is usually determined in reference to a stable pH gradient formed during isoelectric focusing. Here the pH gradient forms early while the proteins are beginning to migrate. Only when the proteins stop moving can their isoelectric points be determined in relation to the pH gradient. For example, the more acidic proteins in the pH 3.5–10 gels which stop migrating (see Figure 4A) are likely to be at their isoelectric points. The more basic proteins, however, are slow to stop migration. Thus although it is probable that even the basic proteins are migrating relative to their isoelectric points, their isoelectric points cannot be determined accurately from the pH gradient. It is suggested that the positions of protein in NEPHGE gels be referred to relative to other proteins to avoid the inaccuracies of using a physical designation of unknown precision. Furthermore, the reproducibility and accuracy with which the position of migration of a standard protein can be determined are much greater than that of pH measurements.

Other properties of high resolution two-dimensional electrophoresis (that is, loading capacity, sensitivity of detection, streaking, charge heterogeneity and possible artifacts) have already been discussed (O'Farrell, 1975) and apply to NEPHGE. Single change modifications of most acidic proteins are resolved by NEPHGE gels, but the spacing is less than in IEF gels. Single charge modification (acetylation) of the very basic histones, however, did not produce a detectable change in mobility.

To avoid confusion in the literature, it is suggested that protein patterns be displayed in a standard fashion as presented here with basic proteins on the left and acidic proteins on the right.

It is hoped that the present technique will be useful for investigators studying protein patterns both as a one-dimensional procedure for some applications, and in combination with SDS slab gel electrophoresis as a high resolution two-dimensional system.

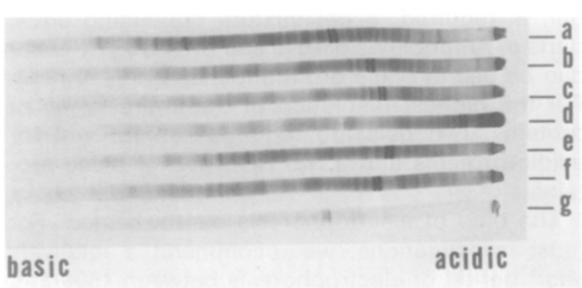


Figure 7. Changes in Mobility of Proteins on NEPHGE Gels as a Function of Addition of Ionic Compounds

^{35}S-methionine-labeled monkey cells were lysed in lysis buffer, and the extracts were adjusted to contain various additives: (a) control, no additions; (b) 0.25 M NaCl; (c) 0.50 M NaCl; (d) 1.0 M NaCl; (e) 0.25 M Tris (pH 7.4); (f) 0.25 M sodium phosphate buffer (pH 7.4); and (g) 0.25% SDS.

Experimental Procedures

Chemicals

All chemicals for electrophoresis and their suppliers were as described previously (O'Farrell, 1975). LKB Ampholines with nominal ranges of pH 7–10 and pH 3.5–10 were used as carrier ampholytes. We subsequently learned that Ampholines pH 7–10 are no longer available, but an equivalent mixture for these purposes can be generated by using equal parts of the available pH 7–9 and pH 8–10 ranges. Ampholytes from other suppliers are not equivalent.

Preparation of Radioactive Cell Extracts

African green monkey kidney cells (CV-1) in multiwell dishes (15 mm diameter wells) at 10^5 cells per well were labeled with 50 μCi ^{35}S-methionine (spec. act. >200 Ci/mM; Amersham #SJ-204) for 3 hr at 37° C in 0.25 ml of methionine-free media and prepared for electrophoresis as described (O'Farrell and Goodman, 1976). E. coli 1100 at 2×10^8 cells per ml was labeled in M9 media with 10 μCi ^{35}S-methionine for 10 min at 30°C. The cells were collected by centrifugation and lysed by sonication, and the lysate was nuclease-treated and prepared for electrophoresis as described (O'Farrell, 1975). The specific activities of both radioactive extracts were $>10^6$ cpm/μg of protein.

Electrophoresis

The stock solutions and most procedures were as described previously (O'Farrell, 1975; O'Farrell and O'Farrell, 1977). The only difference occurred in preparation and electrophoresis of the first dimension, which is described below. The first dimension gels were poured to a height of 12 cm in glass tubes (130 mm × 2.5 mm inside diameter) with a gel mixture composed of 9.2 M urea, 2% Nonidet P-40 (NP-40), 4% acrylamide/bisacrylamide (from a 30% stock solution comprised of 28.4% acrylamide and 1.6% bisacrylamide) and 2% Ampholines. We used either Ampholines pH 7–10 or pH 3.5–10 in the gel mixture, depending upon which pH range of proteins was to be observed. To ensure reproducible separation, the height of the gels should be uniform. Gels containing basic Ampholines tend not to polymerize well. Wheres IEF gels were polymerized with 10 μl of ammonium persulfate (10%) and 7 μl of N, N, N', N'-tetramethylethylenediamine (TEMED) per 10 ml of gel mixture, NEPHGE gels containing pH 3.5–10 or pH 7–10 Ampholines were polymerized with 20 μl of ammonium persulfate and 14 μl of TEMED per 10 ml of gel mixture. The gels were overlayed with water and allowed to polymerize for 1–2 hr. The lower reservoir was filled with 0.02 M NaOH, and the tubes were placed in the electrophoresis chamber. The samples were loaded and overlayed with 20 μl overlay solution (8 M urea and a mixture of 0.8% pH 5–7 and 0.2% pH 3.5–10 Ampholines), and the tubes were filled with 0.01 M phosphoric acid. The upper reservoir was filled with 0.01 M phosphoric acid. The gels were electrophoresed without a prerun, and with the cathode on the bottom and the anode on the top (that is, the

polarity was the reverse of that used for isoelectric focusing). Electrophoresis was usually for 4–5 hr at 400 V for a total of 1600–2000 V hr, but the time was varied depending upon the application (see Results and Discussion). At the end of the run, the gels were removed by careful extrusion; gels containing basic Ampholines are more fragile than those used for IEF. The NEPHGE gels were equilibrated for 2 hr in SDS sample buffer, frozen, stored at minus 70°C and run in the second dimension as described (O'Farrell, 1975).

The pH gradient formed during electrophoresis in the first dimension was measured with a Biorad micro pH electrode (propHiler). Immediately after electrophoresis, the gels were extruded into water, and the pH along the length of the gel was measured.

The isoelectric focusing gels were prepared, electrophoresed for 6800 V hr and run in the second dimension as described previously (O'Farrell, 1975).

To determine the average of the molecular weights of the detected proteins, the pattern was divided into a series of molecular weight zones: 0–11,000 daltons; 11,000–14,000; 14,000–18,000; 18,000–22,500; 22,500–29,000; 29,000–38,000; 38,000–48,000; 48,000–60,000; 60,000–78,000; 78,000–100,000; 100,000–140,000; and 140,000 daltons and greater. The number of proteins in each zone was determined and multiplied by the molecular weight corresponding to the center of the zone. For example, the number of proteins with SDS electrophoretic mobilities between 38,000 and 60,000 daltons was determined, the molecular weight was approximated as 44,000 daltons, and the product of the total number of proteins and the approximate molecular weight was taken. These products were summed and divided by the total number of proteins to obtain the average molecular weight. The gels were calibrated using the following molecular weight standards: the β subunit from E. coli RNA polymerase (145,000 daltons); β-galactosidase (116,000 daltons); the σ subunit from E. coli RNA polymerase (86,000 daltons); albumin (68,000 daltons); gamma immunoglobin (50,000 daltons); actin (45,000 daltons); the α subunit of E. coli RNA polymerase (41,000 daltons); tropomyosin (35,000 daltons); and the E. coli ribosomal proteins L7 and L12 (11,000 daltons).

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