Two-dimensional Gel Electrophoresis and Computer Analysis of Proteins Synthesized by Clonal Cell Lines*

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An improved method of two-dimensional gel electrophoresis has been developed which produces high resolution, reproducible images suitable for computer analysis. In the images that are presented, more than 800 proteins have been resolved without significant overlap, and many more proteins can be detected after longer exposures. To establish the usefulness of such methods for detailed quantitative comparisons of cultured cells, extensive controls have been carried out to test the reproducibility of the electrophoretic procedures, the sample preparation procedures, and the cell culture conditions.

A computerized scanning system has been developed which can automatically detect and integrate the densities of the spots on a two-dimensional fluorogram or autoradiogram. The corresponding proteins from two or more samples can then be matched and their intensities compared. Several types of graphic output have been used to display the number and magnitude of the differences between the compared samples.

These methods were used to study the patterns of protein synthesis in the nerve cell line B103 and the glial cell line B9. Both exponentially dividing and stationary cultures were analyzed and the relative rates of synthesis of approximately 300 proteins were compared by computer. For each cell line, no major qualitative differences were found between dividing and stationary phase cells although numerous quantitative differences of up to 15-fold were detected. The proteins that were increased or decreased in rate of synthesis as B103 cells became confluent were in general not the same proteins that were increased or decreased in rate of synthesis as B9 cells reached confluence, indicating that most of the changes do not reflect growth control responses common to all cells. When the two cell lines were analyzed in the same state of growth and compared by computer, qualitative differences were found in approximately 5% of the proteins analyzed, and at least 40% of the shared proteins were involved in quantitative differences of 2-fold or more. The rates of synthesis of the shared proteins were more divergent between the two cell lines than between dividing and stationary phase cells of either line. These studies show, therefore, that these cell lines can be distinguished, regardless of growth state, by their cell-spe-

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The understanding of complex biochemical phenomena, such as growth regulation and the expression of tissue-specific functions, will require a much more complete knowledge of the proteins which mediate and regulate these functions. Studies of exponentially dividing and stationary cells have revealed qualitative differences in the rate of histone synthesis (1) and quantitative differences in the activities of certain enzymes, such as thymidine kinase (see Refs. 2 and 3 for recent reviews), but very little is known about the role of intracellular proteins in the control of growth rate and cell division. Likewise, most studies of the proteins involved in tissue-specific functions have been limited to the major proteins and proteins with known enzymatic activities. Until recently it has not been possible to estimate how many of the yet uncharacterized proteins of the cell are involved in the control and expression of these functions, and there has been no general method by which to identify these proteins.

The technique of two-dimensional gel electrophoresis (4) allows the direct detection of a large number of cellular proteins, many of which have never been previously characterized. Although two-dimensional gel electrophoresis cannot be used to directly determine the functions of the uncharacterized proteins, it can be used in at least two indirect ways to study the involvement of the uncharacterized proteins in particular cellular activities. First, it can be used to gauge the overall magnitude of a biological response in terms of the number and amplitude of the changes of protein synthesis. Studies of this nature have been carried out for differentiating muscle cells (5, 6), for a cell line before and after viral transformation (7), for a cell line in different growth states (8), and for cell lines before and after hormone treatment (9, 10). The greatest responses were found in the differentiating systems, where major qualitative and quantitative changes occurred (5, 6), and the smallest responses observed were those resulting from the hormone treatment, where no qualitative changes and only limited quantitative changes were found (9, 10). Although these studies provide some insight into the magnitude of each biological response, most have been limited by either lack of high resolution or lack of rigorous quantitative analysis. Since the conclusions of such studies cannot be extrapolated beyond the proteins actually analyzed, their significance depends heavily upon both the degree of resolution obtained and the accuracy of the quantitative analysis.

The second application of two-dimensional gel electrophoresis is the comparative analysis of data from multiple samples in order to correlate the presence of particular proteins with specific biochemical functions. For example, the comparison of many cell types could reveal those proteins which are
Fig. 1. Separation of proteins from growth phase B9 cells. This montage of four fluorograms shows the full resolving power of the system. The first dimension gels contained either pH 5 to 7 ampholytes or pH 6 to 8 ampholytes, and the second dimension slabs contained either 7.5% or 12% acrylamide. Most, but not all, of the overlapping regions of these films was removed in making the montage. The overall dimensions of the pattern (excluding overlap) are 30 cm horizontal by 26 cm vertical. The cells were labeled for 2 h with \[^{35}S\]methionine in complete medium and prepared for electrophoresis as described in the text. Five to ten micrograms of protein containing 150,000 cpm were applied to the gels. The 7.5% gels were exposed to film for 10 days, and the 12% gels were exposed for 20 days. The latter were exposed longer because the efficiency of detection from 12% gels is lower and because the smaller proteins incorporate fewer counts. Shorter (1½ to 2 day) exposures were also obtained for quantitation of the most intense proteins. The films were photographed at normal contrast. More than 800 spots are visible at this exposure level. The same labeling, loading, and exposure conditions were used for all of the fluorograms shown in this paper except where noted.

involved in cell-specific functions, and the comparison of the responses of different cell types to growth inhibition could reveal those proteins which mediate or regulate common growth control mechanisms. The proteins identified by such studies would be useful marker proteins and would aid in the interpretation of subsequent two-dimensional gel patterns. These proteins could also provide starting points for efforts to purify and characterize new proteins which play important roles in particular cellular functions. This application of two-dimensional gel electrophoresis has been limited primarily by the difficulty of quantitating and correlating the large amounts of data involved.

In this paper, an improved method of two-dimensional gel electrophoresis and a system for computerized analysis have been used to compare two different cell types, each in two different states of growth. The B103 nerve cell line and the B9 glial cell lines were chosen for study because they represent different cell types, yet they have very similar origins and histories of growth in culture. Both lines were derived from the same inbred strain of rats in the course of the same tumorigenesis program (11). These lines have been characterized as nerve and glial cells by biochemical (11, 12), antigenic (13), and electrophysiological criteria (12). Since they have always been grown under similar conditions in the same laboratory, they have not been subjected to known differential selective pressures.

The first objective was to obtain a quantitative measure of the differences of protein synthesis between the two cell lines and between cells in the two states of growth. The results were used to judge the relative importance of cell type and growth state on the overall patterns of protein synthesis. The second objective was to analyze multiple samples simultaneously in order to determine (a) what fraction of the changes of protein synthesis that occur during the transition from the exponentially dividing state to the confluent state are common to both cell lines, and (b) what fraction of the qualitative and quantitative differences of protein synthesis that distinguish the two cell lines during exponential growth are still detectable when the cells have reached confluence. These results have shown that most of the changes that occur as the proliferating cells reach confluence are not shared by both cell lines; however, despite these divergent responses to confluence,
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Materials and Methods

Cell Culture—The glial cell line B9 and the nerve cell line B103 were originally derived from the BDIX strain of inbred rats by transplacental tumorigenesis using nitrosourea urea (11). All cell cultures were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Except where noted, cells were plated on 50-mm diameter Falcon tissue culture dishes at 5000 cells/cm² and were not refed. Exponentially dividing cells were harvested 2 to 5 days after plating. Stationary phase cells were harvested as late as possible after confluence (10 to 12 days) before the medium became acidic. The times of harvest are indicated for each experiment. In all cases confluent cells had been at maximum density at least 3 days prior to harvest.

Two-dimensional Gel Electrophoresis—These methods are presented in detail in miniprint supplement A.

Methods of Computer Analysis—These procedures are presented in detail in miniprint supplement B.

Results

Important Features of the Gel System—the two-dimensional gel procedure employed here differs substantially from the procedure of O’Farrell (4). Those features most essential to the interpretation of the results are mentioned below, and the full details are described in miniprint supplement A.

To enhance the reproducibility of the gel system, the number of steps has been minimized and the procedure has been simplified as much as possible. The cells were labeled in complete medium in order to avoid artifacts due to special medium or changes of medium. The sample preparation was performed in the cold in less than 1 min to avoid possible degradation or modification of proteins after cell lysis. The isoelectric focusing gels were applied directly to the surface of the slab gels, and stacking gels were not used. Since stacking gels were not necessary, the isoelectric focusing gels were equilibrated in an SDS solution that is similar in pH and ionic strength to the buffer inside the second dimension slab gel. Very wide slab gels (80 cm) were used so that each could accommodate four isoelectric focusing gels. After completion of electrophoresis, each large slab was cut into four separate two-dimensional gels. The large slab gels are easier to form and to load than an equivalent number of smaller slab gels; the problem of current inhomogeneities near the edge of a slab gel is reduced; and each set of four gels is run under identical second dimension conditions.

The resolution of the gel system has been improved by the use of long (20 cm) isoelectric focusing gels and by the use of narrow range ampholytes. The resulting pH gradients have an average slope of about 0.01 pH unit/mm. Since the proteins appear to focus as tightly on these shallow gradients as they do on steeper gradients, resolution is increased in proportion to the length of the gel. Once the proteins have been focused in the first dimension, they can be transferred to the second dimension gel without significant loss of resolution due to diffusion. The isoelectric focusing gels were cooled to precipitate the urea as soon as focusing was complete. In the precipitated state, there was no detectable diffusion of the focused proteins for several hours. Each gel was held in the precipitated state until it was pushed from its tube into SDS equilibration buffer. Since the gels were thin (1.2 mm) and porous (2.7% acrylamide), equilibration could be achieved in less than 5 min. Each equilibrated isoelectric focusing gel was then placed directly onto the surface of the slab gel for electrophoresis in the second dimension. Because the proteins were transferred rapidly and directly from the first to the second dimension, the resulting spots are nearly circular in outline.

The fluorograms in Fig. 1 show the complete pattern of proteins resolved from B9 cells labeled with [35S]methionine in growth phase. The isoelectric focusing gels contained either pH 5 to 7 or pH 6 to 8 ampholytes, and slab gels of 7.5% and 12% acrylamide were used in the second dimension. Much, but not all, of the overlap has been removed in making this montage. There is no aggregation of proteins at the basic ends of these gels although some basic proteins do not enter. Very little protein is seen at the acidic end of the gel where the SDS accumulates. The composite (nonoverlapping) size of this pattern is 30 cm in the first dimension by 26 cm in the second dimension. Over 800 different proteins are detectable on these films, and well over 2000 can be detected after longer exposures.

Reproducibility of Sample Preparation and Cell Culture—For comparison of differentiated cell lines grown in vitro it is essential to know that any differences observed were not introduced during sample preparation, either by artifactual modification or incomplete solubilization. Protein modification or degradation by cellular enzymes was minimized by preparation of samples at 4°C and by freezing the samples within 1 min after cell lysis. Thorough solubilization of pro-

![Fig. 2. Tests of sample preparation conditions (acidic range). A, B9 cells were harvested 1 week after plating (late growth phase) and used to prepare four samples as described below. The samples were electrophoresed on pH 5 to 7 isoelectric focusing gels with 7.5% slab gels. The corresponding pH 6 to 8 gels are shown in Fig. 3. These fluorograms were exposed for only 35 h to avoid overexposure of the major proteins. The indicated proteins are tubulin (T) and the major 100-A filament protein (cs). The dark spots at the lower right are β and γ-actin. The sample preparations were by (A) standard procedure as described under “Materials and Methods,” (B) omission of SDS presolubilization, DNase-RNase digestion, and lyophilization although SDS was added to the final sample, and (C) standard procedure with a 5-min incubation at 37°C before lyophilization. The sample electrophoresed in D was an aliquot of A that had been incubated 45 min at 37°C in sample buffer. The arrow in panel C points to a spot that increased during incubation in SDS.](http://www.jbc.org/)
teins and degradation of the nucleic acids were accomplished by the use of SDS and nucleases. The cells were broken in a solution of staphylococcal nuclease, which has specificity for both double- and single-stranded DNA and RNA (14). This nuclease does not digest chromatin to completion (15), but it does prevent the sample from becoming extremely viscous when SDS is added. The broken cells were then immediately solubilized in SDS and β-mercaptoethanol, followed by a brief treatment with pancreatic DNase and RNase. These enzymes are active in SDS, and the disruption of protein-nucleic acid interactions by the detergent actually enhances their effectiveness. Greater than 95% of the DNA was made acid soluble at this step. The sample was then frozen, lyophilized, and finally dissolved in sample buffer containing urea, NP-40, and dithiothreitol.

Several experiments were carried out to test the reproducibility of the sample preparation procedures. The first experiment is shown in Figs. 2 (pH 5 to 7 gels) and 3 (pH 6 to 8 gels). A monolayer of B103 cells was scraped from the dish into staphylococcal nuclease solution and divided into three aliquots. The first aliquot (Figs. 2A and 3A) was prepared by the normal sample preparation procedure. The second aliquot (Figs. 2B and 3B) was not presolubilized in SDS but was diluted directly into sample buffer containing 0.3% SDS and stored frozen until electrophoresis. These patterns are generally similar to the control patterns; however, certain proteins are missing or reduced in intensity. These include tubulin (marked t) and a major cytoskeletal protein (marked cs). The latter protein is similar to the 100-Å filament protein identified by Brown et al. (16). The loss of major structural proteins seemed to indicate that solubilization with SDS was a necessity.

The third aliquot was prepared in a manner designed to enhance any time- or temperature-dependent processes that might cause irreproducibility. The sample was incubated at 37°C for 5 min after the addition of SDS and nucleases. It was then lyophilized, redissolved in sample buffer as usual, and electrophoresed. These gels (Figs. 2C and 3C) indicate that a few spots become more intense during the incubation (see arrows), but the majority of the spots were unchanged. These enhanced spots could represent proteolytic fragments of proteins; they could represent proteins that are slowly

Fig. 3. Tests of sample preparation conditions (basic range). The samples electrophoresed were the same as in Fig. 2, except that here the pH 6 to 8 gels are shown. These fluorograms were exposed for 10 days. The arrows in panel C indicate spots that increase during incubation in SDS.
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**FIG. 4.** Solubilization versus SDS concentration and temperature. Confluent B9 cells were harvested and lysed in staphylococcal nuclease at a cellular protein concentration of 4.7 mg/ml. Sample A was prepared by a 10-fold dilution of lysate into sample buffer without SDS. Samples B, C, D, and E were prepared by the usual procedures after the protein concentration had been adjusted by addition of more staphylococcal nuclease solution to 4.7 mg/ml, 2.4 mg/ml, 1.2 mg/ml, and 0.6 mg/ml, respectively. Sample D was heated to 100°C for 2 min prior to lyophilization. Sample F was prepared similarly to Sample D except that it was incubated at 37°C for 5 min prior to lyophilization. Approximately 60,000 cpm of each sample was applied to pH 5 to 7 isoelectric focusing gels. 7.5% slab gels were used in the second dimension. The gels were exposed to film for 11 days. The arrows in Panel B indicate proteins that are more intense when SDS presolubilization is omitted and when the SDS to protein ratio is low during presolubilization. The arrows in Panel D indicate proteins that do not solubilize adequately without SDS.

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**FIG. 5.** Reproducibility of cells from separate platings. Growth phase B9 cells were harvested from exponentially dividing cultures 2 to 3 days after plating at 3000 cells/cm². The cells used for solubilized; or they could be the result of some other chemical or enzymatic modification. The slow solubilization hypothesis seems least likely since these spots have not been seen in other experiments in which samples were heated to 100°C in SDS (not shown). Furthermore, none of these spots appears in the gels shown elsewhere in this paper, indicating that these spots can be consistently and completely avoided. Their presence in the control sample (Figs. 2A and 3A) is probably due to delays of sample preparation caused by processing three aliquots in parallel.

To determine whether further chemical or enzymatic modifications could occur once the proteins were dissolved in sample buffer, a portion of the control sample from the experiment described above was incubated at 37°C for 45 min in complete sample buffer before electrophoresis. The resulting patterns (Figs. 2D and 3D) are virtually identical with the unincubated controls (Figs. 2A and 3A). Although proteins in urea are subject to carbamylation reactions that would alter their charge (17), this and other experiments (not shown) indicate that the proteins are protected for at least several hours at 37°C by the high concentration of reducing agent and/or the presence of ampholytes in the sample buffer.

The upper gel were grown 16 days earlier than the cells used for the lower gel, and the gels were run at different times. The culture conditions and electrophoretic conditions were identical. Each protein was detected on both gels; however, a couple of proteins which focused tightly in the first sample were detected only as faint streaks in the second sample.
Fig. 6. Comparison of exponentially dividing and stationary phase cells (acidic range). Shown here are the proteins resolved by pH 5 to 7, 7.5% acrylamide gels. The samples were prepared from (A) exponentially dividing B9 cells, (B) confluent B9 cells, (C) exponentially dividing B103 cells, and (D) confluent B103 cells. The proteins resolved from these samples using pH 6 to 8 ampholytes are shown in Fig. 7. The exponentially dividing cells were harvested 3 days (A) and 5 days (C) after plating; the confluent cells were harvested 10 days (B) or 12 days (D) after plating. The confluent cells had formed complete stationary monolayers in which mitotic cells were very rare. Although several major differences are apparent between exponentially dividing and stationary B9 cells, examination of the original films and films from longer exposures revealed no qualitative differences.

Since SDS seemed essential for solubilization of certain proteins, it was important to test whether the amount of SDS added was sufficient (especially when solubilizing confluent cultures), and that the SDS could effectively solubilize proteins at 4°C. To clarify these points, confluent B9 cells were harvested at 4.7 mg of cell protein/ml of staphylococcal nuclease buffer. An SDS-free sample was first prepared by a 10-fold dilution of the cell lysate directly into sample buffer without SDS (Fig. 4A). Other samples were subsequently prepared by addition of SDS (to 0.3%) and pancreatic nucleases either directly to the concentrated lysate (Fig. 4B) or to aliquots of the lysate which had been diluted with further staphylococcal nuclease solution to 2.4 mg/ml (Fig. 4C), 1.2 mg/ml (Fig. 4D), or 0.6 mg/ml (Fig. 4E). The sample shown in Fig. 4D was heated to 100°C for 2 min in the presence of SDS and nucleases. Each SDS-containing sample was lyophilized, redissolved in sample buffer, and adjusted to the same final protein and SDS concentration. Comparison of Fig. 4A to Fig. 4, C to E confirms that a number of major acidic proteins (marked by arrows in Fig. 4D) do not solubilize adequately without SDS. The samples prepared by usual methods at moderate to low protein concentrations (Fig. 4, C to E) are practically indistinguishable from one another. However, the sample prepared at highest protein concentration (Fig. 4D) resembles the SDS-free sample in that the tubulin is again underrepresented. Those proteins that require SDS for solubilization were completely solubilized at 4°C since their intensities were not enhanced in the heated sample (Fig. 4D).

Further examination of the gels in Fig. 4 shows that seven spots are more intense when SDS solubilization is omitted (Fig. 4A) and when the SDS to protein ratio is low during solubilization (Fig. 4B). These are marked by arrows in Fig. 4B. The same phenomenon can be observed in Fig. 2 (compare...
The reason for this is not understood, but samples prepared over the normal range of SDS to protein ratios were identical (Fig. 4, C to E), and incubation in SDS at 37°C for 5 min did not further reduce the intensities of these proteins (Fig. 4F). Although the subtleties of sample preparation are not yet fully understood, the above experiments show that the procedure described here is reproducible and generally free of artifacts.

If the sample preparation conditions and electrophoretic procedures were perfect, it would still be impossible to do meaningful studies of tissue culture cells if the cells themselves failed to show a consistent spectrum of protein synthesis from experiment to experiment. To examine the reproducibility of the cells and culture conditions, two cultures of exponentially dividing B9 cells, grown 16 days apart, have been compared. A close-up of the most crowded region of each is shown in Fig. 5. These patterns are extremely similar although a few quantitative differences can be found. Further controls, and a quantitative measure of the overall reproducibility of the system, are presented below in the section on computer analysis.

Comparison of Protein Synthesis in Two Cell Lines—The methods outlined above have been used for a comparative study of protein synthesis in the B9 glial and B103 nerve cell lines. The data have been examined to determine 1) the degree of qualitative and quantitative variation of protein synthesis between the two cell types, and 2) how much the rates of protein synthesis differ between exponentially dividing cells from low density cultures and stationary phase cells from confluent cultures. Since the latter comparison measures not

![Image of gel electrophoresis results](image_url)

**Fig. 7. Comparison of exponentially dividing and stationary phase cells (basic range).** The proteins shown were resolved on pH 6 to 8, 7.5% acrylamide gels from the samples described in Fig. 6. The arrows point to the qualitative differences between B9 and B103 cells, as determined by visual inspection and quantitative analysis.
just the changes related to growth control, but rather the whole spectrum of cellular responses to the confluent culture environment (higher cell density, conditioned medium, partially depleted medium and serum), these changes will be called the “responses to confluence.” The data were further examined to determine what fraction of the responses to confluence are common to both the nerve and glial cell lines and whether the responses to confluence are more or less extensive than the differences between the two cell types.

The patterns for dividing and stationary B9 and B103 cells are shown in Fig. 6 (pH 5 to 7 gels) and Fig. 7 (pH 6 to 8 gels). Only gels run on 7.5% slabs are shown. Most proteins are common to all four samples; however, several major cell-specific proteins can be observed (see arrows). These are present in both dividing and stationary cells. There are no major qualitative changes between dividing and stationary cells although numerous quantitative changes occur. One of the latter is a decrease in the rate of tubulin synthesis. This agrees with the observation of Ivarie and O'Farrell (9) who found a decrease of tubulin synthesis in a hepatoma cell line at high cell densities. Another quantitative change is an increase in the rate of synthesis of the major series of spots at about 180,000 daltons, pH 6.1. These have been identified elsewhere (6) as forms of collagen. Although it is easy by visual analysis to detect changes in individual proteins, it is much more difficult to determine the magnitude of the changes and to gauge the overall similarity or difference between these complex patterns. To obtain this information, computer analysis is required.

**Computer Analysis of Two-dimensional Gel Patterns—**

The description of the scanning equipment and computer programs is given in miniprint supplement B. The function of the computerized scanning system is to scan a section of a two-dimensional gel fluorogram, detect the spots, and integrate their densities. This information is stored on a floppy disc, and it can be plotted graphically. Fig. 8A shows a fluorogram that was scanned, and in Fig. 8B is the plotted representation. Each cross represents a spot and the area of the cross is proportional to the intensity of the spot. The system can resolve most overlapping spots, and it is relatively insensitive to streaks and noise on the film. Further programs have been written (as described in miniprint supplement B) to allow spot for spot matches (with operator assistance) of up to four patterns at once. When patterns are matched, a table is constructed in memory with one row for each protein and one column for each of the samples matched. From this table of matched data, the number of changes and their magnitudes can be quickly determined.

As can be seen from Fig. 8 and from the controls presented below, the image analysis programs can accurately and reproducibly detect and integrate the spots. Unfortunately, the computer and scanning equipment used in these experiments are much too slow to cope with the full information content of the gels run. For that reason, only the region of the pH 6 to 8, 7.5% acrylamide gels corresponding to that shown in Fig. 8 has been analyzed. As will be seen, this limited region of the full pattern provides sufficient data for meaningful and statistically significant conclusions.

To examine the reproducibility of the spot detection and integration procedures, a fluorogram representing the proteins of growth phase B9 cells has been scanned twice. The matched data from the duplicate scans are plotted as a histogram of ratios in Fig. 9A. The distribution is narrow with a standard deviation of 0.21 division on the abscissa, which is a log base 2 scale. In other words, individual protein ratios are within 1 S.D. of the mean if the ratio is between $2^{-0.21}$ and $2^{0.21}$ (ratios of 0.86 to 1.16). In Fig. 10A these data are expressed as a scatter plot in which each protein is plotted according to its intensity in the first scan (x axis) versus its intensity in the second scan (y axis). If the results of the two scans were identical, the points would all fall on the diagonal line. This type of plot is useful because it displays both the deviations from a ratio of 1 and the intensity of each spot. It can be seen that the points from duplicate scans are tightly clustered about the diagonal. In a second control experiment (Figs. 9B and 10B), duplicate gels were run from the same sample and a fluorogram of each scanned. In this comparison the standard deviation is a bit greater (0.28) and the points on the scatter plot are not quite as tightly clustered. The third control (Figs. 9C and 10C) is the comparison of duplicate samples of dividing B9 cells, prepared from cells grown at different times. This control includes all experimental errors including the variabilities of the cells and culture conditions. The standard
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![Diagram](https://via.placeholder.com/150)

**Fig. 9. Ratio histograms.** In each panel, the computer-analyzed data from two scans (similar to that shown in Fig. 8) have been compared, and histograms of the protein intensity ratios have been constructed. The ratios are plotted on the abscissa, which is a log (base 2) scale, and the number of proteins in each interval is plotted on the ordinate. Ratios were determined only for proteins having integrated densities of greater than 0.1 in both scans (see Fig. 10). The number of proteins represented in each histogram ranges from 243 to 279. Most proteins were quantitated from fluorograms that had been exposed for 10 days, but some of the most intense spots were quantitated from shorter (1 to 2 day) exposures. The scans compared deviation is 0.39 on the log scale.

The computer analysis of the response to confluence of each line (early versus late times after plating) shows that these responses (see Figs. 9D and 10D for B9 and Figs. 9E and 10E for B103) are significantly greater than the differences observed between duplicate cultures (compare to Figs. 9C and 10C). The standard deviations of the histograms are 0.96 and 1.03, respectively, indicating that about one-third of the proteins of each are altered in rate of synthesis by a factor of 2 or more. The maximum change among the proteins analyzed was about 15-fold.

A comparison of B9 and B103 cells, each in the exponentially dividing state, is shown in Figs. 9F and 10F. Here the quantitative differences are even greater (S.D. = 1.27) and qualitative differences are apparent. Only the shared proteins were used to construct the histograms, thus, the standard deviation does not reflect the qualitative differences. The number of qualitative changes in protein synthesis depends on our definition since we cannot establish that the rate of synthesis of any protein is zero. By the definition used here, a qualitative difference exists whenever a protein has intensity at least 16-fold greater than the threshold of detectability in one sample and is not detectable in the other sample. These proteins are plotted on the scatter plot at the threshold of detectability of the eye (taken to be 0.05 unit of density × mm²). They form the outer series of horizontal and vertical points seen in Fig. 10F. In other cases that arise during matching, the eye can detect a faint spot that the scanner did not report. (The scanner sensitivity could be set to detect everything the eye can see, but this was not done to avoid picking up excessive background noise.) The true intensity of these spots is, therefore, somewhere between the eye threshold and the computer threshold. These are plotted on the scatter plot at the computer threshold (set at 0.1 unit of density × mm²). These entries form the second horizontal and vertical series of points on the scatter plot (see Fig. 10F). The qualitative changes are easy to detect on the scatter plot; they are simply those proteins at the eye threshold that are more than a factor of 16 from the diagonal. Many qualitative differences are evident in the comparison of B9 and B103 cells (see boxes in Fig. 10F), but none are observed in the comparison of growing and confluent cells (Fig. 10, D and E).

A summary of the pairwise comparisons is given in Table I. The standard deviation from the histogram of ratios is re-
Fig. 10. **Scatter plots.** Each panel represents the same data that was compared in histogram form in the respective panel of Fig. 9. Here the data is presented as scatter plots in which each protein is plotted according to its intensity in the first sample on the x axis and according to its intensity in the second sample on the y axis. Each axis represents integrated film density in units of absorbance times mm². Some of the more intense spots were quantitated from shorter exposures and multiplied by a calibration factor so they could be plotted on the same scale. The calibration factor relating the long and short exposures was determined by averaging the ratios of about 30 proteins that were detected without saturation on both exposures. In the lower right panel, the boxes enclose proteins that fall within the definition of qualitative differences. These are proteins that were not detectable by computer or by eye in one sample (plotted at the threshold of sensitivity of the eye), but were present at 16-fold or more above the threshold of sensitivity of the eye in the other sample. The eye threshold is taken to be 0.05 unit of integrated density. Certain other spots were detected by eye, but not by the computer. These were placed at the computer’s threshold of sensitivity (taken to be 0.1 unit of integrated density).

Although the pairwise comparisons have allowed us to determine the relative magnitudes of the responses to confluence and to the cell type difference, several other questions were addressed by simultaneously comparing the data from more than two samples. Are the responses to confluence seen in B9 cells largely the same as or mostly different from the responses to confluence in B103 cells? Are the proteins that distinguish B9 cells and B103 cells in the growth phase also the same proteins that distinguish these lines at confluence? To answer these questions, it was necessary to correlate the changes found in one pairwise comparison with the changes found in a second pairwise comparison. This was done by using tables of matched data in which four samples were simultaneously matched.

Correlation plots were constructed as shown in Fig. 11. In each correlation plot, the changes of protein synthesis from one of the pairwise comparisons (from Table I) were plotted as ratios on the horizontal axis, and the changes in protein synthesis from a second pairwise comparison were plotted as ratios on the vertical axis. Each plotted point represents a single protein. Both axes are log scales marked in factors of 2. Fig. 11A shows the correlation between pairwise comparisons 5 and 6 (cf. Table I). Since each axis in this example represents the response to confluence of B9 cells, the correlation is high (correlation coefficient 0.81). The failure to correlate perfectly is due to the experimental errors in the system.

Fig. 11B shows another control in which pairwise comparisons 3 and 4 are correlated. Here each axis represents a comparison of duplicate samples, and the only deviations from the center are the experimental errors. These are limited and uncorrelated.

The correlation of the responses to confluence of B9 and B103 cells (pairwise comparisons 5 versus 7) is given in Fig. 11C. These responses are weakly correlated, with a correlation coefficient of 0.35. The correlation plot shows that about 10% of the proteins were altered more than 2-fold and in the same direction in both cell lines at confluence. Some of these may represent changes that occur in all cell lines that cease to divide at confluence. Another 30% of the proteins were altered in rate of synthesis by 2-fold or more in only one cell type or in both cell types but in different directions. These represent...
In each comparison, proteins from pH 6 to 8, 7.5% acrylamide gels (as shown in Fig. 8) were quantitated and matched. Both a long and a short exposure of each gel were analyzed in order to detect both the faint and the most intense spots without film saturation. The intensity ratios of the matched proteins were plotted on histograms as shown in Fig. 9. For each histogram, the standard deviation of the ratios about the mean (in units of log base 2) and the actual ratios corresponding to 1 S.D. are reported. The qualitative differences are those proteins in the region analyzed by computer which were more than 16-fold above the limit of sensitivity in one sample and not detected in the other sample. In each case, this number has been compared to the total number of proteins in the region analyzed that were more than 16-fold above the limit of sensitivity in one or both samples.

### Table 1
Summary of pairwise comparisons

<table>
<thead>
<tr>
<th>Comparison No.</th>
<th>Histogram of ratios</th>
<th>Data compareda,b</th>
<th>Proteins matched</th>
<th>S.D.</th>
<th>Ratios (±1 S.D.)</th>
<th>Differences &gt;2-fold</th>
<th>Qualitative differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fig. 9A</td>
<td>Duplicate scans</td>
<td>275</td>
<td>0.21</td>
<td>0.86–1.16</td>
<td>0</td>
<td>0/151</td>
</tr>
<tr>
<td>2</td>
<td>Fig. 9R</td>
<td>Duplicate gel</td>
<td>275</td>
<td>0.29</td>
<td>0.80–1.21</td>
<td>0</td>
<td>0/172</td>
</tr>
<tr>
<td>3</td>
<td>Fig. 9C</td>
<td>B9 exp1 versus B9 exp2</td>
<td>277</td>
<td>0.39</td>
<td>0.76–1.31</td>
<td>13 (5%)</td>
<td>0/158</td>
</tr>
<tr>
<td>4</td>
<td>B9 stat1 versus B9 stat2</td>
<td>276</td>
<td>0.52</td>
<td>0.70–1.43</td>
<td>18 (7%)</td>
<td>0/184</td>
<td></td>
</tr>
</tbody>
</table>

#### Controls

- The data for comparisons 1 and 2 were taken from gels of exponentially dividing B9 cells.
- The samples for comparisons three to nine were as follows. B9 exp1, plated at 50,000 cells/dish; harvested on Day 3, 89 pg of protein/dish; B9 exp2, plated at 100,000 cells/dish, harvested on Day 2, 119 pg of protein/dish; B9 exp3, plated at 100,000 cells/dish, harvested on Day 5, 370 pg of protein/dish; B9 stat1, plated at 50,000 cells/dish, harvested on Day 12, 684 pg of protein/dish; B9 stat2, plated at 100,000 cells/dish, harvested on Day 10, 702 pg of protein/dish; B103 stat, plated at 100,000 cells/dish, harvested on Day 12, 942 pg of protein/dish.

#### Responses to confluence

- The histogram for this comparison is not shown.
- The correlation coefficient is 0.76. We have already observed that these lines can be distinguished regardless of growth state by their cell-specific proteins.
- The remaining 60% of the proteins did not change by more than 2-fold in either cell line during the transition from the dividing to the stationary state.

### Discussion

This study has shown that cultured cell lines can be analyzed reproducibly and quantitatively by two-dimensional gel electrophoresis. Improved electrophoretic methods have been introduced for higher resolution and reproducibility, and new computer methods have been developed for quantitative analysis. These methods have been used to quantitatively compare the patterns of protein synthesis in a nerve cell line and a glial cell line and to study the changes of protein synthesis in each line as a function of growth state. Since the significance of the results depends upon the quantitative reproducibility of the methods used, the experimental errors of the system will be discussed first.

Each step of the analysis, from cell culture to computer analysis, is a potential source of quantitative variability. A particularly critical step is the preparation of samples for electrophoresis. Incomplete solubilization of the cellular proteins could cause lack of reproducibility, and artifacts could be introduced by enzymatic or chemical modification of the proteins. Most previous studies have offered no controls to rule out these possibilities. Indeed, it was found in this study that protein modifications and incomplete solubilization of certain proteins can occur under conditions similar to those commonly used; however, these problems were reduced to insignificant levels when the samples were prepared quickly at 4°C in the presence of SDS and nucleases.

Another major concern in these studies was the reproducibility of the cells and cell culture conditions. Ivarie and O'Farrell (9) have reported variability in the basic pattern of protein synthesis in a hepatoma cell line and in the response of these cells to hormonal stimulation. Lee and Engelhardt (8) have reported a continuously variable pattern of protein synthesis in Vero cells as their density increases in culture. In contrast to these reports, I have found that exponentially dividing B9 cells derived from separate platings have quite similar patterns of protein synthesis (Fig. 5), even though the cultures compared differed somewhat in density and culture age when harvested (see Table I). It was also found that the changes that occur when exponentially dividing B9 cells reach confluence are reproducible (Fig. 11).

Quantitative estimates of the experimental errors were obtained by comparison of (a) two scans of the same film, (b) scans of two films representing proteins from the same sample resolved on two independent gels, and (c) scans of two films representing proteins from samples prepared from two independently plated cultures of the same cell line grown under similar conditions. For each comparison, the corresponding spots were matched and the ratios of these intensities were plotted as a histogram (Fig. 9). The standard deviation of each histogram is a measure of the overall quantitative differences between the two sets of compared data. The standard deviations from these three control comparisons correspond to quantitative errors of approximately (a) 15%, (b) 20%, and...
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**Fig. 11. Correlation plots.** In each panel, two pairwise comparisons (from Table I) are plotted against one another. Each axis is a log scale marked in factors of 2. Each protein is plotted on the x axis according to how much it increased (right of center) or decreased (left of center) in the first pairwise comparison and on the y axis according to how much it increased (above center) or decreased (below center) in the second comparison. If a protein increased or decreased in both pairwise comparisons, this plot will reveal the correlation. A, duplicate samples (c) 30 to 40%. The first error estimate gives the reproducibility of the scanning and integration procedures. This error is reasonably small considering that the proteins quantitated vary by about 100-fold in rate of synthesis, and it is likely that future improvements of the methods of quantitative analysis will reduce this value even further. The second error estimate includes the errors of scanning and integration and the additional errors involved in comparing data obtained from two different gels of the same sample. The additional errors are small, indicating that the procedures for electrophoresis and fluorographic detection of the proteins are highly reproducible. The third error estimate represents the overall errors of the system since all steps of the procedure including cell culture were repeated. About one-half of the overall error is due to differences between samples prepared from cells grown at different times. It is likely that more careful attention to the exact density and culture age of the cells compared could reduce this error. Nevertheless, the overall experimental errors were substantially smaller than the changes in protein synthesis that were found in comparisons of exponentially dividing and stationary cells or in comparisons of nerve and glial cells (Table I).

The comparison of the nerve cell line B103 and the glial cell line B9 has revealed both qualitative and quantitative...
differences. Approximately 5% of the proteins analyzed were defined as qualitative differences, and about 40% of the proteins differed quantitatively in relative rate of synthesis by more than a factor of 2 (Table I). The discovery of major qualitative differences is consistent with previous studies (11-13) which indicate that these lines represent distinct cell types. It is not yet known, however, whether any of these qualitative differences represent cell-specific proteins characteristic of all nerve or glial cells. Analysis of more nerve and glial cell lines and cells of other types should help to define the cell-specific proteins.

Comparison of exponentially dividing and stationary cells has not revealed qualitative differences, and the quantitative differences are somewhat less pronounced than those observed in comparison of the two cell types. These conclusions can be applied, however, only to the proteins analyzed and do not imply that no qualitative changes occur as a function of growth state in these cells. Many proteins, including the histones, were too basic to be detected on these gels, and the great majority of the cellular proteins were still below the sensitivity of the methods used. Furthermore, the changes that were found should not be interpreted strictly as differences between dividing and nondividing cells since what was in fact measured was the total cellular response to two very different cellular environments. Nevertheless, the results do indicate that the detectable changes of protein synthesis that occur as these cells pass from one extreme of the cell culture environment (low density, fresh medium) to the other extreme (high density, conditioned medium) are less extensive qualitatively and quantitatively than the differences between the two cell lines.

In contrast to these general comparisons of protein synthesis between pairs of samples, another application of two-dimensional gel electrophoresis is to identify particular proteins which may be involved in specialized cellular functions. This can be done only when computer methods are available for the correlation of data from multiple samples. Although the present methods are still limited, they have been used to correlate the data from four samples simultaneously in order to determine which proteins are increased or decreased in both B9 and B103 cells at confluence (Fig. 11C). The proteins which satisfy this criterion may have important roles in the mechanism of growth control in many different cell types. It was surprising to find that, although about 40% of the proteins analyzed were altered in relative rate of synthesis by a factor of 2 or more in one or both cell lines at confluence, only about 10% of the total proteins analyzed were changed by this magnitude and in the same direction in both cell lines. This indicates that most of the proteins which are altered substantially in rate of synthesis as these cells reach confluence are not involved in a common growth control mechanism. Further analysis of more cell lines should help to reveal which if any of the changes common to B9 and B103 cells also occur during growth regulation in other cell types.

In another multisample comparison, a significant correlation was found when the differences between exponentially dividing B9 and B103 cells were compared to the differences between stationary B9 and B103 cells. This indicates that these lines can be distinguished, regardless of growth state, not only by the qualitative differences, but also by characteristic patterns of protein synthesis among the shared proteins. This study has shown that the methods of gel electrophoresis, sample preparation, and cell culture are reproducible enough to warrant detailed quantitative analysis of the data. Furthermore, these studies have shown that a computerized scanning system can be used to obtain quantitative results concerning protein synthesis in differentiated cells that would have been difficult or impossible to obtain otherwise. Further development of the methods of quantitative analysis, incorporating faster scanning and computing equipment, should make possible the quantitation and correlation of data from many different samples, leading to a better understanding of the proteins involved in the control and expression of specialized cellular functions.

Acknowledgments—I would like to thank Dr. David Schubert for support and encouragement throughout this work, Mr. Jack Bendel for helping to interface the computer and microscope scanner, and Dr. Bart Sefton for generously loaning the microscope with scanning stage. I also wish to thank Jim Patrick, Joe Henry Steinbach, Tony Hunter, John Merlie, and Kate Barald for advice and encouragement.

REFERENCES

Additional references are found on p. 7974.
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Introduction

Two-dimensional gel electrophoresis (2-DE) is a powerful tool for the separation and analysis of proteins. It allows for the detection and quantification of proteins with high sensitivity and resolution. This method is widely used in biomedical research, drug discovery, and proteomics.

The process involves a two-step electrophoretic process: isoelectric focusing in the first dimension, followed by SDS-PAGE in the second dimension. The proteins are separated based on their isoelectric point in the first dimension and their molecular weight in the second dimension.

Methods

Preparation of Samples

Samples are usually prepared by solubilizing the proteins in a sample buffer containing a denaturing agent such as SDS and reducing agent such as DTT. The sample is then boiled for a few minutes to ensure complete denaturation and unfolding of the proteins.

Electrophoresis

The proteins are separated in the first dimension by isoelectric focusing, usually using a pH gradient ranging from 3 to 10. After the first dimension, the proteins are transferred to a second gel for SDS-PAGE, where they are separated based on their molecular weight.

Analysis

The separated proteins are visualized on the gel using Coomassie blue staining or silver staining. Quantitative analysis is performed using ImageJ software to determine the intensity and amount of each protein band.

Applications

2-DE is widely used in various fields, including proteomics, drug discovery, and disease research. It has been instrumental in identifying protein biomarkers for various diseases and in understanding the molecular mechanisms underlying these diseases.

Conclusion

Two-dimensional gel electrophoresis is a versatile and powerful method for the separation and analysis of proteins. Its ability to separate proteins based on both their isoelectric point and molecular weight makes it an invaluable tool in biomedical research.

References


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The computer program used contains some projections which can be rotated to improve the view of the protein pattern. The program can save the image in a variety of formats, including JPEG, PNG, and PDF. This allows for the creation of high-quality images for publication or presentation.

The data can be further analyzed using various statistical tools, such as the two-way ANOVA test, to determine if there are significant differences between the groups. This analysis can help researchers understand the biological significance of the protein expression patterns.

In conclusion, quantitative two-dimensional gel electrophoresis is a powerful tool for the analysis of complex protein mixtures. With the availability of advanced software and hardware, this technique is becoming increasingly accessible and user-friendly. Researchers can now easily perform high-resolution protein analysis and gain valuable insights into the molecular mechanisms underlying various biological processes.
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Figure 8.1: Computer analysis of a small section of a two-dimensional gel electrophoresis. The gel was stained with Coomassie blue and scanned. The gel was then analyzed using image analysis software to quantitate the intensity of each band. The intensity of each band was plotted against its position on the gel. The software was able to accurately quantitate the intensity of each band even when the bands were closely spaced.

Calibration curves were generated using a semiautomatic procedure. The intensity of each band was plotted against its position on the gel. The software was able to accurately quantitate the intensity of each band even when the bands were closely spaced.

Figure 8.2 (top): Lines of data generated by consecutive scans across the gel shown in Figure 8.1. Each line contains about 300 readings of pixel intensity. The data were obtained by scanning the gel in the horizontal direction of the scanning machine. The intensity of each pixel was calculated by averaging the intensity values of the pixels in the region that corresponded to the same area on the gel.

Program Listings

Fully documented program listings, written in Basic, will be provided by the author on request.
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Integrated Density

Figure 3d (left). Calibration of film response. Calibration strips were processed for two-dimensional gel electrophoresis, and exposed to the same film as the gels to be calibrated. Shown here are scans of the film image of one strip after 36, 128, and 241 hr of exposure. Each point represents one segment of the strip. The overall density of each segment (minus background) is plotted versus the known specific activity in each segment. The best straight line fit to each set of points is shown. The inverse slopes (representing time per unit of integrated density) are shown above each point. The sensitivities of the method (in density units) are: A) 51.3, -0.35, B) 14.5, -0.06, C) 0.8, -0.06.
Two dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines.
J I Garrels