An Attempt to Resolve All the Various Proteins in a Single Human Cell Type by Two-Dimensional Electrophoresis: I. Extraction of All Cell Proteins

J. Klose and E. Zeindi

A concept is presented for estimation of the total number of different proteins in a single human cell type (exemplified here by Hep cells) by use of two-dimensional electrophoresis (2DE). This concept includes three problems, the first, investigated in this study, being the transfer of all protein species of the cells into a sample useful for separation by 2DE. Five different extraction media containing—in various combinations—urea, Nonidet P-40, Zwittergent, mercaptoethanol, dithiothreitol, and sodium dodecyl sulfate were used step by step in three different extraction procedures to extract the cell proteins. The amount of radiolabeled proteins in each extract was measured. Each extract was subjected to 2DE. From the total mass of cell proteins, 99.99% could be extracted in two steps: 96% were extracted with urea/β-mercaptoethanol solution, the remaining 4% with sodium dodecyl sulfate/urea/β-mercaptoethanol solution. A special class of proteins assumed to be present in the latter fraction was not detected. Thus this fraction can be omitted from the further analysis of all cell proteins by 2DE. Protein classes that possibly remain undetected by the described extraction procedures are mentioned.

The total number of different proteins (protein species) that may be present in a single cell type of a higher organism is of fundamental interest for the understanding of the biology of the cell. Proteins are involved in all biological processes of cells. Therefore, the elucidation of these processes largely depends on detection and examination of proteins specifically involved in these processes. However, the problem of detecting such proteins and assessing their cell-biological significance often include the problem of the number of proteins.

A simple example may help illustrate this. Let us suppose the investigation of a certain genetic disease has led to the detection of one abnormal protein among 500 proteins that were electrophoretically resolved. If the 500 proteins were selected by chance from the total proteins of the organism investigated, it could be expected that 20 abnormal proteins exist in the organism bearing this disease, if the total number of proteins per cell is 10,000, and 100 or 200 abnormal proteins may be present if the total number of proteins of this organism is 50,000 or 100,000, respectively. If it is assumed that the total number of proteins per organism is very low, the abnormal protein that was in fact found is most likely to be directly related to the development of the clinical phenotype of the disease. It possibly represents the primary product of the gene affected by this disease. If, however, the total number of proteins per organism is very high, many other abnormal proteins may exist in this disease, and the only one found is not necessarily the most important one. This variant protein might be one of the many secondarily altered proteins that possibly result from the physiological and morphological abnormalities that have developed in the ill organism. Most of these proteins probably do not play a central role in the development of the genetic disease, and many of these may not even occur consistently among patients with this disease. Yet another possibility is that several other diseases are accompanied by this abnormal protein.

If one ignores the post-translational modifications of proteins, the number of different proteins in a given cell type should correspond to the number of mRNA species that are translated in the cells. The complexity of this class of mRNA has been extensively studied by mRNA/DNA hybridization. The number of mRNA species found in mammalian cells ranged, depending on the hybridization technique used (1) and on the cell type or tissue investigated, between 6000 and 15,000 (2–7), 26,000 and 57,000 (8–10), and 50,000 and 240,000 (9, 11).

A way to determine the number of cell proteins directly, by making visible and counting discrete proteins, became feasible when the first attempts were made by Klose (12, 13) and O'Farrell (14) in 1975 to separate the proteins of crude cell extracts by two-dimensional electrophoresis. The number of proteins (polypeptides) found with this method lies in the range of 1000 per 2DE pattern and per cell, and ranges up to approximately 2000 per cell if the first dimension of the 2DE is performed in both directions (CIF and AIF) and the numbers of protein spots found in the two 2DE patterns are added (15). Duncan and McConkey (16) compared the relative amount of the rare mRNA species in mammalian cells with the relative amount of the weakest protein spots still detectable in a 2DE pattern of HeLa cells. They found that the translation products of mRNAs would be easily detectable in the 2DE pattern if all mRNAs were translated in proportion to their abundance. They concluded that a typical mammalian cell may contain not more than 2000 proteins (primarily polypeptides), a number that corresponds closely to the number of polypeptides that have been resolved from mammalian cells by use of the usual 2DE techniques (14, 15).

When we used our 2DE technique adapted to large gels (17) and when fluorography was carried out by exposing the x-ray film for different time periods, we found 4300 proteins spots in a preliminary investigation of total protein extracts of "Hep" cells (18). Voris and Young (19), using their "giant gel" technique to separate total cell proteins, found about 3000 protein spots when the first-dimension resolution was performed by CIF and about 1800 more spots when AIF was used in the first dimension (reported in this issue). When one attempts to determine the total number of proteins in a single cell type by 2DE, one has to consider three problems: (a) the extraction of all protein species that are present in the cells, (b) the presence of all of them in the

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1 Nonstandard abbreviations: 1DE, one-dimensional electrophoresis; 2DE, two-dimensional electrophoresis; IEF, isoelectric focusing; AIF, CIF, isoelectric focusing, sample applied to the gel on the anode or cathode side, respectively; SDS, sodium dodecyl sulfate; NP-40, Nonidet P-40 detergent; ME, β-mercaptoethanol; and DTT, dithiothreitol.

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Institut für Humangenetik, Institut für Toxikologie und Embryonal-Pharmakologie, Freie Universität Berlin, Garsstrasse 5, D-1000 Berlin 33, P.R.G.

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gel, and (c) making all of them visible in the gel. We investigated these three steps systematically, using Hep cells, an established human cell line. The results obtained from the investigation of the first step are reported here; our investigation of the other two steps will be the subject of two other papers. Preliminary results have shown that at least 10 000 different polypeptides can be revealed by 2DE in a single human cell type.

Study of the number of proteins in a cell by use of 2DE includes the problem of the resolving power of 2DE. The question of whether 2DE can reveal all cell proteins is not only important for the protein-number problem, it is of interest for all investigations in which 2DE is used. If, after 2DE, not all proteins of the cell are in fact detected, the method may also not detect all protein classes of the cell. Because different protein classes may have, on the average, different concentrations in the cell, it cannot be expected that all protein classes are seen in the 2DE pattern in the same proportion as in vivo. Protein classes of high concentrations are certainly over-represented in a 2DE pattern—while protein classes of low concentrations are under-represented or even completely absent, even though they may be of particular biological significance (e.g., regulator proteins, receptors, inducers). Hence, if 2DE patterns are analyzed which reveal only some of the most abundant protein classes, interpretations may be inconsistent with reality.

Materials and Methods

Cell Culturing

The "Hep" cell line is an established epithelial-like human larynx carcinoma cell line. Cells of this type were cultured in plastic Petri dishes with Eagle’s Minimal Essential Medium (Seromed GmbH, München, F.R.G.) supplemented with antibiotics and 50 mL of fetal calf serum (Seromed) per liter. The cells were incubated in a humidified atmosphere (CO2/air, 5/95 by vol) at 37 °C. We passaged the cells growing as monolayers when they became confluent, using 500 mg of trypsin (EC 3.4.21.4) and 200 mg of EDTA per liter of phosphate-buffered saline (Seromed). The culture medium was changed every three days.

Radiolabeling of Proteins

The Hep cells were seeded with 18 mL of culture medium (see above) into a 15-cm (diameter) Petri dish at a density of 4 × 10^6 cells per dish and incubated for 24 h. When the cell density became approximately 5 × 10^6 cells per cm², we completely removed the culture medium. We mixed 14.4 mL of this medium ("conditioned medium") with 0.6 mL of uniformly 14C-labeled amino acid mixture ([U14C]protein hydrolysate, code CFB 25, 56 mCi per milligram of carbon, 1 Ci/L; Amersham Buchler, England) and replaced this mixture onto the cells. The cells were then incubated for 20 h. Then the radioactive medium was removed and the cells were detached from their support with trypsin/EDTA solution as described above. The cells were freed from remaining radioactive amino acids and culture medium by washing them twice with cold physiological NaCl solution, then resuspended in 1 mL of the NaCl solution and centrifuged in an Eppendorf tube. The supernatant fluid was completely removed with filter strips and the pellet was stored in liquid nitrogen.

Protein Extraction

We used three different procedures to extract the proteins from the cultured and radiolabeled Hep cells.

Procedure A: The cell pellet, consisting of about 10 × 10^6 Hep cells, was suspended in one volume of water and homogenized by use of a wire loop connected to a motor. The homogenate was frozen and thawed twice, then centrifuged at 145 000 × g for 40 min. The supernate ("water extract") was stored in liquid nitrogen. The pellet was washed twice with water by homogenization and centrifugation (see above). The wash solutions (supernates) were combined and stored. The pellet was suspended in 0.5 volume of water, and urea and β-mercaptoethanol were added to yield final concentrations of 9 mol of urea and 50 g of β-mercaptoethanol per liter. The mixture was homogenized for 30 min and centrifuged as indicated above. The supernatant fluid ("urea/ME extract") was stored in liquid nitrogen. The pellet was again washed with urea/ME solution as indicated above. The washed pellet was suspended in two volumes of an aqueous solution containing, per liter, 9.5 mol of urea, 5 mmol of K2CO3, 20 mL of Nonidet P-40 detergent (Serva, Heidelberg, F.R.G.) and 5 g of 4N-dithiothreitol (Sigma Chemical Co., St. Louis, MO) (20), homogenized for 30 min, and centrifuged (see above). The supernatant ("urea/NP-40/DTT/K2CO3 extract") was stored in liquid nitrogen. The pellet was washed with urea/NP-40/DTT/K2CO3 solution and then suspended in three volumes of an aqueous solution containing, per liter, 10 g of sodium dodecyl sulfate (Sigma), 9 mL of urea, and 50 g of mercaptoethanol. The suspension was homogenized for 30 min and centrifuged (see above). The supernatant ("SDS/urea/ME extract") was stored in liquid nitrogen. The pellet was washed with SDS/urea/ME solution, then suspended in water by homogenization.

Procedure B: Procedure B differed from procedure A in the following respect: the cell pellet was suspended in three volumes of water instead of one volume, to improve the conditions for cell lysis. The cells were homogenized more intensively by using a glass homogenizer. For extraction with the urea/NP-40/DTT/K2CO3 medium we substituted extracting the cells with an aqueous 40 g/L solution of "Zwittergent 3-12" (Calbiochem-Behring Corp., La Jolla, CA). After homogenizing the cell pellet in two volumes of this solution, we added one volume of an aqueous solution containing 8 mol of urea and 40 g of NP-40 per liter and again homogenized. After centrifugation, the supernatant fluid ("Zwittergent/urea/NP-40 extract") was stored in liquid nitrogen.

Procedure C: The cell pellet was suspended in nine volumes of water. The cell suspension was sonicated for five 12-s intervals in a "Sonifier B-12" (Branson Sonic Power Co., Danbury, CT) and then mixed with a solution containing 9 mol of urea and 50 g of ME per liter (final concentrations). The mixture was stirred for 1 h, then centrifuged as indicated for procedure A. The supernatant fluid was stored; the pellet was washed and treated with SDS as described (Procedure A).

Table 1 shows the number of experiments performed. The radioactivity of each extract and each wash solution was measured in an LKB 1217 Rackbeta liquid scintillation counter (LKB, Bromma, Sweden). The proteins of each extract were separated by 2DE. The protein sample used for one electrophoretic run contained between 1.5 × 10^6 and 2.0 × 10^6 dpm of 14C-labeled amino acids.

Two-Dimensional Electrophoresis

Our original 2DE technique (12, 13) was substantially modified to adapt the technique to larger gels. For IEF we used 32 cm × 0.9 mm (i. d.) glass tubes, the length of the gel was 30 cm. For electrophoresis of the proteins in the second dimension, we used a Model 1120 Super Slab Gel (Bio-Rad Labs., Richmond, CA). The size of the glass plates used in this apparatus was 43.4 cm (height) × 37 cm, the slab gels were 42 cm × 33 cm × 0.85 mm. The protein spots were
made visible by fluorography. A detailed description of the large-gel 2DE technique used in this study is given elsewhere (17). 2

One-Dimensional SDS Gel Electrophoresis

One-dimensional SDS gel electrophoresis was carried out under the same conditions as the separation of proteins in the second dimension of the 2DE technique indicated above. However, we used an apparatus (DESGA, Heidelberg, F.R.G.) with gel cells 30 cm (height) × 25 cm instead of the Bio-Rad apparatus.

Results

The proteins of the Hep cells were extracted by treating the same cell sample sequentially with all the different extraction media indicated for an extraction procedure (A, B, or C). The radioactivity of each successive protein extract was measured. The dpm values obtained for each extract and for each experiment are indicated in Table 1, in percentages, 100% being the sum of the values obtained from all extracts investigated in one experiment. The results show that each medium extracted a certain amount of proteins which, apparently, could not be extracted with the medium used in the preceding extraction step. However, the relative amount extracted with each medium differed depending on the extraction procedure used. With procedure A, almost 90% of the proteins could be extracted with the first two steps, water and urea/ME. From the cell pellet that remained we could extract about 5% with urea/NP-40/DTT/K2CO3, and then almost all the remaining activity with SDS/urea/ME. Our extraction procedure B showed that the presence of the zwitterionic detergent, "Zwittergent 3-12," contributed only negligibly to the total extracted proteins.

Our results with Procedure B also showed that the high amount of radioactivity found in the first wash solution can be decreased by more extensive homogenization and lysis of the cells. Our results with procedure C showed that 96% of the total cell proteins can be extracted in one step with urea/ME if the cells are sonicated before treating with the extraction media. The proteins which remained in the pellet after this procedure, comprising 3 to 4% of the total amount of proteins, could be solubilized with the SDS/urea/ME medium.

The four protein extracts, obtained by use of Procedure A, were investigated by 2DE. This analysis should reveal whether each extract contains a protein class (i.e., category) of its own, a question of particular interest with regard to the SDS extracts. The results of extraction experiment C (Table 1) suggest that the SDS extract does contain a class of proteins that cannot be resolved by any of the other extraction media. The protein patterns obtained from the water extract and from the urea/ME extract are shown in Figures 1 and 2. About half the protein spots differ in these two patterns. Each extract therefore contains a protein class of its own. The protein pattern of the urea/NP-40/DTT/K2CO3 extract comprised relatively few spots, and these same spots were also detected in the pattern for the water or urea/ME extract. The pattern showed horizontal and vertical streaks, particularly in the region below the sample application site.

Apparently, most of the proteins of the urea/NP-40/DTT/K2CO3 extract were not clearly resolved in the 2DE-pattern. A similar pattern was found when the SDS/urea/ME extract was separated by 2DE. All protein spots of this pattern were also present in the pattern of the water extract or in that of the urea/ME extract. However, most of the proteins were not well resolved but formed streaks, particularly in the region of the basic proteins. Similar 2DE patterns were obtained when we applied the SDS extract, (or the urea/NP-40/DTT/K2CO3 extract) alternatively to the anodic end of the IEF gel or to the cathodic end.

While the protein spots of the two 2DE patterns just described indicated that neither the urea/NP-40/DTT/K2CO3 nor the SDS/urea/ME extract contained a special class of proteins, the possibility existed that the streaks in these patterns represented fraction-specific proteins not clearly separated by the 2DE procedure. To test this possibility we separated the two extracts, and, as a control, the urea/ME extract by one-dimensional SDS gel electrophoresis. All three samples contained 2 g of SDS per liter. If the

Table 1. Radioactivity in Different Protein Extracts Obtained from Hep Cells Cultured in the Presence of 14C-Labeled Amino Acids

<table>
<thead>
<tr>
<th>Protein extraction media</th>
<th>Procedure A</th>
<th>Procedure B</th>
<th>Procedure C</th>
<th>Total and average values</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 3</td>
<td>Exp. 1</td>
</tr>
<tr>
<td>1. Water:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Extract</td>
<td>13.40</td>
<td>14.02</td>
<td>15.65</td>
<td>21.09</td>
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<td>Wash solution</td>
<td>47.39</td>
<td>44.81</td>
<td>45.25</td>
<td>37.23</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>24.11</td>
<td>22.41</td>
<td>20.12</td>
<td>16.56</td>
</tr>
<tr>
<td>Wash solution</td>
<td>4.89</td>
<td>7.18</td>
<td>6.00</td>
<td>7.47</td>
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<td>3. Urea/NP-40/DTT/K2CO3:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>2.63</td>
<td>3.29</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>Wash solution</td>
<td>1.88</td>
<td>2.21</td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td>4. Zwittergent/urea/NP-40:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td></td>
<td></td>
<td></td>
<td>1.27</td>
</tr>
<tr>
<td>Wash solution</td>
<td></td>
<td></td>
<td></td>
<td>1.33</td>
</tr>
<tr>
<td>5. SDS/urea/ME:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>4.40</td>
<td>4.50</td>
<td>5.41</td>
<td>9.17</td>
</tr>
<tr>
<td>Wash solution</td>
<td>0.62</td>
<td>0.70</td>
<td>0.85</td>
<td>3.24</td>
</tr>
<tr>
<td>6. Insol. residue:</td>
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<td>0.88</td>
<td>1.19</td>
<td>2.64</td>
</tr>
<tr>
<td>Sum:</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

* The cells were sequentially extracted with different media in the order (1-6) indicated in the Table.

** Cells sonicated.

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2 Reprints available from the author.
urea/NP-40/DTT/K₂CO₃ extract and the SDS/urea/ME extract largely consisted of extract-specific proteins, then one would expect to find in the SDS gel pattern some major bands characteristic of each of these two extracts, but no corresponding bands in the pattern of the urea/ME extract. Figure 3 shows that almost all the protein bands are seen in all three patterns, and that the two extracts we tested therefore did not consist of a number of major bands that did not occur (or only weakly) in the control pattern. The IDE pattern suggests that the urea/NP-40/DTT/K₂CO₃ extracts as well as the SDS/urea/ME extracts consisted of protein classes that, more or less, were also present in the urea/ME extract.

When one considers the radioactivity that remained in the rest of the cells when all the extraction steps had been performed, it becomes obvious that the total mass of proteins of the Hep cells was in fact solubilized by our extraction procedures. Under the most favorable conditions only 0.01% of the radioactivity remained in this final pellet. Moreover, we considered the possibility that some protein species or classes may get lost on the way from the cell culture to the cell pellet used for the various extraction steps. Possibly, washing the cells with trypsin/EDTA solution and with NaCl solution after cell culturing may remove some of the cell-surface proteins. When the radioactivity was measured in the used wash solution, high values were found: the dpm of all wash solutions relative to the dpm of all cells was 1:4.7. However, residues of the culture medium and broken cells may be the sources of this high radioactivity in the wash solution. The problem of losing cell-surface proteins during washing of the cultured cells will be investigated by comparing 2DE patterns from washed and non-washed cells.
Discussion

This investigation shows that the total mass of proteins of a certain human cell type can be completely extracted. However, two extraction steps are necessary: extraction with urea/ME followed by extraction with SDS/urea/ME. Several investigators have shown that SDS must be present if all proteins of animal cells are to be extracted (21, 22). On the other hand, protein samples containing SDS interfere with the IEF-step of the 2DE, with the result that a certain portion of the proteins cannot be resolved by 2DE (20, 23).

If SDS extracts a special class of proteins that cannot be extracted in any other way, then this would immediately restrict our attempt to detect all cell proteins by 2DE. However, our investigation has shown that only 3 to 4% of the cell proteins are not extracted in the absence of SDS. Moreover, most if not all of the protein species represented by this small proportion are also present in the urea/ME extract. Complete disruption of all cells and organelles is, however, an important prerequisite for extracting all the cell proteins in this way. Thus, it can be concluded that urea/ME will extract all protein species of the cell, but SDS is required for complete solution of certain protein species.

The possibility that cell-surface proteins get lost during washing of the cultured cells is still under investigation. Another class of proteins that would remain undetected, as has been taken into consideration (16), would be any proteins that have extremely short biological half-lives.

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Fig. 3. Proteins extracted from Hep cells and separated in an SDS gradient gel as described in the text.

A: proteins extracted with SDS/urea/ME solution, B: proteins extracted with urea/ME solution, C: proteins extracted with urea/NP-40/DTT/K₂CO₃ solution. The proteins were labeled with [³⁵S]amino acids and made visible by fluorography, with exposure for four days (left) and two days (right). No extra bands occur in patterns A and C compared with B. There is no evidence that SDS/urea/ME (A) and urea/NP-40/DTT/K₂CO₃ (C) extract special categories of proteins, i.e., protein classes not extractable with urea/ME (B).

References

8. Bishop JO, Morton JG, Rosbash M, Richardson M. Three abun-