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OVER TWO HUNDRED POLYPEPTIDES RESOLVED FROM THE HUMAN ERYTHROCYTE MEMBRANE

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Summary

A modification of O'Farrell's method of two-dimensional polyacrylamide gel electrophoresis has allowed for the resolution of erythrocyte membranes showing up to 200 individual components. Data is presented which indicates that this protein heterogeneity is not produced by artifactual protein-protein aggregation, endogenous protease activity or secondary charge modification. Similar patterns are obtained when the samples are added to the unpolymerized isoelectric focusing gel, and isolated and stored in protease inhibitor. Individual spots could be eluted off of stained gels, resolubilized under extreme detergent solubilization conditions and run on one-dimensional gels; these run as individual bands. One of the advantages of the method is the presence of sodium dodecyl sulfate in the solubilization procedure. The method chosen for solubilization prior to isoelectric focusing appears to cause selective aggregation of all or most of the spectrin and band 3 proteins. This further allows for excellent resolution of minor components.

Introduction

The use of two-dimensional polyacrylamide gel electrophoresis for the analysis of complex mixtures of proteins has increased dramatically in the past few years. With the advent of the development by O'Farrell of an isoelectric focusing-sodium dodecyl sulfate system of great reliability [1], the full potential of this type of method seems to have been reached. These types of methods can now be employed to analyze the degree of protein heterogeneity of commonly studied membrane preparations. Here we report a modification of the O'Farrell method and its applicability to membrane studies. The results suggest that the commonly described one-dimensional sodium dodecyl sulfate

Abbreviation: SDS, sodium dodecyl sulfate.

protein pattern of human erythrocyte membranes may represent an oversimplification of the numbers of individual polypeptides present within this structure.

Materials and Methods

Sample preparation and electrophoresis

Two-dimensional gel analysis of human erythrocyte membranes was carried out by the method of O'Farrell [1] with the following exceptions. The first dimension was run in a 3.5 mm inside diameter cylindrical tube, the second dimension slab gel was made to a thickness of 0.75 mm. Sample preparation proved to be of critical importance and the following protocol was followed. The membranes were spun at 50 000 $\times g$ for 10 min in the final wash. The supernatant was carefully removed and a preweighed tube weighed a second time to determine the weight of the membrane pellet. The weight in grams times 10 gives an approximate value for the numbers of mg of protein in the sample. This value was then multiplied by 30 to provide the value of the number of μl of stock grinding solution (1% SDS/10% β-mercapetoethanol/ 9.5 M urea; pH 7.4) to be added to the sample. The sample was then shaken at room temperature for 30 s until the membrane material was put completely into suspension and then lysis supplement (4 ml of 10% nonionic detergent/ Nonidet-P40/0.08 ml 3/10 ampholines/0.32 ml 5/7 ampholines) was added in an amount equal to 10 times the original number of milligrams protein. 30 s of shaking at room temperature was finally followed by the addition (in μl) of 40 times the original number of mg of protein of lysis buffer (5% β-mercaptoethanol/9.5 M urea/20% of 10% NP40/1.6% 5/7 ampholines/0.4% 3/10 ampholines). Some of these methods were described previously [2]. In this way, a realtively constant amount of protein per unit volume of lysed sample could be obtained. Varying this solubilization procedure frequently resulted in failure to solubilize all the membrane components as evidenced by turbidity in the sample. When this procedure is followed precisely, no observable pellet was obtainable after spinning the sample for 1 h at 100 000 $\times g$. This was taken as evidence of complete membrane solubilization. The addition of SDS in the original stock grinding soluion was of particular importance in obtaining repeatable two-dimensional gel patterns. 40—135 μl of sample was applied to each cylindrical gel. The resolution of minor components required the higher concentration. The single-dimension SDS slab gels were run using the system of Laemmli [3] with the exception that the acrylamide concentration was 12% and the slab gel thickness was 0.75 mm. Samples were denatured by heating at 100°C for 2 min in 5% mercaptoethanol/2% SDS/0.0625 M Tris, pH 6.8 (SDS sample buffer). Prolonged storage at —80°C provided pronounced changes in the protein patterns. Therefore, all samples were run within one week of obtaining the fresh erythrocytes. For the protease inhibitor experiments whole blood was obtained and immediately washed in phosphate-buffered saline (0.15 M NaCl/5 mM sodium phosphate, pH 7.5) containing the protease inhibitor α-toluenesulfonyl fluoride (100 mM). This inhibitor was also placed in all the subsequent wash solutions and in the solubilization solutions.

A parallel run was made from the sample of blood in which the membranes

were treated in an identical manner with the exception that no protease inhibitor was added. Parallel gel runs were made of these two samples and a direct comparison of all resolvable spots from the two preparations on the slab gels were made.

Membrane isolation

For the removal of stained spots from the second-dimension slab gel, the stained spots were cut out of the gel, the gel piece smashed, and the resulting preparation dialyzed for 24 h against 1 l 5 mM sodium phosphate buffer (pH 7.5) in 0.1% SDS. The preparation was then spun at 10 000 × *g* for 5 min and the supernatant removed. This supernatant was then made 0.2 M in KCl which precipitated the SDS · protein complex. These samples were spun at 10 000 × *g* for 10 min at 23°C and the pellet resolubilized in SDS sample buffer as described above.

Human whole blood was spun on a clinical centrifuge and the serum and buffy-coat aspirated off. The red blood cells were then washed 3 times in phosphate-buffered saline. At each washing, the top layer of the cells was removed. The cells were then lysed twice in 5 mM sodium phosphate buffer, pH 8.0, and the membranes were spun at 10 000 × *g* for 10 min. The membranes were then resuspended in 10 mM Tris buffer, pH 8.0, and spun a second time at 10 000 × *g* for 10 min. The membranes were then washed twice in 56 mM sodium borate buffer pH 8.0, and spun at 10 000 × *g* for 10 min the first time and at 50 000 × *g* the second time. The final pellet was solubilized as described above.

Results

The major changes in this two-dimensional gel system over the method of O'Farrell were in the nature and sequence of sample preparation and the addi-

Fig. 1. One-dimensional SDS slab gel electrophoresis of human erythrocyte membrane proteins. From left to right the samples were treated as follows. Sample 1 shows a preparation solubilized in the lysis buffer of O'Farrell. Well 2 gives the pattern of a sample solubilized in lysis buffer containing 2% SDS. The third sample was prepared using our standard two-dimensional gel membrane solubilization procedure (see Materials and Methods). Arrows denote spectrin (well 1) and Band 3 (well 2).

tion of SDS to the sample solution. The use of SDS was suggested by experiments in which membranes were initially solubilized in the lysis buffer of O'Farrell [1] and then run on SDS gels. It was found that much of band 3 was missing when these gels were compared to those for which the samples were solubilized in SDS/Tris sample buffer (Fig. 1). Spectrin was also frequently absent from the one-dimension SDS gels when the samples were solubilized and stored in O'Farrell's lysis buffer. Spectrin was also absent from the standard two-dimensional or one-dimensional gels prepared with SDS in the stock grinding solution as described above (Fig. 1). A given membrane preparation could be split into two aliquots both of which were first solubilized using our standard two-dimensional gel method. If one of these was then diluted 1 : 1 with SDS sample buffer and both preparations run on the Laemmli one-dimensional gel system the sample run with SDS sample buffer showed both spectrin and band 3 while the other, even though 1% SDS was present in the stock grinding solution, lacked both spectrin and band 3 (Fig. 1). Apparently, a minimum SDS concentration or SDS to protein or NP40 ratio is required to solubilize spectrin and band 3. Occasionally, though insufficient SDS was present to allow spectrin and band 3 to enter the gel, it was found that heating at 100°C for 2 min produced a pattern containing both band 3 and spectrin. However, the absence of these proteins which make up a major portion of the total protein content of the membrane allowed the resolution of numerous spots which would normally be masked by these two major components (Figs. 2 and 3). On two-dimensional gels the absence of SDS in the original sample prevented much of the protein from entering the first-dimension gel. Those proteins that did enter were frequently smeared and the patterns differed greatly from preparation to preparation. Therefore, all two-dimen-

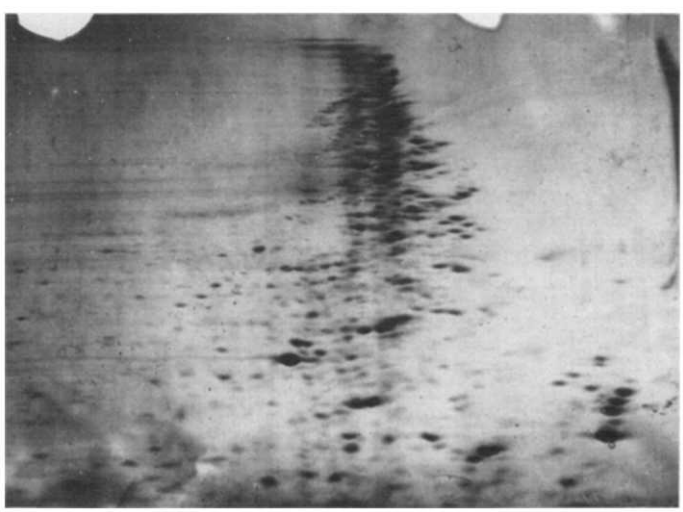

Fig. 2. A pattern of protein spots on a standard preparation of the erythrocyte membranes. 40 µl of material were placed in the first dimension cylinder. Horizontal dimension is the isoelectric focusing separation with the anode to the right, the vertical direction indicates the molecular weight separation with the low molecular weight region and the anode down.

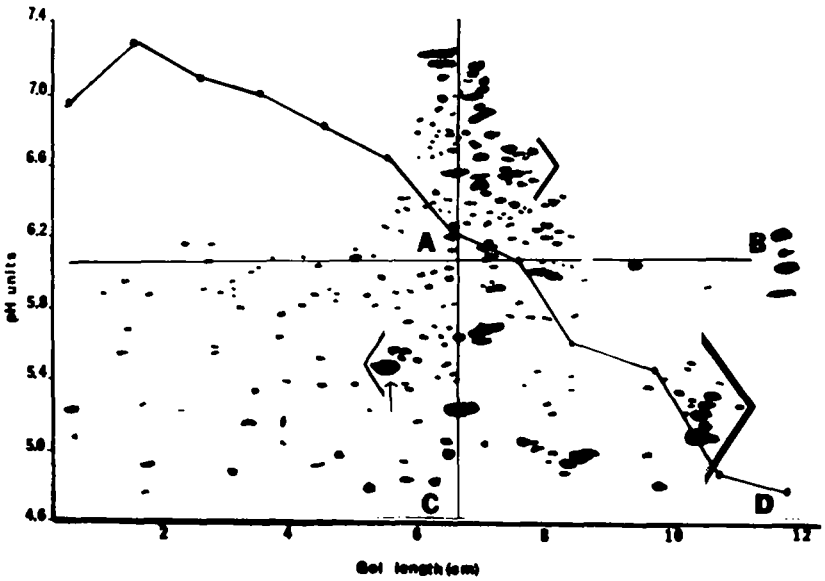

Fig. 3. Diagrammatic representation of the same gel shown in Fig. 2 with the addition of a standard pH gradient measured by dialyzing sections of a replicate run containing the same amount of sample as shown in Fig. 2. Brackets denote reference groupings. Arrow points to actin spot.

sional gel samples were prepared with SDS in the stock grinding solution as described above.

The basic pattern observed for whole solubilized human erythrocyte membrane proteins on the two-dimensional gel system is shown in Figs. 2 and 3. As many as 200 individual spots could be counted from any one gel. However, many of these were very minor in amount and difficult to resolve. From preparation to preparation, approximately 150 individual spots were directly comparable from one gel to another.

Due to the extreme heterogeneity observed in the protein content of the erythrocyte membrane using this method, it is difficult to identify individual spots rapidly when comparing one preparation to another. This process was facilitated by identifying a few easily recognizable groups of spots that were always present in an identical pattern. These included some of the groupings bracketed in Fig. 3. Once these various groupings in the four quadrants of the gel were identified, it became much simpler to compare individual spots. In this way, each component is identified by its relative position to other spots and recognizable landmark groups.

The extreme protein heterogeneity suggested by these patterns made direct comparison with previously published one-dimensional gel analysis of human erythrocyte membrane proteins impossible. The potential sources of artifact fall into three categories. The first is the possibility of protein-protein aggregation producing more than one spot for each polypeptide. The second is multiple charge artifacts resulting from sample preparation [1]. Lastly, there is the possibility of protease activity in the preparation, inducing protein degradation during membrane isolation or sample preparation and storage. We have

attempted to test for all of these possibilities in order to verify the validity of the method.

To detect the possibility that protein-protein aggregation produced the heterogeneity observed in the patterns, a standard membrane preparation was isolated, solubilized and run under normal conditions. It was then stained with Coomassie Brilliant Blue and individual spots of varying molecular weights were cut out of the gel, eluted off and solubilized under extreme conditions of solubilization (100°C temperature, 2% SDS, 5% mercaptoethanol). These were run on one-dimensional gels, the results are shown in Fig. 4. Clearly, these spots represent components which run as single bands under conditions of harsh denaturation. It is still possible that aggregation phenomena are occurring in the first dimension. We feel this is minimal as evidenced by the dramatic increase in clarity and reduction of smearing when SDS is included in the sample during the first-dimension run. However, there is still a possibility of aggregation at the interphase of the first-dimension cylinder as the proteins are concentrating in a small area in the beginning of the run. This was further suggested by the fact that the majority of the high molecular weight spots is congregated in one region of the isoelectric focusing gel suggesting some interaction within the first dimension gel. To further test the possibility of aggregation or peptide interaction in the first dimension, the membrane pellet was added directly to the unpolymerized polyacrylamide isoelectric focusing solution with the normal amounts of SDS added. The gels were then run without the prerun and handled subsequently in a normal manner.

The resulting spot pattern, although badly smeared in the isoelectric focusing dimension (most likely due to the lack of a pre-run) was essentially identical to the normal pattern. As was the case for the normal patterns, little or no spectrin or band 3 was observed.

To test whether the process of focusing and separation in the second SDS

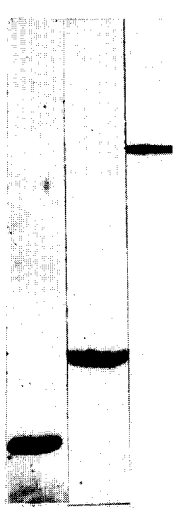

Fig. 4. One dimensional slab gel electrophoresis of low and high molecular weight spots removed from stained two-dimensional slabs, subsequently eluted and solubilized in sample buffer at 100°C.

dimension produced artifactual secondary charge modifications, spots were recovered from the second-dimension slab which was lightly stained and these were re-run on the micro-isoelectric cylinder. In all cases they focused at the same pH as originally observed in the previous two-dimensional run.

Also tested was the effect of the amount of protein applied to the first-dimension cylinder on the nature of the patterns obtained. It was found that within a wide range of protein values (as much as a 4-fold increase in total amount of material applied) the patterns remained unchanged. Virtually 90% of the spots could be observed as being homologous from a sample in which only 40 μl were applied as compared to one in which 160 μl were applied (Figs. 2 and 5). Reducing the amounts also did not affect the heterogeneity of the patterns, although below 30 μl the most minor components become difficult to resolve. One of the most interesting results of this series of gels was the demonstration that the addition of more material did not necessarily result in an increase in staining of all the individual spots. It would appear that the system had been saturated such that increasing the sample beyond a certain point did not produce an increase in the amount of protein entering the gels or at least for some spots did not produce an increase in the amount of material focusing on the first dimension gel. The only benefit obtained by increasing the amount of sample was in the resolution of very minor components in quadrants C and D. However, samples greater than 150 μl introduced severe smearing which masked many of the high molecular weight spots.

The most probable artifactual source of protein heterogeneity was throught to be protease activity present in the membrane preparation during membrane isolation or sample treatment and storage. As we observed no increase in heterogeneity of the protein patterns during storage of the sample, we tested the possibility that there was protein degradation occurring during membrane isolation or even during the preparation and solubilization of the membrane samples. This was done initially by lysing cells rapidly and allowing them to

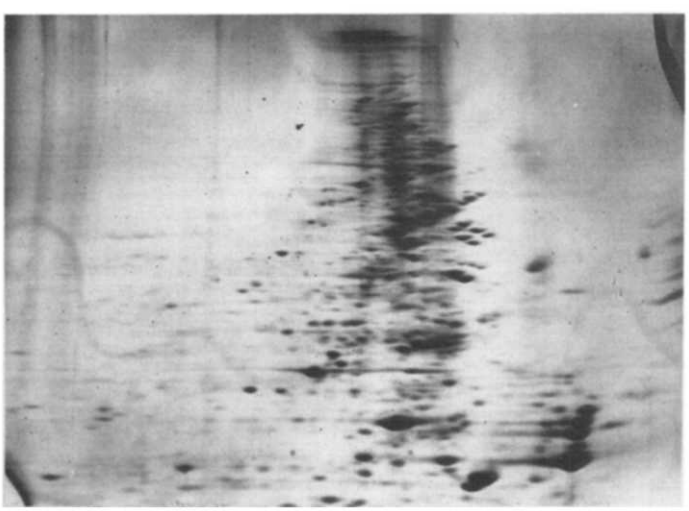

Fig. 5. Two-dimensional gel pattern of the same sample as shown in Fig. 2 with the exception that 160 μl of solubilized material was added to the first dimension cylinder.

stand at room temperature for a period of hours. The samples were then solubilized and run on the gel as previously described. Fig. 6 shows some of the results of these experiments. Clearly, no change in the overall pattern could be observed after prolonged periods of time. These gels which were overloaded to reveal minor components had 163 individual spots which could be identified on each of the four stored preparations. Each of these spots was present in the zero time sample and the one taken at 3.5 h. However, many of the spots had changed in relative staining intensity possibly indicating some generalized

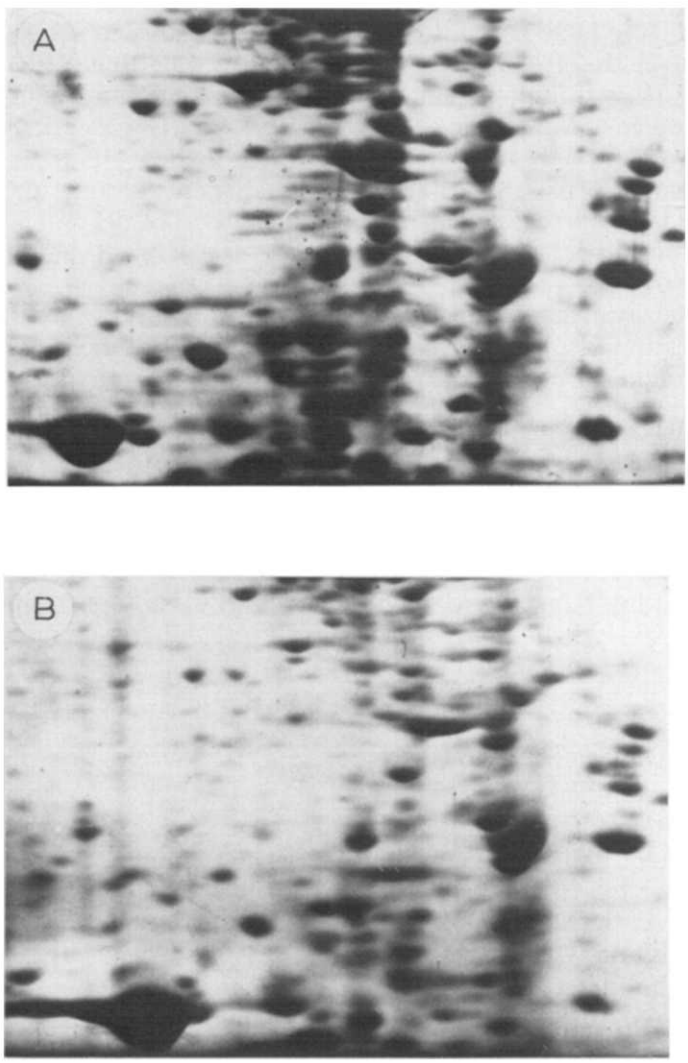

Fig. 6. Two two-dimensional gel runs in which the samples were solubilized in a standard way immediately (A) or allowed to incubate in borate buffer for 3.5 h (B). These are magnifications of low molecular weight regions of the gel in quadrants C and D. They show minor components not clearly visible in the entire gel pattern shown in Fig. 2. The differences in the patterns from A and B are typical of the type of differences observed from any preparation run on two separate occasions and probably reflect slight variations in the pH gradient in the first dimension from gel to gel.

degradation. To test the possibility of protease activity occurring prior to membrane isolation or during the period of solubilization of the membranes in the NP40/SDS buffer, two-dimensional gel runs were made of membranes with and without a protease inhibitor. As was the case in the previous time sequence experiments, no consistent differences between the two preparations could be detected even though the resolution of individual spots allowed a comparison of over 150 components.

An alternative explanation for the positioning of the high molecular weight components in a relatively narrow region of the isoelectric focusing gradient would be that the pH gradient had broken down in this region of the gel due to ampholine interaction with the large amount of protein run through this area or as a result of the SDS in the original sample. To test this, one isoelectric focusing gel was run with and without the standard solubilized membrane material. 1-mm sections were taken of the gel and extracted with water. The resulting curves were essentially identical. The gradients from protein loaded gels were linear over a pH range from 4.75 to 7.3 (see Fig. 3). The relative low value for the most basic section was thought to be an artifact of the method of pH determination as micro pH probes placed into the center of the gels produced values greater than 8.0 at this are of the gel.

The final type of artifact associated with this system is the phenomenon of secondary charge modification resulting in multiple spotting in the isoelectric focusing dimension. As pointed out by O'Farrell [1], these types of artifacts are readily recognizable as multiple spots that are seen to be equally spaced in the lateral direction on the second dimension slab gel. Each spot represents a similar change in net charge. As can be seen in Fig. 1, this type of multiple spotting phenomenon is very rare in these gels, these account for less than 5% of the total numbers of spots. Using conditions of careful sample preparation, multiple spotting can be almost entirely eliminated. It is essential to keep the samples frozen at $-80°$ during storage and to never allow the samples to be warmed beyond 23°C.

Discussion

Over the past few years, numerous two-dimensional gel systems for the analysis of membrane proteins have been described. Frequently, urea-polyacrylamide cylindrical gel electrophoresis was followed by the standard method of SDS electrophoresis. For example, Conrad and Penniston [4] report the resolution of approx. 25 individual components from human erythrocyte membranes using such a method. Others report the use of isoelectric focusing followed by SDS discontinuous polyacrylamide gel electrophoresis. Falk et al. [5] recently reported the resolution of 30 individual components from human erythrocyte membranes using a urea-NP40 isoelectric focusing followed by SDS electrophoresis. In a similar report [7], 30 components were resolved from erythrocyte membranes using Triton X-100 and urea in the isoelectric focusing dimension. Studying bacterial membranes, Ames and Nikaido [6] were able to resolve 150 individual components from *Escherichia coli* cell envelopes using a modification of the O'Farrell system. They demonstrated that SDS can be

present in the isoelectric focusing sample and still allow good focusing of the solubilized proteins. They indicated that the ratio of SDS to NP40 was an important factor in spot resolution. This is also suggested by our results. We have employed another method of sample preparation which also utilizes SDS during sample preparation. Using our method we are able to resolve as many as 200 individual spots from normal human erythrocyte membranes. This is of the same order of magnitude as reported by Ames and Nikaido for *E. coli* cell envelopes, but is much less than they report for the plasma membranes of HeLa cells.

The fact of the dramatic heterogeneity of the erythrocyte protein patterns coupled with the localization of many of these components in a narrow pH range suggest the possibility that this heterogeneity is in part artifactual. We have tested the major causes of electrophoretic artifact, protein aggregation and degradation. The results suggest that individual spots are single polypeptides, and that protein-protein aggregation and protease activity are not responsible for the bulk of the complex pattern observed.

One untested possible source of artifact is the binding of proteins to the ampholines during protein solubilization or isoelectric focusing. The presence of SDS from the beginning of the solubilization procedure may reduce this potential problem. Another potential cause of artifact is the possibility of deamination, especially at acid pH. As mentioned above and discussed by O'Farrell, this is the type of artifact that can result in multiple linear spotting. This phenomenon was largely absent from our patterns although we can not eliminate the possibility of deamination artifact.

The possibility of protein-protein aggregation producing these patterns is unlikely. The patterns are still obtained when the sample is polymerized within the gel, where SDS is included in the sample preparation procedure, and individual spots, when eluted from the gel, could be seen to run as single bands after further denaturation in a high concentration of SDS and mercapto-ethanol. Lastly, it is apparent that proteolytic degradation is not a major factor in theproduction of the patterns observed. This is suggested by the failure to alter the patterns by the addition of a protease inhibitor during the isolation and solubilization and storage procedures.

However, no protease inhibitor is completely effective and the one used here is generally ineffective against cathepsins. Perhaps a better indication of the lack of serious protease induced artifact was the failure to alter the heterogeneity of the pattern upon storage at room temperature. It also appears that the positioning of the bulk of the higher molecular weight polypeptides in the narrow pH range reflects actual isoelectric characteristics of each of these polypeptides since the pH gradient is still linear at this region of the gel (see Fig. 2).

Apparently, one of the major contributing factors to the resolution of this complex pattern was the selective removal of the proteins spectrin and band 3. Their removal presumably by selective aggregation prior to entrance into the gel, reduces the overloading phenomenon that we previously observed and allows minor components to enter and focus properly during the isoelectric focusing run. It is entirely possible that polypeptides other than band 3 and spectrin have also aggregated and failed to enter the gel and, therefore, the

pattern that we obtained probably represents a conservative estimation of the heterogeneity of the human erythrocyte membrane proteins. The tendency for spectrin to aggregate has been frequently studied.

On of the difficulties with the use of this system was comparison of the patterns from preparation to preparation. In a number of regions of the gel the patterns are very complex and it is difficult to compare individual spots. There is also variation from run to run in the characteristics of the isoelectric focusing gradient. A typical type of variation from run to run is shown in Fig. 6. In this case, almost every spot from Fig. 6A can be found in Fig. 6B, however, they are somewhat more spread out in Fig. 6B. This detailed enlargement also indicates the excellent resolution of the system which is not as apparent in low magnification photographs of the entire gel pattern. The division of the entire pattern into quadrants and the subdivision of the patterns into recognizable groups makes identification of individual spots from run to run possible. Our results on the addition of increasing amounts of material to the first-dimension gel do suggest that great caution should be made when attempting quantitative evaluations of the amount of any particular spot. Clearly, there are a number of polypeptides in the preparation which do not increase in staining intensity upon increasing the total sample volume. These may precipitate at certain concentrations in the solubilization process at the interphase of the first dimension. Nonetheless, overall we feel this method represents a dramatic improvement in the quality of membrane protein analysis. It is concluded that the patterns observed do represent a valid indication of erythrocyte membrane protein heterogeneity and that the high molecular weight proteins of this membrane have a similar isoelectric point.

Acknowledgements

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