DISCONTINUOUS GEL ELECTROPHORESIS OF REDUCED MEMBRANE PROTEINS

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SUMMARY. Fractionation of reduced membrane proteins in discontinuous sodium dodecyl sulfate gel electrophoresis is affected by reconstitution of disulfide bridges, by dissociation of dodecyl sulfate-protein complexes through sieving in the gel matrix, and by changes in mobility due to alterations in ionic environment of the sample.

Shapiro et al. (1), using SDS* for fractionation of reduced proteins, have discussed the reconstitution of disulfide bridges in polyacrylamide gel electrophoresis. The possibility of artificial protein banding has been vaguely mentioned by other authors (2, 3, 4). In this communication we want to demonstrate that formation of disulfides constitutes a source of error which should not be neglected when interpreting results obtained in SDS electrophoresis. This is particularly valid while fractionating proteins derived from biological membranes of which the electrophoretic patterns can hardly be examined by independent methods (5, 6). It is also shown that the ionic environment is altered by the presence of reducing agents in the sample. Likewise, sieving-induced disintegration of SDS-protein complexes can lead to erroneous interpretations, especially with respect to discontinuous buffer systems in gels of constant pore size.

MATERIALS AND METHODS

The membraneous fraction of rat forebrain homogenate was prepared as described (7). Human erythrocyte ghosts were prepared according to Dodge et al. (8). Membrane proteins were extracted with 0.4 % (w/v) SDS - 28 mM Tris-sulfate (pH 8.4)(0.5 mg protein/ml). Cytochrome C from horse heart (MW 12,100; Sigma), ovalbumin (MW 45,000; Sigma), conalbumin

*SDS, sodium dodecyl sulfate

(MW 80,000; Sigma), bovine serum albumin* (MW 67,000; Behring), and human serum albumin* (MW 69,000; Serva) were used as molecular weight markers. If not mentioned otherwise these marker proteins were kept in buffered SDS solution used for extraction of membrane-bound proteins at concentrations of maximally 0.5 mg/ml. All samples were heated under nitrogen in a boiling water bath for 10 min either in presence or absence of 1 % or 5 % (v/v) 2-mercaptoethanol. For carboxymethylation a twofold molar excess of iodoacetamide over mercaptoethanol was added to the sample, which was kept at 37° C for 30 min, and was then dialyzed overnight against 0.4 % SDS (w/v). SDS electrophoresis was carried out in following discontinuous buffer system in 12 % acrylamide gels (9), altered as described (7): upper buffer, 0.05 M Tris-glycine (pH 8.9) plus 0.1 % SDS (if not stated otherwise); lower buffer, 0.1 M Tris-Cl (pH 8.1); gel buffer, 0.372 M Tris-Cl (pH 8.9). No differences were found in electrophoretic patterns whether potassium ferricyanide was incorporated in the gels or not. Occasionally, mercaptoethanol was added at different concentrations to the upper buffer (see legends to the Figures). The presence of SH groups was tested with sodium nitroprusside (3).

RESULTS AND DISCUSSION

Most conspicuous in the electrophoretic patterns of mercaptoethanol-reduced samples is the occurrence of large gaps in the upper portion of the gels, completely devoid of protein bands (Fig. 1, gels 2-4)(8, 11, 12). Despite constant amount of protein these gaps can be enhanced by increasing the total amount of mercaptoethanol. They are due to a decrease in conductivity of the sample, leading to an acceleration of the SDS-protein complexes in the anodal direction. Since the reducing agent

*BSA, bovine serum albumin; HSA, human serum albumin

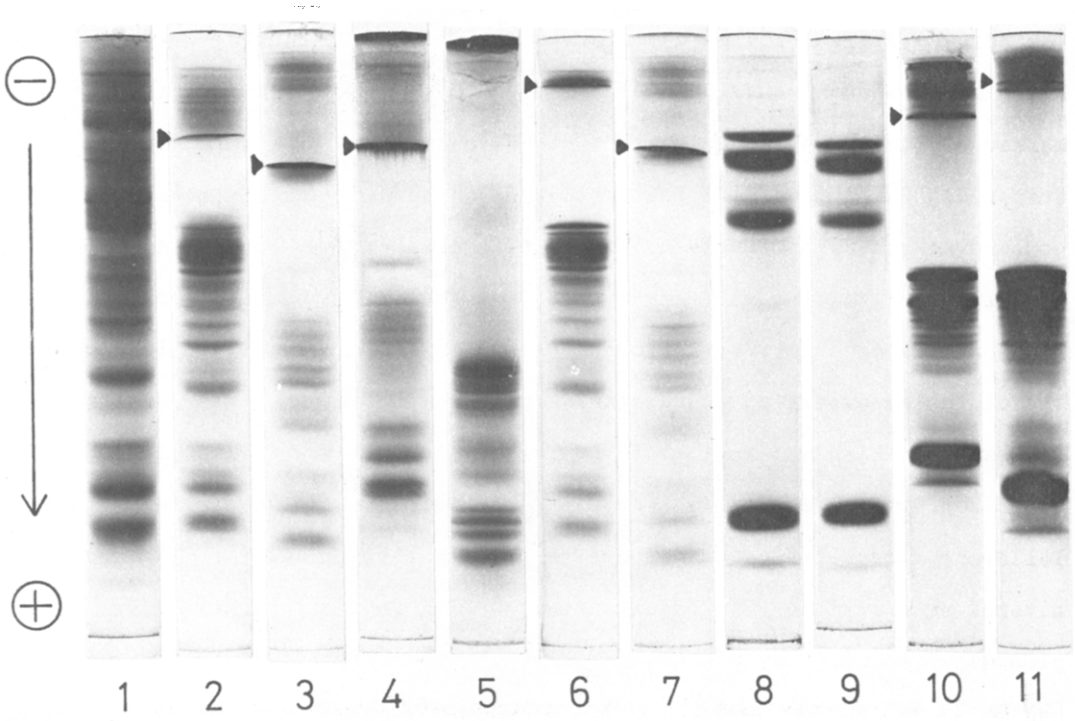

Figure 1. Gels 1-7, polyacrylamide gel electrophoresis of SDS-extracted particulate fraction from rat brain (TH).
1, profile of unreduced TH, standard buffer system; 2, profile of reduced TH (1 % mercaptoethanol), standard buffer system. Decrease in number of bands, shift in anodal direction, SDS-deficient band in upper portion of gel (▶); 3, profile of reduced TH (5 % mercaptoethanol). Same effects as in 2, however, more pronounced; 4, same as 3. Reoxidation of sample during reductive treatment leads to ill-defined material at the top of the gel. SDS-deficient band is very pronounced; 5, same as 4, except for 0.4 % SDS in upper buffer, disappearance of SDS-deficient band; 6, same sample as in 2, upper buffer contains 0.1 % mercaptoethanol. As compared to 2 several bands in the upper portion of the gel have disappeared while the residual profile remains the same; 7, same sample as in 3, upper buffer contains 0.1 % mercaptoethanol. No remarkable difference as compared to 3. Reduction with 5 % mercaptoethanol leads to stabilized pattern, in contrast to 1 % mercaptoethanol. Addition of mercaptoethanol to the upper buffer appears to be unnecessary.

Gels 8-10, polyacrylamide gel electrophoresis of reduced marker proteins, from top to bottom: conalbumin, HSA, ovalbumin, cytochrome C.
8, separation in standard buffer system; 9, same as 8 except 0.1 % mercaptoethanol in upper buffer. In contrast to TH (gels 2 and 6) no change in profile is noted; 10, same sample as 8, however diluted 1:4 with a mixture of 0.4 % SDS and 0.1 % mercaptoethanol. The sample volume thus applied corresponds to that of TH (gels 2-7, 11), the anodal shift is obvious. The SDS-deficient band in the upper portion is more pronounced, consisting predominantly of conalbumin which is virtually absent in the other portion of the gel. Several small bands close to the marker proteins can be ascribed to partial SDS-stripping; 11, mixture of reduced TH (gel 2) and reduced marker proteins. Upper buffer contains 0.1 % mercaptoethanol. Anodal shift and SDS deficiency similar to that of gel 10.

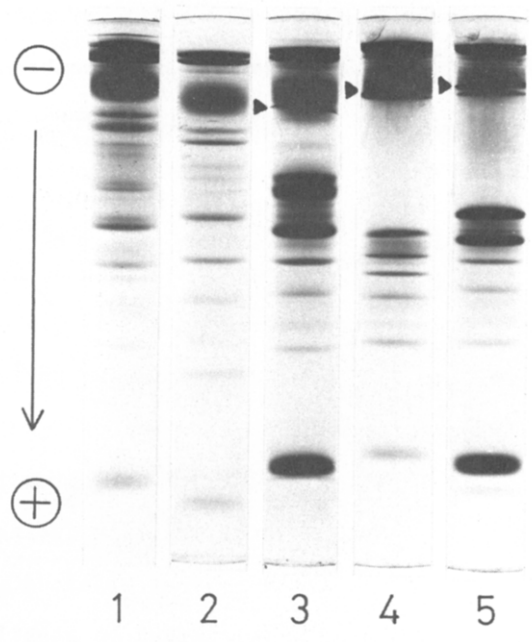

Figure 2. Polyacrylamide gel electrophoresis of SDS-extracted erythrocyte ghosts (G).
1, profile of unreduced G, standard buffer system; 2, profile of reduced G (1 % mercaptoethanol), standard buffer system. In contrast to gel 2 (Fig. 1) no anodal shift of bands is observed except for a slight reduction of some proteins; 3, same as 2, reduced marker proteins added (cf. Fig. 1, gel 8). Compared to 2 the anodal shift is obvious. Besides the occurrence of an SDS-deficient band several bands have disappeared from the upper portion of the gel; 4, same as 2, upper buffer contains 0.1 % mercaptoethanol, marked anodal shift. Some bands in the middle of the gel have disappeared through reduction during electrophoresis (see gel 2). Aside from the SDS-deficient band two high molecular weight bands are conspicuous in the uppermost portion of the gel which are not altered through reduction (6); 5, same as 2, reduced marker proteins added, 0.1 % mercaptoethanol in upper buffer. Except for anodal shift of pattern and disappearance of conalbumin (cf. gel 3) the SDS-deficient band contains large quantities of precipitated conalbumin. HSA migrates in approximately the same position as the largest protein of the reduced pattern (gel 4) with the exception of the two high molecular weight bands.

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should not be removed by dialysis prior to electrophoresis (10) the anodal shift in migration has to accepted in order to maintain the reduced state of proteins unless free sulfhydryl groups are protected with a blocking agent, e.g., iodoacetamide or acrylonitrile (11, 12). Carboxymethylation, however, implies a chemical modification which in most cases prevents appropriate conditions for analysis of the proteins

under consideration. Carboxymethylation, carried out according to the literature (2), lead to inconsistent results with membrane proteins. By contrast, good results were obtained with water-soluble proteins, similar to those of the above-mentioned authors. Despite carboxymethylation the membrane proteins still contain a certain portion of unreduced protein (Fig. 3, gels 1 and 2). Commonly, the sample - reduced but not alkylated - is fractionated in a buffer system devoid of reducing agent (for continuous buffer systems, see 2, 3; for discontinuous buffer systems, see 2, 13-15). Under these conditions reconstitution of disulfide bridges can be demonstrated during electrophoresis, inasmuch as the difference in electrophoretic mobilities of proteins and of reducing agent results in a separation of components, similar to that attained while dialyzing the sample against SDS. Therefore we have tried to stabilize the state of reduction during electrophoresis in the discontinuous buffer system by constantly supplying mercaptoethanol in the cathodal buffer reservoir at different concentrations. Similar attempts have been made by other authors applying continuous phosphate buffer systems of neutral pH (16). Under these conditions, however, the electrophoretic mobilities of SDS-protein complexes by far exceed that of the virtually uncharged reducing agent. This can be shown with the nitroprusside test (see 3) which in our case serves to demonstrate the presence of mercaptoethanol within the gel matrix. In the neutral phosphate buffer system a reducing agent of higher electrophoretic mobility, e.g., thioglycolic acid, may be used. Reduction of initially unreduced samples during discontinuous electrophoretic fractionation is possible (Fig. 3, gels 7, 8, 10, 11) provided that sufficient quantities of reducing agent are supplied in the cathodal buffer, thereby preventing reoxidative artefacts which can be formed during the sample preparation prior to electrophoresis (Fig. 1, gels 3 and 4).

In continuous gradient gels partial dissociation of SDS from protein

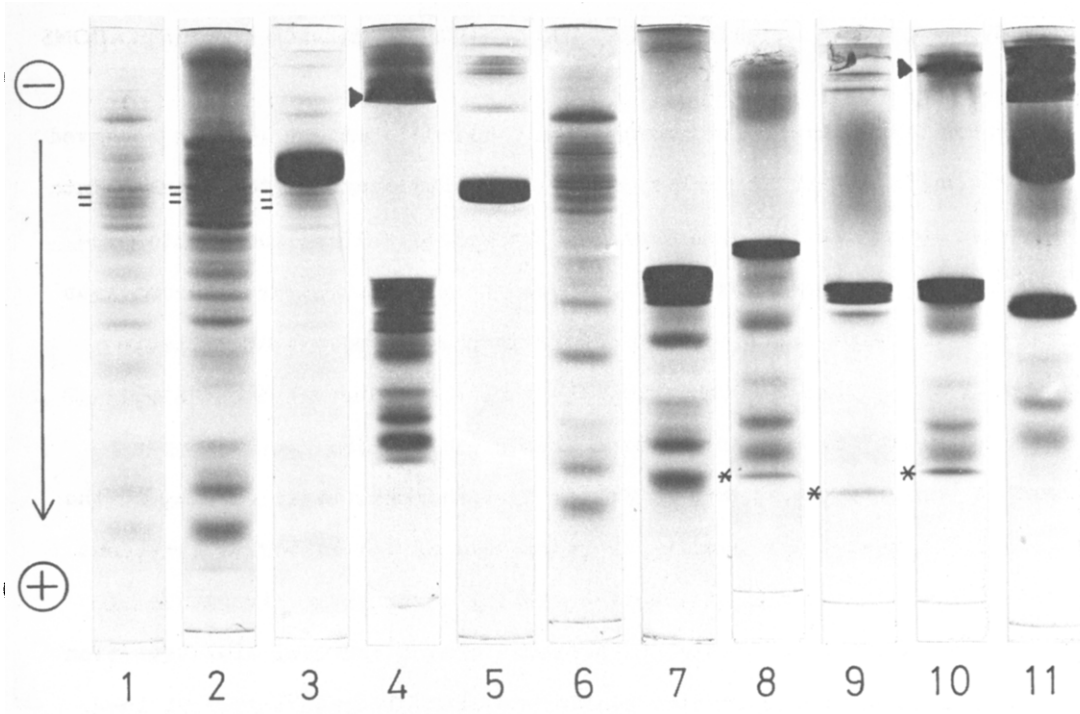

Figure 3. Gels 1-4, polyacrylamide gel electrophoresis of SDS-extracted particulate fraction from rat brain (TH), reduced and carboxymethylated. 1, profile after fractionation in standard buffer system. In comparison to gel 1 (Fig. 1) only small differences are observed; 2, same sample as 1, 0.1 % mercaptoethanol in upper buffer. While comparing this pattern with that of gel 1 it is evident that contact with anodally-migrating mercaptoethanol leads to disappearance of some bands in the upper portion of the gel. The remaining pattern, however, corresponds entirely to that of gel 1, i.e., reducable proteins are still present despite carboxymethylation; 3, same as 1 with BSA added. The position of the BSA marker band relative to the TH pattern can be recognized by the triple band (≡). In comparison with gel 5 further bands are identifiable; they can be ascribed to partly SDS-stripped BSA oligomers; 4, same as 1, with 1 % mercaptoethanol added. The decrease in number of bands is both due to further reduction of the sample and to precipitation of protein as a consequence of SDS deficiency; 5, unreduced BSA in the standard buffer system. Owing to partial dissociation of SDS through severe sieving a large number of bands is found in the region of BSA oligomers (10).

Gels 6-8, 10, 11, reduction of initially unreduced proteins during electrophoresis. 6, fractionation of TH in standard buffer system with 0.1 % mercaptoethanol. As compared to gel 1 (Fig. 1) only slight reduction is seen; 7, same as 6 except for 0.5 % mercaptoethanol. Marked decrease of bands through drastic reduction. The upper portion of the gel is virtually free from large proteins; 8, same as 7 except for 0.2 % SDS in the upper buffer to abolish any hidden SDS-deficient bands. However, an additional artificial band (*) is formed at the anodal end; 9, reduced BSA, fractionated under conditions of 8; 10, unreduced TH with reduced BSA added. Same electrophoretic conditions as 8. BSA comigrates with the largest protein derived from TH. Despite 0.2 % SDS, resulting in an artificial band (*) near the anodal front, an SDS-deficient band is formed near the top of the gel in the region of BSA oligomers, i.e., both artefacts are simultaneously formed; 11, TH plus BSA, both unreduced. Same conditions as in 7. Comparison with gel 10 shows that the conditions for reduction during electrophoresis are not unlimited.

has been found during electrophoresis (10). This effect is also observed in the uniform gels as applied here. SDS deficiency has to be taken into account on one hand, leading to artificial protein banding due to precipitation (Fig. 1, gels 2-4, 6, 7, 10, 11); on the other hand numerous and inconspicuous bands will appear, possibly representing variable loading of identical proteins with SDS (17)(Fig. 3, gel 5). Protein precipitation can be avoided by supplying enough SDS via the upper buffer (Fig. 1, gel 5) which however produces artificial banding at the anodal front (Fig. 3, gels 8-10). Formation of the latter is dependent on the state of aggregation of SDS as found with the distribution of detergent in protein-free gels (10). In order to prevent disintegration of SDS-protein complexes through sieving (Fig. 3, gel 5) gels of increased pore size should be used; this, however, will lead to inferior resolution.

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