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Dodecyl Sulphate/Polyacrylamide-Gel Electrophoresis at Low pH Values and Low Temperatures

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A simple method is described for dodecyl sulphate/polyacrylamide-gel electrophoresis of pH- and temperature-labile biological intermediates. The method is based on a catalyst system that works at temperatures of 2–4°C and pH values of 2–4 and an appropriate buffer system containing Li⁺ or Tris [CH₂OH–C(CH₂OH)₂–NH₃⁺] instead of Na⁺. This system does not lead to the precipitation of 1% dodecyl sulphate.

Certain biological intermediates, e.g. the phosphorylated intermediate of the (Na⁺+K⁺)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) or of the high-affinity Ca²⁺-dependent ATPase are labile under conditions normally used in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, i.e. room temperature and pH 7.0 (Weber & Osborn, 1969; Fairbanks *et al.*, 1971). Nevertheless, they are quite stable at low pH values (2–4) and at temperatures of 2–4°C (Bader *et al.*, 1966).

Under these conditions, however, gel electrophoresis cannot normally be carried out because of a marked precipitation of sodium or potassium dodecyl sulphate at temperatures below +15°C (Hokin *et al.*, 1973; Knauf *et al.*, 1974; Schuurmans Stekhoven *et al.*, 1976), although several catalyst systems are known for gel electrophoresis at low pH values (Choules & Zimm, 1965; Narayan *et al.*, 1965; Jordan & Raymond, 1969; Avruch & Fairbanks, 1972; Fairbanks & Avruch, 1972). Even a 1% sodium dodecyl sulphate solution forms a precipitate at pH 2.4 and 2–4°C.

To increase the solubility of dodecyl sulphate at low temperatures, we attempted to substitute another cation for Na⁺; substitution by K⁺ does not increase the solubility of dodecyl sulphate (Hokin *et al.*, 1973).

We found that both Li⁺ and Tris [CH₂OH–C(CH₂OH)₂–NH₃⁺] were suitable cations for carrying out dodecyl sulphate/polyacrylamide-gel electrophoresis at low temperatures and low pH values. In the latter case, Tris acts merely as a counterion, not as a buffer.

Materials and Methods

Materials

Acrylamide and *NN'*-methylenebisacrylamide were purchased from Bio-Rad, Richmond, CA, U.S.A.; dithiothreitol from Calbiochem, San Diego, CA, U.S.A.; lithium dodecyl sulphate from BDH

Chemicals Ltd., Poole, Dorset, U.K.; 2-mercaptoethanol, *NNN'*-tetramethylethylenediamine, ammonium persulphate, Coomassie Brilliant Blue G-250 and Pyronin Y from Serva, Heidelberg, Germany, and all other chemicals from Merck, Darmstadt, Germany. The water was twice-distilled in a quartz apparatus.

Marker proteins. Carbonic anhydrase (carbonate dehydratase; EC 4.2.1.1) was purchased from Serva; catalase (EC 1.11.1.6) from Boehringer, Mannheim, Germany; and β -galactosidase (EC 3.2.1.23) and phosphorylase *a* (EC 2.4.1.1) from Sigma München, Germany.

Electrode buffer. The buffer contained 93.8 mM-citric acid, 12.4 mM-phosphoric acid, 12 mM-Tris and 1.0% lithium dodecyl sulphate.

Gel buffer. Electrode buffer (without lithium dodecyl sulphate) at double concentration was used as gel buffer.

Protein-solubilization solution. The pH 2.4 (pH 7.0) solution contained 2.5 ml of electrode buffer, pH 2.4 (pH 7.0), 1 ml of lithium dodecyl sulphate (10%, w/v) and 1.25 ml of 0.1 M-EDTA. The pH 9.3 solution contained 10 mM-NaOH, 10 mM-dithiothreitol and 1% (w/v) lithium dodecyl sulphate.

Enzyme. Membrane-bound high-affinity (Ca²⁺+Mg²⁺)-dependent ATPase was prepared from human erythrocytes by the method of Wolf (1972).

Myosin. Myosin was prepared by the method of Perry (1955).

Methods

Polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate was performed as described by Weber & Osborn (1969), with gels of 120 nm length and 6 mm diameter.

To prepare gels at pH 2.4, polymerization was achieved by the method of Jordan & Raymond (1969),

by using an appropriate buffer system (McIlvaine, 1921) in which Tris was substituted for Na^+ .

Each 30 ml of gel solution contained the following components (initial concentrations) added sequentially: 1.68 g of acrylamide, 0.063 g of *NN'*-methylene-bisacrylamide, 15.9 ml of water, 15 ml of gel buffer, 3 ml of lithium dodecyl sulphate (10%, w/v), 0.3 ml of ascorbic acid (8.33%, w/v), 0.3 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025%) and 0.3 ml of H_2O_2 (2.5%, w/v). The last three solutions were freshly prepared throughout.

The tubes for gel preparation were filled with about 1 ml of ice-cold 2M-sucrose containing the last three components mentioned above and at the same final concentrations as in the gel. The tubes were filled with the ice-cold gel solution and overlaid with a few drops of the electrode buffer. The gel polymerized at 2°C within 1 h.

After polymerization the gels were turned upside down and were rinsed with electrode buffer to remove sucrose solution. This should be done immediately after polymerization, since the sucrose solution is strongly hyperosmotic and therefore would cause a concave curvature of the surface of the gels during the succeeding hours.

Gels were stored 12–24 h before use. They were pre-run for 2 h at 3 mA/tube (pH 2.4) or 11 mA/tube (pH 7.0) to remove excess catalysts and non-polymerized material (Choules & Zimm, 1965; Tombs, 1965; Hjerten *et al.*, 1965).

The solubilization of human erythrocyte membranes or of the marker proteins was performed by mixing them 1:1 (v/v) with the solubilization solution at pH 2.4 or pH 7.0. To 200 μl of these mixtures was added 10 μl of 2-mercaptoethanol, followed after 15 min by 200 μl of 2M-sucrose (all solutions were at room temperature). A portion (20 μl) of the mixture was carefully layered on top of the tubes, followed by 10 μl of tracking dye (Pyronin Y; 10 $\mu\text{g}/\text{ml}$ in 2M-sucrose). The sample buffer is hypo-osmotic with respect to the electrode buffer, which aids focusing the protein in this layer (Choules & Zimm, 1965); this replaces the spacer gel.

The phosphorylated intermediate of the Ca^{2+} -dependent ATPase, which had been precipitated with 10% (w/v) trichloroacetic acid, was solubilized with 200–500 μl of solubilization solution at pH 9.3. (This slightly increased pH value was necessary to dissolve the trichloroacetic acid-precipitated proteins.) The solution did not clarify completely within 10 min at room temperature, but, considering the pH- and temperature-sensitivity of the acyl phosphate linkage (Bader *et al.*, 1966), this was a tolerable source of error. To 200 μl of the mixture, 20 μl of 2M-sucrose were added and 100 μl of this mixture were carefully layered on top of the tubes as described above.

Electrophoresis was performed for 5 min at 1 mA/tube (pH 2.4) or 3 mA/tube (pH 7.0), and then for 5.5 h at 3 mA/tube (pH 2.4) or 3 h at 11 mA/tube

(pH 7.0), until the tracking dye had reached the distance of 7.3 cm. The temperature was always 2°C.

The gels were cut below the tracking dye. Fixation, staining and destaining of gels were performed as described by Fairbanks *et al.* (1971).

Results and Discussion

The phosphorylated intermediate of the high-affinity ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase of human erythrocyte membranes has already been identified by Drickamer (1975) by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Avruch & Fairbanks (1972). Under these conditions the electrophoresis cannot be carried out below 15°C, since dodecyl sulphate precipitates with Na^+ or K^+ ions at lower temperatures (Hokin *et al.*, 1973; Knauf *et al.*, 1974; Schuurmans Stekhoven *et al.*, 1976). According to Bader *et al.* (1966) the phospho-enzyme bond is most stable at pH 2–4 and low temperatures. Therefore we used a combined citrate/phosphate buffer at pH 2–4, and attempted to work at temperatures around 2°C. To avoid any precipitation of the dodecyl sulphate with the Na^+ ions of the buffer, we had to substitute other cations for Na^+ . K^+ ions are not suitable, because they precipitate dodecyl sulphate more easily than do Na^+ ions. On the other hand, Li^+ as counterion for

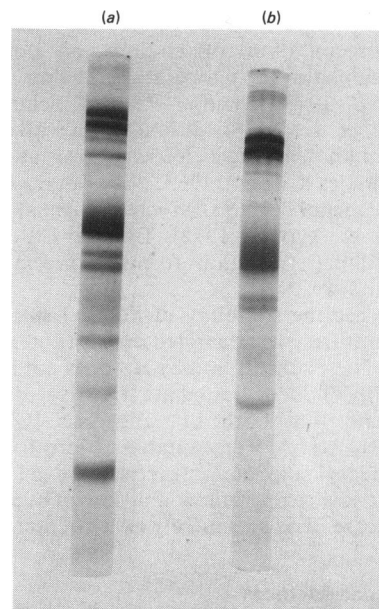


Fig. 1. Dodecyl sulphate/polyacrylamide-gel electrophoresis of human erythrocyte membranes. Gel (a) was prepared as described by Weber & Osborn (1969) at pH 7.0; gel (b) was run at pH 2.4 under the present conditions.

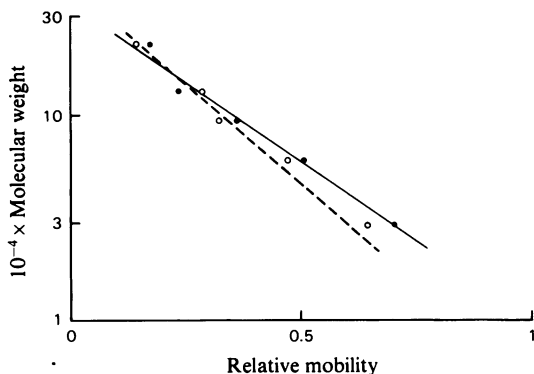


Fig. 2. Calibration curves obtained with various polypeptide chains (myosin, β -galactosidase, phosphorylase a, catalase and carbonic anhydrase) on pH 7 (○) and pH 2.4 (●) gels. The mobility is relative to that of the tracking dye (= 1).

dodecyl sulphate (Noll & Stutz, 1968), and Tris as counterion for the buffer system do not precipitate dodecyl sulphate. In the buffer system we therefore replaced Na^+ by Tris, which does not serve as a buffer at pH 2.4, but only as a counterion.

If the catalyst system of Avruch & Fairbanks (1972) is used at the original concentrations at 2–4°C, the polymerization velocity is far too slow, and this causes an uneven polymerization, so producing curved protein bands. An increase of the concentration of Fe^{2+} or of ascorbic acid by the factor of 10, or the saturation of the ice-cold gel solution with purified N_2 for 15 min sufficiently increases the polymerization velocity. The fastest (1 h) and most even polymerization was obtained after increasing the concentration of the ascorbic acid to ten times that used in the method of Avruch & Fairbanks (1972).

Fig. 1 shows the results of dodecyl sulphate/polyacrylamide-gel electrophoresis of human erythrocyte membranes at different pH values (a, pH 7.0; b, pH 2.4). Generally, the values of the relative mobilities are slightly higher at pH 2.4 than at 7.0. This might be due to a different pH-dependence of the charge of proteins and tracking dye (Pyronin Y).

Additional experiments with the present method were described previously (Wolf *et al.*, 1977). However, in those experiments we used a dodecyl sulphate concentration of 0.2%, which does not lead to a precipitation at 2–4°C. In that case we had to use erythrocyte-membrane proteins of known molecular

weights as calibration proteins, since the molecular weights of erythrocyte proteins obtained at pH 2.4 differed markedly from those reported in the literature after calibration by conventional marker proteins. This effect was abolished when the dodecyl sulphate concentration was increased to 1%. As a consequence we had to replace Na^+ by Li^+ and/or Tris as counterions.

As Fig. 2 shows, the plot of the molecular weights of the marker proteins against their relative mobilities also yields a straight line in the case of dodecyl sulphate/polyacrylamide-gel electrophoresis at pH 2.4. The molecular weights of the erythrocyte-membrane proteins are in good agreement with those reported in the literature. Therefore the present method would appear to be suitable for the identification and estimation of the molecular weights of pH- and temperature-labile intermediates.

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