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A Novel System for the Two-Dimensional Electrophoresis of Membrane Proteins

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Membrane proteins were resolved in two dimensions by a novel technique that uses discontinuous electrophoresis in both directions. After electrophoresis in the first direction in chloral hydrate, the membrane proteins were further resolved by a novel system that used organic-base dodecyl sulphates to stack and then resolve them. This latter system has several advantages over conventional electrophoresis in sodium dodecyl sulphate, notably that it avoids the production of artifacts generated by other systems.

Two-dimensional electrophoresis is becoming an increasingly popular method to improve the resolution of membrane proteins obtainable by one-dimensional electrophoresis in sodium dodecyl sulphate. Ames & Nikaido (1976) have used isoelectric focusing as the additional dimension, a combination that gives very high resolution. Isoelectric focusing has the drawback that microheterogeneity of charge in a membrane glycoprotein can produce bands from a single membrane component (Ames & Nikaido, 1976).

Other workers have sought alternatives to sodium dodecyl sulphate to solubilize the membrane for electrophoresis in the additional dimension. However, these too have disadvantages, since they may fail to solubilize all the proteins in the membrane (e.g. Bhakdi *et al.*, 1975) or fail to convert oligomeric proteins into monomers (e.g. Anselstetter & Horstmann, 1975). Perhaps the best solution so far to this problem is the system of Conrad & Pennington (1976), which uses electrophoresis in sodium dodecyl sulphate in the first dimension followed by electrophoresis in phenol/urea/acetic acid in the second. However, acrylamide cannot be polymerized in the latter solvent, necessitating lengthy soaking of previously prepared gels. Furthermore, discontinuous electrophoresis cannot be performed in this solvent, thus limiting the possible resolution.

The system described in the present paper appears to overcome these disadvantages. The first dimension uses chloral hydrate, which gives full solubilization of the membrane (Ballou *et al.*, 1974), and this is followed by a novel discontinuous system using the dodecyl sulphates of Tris and 4-picoline, which gives good resolution without generating artifacts.

Materials and Methods

Agarose and Tris were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. 3-Bromopyridine, *NNN'*-tetramethylethylenediamine and *NNN'*-tetramethyl-2-thiourea were from Aldrich Chemical Co., Gilling-

ham, Dorset, U.K. Other chemicals were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Pig kidney microvilli were prepared as described previously (Booth & Kenny, 1974). Rabbit skeletal-muscle actin and α -actinin were prepared by the methods of Spudich & Watt (1971) and Goll *et al.* (1972) respectively. Pig kidney dipeptidyl peptidase IV was prepared by Dr. A. J. Kenny as previously described (Kenny *et al.*, 1976).

The dodecyl sulphates of Tris and 4-picoline were prepared by dissolving 0.5 mol of the organic base and 0.5 mol of sodium dodecyl sulphate in 500 ml of boiling ethanol. Concentrated HCl (0.5 mol) was slowly added and the mixture was stirred under reflux for 10 min. After filtering off the precipitated NaCl, the product was crystallized from the filtrate at -20°C .

For electrophoresis the following solutions were prepared. Solution A: 200 g of chloral hydrate and 1.56 ml of triethanolamine in 66.7 ml of 0.6 M-taurine (the pH of this solution was adjusted to 3.0 with lactic acid before making its volume up to 200 ml with water). Solution B: 7 ml of solution A diluted with 3 ml of water. Solution C: 50 g of chloral hydrate and 0.15 ml of 3-bromopyridine in 16.7 ml of 0.3 M-taurine (after adjusting the pH of this solution to 2.8 with lactic acid, its volume was made up to 50 ml with water). Solution D: 0.2 M-taurine in 60 mM-3-bromopyridine adjusted to pH 3.0 with lactic acid. Solution E: 0.2 M-taurine in 56.8 mM-triethanolamine adjusted to pH 3.0 with lactic acid. Solution F: 0.14 M-Tris dodecyl sulphate/2.43 M-Tris base. Solution G: 0.14 M-4-picolinium dodecyl sulphate/0.14 M-4-picoline. Solution H: 70 mM-4-picolinium dodecyl sulphate/70 mM-4-picoline containing 1% (w/v) 2-mercaptoethanol. Solution J: 31.08% (w/v) acrylamide/0.42% (w/v) *NN'*-methylenebisacrylamide. Solution K: 16.0% (w/v) acrylamide/0.43% (w/v) *NN'*-methylenebisacrylamide. Solution L: 0.35 M-boric acid/0.39 M-Tris base/30 mM-Tris dodecyl sulphate. Solution M: 0.18% (w/v) ammonium persulphate (freshly prepared each time).

Electrophoresis in chloral hydrate

Acrylamide (2g) and *NNN'*-methylenebisacrylamide (0.2g) were dissolved in 40ml of solution A. *NNN'*-Tetramethyl-2-thiourea [150 μ l of a 10% (w/v) solution in chloroform] was added and the chloroform was removed under vacuum. After thoroughly gassing this solution on ice with N_2 , 50 μ l of 30% (w/v) H_2O_2 was added and the solution was swiftly transferred via a 5ml syringe, fitted with a 14-gauge needle, to N_2 -filled glass tubes (3mm internal diam.) sealed at their lower ends with Parafilm. After overlaying with solution B previously gassed with N_2 , the gels were left to set for at least 3h.

The sample was prepared by adding 10 μ l of 2-mercaptoethanol to 1–5mg of membrane protein suspended in 200 μ l of water. The volume was adjusted to 1ml with solution C and 0.5ml of chloral hydrate was dissolved in this solution. Pyronine G [50 μ l of a 0.01% (w/v) solution] was added as tracking dye.

When set, the overlay and Parafilm were removed from the gels and their upper surfaces were washed with solution C. The tubes were placed in the electrophoresis apparatus with their lower ends dipping into glass specimen tubes (50mm $\times$ 10mm) containing solution A. The lower reservoir was then carefully filled with solution E. The samples (100 μ l) were loaded on to the gels and overlaid with a mixture of solutions C and D (7:1, v/v), which was in turn overlaid with solution D, the upper reservoir buffer. Electrophoresis, with the cathode as the lower electrode, was carried out at 100V for 2h and then 350V for 18h.

Equilibration of the first-dimension gels

The gels were removed from their tubes and soaked in 5ml of solution H. After 1h the proteins became visible as precipitates within the gels and the best gel was selected for electrophoresis in the second dimension. The solution was changed after 1 and 2h. A total equilibration time of 3h was used.

Electrophoresis in organic-base dodecyl sulphates

Electrophoresis in the second dimension was carried out in an apparatus similar to that described by O'Farrell (1975), using 1.5mm spacers. For 40ml of resolving-gel mixture, 20ml of solution F, 10ml of solution J and 10ml of solution M were mixed and degassed under vacuum. *NNN'*-Tetramethylethylenediamine (50 μ l) was added and the mixture was poured into the gel plate and overlaid with water. When the resolving gel had set, the overlay was removed and replaced with the stacking-gel mixture. This consisted of a mixture of 4ml of solution G, 2ml of solution K, 2ml of solution M and 10 μ l of *NNN'*-tetramethylethylenediamine; the

solution was degassed before use. When this had set, the overlay, again water, was removed and the first-dimension gel, after rinsing with solution H, was laid along the top of the gel plate leaving a gap of about 15mm at either end. It was sealed in place with hot (90°C) 1% (w/v) agarose dissolved in solution H. When the seal had set, rectangular notches were cut in it at the ends of the first-dimension gel. These were filled with 50 μ l of the sealing mixture containing either 50 μ g of membrane protein or marker proteins (10 μ g of each). The gel plate was placed in the electrophoresis apparatus and the reservoirs were filled with electrode buffer (solution L diluted 10-fold). Bromophenol Blue was added to the upper reservoir as tracker dye. Electrophoresis was carried out at 40V for 18h (anode as lower electrode). The gels were stained as described previously (Kenny *et al.*, 1976).

Results and Discussion

First dimension: electrophoresis in chloral hydrate

The procedure used for the first-dimension separation was modified from that of Ballou *et al.* (1974), who showed that chloral hydrate, buffered with bromopyridinium lactate, is capable of completely solubilizing the proteins of various membranes. Furthermore, when electrophoresed in this solvent, the proteins separated according to size and charge. Unlike phenol in urea and acetic acid, chloral hydrate permits the polymerization of acrylamide, thus simplifying the preparations of polyacrylamide gels containing this solvent. However, membrane proteins, after electrophoresis by the method of Ballou *et al.* (1974), have low mobilities relative to the dye front. For two-dimensional electrophoresis it is desirable to have the proteins spread along the length of the first-dimension gel. In the present method this was achieved by incorporating a discontinuous buffer system, by using triethanolamine, whereby the mobilities of the proteins may be controlled. Their mobilities increase (but their solubility falls) as the pH value of the gel buffer is increased.

In the system described, the proteins run in 100% (w/v) chloral hydrate buffered with 50mM-3-bromopyridine/0.283M-lactic acid, pH 2.9.

Second dimension: electrophoresis in organic-base dodecyl sulphates

The pK_a of 3-bromopyridine is 2.84, a value close to the pH of the first-dimension gel. This buffer, which is present at a relatively high concentration, must be either removed or overwhelmed before electrophoresis in the second dimension. Complete removal of this buffer is difficult and it is sufficient to overwhelm it by soaking the first-dimension gel in a

EXPLANATION OF PLATE I

Electrophoresis of kidney-microvillus proteins

(a) Electrophoresis of kidney-microvillus proteins (300 μ g) in the system of Davis (1964) with 0.1% (w/v) sodium dodecyl sulphate in all buffers. The sample contained 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercapto-ethanol. Electrophoresis in the second dimension was carried out in organic-base dodecyl sulphates as described in the Materials and Methods section. A first-dimension gel is shown at the top. Note the step in the diagonal pattern; this indicates that the band arrowed in the first-dimension gel is an artifact composed of several unresolved proteins. These proteins are resolved by electrophoresis in organic-base dodecyl sulphates. The left-hand strip of the gel shows the electrophoresis of the membrane protein in the second dimension only. (b) Electrophoresis of kidney-microvillus proteins (300 μ g) in chloral hydrate, followed by electrophoresis in the second dimension in organic-base dodecyl sulphates. The left-hand strip of the gel shows the electrophoresis of the membrane protein in the second dimension only and the right-hand strip shows the subunit mol. wt. markers, which are dipeptidyl peptidase IV (130000), α -actinin (95000) and actin (42000). Both two-dimensional gels were dried before being photographed.

dodecyl sulphate-containing buffer. In early experiments, the sodium dodecyl sulphate system described previously (Kenny *et al.*, 1976) was used. However, this system, which is a modification of the continuous electrophoretic system of Weber & Osborn (1969), was found to give too small a voltage gradient to remove the proteins from the highly-cross-linked first-dimension gel without trailing. Clearly, the steeper voltage gradient generated by a buffer system was required. Unfortunately, all the discontinuous electrophoretic systems containing sodium dodecyl sulphate that have been published to date (e.g. Laemmli, 1970; Neville, 1971) stack proteins from solutions of very low buffering capacity. Furthermore, when used with highly-cross-linked spacer gels, these systems may produce artifactual bands if the sample contains a high concentration of dodecyl sulphate. Although this phenomenon is not as yet fully understood, it seems to be connected with the fact that these systems generate two moving boundaries, one at the leading-ion/dodecyl sulphate boundary and the other at the dodecyl sulphate/trailing-ion boundary. Electrophoresis in organic-base dodecyl sulphates eliminates the first of these boundaries, by using dodecyl sulphate as the leading ion, and as a consequence eliminates the artifact. Plate 1(a) shows that electrophoresis in organic-base dodecyl sulphates can separate the proteins unresolved by electrophoresis in the system of Davis (1964) modified by the addition of 0.1% (w/v) sodium dodecyl sulphate to all buffers.

In designing the system to eliminate this artifact, the theory of electrophoresis in discontinuous buffers developed by Ornstein (1964) and Jovin (1973a,b,c) was particularly valuable. A consequence of Jovin's (1973) eqn. (26) is that the stacking efficiency at a moving boundary is indirectly proportional to the square-root of the antilogarithm of the difference between the pK_a values of the trailing ion and the counterion. This fact has been used to achieve stacking of protein-dodecyl sulphate complexes by a novel method. With dodecyl sulphate as the leading ion, borate (pK_a 9.24) was chosen as trailing ion. The resolving gel contains Tris (pK_a 8.08) as counterion, which is replaced in the stacking gel by 4-picoline (pK_a 6.02). This substitution has the effect of increasing the stacking efficiency in the stacking gel to such an extent that the protein-dodecyl sulphate complexes stack adequately when the gel is buffered at pH 6.02. Thus the sample is stacked from a maximally buffered solution, a condition required for two-dimensional electrophoresis as described above. Furthermore, the protein-dodecyl sulphate complexes are stacked from a solution containing 70 mM-dodecyl sulphate, even in highly-cross-linked gels, without producing artifacts.

The system described stacks at about pH 8.3 and resolves at about pH 9.5. It gives good resolution of

proteins of mol.wt. greater than 15000, but smaller proteins run with the dye front. However, the relative mobilities of the proteins may be controlled by either altering the acrylamide concentration in the resolving gel or by altering the pH value of the resolving gel buffer. In the latter case it should be noted that a decrease in the pH of this buffer increases the relative mobilities of the proteins.

Two-dimensional electrophoresis of kidney microvillus membrane proteins

Plate 1(b) shows the result of electrophoresis in chloral hydrate followed by electrophoresis in organic-base dodecyl sulphates in the second dimension. The sample contained 300 μ g of kidney microvillus proteins. More than 30 spots were visible at this loading. Several bands visible on the one-dimensional reference strip were resolved further after two-dimensional electrophoresis. Both the resolution and the spread of the proteins obtained by this method are intermediate between those obtained by the electrophoretic method of Conrad & Pennington (1976) and the isoelectric-focusing method of Ames & Nikaido (1976). The reproducibility does not depend on the composition of any batch of chemical, unlike isoelectric focusing in which the final pattern depends on the composition of the ampholytes used. Thus the method described above should find applications in the 'mapping' of membrane proteins as an alternative to the method of Ames & Nikaido (1976).

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