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Fractionation of the Insoluble Brain Proteins with Acrylamide Electrophoresis[1]

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Polyacrylamide gel electrophoresis as originated by Ornstein and Davis (1, 2) and Raymond (3) is an excellent method for the fractionation of soluble proteins. On the other hand, the separation of membrane proteins poses considerable problems because of their low solubility in water and because of their large particle size. In this respect the following agents have been employed to solubilize or disaggregate complex proteins for acrylamide electrophoresis: urea (4–6), Triton X-100 (7, 8), sodium dodecylsulfate (9, 10), phenol (11, 12), and β-mercaptoethanol (7–10). However, careful evaluations should be made before applying such procedures to the insoluble proteins of the brain because of the complexity of membrane organization and the high lipid content in this organ.

Several methods for fractionating insoluble brain proteins with acrylamide electrophoresis have been described. That of Cotman and Mahler (13, 14) uses phenol, acetic acid, water, and urea. That of Mehl (15) uses phenol, formic acid, and water. A method devised by Shooter (16) and his associates consists of solubilizing the proteins with Triton X-100, which is subsequently replaced by sodium dodecylsulfate before electrophoresis. In this report we evaluate the effects of the various solubilizing agents and the gel conditions, either singly or in combination, on the acrylamide electrophoretic pattern of the insoluble brain proteins. The resulting technique is mainly a variation of the conditions prescribed by Neville (17) originally employed for the plasma membranes of the liver cells.

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METHOD

Protein pellets are dissolved in a "sample solvent" consisting of 50 m*M* K_2CO_3, 8 *M* urea, 10% (v/v) mercaptoethanol, and 5% Triton X-100. Separation is effected by electrophoresis in polyacrylamide gels containing 5 *M* urea and 0.25% Triton X-100 under a discontinuous acidic pH. The complete ingredients of the stacking (upper) part and the separation (lower) part of the gel, together with the procedure to prepare them, are presented in Table 1. Each gel segment is layered with water and photopolymerized with a 30 W daylight fluorescent lamp for 1 hr. The upper electrode buffer (pH 4.0) contains 110 m*M* glycine, 16 m*M* acetic acid, and 5 *M* urea. The lower buffer (pH 2.7) contains 4.6 *M* acetic acid and 60 m*M* KOH. After layering the sample and the addition of the tracking dye (methyl green), electrophoresis is conducted at room temperature with the protein bands migrating toward the cathode, using a current of 0.75 mA for stacking and 1.5 mA for separation for each cylindrical gel of 5 mm diameter. This procedure differs from Neville's (17) in the fol-

TABLE 1

Ingredient	Small-pore gel solution (pH 2.7) Amt. used	Final concn. in gel	Large-pore gel solution (pH 5.9) Amt. used	Final concn. in gel
	(A)		(A)	
Acrylamide	7.00 gm	7.00%	1.75 gm	1.75%
BIS	0.20 gm	0.20%	0.20 gm	0.20%
10 *M* acetic acid	5.80 ml	0.58 *M*	0.65 ml	65.00 m*M*
1 *N* KOH	0.75 ml	7.50 m*M*	6.00 ml	60.00 m*M*
Urea	30.00 gm	5.00 *M*	30.00 gm	5.00 *M*
25% Triton X-100	1.00 ml	0.25%	1.00 ml	0.25%
Water	to 90.00 ml	-	to 90.00 ml	—
	(B)		(B)	
Riboflavin	2.00 mg	4×10^{-4} %	2.00 mg	4×10^{-4} %
TEMED	0.40 ml	8×10^{-2} %	—	—
Water	to 50.00 ml	—	to 50.00 ml	—
	(B′)		(B′)	
Ammonium persulfate	25.00 mg	5×10^{-2} %	25.00 mg	5×10^{-2} %
Solution B	to 5.00 ml	-	to 5.00 ml	—

Solutions A and B are stocks. Solution A is kept at room temperature and does not deteriorate for at least 3 weeks. Solution B is kept at 4°. Solution B′ is made up before each use. The final gel solution consists of 9 parts A and 1 part B′.

lowing respects. Instead of dissolving proteins first in K_2CO_3, we dissolve the pellet directly in a solution containing all the ingredients mentioned above, including the Triton. In this way not only is a greater portion of protein solubilized, but also more bands are observed in the gel. Up to 10 mg of membrane protein can be dissolved in 1 ml of the solution, as is evident by the clarity of the mixture and the lack of visible pellet after high-speed centrifugation. We incorporate 0.25% Triton into both parts of the gel (stacking and separation), whereas the urea concentration in the gel is reduced. The low urea concentration facilitates handling of the gel solutions, since gel solutions containing 7–9 *M* urea frequently solidify even at room temperature. The presence of urea in the upper buffer is necessary for good results, although its concentration can be lowered from 5 to 3 *M* in order to ensure a density difference between the upper buffer and the sample solution to be layered. The presence of Triton promotes gelation and also enhances the separation of bands. Too much Triton can cause the protein to streak and the gel to slip out of the tube during electrophoresis.

RESULTS AND DISCUSSION

The protein pellet obtained after an exhaustive extraction of a goldfish brain homogenate was used to demonstrate the effectiveness of the method. When the pellet was solubilized and fractionated by the procedure described, as many as 30 protein bands were observed (Fig. 1, lower portion, gel F). The bands were sharply delineated and widely distributed throughout the entire gel. No bands could be seen when the same sample was applied to the standard alkaline gel system of Davis (2) (Fig. 1, upper portion, gel F). Figure 1 further shows that, at the various extraction steps, much protein that would have been excluded from the gel when using the Davis system was fractionated by our present procedure (gels A to C). The freeze-thawing and the sonication steps were essential for obtaining good separation of fraction F.

Several of the present conditions were altered to determine their importance for optimal separation of the protein bands. Figure 2 shows that the protein pattern having the greatest number of bands was obtained only in an acidic gel and only when all the ingredients were present (Fig. 2, J). When compared with related methods, such as that of Neville (17), that of Takayama *et al.* (12), or the procedure of Cotman and Mahler (13, 14) (Fig. 2, I, K, L, respectively), the technique we described still yields the best result for the fish brain. The last cited method has been used for brain proteins (13, 14) but in our experience it carries some obvious drawbacks: (1) the protein bands are usually ill-defined and the background hazy; (2) a prolonged soaking step is necessary to incorporate phenol into the gel; (3) a discontinuous buffer

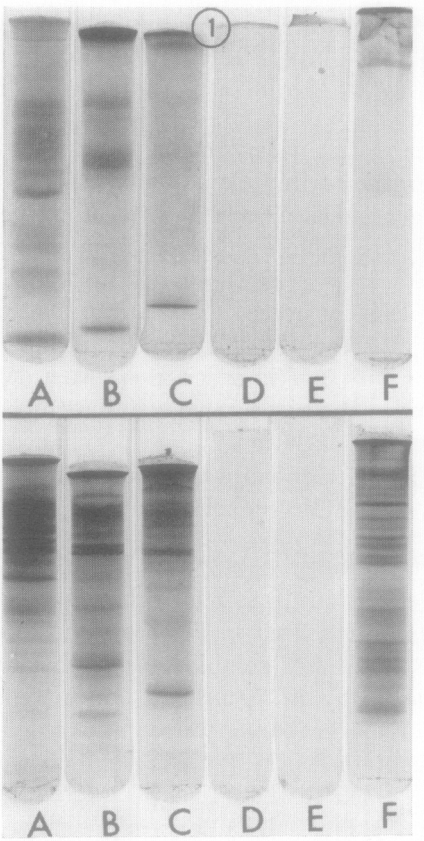

FIG. 1. Electrophoretic patterns of protein fractions obtained from a sequential extraction procedure. A 10% goldfish brain homogenate in isotonic saline was spun at 100,000*g* for 30 min to obtain a supernatant fraction (A) and a pellet, which was subsequently extracted with water by similar centrifugations after freeze-thawing (B) and sonification (C). Thorough washing was carried out after each extraction. The residue was again extracted twice with water (D and E) in order to ensure the complete removal of water-soluble material, and the remaining pellet, taken as "insoluble brain protein," was then dissolved in the "sample solvent" as described in the text (F). The amounts of protein extracted in the various steps, expressed as the percentage of the total in the homogenate, were 40, 15, 15, 0.1, 0.05, and 30, in that order; and the amounts applied to the polyacrylamide gels were 500, 500, 500, 10, 10, and 500 μg, respectively. Protein patterns in the upper half of the figure were obtained according to the usual conditions of Davis (2), whereas those in the lower half were achieved by the authors' method of sample solubilization and electrophoresis. The same amount of protein was processed under both conditions in gels having the same alphabetical designation. All gels were stained with 1% amido black in 7% acetic acid and destained by soaking in several changes of 7% acetic acid.

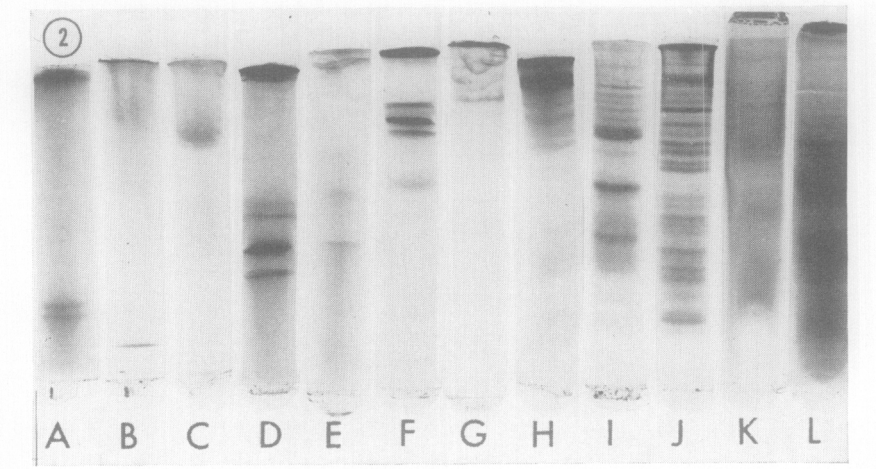

FIG. 2. Comparison of various methods for the fractionation of insoluble brain proteins. The same amount (500 μg) of the insoluble brain protein (see Fig. 1) was extracted by the different methods for protein solubilization and fractionated by polyacrylamide gel electrophoresis under various conditions. In the following system of designation, letters to the left of the bar indicate the type of solution used for extraction, and those to the right denote the ingredients added in the gel (in both the stacking and the separation parts). Unless otherwise stated, in the extraction solution, T = 5% Triton X-100, U = 8 *M* urea, P = 50 m*M* potassium carbonate, M = 10% (v/v) mercaptoethanol; in the gel, T = 0.5% Triton X-100, U = 5 *M* urea. The pH in gels A to G was the discontinuous alkaline system of Davis (2). That in H to J was the discontinuous acidic system of Neville (17). (A) T/T; (B) U/U; (C) TU/TU; (D) TUP/TU; (E) TUM/TU; (F) TUMP/TU; (G) TUMP/no addition; (H) TUMP/T; (I) TUMP/7 and 9 *M* urea in stacking and separation parts, respectively; (J) TUMP/0.25% Triton X-100 plus 5 *M* urea; (K) the extraction and gel system of Takayama *et al.* (12); (L) the extraction and gel system of Cotman and Mahler (13, 14). The conditions for gel I were the original specifications of Neville (17), those for gel J were authors' modifications, which were identical with those for gel F in the lower part of Figure 1. Gel G is the same as gel F in the upper part of Figure 1. All gels were stained and destained as in Figure 1.

system cannot be employed. The use of solubilizing agents compatible with the polymerization step and with the formation of a discontinuous buffer system is a distinct advantage in our method as well as in Neville's (17).

In order to demonstrate the reproducibility of the band pattern, protein samples were simultaneously electrophoresed in a gel slab system. The gel was cast in the apparatus previously described (18) using the reagents for the current method, with the following exceptions. The ammonium persulfate in the stacking gel was raised to 0.10% to obtain better sample slots (alternatively the monomer concentration could be

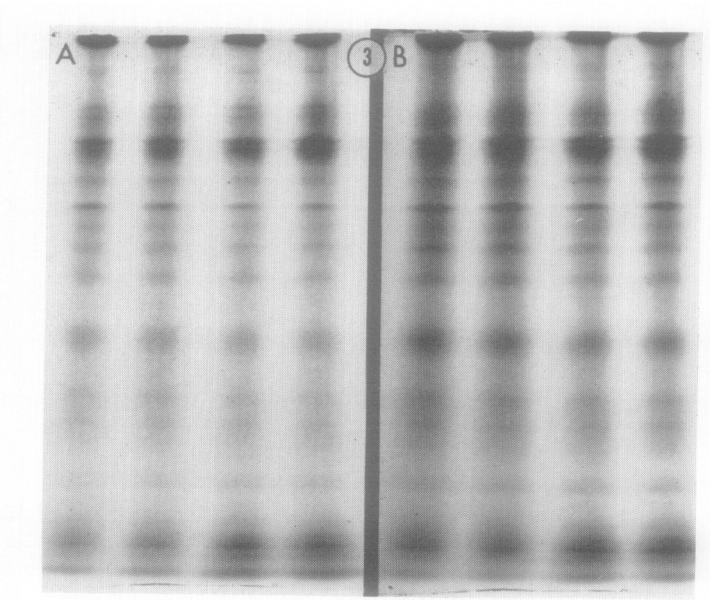

FIG. 3. Replicability of protein patterns in an acrylamide gel slab. (A) The samples used for the two left slots were duplicates taken from a single pool (20 fish) of insoluble brain protein. Those for the two right slots were from another pool of 20 fish treated similarly. The gel system used for J in Figure 2 was cast in the gel slab apparatus described earlier (18). The procedure used for obtaining insoluble protein in this experiment was different from that described in Figures 1 and 2. Instead of subjecting to stepwise centrifugations and washings, the entire homogenate was freeze-thawed and sonicated, and then centrifuged. The pellet obtained was thoroughly washed and solubilized. 500 μg of this protein was applied to each slot. The gel was stained with amido black as with the cylindrical gels. (B) The same piece of gel stained with Coomassie brilliant blue according to the method of Fazekas de St. Groth (see Chrambach) (19) for the intensification of bands.

increased). Since an edge effect was sometimes encountered with this gel system when used in the slab form, wedge-shape spacers were used to increase the width of the front as it traversed downward. Figure 3 shows that not only are protein patterns similar between duplicate samples taken from the same brain pool, but also they are comparable between different groups of animals when treated similarly. Such high degree of reproducibility makes the method suitable for rigorous comparison of individual protein bands.

SUMMARY

A method for fractionating the insoluble proteins of the brain with polyacrylamide electrophoresis is described. The procedure consists of dissolving the membrane proteins in a solution of urea, alkali, mercapto-

ethanol, and Triton, and then conducting the separation in gels containing urea and Triton in discontinuous acidic buffers. As many as 30 sharp bands are discernable in the gel out of an insoluble brain fraction. The reproducibility of the band pattern is demonstrable in a gel slab system.

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