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A rapid method for preparation of sarcolemma from frog skeletal muscle

S. I. M. ZAIDI, R. N. PANDEY, A. M. KIDWAI and C. R. KRISHNA MURTI

Industrial Toxicology Research Centre, Lucknow 226 001

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Abstract. A rapid method for the preparation of sarcolema from frog skeletal muscle has been described. The purified cell segments were transparent and devoid of contractile material. The Na^+, K^+ -ATPase and 5'-nucleotidase activities in sarcolemma purified by this method were comparable to those reported for sarcolemmal preparations purified by density gradient centrifugation. The preparation also possessed acid phosphatase, alkaline phosphatase and K^+-activated, ouabain-sensitive p-nitrophenyl phosphatase activities. The cholesterol to phospholipid ratio of the sarcolemma was 0.33, indicating its high purity; further, the preparation was free from mitochondria and contractile proteins.

Keywords. Skeletal muscle; plasma membrane; sarcolemma.

Introduction

Skeletal muscle sarcolemma can be prepared from rat (Kono and Colowick, 1961; McCollester, 1962; Kono *et al.,* 1964; McCollester and Semente, 1964; Rosenthal *et al.,* 1965; Peter, 1970; Kidwai *et al.,* 1973) and frogs (Koketsu *et al.* 1964; Abood *et al.* 1966; Boegman *et al.,* 1970; Narhara *et al,* 1979) by a variety of techniques. Among the more extensively used procedures are the ones which involve prolonged extraction with aqueous salt solutions and drastic homogenization to obtain vesicles of different sizes and separation of the latter by density gradient centrifugation.

Prolonged extraction with aqueous salt solutions results in significant denaturetion rendering the preparations unfit for enzymatic studies. When membranes are prepared on the other hand, by drastic homogenization, contamination by intracellular membranes arising out of endoplasmic reticulum or mitochondria is unavoidable. Further more, during homogenization depending upon the composition of the medium, the sarcolemmal vesicles are turned "inside out" or remain "outside out" (Steck *et al,* 1970).

A method is described in this paper which, within 5 h, yields hollow sarcolemmal tube-like structures with good marker-enzyme activities. The method named as "toluene-lithium bromide method" takes advantage, for the first time, of the

property of toluene to increase permeability of muscle cells of frog and shorten the time of release of intra-cellular material.

Materials and methods

Adult frogs (*Rana tigrina*) were purchased from local suppliers and kept in a pond for a few days before use. All the operations were carried out between 0-5°C, unless otherwise specified.

Chemicals

Tris, RNA, DNA, bovine serum albumin and enzyme substrates were from Sigma Chemical Co., St. Louis, Missouri, USA, Sucrose (AR) from Polypharma, Bombay, lithium bromide from Loba Co., Bombay and toluene from British Drug House Ltd., Bombay. All the reagents were of Analytical grade and of the highest purity available.

Light microscopy

The preparations were observed with a phase contrast Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan). A 0.1% solution of Janus green was used for staining cell segments during the extraction.

Enzyme assays

5'-Nucleotidase (E.C.3.1.3.5) was assayed using glycine-NaOH buffer (pH 9.0), 100 mM; $MgCl_2$, 5 mM; AMP, 5mM;in a reaction mixture of final volume 1 ml. A suitably diluted sarcolemmal preparation was added and incubated for 15 min at 37°C. The reaction was stopped by adding 1 ml cold, 10% trichloroacetic acid, the mixture centrifuged and inorganic phosphate determined in the supernatant by the method of Fiske and Subba Row (1925). Na^+, K^+-ATPase was determined in Tris-HCl buffer (pH 7.5), l00 mM; NaATP, 3 mM; $MgCl_2$, 3mM; NaCl, l00mM; KCl, l0mM and suitably diluted membrane preparation in a total volume of 1 ml. After incubation at 37°C for 30 min, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The tubes were kept cold and centrifuged to sediment protein and the supernatant was used for phosphate determinations. Ouabain (1mM) was used to study the inhibition of Na^+, K^+-ATPase.

Phosphomonoesterases (E.C.3.1.3.1) and (E.C.3.4.1.1) at acidic and alkaline pH (5.5 and 7.5) were assayed using *P*-nitrophenyl phosphate as substrate as described earlier (Kidwai *et al.* 1971). Succinic dehydrogenase (E.C. 1.3.99.1) was assayed by the ferricyanide method (Rodriguez De Lores *et al.* 1962) and myosin ATPase (E.C.3.6.1.3) was extracted and assayed by, the method of Perry (1955).

Preparation of the muscle cell fragments

Adult frogs (*Rana tigrina*) were stunned, decapitated and the muscles from hind limbs were removed and kept in ice-cold 50 mM calcium chloride (pH 7.0); pH of the solution was adjusted with 100 mM sodium bicarbonate. Connective tissue and nerves were dissected out. About 50 g of muscle were minced and homogenized for one min in a Sorvall omni-mixer at a speed setting of 240. The

homogenate was filtered through a filter described earlier by Kidwai (1978). The residual tissue was rehomogenized, filtered and the muscle cell fragment suspensions pooled and centrifuged at 70 *g* for 5 min in the cold. The sediment was resuspended in ice-cold 0.25 M sucrose (pH 7.0, adjusted with Tris). The absorbance of this suspension was adjusted to a reading of 160 using a red filter in a Systronic Colorimeter. Under phase contrast, the cell segments were dark and of different sizes.

Toluene treatment

Five per cent toluene (v/v) was added to the cell suspension and gently mixed by tilting for exactly 3 min and allowed to settle in the cold for 5 min. The supernatant was decanted and the cell suspension centrifuged at 70 g for 5 min.

Lithium bromide treatment

The sediment was suspended in 150 ml of 0.4 M lithium bromide, pH 8.4, adjusted with 1 M Tris. This suspension was stirred at low speed in the cold for 90 min. The material was centrifuged at 900 *g* for 5 min, the sediment suspended in lithium bromide and stirred for the same period, and once again centrifuged. The resulting sediment was resuspended and stirred for a period of 120 min. The final sediment was suspended in 0.25 M sucrose the pH adjusted to 7.0 by Tris, centrifuged at 900 *g* for 5 min, the sediment resuspended in 0.25 M sucrose and used for enzymatic and other studies (figure 1).

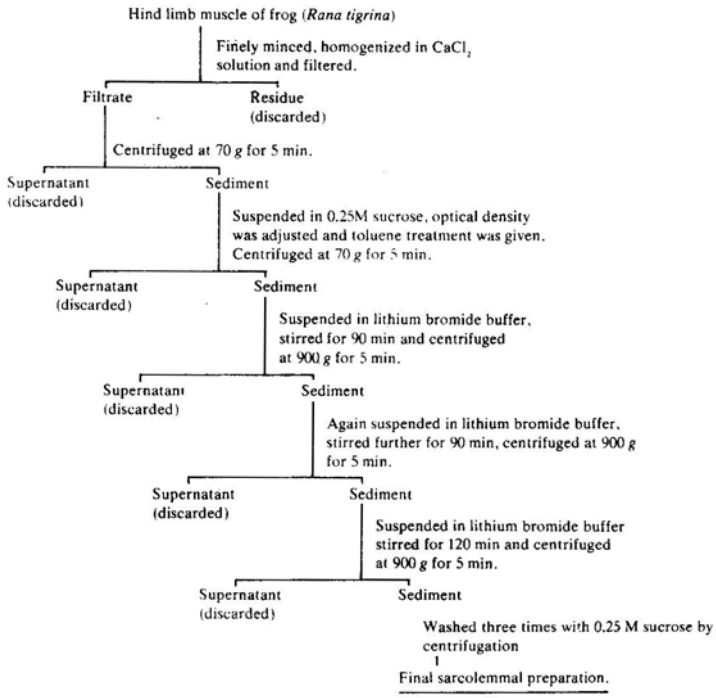

Figure 1. Isolation procedure of frog skeletal muscle sarcolemma.

Light microscopy

Cell segments were examined under phase contrast microscope and also by staining with 0.1% Janus green. Figure 2A is a representative phase contrast microscopic picture of a frog muscle cell segment just after homogenization. Cell segments of various sizes were present and all of them were long, cylindrical and broken at the ends. Very little unidentified material was present. Figure 2B is an

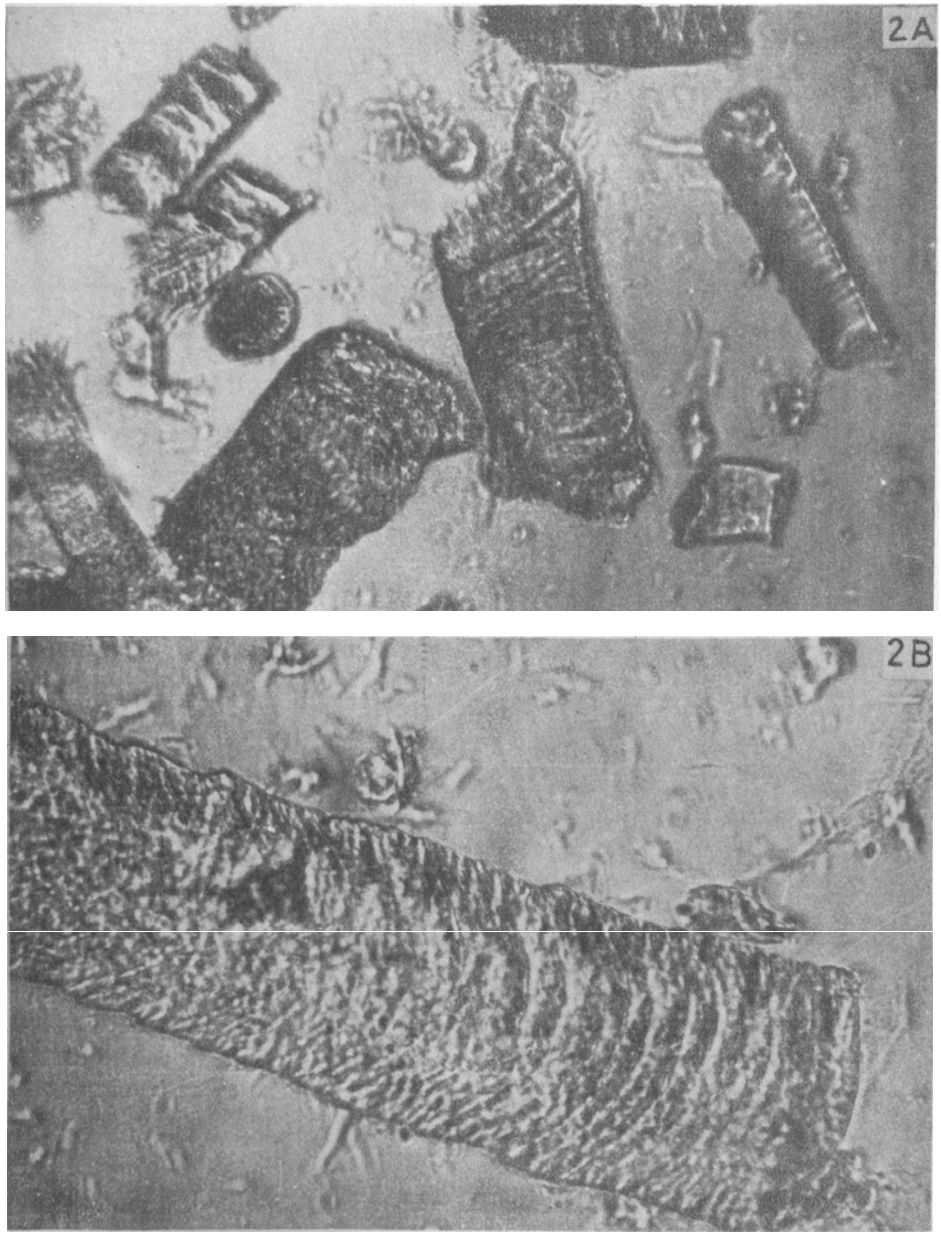

Figure 2. A. Phase contrast photomicrograph of cell segments as seen after homogenization and adjusting the absorbance before toluene treatment (thickness 81 μ). B. A single cell segment after enlargement (thickness 81 μ).

enlarged single cell segment. The cross striations were prominent and the cell segment was dark in appearnce. These cell segments were stained heavily by Janus green. During the first hour of extraction (figure 3A, B), the intracellular

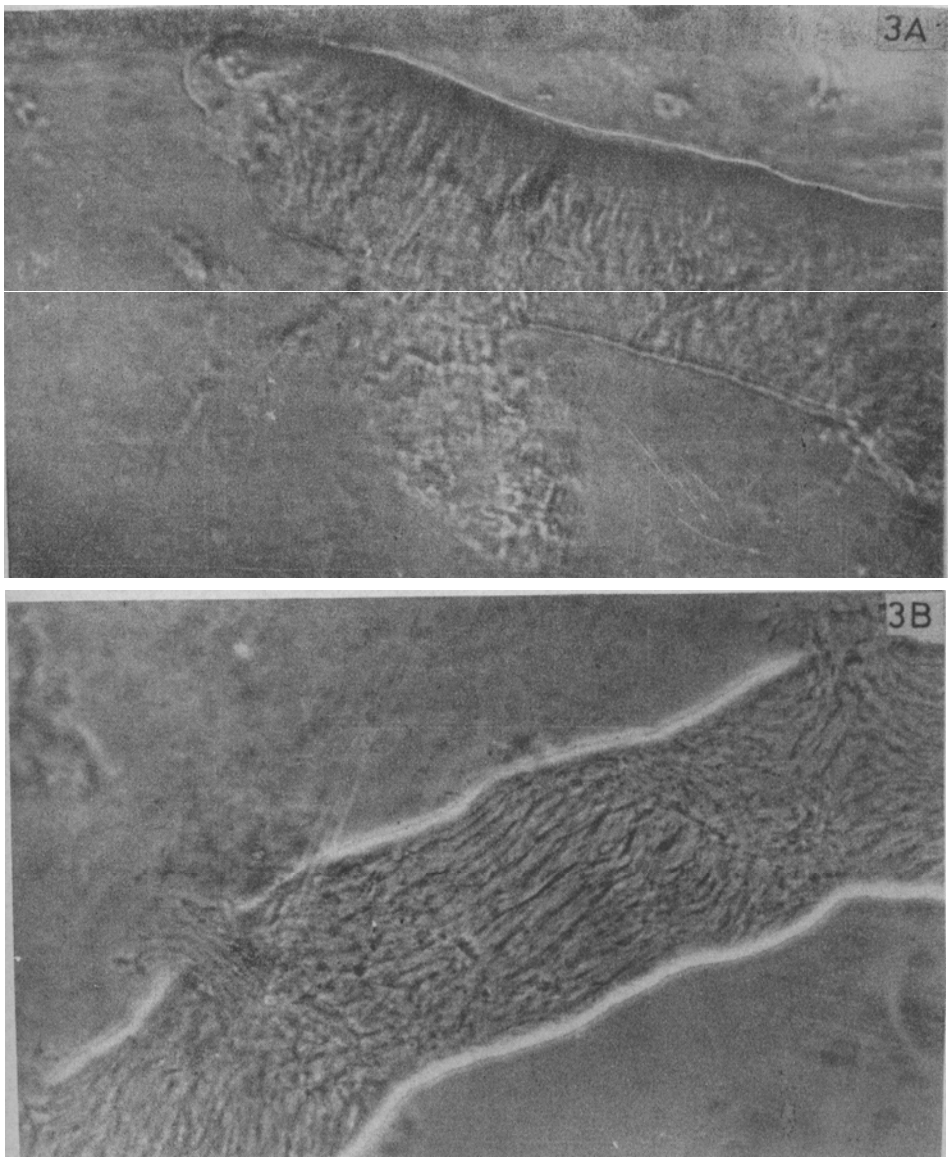

Figure 3. A. Phase contrast photomicrograph of a single cell segment after toluene treatment and immediately after starting extraction with LiBr (thickness 73 μ). B. Protein coming out from the side holes made by toluene (thickness 75 μ).

material started coming out of the holes created by toluene. After 2 h of lithium bromide extraction, the cross striations disappeared and in some cell segments the vicinity of the toluene hole was lighter (figure 4). The intracellular material was seen coming out from the ends of the cell segments as well.

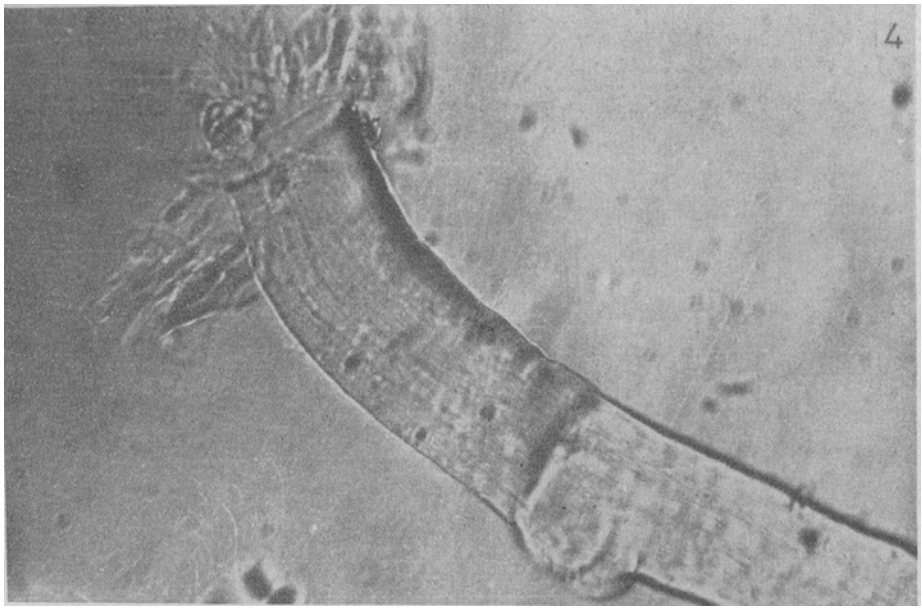

Figure 4. Phase contrast photomicrograph of a single cell segment after 2 h extraction (thickness 54 μ).

At the end of the lithium bromide treatment, the cells were not dark any more (figure 5A, B, C) and gave a cellophane-tube like appearance with one or more holes on the side of these cell segments. The cellophane-tube like structures were not stained by Janus green. The diameter of the sarcolemonal tubes was approximately 80 μ.

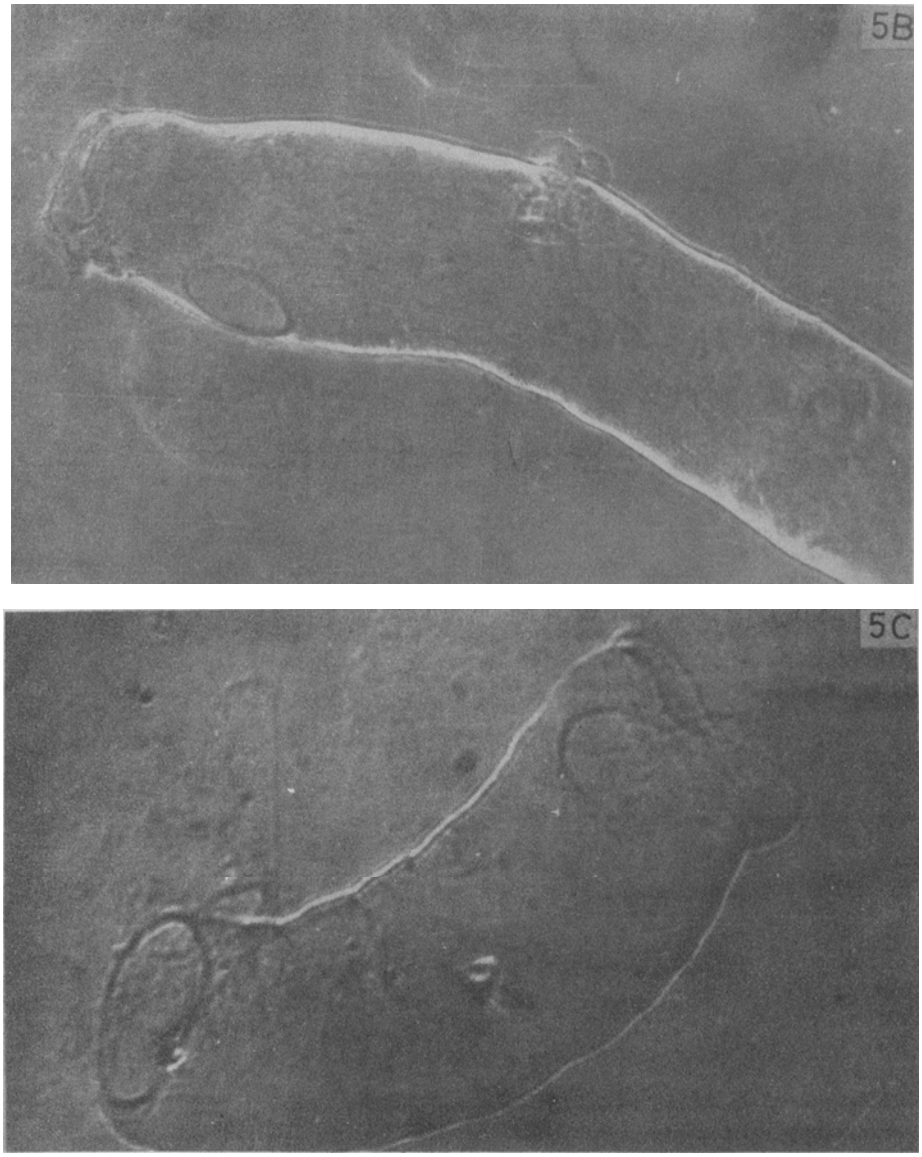

Figure 5. A. Phase contrast photomicrograph of empty cell segments after complete extraction (thickness 76 μ). B-C. Single segment showing holes clearly.

Results

Yield of sarcolemma

The yield of sarcolemma was equivalent to 2 mg protein from 100 mg protein of the homogenate consisting of cell segments. About 0.3 mg protein was recovered from 1 g wet weight of muscle.

Chemical composition

Table 1 indicates the cholesterol, phospholipid RNA and DNA contents of the sarcolemmal preparation.

Table 1. Chemical composition of frog skeletal muscle sarcolemma.

Chemicals	µmol/mg protein	
Cholesterol	0.25±0.092	(4)
Phospholipid	0.85±0.17	(4)
Cholesterol: phospholipid ratio	0.33±0.94	(4)
RNA-phosphorus	0.28±0.09	(4)
DNA-phosphorus	0.12±0.05	(4)

Nucleic acids are expressed as µmol of nucleic acid inorganic phosphate per mg protein, µg lipid phosphate × 25 = µmol phospholipid.

Number in parentheses are the numbers of experiments performed on separate preparations.

Salt-soluble proteins

When purified sarcolemma was treated wih 1 M NaCl for 20 min with constant stirring and centrifuged, no protein could be recovered in the supernatant. However, KCl (100 mM) extracted about 17% of the protein from the preparation.

Deoxycholate treatment

The purified sarcolemma was treated with 0.5% deoxycholate for 20 min with occasional mixing. About 43% of the membrane protein was solubilized and the membrane looked more transparent under light microscopy. Besides holes created by toluene were more clearly visible.

Enzyme markers

In table 2, are given specific activities of Na^+, K^+-ATPase. K^+-activated *p*-nitrophenyl phosphatase (sensitive to ouabain), Mg^{2+}-ATPAse, acid and alkaline phosphatase. Succinic dehydrogenase activity of the preparation was negligible (specific activity 0.02), Myosin ATPase was not detectable suggesting that our preparation was free from mitochondria and contractile proteins.

Table 2. Specific activities of enzymes of frog skeletal muscle sarcolemma.

Enzyme	Specific activities
Mg^{+2}-ATPase	83.52 (6)
Mg^{+2}, Na^{+}, K^{+} [a]	95.06 (6)
Mg^{+2}, Na^{+2} K^{+}, ouabain (1 mM)	84.66 (6)
5'-Nucleotidase	2.96 (6)
K^{+}-Activated *p*-nitrophenyl phosphatase	0.11 (6)
p-Nitrophenyl phosphatase (alkaline)	2.26 (6)
p-Nitrophenyl phosphatase (acidic)	5.90 (6)
Succinic dehydrogenase	0.02 (6)
Myosin ATPase	0.00 (6)

[a] Specific activities are expressed as μmol phosphorous released/mg protein/h.

Specific activites of ATPase and 5'-nucleotidase are expressed as μ mol phosphorus released per mg protein per h at 37°C. Specific activites of phosphatases are expressed as μmol of *p*-nitrophenyl phosphate hydrolyzed per mg protein per h at 37°C. and the specific activity of succinic dehydrogenase expressed as change in absorbance (due to reduction of ferricyanide) per mg protein per h at 30°C. Number in parenthesis is the number of experiments performed on separate preparations.

Discussion

Earlier methods of isolation of skeletal muscle sarcolemma required extraction for 16-24 h which was followed by drastic treatments. In the present method, the cells were made permeable to charged ions and large molecules by the use of toluene. Toluene is known to make membranes permeable in bacteria, nuclei, hepatocytes and mitochondria (Jackson and DeMoss, 1965; Serrano *et al.* 1973; Hilderman and Deutscher, 1974; Matlib, *et al.* 1979). The muscle cell segments were made permeable to lithium bromide presumably by the removal of sarcolemmal patches from the cell segments. Protein emerged from the ends and also from the holes created by toluene. At the end of the extraction period, the cell segments could not be stained by Janus green. The diameter of the tube-like structure was 100 μ as reported by Koketsu *et al.* (1978).

The preparation retained enzymes known to be present in the plasma membrane from various sources. The activity of Na^{+}, K^{+}-ATPase was very similar to the value reported by Narhara *et al.* (1979) using density gradient centrifugation. The specific activity of 5'-nucleatidase in the present preparation was 3.0 compared with a value of 5.0 reported in rat skeletal muscle (Kidwai *et al,* 1973).

Another criterion for the purity of plasma membrane is a high cholesterol phospholipid ratio. The present preparation had a cholesterol: phospholipid ratio of 0.33. In rat skeletal muscle preparations the values were in the range of 0.11-0.24, (Ashworth and Green 1966; Kidwai *et al,* 1973). The amount of DNA was negligible but a significant amount of RNA was found in agreement with the results

as reported by others (Benedetti and Emmelot, 1968). The yield of sarcolemma was much higher than obtained by earlier methods (Kono and Colowick, 1961; Peter, 1970; Kidwai *et al.* 1973), Toluene treatment did not appear to have damaged the surface membrane except to remove patches of membrane and leaving holes.

The method described in this paper has the advantages of yielding sarcolemmal tubes, retaining the shape of the outer membrane with the enzyme activities intact in comparison to earlier preparations (Wattiaux *et al,* 1971).

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