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Chemical Composition of Isolated Rat Skeletal Sarcolemma

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Summary. Isolated rat skeletal muscle sarcolemmal tubes contained 78.1% protein, 13.1% lipid and 3.3% neutral carbohydrate. Gas-liquid chromatography of the total sarcolemmal carbohydrates showed the presence of fucose (0.28%), mannose (0.44%), galactose (0.95%), neuraminic acid (0.31%) and larger quantities of glucose (1.58%), glucosamine (2.59%) and galactosamine (2.03%). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed 30 to 35 protein bands including three major bands at 170,000, 140,000 and 44,000 mol wt. Amino acid analysis showed the sarcolemma to contain small amounts of proteins characteristic of collagen and relatively large amounts of acidic amino acids (21.5% glutamic and aspartic acids). Lithium 3,5-diiodo salicylate (LIS) extracted a fraction of the external lamina of the sarcolemma (designated ELS). The ELS was rich in glucuronic acid and hexosamines as well as having an increased proportion of acidic amino acids. The ELS contained no hydroxyproline and had less lipid than whole sarcolemma.

Cell coats containing carbohydrates and proteins have been found to play an important role in membrane permeability [6, 10], cell adhesion [14], antigenic specificity [21] and cell recognition [12, 30]. Whereas nearly all cells have a thin cell coat (approximately 200 Å) external to the unit membrane [29], Schwann cells and skeletal myofibers have a wider (800 to 1,000 Å) external lamina (EL)[1] [8]. The chemical composition of isolated sarcolemma has been studied by Kono and Colowick [15], Kono *et al.* [16] and Madeira and Madeira-Antunes [20] (rat) and by Abood *et al.* [1] (frog) after extraction with strong salt solutions (0.4 M LiBr and/or 1.0 M KCl). To avoid possible solubilization of EL constituents under these conditions, we used physiological salt concentrations in a modified McCollester [24]

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1 *Abbreviations:* LIS, lithium 3,5-diiodo salicylate; SDS, sodium dodecyl sulfate; EL, external lamina; ELS, external lamina substance; RR, ruthenium red.

procedure. With this method, we isolated sarcolemmal tubes, emptied of contractile proteins and free of major contaminants, with cytochemical and ultrastructure characteristics including those of the EL similar to those of the sarcolemma of intact myofibers. Others have shown that similar gentle procedures preserve enzymatic activity in isolated sarcolemma [28].

In addition to analysis of the chemical composition of the emptied whole sarcolemmal tubes, lithium 3,5-diiodo salicylate (LIS) was used for extraction of ruthenium red positive material from the sarcolemmal tubes. This LIS-soluble external lamina substance (ELS) was also chemically analyzed. This study, which has previously appeared in abstract form [33], extends and quantitates our previous cytochemical observations [37, 38].

Materials and Methods

Reagents

Lithium 3,5-diiodo salicylate, lot no. 711–3, was purchased from Eastman Kodak Company. L(−)-fucose and D(+)-galactose were purchased from Nutritional Biochemical Corp. D-glucuronic acid (grade I), D(+)-glucosamine-HCl, D(+)-xylose, D(+)-mannose, N-acetyl neuraminic acid (type IV), hyaluronic acid (grade III-P), sodium dodecyl sulfate (SDS), avidin and transferrin were obtained from Sigma Chemical Company. D(+)-glucose was a Baker Analyzed Reagent and N-acetyl-D-galactosamine was purchased from Schwarz-Mann. Reagent grade acetic anhydride, pyridine, hexamethyldisilazane and mannitol were purchased from Analabs Inc. Trimethyl chlorosilane (GC grade) was purchased from Pierce Chemical Company. All reagents used for disc electrophoresis were obtained from Canalco Inc. β-galactosidase and ovalbumin were purchased from Worthington Biochemical Corp.

Preparation of Sarcolemmal Tubes

Sarcolemmal tubes from rat leg muscle were prepared by the method of McCollester [24] with minor modifications which we have previously described [38]. These consisted of several additional cycles of washing and resedimentation monitored by phase-contrast microscopy. For assays on whole sarcolemma the isolated tube preparations were lyophilized and stored at −20 °C until analyzed.

Extraction with Lithium Diiodo Salicylate

The sarcolemmal tubes were extracted with lithium 3,5-diiodo salicylate immediately after isolation by the method of Marchesi and Andrews [22]. An equal volume of 0.6 M LIS in 0.10 M tris hydrochloride, pH 7.5, was added to the tube suspension (approximately 5 to 7 mg in 3 ml distilled water) and vigorously stirred for 15 min at room temperature and for 10 min at 4 °C. The residue was removed by centrifugation for 30 min at 40,000 × g at 4 °C and the supernatant filtered twice through No. 42 Whatman filter paper. The supernatant was dialyzed at 4 °C against distilled water for 72 to 96 hr with frequent water changes until no detectable LIS was found in the dialysate by UV

spectrophotometry at 323 nm. As the LIS concentration fell, a flocculent white precipitate of ELS material formed in the dialysis bag. The dialyzed suspension was lyophilized and stored at −20 °C until analyzed.

Determinations

The protein content of the sarcolemmal tubes, ELS and LIS-insoluble residue was determined by the method of Lowry *et al.* [17] with bovine serum albumin as the standard. The lipid content was determined by thrice extracting with chloroform-methanol (2:1 v/v), drying the extracted material and weighing it on a Cahn Ratio Electrobalance. Carbohydrates were analyzed as trimethylsilyl derivatives on a Hewlett-Packard 5750 Research Chromatograph equipped with a flame ionization detector. The glass columns (240 × 0.4 cm) that were used contained a stationary phase of SE-30 (3% w/w) on 80- to 100-mesh siliconized, acid-washed Chromosorb W (Hewlett-Packard) and the chromatograph was temperature-programmed from 140 to 200 °C at 1.0 °C/min and held at 100 °C until the final peak was eluted. Samples were methanolyzed in 1 N HCl/methanol at 100 °C for 24 hr under N_2, re-N-acetylated and trimethylsilylated according to the method of Clamp *et al.* [2]. Mannitol was used as the internal standard and the carbohydrates were quantitated using linear calibration curves obtained for sugar standards. Peak areas were determined by planimetry.

Amino acid analyses were performed on samples of whole tubes and ELS hydrolyzed at 105 °C for 24 hr with 6 N HCl using a Technicon TMS Analyzer[2].

Gel Electrophoresis

Proteins and glycoproteins were studied by SDS polyacrylamide gel electrophoresis using a sulfate-borate discontinuous buffer system at pH 9.5 after solubilizing the samples with 2% SDS-10% β-mercaptoethanol in 50 mM Na_2CO_3 for 10 min at 25 °C according to the methods of Neville [26] and Neville and Glossman [27]. Proteins in the gels were stained with Coomassie blue [35]. A variety of methods [7, 11, 36] employing the periodic acid Schiff reaction were used in an attempt to locate glycoproteins. Molecular weights were estimated by using a calibration curve prepared from standard proteins and glycoproteins of known molecular weight including β-galactosidase, ovalbumin, trypsin-diisopropyl fluorophosphate, avidin and transferrin.

Results

An advantage to the procedure used for isolating sarcolemmal tubes is that it produces readily identifiable structures. But methods such as this, where there is limited tissue degradation, must cope with the problem of contaminants as an integral part of the final product. For example, collagen microfibrils are intimately associated with the surface of the myofiber external lamina [32, 38] and whether they are considered to be an integral part of the sarcolemma or a contaminant is a matter of definition. Although

2 Determinations were done by Dr. H. Bates, Center for Laboratory Medicine, Metuchen, N.J.

McCollester [24] considered capillaries to be a significant contaminant of his sarcolemmal tube preparations, addition of several cycles of washing and resedimentation has eliminated capillaries. Extensive electron microscope analysis of the myotube preparations showed them to consist of 98 to 99% emptied sarcolemmal tubes rarely contaminated by other components. Two possible major contaminants are mitochondrial and nuclear membranes. By phase-contrast microscopy, it was possible to distinguish the circular or longitudinal profiles of sarcolemmal tubes and to assess whether they contained significant quantities of unextracted sarcoplasm. In the EM, myofiber membranes could be distinguished from mitochondrial and nuclear membrane remnants because of the thick coating of external lamina of the sarcolemma. Nuclear membranes had adherent clumps of chromatin and mitochondrial membrane material occurred as intact or slightly disrupted profiles with recognizable cristae. These criteria were used to assess the homogeneity of the sarcolemmal tube preparations assayed. Cytochemical and ultrastructural studies demonstrated occasional collagen microfibrils embedded in the surface of the sarcolemmal tubes but they did not constitute a regular layer as reported by Kono *et al.* [16].

Our preparations yielded 5 to 7 mg of lyophilized sarcolemmal tube membrane from 25 to 35 g (wet weight) of muscle. LIS extracted 20 to 30% of the dry weight of the sarcolemmal tubes. Our previous ultrastructural studies [38] have shown that LIS does not disrupt the continuity of the EL but causes a general loosening of its structure and approximately 50% reduction of its width. The ruthenium red staining material which is responsible for sarcolemmal staining in tissue sections and which is extracted by LIS from the EL covering isolated sarcolemmal tubes [38] was recovered in the flocculent ELS fraction after dialysis. Also, collagen microfibrils appeared unravelled after LIS extraction [32, 38].

Table 1 compares the chemical composition of the sarcolemmal tubes, ELS and LIS-insoluble residue. Whereas the whole sarcolemmal tubes contain 3.3% neutral carbohydrate (as determined by quantitative gas chromatography), ELS has almost no detectable *neutral* carbohydrate; these carbohydrates were recovered in the LIS-insoluble residue. Fig. 1 illustrates a gas chromatograph obtained from a typical sarcolemmal tube preparation. It shows the neutral carbohydrates plus a relatively high proportion of glucosamine and galactosamine. N-acetyl-neuraminic acid is also found in the sarcolemma, but is eluted after glucosamine and hence is not shown on this portion of the chromatograph. No uronic acids were found in these hydrolysates. Table 2 shows the per cent carbohydrate composition of the sarcolemmal tubes as determined by gas chromatography.

Table 1. Chemical composition of sarcolemmal, external lamina substance (ELS) and LIS-insoluble residue [a]

	Sarcolemma	% Dry weight	
		ELS	LIS-insoluble residue
Protein	78.1	94.3	86.1
Lipid	13.1	6.5	4.8
Neutral carbohydrate	3.3	<0.5	3.3 [b]
Hexosamine	4.6	N.D. [c]	3.7

[a] Mean of three separate preparations, in duplicate.
[b] Value does not include fucose.
[c] N.D. = Not Determined.

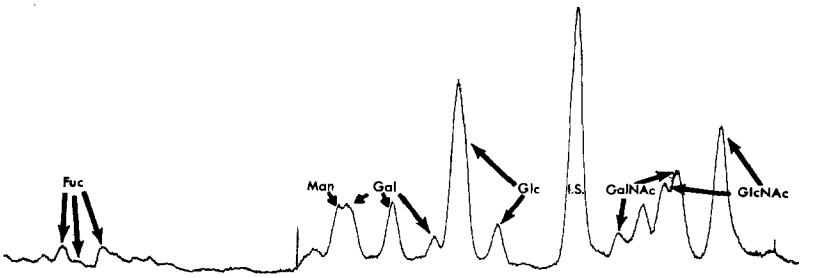

Fig. 1. Gas chromatograph of carbohydrates in isolated sarcolemma. N-acetyl-neuraminic acid was detected but elutes after glucosamine and is not shown. Abbreviations: Fuc, Fucose; Man, Mannose; Gal, Galactose; Glc, Glucose; I.S., Internal Standard (mannitol); GalNAc, N-acetyl galactosamine; GlcNAc, N-acetyl glucosamine

Table 2. Carbohydrate content of sarcolemmal tubes and LIS-insoluble residue [a]

Monosaccharide	Sarcolemmal tubes		LIS-insoluble residue	
	nmoles/mg membrane	% dry wt membrane	nmoles/mg membrane	% dry wt membrane
Fucose	16.8	0.28	[b]	[b]
Mannose	24.5	0.44	37.8	0.68
Galactose	52.7	0.95	44.7	0.81
Glucose	87.6	1.58	96.4	1.74
N-acetyl-glucosamine	117.3	2.59	52.0	1.15
N-acetyl-galactosamine	92.0	2.03	70.1	1.55
N-acetyl-neuraminic acid	10.0	0.31	0.0	0.0

[a] Mean of three separate preparations, in duplicate.
[b] Recovery masked by unknown peaks (*see* Fig. 3).

Fig. 2 is a representative gas chromatograph obtained from ELS. With the exception of glucose there is very little neutral carbohydrate. However, large amounts of glucuronic acid and glucosamine are present,

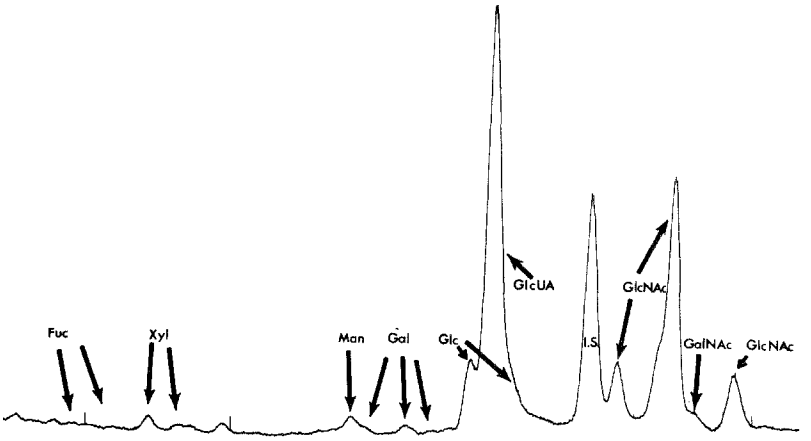

Fig. 2. Gas chromatograph of the LIS extract external lamina substance. Abbreviations are the same as in Fig. 1 with the addition of Xyl, xylose, and GlcUA, glucuronic acid

and there is a moderate amount of galactosamine. Xylose (the common binding sugar of glycosaminoglycans to protein) [9] is also present in ELS. Iduronic acid was not found. It is notable that there is a large amount of glucuronic acid in the ELS which was not detected in the sarcolemmal tubes. The probable explanation is that the hydrolysis conditions used are not sufficient to solubilize the uronic acids in the intact sarcolemmal tubes. We found that only 75% dry weight of the sarcolemmal tube preparation could be solubilized by the 1 N HCl/methanol hydrolysis method used. It seems probable that the uronic acids are in the insoluble portion. This inaccessibility of uronic acids to hydrolysis is also found in enzymatic studies [32, 38] with the sarcolemma being resistant to hyaluronidase digestion. Quantitation of the uronic acids shows that ELS preparations consist of up to 8% of acid mucopolysaccharides. This figure may, however, be increased after further experiments to find the optimal conditions for hydrolysis.

Fig. 3 is a representative chromatograph of the carbohydrates left in the residue after the extraction of sarcolemma with LIS. Examination of Table 2 shows that neutral sugars have not been destroyed by the extraction technique, but are left in the residue. The most striking decrease in the residue as compared with the original tubes is in glucosamine. This agrees with its occurrence as a major constituent in ELS. The only component which is lost is N-acetyl neuraminic acid. A new peak, not seen in LIS extract or sarcolemmal tube preparations, appears in chromatographs of LIS residue with a similar elution time for fucose. However, we have not yet been able to identify it.

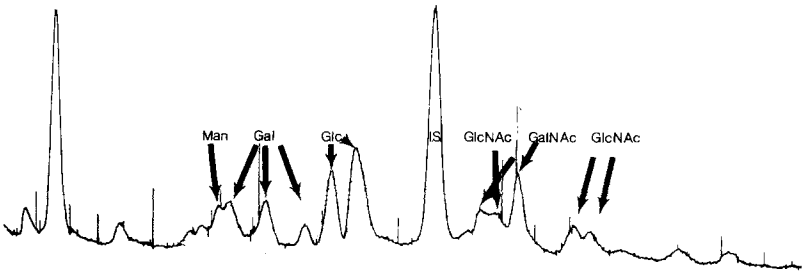

Fig. 3. Gas chromatograph of the LIS-insoluble residue. Abbreviations are the same as in Fig. 1

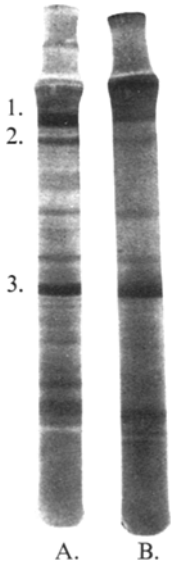

Fig. 4. SDS-gel electrophoresis of isolated sarcolemma (*A*) and the LIS extract external lamina substance (*B*). Gels are stained with Coomassie blue. The molecular weight of the major proteins are: (1) 170,000, (2) 140,000, and (3) 44,000

Fig. 4 shows electropherograms of whole sarcolemmal tubes and ELS prepared and solubilized by the methods described above. Using this method, 80 to 90% of the material was solubilized. Longer incubations at higher temperatures (up to 48 hr at 60 °C) did not increase the amount of soluble material or change the electrophoretic pattern. Although 30 to 35 bands could be seen in electropherograms of sarcolemmal tubes (*A*), there were only three major bands with approximate molecular weights of 170,000, 140,000 and 44,000. Three major protein bands were also obtained by Edidin and Fambrough [5] after reduction and alkylation of isolated sarcolemma. The ELS (*B*) contains fewer bands. In particular, there are fewer

Table 3. Amino acid analysis of sarcolemma and ELS[a]

	Residues per 100 total amino acid residues	
	Sarcolemma	ELS
Aspartic acid	8.4	11.0
Threonine	4.3	5.4
Serine	5.6	5.1
Glutamic acid	13.1	16.2
Proline	5.2	5.2
Glycine	19.9	8.3
Alanine	6.0	6.3
Valine	4.1	8.5
Cysteine	0.8	0.3
Methionine	1.5	3.5
Isoleucine	3.3	3.8
Leucine	7.3	8.8
Tyrosine	2.2	2.3
Phenylalanine	3.0	2.9
Lysine	4.8	6.7
Histidine	1.5	0.7
Arginine	5.8	4.8
Hydroxyproline	3.0	0.0

[a] Mean of three determinations on three separate preparations.

of the less intensely stained bands and the band of 170,000 mol wt protein is reduced. The major band at 44,000 mol wt is still present and a very heterogeneous, high molecular weight material is apparent at the top of the gel. PAS staining of the gels to demonstrate glycoproteins was unsuccessful because of the presence of SDS bound to the protein bands. Nearly all of the protein bands stained with PAS, even without periodic acid oxidation.

The amino acid contents of sarcolemmal tubes and ELS are given in Table 3. These data are not given to characterize any specific protein but to assess the relative number of functional active sites for ion binding contributed by the polyacidic and polybasic amino acids. It also gives information on the relative abundance of collagen or collagen precursors in whole sarcolemmal tubes and ELS. The sarcolemma is characterized by a large proportion of acidic amino acid (21.5% aspartic and glutamic acid) and quantities of glycine, proline and hydroxyproline intermediate between those characteristics of collagenous and noncollagenous protein. The amino acid content of the ELS is similar to the whole sarcolemma but has some significant differences. In particular, the ELS contains a greater proportion of acidic amino acid residues (27.2%), a lower glycine and proline content and no hydroxyproline.

Discussion

Membranes are polyphasic systems with components bound together by a variety of bonds including very weak noncovalent linkages. Hence, the method of isolation will modify the results of analyses. Although the method used in this study was relatively gentle, since it avoided strong salt concentrations and extremes of pH, an unknown percentage of loosely bound and water-soluble materials must inevitably have been lost. Despite this, the isolated sarcolemmal tubes were morphologically intact.

We found rat sarcolemma to be relatively free of collagen microfibrils though a small number of collagen microfibrils are attached to the *surface* layer of the EL in all our preparations. The amino acid analysis reported here confirms and extends our previous findings [32, 38] that collagen is not a major constituent of sarcolemmal tubes, in agreement with the results of Abood *et al.* [1] and Madeira and Madeira-Antunes [20]. The protein structure of EL is therefore markedly different from the specialized form of collagen which characterizes typical basement membranes [13], although the two are similar morphologically and histochemically.

We have observed previously that LIS extracts the ruthenium red staining material in rat sarcolemma [32, 38]. ELS was found to contain large quantities of uronic acid though such constituents had not been found in whole tube preparations. Margolis and Margolis [23] have also found that LIS is effective in solubilizing uronic acid containing components that are insoluble by other methods.

Data obtained in this study may be correlated with previous cytochemical observations on myofiber EL and possibly with some of the physiological properties of the sarcolemma. A notable characteristic of unfixed EL is binding of metallic cations, particularly in the region of the subneural apparatus of the neuromuscular junction [3, 25, 31] and selective binding of the polycationic dye, ruthenium red [18]. Although RR was originally thought to be a specific stain for acid mucopolysaccharides, Luft [18] has shown that it also precipitates polyglutamic and polyaspartic acids. Since we have previously shown that ELS contains the RR-staining material [32, 38], it is of considerable interest that the isolated ELS contains a high proportion (27.2%) of acidic amino acids. These acids may be partially responsible for the binding of metallic cations and RR. Gas-liquid chromatography has shown the presence of considerable quantities of uronic acids in LIS-extracted ELS. Whether the uronic acids are available for binding of both RR and cations in the whole tube is an open question since at least the glycosidic linkages are unavailable to hydrolytic agents. It is

probable that the acid mucopolysaccharides are bound to protein in an organized complex whose tertiary structure determines the number of available groups. In this context our observation [32] that the number of ionizable acid groups in ELS is increased by very slight shearing forces may be significant. It suggests that the binding *properties* of the EL may be susceptible to alterations in the course of physiological function. A reasonable hypothesis is that the acidic groups that are responsible for both metallic cation and RR binding under physiological conditions are occupied by Ca^{++}. In this connection, it is of interest that the Ca^{++} binding protein present in large quantities in the sarcoplasmic reticulum [19] has the same mol wt (44,000) and electrophoretic characteristics as band 3 observed in electropherograms of whole sarcolemmal tubes and extracted ELS (Fig. 4). Also supporting this hypothesis is the observation that RR specifically inhibits Ca^{++} ATPase activity [34]. The selective binding of metallic cations and the intense RR staining of the EL within the subneural apparatus may indicate increased amounts of accessible glutamic and aspartic acid residues and possibly uronic acid-containing macromolecules in this region which are capable of binding large amounts of Ca^{++}. Alterations in the configuration of the complexes associated with the passage of the nerve impulse into the neuromuscular junction might then be responsible for the release of Ca^{++} from this area which has been observed to accompany the passage of the stimulus [4].

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