Sizes of Components in Frog Skeletal Muscle Measured by Methods of Stereology

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ABSTRACT Stereological techniques of point and intersection counting were used to measure morphological parameters from light and electron micrographs of frog skeletal muscle. Results for sartorius muscle are as follows: myofibrils comprise 83 % of fiber volume; their surface to volume ratio is 3.8 μm^{-1}. Mitochondria comprise 1.6 % of fiber volume. Transverse tubules comprise 0.32 % of fiber volume, and their surface area per volume of fiber is 0.22 μm^{-1}. Terminal cisternae of the sarcoplasmic reticulum comprise 4.1 % of fiber volume; their surface area per volume of fiber is 0.54 μm^{-1}. Longitudinal sarcoplasmic reticulum comprises 5.0 % of fiber volume, and its surface area per volume of fiber is 1.48 μm^{-1}. Longitudinal bridges between terminal cisternae on either side of a Z disk were observed infrequently; they make up only 0.035 % of fiber volume and their surface area per volume of fiber is 0.009 μm^{-1}. T-SR junction occurs over 67 % of the surface of transverse tubules and over 27 % of the surface of terminal cisternae. The surface to volume ratio of the caveolae is 48 μm^{-1}; caveolae may increase the sarcolemmal surface area by 47 %. Essentially the same results were obtained from semitendinosus fibers.

INTRODUCTION

Quantitative information on the volume and surface area of a muscle fiber and its ultrastructural components is often necessary in interpreting the results of experiments and in formulating theories of physiological processes in muscle. Stereology, the three-dimensional interpretation of two-dimensional images using methods of geometric probability (Weibel and Elias, 1967), has been used to obtain quantitative information on the structures of cardiac muscle (Page et al., 1971), cardiac Purkinje fibers (Mobley and Page, 1972), and mammalian skeletal muscle (Eisenberg et al., 1974). We have used stereological techniques, point and intersection counting (Elias et al., 1971), in determining morphological parameters of frog skeletal muscle from light and electron micrographs of that tissue.

Peachey (1965) measured some morphological parameters of frog sartorius

muscle, and those measurements have indeed been important in the study of the physiology of muscle. However, since several of the parameters determined by Peachey were possibly in error by as much as a factor of two or three (Peachey, 1965), we measured parameters that were originally measured by Peachey as well as some additional ones. We were particularly interested in those parameters that seem relevant to excitation contraction coupling, the fiber surface, the transverse tubules, and the sarcoplasmic reticulum.

METHODS

Symbols

The following lists explain the symbols used in this paper.

Stereological symbols:

Surface	S
Volume	V
Point counts	P
Intersection counts	C
Number	N

Anatomical symbols:

Muscle fiber	f
Myofibrils	mf
Mitochondria	mit
Transverse tubules	t
Total sarcoplasmic reticulum	sr
Terminal cisternae of the sarcoplasmic reticulum	tc
Longitudinal tubules, intermediate cisternae, and fenestrated collar of the sarcoplasmic reticulum are treated as a unit called longitudinal sarcoplasmic reticulum	lsr
Sarcoplasmic reticulum that forms bridges between sarcomeres at the Z line	zsr
Close apposition of transverse tubules and terminal cisternae of the sarcoplasmic reticulum (T-SR junction)	t-sr
Caveolae	cav
Nuclei	nuc
Lipid droplets	lip
A surface that appears smooth in the light microscope	smooth

Fixation, Dehydration, and Embedding

A sartorius muscle was isolated from each of five frogs, *Rana pipiens*, which measured approximately 5 cm between the knee and the pelvis. Thread was tied to a tendon at the knee and to a piece of bone at the pelvic end of the muscle, and the muscle was mounted on a rod at approximately rest length. Each frog was chosen from a different batch of frogs. The procedure for fixation was essentially the same as Peachey's (1965). Muscles were fixed for 1.5 h in 5% glutaraldehyde, 0.1 M sodium cacodylate, and 2 mM calcium chloride solution (pH = 7.2, osmolality = 650 mosmol/kg-H_2O). Muscles were then rinsed 0.5 h in 10% sucrose, 0.1 M sodium cacodylate, and 2 mM

$CaCl_2$ (pH = 7.2, osmolality = 450 mosmol/kg-H_2O) and postfixed for 2 h in 1% osmium tetroxide and 0.1 M sodium cacodylate (pH = 7.2, osmolality = 170 mosmol/kg-H_2O). The whole muscle was stained 1 h in uranyl acetate (Kellenberger et al. 1958) (osmolality = 350 mosmol/kg-H_2O) after which 10–20 small blocks were cut from both edges of the muscle midway between the knee and pelvic ends and stained for a second hour with uranyl acetate. The blocks of tissue were dehydrated 15 min each in 70, 95, and 100% solutions of ethanol, 100% ethanol and propylene oxide (1:1), propylene oxide, and then in Epon overnight. All of the above procedures occurred at room temperature, 20–22°C; polymerization of Epon was at 60°C the next day.

Sectioning and Sampling of Fibers

Thick (approximately 0.5 μm) and thin longitudinal sections of silver-gray interference color (600–900 Å) were cut from two blocks randomly selected from each muscle. Thick cross sections were cut from two additional blocks that were also randomly selected from each muscle. Sections were cut with an LKB-Huxley microtome (LKB Instruments, Inc., Rockville, Md.) using glass and diamond knives for the thick and thin sections, respectively. Thick sections were stained with toluidine blue, and thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Longitudinal sections were taken at three spatial intervals or steps (greater than 100 μm) from each block embedded for longitudinal sectioning. A thick and thin section was taken at each step, and a surface and an adjacent fiber were sampled in each section. Therefore 6 fibers were sampled from each of the two blocks, 12 fibers were sampled from each frog, and a total sampling of 60 fibers was obtained from the five frogs used in the study. One thick section was taken from each of the blocks embedded for cross-sectioning. The total cross-sectional sampling was 117 fibers (surface plus adjacent) from the five frogs.

Light Microscopy

Thick sections were observed on a Zeiss RA microscope (Carl Zeiss, Inc., New York) at a magnification of × 400. A square grid with a 25-μm spacing at the given magnification was mounted in one of the oculars. The grid was used to count the points that overlay fibers in the two most superficial layers of the inner surface of the muscle, P_f, and to count the intersections with the surfaces of those fibers, C_f. When longitudinal sections were counted, the grid was oriented so that its lines were 19 and 71° with respect to the axes of the fibers (Sitte, 1967); hence the number of intersections counted was the mean number of intersections that would have been counted if all orientations of grid to fiber axis had been used. Fibers cut in cross section were acceptable if the sarcomere pattern repeated no more than once in 5 μm. The ratios of intersection to point counts (C_f/P_f) obtained from cross sections when compared to the ratios obtained from longitudinal sections determined an empirical orientation constant (see Equations and Calculations, Eq. 3).

Electron Microscopy

Micrographs were taken with a Siemens Elmiskop 1A (Siemens Corp., Iselin, N. J.) operated at 80 kV; the microscope had the added convenience that the currents in

the objective, intermediate, and projector lenses could be monitored with a digital ammeter (Dunn and Preiss, 1974). Twelve micrographs of each section were taken at × 10,000 and printed at a three-times enlargement giving a total magnification of × 30,000. Three micrographs of sarcoplasm were taken from each fiber (Fig. 1). These micrographs were taken of random areas, i.e. areas at a corner of the copper supporting grid (Weibel et al., 1966). This procedure sampled the sarcolemma in-

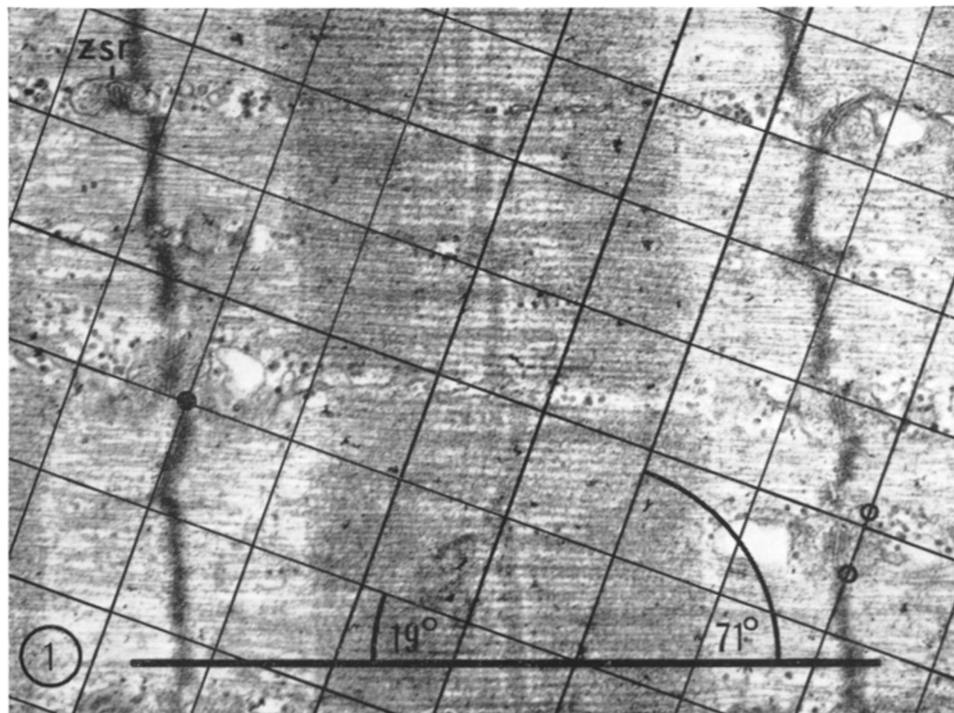

FIGURE 1. Electron micrograph of a longitudinally sectioned frog sartorius muscle. A test grid is superimposed on the micrograph at 19 and 71° to the fiber axis. The solid circle is an example of a point count, P_{tc}. The two open circles are examples of intersection counts, C_{tc}. The grid lines are spaced $d = 0.5$ μm apart. Sarcoplasmic reticulum crossing the Z line is labeled zsr. × 30,000.

sufficiently. Therefore random segments of sarcolemma from each fiber were also sampled; three micrographs of sarcolemma from each fiber were taken where the sarcolemma intersected the copper grid bars. In all, 360 micrographs were taken; a total of 9,000 μm^2 of fiber area and 1,700 μm of sarcolemmal length were analyzed.

Grids and Counting

The choice of the spacing on the test grids was made so that the error of all volume fractions on each frog would be less than 7 % (Eq. 6.53, Weibel, 1973). No comparable equation was available to predict errors in quantities which include surface areas; therefore we measured these quantities from the same micrographs we used for the

volume fractions. The components in muscle occur in such sizes and with such spatial frequencies that three grids with three spacings of test lines, 1.67, 0.500, and 0.167 μm, were used for volume fractions greater than 10, 1, and 0.1%, respectively, of fiber volume.

The grid with test lines spaced 1.67 μm apart was used to count points that overlay myofibrils and to count intersections with the surface of myofibrils. The grid with test lines spaced 0.500 μm apart was used to count points that overlay mitochondria, longitudinal sarcoplasmic reticulum, and terminal cisternae and to count intersections with the surface of longitudinal sarcoplasmic reticulum (see Fig. 1). Finally, the grid with test lines spaced 0.167 μm apart was used to count points that overlay transverse tubules, Z-bridge sarcoplasmic reticulum, caveolae, nuclei, and lipid droplets and to count intersections with the surface of terminal cisternae, transverse tubules, T-SR junction, Z-bridge sarcoplasmic reticulum, sarcolemma, and caveolae. All grids were oriented so that the lines made angles of 19 and 71° with the axes of the filaments (Fig. 1).

Twenty micrographs were randomly selected from the total, and intersections of the finest grid (test lines 0.167 μm apart) with the surface of longitudinal sarcoplasmic reticulum, terminal cisternae, and transverse tubules were counted a second time. This time, however, the lines of the grid were oriented parallel and perpendicular to the axes of the filaments. The degree of orientation of the surfaces of these three structures was calculated using the model of linear orientation for S_{lsr} and S_{tc} and the model of planar orientation for S_t (DeHoff and Rhines, 1968, p. 118). This measured degree of orientation was also used to justify the use of Eqs. 2 and 3 (presented later) in calculating surface areas of longitudinal sarcoplasmic reticulum, terminal cisternae, and transverse tubules per fiber volume.

Equations and Calculations

A volume fraction, i.e., the volume of a particular component, V_i, contained in a test volume, V_{test}, is calculated from the ratio of grid points overlaying the component, P_i, and test areas, P_{test}, in the micrograph (Weibel and Elias, 1967),

$$\frac{V_i}{V_{test}} = \frac{P_i}{P_{test}}. \tag{1}$$

The surface area of a particular component, S_i, per unit test volume, V_{test}, was calculated from the equation:

$$\frac{S_i}{V_{test}} = \frac{C_i}{dP_{test}}, \tag{2}$$

where d is the distance between the test lines on the grid (micrometers) (Weibel and Elias, 1967). Eq. 2 is valid for objects with isotropic surfaces, namely surfaces with random orientation where the number of intersections counted depends neither on the sectioning angle, ϕ, nor on the angle θ of the grid with respect to the structure in the micrograph. If the component of interest is anisotropic in the test volume and the sectioning angle, $\phi = 0$ (longitudinal sections), we used the equation:

$$\frac{S_i}{V_{test}} = 1.15\,\frac{C_i}{dP_{test}}, \tag{3}$$

where the constant 1.15 was determined experimentally by the following procedure: $S_{f(\text{smooth})}/V_f$ was determined exactly by counting the thick cross sections and using the equation,

$$\frac{S_{f(\text{smooth})}}{V_f} = \frac{\pi}{4}\frac{C_{f(\text{smooth})}}{dP_f}, \tag{4}$$

(Sitte, 1967). The ratio $C_{f(\text{smooth})}/d\,P_f$ for the 60 fibers used in our analysis was then determined by counts from the thick longitudinal sections. The factor that was required to make $C_{f(\text{smooth})}/d\,P_f$ obtained from longitudinal sections equal the correct number, $\pi\, C_{f(\text{smooth})}/4dP_f$, obtained from cross sections and Eq. 4 was 1.15. This empirically derived constant was used in Eq. 3 to determine S_{mf}/V_{mf}, S_{lsr}/V_f, S_{zsr}/V_f, since myofibrils, longitudinal sarcoplasmic reticulum, and Z-bridge sarcoplasmic reticulum are all oriented longitudinally (see Assumptions and Errors). We are not aware of a theoretical derivation that would enable one to calculate S_i/V_{test} of a longitudinally oriented, nonuniform and noncylindrical structure which is sampled randomly with longitudinal sections; several derivations do exist for S_i/V_{test} of uniform right circular cylinders (Weibel, 1972; Whitehouse, 1974, D. F. Davey [personal communication]). A similar empirical approach in the choice of the correct constant was used for the sarcoplasmic reticulum of guinea pig muscle and found to be 1.06 (calculated in Eisenberg et al., 1974 and used in Eisenberg and Kuda, 1975).

A surface fraction is the ratio of one surface area, S_i, to another, S_{test}. The ratio of the intersections of a grid with each surface (which appears as a line in the micrograph) were counted and C_i and C_{test} were obtained. Then,

$$\frac{S_i}{S_{test}} = \frac{K_i C_i}{K_{test} C_{test}}. \tag{5}$$

If both surfaces had the same orientation: $K_i = K_{test} = 1$ (e.g., $S_{t\text{-}sr}/S_t$, $S_{t\text{-}sr}/S_{tc}$, $S_f/S_{f(\text{smooth})}$). If the surfaces were oriented differently, i.e., one was isotropic and the other anisotropic, then K_i or K_{test} equaled 1.15 (from Eq. 3) while the other equaled 1 (e.g., $S_{cav}/S_{f(\text{smooth})}$ and S_{cav}/S_f; $K_{test} = 1.15$ and $K_i = 1$). Intersection counts with an apparently smooth sarcolemma were obtained by counting intersections of the grid with a straight line drawn between the two end points of the sarcolemma in a micrograph. In the statistical analysis, $N = 5$, the number of frogs.

Assumptions and Errors

Perhaps the most serious error in the analysis arises from an unmeasured alteration of the ultrastructure upon fixation. A study of this error on single semitendinosus fibers (Eisenberg and Mobley, 1975), showed that a striking net decline in the volume of the cell $\simeq 45\%$ occurred during the steps after osmium fixation. Of course it is impossible to monitor changes in individual components comprising the fiber under the same circumstances. The large fluctuations in volume and the extensive shrinkage observed in single semitendinosus fibers probably did not occur in the fibers of whole muscles or bundles studied here. However, this suspicion is based only on a comparison of the quality of the final embedded material and not on direct observation or

testing of the tissue in this case. It should be noted that if shrinkage in the fiber volume or its components occurred then the calculated parameters, though accurate for fixed material, might not accurately describe the living fibers studied in physiological experiments.

Before the analysis we recognized that there could be subjective differences in the identification of certain components, particularly longitudinal sarcoplasmic reticulum. Therefore, each of us counted several of the same micrographs without moving the grid. Our data differed by less than 10% for any component. This procedure was repeated after the general analysis and the same results were obtained. Data then are presented as means derived from total counts from each animal.

Eq. 3 and the experimentally determined constant, 1.15, probably give accurate results for S_{mf}/V_{mf} since the myofibrils seem fully oriented; their shape is not unlike that of the fibers and the shape is retained over significant longitudinal distances. Eq. 3 is somewhat less accurate in estimating S_{lsr}/V_f because, as is presented in the Results, longitudinal sarcoplasmic reticulum is not fully oriented (only 84%). If the longitudinal sarcoplasmic reticulum were fully oriented and the same cross section were maintained along its entire length, i.e., if there were no fenestrated collar, then accurate results could be obtained by counting the structures in cross section. Since neither of these conditions holds, our estimate of S_{lsr}/V_f, though not quite correct, is probably the best that can be achieved at this time.

The sartorius fibers that were tested were taken from the edges of the muscles and from either the inner surface of the muscle (where fibers are chosen for experiments with microelectrodes) or one fiber away from the surface. We assume that if fibers in the core of the muscle could have been fixed and sampled that the results would have been the same as we report here. The semitendinosus fibers that were tested came from the core of the muscle.

Semitendinosus Muscle

A similar study was also performed on fibers from the semitendinosus muscle of frogs. A bundle of 50–100 semitendinosus fibers was isolated from the muscle of two frogs. Fixation and analysis were similar to that performed on sartorius muscles. Four micrographs, two of sarcoplasm and two of the surface, were taken from eight fibers from each of the two frogs. The purpose of this study was to call attention to quantitative differences in the structure of semitendinosus and sartorius muscles.

RESULTS

Myofibrils

Quantities in Tables I and II were determined directly from the point and intersection counts on the electron micrographs; the standard error of each quantity is also given in the tables. The fraction of the fiber volume that is composed of myofibrils is: $V_{mf}/V_f = 83\%$ and the surface to volume ratio of the myofibrils is $S_{mf}/V_{mf} = 3.8\ \mu m^{-1}$.[1] Assuming that myofibrils are right circular cylinders, then their mean diameter is 1.05 μm.

[1] $\mu m^2/\mu m^3$ is abbreviated μm^{-1}.

Transverse Tubules

The fraction of the fibers that is composed of transverse tubules is very small, $V_t/V_f = 0.32\%$ (Table I); the surface area of the transverse tubules per unit volume of fiber is $S_t/V_f = 0.22\ \mu m^{-1}$ (Table I). The surface to volume ratio of the T system itself, S_t/V_t, is important when considering the electrical properties of the T system and accumulation of ions in the system.

$$\frac{S_t}{V_t} = \frac{S_t/V_f}{V_t/V_f} = 69\ \mu m^{-1}.$$

TABLE I

STRUCTURAL PARAMETERS DETERMINED FROM FROG SARTORIUS FIBERS USING POINT AND INTERSECTION COUNTING AND STEREOLOGICAL ANALYSIS

	Volume as % of fiber volume*		Surface area per unit test volume (μm^{-1})‡		
Component	Symbol	Mean ± SEM§	Symbol	Mean ± SEM§	Equation (Methods)
Myofibrils	V_{mf}/V_f	83±2	S_{mf}/V_{mf}	3.8±0.2	3
Mitochondria	V_{mit}/V_f	1.6±0.1	—	—	—
Nuclei	V_{nuc}/V_f	0.09±0.06	—	—	—
Lipid droplets	V_{lip}/V_f	0.06±0.03	—	—	—
Transverse tubules	V_t/V_f	0.32±0.02	S_t/V_f	0.22±0.01	2
Longitudinal sarcoplasmic reticulum	V_{lsr}/V_f	5.0±0.3	S_{lsr}/V_f	1.48±0.08	3
Terminal cisternae	V_{tc}/V_f	4.1±0.4	S_{tc}/V_f	0.54±0.04	2
Z-bridge sarcoplasmic reticulum	V_{zsr}/V_f	0.035±0.007	S_{zsr}/V_f	0.009±0.002	3
Caveolae	V_{cav}/V_f	—‖	S_{cav}/V_{cav}	48±2	2

* Quantities calculated using Eq. 1 (Methods).
‡ $\mu m^2/\mu m^3$ is abbreviated μm^{-1}.
§ $N = 5$, the number of frogs.
‖ Not calculated directly. See text. $V_{cav}/V_f = 0.06\%$.

Sarcoplasmic Reticulum

Sarcoplasmic reticulum can be divided into three components. Terminal cisternae and longitudinal sarcoplasmic reticulum (see Methods) were considered separately because the two may serve different functions during contraction and relaxation of muscle (Winegrad, 1968). The component of sarcoplasmic reticulum which appears to connect terminal cisternae on either side of a Z disc is called zsr. Measuring longitudinal links formed by zsr may be of help in determining whether the sarcoplasmic reticulum is electrically continuous longitudinally throughout a muscle fiber. Z-bridge sarcoplasmic reticulum appear not to be randomly distributed throughout the fibers; some

areas had several connections close to each other, but many micrographs showed no zsr at all. The fraction of the total number of triads that contained zsr is 6 ± 1%.

The fraction of a fiber that is composed of each of the three components of sarcoplasmic reticulum is $V_{lsr}/V_f = 5.0\%$, $V_{tc}/V_f = 4.1\%$ and $V_{zsr}/V_f = 0.035\%$ (Table I) and the fraction of a fiber composed of sarcoplasmic reticulum is the sum of the previous numbers, $V_{sr}/V_f = 9.1\%$. The surface area of each component per unit volume of fiber is $S_{lsr}/V_f = 1.48\ \mu m^{-1}$, $S_{tc}/V_f = 0.54\ \mu m^{-1}$, and $S_{zsr}/V_f = 0.009\ \mu m^{-1}$ (Table I) while the total surface of the sarcoplasmic reticulum per unit volume of fiber is the sum of the three, $S_{sr}/V_f = 2.03\ \mu m^{-1}$. Knowing $V_{mf}/V_f = 83\%$, we calculate that

$$\frac{V_{lsr}}{V_{mf}} = 6.0\,\%, \quad \frac{V_{tc}}{V_{mf}} = 4.9\,\%, \quad \frac{V_{zsr}}{V_{mf}} = 0.04\,\%, \quad \frac{V_{sr}}{V_{mf}} = 10.9\,\%;$$

$$\frac{S_{lsr}}{V_{mf}} = 1.78\ \mu m^{-1}, \quad \frac{S_{tc}}{V_{mf}} = 0.65\ \mu m^{-1}, \quad \frac{S_{zsr}}{V_{mf}} = 0.01\ \mu m^{-1}, \quad \frac{S_{sr}}{V_{mf}} = 2.44\ \mu m^{-1}.$$

The previous numbers combined with the size of the myofibrils could be useful when considering gradients of the concentration of calcium ions between the myofibrillar space and sarcoplasmic reticulum, and when considering calcium fluxes into and out of the sarcoplasmic reticulum. The ratio of the surface of each component to its own volume was also calculated since the fraction of the volume of the fibers composed of that component was known.

$$\frac{S_{lsr}}{V_{lsr}} = 30\ \mu m^{-1}, \quad \frac{S_{tc}}{V_{tc}} = 13\ \mu m^{-1}, \quad \frac{S_{zsr}}{V_{zsr}} = 25\ \mu m^{-1}, \quad \frac{S_{sr}}{V_{sr}} = 22\ \mu m^{-1}.$$

T-SR Junction

A fraction of the surface of the transverse tubules is in close apposition to a fraction of the surface of the terminal cisternae from sarcoplasmic reticulum. This junction between the membranes of the transverse tubules and terminal cisternae is called the T-SR junction, and its structure has been studied (Peachey, 1965; Franzini-Armstrong, 1970). This junction probably mediates the electrical signal of the transverse tubules with the sarcoplasmic reticulum. The fractional surface of the transverse tubules that is closely apposed by terminal cisternae is $S_{t\text{-}sr}/S_t = 67\%$ (Table II). The fractional area of the terminal cisternae that is closely apposed by the transverse tubules is $S_{t\text{-}sr}/S_{tc} = 27\%$ (Table II).

Sarcolemmal Area and Caveolae

The mean surface (smooth) to volume ratio of 117 fibers from the five frogs studied, $S_{f(smooth)}/V_f$, is 0.060 μm^{-1}, a number obtained using Eq. 4 (Methods) and the point and intersection counts from thick cross sections. If one

TABLE II

RATIOS OF THE SURFACE AREAS OF VARIOUS STRUCTURAL COMPONENTS IN FROG SARTORIUS FIBERS*

Component	Symbol	Mean ± SEM‡
		%
T-SR junction	S_{t-sr}/S_t	67±1
	S_{t-sr}/S_{tc}	27±1
Sarcolemmal folding§	$S_f/S_{f(smooth)}$	107±2
Caveolae§	$S_{cav}/S_{f(smooth)}$	50±6
	S_{cav}/S_f	47±5

* All quantities calculated using Eq. 5 (Methods).

‡ $N = 5$, the number of frogs.

§ The mean sarcomere length for the five frogs was 2.6 μm ± 0.1.

assumes that the fibers are right circular cylinders, which they clearly are not, their mean diameter is 67 μm. The light microscope reveals only a smooth surface for cells. If this smooth surface enclosed the fiber volume, then $S_{f(smooth)}/V_f = 0.060\ \mu m^{-1}$.

The caveolae and the folding of the sarcolemma have recently been investigated by Dulhunty and Franzini-Armstrong[2] and by Zampighi et al.[3] Folding of the sarcolemma should be related to the length of the sarcomeres. The surface area of the sarcolemma divided by the apparent surface area that would appear in the light microscope is given in Table III for the frogs studied. Unfortunately the correlation of folding with sarcomere length is not consistent. Perhaps the sample was too small or the folding is variable along the length of a fixed fiber; in addition, wrinkling due to an artifact of fixation, dehydration, and embedding (Eisenberg and Mobley, 1975) may have been observed. Dulhunty and Franzini-Armstrong[2] noted that folding of sarcolemma in frog skeletal muscle occurs only along the longitudinal axis; therefore the ratio of folded to smooth surface area can be determined from longitudinal sections. The mean degree of folding for all five frogs, $S_f/S_{f(smooth)} = 107\%$ (Table II), at a mean sarcomere length of 2.6 μm (Table II). The surface to volume ratio of the fibers, S_f/V_f, can be calculated, $S_f/V_f = (S_f/S_{f(smooth)}) \times (S_{f(smooth)}/V_f) = 0.064\ \mu m^{-1}$.

The surface area of the caveolae compared to an apparently smooth sarcolemma, $S_{cav}/S_{f(smooth)}$, and compared to the real folded sarcolemma, S_{cav}/S_f, are also presented in Table III as a function of sarcomere length. Since the

[2] Dulhunty, A. F., and C. Franzini-Armstrong. 1975. The relative contributions of the folds and caveolae to the surface membrane of frog skeletal muscle fibers at different sarcomere lengths. In press.

[3] Zampighi, G., J. Vergara, and F. Ramón. 1975. On the connection between the T-tubules and the plasma membrane in frog semitendinosus muscle. *J. Cell Biol.* **64**:734.

TABLE III
QUANTITIES RELATED TO THE SURFACE OF FROG SARTORIUS FIBERS AS A FUNCTION OF SARCOMERE LENGTH*

Sarcomere length	$S_f/S_{f(smooth)}$	$S_{cav}/S_{f(smooth)}$	S_{cav}/S_f
2.3	113	62	54
2.4	106	55	52
2.5	104	48	46
2.6	104	43	42
2.7	106	43	41
2.8	107	49	46
2.9	104	43	42

* All quantities were calculated using Eq. 5 (Methods) and are given in percentage.

sarcomere length of all of the fibers were less than the critical length at which the surface of caveolae apparently become part of a smooth sarcolemma (Dulhunty and Franzini-Armstrong),[2] S_{cav}/S_f should be and indeed seems to be constant even though the number of caveolae per micrograph was quite variable. $S_{cav}/S_f = 47\%$ for the five frogs (Table II).

The surface to volume ratio of the caveolae themselves, S_{cav}/V_{cav}, has a mean of 48 μm^{-1} (Table I). If the caveolae are assumed to be spherical then the mean diameter of the caveolae would be 125 nm. Checking that the fractional volume of the fibers composed of caveolae was insignificant, we find $S_{cav}/V_f = (S_{cav}/S_{f(smooth)}) \times (S_{f(smooth)}/V_f) = 0.030\ \mu m^{-1}$ where $S_{cav}/S_{f(smooth)} = 50\%$ (Table II). The fraction of the volume of the fibers contributed by the caveolae is then $V_{cav}/V_f = (V_{cav}/S_{cav}) \times (S_{cav}/V_f) = 0.06\%$ which is indeed insignificant.

The number of caveolae per unit sarcolemmal area can be calculated if the caveolae are assumed to be spherical.

$$\frac{N_{cav}}{S_f} = \frac{S_{cav}}{S_f} \times \frac{N_{cav}}{S_{cav}} = 10 \text{ caveolae}/\mu m^2 \text{ sarcolemma},$$

where S_{cav}/N_{cav} is the surface area of a spherical caveolae 125 nm in diameter.

Orientation of Surfaces

Assumptions about the orientation of the membranes of longitudinal sarcoplasmic reticulum, terminal cisternae, and transverse tubules with respect to the axes of the fibers were critical in choosing equations to calculate S_{lsr}/V_f, S_{tc}/V_f, and S_t/V_f (see Methods and Table I). In addition, data on the orientation may be useful in studies of these membranes with optical techniques. 84% of S_{lsr} and 9% of S_{tc} is oriented parallel to the fiber axis, and 17% of S_t is oriented perpendicular to the fiber axis. The remaining fraction of the total surface area of each of the three structures is isotropic.

Mitochondria, Nuclei, Lipid Droplets, and Aqueous Sarcoplasm

Mitochondria occupy 1.6% of fiber volume. Other cellular constituents are nuclei, V_{nuc}/V_f = 0.09% and lipid droplets V_{lip}/V_f = 0.06%. Aqueous sarcoplasm between the myofibrils was calculated as 6% of fiber volume by subtracting all of the other volumes of cellular constituents from the total fiber volume.

Semitendinosus Fibers

Table IV gives the results we obtained from a limited study of semitendinosus fibers. Comparisons of results from sartorius and semitendinosus fibers can be made by comparing the corresponding numbers from Tables I and II with IV. Standard errors are not given in Table IV because only two muscles were

TABLE IV

STRUCTURAL PARAMETERS DETERMINED FROM FROG SEMITENDINOSUS FIBERS USING POINT AND INTERSECTION COUNTING AND STEREOLOGICAL ANALYSIS

	Volume as % of fiber volume*		Surface area per unit test volume		
Component	Symbol	Mean‡	Symbol	Mean‡	Equation (Methods)
				μm^{-1}§	
Myofibrils	V_{mf}/V_f	85	S_{mf}/V_{mf}	3.8	3
Mitochondria	V_{mit}/V_f	1.0	—	—	—
Transverse tubules	V_t/V_f	0.25	S_t/V_f	0.17	2
Longitudinal sarcoplasmic reticulum	V_{lsr}/V_f	5.3	S_{lsr}/V_f	1.63	3
Terminal cisternae	V_{tc}/V_f	3.5	S_{tc}/V_f	0.35	2
Z-bridge sarcoplasmic reticulum	V_{zsr}/V_f	0.011	S_{zsr}/V_f	0.003	3
Caveolae	—	—	S_{cav}/V_{cav}	48	2

Surface areas per unit test surface area‖

Component	Symbol	Mean
		%
T-SR junction	S_{t-sr}/S_t	51
	S_{t-sr}/S_{tc}	24
Sarcolemmal folding¶	$S_f/S_{f(smooth)}$	104
Caveolae¶	$S_{cav}/S_{f(smooth)}$	36
	S_{cav}/S_f	35

* Quantities calculated using Eq. 1 (Methods).
‡ N = 2, the number of frogs.
§ $\mu m^2/\mu m^3$ is abbreviated μm^{-1}.
‖ Quantities calculated using Eq. 5 (Methods).
¶ The mean sarcomere length for the two frogs was 3.4 μm.

studied. We conclude that the two types of muscles are identical with respect to the quantities presented in the tables. Given that the analysis of sartorius fibers was more extensive than the analysis of semitendinosus fibers, the numbers from Tables I and II should be used for both types of muscle except of course where sarcomere length is an important consideration.

DISCUSSION

We have measured and calculated morphological parameters from fibers of frog skeletal muscle; the parameters may be useful in interpreting physiological experiments and in developing physiological models in muscle. We tested fibers from both the sartorius and semitendinosus muscles and found no significant difference in the parameters of these two muscles. Fig. 2 gives a summary of the fundamental data for terminal cisternae, longitudinal sarcoplasmic reticulum, and transverse tubules.

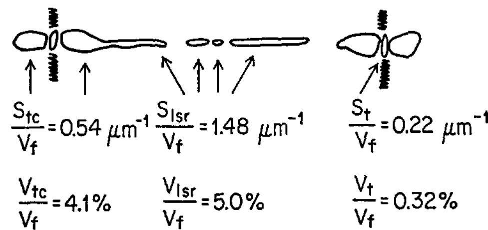

FIGURE 2. Representation of terminal cisternae, longitudinal sarcoplasmic reticulum, and transverse tubules in a sarcomere. The surface areas and volumes of these components per unit volume of fiber are given.

Earlier Peachey (1965) determined the volume and surface areas of transverse tubules and sarcoplasmic reticulum in frog sartorius fibers. He did this by making an estimate of the average size and shape of each component and then multiplying by the average number of times a component occurred around an average-sized myofibril. The quantities that Peachey measured on frog sartorius that can be compared with quantities we determined are given below; our notation is used to facilitate comparisons:

$$\frac{V_t}{V_f} = 0.3\%, \qquad \frac{V_{lsr}}{V_f} = 8\%,$$

$$\frac{V_{tc}}{V_f} = 5\%, \qquad \frac{S_{t-sr}}{S_t} = 80\%.$$

The overall agreement between our data and Peachey's is good considering the difference in the methods. The lower volume of sarcoplasmic reticulum, particularly longitudinal sarcoplasmic reticulum which we measure (Fig. 2), is probably significant. The surface areas of components which Peachey mea-

sured are expressed in terms of a radius of the fiber, i.e., Peachey assumed the fibers are right circular cylinders. With this assumption and the volume fractions given above, several additional parameters can be calculated from Peachey's data.

$$\frac{S_t}{V_f} = 0.28\ \mu\text{m}^{-1}, \quad \frac{S_{tc}}{V_f} = 1.4\ \mu\text{m}^{-1}, \quad \frac{S_{lsr}}{V_f} = 4\ \mu\text{m}^{-1},$$

$$\frac{S_t}{V_t} = 93\ \mu\text{m}^{-1}, \quad \frac{S_{tc}}{V_{tc}} = 28\ \mu\text{m}^{-1}, \quad \frac{S_{lsr}}{V_{lsr}} = 50\ \mu\text{m}^{-1}.$$

Each of the parameters of the T system given above is about one-third larger than the results we obtained. If we calculate the surface to volume ratio of an elliptical T system whose major axis is 800 Å and minor axis is 260 Å (Peachey, 1965), then $S_t/V_t = 114\ \mu\text{m}^{-1}$, which is two-thirds larger than we obtained. The four parameters dealing with sarcoplasmic reticulum range between 1.7 and 2.7 times larger than the parameters we measured.

In modeling the T system as a distributed electrical network, Adrian et al. (1969) defined two basic geometric constants of the T system: ρ, the fraction of fiber volume occupied by tubules, V_t/V_f, and ζ, the volume to surface ratio of tubules, V_t/S_t. Adrian et al. (1969) used Peachey's data in determining the approximate magnitude of those constants, $\rho = 3 \times 10^{-3}$ and $\zeta = 10^{-6}$ cm. Our data would make the constants to be $\rho = 3.2 \times 10^{-3}$ and $\zeta = 1.45 \times 10^{-6}$ cm.

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