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CHAPTER 11

Impedance measurement of the electrical structure of skeletal muscle

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MUSCLES ARE COMPLEX STRUCTURES that use chemical energy to perform mechanical work. The mechanisms that control the transduction of chemical energy into mechanical work are associated with the membrane systems of muscle fibers and so are easily studied by electrophysiological techniques designed to measure the properties of membranes.

The membrane systems of skeletal muscle are quite complex and have obviously been specialized by evolution to help perform the control mechanisms required. I briefly discuss the evolutionary specialization of muscle structure in the light of molecular and developmental biology before dealing with our main subject. After all, it is much easier to figure out how a machine works if you have some idea of how and why it was designed! Structural complexity extends beyond muscle; the basic structural patterns of membranes are repeated in many tissues (26). Therefore impedance techniques, the main subject of this chapter, are potentially useful in many other areas of biology, wherever structural specialization helps a tissue to function.

The structural specialization of muscle is only one example of the mechanisms of gene expression and cellular differentiation that allow a genome, common to all cells in the body, to produce cells highly specialized for particular functions. Evidently, it is much easier for evolution to modify an existing gene or gene product than it is to create a new one. The fundamental organelles and macromolecules of all cells are quite similar. Tissues are specialized more often by elaboration of common organelles and macromolecules than by synthesis of new ones. Many of the enzymes, structural proteins, and organelles of muscle fibers are not very different from those of other cells, but their structural organization is strikingly specialized.

Conservation of genetic information may be an important evolutionary principle; put another way, creation of new genetic information is evolutionarily difficult. That is probably why lower eucaryotes have a segmented structure (basically a periodic repeat of the expression of a single set of genes) and why even higher vertebrates have so much symmetry in their anatomy. Surprisingly large changes in final structure (and function) can be produced by simple changes in the rate of growth of a particular substructure. For example, Hampé [as described by Gould (50)] showed that "minor quantitative changes in the timing of development [could lead to] a change in arrangement of the entire ankle area." Similarly many structural complexities of muscle fibers may be the result of minor changes in the rate or pattern of expression of genes. Thus much structural specialization could be produced from a rather limited genome. The similar specialization of membranes in many tissues—the similar use of invaginations and infoldings with different distribution of ionic conductances and active transport systems—argues for a related genetic and developmental origin. Techniques for analysis of structural complication [see Eisenberg and Mathias (35)], including impedance measurements, are thus of general biological interest because structural complexity is a common evolutionary strategy for specialization and adaptation.

Skeletal muscle fibers illustrate the uses of structural complexity. They have a variety of membrane systems, most with specific and well-known functions

directly related to contraction. The three main membrane systems are the outer membrane, the T system, and the sarcoplasmic reticulum (SR). The electrical properties of the surface and T-system membrane, and of the solution within the T system, are important determinants of the shape, conduction velocity, and radial spread of the action potential and so are of direct physiological interest. Many of these properties can be determined by impedance measurements.

Even the outer membrane of skeletal muscle fibers is structurally complex. It is not a smooth cylinder; it has irregular shallow folds that allow the fiber to stretch and infolded pockets called caveolae (27, 105) of unknown function.

The T system of skeletal muscle is formed by tubular infoldings of the outer membrane; these tubules branch predominantly in the transverse plane (hence the name T system) to surround the myofibrils and to be in close contact with the SR. Some of the caveolae seem independent of the T system, but a substantial fraction of the caveolae are part of the T system and connect the tubular system and the extracellular space. Indeed tubules probably open into caveolae and are not otherwise connected to the surface membrane.

The sarcoplasmic reticulum is a separate compartment within the muscle; its membrane is not continuous with that of the T system. A substantial gap separates the T membrane and the adjacent SR membrane, a gap accessible to solutes in the sarcoplasmic solution [Eisenberg, Mathias, and Gilai (30); Franzini-Armstrong (41)]. The gap between T system and SR is spanned by well-delineated structures called *pillars* [Eisenberg and Gilai (29); Eisenberg, Mathias, and Gilai (30); Somlyo (96); Eisenberg and Eisenberg (28a)] and amorphous material called *feet* [reviewed in Franzini-Armstrong (42)]. The lumen of the SR is inaccessible to large molecules in the bathing solution that diffuse easily into the lumen of the T system. The possibility that small ions can flow and carry electrical current from T system to SR has recently been discussed at length by Mathias, Levis, and Eisenberg (68). Whatever the validity of their speculations, clearly the SR must be considered a separate compartment from the T system, electrically linked to the T system, if at all, by a specialized system with a specialized molecular structure and function.

The function of each membrane system of muscle is important for contraction. The surface membrane conducts the action potential longitudinally down the fiber, allowing nearly simultaneous contraction of longitudinally distant sarcomeres. The T system conducts the action potential radially into the depths of the fiber, allowing nearly simultaneous contraction of radially distant myofibrils. The T system profoundly alters the shape and longitudinal conduction velocity of the action potential (4, 47). The SR releases calcium in response to an action potential, thus initiating contraction; subsequently it reaccumulates calcium for another twitch.

Each of the membrane systems thus has a distinct functional role; each membrane must therefore be expected to have distinct electrical properties resulting from the differences in molecular structure and in the currents that cross the membranes. The different membrane systems have distinct lipid and protein composition. The density and even type of macromolecular conductors (e.g., sodium channels) must be expected to differ in various membranes.

One must then measure the electrical properties of each membrane. Someday electrical measurements will be made on preparations isolated by biochemical techniques. Eventually the individual membranes and membrane macromolecules will be recovered and purified from homogenized muscle, just as enzymes are recovered and purified today. In the meantime, and even then, there are some advantages to measuring the properties of the membranes of muscles in their natural state within a living muscle fiber.

The electrical properties of muscle fibers can be divided into two categories, linear and nonlinear. The distinction is made because of different analytical techniques. Linear properties (which are independent of the size of the signal used to measure them, like the capacitance of lipid bilayers) can be analyzed with the general theory of linear systems, which is a highly developed and powerful branch of electrical engineering and applied mathematics [see Cooper and McGillem (22) for an introductory text]. Most of the important derivations and results of linear-system theory are in the frequency domain, with the idea of impedance used to relate the sinusoidal output to the sinusoidal input of the linear system. Unknown linear systems are usually best characterized by impedance functions because of the central role of impedance functions in linear-system theory.

Nonlinear properties (which vary with the size of the applied signal, like the ionic conductances of nerve fibers) are much harder to analyze because no general theory describes their complex behavior. Indeed nonlinear systems have such a variety of behavior that a general theory may not be possible. [For example, the general theory of nonlinear systems called Wiener kernel analysis (63, 93, 93a) has been shown to have serious intrinsic limitations (80, 81).] Nonlinear systems are not usually measured in the frequency domain or characterized by impedance functions because such functions do not easily describe complex nonlinear behavior. (See refs. 45, 63, and 104 for exceptions to this statement.) Rather, the behavior of nonlinear systems is usually measured in the time domain, where it is more easily described.

Why should one be interested in the linear electrical properties of biological systems, when their natural activity is usually nonlinear? Linear properties are interesting for several reasons. They describe the biophysical properties of resting membranes; they describe all the electrical properties of some cellular components, like the lumen of the T system; and they

are the resting basis for natural activity. Linear properties can also be analyzed in much more detail than nonlinear properties because a general theory of linear properties is known. Thus the contribution of each membrane system to the linear properties of a muscle fiber can be determined, but it has not yet been possible to perform such a structural analysis of nonlinear properties.

A structural analysis has several steps (35): the structure must be described and measured, assumptions must be made concerning the qualitative properties of each membrane, the expected properties of a cell with that structure and those membranes must be predicted theoretically, the electrical properties of the cell must be measured, and the measurements must be compared with theoretical predictions. If the confrontation of structure, theory, and measurements is satisfactorily resolved, the measurements can then yield reliable estimates of the resistance and capacitance of the various components, membrane systems, and extracellular spaces of the cell. Several of the steps are far more difficult for nonlinear systems than linear systems. In particular the qualitative assumptions concerning membrane properties, the theoretical prediction of electrical properties, and the measurement of electrical parameters with high resolution are very difficult with a nonlinear system. Analysis of linear systems is much easier; they have much less diverse behavior than nonlinear systems. The powerful techniques of linear-system theory are also a great help. Therefore structural analysis so far has been confined to linear systems.

The linear electrical properties of biological preparations can be measured in a number of ways. Sometimes the properties can be deduced from the natural electrical activity of the preparation. For example, the shape and conduction velocity of the action potential can itself be used to estimate some of the linear properties of a muscle fiber [reviewed in Jack, Noble, and Tsien (58)]. Usually, however, the system must be artificially perturbed to measure the electrical properties. For impedance measurements with microelectrodes, current is usually applied and the resulting voltage is recorded. Voltage can also be applied, with a voltage-clamp system, and the resulting current recorded [Fishman, Poussart, and Moore (40) is a recent reference to this technique applied to axons]. The voltage-clamp technique has recently been used for microelectrode recording of impedance (J. L. Rae, R. T. Mathias, and R. S. Eisenberg, unpublished observations) and has advantages.

Whether the perturbation applied to the system is a sinusoidal current or voltage, the result is an impedance measurement: as originally defined, impedance (or its reciprocal admittance) measures the response to experimentally applied sinusoidal currents or voltages, or at least that was the original definition. The definition now must be generalized to include the response to perturbations that are the sums of sinusoids, for example, the response to white noise. The essential characteristic of impedance measurements is that they are made in the frequency domain, whether the perturbing signal is a simple sinusoid or a more complex waveform. Impedance analysis does not analyze directly the time course of the applied signal and its response; rather, the applied signal and response are decomposed into their sinusoidal components by Fourier analysis, and the frequency dependence of the amplitude and phase delay of those components is studied.

Analysis in the frequency domain is justified, despite its apparent complexity (in algebra, if not logic), by the resulting sensitivity and resolution of the measurements. Measurements in the frequency domain typically provide more resolution, by at least an order of magnitude, than direct analysis of time-varying signals.

Impedance measurements are thus made by recording the sinusoids resulting from sinusoids applied to the preparation. Sinusoids are characterized by three parameters: size (i.e., amplitude), phase, and frequency. The amplitude of the sinusoid is its peak value [or equivalently, its root-mean-square value]; the phase angle is the normalized delay between applied current and recorded voltage. In a linear system, the frequency of the sinusoidal response measured is the same as the frequency of the sinusoidal perturbation applied to the system. The frequency of the applied signal is known and thus does not need to be measured. Impedance measurements then involve two quantities: the ratio of the amplitude of recorded voltage to applied current and the normalized delay (phase angle) between the same two signals.

The amplitude of the impedance and its phase angle depend on the frequency of applied current, because the properties of the components of a muscle depend on frequency. The capacitive properties of membranes ensure that both the amplitude and delay (i.e., phase angle) of currents are different at different frequencies. Impedance must therefore be measured at a variety of frequencies if the electrical properties are to be completely specified. The phase angle and magnitude of the impedance (i.e., the amplitude) are functions of frequency. To specify the electrical properties of the muscle fiber one must measure those functions over a band of frequencies wide enough to determine the biologically significant behavior of the muscle. Typically this band of frequencies has been from 0.1 Hz to 10 kHz, although information over a wider bandwidth might be of interest.

The experimental impedance data may be treated in a variety of ways. Measurements of phase angle and magnitude can be converted into other forms, with various algebraic formulas. Such conversions are quite natural and proceed without effort because of a convenient property of impedance functions: they can be described by complex numbers. The phase angle between sinusoidal current and voltage can be written

as the phase angle of a complex number, and the relative amplitude of the sinusoids can be written as the magnitude of the same complex number. The impedance of a preparation is simply a complex function of frequency. At any one frequency the phase angle of that complex function is the normalized delay between sinusoidal current and sinusoidal voltage of that frequency; the magnitude is the relative magnitude of the same two signals. The use of complex numbers and complex algebra to describe impedance functions is well described in textbooks of electrical engineering [e.g., Desoer and Kuh (25)]. Although the algebra has the name "complex," and that name is often thought appropriate by biologists, the rules of complex arithmetic and algebra are the same as, and thus as simple as, those of real arithmetic and algebra. Indeed, complex numbers were discovered (or should we say invented?) because without them one sometimes cannot solve equations involving only real quantities. For example, the solutions of quadratic equations usually involve the square root of minus one.

The reader unfamiliar with complex arithmetic will find that the few days of work necessary to learn to manipulate complex numbers and penetrate the psychological energy barrier will make many disciplines accessible. Indeed, without the knowledge of complex arithmetic little biological use can be made of many results of physics, engineering, or applied mathematics. Practical exercises and the needed theory of complex arithmetic are presented in many texts of college algebra.

Some confusion has been produced by the different descriptions of complex numbers and thus impedance functions. Early workers preferred to describe the rectangular components of complex numbers and impedances, whereas later workers prefer to use the amplitude and phase angle. The relation of these two representations is straightforward. Just as any point can be described by its rectangular coordinates x and y or its polar coordinates ρ and θ, so can any impedance or complex number be described by its real and imaginary component or its amplitude and phase. (The real component is neither more nor less tangible than the imaginary component. The name *real* is simply an unfortunate historical synonym for x coordinate; the name *imaginary* is a synonym for y coordinate.) Later workers and electrical engineers prefer to describe impedance functions by their amplitude and phase for a number of practical reasons, particularly because phase plots are such a sensitive measure of the properties of many systems. Conversion of impedance data from one representation to another is no more complicated than conversion from polar to rectangular coordinates.

In fact the question of the proper representation of impedance functions is somewhat academic now. Measurement, computation, and theory are done largely by computers that directly perform complex arithmetic without explicit reference to the components of the complex number. Only the output programs, meant for human evaluation, convert these complex numbers into phase angles and magnitudes or real and imaginary parts.

Figure 1 illustrates the sensitivity of impedance measurements compared with transient measurements and the sensitivity of phase plots compared with other impedance plots. I calculate the response of a simple resistance-capacitance (RC) circuit that contains the essential features of many biological preparations. The circuit contains a series resistance (R_s), a "membrane" resistance of 2,000 Ω, and a "membrane" capacitance of 6 μF. Plots are given in Figure 1 for two different values of R_s, 0 and 100 Ω. The responses shown are either to sinusoids or to a step function of current. The response to sinusoids is plotted in three ways: *1*) as the phase angle of the impedance versus log frequency, *2*) as the magnitude versus log frequency, and *3*) as the real part of the impedance versus the imaginary part of the impedance (often called a Cole-Cole or Nyquist plot). The response to a 1-μA step function of current is also shown.

Consider first the response to a step function. The effect of R_s is rather small and can be seen most clearly only at very short times. Indeed the shape of the transient response is independent of the value of R_s. The constancy of shape causes serious difficulties in the real world. If there is instrumental confusion at short times (as is usually the case) or if the system is somewhat distributed (either spatially or statistically), the initial jump in the waveform is obscured. Then the value of R_s cannot be determined with any accuracy.

Figure 2 shows the responses of a more relevant circuit, one that is a lumped approximation to the electrical properties of 1 cm^2 of the outer membrane of a frog muscle fiber together with its associated T system. The two curves shown are for two different values of R_s, 200 and 300 Ω.

Figures 1 and 2 clearly show that the phase plot is much more sensitive than the other plots. The size and more importantly the shape of the phase plot vary substantially as parameters change value. This is not a special property of the circuits illustrated but is a rather general finding. In fact as circuit complexity increases, the advantages of the phase plot increase, and the advantages of impedance measurements in general also increase. This point is discussed at some length in Eisenberg and Mathias (35), where reference is made to some of the applied mathematics literature comparing analysis of frequency domain and time domain. We also consider integrals of transients and show that such integrals may be more helpful than the transients themselves in characterizing linear systems. In fact integrals of transients may sometimes be more helpful than impedance measurements.

Impedance measurements are important in muscle physiology. They allow specification of the linear prop-

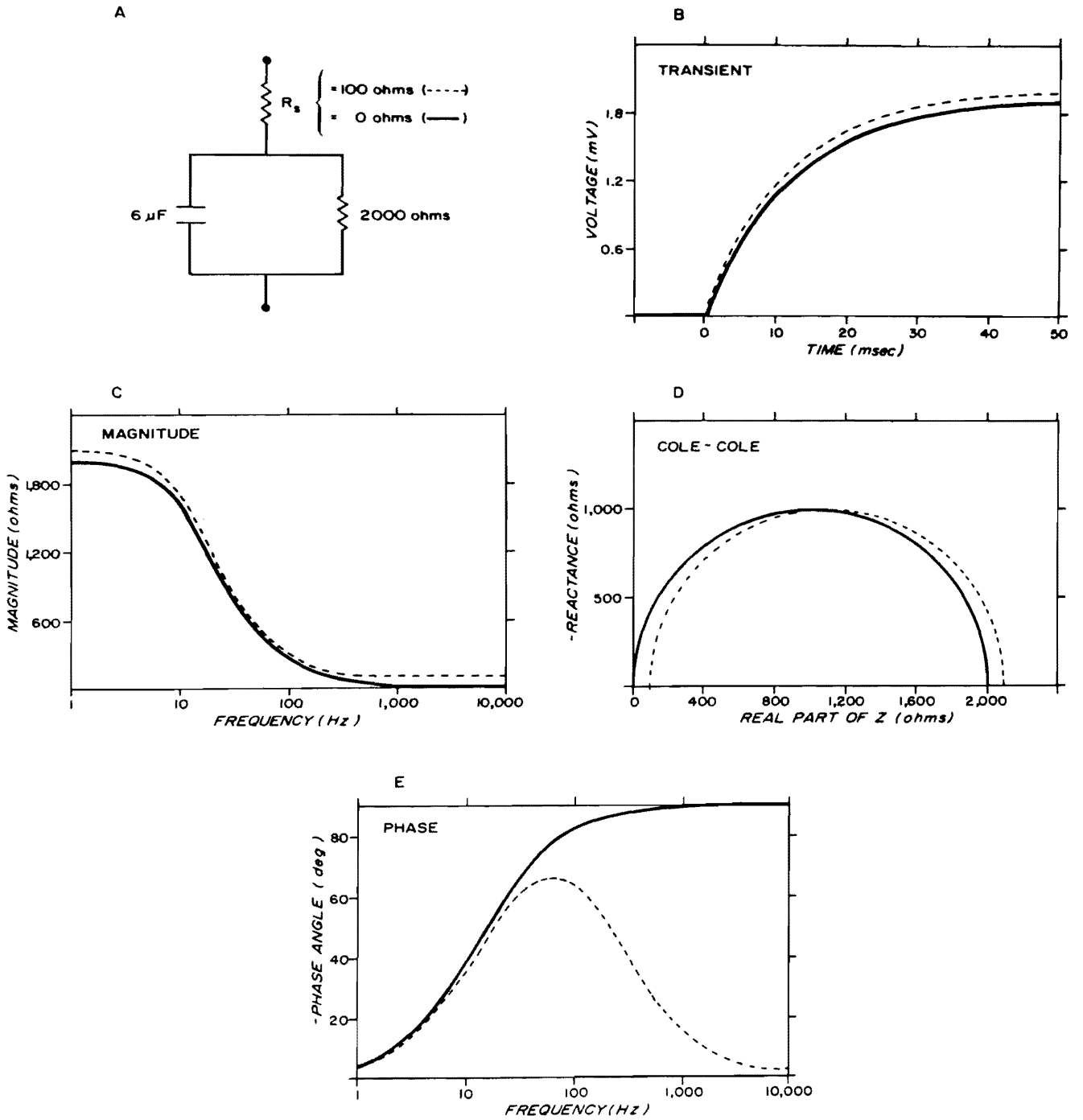

FIG. 1. *A*: simple circuit with 2 different values of series resistance. Other panels show response of the circuit plotted in different ways. *B*: transient response to a step function of current (1 μA) applied at time 0. *Dashed line*, response with 100-Ω series resistance, is a vertical displacement of response with no series resistance (*solid line*). Therefore these responses are hard to tell apart and are hard to use to measure the series resistance. *C*: magnitude of impedance of circuit measured with sinusoidal currents of the frequency shown on the abscissa. The effect of a series resistance is upward displacement of the curve without change in shape. *D*: plot of imaginary part of the impedance vs. real part of the impedance. Although frequency is not an explicit variable, variation of frequency and the subsequent variation in the real and imaginary parts of the impedance produce the curve. Again the effect of a series resistance is a simple translation of the curve without change in shape. *E*: phase angle between sinusoidally applied current and voltage. Effects of series resistance are substantial, making it easy to measure series resistance.

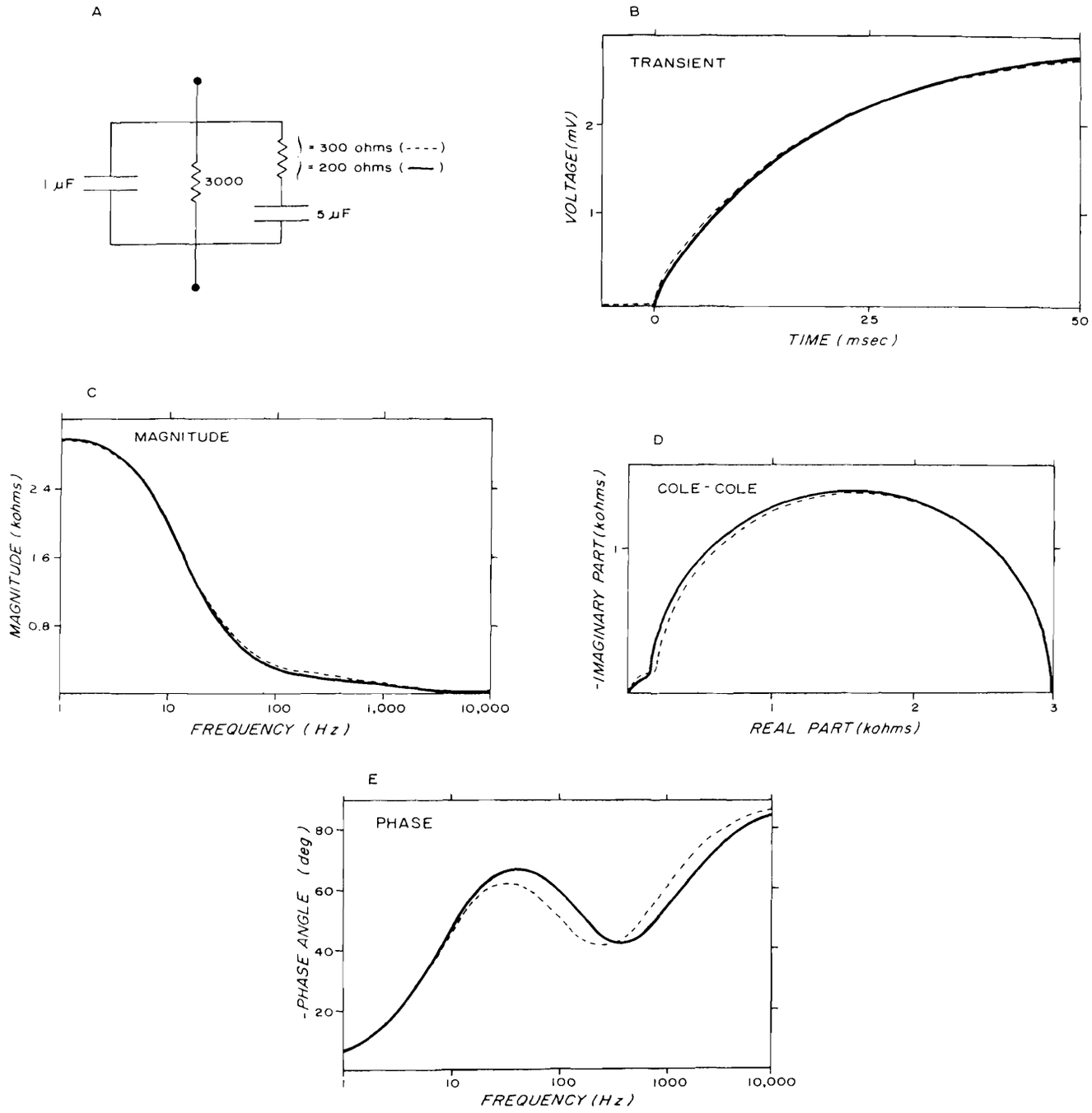

FIG. 2. *A*: lumped approximation to properties of 1 cm^2 of surface membrane and associated T system of frog skeletal muscle with 2 different values of series resistance indicated. *B*: transient response of lumped circuit to a step function of applied current. Tiny difference between the 2 curves makes measurement of series resistance difficult from transient responses. *C*: magnitude of the response to sinusoidal currents of different frequencies. Effect of series resistance is small, making measurement difficult. *D*: a plot of imaginary vs. real part of the impedance. Effects of series resistance and of the 1-μF capacitor are hard to see in this plot. *E*: phase angle between sinusoidally applied current and voltage. Effects of series resistance are substantial, making it easy to measure series resistance.

erties of the muscle fiber with a high degree of resolution. Combined with the appropriate morphological measurements and theory, impedance measurements can determine the resting electrical properties of several components of a muscle fiber—the surface membrane and T system, at least. Knowledge of the resting properties is important in itself and also gives insight into many of the active properties.

METHODS AND TECHNIQUES

The main advantage of impedance measurements is their accuracy and resolution. This advantage, however, can also be a disadvantage. Impedance measurements are just as sensitive to the properties of the recording system as they are to the properties of a muscle fiber. Artifact is more apparent in the frequency domain than in the time domain. Techniques and recording systems developed for transient measurements are usually unsatisfactory for impedance measurements.

It has taken a number of years and the work of many laboratories to develop reliable methods of measuring impedance from single cells with microelectrodes. Other methods are undoubtedly possible but have not yet been tested on previously studied preparations. I therefore pay most attention to microelectrode methods that have been extensively used and tested by several laboratories.

Microelectrode Techniques

The basic plan of measurement was introduced by Falk and Fatt (38). They inserted one microelectrode into a muscle fiber to pass sinusoidal current from the fiber interior to an external electrode in the bath. They recorded the induced potential with another microelectrode inserted into the fiber. The phase angle and relative delay of the two signals determined the input impedance $Z(j\omega)$; Z (in Ω) is a complex number, a function of $j\omega$ (in rad/s). The angular frequency ω equals $2\pi f$, where f is the frequency (in Hz), and $j = (-1)^{1/2}$. The input impedance is a property of the whole length of the muscle fiber, the sarcoplasm, the outer membrane, and the inner (i.e., tubular and perhaps SR) membranes as well. Moreover Z is simply related to r_i (in Ω/cm), to the resistance of the sarcoplasm in a unit length of fiber, and to the shunt admittance y in a unit length of fiber (in S/cm)

$$Z(j\omega) = \tfrac{1}{2}\,(r_i/y)^{1/2} \qquad (1)$$

the resistance r_i is related to the resistivity R_i (in $\Omega \cdot$ cm) by $r_i = R_i/\pi a^2$, where a is the radius (in cm).

Falk and Fatt (38) interpreted the shunt admittance y as the result of all current flows from sarcoplasm to the extracellular space. They allowed it to have two components, the admittance y_s of the surface membrane (in S/cm) and the admittance y_e of the inner membranes (also in S/cm)

$$y = y_s + y_e \qquad (2)$$

where $y_s = G_s + j\omega C_s$; that is, the admittance of the surface membrane consists of the conductance of the surface membrane G_s (in S/cm) and the capacitance C_s of the surface membrane (in F/cm). The linear properties of the T system are given by y_e, which is properly described later.

The formula of Falk and Fatt (38) requires some justification. First, it must be understood that their equation is assumed and not derived. A derivation must proceed from the fundamental laws of the electric field and statements of the structure of a muscle fiber; such a derivation for a tubular system is only recently available [R. T. Mathias, personal communication; see Peskoff (86) for a related derivation for a system of clefts]. Second, Falk and Fatt (38) assumed that the only significant electrical property of the sarcoplasm was its resistance—they did not allow the sarcoplasm or SR to have an impedance. This assumption has been shown to be correct experimentally by Schneider (94) and Mobley, Leung, and Eisenberg (72, 73).

Falk and Fatt (38) assumed, for the most part, that the membrane capacitance behaved ideally, with a phase angle of 90°. This assumption has been questioned on the basis of early impedance measurements on suspensions of cells [reviewed in Cole (20)], which were interpreted as evidence for a membrane capacitance with a reduced phase angle of about 70°. Hanai and co-workers (6, 51) have shown, however, that the theory used by early workers to interpret the impedance of suspensions was incorrect: it did not properly describe conductors with thin membranes, and it did not properly describe cells of nonspherical shape. When a revised theory is used, the impedance data do not require a membrane capacitance with a reduced phase angle. Membrane capacitors of 90° phase angle produce essentially perfect fits to impedance data from suspensions of cells.

Early impedance measurements on axons were also taken as evidence for a reduced phase angle of the membrane capacitance. The recent results of Fishman, Poussart, and Moore, and other references cited therein (40), together with the work of Takashima and Schwan (99), show that the linear properties of squid axons are highly sensitive to corrections for series resistance, to the length of the axon from which measurements are made, and to reactive current that flows through the sodium (i.e., tetrodotoxin-sensitive) and potassium (i.e., tetraethylammonium-sensitive) channels. (A reactive current is one that is delayed or advanced in time from its driving voltage: it has a phase shift. Currents through capacitors and inductors are reactive, whereas currents through resistors are not. Currents through time-dependent ionic conductances are generally shifted in phase. If the perturbing signal is sufficiently small to make the time-dependent ionic conductance quasi-linear, the current through an ionic channel can be described as an ionic reactance.)

The deviations from a phase angle of 90° reported by Cole and colleagues can probably be explained by the effects just listed, without invoking an imperfect membrane capacitance.

There are certain important technical difficulties in the measurements of Falk and Fatt (38). The first is the difficulty of interpreting results in which the source of current is very small, almost a point. The resulting three-dimensional effects are not trivial and can be made negligible only by the special precautions

taken by Mathias, Eisenberg, and Valdiosera (67). Little more is said here about such effects since they have been extensively analyzed and reviewed (3, 32, 34, 87).

The other type of problem arises from the stray capacitances around the microelectrode and in the devices attached to the microelectrode. Since microelectrodes have impedances of the order of tens of megohms, and measurements are needed to frequencies of many kilohertz, stray capacitances of tenths of picofarads are serious problems. Indeed one capacitance—the capacitance between the top of the current microelectrode and the top of the voltage-recording electrode—needs to be smaller than 10^{-15} F to be negligible! Falk and Fatt (38) and subsequently Eisenberg (31) (their graduate student) faced these difficulties without operational amplifiers. They refined the standard microelectrode recording apparatus [Fatt and Katz (39); Nastuk and Hodgkin (77)] and made corrections (determined by measurement to a large extent) for the capacitive artifact. Despite the care with which the corrections were made (both experimentally and analytically), the size of the correction at frequencies above 1 kHz was disquieting and must bring into question the accuracy of their measurements. Freygang et al. (46) used a new recording circuit [suggested by A. Bak (46)] that, at least in principle, was much less sensitive to capacitive artifact. They did not extend their measurements beyond 1 kHz, however, because of difficulties in implementation. Without measurements in the frequency range 1–10 kHz, it is difficult to be sure of the amount of artifact in the lower frequency ranges. Artifact is often obvious in the higher frequency range because it introduces spurious maxima/minima into impedance plots or produces "unallowed" (i.e., unanticipated) behavior. In the lower frequency range the artifact does not produce such dramatic behavior and thus is not so obvious. Qualitatively invisible artifacts can be quantitatively quite significant, and one cannot be sure of the quantitative reliability of data taken with a limited frequency range. Although these are fairly serious criticisms, Gilai (48), Nicolaysen (78), and Schneider (94), all of whom used versions of the circuit of Freygang et al. (46), seem not to have substantial error in results from the frequency range below 800 Hz.

Circuits have also been made that try to compensate for some or all of the stray capacitances. Such circuits have not been very successful, because of *1*) difficulties in implementing an accurate negative capacitance, *2*) difficulties in adjusting the value of the negative capacitance to precisely compensate the actual stray capacitance, and *3*) the instability of the stray capacitances in and around microelectrodes. Even when implemented with modern electronics [e.g., Suzuki, Rohlicek, and Fromter (98)], the reliability of circuits with negative capacitance must be demonstrated by measurements in the frequency domain, preferably of phase angle.

Valdiosera, Clausen, and Eisenberg (100) modified the recording circuit of A. Bak (46) and made measurements with small and measured artifact up to 10 kHz. They showed that all the artifacts identified by Falk and Fatt (38) could be made negligible but that a significant artifact remained. The significant artifact was caused by capacitive coupling between the interior of the microelectrodes and the bathing solution. This artifact had not been previously recognized because its frequency dependence is similar to that of the interelectrode artifact. The current through the capacitances between the microelectrode interior and bath flows through the resistance of the bathing solution, produces a substantial extracellular potential, and thus distorts the recorded potential. Coupling into the bath could be limited but not removed by painting the microelectrodes with a conducting silver paint. The painting technique, introduced (I believe) by Valdiosera, Clausen, and Eisenberg (100), also reduces the interelectrode capacitance to negligible amounts.

The circuitry used by Valdiosera, Clausen, and Eisenberg (100) was accurate but inconvenient. It requires the repeated measurement of the resistance of the voltage microelectrode, since the gain of the circuit depends on the microelectrode resistance. In their circuit the microelectrode resistance also appears as a resistive load on the muscle fiber, typically drawing some 10 nA of DC from the fiber. The DC can be removed in a more complex version of the circuit, independently developed by R. T. Mathias (personal communication) and Sachs and Specht (91). Those workers applied feedback to the noninverting input of the voltage-recording amplifier [marked $\mathbf{A}(j\omega)$ in Fig. 2 of ref. 100]. The feedback was derived from the DC component of the output of that amplifier. By sensing and feeding back only the DC component (with, for example, a low-pass filter circuit with bandwidth from DC to 0.01 Hz), Mathias and Sachs removed the resistive load on the muscle fiber.

The circuit of Valdiosera, Clausen, and Eisenberg (100), either in its original or modified form, gives the widest reported bandwidth with microelectrodes because it is the only circuit that permits the bath to be at ground potential while the top of the voltage-recording microelectrode is also held close to ground potential. This arrangement minimizes current through the stray capacitance from the inside of the microelectrode to the bath and ground. One early implementation had a bandwidth (−3-dB point) of some 300 kHz with microelectrode attached, and this bandwidth undoubtedly could be extended with newer amplifiers if necessary.

Despite the spectacular bandwidth of the Valdiosera circuit, it remains inconvenient. If the top of the microelectrode is at virtual ground while the bath is also close to ground, there must be current flow through the microelectrode (driven by the membrane potential of the muscle fiber). In fact the Valdiosera circuit actually measures that current flow and not the membrane potential! Thus determination of the size

of the membrane potential with this circuit requires the microelectrode resistance to be known, a serious inconvenience. Mathias, Rae, and Eisenberg (69, 70) therefore developed another wide-band circuit, which keeps the top of the voltage-recording microelectrode close to ground potential. Their circuit avoids the practical difficulties of the Valdiosera circuit because it does not require current to flow through the microelectrode, but the new circuit has some limitation in bandwidth.

Mathias, Rae, and Eisenberg modified the feedback-follower circuit of Eisenberg and Gage (33) first by shielding the microelectrode with conductive paint (69) and then by adding circuitry to allow direct recording of the current flowing through the preparation (70). The article by Mathias, Rae, and Eisenberg (70) contains extensive discussion and detailed diagrams of a practical circuit that allows measurement to some 10 kHz with little or no capacitive artifact. With this circuit it is possible to measure impedance reliably, conveniently, and over a sufficiently wide bandwidth.

Of course microelectrodes are not the only way to record potential within a muscle fiber. Hille and Campbell (53), for example, have recorded intracellular potential with a modification of the Vaseline-gap apparatus previously used on vertebrate nerve. One might expect that such a system could give an even wider bandwidth than the circuits previously discussed because high-impedance microelectrodes are not involved, and such is probably the case. The history of recording with microelectrodes suggests that a certain degree of caution is appropriate, however: between the principle and the reality lurks the hidden artifact! Until the artifacts of the Hille-Campbell arrangement are measured and analyzed in the frequency domain, it will not be safe to assume they are negligible.

Analysis of Sinusoidal Data

The earliest records of impedance with microelectrodes (31, 38, 94) were measured with oscilloscopes using Lissajous patterns photographed and analyzed by hand. This procedure was not speedy, typically requiring 2 days of film reading and computation with tables of trigonometric functions to acquire 24 data points. Further, although the variance of the estimates appeared quite small, even with signal-to-noise ratios of 2–4, the possibility of systematic errors was always present. Thus it seemed wise to use instrumentation rather than eye and brain to measure phase angle and amplitude.

A variety of instruments can be used for this purpose, ranging from the phase meter of Freygang et al. (46), to the phase-sensitive detectors of Valdiosera, Clausen, and Eisenberg (100), to the computer programs of Nicolaysen (78). I do not discuss these implementations, since they undoubtedly will be supplanted by the time this chapter is published. The instrumentation to measure impedance (phase and amplitude or real and imaginary parts) clearly should be accurate, insensitive to contaminating noise, and quick and easy to operate. Automation in data acquisition is desirable since the results must be entered into a computer for subsequent analysis. Of course the cheaper and less complex the instrumentation, the better. Although many of these characteristics are obtainable from the instrumentation that measures sinusoids, others are not. It is particularly difficult to measure rapidly with sinusoids, because all measurements must be made one frequency at a time with each measurement delayed until a steady state is reached and noise is averaged out. Averaging the response at one frequency takes a substantial amount of time, depending on the signal-to-noise ratio. Even if the signal far exceeds the noise, one must average for something like $10/f$ seconds, where f is the frequency of the sinusoid being measured. Thus the total duration of the measuring period is mostly determined by the low-frequency points, and measurements at many frequencies must take a long time [see Bendat and Piersol (ref. 10, chapt. 8) and Magrab and Blomquist (ref. 62, p. 81) for a quantitative analysis of the duration of analog measurement].

The requirement for a long duration of measurement is an example of a law familiar to experimenters called "the conservation of troubles." The price paid for the improved sensitivity and resolution of sinusoidal methods is the longer duration of the measurements. Sinusoidal measurements provide much more accurate information about a system but are much slower than transient measurements.

The problems associated with measurements of long duration are serious in biology, particularly when microelectrodes are used. Most biological preparations have random drift in their properties on a slow time scale; most physiological preparations also drift systematically, toward death. Because penetration by microelectrodes accentuates both random and systematic drifts, the measurements of biological impedance need to be done as quickly as possible. The faster the measurements are made, the more successful experiments can be done, and the greater are the range of experimental conditions that can be investigated, including conditions that eventually damage the fiber.

The speed of measurement of sinusoids can be improved considerably by swept-frequency methods (48) and by automating those measurements with a digital computer (78). These changes, however, do not provide a qualitative improvement in the measurement of sinusoids. The most important limitation on the speed of measurement of sinusoids is inherent in the method, as already pointed out: measurement at a given frequency requires many periods of the sinusoid at that frequency. Thus the total duration of the experiment is set largely by the lowest frequency points measured. These in turn are set by the longest functionally important time constants of the system and the resolution (i.e., number of frequency points) required to determine the experimental properties of the system, as well as the signal-to-noise ratio at each

frequency. In practice, measurement of 24 data points at frequencies between 1 Hz and 10 kHz takes many minutes.

Impedance Analysis With Fourier Techniques

One way to decrease the duration of the experiment is to apply all sinusoids at once. If the perturbation applied to the system were the sum of sinusoids of many frequencies, it would be possible, at least in principle, to measure the response to that single perturbation. Then one could determine the response to all the frequencies represented in the perturbing signal. In other words, if a broad-band signal is applied to a muscle fiber and the resulting output is analyzed into its component frequencies, one should be able to measure the response (of a noise-free system) over the entire frequency range from a single measurement. The duration of that measurement would be the duration necessary to determine the response to the lowest frequency component. For example, to measure the response of a muscle fiber from 1 Hz to 10 kHz (or to any higher frequency for that matter), it would be necessary to take data just long enough to determine the response at 1 Hz.

The preceding paragraph describes the principles of Fourier analysis with white-noise input. If the perturbing signal is noise, it contains energy at all frequencies. If the response to that applied signal is decomposed by Fourier analysis into its frequency components, then sufficient data are available to determine the broad-band response, even from a short-duration record. One must acquire enough data to determine the lowest frequency point, but the higher frequency points are free, as it were.

Of course the higher frequency points are only free in principle. The implementation of this Fourier analysis is not trivial, and the number of frequency points computed is an important determinant of the cost of the Fourier analysis.

The Fourier analysis of signals is well described in the engineering literature, although perhaps it is not so well known to physiologists. Therefore a fairly extensive listing of references may be useful. Cooper and McGillem (22) provided a textbook that assumes little background and provides a clear and elementary introduction to the analysis of linear systems, including a few chapters on Fourier analysis. The other references concentrate on Fourier analysis. The book by Koopmans (60) is moderately difficult but very useful and should be accessible to most readers comfortable with calculus. The books by Papoulis (82, 83) are advanced with a rather personal approach, but contain a wealth of useful information. The book by Oppenheim and Schafer (79) is advanced, elegant, but rather abstract. Brillinger (15) and Hannan (52) provide statistical analysis and proofs of the relevant theorems; despite their rigor the results of the analysis can be read by the nonstatistician, although the proofs of the theorems are more difficult. A number of books are excellent on specialized subjects. For example, Brigham (14) includes an outstanding discussion of the digital (i.e., discrete) Fourier transform and presents ingenious and useful graphs to explain the relation of the discrete and continuous Fourier transforms; For a long time, Bendat and Piersol (10, 11) provided the only references with a reasonably complete and practical discussion of impedance measurements with Fourier techniques. Carter (15a) and Kay and Marple (58a) have recently added significantly to this literature.

This is not the place, and I am certainly not the author, to attempt a brief synthesis of this literature. There are several critical results, however, that seem important enough to present here, even though they are not original. A practically oriented tutorial treatment of impedance measurements with Fourier techniques would be helpful to both physiologists and engineers. Perhaps when the technology of Fourier measurements reaches maturity, such a review will appear in the engineering literature and supplant the next few paragraphs.

The first step in a Fourier analysis is the conversion of the analog signal into numbers. The conversion process involves many pitfalls, most of which have been removed from presently available instrumentation. One problem for digital measurements remains in much equipment designed for transient measurements. Impedance measurements require simultaneous measurement of the input and output to determine phase (i.e., time delay), whereas transient measurements often do not. It is not sufficient to measure one signal, then the other (as is often sufficient for transient measurements). Rather, the jitter and delay between the measurements must be small compared to the period of the signal with the highest frequency of interest. That is the only way to avoid artifactual phase errors. Analog-to-digital (A/D) converters are available with the feature of "simultaneous sample and hold" and the jitter and delay in such converters are negligible in the frequency range of interest here.

The central difficulty in digital Fourier analysis is the discrete nature of the numbers used to represent a continuous signal. The numbers are measured and computed with a finite number of digits, and the numbers represent samples at a finite number of times. In contrast, the analog signals from the muscle fiber are continuous in both amplitude and time. The quantization of the amplitude of digital signals need not be a problem now, although early A/D converters (still found on some instruments) often were not sufficiently linear for phase measurements. The linearity of present 12-bit A/D converters and the word length of minicomputers are sufficient (if properly used) to make this source of error negligible. The programmer should be aware, however, that computations of the power in a waveform usually require the temporary use of double precision, and power computations are needed for estimates of impedance.

Quantization in the time domain is more serious,

however. Here the physiological investigator reaches limits set by the speed of A/D converters, the size and cost of memory, and the speed of Fourier analysis of blocks of many time points. These factors set the practical limits of resolution and bandwidth for impedance measurements.

The fundamental difficulty caused by sampling (i.e., quantization) in time is aliasing, the inability to distinguish different waveforms ("aliases") if insufficient samples or too low a sampling rate is used. In fact one might expect that no finite amount of sampling could allow waveforms to be distinguished, since no finite number of (x, y) coordinates can specify a function! Luckily, however, the physical origin of our analog waveforms avoids this problem. If a waveform of physical origin (waveforms that are a tiny subset of all possible functions) contains no energy above the so-called folding frequency F_f (which equals half the sampling rate S), then it can be reconstructed uniquely from its samples, according to the sampling theorem (see engineering references 10, 11, 14, 15, 22, 52, 60, 79, 82, 83 already cited). Ambiguity arises only if there is energy in the analog waveform above the folding frequency. If there is energy above the folding frequency, however, the ambiguity produced by aliasing is a necessary consequence of sampling *and cannot be removed once the digital samples are taken* without a priori knowledge of the signal sampled.

Figure 3 illustrates the problem. If digital samples are taken at a rate of $S = 8$/s (samples shown as filled circles in Fig. 3), a sinusoid with a frequency of 4 Hz cannot be distinguished from a sinusoid of 0 Hz (horizontal line shown). Similarly, sampling at 5/s (open circles) cannot distinguish a sinusoid of 1-Hz frequency from a sinusoid of 4-Hz frequency. A sinusoid of frequency greater than half the sampling rate cannot be distinguished from a sinusoid of frequency less than half the sampling rate. Similarly a waveform with energy at frequencies above half the sampling rate will not be distinguishable from a waveform with all its energy below half the sampling rate. In fact the energy

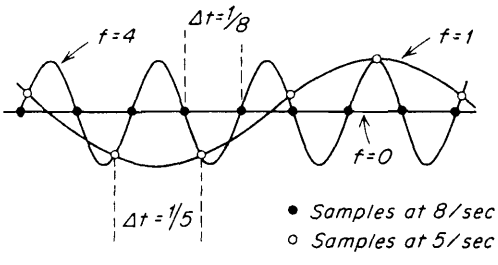

FIG. 3. Three sinusoids show effects of digital sampling. Samples taken at 8/s (*filled circles*) from a sinusoid of 4-Hz frequency are the same as samples taken from a sinusoid of 0 Hz (i.e., DC). Thus samples taken at this rate cannot distinguish between the 2 sinusoids—the sinusoids are said to be aliases of one another. Similarly samples taken at 5/s (*open circles*) from a sinusoid of 1-Hz frequency cannot be distinguished from samples from a sinusoid of 8 Hz; they also are aliases. Aliasing is a direct consequence of sampling. Once digital samples are taken, aliases cannot be distinguished. Precautions can be taken before sampling to minimize problems of aliasing.

density $E(f_0)$ at a frequency f_0 (in Hz, with $f_0 \leq S/2$) cannot be distinguished from energy at the frequencies $S \pm f_0$, $2S \pm f_0$, ..., $nS \pm f_0$, and so on, where n is any integer. The total energy $\hat{E}(f_0)$ is the sum of the energies $E(f_n)$ at all the frequencies $f_n = nS \pm f_0$ (summation over n) and not the energy density $E(f_0)$ at the single frequency f_0 that one would wish to measure.

Sampling at an infinite rate would clearly remove the problem of aliasing. This is not possible, however, nor can it be approached with present-day technology. There are serious technological limitations on the speed of high-resolution A/D converters, although these will disappear with the growth of technology. There is also a limit on the number of data points that can be handled in subsequent digital Fourier analysis, however, and this limitation is unlikely to disappear in the next few decades. If a waveform is sampled at high speed, a large amount of data is acquired, perhaps more than is actually needed and more than can be easily stored or rapidly computed. Thus samples should be taken as frequently as necessary but as seldom as possible.

One method of sampling has been suggested that might avoid aliasing of a waveform containing energy at all frequencies. If samples are taken at random time intervals [see Masry and Lui (65)], aliasing does not occur. Masry's scheme requires sampling at arbitrarily short intervals (i.e., at arbitrarily high rates) and eventually produces an infinite number of samples in a given frequency range. The theoretical advantages of random sampling may not survive the practical restrictions of a limited sampling rate and a finite number of samples in a given frequency range. In any case, simulations including these and other practical limitations are needed before Masry's scheme could be safely applied to unknown systems.

Although manipulation of periodic sampling cannot avoid aliasing, manipulation of the analog waveform can. If the analog signals that will be digitized contain no significant energy above the so-called folding frequency F_f, they can be uniquely specified by their samples. There will then be no aliasing. Filtering of the input signals (in steep low-pass, so-called antialiasing filters) circumvents aliasing, because after the filtering, there is no energy present to alias. The use of such filters has an added benefit, which applies to transient measurements as well. The steep low-pass filters decrease the amount of noise energy at irrelevant frequencies. Thus the filtering used to remove aliasing also significantly improves signal-to-noise ratios.

Some of the details of filtering, which are critical to the implementation of impedance measurements, are not well discussed in the literature, at least to my knowledge. I present analysis only of analog low-pass filtering. Instruments recently made by Hewlett-Packard (e.g., model 5420A) use a combination of analog and digital low-pass filters with great success (as determined by direct measurement), but the techniques

involved are largely proprietary and so I am not able to discuss them in a critical manner. [See Patkay, Chu, and Wiggers (84) for an outline presentation of the digital technique.]

An idealized low-pass filter is shown in Figure 4 in a conventional logarithmic plot. The steep filters actually used in most antialiasing schemes have more complex characteristics, but the idealized filter characteristic shows the important points. The ordinate of the plot represents the gain of the filter (on a logarithmic scale), and the abscissa represents the log frequency. The bandwidth of the filter is called B_w and the cutoff frequency is called $F_A = kB_w$, where k is a constant determined by the steepness of the filter. Where M is the average slope of the filter characteristic between B_w and F_A in decibels per octave, $k = 2 \times 10^{A/M}$. The cutoff frequency is the frequency F_A at which a signal is attenuated by A, a value large enough to ensure no significant effects of aliasing compared with other errors. If $-A$ is 70–100 dB (i.e., 0.03%–0.001%), most workers assume that there will be no significant effect of aliasing from frequencies above F_f. Aliasing is caused by the total energy of the incoming signal at frequencies above F_f; therefore the low-pass filter must attenuate the total energy above F_f sufficiently to eliminate significant aliasing at any one frequency below F_f. This is not a stringent requirement in typical physiological situations but can be limiting in some applications where there is a tremendous concentration of energy in a small frequency band somewhere above F_f.

Figure 5 shows the optimal relationship between the filter characteristics and the sampling rate, with linear scales. A linear scale is used because the distribution of aliases (described previously) occurs on a linear frequency scale, not a logarithmic one. The optimal relationship between sampling and filter characteristics is determined by two conditions: *1*) that signals in the usable band, extending to B_w, be free of aliasing and *2*) that the sampling frequency S be as close to B_w as possible. These conditions are met by setting the folding frequency as shown, with F_f lying between B_w and F_A. The relationship illustrated of $F_f = \frac{1}{2}(F_A + B_w)$ satisfies both conditions optimally.

The sampling rate $S = 2F_f = F_A + B_w$ must be much larger than twice the usable frequency B_w because the low-pass filter is not infinitely steep. Consequently many of the samples taken and many of the computations subsequently made on those samples are wasted. The wasted samples must be taken and the computations made to perform Fourier analysis on the frequencies below B_w. The wasted samples are not useful, however, because they give results in the frequency range between B_w and F_A, a range in which there is significant aliasing. Indeed the fraction of usable frequency points (those uncontaminated by aliasing) is simply $2/(1 + k)$. If the filter were infinitely steep, k would equal 1 and no samples would be wasted. If the low-pass filter were a typical Butterworth filter with a slope of 24 dB/octave ($k = 7.5$), only about 20% of the computed frequency points

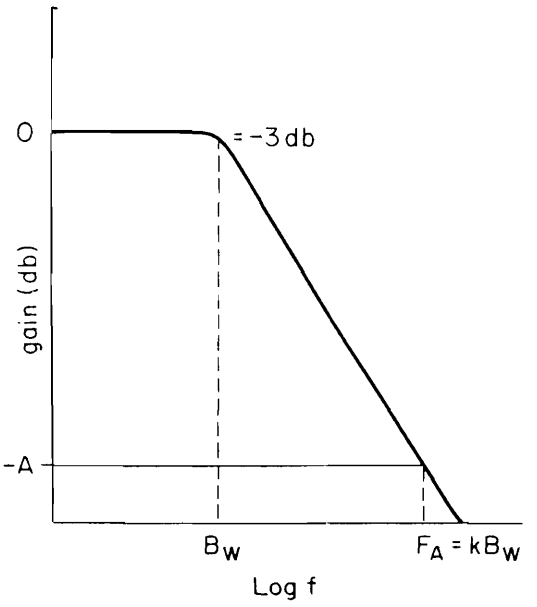

FIG. 4. Idealized filter response shown in a log-log plot. Ordinate is the gain in dB. Frequency at which the gain is −3 dB is shown as B_w; frequency at which the gain is down $-A$ dB is shown as $F_A = kB_w$. Filters recommended for use in impedance measurements have more complicated responses, with ripple in the pass band (DC to B_w), nonlinear dependence on frequency, and a finite amount of gain in any range of frequencies. All these features of practical filters are introduced to allow a very steep dependence of gain on frequency, i.e., as small a value of k as possible.

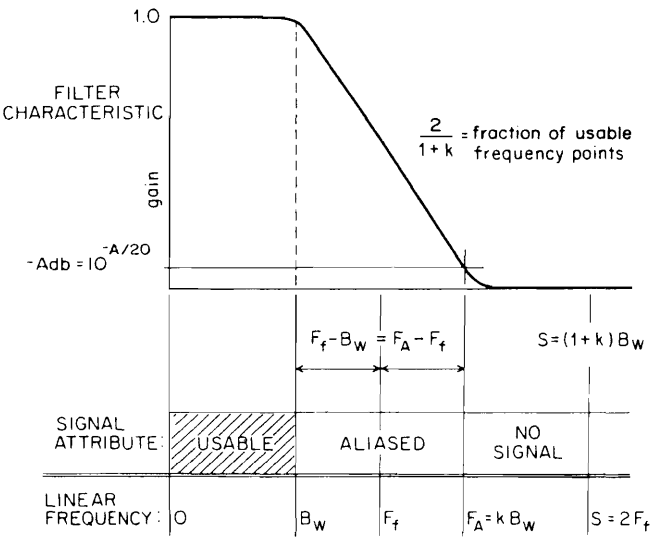

FIG. 5. Optimal relation between sampling rate, bandwidth, and folding frequency. All signals above B_w contain aliased energy. Adjustment of the folding frequency to halfway between B_w and F_A allows closest approach of F_A to B_w without introduction of aliased energy below B_w. This adjustment maximizes bandwidth and fraction of usable frequency points. Linear plot is used for convenience. Filter is shown with a limiting gain at high frequencies to be more realistic.

would be useful for $A = -70$ dB. Even if the filter slope were 48 dB/octave ($k = 2.7$), which is the steepest Butterworth filter available commercially (to my knowledge), only some 50% of the points would be useful. To minimize unnecessary computation and thus minimize cost while maximizing bandwidth and resolution, it is important to have the steepest filter possible. These are called Cauer elliptic filters (61) and are currently available with slopes of 110 dB/octave ($k = 1.6$), allowing about 75% of the computed points to be used.

Instrumentation Noise

With the procedures just discussed, it is possible to measure impedance with wide-band signals analyzed by Fourier methods. The only quantities directly used to estimate impedance are the power and cross-power spectra. These have been computed in two different ways. In the early engineering literature, before the discovery of the fast Fourier transform algorithm, power spectra were computed from correlation functions. That is, correlation and cross-correlation functions (defined in engineering texts previously cited) were computed from the input- and output-voltage waveforms. Then a Fourier transform of these correlation functions was taken to determine the power and cross-power spectra.

After the discovery of the fast Fourier transform, it was much faster to measure the power spectra directly from the discrete Fourier transforms of the input and output signals. Thus almost all measurements of power spectra with Fourier transforms use the following formulas

$$\begin{aligned} G_{xx} &= X(f) \cdot X^*(f) \\ G_{yy} &= Y(f) \cdot Y^*(f) \\ G_{xy} &= X^*(f) \cdot Y(f) \end{aligned} \tag{3}$$

where f is the frequency; a superscript * indicates the complex conjugate of the Fourier transforms $X(f)$ and $Y(f)$ of the analog waveforms $x(t)$ and $y(t)$; G_{xx}, G_{yy}, and G_{xy} indicate the power spectra of the input, the output, and the cross power between input and output, respectively.

The power spectra defined in Equation 3 are computed for a single series of time points, usually between 128 and 2,048 points, called a block of data. To measure impedance in the presence of contaminating noise, it is necessary to average several such blocks of data; we use the notation G_{xx}^k to indicate the kth block of data. Certain other difficulties arise if the signals are contaminated by instrumentation noise; these are worth mentioning, since they have not been well reviewed in the literature, at least to my knowledge.

Figure 6 shows a typical physiological situation in which an input signal $x(t)$ is applied to a muscle fiber. The Fourier transform of the input signal is called $X(f)$, and the muscle fiber is described by its transfer function $H(f)$, i.e., its impedance. The input is supposed to be measured without the addition of contaminating noise, but the output is contaminated with noise. The idealized output, free of noise, is called $y(t)$, and the Fourier transform of that output is called $Y(f)$. The recording amplifier and associated electronic devices introduce a noise $n(t)$, giving the output $\hat{y}(t)$ actually available for measurement; $\hat{y}(t)$ is the best estimate available of the true output $y(t)$. Figure 6 distinguishes between the effects of uncorrelated contaminating noise arising in the instrumentation, called $n(t)$ or "noisy noise," and the input signal $x(t)$, which may or may not be stochastic (i.e., noise), depending on the choice of the investigator. The input signal $x(t)$ must be wide band, with energy at many frequencies, but whether it is stochastic or periodic, with energy at many frequencies (i.e., pseudorandom), is not significant here.

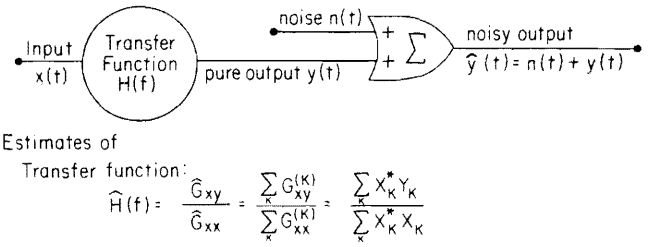

FIG. 6. Instrumentation noise in the output signal. Transfer function $H(f)$ might be impedance of a muscle fiber. Input $x(t)$ is applied current. Noise-free measurements of $x(t)$ are assumed to be available. Pure output $y(t)$ is voltage that would be measured in the absence of instrumentation noise. Noise $n(t)$ is noise introduced by voltage-recording amplifier and associated electronic devices. Noisy output $\hat{y}(t)$ is the sum of pure output and noise; $\hat{y}(t)$ is the best available estimate of membrane voltage. Transfer function should be estimated from estimates $\hat{G}_{xy}(f)$ and $\hat{G}_{xx}(f)$ of cross-power and power spectra, respectively. Those estimates should be the mean of cross power and power recorded from k blocks of data, as indicated. Cross power and power of each block of data are determined from the Fourier transforms $X(f)$ and $Y(f)$ of signals $x(t)$ and $y(t)$, respectively. *Complex conjugate. Other estimates of the transfer function (e.g., made by dividing the power spectra and then averaging) lead to incorrect results, as discussed by Bendat and Piersol (10).

The proper method of estimating impedance functions in the presence of noise is given by Bendat and Piersol (10, 11). They have shown that the only correct estimate of the transfer function, i.e., the impedance (in the statistical sense that it is unbiased and of reasonable variance), is the one shown with a circumflex in Figure 6. In this estimate of impedance the power spectra G_{xx} and G_{xy} of each block of data must be computed; the estimates of the power spectra of each block are then averaged to form the estimate of the power spectra. The only correct estimate of impedance is the ratio of the averaged power spectra shown in Figure 6. One must not average the Fourier transforms and then compute the transfer function, nor can one compute transfer functions from each block of data and then average those estimates.

Bendat and Piersol (10, 11) also analyze systems where the measurement of the input signal $x(t)$ is corrupted by noisy noise, a noise that is not an input

to the system being studied (see Eq. 6.122 in ref. 10). Some setups used to measure the impedance of muscle fall into this category, where the current flowing through the muscle fiber is measured with circuits that introduce noise. In those setups, estimates of the amplitude of an impedance can be biased by the noisy noise, but estimates of phase are not. Bendat and Piersol show that a signal-to-noise ratio of 10 (in power terms) can produce about a 10% underestimate of the amplitude of the impedance.

The discussion just presented assumes, of course, that both the current applied and the voltage recorded from the muscle fiber are measured and that both are used to compute the power spectrum and cross-power spectrum. When the input signal is known in advance and thus need not be measured each time it is applied to the preparation, impedance can be measured without simultaneous measurement of both input and output and without computation of input, output, and cross-power spectra [see, e.g., the work of Poussart and colleagues (89) and Clausen and Fernandez (18)]. When microelectrodes are used, however, it is not possible to know the input signal beforehand. The microelectrode resistance fluctuates substantially during the experiment, and these effects cannot be removed with sufficient accuracy at high frequencies by a constant-current circuit (70). Thus microelectrode measurements of impedance require measurement of the current applied to the muscle fiber. The classic methods described here therefore seem advisable and the simpler methods seem likely to give difficulty.

Manipulation of Impedance Data

A number of manufacturers make instruments that measure impedance by the Fourier techniques just described. These instruments allow measurements at between 100 and 800 frequency points (corresponding to between 256 and 2,048 time points in each block of data) and at frequencies up to ~50 kHz. The major difficulty with such machines is the embarrassment of riches. They provide more data than can be dealt with easily. The techniques for reducing the amount of data and putting the data into conventional formats are important and have been described elsewhere (69, 70).

Theory and Curve Fitting

The next steps in impedance analysis are the fitting of theory and the determination of circuit parameters. Both require a theoretical description of the electrical structure of the preparation. For skeletal muscle there is wide agreement concerning the appropriate theory. The longitudinal spread of current away from the current microelectrode is accurately described by traditional one-dimensional cable theory [reviewed by Jack, Noble, and Tsien (ref. 58, chapt. 3)]. The frequency dependence of the impedance data and the presumptive radial spread of potential in the T system require more analysis (see ref. 58, chapt. 6).

The radial spread of current within the T system of muscle is governed by cable equations similar to those governing longitudinal spread down unmyelinated nerve. The potential change across the T-system membrane is not uniform, just as the potential across a nerve fiber is not uniform and for a similar reason: significant potential drops occur in long narrow volumes of saline and across membranes. In nerve fibers the potential drops occur longitudinally within the saline solution that fills the axon. In the T system the potential drops occur radially within the saline solution that fills the extracellular lumen of the T system. The radial variation of potential in the T system is produced by current flow in the high resistance of an extracellular space; the radial variation of potential within the sarcoplasm is negligible. Thus the longitudinal potential drops in nerve fibers occur intracellularly, whereas the radial potential drops in the T system occur extracellularly. This difference between axonal and T-system cable theory is important conceptually but trivial analytically. It does not change the form of the cable equations.

A more important difference is produced by the shape of the T system. The T system is embedded in a roughly cylindrical muscle fiber, which gives the system a roughly circular outer boundary. This geometry implies that the radial spread of potential will be described by cylinder functions instead of exponentials; the cylinder functions are in fact the modified Bessel functions I_n and K_n of integral order, usually with order $n = 0$ or 1.

Cable theory for the T system and cable theory for an unmyelinated fiber are conceptually different. The nerve fiber is usually considered infinitely long, at least in the mind's eye, whereas the diameter of a muscle fiber, and thus of the outer boundary of the T system, is finite. The T system is analogous to a nerve fiber, but a nerve fiber of finite length. The length of the axon analogous to the T system (measured in units of axonal length constants) is equal to the radius of a muscle fiber, measured in units of T-system length constants. The finite size of the axon and T system constrains the flow of current and makes the spread of potential quite different from that in axons of infinite length. In an axon of finite length, or a T system, current cannot flow away to infinity. Rather it is reflected off the end (or center) of the system. These reflections add to the potential that would be present in an infinitely long axon. Thus there is much less decrement of potential in an axon of finite length or a T system than there would be in an axon of infinite length.

Of course the T system is not an axon of finite length. It has radial symmetry and so the spread of potential in the T system is described by Bessel functions, whereas an axon of finite length is described by exponentials. This radial symmetry forces a convergence of current flow near the center of the fiber, which is not present in the axon of finite length.

Nonetheless the electrical properties of finite lengths of terminated axon are surprisingly close to those of radially symmetric T systems if the comparison is made between properly normalized systems, systems normalized to have the same total amount of membrane (R. A. Levis, R. T. Mathias, and R. S. Eisenberg, unpublished observations). In fact these systems are sufficiently similar that they may be difficult to distinguish with measurements at experimentally accessible frequencies.

Electrical Models of the T System

The fundamental cable equations describing current flow in the T system are used to compute the admittance y_e in Equation 2. These equations were first given by Falk and Fatt (38) in their "distributed" model of the T system. Falk and Fatt described the T system as if it were a disk, the faces of the disk being the tubular membrane and the space between the faces being filled with a saline solution. The circumference of the disk coincided with the circumference of the muscle fiber. Adrian, Chandler, and Hodgkin (2) and Schneider (94) described the T system more realistically as a branching network of tubules. Such a network can be described as a disk (if the amount of membrane area and luminal volume is properly written), but only crudely, since current flow in a network clearly cannot be the same as current flow in a disk. The branching of the tubules obviously will have important effects on the radial flow of current. For example, *1*) a branched network will have a larger radial resistance than the disk; and *2*) when the frequency is high enough, or the length constant short enough, the discrete nature of a network of branching tubules will dominate, and a tubular network cannot be described as a disk even approximately. Adrian et al. (2) dealt with the first effect by introducing a tortuosity factor to describe the extra radial resistance to current flow. They computed this tortuosity factor for several regular models of the T system and used it to analyze the resistivity of the solution in the lumen of the tubules.

The tortuosity factor of Adrian et al. (2) is certainly needed to reconcile the properties of a network with the disk equations, but their factor is incomplete. The radial resistance of the T system must depend on the amount of twisting of the tubules in the T network. The path length for radial current flow will clearly be much longer in a network of convoluted tubules than in a network of straight tubules; thus the radial resistance must be higher in the convoluted network. Since the length of the tubules did not enter into their equations or definition of tortuosity, a factor is obviously missing.

A simple derivation of the missing factor can be found in Eisenberg, Mathias, and Rae (36). We related the effective radial resistance (and by inference the tortuosity factor) to the morphometric parameters of the T-system network, including the length of the tubules. Our simple derivation is justified mathematically by the rigorous work of Mathias, Eisenberg, and Valdiosera (67), who considered the distribution of potential in a random network made of branching tubules.

Mathias, Eisenberg, and Valdiosera (67) treated the T system as a random branching mesh that could be divided (by an explicit geometrical construction) into concentric shells. If the potential within each shell was averaged over the circumferential location, the potential within the T system (and thus its impedance) could be calculated accurately, with no other assumptions. In this case the T-system admittance (the reciprocal of impedance) is described by a first-order nonlinear difference equation (ref. 67, Eq. 36), which can be dealt with rapidly and trivially by computers, since a difference equation is a computer program.

Mathias, Eisenberg, and Valdiosera (67) compared their mesh model of the T system with earlier models by constructing accurate analytical approximations to the solution of their difference equation. They showed that the mesh model was a simple generalization of the disk model of Falk and Fatt (38) and later workers (2, 94). The mesh model included the effects of the branching and wiggling of the tubules that might be expected. When the length constant of the T system was large compared with the mesh size, the mesh expression had precisely the same form as the disk expression, but there was no explicit tortuosity factor requiring separate analysis, as in the work of Adrian et al. (2). Rather, the morphometric properties of the T system appeared directly in the solution of the mesh equations. If one then adopts the definition of a tortuosity factor introduced by Adrian et al. (2), one can compute the tortuosity factor of any network from its morphometric parameters. The resulting expression for the tortuosity factor (ref. 67, Eq. 29) includes the dependence on the length of tubule and the number of branches per node that one might intuitively expect.

When the length constant of the T system is sufficiently short, the analysis of the mesh clearly must differ from that of the disk either in the form presented by Falk and Fatt (38) or in the form presented by Adrian et al. (2) and Schneider (94). The mesh expression must approach the admittance of a number of unbranched tubules in parallel, whereas disk expressions—even those including a tortuosity factor—cannot have this property. In fact the expression derived by Mathias, Eisenberg, and Valdiosera (ref. 67, Eq. 43) has just the required behavior under conditions of short length constant or high frequency.

Despite the involved mathematics of the derivation of the mesh model (67), the result is pleasingly simple. At low frequencies, when the length constant is long, the mesh model has the same form as the disk model, with a generalization of the definition of the tortuosity factor. At high frequencies, when the length constant is short, the mesh model behaves like a set of tubules

in parallel. In fact the complexity of the mathematics of the mesh model is misleading, since its main result can be derived (36) just as simply as the disk model itself.

There is practical significance to the behavior of the T system and mesh model at high frequencies or short length constants. During an action potential, both conditions occur and the spread of current within the T system might be expected to be better described by a mesh than by a disk model. Indeed preliminary calculations of propagating action potentials (R. A. Levis, R. T. Mathias, and R. S. Eisenberg, unpublished observations) have shown sensitivity to the properties of the mesh and give results different from calculations with a disk model.

Other models of the T system have been suggested. Falk and Fatt (38) used a lumped model in which all the membranes of the T system were supposed to be in series with the same resistance. They assumed that the radial drop of potential in the resistance of the lumen of the tubules was negligible compared with the drop of potential in a resistor elsewhere, presumably at the mouth of the tubules. The lumped model was certainly a useful (if crude) approximation and is still useful [see, e.g., ref. 67, which shows how the lumped model is an analytical approximation to the mesh or disk model]. The existence of significant decrement of potential within the T system under many physiological conditions has always been apparent, however, both from qualitative experimental results [e.g., Huxley and Taylor (57); Endo (37); Gonzalez-Serratos (49); Adrian, Costantin, and Peachey (3)] and from consideration of the shape and size of the tubules. The shape and size of the lumen of the T tubule guarantee significant potential decrement in the radial direction under many physiological conditions.

Peachey and Adrian (4, 85) generalized the lumped model by placing a single large resistance at the mouth of the tubules. This access resistance was supposed to be of the same order as the total radial resistance of the rest of the T system. Thus approximately half the radial drop in potential would occur across the access resistance and approximately half would occur across the radial dimension of the fiber. Mathias, Eisenberg, and Valdiosera (ref. 67, Eqs. 47–49) give general expressions for the effect of an access impedance on the impedance of any model of the distributed T system. In *Impedance Measurements of Normal Frog Fibers*, p. 317, I discuss the experimental evidence concerning the access resistance and show that if the access resistance exists at all, it must have a value much smaller than the effective radial resistance of the tubular meshwork.

A generalization of the analysis of the T system considers it a branching network of extracellular space that penetrates the muscle fiber. As such it can be described in much the same way as the extracellular space within a syncytial tissue. (A syncytial tissue is made of many cells electrically coupled together with a narrow extracellular space between the cells.) Eisenberg, Barcilon, and Mathias (32) derived the partial differential equations and boundary conditions that describe syncytial tissues, and these have been solved by Peskoff (86) for cylindrical syncytia with clefts between cells. R. T. Mathias (personal communication) has solved the syncytial equations for cylindrical tissues containing tubular networks and has shown that the disk or mesh model appears in the solution naturally, without further assumptions. Mathias finds that the potential close to the current microelectrode is quite complex, much more so than assumed by Valdiosera et al. [(101); but see the caption of their Fig. 2]. The solution for the radial variation of potential away from the current electrode (and thus impedance) assumes the form used by Falk and Fatt (38) and later workers (2, 94) in the field, namely, the form given in Equations 1 and 2.

The mesh theory of the T system thus seems an adequate description of the known structural complexities. All the parameters of the theory can be determined experimentally by a combination of electrical and morphometric measurements. Measurements of the frequency dependence of the muscle impedance can then be used to determine the parameters of the theory and thereby check its validity.

Necessity for Morphometry

The electrical properties of muscle fibers depend on the structure and organization of their membrane systems as much as on the properties of the individual membranes themselves. This fact, which applies to the electrical properties of all cells and tissues (35), implies the importance of structural analysis, both description of the qualitative structure and measurement of the amounts of the various structures.

For example, the electrical properties of muscle fibers depend in a critical way on the amount of T membrane and of surface membrane. In a muscle fiber of small diameter, there is much less T membrane in relation to outer membrane than in a fiber of large diameter. Thus the T system influences the electrical properties of small fibers less than large fibers. Some of the electrical properties can be measured independent of impedance measurements [Hodgkin and Nakajima (55, 56)].

The importance of the total amount of membrane in determining electrical properties is obvious, but the theoretical implications are sometimes forgotten. When one compares different models of the same tissue, it is essential that the different models contain the same amount of membrane area. If the disk model is compared with a mesh model, if a model with branching tubules is compared with one without branching, or if a model containing clefts is compared with one containing tubules, care must be taken to ensure that the amount of membrane is the same in all cases. The easiest way to ensure morphological

comparability is to write the models in terms of morphological parameters. Morphometric parameters and electrical parameters are of equal importance in determining the measured properties, and suppression of morphometric parameters by combination into "effective" parameters is more likely to hide errors than to reveal truth.

The morphometric parameters of interest in a particular preparation and their measurement are an important subject beyond the scope of this chapter. This subject is discussed, with reference to the appropriate stereological literature, in the chapter by B. R. Eisenberg in this *Handbook* and by Eisenberg and Mathias (35).

RESULTS OF IMPEDANCE MEASUREMENTS

Impedance Measurements of Normal Frog Fibers

Many of the results of early impedance measurements are reviewed by Cole (20) and by Schanne and Ruiz-P.-Ceretti (92). Falk and Fatt (38) first showed that the impedance of muscle fibers could not be explained without reference to the T system. The dependence of impedance on frequency was far more complex than that of an axon and showed the general kind of behavior expected from a T system connected in parallel with a surface membrane. This result is now taken for granted but was quite important at the time it was made, since the patency of the T system was still then in question.

Comparison of impedance results from several laboratories is complicated by the evolution of techniques, theories, and interpretations through the years. Nonetheless the measurements by Falk and Fatt (38) and Freygang et al. (46) of the properties of fibers in normal solutions have been largely supplanted by the results of Schneider (94), Valdiosera, Eisenberg, Mathias, and Clausen (67, 100–102) because the earlier measurements had a significant possibility of artifact, did not extend over a sufficient frequency range, and were done at an unknown sarcomere length. Indeed the results of Valdiosera, Clausen, and Eisenberg (100–102) are also subject to serious criticism because the microelectrodes were placed close together. This placement of microelectrodes is not appropriate for the theory they used.

The discussion in this chapter is based on the results of Mathias, Eisenberg, and Valdiosera (67) without meaning to slight the significance of earlier work. They measured the impedance of single muscle fibers with microelectrodes placed to minimize three-dimensional effects. They interpreted their results with the mesh model of the tubular system and made measurements from 1 Hz to 10 kHz with small and measured artifact. Their main findings, which in most cases confirmed the qualitative results of earlier papers, were the following:

1. The electrical properties depended steeply on sarcomere length. The biological variance in measurements was too large to allow detailed interpretation of this dependence on sarcomere length. Furthermore morphometric data were not (and are not) available at different sarcomere lengths.

2. The capacitance of the surface membrane measured 1.1 $\mu F/cm^2$, where the area implicit in the measurement is that of a smooth unfolded outer membrane. Although this value seems large, it is easy to explain if a substantial fraction of the caveolae appear as part of the surface membrane.

3. The specific capacitance of the wall of the tubules is 1.4 $\mu F/cm^2$ of tubular membrane area. This is substantially larger than expected, implying either that the T membrane has unusual thinness or dielectric constant, or that the area measurements used were incorrect, or that some current is flowing across membranes other than the T system, for example, through the membranes of the SR.

4. The conductance of the tubular membrane could not be determined because potential decrements within the T system are small at DC. Under those conditions any distributed model of the T system—disk, mesh, or whatever—approaches the lumped model and shares the property that parallel conductances (i.e., the conductance of the surface membrane and the conductance of the tubular membrane) cannot be separated. This is an inconvenient property of resting muscle that can be modified by changing conditions. When the DC tubular length constant is shortened, usually by increasing the resistance of the lumen of the tubules, radial decrement can be large enough to allow measurement of the tubular conductance (1).

5. The resistivity of the tubular lumen was ~140 $\Omega \cdot cm$ compared with the resistivity of ~90 $\Omega \cdot cm$ for the extracellular bathing solution. The difference is certainly significant and requires discussion. Errors in the electrical measurements are unlikely to produce this difference, since estimates of the resistivity are determined by the low- to middle-frequency data and so are quite reliable. Furthermore a similar discrepancy appears in a wide variety of measurements of the T system [see Nakajima and Bastian (74) for references and discussion]. Errors in the morphological measurements used to compute the resistivity are possible and may explain the discrepancy in estimates. It seems more likely, however, that some structural property of the muscle fiber was not properly included in the theory used to interpret the data. One possibility is that some of the apparent radial resistance comes from current flow into the SR (68). Another possible source of error in the estimate of radial resistance comes from structural complexities in the T system itself. S. Page and L. D. Peachey (personal communication) found that the T system has a small circular cross section in regions where it is not associated with the terminal cisternae of the SR. Such regions might have more radial resistance than predicted by the

mesh model, which assumes all tubules have the same cross section. In fact the mesh model assumes all tubular parameters are radially uniform. Even if the volume fraction and surface-to-volume ratio of these circular tubules are measured correctly by the procedures of Mobley and Eisenberg (71), use of their average parameters in the mesh model may not properly describe the radial resistance of a nonuniform T system.

The impedance measurements of Valdiosera, Eisenberg, Mathias, and Clausen (67, 100–102) bear directly on the value of the access resistance to the T system postulated by Peachey and Adrian (4, 85). Valdiosera, Clausen, and Eisenberg (102) showed that the impedance data were incompatible with a substantial value of the access resistance; only a small amount (1/7) of the total radial resistance was lumped into an access resistance at the mouth of the tubules. Mathias, Eisenberg, and Valdiosera (67) then showed that even this small amount of access resistance produced a significant misfit to experimental data taken with precautions to minimize three-dimensional effects. Thus there is clearly no experimental reason to postulate the existence of an access resistance.

Other Preparations of Skeletal Muscle

Impedance measurements have now been performed on crab skeletal muscle (31), scorpion muscle (48), and hagfish muscle (78). The work on crab muscle is difficult to interpret because of the dearth of structural information and the plethora of potential artifact. The work on scorpion and hagfish muscle is notable because in these preparations the T system consists of short unbranched tubules. The finding that the resistivity of the tubular lumen in the scorpion is close to that of Ringer solution is striking and if confirmed would suggest that the different result in frog muscle is caused by structural complication. Whether the relevant structural complexity of frog muscle is the branching, the density of T-SR junctions, or the variation of diameter of the tubules is not clear and deserves further investigation.

Impedance Measurements of Muscle Fibers in Various Conditions

Freygang et al. (46) were the first to study the impedance of muscle fibers under various experimental conditions. They were particularly interested in the effects of hypertonic solutions, presumably because hypertonic solutions paralyze muscle fibers. Rather surprisingly Freygang et al. (46) found that the capacitance of the T system increased markedly in hypertonic solutions [confirmed by Valdiosera, Clausen, and Eisenberg (102)]. Indeed, Almers [(5) and personal communication] has confirmed the result by measuring integrals of transients from the same fiber in both normal and hypertonic Ringer's solutions.

There may be some artifact or error in the measurements in hypertonic solutions, perhaps associated with the general state or low resting potential of such fibers. Most likely, however, the increased capacitance in hypertonic solutions reflects an increase in the area of membrane across which current can flow. Morphological measurements are needed to see if the increase in area is in the T system. If not, one might suspect that current was flowing into the SR under these hypertonic conditions. A combination of impedance measurements under voltage-clamped conditions (to control possible effects of depolarization in hypertonic fibers) and morphometric measurements might resolve these questions. The qualitative morphological findings of Franzini-Armstrong et al. (43) and the microprobe measurements of Somlyo et al. (97) do not rule out this possibility, in my opinion. Other interpretations of their (43) images seem possible, and microprobe measurements (97) are probably not sensitive enough to detect concentration changes produced by leakage through the small ionic conductance that has recently been postulated (68) to link the T system and SR.

Impedance has also been measured in solutions of various conductivities (67, 78, 102). These measurements show that the conductivity of the lumen of the tubules varies linearly with the extracellular conductivity and so provide additional evidence, if such were needed, that the lumen is filled with extracellular solution. The absolute value of the luminal conductivity in these solutions is not explained entirely; the luminal conductivity is less by almost a factor of 2 than that of the bathing solution. The possible explanations for this discrepancy have already been discussed.

Finally, impedance has been measured in glycerol-treated fibers in which the T system has been substantially disrupted (102). Eisenberg and Eisenberg (28) [confirmed by Franzini-Armstrong, Venosa, and Horowicz (44)] have shown that only a small fraction of the T system is accessible to extracellular marker under these conditions. The possibility had been raised [Nakajima, Nakajima, and Peachey (76)] that glycerol treatment drastically increases the radial resistance to current flow without actually disrupting the tubular system. The increase in radial resistance would be enough to prevent the diffusion of extracellular marker but might not represent an actual disconnection of the T system from the extracellular space. That is, current (carried by small ions) might penetrate where markers did not.

Surprisingly, impedance measurements differentiate between a muscle fiber containing a T system with a very high radial resistance and a muscle fiber containing a disrupted T system [Valdiosera, Clausen, and Eisenberg (ref. 102, Fig. 10)]. The best fit to impedance data from glycerol-treated fibers gave a striking result: the tubular capacitance (per unit area of outer membrane) is only 0.4 $\mu F/cm^2$, suggesting that some 90% of the T system is unavailable for current flow, in reasonable agreement with the mor-

phological results of Eisenberg and Eisenberg (28) and in close agreement with those of Franzini-Armstrong et al. (44). Furthermore Valdiosera, Clausen, and Eisenberg (102) showed that the impedance data could not be fitted with a model including the capacitance of a resting fiber and a series resistance, no matter how large the value of that series resistance.

The impedance measurements of Valdiosera, Clausen, and Eisenberg (102) give estimates of the radial resistance in glycerol-treated fibers that allow direct evaluation of the suggestion of Nakajima et al. (76) that the radial resistance is very high in glycerol-treated fibers. Valdiosera, Clausen, and Eisenberg (102) found that the effective radial resistance, computed using a lumped model of the T system in glycerol-treated fibers, was some 70 times larger than that in normal fibers. Of course the effective radial resistance depends linearly on the volume fraction of tubules. Thus the effective radial resistance would be expected to be some 10 times higher in glycerol-treated fibers than in normal fibers, because only 10% of the T system is left in such fibers. Thus the radial resistivity (as opposed to effective radial resistance) apparently is increased by a factor of 7 in glycerol-treated fibers. This increase is probably the result of collapse of the tubular lumen and could explain the slow diffusional phenomena observed by Nakajima et al. (76).

The capability of impedance measurements to distinguish between similar but distinct explanations of the properties of glycerol-treated fibers is a striking example of the utility of the method, at least under these circumstances.

Comparison With Other Results

Different kinds of measurements of the linear electrical properties of skeletal muscle agree quite well [see Nakajima and Bastian (74) and Chandler and Schneider (17) for some numerical comparisons]. Measurements with transient techniques (1, 17, 55, 56), measurements with estimates of diffusion constants (7, 37, 59, 75), and impedance measurements (67, 102) are in good agreement, well within the range expected because of different experimental conditions, different morphometric assumptions, and possible experimental errors.

Thus measurements of the properties of the T system derived from the properties of the whole muscle fiber seem to have reached a certain state of maturity. The maturity of the measurements of T-system properties does not imply a similar development in their interpretation, however.

DISCUSSION

Electrical properties of skeletal muscle have been studied intensely for perhaps two logical reasons, as well as the psychological reason that such study was a natural and seductive sequel to earlier work on nerve fibers. The logical reasons are *1*) to determine the parameters of the various membrane systems and compartments of a muscle fiber and *2*) to study the mechanism by which excitation of the surface membrane produces contraction of the myofibrils deep within a fiber, a process called excitation-contraction (EC) coupling.

The first step in EC coupling is the radial spread of potential within the T system. The measurement and the analysis of this spread of potential have a long history, reviewed in Costantin (23, 24) and Nakajima and Bastian (74). The measurement of impedance has contributed importantly to the study of the spread of potential in the T system. The linear properties of a muscle fiber put severe constraints on theories of the radial spread of potential, even though the distribution and properties of nonlinear conductances within the T system must be known to calculate the radial spread of the action potential [Adrian and Peachey (4)].

The radial spread of the action potential probably is not a critical step in determining the physiological aspects of EC coupling, although of course radial spread is an essential step. The system more likely to be a critical determinant of EC coupling is the junction between T system and SR.

Impedance measurements have had a small part in the analysis of the T-SR junction, but may well have an important function in the future. For example, if the mechanism of coupling between T system and SR is simply the flow of ionic current across the T-SR junction (68), one way to measure that current is with impedance measurements. Alternatively, if the mechanism of coupling is by remote control, that is, by a mechanism with the essential features of the rigid-rod model suggested by Chandler, Rakowski, and Schneider (16), impedance measurements still have a role to play. In that model, a component of capacitive current, called nonlinear charge movement, is supposed to arise from the movement of a voltage-sensing macromolecule within the T membrane. The sensor molecule remotely controls calcium release from the distant membrane of the SR and at the same time contributes to the capacitive current flowing across the T membrane. Impedance techniques might measure the capacitive current associated with the movement of the sensor more accurately than transient methods.

Impedance Measurements of Nonlinearities

Of course it is difficult to make impedance measurements of charge movement in muscle. Charge movement in skeletal muscle is nonlinear and my discussions so far have concerned linear systems.

The measurement and interpretation of linear measurements from nonlinear biological systems are difficult problems that have been worked on for a number of years [see the references in Fishman et al. (40)]. Although the results are inconclusive, some of the problems and principles of a linear analysis of a nonlinear system can be mentioned. These problems apply

to two cases of physiological interest: the analysis of nonlinear charge movement by impedance techniques and the analysis of the distribution of nonlinear ionic conductances (e.g., K conductance) by impedance techniques.

Is an impedance analysis of a nonlinear process worth doing? If the measurement of a transient response and the interpretation of that response by traditional methods are able to explain the biological properties of interest, there seems little to be gained by an impedance analysis. On the other hand, if there is a biological need (e.g., in the analysis of nonlinear charge movement in skeletal muscle), then perhaps the extra resolution of impedance measurements will be worth the novelties and difficulties inherent in a linear analysis of a nonlinear system.

An impedance analysis of a nonlinear preparation can be performed if the system is time invariant, if the system can be linearized by the voltage clamp, and if all the membranes in the preparation have the same potential across them. That is, *1*) if the potential in the T system is uniform, *2*) if the perturbing signal is the sum of a step function (to turn on or, as physiologists say, "activate" the nonlinearity) and a sufficiently small wide-band signal, and *3*) if the nonlinear conductances of the membranes reach a steady state after activation, the system is no longer nonlinear under the conditions of a single voltage-clamp measurement and an impedance analysis is possible. These conditions may be hard to achieve in important situations (since a sufficiently small signal is very small indeed in the potential range where ionic conductances vary steeply with potential, near threshold). One still cannot interpret the results of such experiments. If these conditions are met, however, the measurement is meaningful.

These conditions are not met for the most interesting of the biological conductances, the sodium channel, even when that channel is embedded in a nondistributed system like a squid axon containing an axial wire. The conductance there is transient even under voltage-clamp conditions; that is, the conductance turns on (activates), turns off, and inactivates. Such conductances cannot be measured by simply taking the Fourier transform of the transient current because the transform as usually defined does not apply to time-varying systems. (The Fourier transform of a time-varying system confuses the transient properties of the system and the impedance of the system, when impedance is defined as some time-average property of the time-varying system.) The definition of a Fourier transform can be generalized for use in a time-varying system [(64); ref. 82, p. 440–447; ref. 83, p. 304]; other methods are used to process signals derived from human speech (88, 90) and nonstationary current fluctuations (21, 95). The utility of these generalizations for impedance measurements and their advantages over other methods of analysis are not yet known, however. At present an impedance analysis of a time-varying system cannot be made.

Linear measurements from stationary (i.e., time-invariant) nonlinear systems (such as the steady state of the K-conductance system of the squid) can be interpreted, however, if the system is linearized with the voltage clamp and satisfies the conditions previously discussed. The interpretation must proceed in several steps and deal with formidable problems.

The impedance of each membrane must first be separated into its components before the contributions of a particular conductance system can be identified. These components include the linear capacitance, presumably a property of the lipid of the membrane; the ionic conductance through each conductance system; and the "ionic reactance" (as I like to call it) of each conductance system. The ionic reactance is the result of the time delay and consequent phase shift of each ionic conductance. The phase shift ensures that, in the neighborhood of a given potential, each ionic conductance will behave like a combination of capacitors, inductors, and resistors. The ionic reactance is produced by a combination of the nonlinear and active (i.e., energy-requiring) properties of ionic channels. Thus ionic reactances have properties distinct from those of capacitors and inductors or from the properties of lipid bilayers. For example, ionic reactances depend on the concentration of ions moving through the ionic channel (since the concentrations set the chemical potential energy available to drive this active process), and they disappear in the presence of drugs that specifically block ionic movement through those channels. These special properties of ionic reactances make it possible to distinguish them from the linear properties of the rest of the membrane.

The construction of an explicit linearized model of a membrane is straightforward once one has models of the individual ionic reactances. The models of the ionic reactances may not be quite as simple as often assumed, however. Although the simple linearization of the nonlinear properties of an ionic conductance is straightforward [first reported by Hodgkin and Huxley (54)], this linearization is not sufficient to determine an ionic reactance. One must demonstrate, and not assume, that higher-order terms in the expansion of the ionic conductance are uniformly small, that is, are small under all the experimental conditions of interest. This point is not pedantic but practical, since there are many conditions (e.g., near threshold) where one must expect the contribution of higher-order terms to be substantial. Theoretical analysis of the linearization process is clearly needed, but simulations are more convincing. The best check of the linearization process is a mock experiment in which the Hodgkin-Huxley equations are programmed, a sinusoidal (or wide-band) input is applied, and the resulting output is computed. A correct linearization procedure will give precisely the same results as the simulation.

If full advantage is taken of the variety of selective drugs presently available, an impedance analysis of a time-invariant, nondistributed nonlinearity seems possible. Such an analysis has been started by Fishman,

Poussart, and Moore (40) on squid axon and by Mathias, Ebihara, Lieberman, and Johnson (66) on tissue-cultured aggregates of heart muscle.

The extension to a muscle fiber will not be easy, however. First, a method is needed to separate the properties of the measured impedance into the properties of the T membrane and surface membrane. Such a method requires knowledge of the resting capacitance and conductance of the surface and tubular membranes and the conductivity of the tubular lumen. That is, it requires prior structural analysis of the nonionic reactances. Furthermore one must consider the effects of variation in DC potential within the T system. In the nonlinear case the DC potential in the T system is a function of the properties and distribution of the ionic reactances and of the linear properties of the T system. Thus the analysis of ionic reactances of a distributed system like the T system may well require an explicit distributed model of the nonlinearities, a shell model of the sort constructed numerically by Adrian and Peachey (4). Such numerical models are possible, but they present formidable computational problems and have not yet been used to interpret impedance measurements, to my knowledge.

Other Methods

Many of the difficulties of existing impedance measurements on muscle arise from their essentially composite nature and their paucity of spatial information. Impedance measurements have been confined to only a few spatial locations [see, however, Schneider (94) and Mathias, Eisenberg, and Valdiosera (67)] and so have not measured the spatial distribution of potential with much accuracy. Furthermore the impedance measured is always the combination (see Eq. 2) of the properties of the surface and inner membranes. It would be much better if one could measure the T-system potential directly: the measurement would be independent of the properties of the surface membrane and so would no longer be a composite; the spatial resolution would far exceed that available with microelectrodes.

Optical measurements of membrane potential, for example, with potentiometric dyes [see Cohen and Salzburg (19) for a review], hold great promise. They should allow direct measurement of the distribution of potential, although arrangements such as that used by Blinks (13) may be necessary to avoid confusion in the optical path. Optical measurements certainly should allow measurement of T-system properties independent of the surface membrane. Of course the relative amplitude and phase angle between applied current and light signals from different locations are also quite susceptible to impedance analysis as described in this paper.

For these reasons I expect optical measurements of potential in the T system and SR to answer important questions in muscle physiology, and a great deal of work is in progress right now with these techniques to make transient measurements of natural activity (8, 9, 12, 103). These methods will have their ambiguities, mostly arising from the unknown interaction of dye with membranes of different compositions, but optical measurements of impedance may also be successful in teaching us more about how a muscle contracts.

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