LASER RAMAN SCATTERING

A MOLECULAR PROBE OF THE CONTRACTILE STATE OF INTACT SINGLE MUSCLE FIBERS

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ABSTRACT The 500 to 1,800-cm^{-1} region of the Raman spectra of intact single muscle fibers from the giant barnacle are dominated by bands caused by the protein component of the fibers. The frequency and the intensity of the conformationally sensitive bands indicate that the contractile proteins adopt a predominantly α-helical structure and are not affected when the contractile state of the fibers is changed from relaxed to contracted by addition of ATP and Ca. However, the contraction induces a decrease of the scattering intensity of some of the Raman bands caused by the acidic and tryptophan side chains, showing that these amino acids are involved during the generation of tension.

INTRODUCTION

Raman spectroscopy is a two-photon, inelastic scattering process. When monochromatic light is focused on a sample, the frequency of some of the scattered light is shifted away from that of the incident radiation. In vibrational Raman spectroscopy, the frequency shifts are equal to the frequencies of the molecular vibrations induced in the sample, and they are consequently, sensitive to the geometry and rigidity of the scattering molecules. Because spectra can be obtained from small volumes of samples, typically 75 μl, and in almost any physical state including aqueous solutions, Raman spectroscopy has become an increasingly important tool to characterize biological macromolecules (1–3).

Several physical techniques have been used to uncover the molecular mechanism of muscle contraction. However, most of these techniques were not well suited to investigate such complex assemblies as living muscle fibers and they were used mainly with isolated structural proteins in vitro or with various muscle models (4). Raman spectroscopy, on the other hand, is a noninvasive technique and it has recently been shown to be a very useful probe of the molecular structure of the structural proteins of muscles, either isolated in solution (5–8), or in the highly ordered protein lattice of living muscle fibers (7, 9).

We report here on the effect of muscle contraction induced by ATP, Ca, and Mg (10) on the Raman spectrum of intact single fibers of the giant barnacle. The results clearly show for the first time that the intensities of several Raman bands caused by vibrations of some amino-acid residues change when tension is generated in the muscle fiber. On the other hand, the bands caused by the conformationally sensitive vibrations of the protein backbones seem to be unaffected by the contraction of the fiber.

METHODS

We have used in our study the depressor muscle of the giant barnacle (*Balanus nubilus*) because it contains only rather homogeneous, 0.5- to 3.0-mm diameter translucent white fibers (11) that are especially suitable for optical measurements and for various physiological studies (12). Furthermore, each fiber constitutes a single cell and has a low content of nonstructural proteins (13).

The Raman spectra were excited with the 514.5-nm line of a Spectra-Physics model 165 argon ion laser (Spectra-Physics Inc., Laser Products Div., Mountain View, Calif.), and measured with a Spex 1400 double monochromator equipped with a stepping motor (Slo-Syn) and a cooled photomultiplier (RCA-C1034) (Spex Industries, Inc., Metuchen, N.J.). To remove the nonlasing emission lines of the laser plasma, the incident beam was filtered with a diffraction grating as described by Jain et al. (14). After amplification and standardization, the photoelectron pulses emitted by the photomultiplier were counted for a preset-integrating period and the total number of counts at each frequency-shift increment was stored in the memory of a dedicated IMSAI 8080 microcomputer (IMS Associates Inc., San Leandro, Calif.) that also controlled the stepping motor of the monochromator. This microcomputer-controlled Raman spectrometer is described elsewhere (15). To eliminate the possibility of bands caused by grating "ghosts" in the spectra of the fibers, we have also used the 488.0- and 457.9-nm exciting lines of the argon laser, and we have not detected any ghost.

Single muscle fibers from giant barnacles were isolated from the depressor muscle as previously described (13). Each isolated fiber was rinsed in an isotonic sucrose solution and gently pulled into a glass capillary tube of the desired length and of inside diameter slightly smaller than that of the fiber (1–1.5 mm i.d.) to insure that the capillary would be completely filled by the fiber. The excess fiber extending from the capillary ends was trimmed away and the fiber-filled capillary was introduced into the plexiglass holder of the sample cell illustrated in Fig. 1. The relaxing solution containing 100 mM K, 25 mM Tris, pH 7.2, and 0.5 mM EGTA (solution A) was thermostated at 10°C and circulated in the sample cell. The incident laser beam was aligned perpendicularly to the capillary axis and the scattered light was collected at right angle to the incoming radiation. Typical laser power of 150 mW at the sample led to no sign of sample deterioration, even after 24 h of illumination, and highly reproducible spectra were obtained with different fibers.

It should be stressed at this point that in our experimental system, the fibers are neither skinned nor

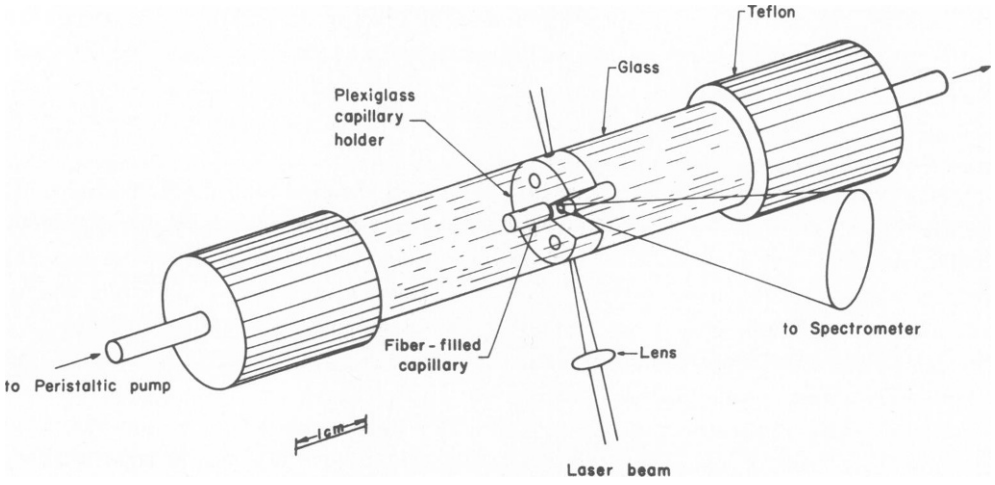

FIGURE 1 Raman cell for fiber-filled capillaries. The plexiglass capillary holder contains holes to enable the circulation of the equilibrating solution and to let the incident laser beam and the transverse scattered light go through. With this arrangement it is possible to change the composition of the bathing solution without disrupting the sample. The diameter of the laser beam at the sample is ~50 μm.

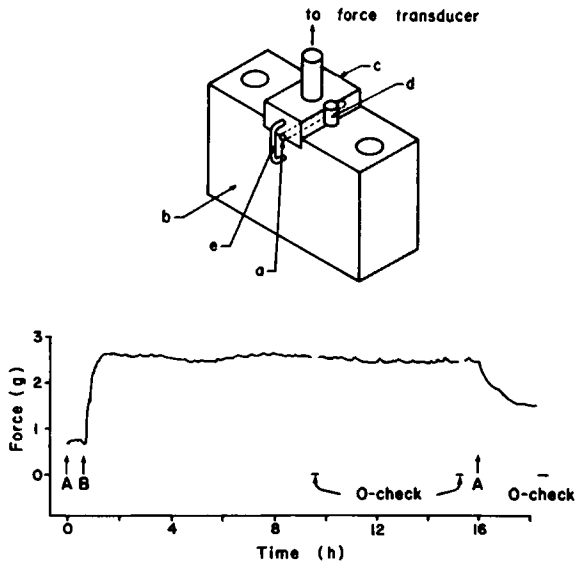

FIGURE 2 Diagram of the split cylindrical cavity used to measure the force of contraction of a single barnacle fiber, and recording of the force exerted on the cavity wall. The cavity (*a*) in which the fiber is introduced is made of two blocks of plexiglass (*b* and *c*). The lower part (*b*) is attached to the bottom of a beaker in which the thermostated equilibrating solution can be circulated. The upper part (*c*) which is guided by a small plexiglass rail (*d*), can move freely in the vertical direction and thus push on the force transducer. The two metal clamps (*e*) which are used to hold both parts of the cavity together when the fiber is introduced in it, are removed during the measurement of the force of contraction. On the recording shown in the lower half of the figure, the vertical arrows indicate the time at which solutions A or B were introduced in the beaker. The composition of these solutions is given in the text.

glycerinated. The cell membrane is intact in the fiber-filled capillary, but the myoplasm is exposed to the external solution at both ends of the capillary because the excess fiber is trimmed away. This system, which is very convenient for a Raman spectroscopic investigation, was developed by Caillé and Hinke (16) to study the physical chemistry of the myoplasm with a good control of the water and ionic content of the fibers.

Furthermore, Caillé (10) has recently shown that the myoplasm contractility is preserved in samples such as those used in the present study, as he was able to induce contraction of 0.75 – 2.0 g amplitude in fibers that had stood in capillary tubes for 24–30 h. To measure these forces of contraction, the fibers were removed from the capillary, attached to an isometric force transducer (Grass Instrument Co., Quincy, Mass., type FT 03), and then exposed to contracting or relaxing solutions. As it is not possible to measure the force of contraction when recording the Raman spectrum of a fiber because it is in a glass capillary tube, we have elaborated the device shown in the upper part of Fig. 2 to determine qualitatively the force of contraction when the myoplasm is in a cylindrical cavity equivalent to the capillary tube we use during the Raman experiments. This device consists essentially of a cylindrical cavity between two blocks of plexiglass. When the cavity filled with a muscle fiber is immersed in a contracting solution, ATP and Ca can diffuse in the fiber interior through the open ends and one would expect an increase of the pressure on the cavity wall caused by the contraction of the myoplasm. Because half of the cavity is mobile, it can be coupled to an isometric force transducer to measure qualitatively the force of contraction. An example of the results obtained with such a system is presented in the lower part of Fig. 2. It is clearly seen on this figure that when the relaxing solution (solution A) is replaced by one containing 100 mM K, 25 mM Tris, pH 7.2, 0.5 mM EGTA, 0.5 mM Ca, and 5.0 mM ATP (solution B) the myoplasm contracts. It is not necessary to add Mg to the contracting solution because the endogenous concentration of this ion is high enough in the barnacle fibers (10). When the relaxing

solution is reintroduced, the myoplasm relaxes again but not completely because there is no restoring force in the direction of the long axis of the fiber.

RESULTS AND DISCUSSION

The Raman spectrum of a fiber-filled capillary in the relaxing solution is shown in Fig. 3 A. It is interesting to note that this spectrum taken approximately 1 h after the dissection is essentially identical to the one we previously published (9) and which had been recorded 36 h after the dissection. However, the intensity of the resonance-enhanced Raman bands at 1,520 and 1,158 cm^{-1} which are attributed to the ν(—C═C—) and ν(═C—C═) stretching vibrations of β-carotene (9) differs in the two spectra because it depends on the amount of the

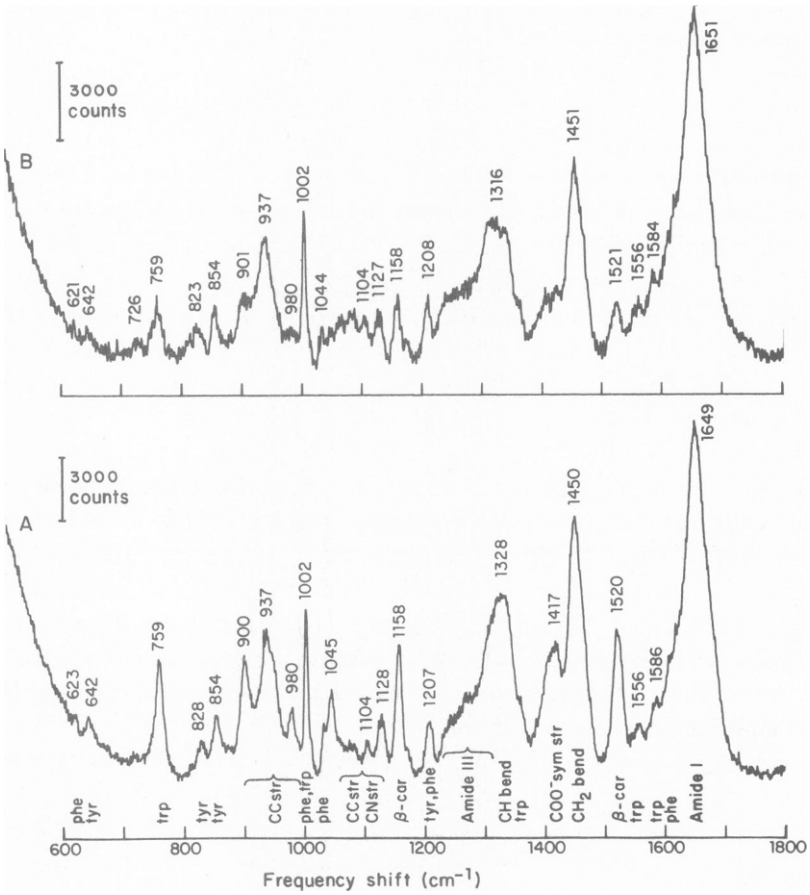

FIGURE 3 Raman spectra of a fiber-filled capillary of 7 mm in length, illuminated at 3 mm from the end of the capillary and bathing in a solution containing 100 mM K, 25 mM Tris, pH 7.2, and 0.5 mM EGTA (curve A), and 100 mM K, 25 mM Tris, pH 7.2, 0.5 mM EGTA, 0.5 mM Ca, and 5.0 mM ATP (curve B). Excitation wavelength, 514.5 nm; laser power at the sample, 125 mW; spectral resolution, 5 cm^{-1}; integration period, 2 s, frequency shift increment, 1 cm^{-1}. Both spectra are from single scans and are unsmoothed. However, each spectrum was corrected for a small fluorescent background by substracting a quadratic polynomial function. Abbreviations: str, stretching; sym, symmetric; bend, bending; CC, carbon-carbon bond; CN, carbon-nitrogen bond; CH, carbon-hydrogen bond; β-car, β-carotene; phe, phenylalanine; tyr, tyrosine; trp, tryptophan.

carotenoid present in each fiber. We have found that fiber-filled capillaries are stable for at least 48 h, after which they progressively lose their translucency. As a result of the high concentration of proteins in muscles (~20% by weight) all the bands shown in the spectrum of Fig. 3 A, except those of β-carotene, are caused by the vibrations of the proteins. Furthermore, a spectrum of the equilibrating buffer solution recorded with the same experimental conditions as those used to obtain the fiber spectrum reproduced in Fig. 3 A did not show any significant band between 500 and 1,800 cm^{-1} except for a broad band near 1,630 cm^{-1} which results from the bending vibration of water. Assignments of the stronger bands from correlations established through the analysis of Raman spectra of amino acids and proteins (1–3) are also given in Fig. 3.

Information on the secondary structure of proteins is obtained from the amide I (1,620–1,680 cm^{-1}) and amide III (1,220–1,320 cm^{-1}) regions of their Raman spectra (1–3). The α-helical conformation gives a strong and sharp amide I band at 1,650 ± 5 cm^{-1} and a weak gives scattering in the amide III region, whereas the antiparalled β-structure is characterized by a strong and sharp amide I band at 1,670 ± 5 cm^{-1} and a strong amide III band at 1,240 ± 5 cm^{-1}. Disordered structures result in a strong and broad amide I band near 1,665 cm^{-1} and a broad amide III band of medium intensity near 1,248 cm^{-1}. The amide I and III regions of the muscle-fiber spectrum of Fig. 3 A clearly indicates that the proteins are mainly in the α-helical conformation. Furthermore, these spectral regions, as well as the C-C skeletal-stretching region which is characterized by a strong band at 937 cm^{-1}, are very similar to those of proteins with high helical content such as myosin (6, 8), tropomyosin (5), avian lens proteins (17), and coat proteins Pfl and fd of bacterial viruses (18).

The spectrum of the same fiber-filled capillary used to obtain that of Fig. 3 A, but in solution B is reproduced in Fig. 3 B. Because the fiber is cut at both ends of the capillary, the solution B containing ATP and Ca can reach the fiber interior and cause contraction of the fiber. However, because this diffusion process in the fiber-filled capillary is slow, it takes time for the illuminated section of the fiber that is located in the center of the capillary to change from one state to another. This is illustrated in Fig. 4 where we have plotted the relative intensity of the 760- and 1,405-cm^{-1} bands against the time elapsed after the addition of the contracting solution. It is seen on this figure that both bands decrease linearly with time and reach a constant value after ~12 h which is an indication that the fiber is then uniformly excited. The spectrum of Fig. 3 B was thus recorded 15 h after adding ATP and Ca, i.e. right after the completion of the spectral changes.

Examination of the two spectra of Fig. 3 suggests that contraction does not significantly change the secondary structure of the contractile proteins. The spectral features in the amide I and III regions are quite similar, and both spectra show a strong band at 937 cm^{-1}. On the other hand, addition of ATP and Ca causes a marked intensity decrease of several bands that arise from vibrations of the amino-acid side chains of the proteins, especially from the acidic and tryptophan residues. It should be noted here that the decrease of the intensity of the bands of β-carotene shown in Fig. 3 is not caused by contraction but rather to the bleaching of the photosensitive pigment by the laser beam after a long illumination period. This effect is quite common with carotenoid pigments in natural systems and has been observed when recording the Raman spectrum of human erythrocyte ghosts (19).

Because the structural proteins of muscles are unusually rich in acidic amino acids (~25%) (4) that are negatively charged at physiological pH, it is not surprising to find at 1,417 cm^{-1} in

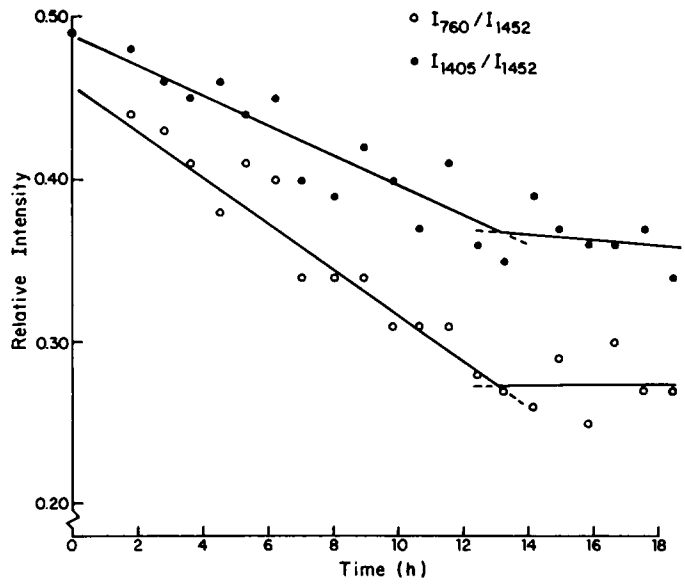

FIGURE 4 Decrease of the intensity of the 760- and 1,405-cm^{-1} bands relative to the 1,452-cm^{-1} methylene band after adding the contracting solution (solution B) to a barnacle fiber. The straight lines were obtained by a least-squares procedure.

the spectrum a rather strong band associated with the symmetric C—O stretching vibration of the carboxylate groups of the ionized glutamic and aspartic acid residues. One of the most striking spectral changes occurring during contraction is the decrease of the scattering intensity of this Raman band. The spectrum of the contracted fiber also reveals that the change in intensity of the 1,417-cm^{-1} band is not accompanied by the appearance of a new band around 1,700 cm^{-1} which would indicate the formation of C═O double bonds. It thus appears that the acidic residues of the proteins remain ionized when ATP and Ca are added, and our results suggest that the change of electronic polarizability associated with the symmetric C—O stretching vibration decreases during contraction, probably as a result of strong interactions between carboxylate groups and either the positively charged counterions of the myoplasm or the basic amino acid residues of the proteins. In fact, Caillé (20) recently showed from electrochemical measurements on barnacle fibers that contraction causes a reduction of 30–50% of the myoplasmic thermodynamically effective charge density, and he concluded that the decrease of the electrostatic repulsion between charged sites on the fiber macromolecules is likely to play an important role in the contraction process.

Another significant spectral change occurring during contraction is the decrease of the intensity of the strongly polarized 759-cm^{-1} band, which is caused by in-phase breathing vibrations of the benzene and pyrrole rings of the tryptophan residues (21). It is interesting to note here that in the spectrum of the relaxed fiber (Fig. 3 A) the intensity of this line relative to the other lines of tryptophan, that at 1,555 cm^{-1} for example, is approximately three times stronger than in the spectrum of free tryptophan in solution. We believe that this effect is the result of a partial orientation of many of the tryptophans in the fiber. For example, if the indole rings were aligned so that the larger diagonal element of the scattering tensor is mostly

involved when the laser beam is polarized along the fiber axis, the bands caused by the totally symmetric modes, such as that at 759 cm^{-1} should be stronger than for randomly oriented molecules. Furthermore, a simultaneous change in the orientation of the tryptophans during contraction of this partially oriented system would certainly affect the intensity of some of their Raman bands. This is observed for the 759-cm^{-1} band as well as in the region of 1,350 cm^{-1} where tryptophan contributes to the scattering intensity (1, 21). Such a simultaneous change in the orientation of the indole rings in muscles has also been detected from the change of the polarization parameter of the fluorescence obtained by exciting muscle tryptophans with light polarized perpendicularly to the long axis of the muscle fiber (22).

The change in intensity of the 759-cm^{-1} line can also be the result of a preresonance effect. It is well known that the strength of the low-lying electronic transitions has a marked effect on the intensity of the Raman bands caused by vibrations that are coupled with these electronic transitions (2, 21). Hirakawa et al (21) have recently shown that the 759-cm^{-1} band derives its intensity solely from the strong $\mathbf{C} \leftarrow \mathbf{X}$ transition of the indole ring at 217 nm. Perturbations of this transition caused by changes in the environment of the chromophores when going from the solution to the relaxed fiber, and then to the contracted fiber would also be reflected in the Raman spectra. However, a shift of the 217-nm indole transition or a change of its molar absorptivity is by no means easy to measure from of an intact fiber since an internal intensity standard has to be introduced in the fiber without changing its physiological state. Furthermore, Hirakawa et al. (21) have shown that the main resonance enhancement of the 759-cm^{-1} band of tryptophan occurs mainly when using UV excitation.

Although the complete structure of muscle cannot be determined by Raman spectroscopy, our preliminary results show that it is a sensitive intrinsic molecular probe of the contractile state of muscle since valuable information regarding the structure of the proteins in this system can be obtained. Contraction also induces spectral changes in the conformationally sensitive region of the skeletal modes involving C—C and C—N (~900–1,125 cm^{-1}) stretching vibrations. It is expected that other structural information will be eventually obtained from this region as it becomes more readily interpretable. Furthermore, it is hoped that improvements in the sample preparation will allow a better control over the diffusion of ATP, Ca, and Mg in the fibers which could enable measurements of spectra after reversible cycles of contraction.

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REFERENCES

1. YU, N-T. 1977. Raman spectroscopy: a conformational probe in biochemistry. *Crit. Rev. Biochem.* **46**:229–280.
2. SPIRO, T. G., and B. P. GABER. 1977. Laser Raman scattering as a probe of protein structure. *Annu. Rev. Biochem.* **46**:553–572.
3. THOMAS, G. J., Jr. 1975. Raman spectroscopy of biopolymers. *In* Vibrational Spectra and Structure. J. R. During, editor. Marcel Dekker, Inc., New York. **3**:239–315.
4. TONOMURA, Y. 1973. Muscle Proteins, Muscle Contraction and Cation Transport. University of Tokyo Press, Tokyo.
5. FRUSHOUR, B. G., and J. L. KOENIG. 1974. Raman spectroscopic study of tropomyosin denaturation. *Biopolymers.* **13**:1809–1819.

6. CAREW, E. B., I. M. ASHER, and H. E. STANLEY. 1975. Laser Raman spectroscopy: new probe of myosin structure. *Science (Wash. D.C.)* **188**:933–936.
7. ASHER, I. M., E. B. CAREW, and H. E. STANLEY. 1976. Laser Raman spectroscopy: a new probe of the molecular conformations of intact muscle and its components. *In* Physiology of Smooth Muscle. E. Bülbring and M. F. Shuba, editors. Raven Press, New York. 229–238.
8. BARRETT, T. W., W. L. PETICOLAS, and R. C. ROBSON. 1978. Laser Raman light-scattering observations of conformational changes in myosin induced by inorganic salts. *Biophys. J.* **23**:349–358.
9. PÉZOLET, M., M. PIGEON-GOSSELIN, and J. P. CAILLÉ. 1978. Laser Raman investigation of intact single muscle fibers: protein conformations. *Biochim. Biophys. Acta.* **533**:263–269.
10. CAILLÉ, J. P. 1977. L'effet du pH, du Mg^{2+} et de l'ATP sur la conductivité électrique du protoplasme musculaire du balane. *Can. J. Physiol. Pharmacol.* **55**:888–894.
11. HOYLE, G., P. A. MCNEILL, and A. I. SELVERSTON. 1973. Ultrastructure of barnacle giant muscle fibers. *J. Cell Biol.* **56**:74–91.
12. BRINLEY, F. J., Jr. 1968. Sodium and potassium fluxes in isolated barnacle muscle fibers. *J. Gen. Physiol.* **51**:445–477.
13. HINKE, J. A. M., J. P. CAILLÉ, and D. C. GAYTON. 1973. Distribution and state of monovalent ions in skeletal muscle based on ion electrode, isotope, and diffusion analyses. *Ann. N.Y. Acad. Sci.* **204**:274–296.
14. JAIN, K., W. T. WOZNIAK, and V. KLEIN. 1975. Diffraction grating use to reject fluorescence from a tunable dye laser. *Appl. Optics.* **14**:811–812.
15. SAVOIE, R., B. BOULÉ, G. GENEST, and M. PÉZOLET. 1979. A microcomputer-controlled Raman spectrometer. *Can. J. Spectrosc.* **24**:112–117.
16. CAILLÉ, J. P., and J. A. M. HINKE. 1972. Evidence for Na sequestration in muscle from Na diffusion measurements. *Can. J. Physiol. Pharmacol.* **50**:228–237.
17. KUCK, J. F. R., Jr., E. J. EAST, and N-Y. YU. 1976. Prevalence of α-helical form in avian lens proteins. *Exp. Eye Res.* **23**:9–14.
18. THOMAS, G. J., Jr., and P. MURPHY. 1975. Structure of coat proteins in Pfl and fd virions by laser Raman spectroscopy. *Science (Wash. D.C.).* **188**:1205–1207.
19. WALLACH, D.F.H., and S. P. VERMA. 1975. Raman and resonance-Raman scattering by erythrocyte ghosts. *Biochim. Biophys. Acta.* **382**:542–551.
20. CAILLÉ, J. P. 1979. Charges fixes du protoplasme des fibres musculaires de balane. *Biochim. Biophys. Acta.* **585**: 300–313.
21. HIRAKAWA, A. Y., Y. NISHIMURA, T. MATSUMOTO, N. NAKANISHI, and M. TSUBOI. 1978. Characterization of a few Raman lines of tryptophan. *J. Raman Spectrosc.* **7**:282–287.
22. DOS REMEDIOS, C. G., R. G. C. MILLIKAN, and M. F. MORALES. 1972. Polarization of tryptophan fluorescence from single striated muscle fibers. *J. Gen. Physiol.* **59**:103–120.