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Polarization of Tryptophan Fluorescence Measurements in Muscle

A Re-Evaluation

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Abstract. The degree of polarization of the intrinsic tryptophan fluorescence of glycerinated single muscle fibres or fibre bundles (rabbit psoas or dorsal longitudinal muscle of Lethocerus maximus) was measured:

a) With sufficiently high (15 mM) ATP concentration or when an ATP regenerating system was used no difference in the degree of polarization of a contracting and a relaxed muscle was detected, whereas a distinct difference was detected between the relaxed and the rigor state. In contrast a distinct difference between the relaxed and contracting state was obtained at low ATP concentrations (5 mM). This difference is interpreted to be caused by an ATP-free core (rigor core) in the centre of the fibre.

b) No change in the polarization degree was detected after a rapid release of the contracting muscle.

c) In rigor state no difference in the degree of polarization of the tryptophan fluorescence was observed in the presence or absence of AMPPNP (concentration 0.5 mM).

These findings and the lack of difference between the polarization degree of the contracting and the relaxed muscle is interpreted to indicate that the polarization degree of the tryptophan fluorescence is not sensitive to the orientation of the cross bridges, or that the cross bridges do not rotate.

Key words: Myosin cross bridge orientation – Tryptophan fluorescence – Actin-myosin interaction.

Introduction

Electron micrographs and X-ray diffraction studies of insect flight muscle preparations suggest that cross bridges stand out from the myosin filament in a perpendicular position in the relaxed state and are attached to actin in an acute angled position in rigor muscle (Reedy et al., 1965). In the latter state the cross bridges are in each half-sarcomere pointing towards the centre of the sarcomere.

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Aronson and Morales (1969) and Dos Remedios et al. (1972a, b) reported that the polarization degree of tryptophan fluorescence was higher in relaxation than in rigor while in contracting muscle it was intermediate between the values for relaxation and rigor. The difference between rigor and relaxed muscle was found to disappear as the actin-myosin overlap zone was diminished by stretching muscle fibres. For this reason and because of the location of a large fraction of fluorescent tryptophan in sub-fragment-1, it was argued that polarization of tryptophan may be a test for cross bridge orientation. Steiger et al. (1973) followed the degree of fluorescence polarization during a stretch-induced contraction of glycerinated insect fibrillar muscle suspended in ATP-salt solution. The observed changes associated with contraction were, however, so small that it seemed desirable to reinvestigate the problem of polarization of tryptophan fluorescence with improved techniques.

Methods

In order to measure the polarization degree and the total intensity of the tryptophan fluorescence in muscle under defined chemical conditions, glycerol-extracted fibres of rabbit psoas or of the dorsal longitudinal muscle (DLM) of Lethocerus maximus were used. The muscles were extracted for 24 h in a 50% glycerol solution buffered with histidine at pH 7.0.

After fixation between a lengthstep generator and a force transducer (cf. Appendix) a single fibre (or a small fibre bundle) was washed for at least 5 min in rigor solution (solution RM in Table 1). After washing, the fibre was immersed into the test solutions (the solutions used are specified in Table 1).

All polarization degrees given in "Results" are defined by

$$P_{\perp} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}, \tag{1}$$

Table 1. Incubation solutions used. In the upper line the symbols of the solutions are given. The solutions marked by a "K" in the first position contain low Ca^{2+} ($< 10^{-8}$ M), those with a "L" contain Ca^{2+} ($\simeq 10^{-5}$ M). Solutions marked by a "R" contain no ATP

	KM (mM)	LM (mM)	RM (mM)	K (mM)	L (mM)	R (mM)	KB[a] (mM)	LB[a] (mM)
KCl	40	40	40	–	–	70	100	60
Histidine	10	10	10	20	20	20	50	50
EGTA	2	–	2	4	–	4	20	20
$MgCl_2$	5	5	5	15	15	–	2	2
ATP	5	5	–	15	15	–	5	5
Ca-EGTA	–	2	–	–	4	–	–	20
NaN_3	–	–	–	10	10	1	1	1
Creatine-phosphate	–	–	–	–	–	–	10	10

[a] + 10 U/ml Creatinephosphotransferase

where $I_{\parallel}$ is the fluorescence intensity polarized parallel to the exciting light and $I_{\perp}$ is the intensity of the fluorescent light polarized orthogonal to the exciting light. The index "$\perp$" at the polarization degree $P_{\perp}$ indicates that the electric vector of the exciting light is orthogonal to the fibre axis.

Intensity and polarization degree of the fluorescent light was measured by a microscope photometer supplied by Zeiss. In spite of the low fluorescence intensity a time resolution of up to 1 ms was achieved. (The measurements reported here are done with a time resolution of 2 ms.) The accuracy of the measurements with high time resolution was mainly determined by the statistical noise of the photon input. For more detailed information see Appendix.

Results

Is the Polarization Degree Affected by Contraction? (Rabbit Psoas)

The difference in the degree of the tryptophan fluorescence polarization in rigor muscle and relaxed muscle (Aronson and Morales, 1969; Dos Remedios et al., 1972a and b) could be confirmed (cf. Figs. 1 and 2, columns 3–5). The polarization degree of a contracting muscle was found by Morales and coworkers to be intermediate between the polarization degree of the relaxed and rigor muscle. In an attempt to confirm these results we obtained typically results as shown in Figure 1: When contraction and relaxation solution contained 5 mM ATP the polarization degree of the contracting state was found indeed to be intermediate between the polarization degree of the relaxed and the rigor state. If, however, the ATP concentration was enhanced to 15 mM the difference between the polarization of the relaxed and the contracted state vanished

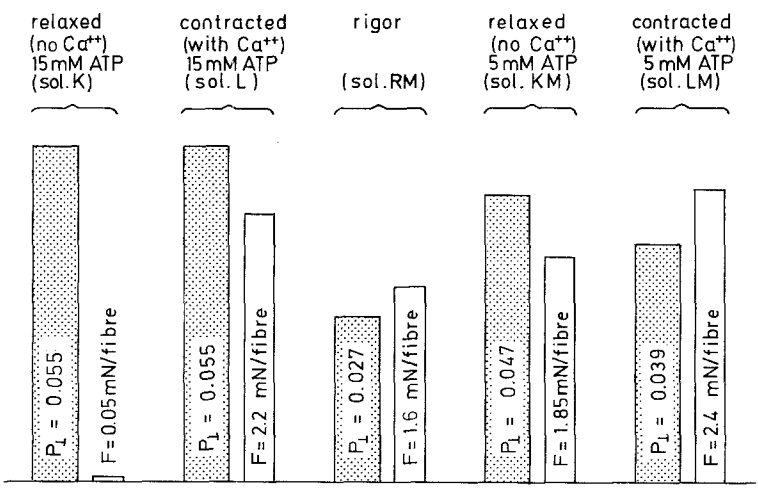

Fig. 1. Influence of the ATP concentration on the polarization degree. The dashed columns show the polarization degree ($P_{\perp}$, Eq. 1), the open columns the simultanously measured force generated by the fibre bundle (12 glycerol-extracted rabbit psoas fibres). The conditions are indicated above the columns (solutions specified in Table 1). Temperature: 28° C. The exciting wavelength was 290 nm

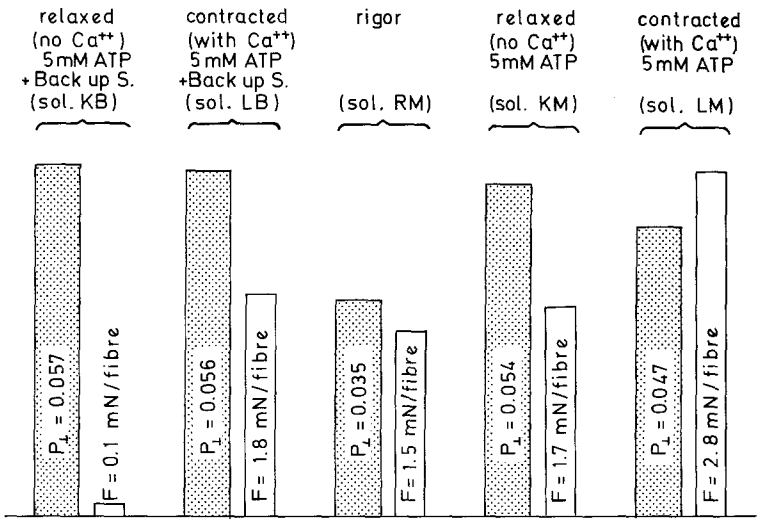

Fig. 2. Influence of an ATP regenerating system on the polarization degree. The dashed columns show the polarization degree ($P_\perp$, Eq. 1), the open columns the simultanously measured force generated by the fibre bundle (6 glycerol-extracted rabbit psoas fibres). The conditions are indicated above the columns (solutions specified in Table 1). Temperature: 28° C. The exciting wavelength was 290 nm

almost completely. However, it should be noted that very small differences of the polarization degree in relaxed and contracted state would not have been detected because of artefacts effected by changing the test solutions: In several experiments the polarization degree in contracted state was even slightly higher than in relaxed state, in other experiments it was slightly lower. But in any case large differences as reported by the Morales group could be ruled out in case of high ATP concentrations or in case of an ATP regenerating system.

It seemed possible that because of diffusional limitations in contracting muscle an ATP free core might have developed in case of low ATP concentrations (5 mM). Therefore we measured the polarization degree in 5 mM ATP solution with and without an ATP regenerating enzymatic system (cf. Table 1). As can be seen from Figure 2 in the absence of the ATP regenerating system a distinct difference in the polarization degree between the relaxed and the contracting state is observed (columns on the right hand side). This difference is abolished in the presence of the ATP regenerating system (columns on the left hand side).

The results shown in Figures 1 and 2 were obtained with a fibre bundle containing 6 or 12 single fibres respectively. Except for the worse signal to noise ratio the results were the same for single fibres.

No Change in the Polarization Degree Induced by Length Changes (Rabbit Psoas)

When a fibre bundle is subjected to a quick release tension drops and than recovers. In order to find out whether the tension changes are associated with a change in the

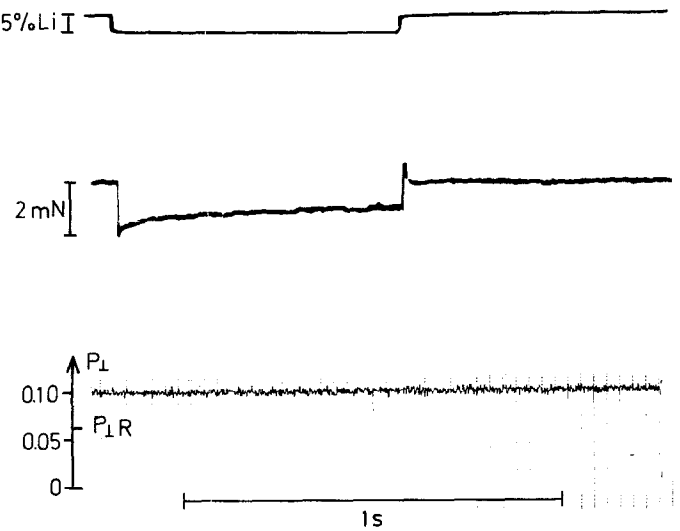

Fig. 3. The polarization degree of a rapidly released and restretched muscle (rabbit psoas). The upper trace shows the length change, the middle trace the corresponding force transient. In the lowest trace the averaged (128 samples) polarization degree is plotted. The time resolution of the polarization degree measurement is 2 ms. $P_{\perp R}$ indicates the polarization degree of the same preparation in rigor state. The temperature was 18° C. The incubation solution is specified in Table 1 under the letter "*L*". The wavelength of the exciting light was 295 nm

polarization degree, the fibre bundle was released and restretched repetitively with an amplitude of 1.5% of the initial length. The time between stretch and release was 0.75 s, the duration of the length change was 4 ms. The polarization degree was averaged over 128 cycles of stretch and release in order to improve the signal to noise ratio. The result is shown in Figure 3. The upper and the middle trace show length change and the force transient of one release and stretch cycle. In the bottom trace the corresponding polarization degree is shown which appears not to be different in the released and in the stretched muscle and no change occurs during the tension recovery after release. A change in the polarization degree during and immediately after release or stretch respectively [which might be caused by cross bridge rotation (Huxley and Simmons, 1971)] is not observed, although the time resolution of the measurement (2 ms) would be expectd to be sufficently high.

Influence of AMPPNP (DLM *of Lethocerus maximus)*

The ATP analogue AMPPNP binds to the cross bridges (half saturation at 0.1 mM AMPPNP; Marston et al., 1976) and is said to alter their orientation (Barrington Leigh et al., 1973; Beinbrech et al., 1976) without causing a detachment. It was of interest whether this change in cross bridge orientation causes a change in the polarization degree of the tryptophan fluorescence (cf. Dos Remedios et al., 1972b).

In the experiments with glycerinated fibrillar insect muscle the exciting wavelength of the fluorescence was adjusted to 275 nm in order to get a sufficiently large difference

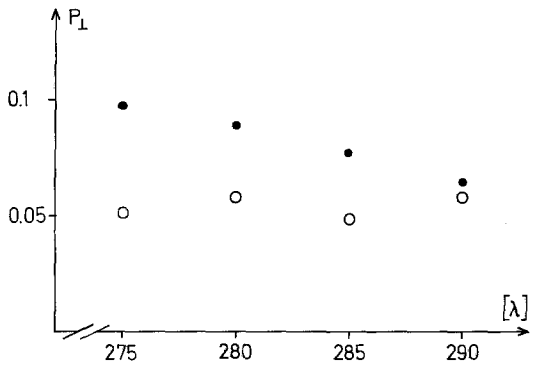

Fig. 4. Dependence of the exciting wavelength on the polarization degree in insect flight muscle (dorsal longitudinal muscle) of *Lethocerus maximus*. The open circles to relaxed state. The electric vector of the exciting light was perpendicular to the fibre axis

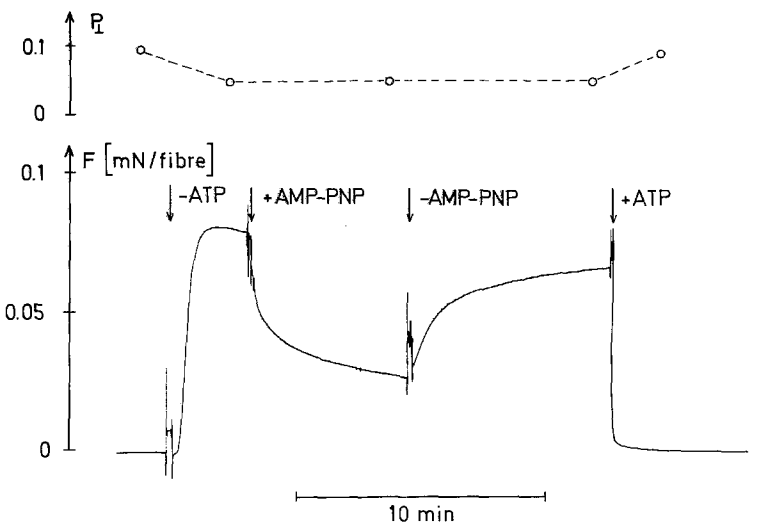

Fig. 5. The effect of AMPPNP on the polarization degree. In the lower trace is plotted the force which is generated by the fibre bundle (8 fibres of the dorsal longitudinal muscle of *Lethocerus maximus*). The corresponding polarization degree is plotted in the upper part of the figure. The exciting wavelength was 275 nm. The electric vector of the exciting light was perpendicular to the fibre axis. Temperature was 20° C

in the polarization degree of the muscle in rigor and relaxed state: In Figure 4 the polarization degree obtained from the insect flight muscle in rigor and in relaxed state is plotted versus the wavelength of the exciting light. As can be seen from the figure, the difference increases continously with decreasing wavelength. Consequently it seems to be advantageous to excite the fluorescence at even shorter wavelengths than 275 nm. However, the fluorescence intensity becomes so low at shorter wavelengths that it becomes difficult to determine the polarization degree.

In Figure 5 it is shown that the addition of AMPPNP in a concentration of 0.5 mM did not affect the polarization degree of the muscle in rigor state, although the fibre bundle relaxed reversibly under the influence of AMPPNP. On the other hand, addition of ATP caused complete relaxation and a drastic increase in the polarization degree (cf. Fig. 5).

Discussion

Influence of Fibre Thickness on the Fluorescence Signal

It could be shown for both insect flight muscle and rabbit psoas muscle that the tryptophan fluorescent light excited at 295 nm originates not only from the surface of the muscle fibre but also from its centre (cf. Appendix). This ensures that processes taking place in the centre of the fibre will be detected if they cause a change in the fluorescence intensity.

Furthermore it could be shown that the extent of depolarization of both the exciting and the fluorescent light due to light scattering or birefringence within the fibre is too small to obscure changes of the polarization degree in the centre of the fibre (cf. Appendix).

The Polarization Degree of Contracting and Relaxed Muscle

It could be shown that both the addition of an ATP regenerating system at 5 mM ATP and the enhancement of the ATP concentration to 15 mM without an ATP regenerating system largely abolished the difference between the polarization degree of the fluorescence in contracting and in relaxed muscle. The simplest interpretation of the phenomenon may be to assume a depletion of ATP in the centre of the preparation in the case of low ATP concentration without an ATP regenerating system: Because of diffusional limitations at 5 mM ATP a rigor core may be formed within the fibre after activation of ATPase activity by Ca^{2+} which lowers the overall polarization degree. Consistent with this interpretation it is reported by Mannherz (1968) that the ATP splitting rate in glycerinated rabbit psoas muscle increased with increasing ATP concentration up to an ATP concentration of 10 mM.

No Effect of Stretch or Release on the Polarization Degree

It is widely assumed that a cross bridge produces force, if it had rotated into the acute angled position thereby straining an elastic element, which is located in the neck of the cross bridge.

If this is so, a considerable number of acute angled cross bridges must be assumed to exist in the contracting muscle. Consequently the polarization degree – if sensitive to the cross bridge orientation – should be lower in the contracting than in relaxed state, where all cross bridges are assumed to be in the perpendicular position. As already reported, this is not observed.

If, however, the elastic elements of the cross bridges are not located in their neck, but rather in the binding site to the actin filament, no difference in the polarization degree between relaxed and isometrically contracted muscle would be expected. In this case a cross bridge rotation into the acute angled position would only occur if the muscle is allowed to shorten. Therefore the polarization degree would also only be expected to change if the muscle is allowed to shorten. Figure 3 shows the polarization degree before and after a rapid release. As can be seen from the figure no change is observed [al-

though the amplitude of the release is small it should be sufficiently large to induce the cross bridge rotation (Huxley and Simmons, 1971; Julian et al., 1974; Kuhn et al., 1979)].

Figure 3 shows furthermore that during the contraction after the release no change in the polarization degree is observed. Because after a large release the stiffness of the muscle is low (Griffiths et al., 1979), i.e., the number of attached cross bridges is reduced, at least a certain fraction of the cross bridges have to start the contraction cycle after a large release again from the beginning, i.e., presumably from a state similar to the relaxed state. Therefore Figure 3 may be interpreted to show again that the transition from the relaxed to the contracted state does not affect the polarization degree.

The Influence of AMPPNP *on the Polarization Degree*

Dos Remedios et al. (1972b) reported under the influence of 5 mM AMPPNP a shift in the polarization degree from the value of the rigor muscle towards higher polarization degrees. Recent investigations of Beinbrech et al. (1976) showed that at this high AMPPNP concentration the muscle stiffness, i.e., the number of attached cross bridges is much smaller than it is without AMPPNP. This may be due to a small quantity of ADP contaminated in the AMPPNP, which – converted by myokinase into ATP – may cause the muscle relaxation. However, in 0.5 mM AMPPNP the stiffness is unchanged, whereas already a considerable fraction (ca. 50%) of the acute angled rigor bridges seem to be transferred into a more upright position (Beinbrech et al., 1976). Under the influence of 0.5 mM AMPPNP it could therefore be possible to observe effects which correspond exclusively to cross bridge rotation independent of cross bridge detachment. However, no change in the polarization degree was detected under the influence of 0.5 mM AMPPNP.

In summary, all experiments reported in this paper seem to indicate that the polarization degree is not sensitive to the orientation of cross bridges, or that no cross bridge rotation takes place during contraction and no cross bridge rotation is induced by AMPPNP.

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Appendix

Measurement of Polarization of Tryptophan Fluorescence in Muscle Fibres with 2 ms Time Resolution

The Photometer. The experimental set up used is schematically shown in Figure 6. The source of the exciting light was a Xenon high pressure lamp provided by Osram (Type

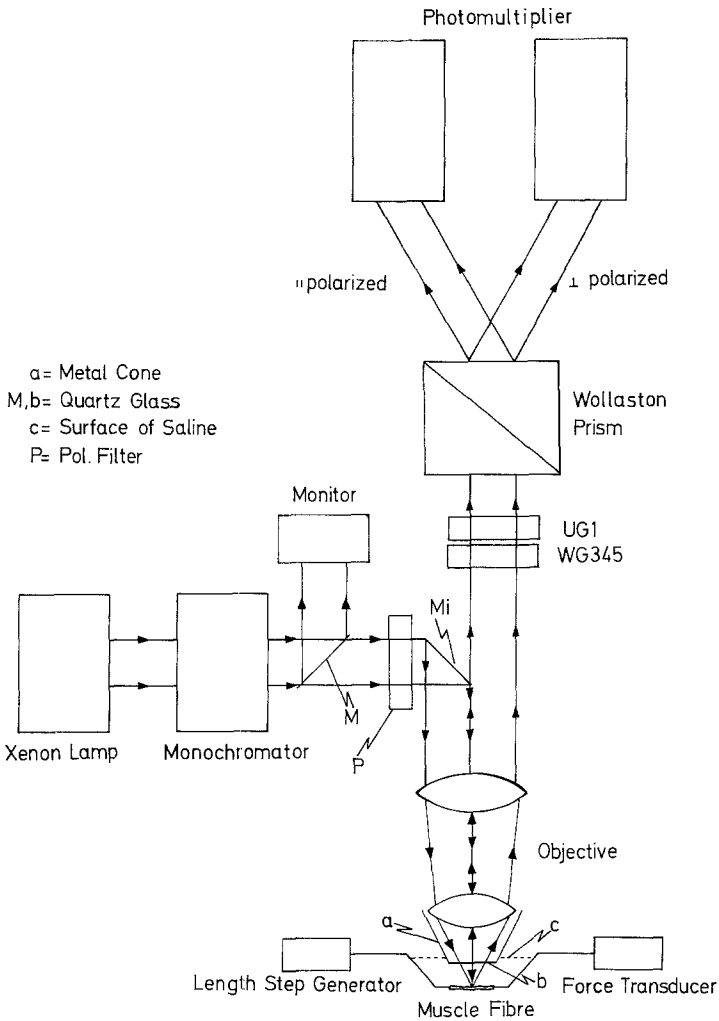

Fig. 6. The exciting light of the high pressure "*Xenon Lamp*" is passed through a "*Monochromator*". The main fraction is reflected at the mirror "*Mi*" and is fed into the "*Objective*", which focusses the light into the "*Muscle Fibre*". The quartz glass "*b*", which is glued onto the tip of the metal cone "*a*", is dipped into the bath solution in order to prevent errors due to a moving liquid surface "*c*". The fluorescent light is sampled by the "*Objective*" and after filtering separated by the "*Wollaston Prism*" into the perpendicular and the parallel (to the fibre axis) polarized fraction. The intensity of the two beams is detected by the "*Photomultipliers*". The intensity of the exciting light is controlled by the "*Monitor*"

XBO 150W/1). The wavelength of the exciting light was adjusted by a prism monochromator (Zeiss, Type MQ3).

In most experiments the bandwith of the exciting light was 15 nm, corresponding to the highest available intensity. For fluorescence intensity measurements the intensity of the exciting light is controlled by monitoring the intensity of the light which is reflected by the quartz glass *M*. The exciting light is polarized by the polarization filter *P* and focussed onto the muscle fibre by the objective of the microscope (objective: Zeiss

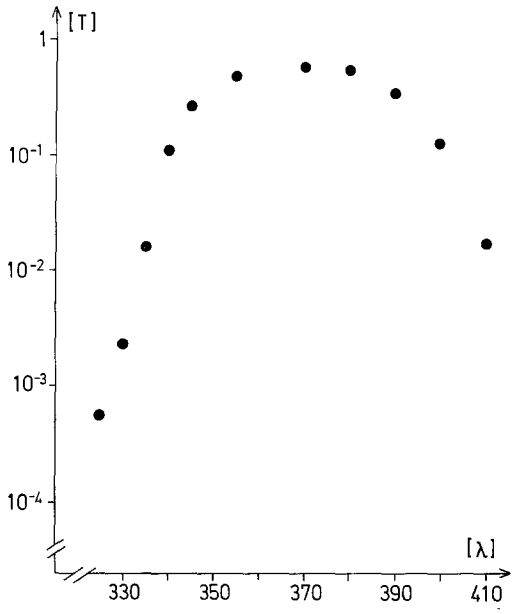

Fig. 7. Transmission of the filter combination UG1 and WG 345, which is used in the microscope photometer shown in Figure 6 to separate the reflected exciting light from the fluorescent light

Ultrafluor 10). Exciting light, which is reflected at the surfaces of the lenses, may disturb the measurement. Therefore it is advantageous to use one half of the objective exclusively for excitation and the other half exclusively for the detection of the fluorescent light. This is achieved by the arrangement shown in Figure 6: The exciting light, which is reflected at the mirror "Mi" enters the objective through the one half, whereas the fluorescent light is detected through the other half.

Exciting light, which is reflected by the muscle fibres or by the surfaces of the incubation saline is filtered out by a filter combination provided by Schott (Type UG1 and WG 345). The transmission curve of the filter is shown in Figure 7. A certain fraction of the exciting light, which is reflected at the surface of the incubation solution will – in spite of the filters UG1 and WG 345 – be detected by the multipliers as a background signal. Motions of the surface of the incubation solution may consequently cause fluctuations of the background. These fluctuations are prevented by the metal cone (a) with the quartz cover (b) at its end, which penetrates the solution (c).

After passing the filters UG1 and WG 345 the fluorescent light is divided into its perpendicular and parallel (with respect to the muscle fibre) polarized component by means of a Wollaston prism.

The intensity of the components are measured by photomultipliers (EMI, Type 625B).

Electronical Data Analysis. The electronical equipment for the data analysis was developed and constructed in cooperation with v. H. + S. Electronic, Schwetzingen.

The duration of the quick tension recovery (quick phase) after a length change applied to a contracting muscle fibre is in the order of 1–2 ms. Because changes in the polarization degree within the quick phase should be detected, a time resolution of at least 2 ms had to be achieved. For a single fibre the number of sampled photons were of the

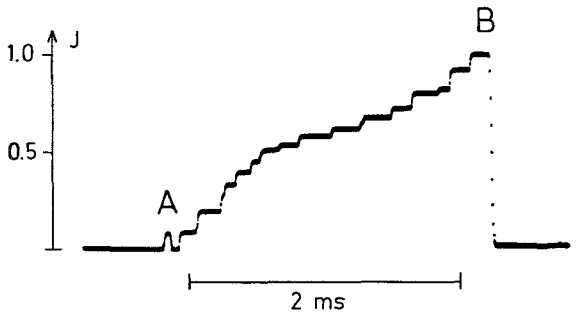

Fig. 8. Integrated photomultiplier current. Each step represents in arbitrary units the number of electrons due to a single sampled photon. The letters *A* and *B* mark the start and reset of the integrator

order of 10^4 s^{-1}. An averaging current measurement of the photomultiplier current with the desired time resolution of 2 ms was consequently not desirable. Thus the photomultiplier current was integrated by a high speed integrator circuit of high sensitivity: on the average a certain fraction of the incoming photons will elicit a burst of electrons at the multiplier anode. Every detected photon causes a step at the output of the current integrator. Figure 8 shows such an integration. The integration is started at *A* and the integrator is reset to zero at the time *B*. During the sampling time, i.e., within the time marks *A* and *B*, 17 bursts of the photomultiplier, each presented by a step, are recorded. Corresponding to the manual of the photomultiplier this corresponds to ca. 85 photons.

Under the assumption that every photon triggers a burst of the same number of electrons at the multipliers anode, amplitude and statistical deviation from the mean of the current integral should only depend on the statistics of the sampled photons. This assumption is not exactly fulfilled, as can be seen from the different step heights in Figure 8. Nevertheless it could be shown that the statistical deviation of the integral from the mean was only by a factor of two greater than the corresponding calculated photon noise.

The intensity of the fluorescent light of a single muscle fibre induced approximately 40 multiplier bursts, if the integration time was set at 2 ms. The corresponding statistical deviation from the mean was consequently at least 15%, whereas the expected deviation due to cross bridge rotation would be less than 3%. Therefore, data analysis by means of averaging over several experiments was necessary. For this purpose the data were stored on a tape recorder for later analysis by a computer. However, the light intensity measurement by integrating over the multiplier bursts is extremely sensitive against an error in time at which the integration value is taken. Therefore this time as well as the start and reset time of the integrator were determined by the same quartz clock. Because the clock pulse was also recorded on the tape errors due to differences between real time and experimental time during transfer of the recorded data to the computer could be completely eliminated in the range of the accuracy of the quartz clock.

Is the Fluorescence Polarization Signal a Probe for the Fibre Surface or for the Whole Fibre?

To ensure that the fluorescence signal measured does not exclusively originate in the vicinity of the surface of the preparation, the tryptophan fluorescence signal of a single

fibre was compared with the signal from a fibre bundle: A single fibre and a fibre bundle (20 fibres) were fixed together under the microscope. It was thus possible to measure the fluorescence output from single fibres and fibre bundles under the same conditions. The distance between the fibre bundle and the single fibre was ca. 1 mm. The diameter of the single rabbit psoas and the single insect flight muscle fibre was 70 μ and 90 μ respectively. The corresponding diameters of the fibre bundles for rabbit psoas were 300 μ and for insect flight muscle (DLM of *Lethocerus maximus*) 320 μ. The fluorescence intensity ratio of single fibre and fibre bundle was 7.1 for insect flight muscle, and 7.5 for rabbit psoas. These ratios are intermediate between the ratio of the volumes and the ratio of the surfaces of the corresponding fibre bundles or single fibres. From these intensity ratios it was estimated that the fluorescence intensity detected from a volume element in the centre of the fibre is about 65% (rabbit psoas) and 50% (insect flight muscle) of the intensity detected from a volume element near the surface.

From this finding alone it cannot be decided whether the polarization degree of the fluorescent light originating in the fibre centre is unchanged by passing through the fibre. Therefore a single fibre was fixed directly above a mirror. A quartz glass covering the fibre (in order to prevent evaporation) was mounted above the fibre at a distance of 100 μ. The space between mirror and quartz glass was filled with rigor solution (solution Type RM in Table 1).

As already described above the fibre can be irradiated by polarized light with adjustable wavelength through the microscope objective. If it is not already absorbed or reflected within the fibre, the light coming from the objective passes the fibre and is reflected at the mirror. After passing the fibre once more it is sampled by the objective and detected by the multipliers. The polarization degree of the sampled light was lowered from 1 to 0.9 for both the exciting and the fluorescence wavelength. This finding proves that neither the exciting nor the fluorescence light is markedly depolarized on its way through the fibre. This result was independent of the orientation of the electric vector of the incident light.

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