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Short Communications

Muscle fluorometry: a determination of the depth of penetration

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Summary. Fluorometric recordings of NADH (nicotinamide adenine dinucleotide) were made on rabbit papillary muscles. The specimens were placed between the UV light source and a small window of the detection stage. As the muscle was moved over the window in a transverse direction, simultaneous measurements could be taken of transmitted UV light and fluorescent light for various thicknesses of tissue. It is concluded that a muscle thickness of 0.65 mm is optimal before absorption of the incident light decreases the fluorescence signal.
Key words. Rabbit papillary muscle; muscle fluorometry; penetration depth.

Following the first investigations of muscular energetics, the need arose for a suitable monitor that could relate the growing pool of myothermic data to specific biochemical reactions. The technique of tissue fluorometry was subsequently developed[1], allowing intracellular oxidation-reduction levels to be monitored continuously in whole muscle preparations.

For most investigations, the experimental method involved focussing a beam of 365 nm light onto the surface of an intact muscle while continuously recording the fluorescent light originating from the same plane[2–4]. As some of the excitation light will be absorbed by the muscle, the depth of penetration from which it is possible to record fluorescence may be limited. While this might not be undesirable for studies involving localized functional regions such as the cortex of the brain and kidney[5], the interpretation of data obtained from whole muscle preparations (particularly those with heterogeneous fibre distributions) might prove difficult if tissue fluorescence were being recorded only from the most superficial cells.

This problem has been approached in two ways. The first was to measure as accurately as possible tissue fluorescence while varying the effective muscle thickness. A fluorometer system was constructed such that the tissue fluorescence was recorded from the opposite side (or 180 degrees to) the beam of incident light. This has the advantage that, by narrowing the field of view to a fraction of a millimetre and moving muscles with a circular cross section horizontally across the field, an indication of the amount of light absorption taking place can be obtained. To verify the experimental results and to simulate other recording conditions, a model of the generation and absorption of fluorescent light was prepared and run on a VAX-11/780 computer.

Right ventricular papillary muscles were removed from adult (1.8 kg) rabbits and placed in modified Krebs-Henseleit solution containing (in mM); NaCl 118.0, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.18 $MgSO_4$ 1.8, $NaHCO_3$ 28.4 and glucose 10.0. The muscles were kept under light tension and equilibrated with 95% oxygen 5% carbon dioxide mixture. Fluorometric measurements were made using a microfluorometer essentially the same as that developed by Jöbsis and Stainsby[6].

For experimentation a suitable muscle was selected and placed in a perspex clamp that held the muscle vertically while allowing it to be moved horizontally across the field of view by a micrometer. The fluorometer field was reduced to 0.3 mm diameter by an aperture in a piece of silver coated with nonfluorescent paint placed as close as possible to the muscle surface. Measurements were performed using two right ventricular papillary muscles approximately circular in cross section and having diameters of 1.4 ± 0.1 mm. Each muscle was placed in the clamp and moved through the field of view until a maximum fluorescence reading was obtained. This was standardized to 100% for both tissue fluorescence and excitation light. The muscles were then repositioned at the edge of the aperture and moved horizontally across the fluorometer field in increments of 0.05 mm. A sample of chart recording for one complete scan showing the outputs of tissue fluorescence and excitation light is presented in figure 1. Four scans were made on each muscle and the data are presented in figure 2. The fluorescence obtained at both zero and maximal horizontal muscle movements originated solely from the fluorometer optics, as the muscles were outside the field of view. The fluorescence output increased rapidly as thicker portions of muscle were brought into the fluorometer field until a plateau was reached. This corresponded to the maximum thickness of the muscle at which point absorption of both incident and fluorescent light became limiting. Presumably, had the diameter of the muscles been larger, a greater reduction of fluorescence would have been evident.

In theory, the absorption of light in a homogenous medium should be described by an exponential function of the type

$$I = I_0 e^{-ax}, \quad (1)$$

where I_0 and I are the incident and tranmitted light respectively, x is the thickness of a particular medium and a is a constant for the absorption of the light through that medium. Thus, it is possible to describe mathematically the present arrangement whereby incident UV light of 365 nm is producing

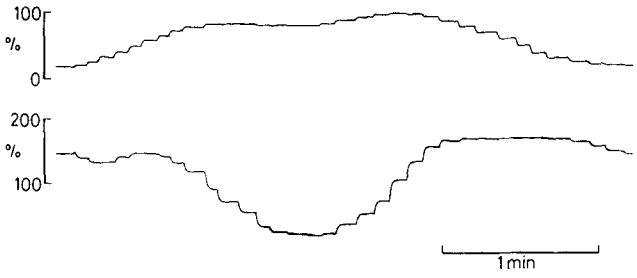

Figure 1. Original chart recording showing fluorescence (above) and excitation light (below) measurements made on a right ventricular papillary muscle 1.4 mm in diameter. Fluorescence and excitation light were standardized at 100% for a region of maximum fluorescence signal. The fluorometer field of view had a diameter of 0.3 mm across which the muscle was moved horizontally in increments of 0.05 mm. Background fluorescence of the fluorometer optics never exceeded 20% of the total signal. The asymmetry of the fluorescence profile is a reflection of the cross section of the muscle.

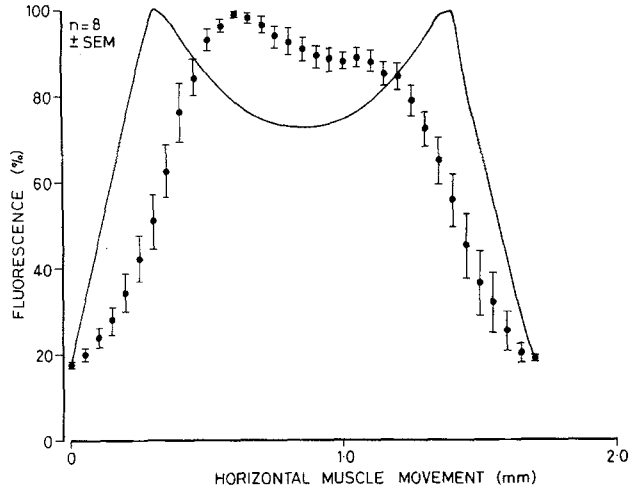

Figure 2. Fluorescence intensity vs horizontal muscle movement obtained from measurements of the type shown in figure 1. All points are the mean of eight measurements on two right ventricular papillary muscles 1.4 ± 0.1 mm in diameter. Error bars are ± SEM. The total horizontal muscle movement (1.7 mm) is the sum of the muscle thickness (1.4 mm) and the diameter of the viewing aperture (0.3 mm). The curve represents a simulation of the fluorescence intensity recorded at different positions across a muscle with a circular cross section and a diameter of 1.4 mm.

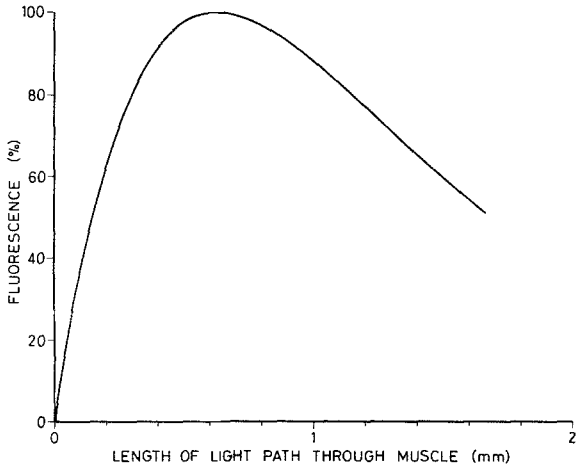

Figure 3. Simulation of the fluorescence intensity vs the length of the light path through the muscle tissue calculated for a muscle with a diameter of 3.7 mm. At a length of equivalent muscle thickness of 0.65 mm fluorescence begins to decline as absorption of both UV and fluorescent light become larger.

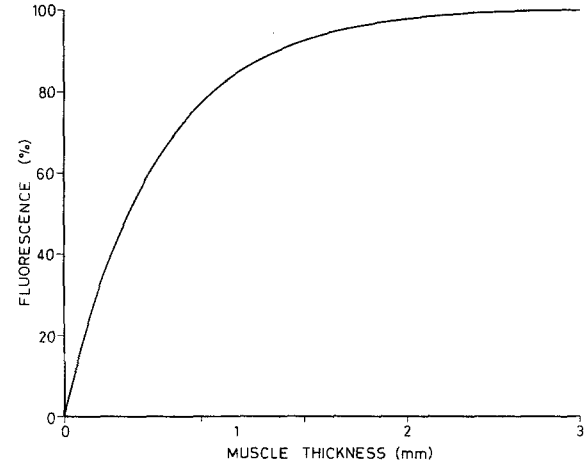

Figure 4. Simulation of fluorescence vs muscle thickness recorded from the same plane as the incident UV light. Tissue layers deeper than 2.0 mm contribute negligible amounts of fluorescence to the total signal.

fluorescent light of 465 nm while passing through a section of muscle by the formula

$$I_{(465)} = \alpha I_{0(365)} \int_0^t e^{-a_1 x} \cdot e^{-a_2 (t-x)}\, dx\,, \qquad (2)$$

where α is a constant relating to the efficiency of excitation of 465 nm light by 365 nm light, $I_{(465)}$ and $I_{0(365)}$ are the intensities of the fluorescent and incident UV light respecitvely, a_1 and a_2 are the absorption constants for 365 and 465 nm light respectively, x is the distance from the surface of the muscle being irradiated by UV light, and t is the thickness of the muscle. The evalulation of the integral between zero to t is necessary to calculate the total fluorescent output recorded from the total muscle thickness, and can be expressed as

$$I_{(465)} = \alpha\, I_{0(365)} \left[\frac{e^{-a_1 t} - e^{-a_2 t}}{a_2 - a_1} \right], \qquad (3)$$

where all expressions have their previous meanings.

Estimations of a_1 and a_2 were carried out as follows. Sections of rabbit ventricle approximately 1.00 mm thick were cut to fit between two glass slides, the spacing of which was set at 0.91 mm. By shining a beam of 365 nm light through the preparation, measuring the intensity at the other side, and substituting the values in equation 1, it was possible to measure the constant of absorption a_1. To minimize any errors owing to inhomogeneity or compression of the muscle, 22 readings from five sections were made. This produced a value for a_1 of 1.37 mm^{-1}. For the calculation of a_2, sections of rabbit ventricle 1.5–2.0 mm thick were placed in the quartz cells of a Varian spectrophotometer and the relative absorption of 365 nm and 465 nm light measured. The ratio of a_1 to a_2 was measured as 1.51, therefore the value of a_2 is 0.91 mm^{-1}.

To simulate the experimental situation, α was set at unity and $I_{0(365)}$ set to a value that resulted in a maximum fluorescence output equalling 100%. The first simulation was run with a muscle diameter of 1.4 mm. This was arranged with a 'sampling window' of 0.3 mm diameter being moved in 0.05 mm increments across the muscle. In addition, a component to sim-

ulate the generation of fluorescence from optics of the fluorometer was included in the model. The result has been superimposed on the data in figure 2. The curve generated by the computer compares well on a qualitative basis with the experimental data, showing maximum absorption of both the excitation and fluorescent light halfway across the muscle. The rate of change in fluorescence as the muscle is brought in and out of the light beam differs slightly from that which the model predicts. This is believed to be a result of the physical limitations of the apparatus and reflection of fluorescent light from the inside surface of the 0.3 mm diameter aperture. Furthermore, the above equations describe the production of fluorescence in a homogeneous medium, but the actual tissue fluorescence will be influenced by many factors including the total number of fluorescent 'sites' in a given volume of tissue, quenching of fluorescence and the light scattering properties of the muscle fibers.

To estimate the thickness of muscle at which absorption should begin to predominate, a simulation was performed plotting fluorescence against the calculated light path through the muscle using a larger muscle diameter of 3.7 mm (fig. 3). The result shows that fluorescence should increase with thickness up to 0.65 mm before absorption of excitation and fluorescent light becomes too great.

To investigate the situation in which the fluorescent light is monitored from the same plane as the incident light, a second model was developed as follows

$$I_{(465)} = \alpha \int_0^t I_{0(365)}\, e^{-a_1 x} \cdot e^{-a_2 x}\, dx\,. \qquad (4)$$

The above formula describes the fluorescence output originating from points which are at a distance x from the surface of a muscle of total thickness t. All the constants have their previous meanings. Evaluating the integral for the complete thickness of the muscle produces

$$I_{(465)} = \frac{\alpha I_{0(365)}}{a_1 + a_2} \cdot \left[1 - e^{-(a_1 + a_2)t}\right]. \qquad (5)$$

Using the same values for a_1 and a_2, a simulation over 50 increments of thickness up to 3.0 mm was performed and the results shown in figure 4. At 1.00 mm thickness approximately 85% of the total fluorescence was being detected. A further doubling of the muscle thickness resulted in increasing the fluorescence signal by the remaining 15%. Thereafter, further increases in muscle thickness produced little effect on the total fluorscence output owing to almost complete absorption of the incident and fluorescent light by the first 2.0 mm of tissue.

It is apparent from both experimental and theoretical considerations that ability to detect fluorescence from deep layers of tissue is severely limited by light absorption. It is equally apparent that the method of epifluorescence as used with cardiac tissue does not exclusively give an answer as to the metabolic state of the surface region since fibers situated as deep as 0.65 mm (or 40 fiber diameters) will contribute appreciably to the recorded signal.

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Morphine selectively facilitates the inspiratory-inhibitory vagal reflex in adult rabbits

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Summary. In rabbits naive to opiates or pretreated with morphine a selective morphine-induced facilitation of the Breuer-Hering inflation reflex is described.
Key words. Breuer-Hering inflation reflex; morphine; naloxone.

Direct central action of opiates on respiration occurs at the level of chemosensitive medullary areas[7] and respiratory neurons[4]. The effects of opiates on the respiratory control system have been recently reviewed by Trippenbach[9], with some remarks on vagal modulation of respiratory timing. Champagnat et al.[3] observed that in animals with bilateral cervical vagotomy, depres-

gram of the C_5 phrenic root was continuously recorded. Series of tracheal occlusions starting randomly at different phases of inspiration were applied. Each occlusion was continued to the onset of the following inspiration. Tracheal occlusions were performed before and after the giving of i.v. doses (3–5 mg) of morphine. For comparison, pentobarbital (5 mg) and chlora-